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Staphylococcus aureus e Estafilococos coagulase-negativa: Virulência, Resistência aos Antimicrobianos e Epidemiologia Molecular

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Texto sistematizado da linha de pesquisa, apresentado ao Instituto de Biociências, como parte das exigências para obtenção do título de Livre-docente em Microbiologia - Bacteriologia

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*"**Deus** quer, o homem sonha, a obra nasce"*

Fernando Pessoa

*A **Deus**, alicerce que me faz forte, persistente e sonhadora...*

Caminho, verdade e vida

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LISTA DE ABREVIATURAS E SIGLAS

Aap	Proteína Associada ao Acúmulo
ab	Quase Negro
AIP	Polipeptídeo autoindutor
agr	Gene Regulador Acessório
ANVISA	Agência Nacional de Vigilância Sanitária
arl	Lócus Relacionado a Autolisina
ATCC	*American Type Culture Collection*
Atle	Autolisina
B	Bordô
b	Negro
Bap	Proteína Associada ao Biofilme
Bhp	Proteína Homóloga Associada ao Biofilme
brd	Bordô
CA-MRSA	*Staphylococcus aureus* Resistente à Meticilina Adquirido na Comunidade
CAPD	Diálise Peritoneal Ambulatorial Contínua
CC	Complexo Clonal

CDC	*Centers for Disease Control*
cDNA	DNA complementar
CFL	Cefalotina
CFO	Cefoxitina
CIM	Concentração Inibitória Mínima
CLSI	*Clinical Laboratory Standards Institute*
CRA	Ágar Vermelho Congo
CVE	Centro de Vigilância Epidemiológica
CTI	Centro de Terapia Intensiva
CVC	Cateter Vascular Central
DP	Diálise Peritoneal
DTP	Método Diferencial de Tempo de Positividade
ECN	Estafilococos Coagulase-negativa
ECTA	Ácidos Teicóicos Extracelulares
eDNA	DNA genômico extracelular
egc	*Enterotoxin gene cluster*
ELISA	Ensaio Imunoenzimático
ERI	Eritromicina

ERIC	*Enterobacterial Repetitive Intergenic Consensus*
ETs	Toxinas esfoliativas
EUA	Estados Unidos da América
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FMB	Faculdade de Medicina de Botucatu
GEN	Gentamicina
GPI	Identificação de Gram-Positivo
HA-MRSA	*Staphylococcus aureus* Resistente à Meticilina de Origem Hospitalar
HACO-MRSA	*Staphylococcus aureus* Resistente à Meticilina de Origem Hospitalar de Início na Comunidade
HC	Hospital das Clínicas
ica	Locus de Adesão Intercelular
ICSRC	Infecção da Corrente Sanguínea Relacionada com Cateter
ID	Identificação
IRAS	Infecções Relacionadas à Assistência à Saúde
IRC	Infecção Relacionada ao Cateter
ITS-PCR	*Intergenic Transcribed Spacers*

ITU	Infecção do Trato Urinário
MH	Ágar Muller-Hinton
mgrA	Regulador Global Múltiplo
MLST	*Multilocus Sequence Type*
MSCRAMMs	*Microbial Surface Components Recognizing Adhesive Matrix Molecules*
MOD-SA	Resistência Modificada à Oxacilina
MRSA	*Staphylococcus aureus* Resistente à Meticilina
MSSA	*Staphylococcus aureus* Sensível à Meticilina
NEC	Enterocolite necrosante
NNIS	*National Nosocomial Infection Surveillance System*
OPT	*Operon Technology*
PB	Preto Brilhante
OXA	Oxacilina
pb	pares de bases
PBP	Proteína Ligadora de Penicilina
PCR	Reação em Cadeia da Polimerase
PEN	Penicilina

PFGE	*Pulsed Field Gel Electrophoresis*
PS	Preta Seca
PIA	Polissacarídeo de Adesão Intercelular
PMNs	Leucócitos Polimorfonucleares
PNAG	Poli-N-Acetilglicosamina
PTs	Toxinas Pirogênicas
PVL	Leucocidina Panton-Valentine
qRT-PCR	Reação em Cadeia da Polimerase – Transcriptase Reversa quantitativo
QS	*Quorum-sensing*
R	Rosa
r	Vermelho
RAP	Proteína Ativadora de RNA
RAPD-PCR	*Random Amplified Polymorphic DNA Based PCR*
REP-PCR	*Repetitive Extragenic Palindromic Sequence Based PCR*
RIA	Radioimunoensaio
RIF	Rifampicina
RIP	Peptídeo Inibidor de RNA

RNAm	RNA mensageiro
RNAt	RNA transportador
RNMPB	Recém-nascidos de Muito Baixo Peso
ROC	Curva de Operação Resposta
rot	Repressor de Toxinas
RPHA	Hemaglutinação Reversa Passiva
RPLA	Aglutinação Reversa e Passiva de Látex
RT-PCR	Reação em Cadeia da Polimerase – Transcriptase Reversa
SAgs	Superantígenos
SaPI	Ilha de Patogenicidade de *Staphylococcus aureus*
sae	Elemento Acessório Estafilocócico
sar	Regulador Acessório Estafilocócico
SCC*mec*	Cassete Cromossômico Estafilocócico
SCOPE	*Surveillance and Control of Pathogens of Epidemiological Importance*
SEs	Enterotoxinas Estafilocócicas
SEA	Enterotoxina Estafilocócica A
SEB	Enterotoxina Estafilocócica B

SEC	Enterotoxina Estafilocócica C
sec-1	Gene da Enterotoxina Estafilocócica C subtipo 1
SEC_3	Enterotoxina Estafilocócica C subtipo 3
SED	Enterotoxina Estafilocócica D
SEE	Enterotoxina Estafilocócica E
SEG	Enterotoxina Estafilocócica G
SEH	Enterotoxina Estafilocócica H
SEI	Enterotoxina Estafilocócica I
SEl	Enterotoxina-like estafilocócica
SElJ	Enterotoxina-like estafilocócica J
SElK	Enterotoxina-like estafilocócica K
SElM	Enterotoxina-like estafilocócica M
SElN	Enterotoxina-like estafilocócica N
SElO	Enterotoxina-like estafilocócica O
SElP	Enterotoxina-like estafilocócica P
SePI	Ilha de Patogenicidade de *Staphylococcus epidermidis*
SElQ	Enterotoxina-like estafilocócica Q
SER	Enterotoxina Estafilocócica R

SES	Enterotoxina Estafilocócica S
SET	Enterotoxina Estafilocócica T
spaTyping	Tipagem do Gene da Proteína A
SRC	Sepse Relacionada a Cateter
srr	Resposta Respiratória Estafilocócica
SSR	Sequência Curta de Repetições
TCP	Aderência em Placa de Poliestireno
TM	Método de Aderência em Tubo de Borossilicato
TP	Tempo de Positividade
TRAP	Proteína Alvo de RAP
TSST-1	Toxina 1 da Síndrome do Choque Tóxico
TSS	Síndrome do Choque Tóxico
UFC	Unidades Formadoras de Colônias
UNESP	Universidade Estadual Paulista
UNIFESP	Universidade Federal do Estado de São Paulo
UTI	Unidade de Tratamento Intensivo
UTINs	Unidades de Tratamento Intensivo Neonatais
V	Vermelha

VAN	Vancomicina
vb	Muito Negro
VISA	*Staphylococcus aureus* Intermediário à Vancomicina
VPP	Valor Preditivo Positivo
vr	Muito vermelho
VRE	Enterococos Resistente à Vancomicina (VRE)
VRSA	*Staphylococcus aureus* Resistente à Vancomicina

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1. Considerações Iniciais

O presente trabalho foi realizado de acordo com o disposto no Artigo 6° da Resolução Unesp-27, de 15-4-2009 que estabelece, como uma das provas realizadas para a obtenção do título de Livre-Docente, "II- defesa de tese original e inédita ou de texto que sistematize criticamente a obra do candidato, ou parte dela, elaborados após o doutoramento".

Neste contexto, optei por discutir os trabalhos vinculados à linha de pesquisa "*Staphylococcus aureus* e Estafilococos coagulase-negativa: virulência, resistência antimicrobiana e epidemiologia molecular". Meu interesse por essa linha de pesquisa surgiu desde a minha iniciação científica na Universidade Estadual de Londrina quando comecei estudar a detecção de enterotoxinas estafilocócicas em *S. aureus* orientada pela Profa. Dra. Elisa Yoko Hirooka. Na pesquisa para dissertação de mestrado continuei a estudar *S. aureus* enterotoxigênicos, porém com ênfase em seu desenvolvimento em leite e extrato de soja. A partir do meu projeto de doutorado já como professora no Departamento de Microbiologia e Imunologia do Instituto de Biociências da UNESP de Botucatu e orientada pelo Prof. Dr. Carlos Alberto de Magalhães Lopes comecei a trabalhar também com as outras espécies do gênero *Staphylococcus*, os Estafilococos coagulase-negativa (ECN), pesquisando desde a identificação dessas espécies, bem como a detecção fenotípica de vários fatores de virulência, incluindo enzimas, biofilme, enterotoxinas e a resistência aos antimicrobianos em amostras isoladas de recém-nascidos da Unidade Neonatal do Hospital das Clínicas (HC) da Faculdade de Medicina de Botucatu (FMB), com a colaboração da Profa. Dra. Ligia Maria S. S. Rugolo e posteriormente também dos

neonatologistas Dr. João César Lyra e Dra. Maria Regina Bentlin, professores pesquisadores do Departamento de Pediatria da Faculdade de Medicina de Botucatu.

A partir dos resultados do meu doutorado e da dificuldade de convencer os revisores da veracidade dos resultados obtidos quanto a toxigenidade de ECN e da necessidade da confirmação dos resultados com métodos genotípicos mais confiáveis, ingressei na área de Biologia Molecular, com a pesquisa de genes de toxinas em amostras de *S. aureus* e estafilococos coagulase-negativa com o meu primeiro projeto financiado pela FAPESP "Detecção de genes de enterotoxinas e toxina 1 da síndrome do choque tóxico em estafilococos, com ênfase em estafilococos coagulase negativa" e com a colaboração do Prof. Dr. João Pessoa Araújo Júnior do Departamento de Microbiologia e Imunologia. A partir desse projeto ainda continuou a dificuldade de publicar, sendo agora questionado o fato de que estas amostras de ECN poderiam ser *S. aureus* mutantes que perderam a capacidade de produzir a enzima coagulase e a cobrança em relação ao fato da PCR detectar somente os genes e não a expressão das enterotoxinas. Para tentar resolver essa questão iniciamos as pesquisas para aprimorar a identificação desses micro-organismos e a utilização de técnicas moleculares para confirmação das espécies, bem como a detecção de RNAm pela técnica de RT-PCR, o que possibilitou o desenvolvimento da Dissertação de mestrado da minha primeira aluna no programa de pós-graduação em Doenças Tropicais e o desenvolvimento do segundo projeto financiado pela FAPESP, com o título "Determinação do perfil toxigênico em *Staphylococcus* pela técnica de RT-PCR".

A partir do meu doutorado também ingressei por convite do Dr. Augusto Cezar Montelli no grupo de pesquisa das peritonites em Diálise Peritoneal juntamente com o

Dr. Pasqual Barretti e a Dra. Jacqueline Teixeira Caramori, professores pesquisadores e responsáveis pela Unidade de Diálise do HC da FMB, com vários projetos já desenvolvidos e outros em andamento, com ênfase para o estudo dos fatores de virulência envolvidos na evolução das peritonites causadas por *S. aureus* e ECN.

Paralelamente a esses projetos de estudos dos fatores de patogenicidade ingressei no estudo da detecção de resistência à oxacilina por métodos moleculares com o desenvolvimento do terceiro projeto de pesquisa aprovado pela FAPESP e minha segunda orientação de mestrado "Resistência à oxacilina em *Staphylococcus aureus* e *Staphylococcus* coagulase-negativa provenientes de pacientes do Hospital das Clínicas da Faculdade de Medicina de Botucatu". A partir daí ingressamos também na área de epidemiologia molecular com a compra do equipamento de Pulsed Field Gel Electrophoresis (PFGE) no quarto projeto financiado pela FAPESP "Epidemiologia molecular e fatores de risco para aquisicão de clones endêmicos de *Staphylococcus aureus* resistente a meticilina (MRSA) em um hospital de ensino", e minha primeira orientação de Doutorado, juntamente com a colaboração do Prof. Dr. Carlos Magno Castelo Branco Fortaleza do Departamento de Doenças Tropicais da FMB.

Sendo assim, continuo nessa mesma linha de pesquisa, com quatro projetos de pesquisa em andamento financiados pela FAPESP e várias orientações, incluindo alunos de iniciação científica, mestrado, doutorado, pós-doutorado, vinculados ao programa de pós-graduação em Doenças Tropicais da FMB ou ao programa de pós-graduação em Biologia Geral e Aplicada do Instituto de Biociências da UNESP de Botucatu.

Os trabalhos publicados e os manuscritos submetidos inseridos nesse texto são portanto, os resultados desta linha de investigação, sendo que os mesmos foram organizados em áreas e não por execução cronológica, por entender que a apreciação dos mesmos será facilitada.

Finalmente, gostaria de ressaltar que, embora eu tenha participado diretamente da execução de todos os trabalhos, seja como coordenadora, colaboradora ou orientadora, só a dedicação, esforço e competência dos alunos e de todos os professores pesquisadores envolvidos permitiram a obtenção desses resultados.

2. O Gênero *Staphylococcus*

O nome *Staphylococcus* tem origem do grego (*staphyle* cacho de uva, e coccus grão ou semente) e foi dado por Alexander Ogston, cirurgião escocês em 1880, quando isolou esse micro-organismo de um abscesso cirúrgico, referindo-se à morfologia e ao arranjo de tais bactérias quando observadas ao microscópio óptico, e conseguiu por meio de vários experimentos demonstrar a importância de estafilococos em infecções humanas purulentas (1). Entretanto, foi somente em 1884 que o pesquisador Rosenbach, estudando micro-organismos isolados a partir de pus, propôs a criação do gênero *Staphylococcus*. Desde a sua proposição por Rosenbach o gênero *Staphylococcus* tem sido classificado dentro da família Micrococcaceae e foi somente na última década com o avanço da biologia molecular, estudos genéticos, perfis de ácidos graxos, composição da parede celular e, principalmente, estudos com RNA ribossômico 16S que o gênero *Staphylococcus* foi incluído na nova família Staphylococcaceae (2,3). Diferentemente de Micrococcaceae, a família Staphylococcaceae pertence ao filo Firmicutes, Classe Bacilli e ordem Bacillales, evidenciando-se, depois de mais de um século desde sua primeira descrição, a distância filogenética entre os gêneros *Staphylococcus* e *Micrococcus* e a importância do avanço da biologia molecular na correta classificação taxonômica dos micro-organismos.

Os estafilococos são cocos Gram-positivos, imóveis, anaeróbios facultativos, apresentando metabolismo fermentativo com produção de ácido e não gás, não fotossintético, não esporulado, catalase-positiva e capazes de crescer em meio contendo 10% de cloreto de sódio. São micro-organismos mesófilos, com temperatura

de desenvolvimento de 7 a 48^{o} C, com ótima de 37^{o} C e pH na faixa de 4,0 a 10,0, com ótimo de 6,0 a 7,0 (4).

Os estafilococos crescem rapidamente na maioria dos meios bacteriológicos. As colônias em meios sólidos são redondas, lisas, elevadas e brilhantes. Produzem um pigmento carotenóide somente em aerobiose, intensificando-se em meios contendo alta concentração salina. A capacidade de se desenvolver em meios contendo alta concentração de NaCl e certa tolerância ao telurito de potássio são aproveitadas para o preparo de meios seletivos.

O gênero *Staphylococcus* compreende diversas espécies e subespécies, que se encontram amplamente distribuídas na natureza, sendo principalmente encontradas na pele e membranas mucosas de aves e mamíferos (4). Atualmente já são 45 espécies descritas dentro do gênero (5, 6), a maioria coagulase-negativa, caracterizando-se a exclusividade da síntese da enzima coagulase as espécies *S. aureus* subsp. *aureus*, S. *aureus* subsp. *anaerobius*, *S. hyicus*, *S. intermedius*, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*, *S. delphini* e *S. lutrae*. A espécie *S. hyicus* é variavelmente coagulase positiva e, frequentemente incluída como coagulase-negativa (5). Cerca de metade das espécies de estafilococos coagulase-negativa (ECN) coloniza naturalmente o homem, incluindo-se dentre eles *S. epidermidis, S. haemolyticus, S. saprophyticus, S. lugdunensis* (7), *S. xylosus* (8), *S. warneri, S. simulans* (9), *S. saccharolyticus* (10), *S. auricularis* (11), *S. caprae* (12), *S. pasteuri* (13), *S. vitulinus* (14), *S. pettenkoferi* (15) e *S. massiliensis* descrita mais recentemente (16). Das 23 subespécies de ECN descritas, o *S. hominis* subsps. *S. hominis*, *S. hominis* subsps. *novobiosepticus, S. capitis* subsps. *capitis, S. capitis* subsps. *urealyticus* (17), *S. schleiferi* subsps. *schleiferi*, *S. cohnii*

subsps. *cohnii* e *S. cohnii* subsps. *urealyticum* (18), também são naturais do homem e de outros primatas.

Staphylococcus aureus sempre foi a espécie mais importante relacionada com uma série de infecções e intoxicações no homem e nos animais. Vários fatores de virulência são responsáveis pelos sintomas e gravidade das infecções causadas por *S. aureus*, adquiridas tanto na comunidade como em hospitais, sobressaindo-se atualmente como um dos maiores problemas clínicos e epidemiológicos em Infecções Relacionadas à Assistência à Saúde (IRAS). *S. aureus* se destaca por sua patogenicidade e alta frequência, causando doenças tanto em indivíduos imunocomprometidos quanto em sadios por sua fácil disseminação intra-hospitalar e sua enorme capacidade de adaptação e resistência a antimicrobianos (19).

Os ECN constituem-se nos mais frequentes integrantes da microbiota do homem (20), podendo atingir de 10^3 a 10^6 UFC/cm^2 na superfície de regiões mais úmidas do corpo, tais como, narinas anteriores, axilas e nas áreas inguinal e perineal. Algumas espécies e subespécies demonstram uma marcante preferência por certos habitats, tais como *S. capitis* subsps. *capitis* encontrado em grandes concentrações na cabeça, fronte, sobrancelha e canal auditivo externo (20). Já *S. capitis* subsps. *urealyticus* é encontrado em menor número nesses sítios, sendo porém mais amplamente distribuído em outras regiões corpóreas (5). *S. auricularis* é uma das espécies mais encontradas no canal auditivo externo de humanos (11), enquanto que *S. saprophyticus* é geralmente encontrado em baixas concentrações e transitoriamente em vários locais orgânicos, mas apresentando aderência especifica as células urogenitais (21). *S. epidermidis* é a espécie predominante em humanos, sendo

encontrada em grande número nas narinas anteriores, axila, área perineal (22). *S. hominis* e S. *haemolyticus* são também numerosos nas regiões mais úmidas do corpo, mas também colonizam regiões mais secas da pele com maior frequência que outras espécies (22). *S. warneri* e *S. lugdunensis* são amplamente distribuídos por todo o corpo, embora sua concentração seja geralmente baixa (5). *S. caprae* e *S. xylosus*, embora, muito distribuídos na natureza, são ocasionalmente isolados da pele de humanos (9).

Contudo, os ECN são também considerados micro-organismos oportunistas, que podem se aproveitar de algumas situações, como rupturas da barreira cutânea por trauma ou pela presença de artigos estranhos, e com isso atingir outros tecidos, proliferar e desenvolver um comportamento patogênico (23). Normalmente estão envolvidos em processos infecciosos em pacientes imunocomprometidos ou em pacientes que fazem uso de cateteres. As principais razões para o aumento da taxa de infecções por ECN nos últimos anos são o aumento da resistência aos antimicrobianos entre os ECN e o uso crescente de dispositivos médicos (24).

3. Significância Clínica de Estafilococos Coagulase-Negativa

Antes dos anos 70, poucos foram os relatos de infecção por ECN, sendo reconhecidos por clínicos e microbiologistas como contaminantes em amostras clínicas e *S. aureus* como a única espécie patogênica dentro do gênero *Staphylococcus* (25). Esta distinção, muito usada para o diagnóstico clínico, entretanto tem sido considerada como um desafio quanto ao papel que esses micro-organismos desempenham em processos infecciosos.

Os dois primeiros relatos na literatura associando ECN a processos patogênicos datam do ano de 1945, quando Herbst e Merricks (26) relataram um caso de septicemia associado a *S. albus* em paciente submetido à cirurgia percutânea em rim para retirada de cálculo renal e no ano seguinte Harry (27) citou um caso de meningite infantil pela mesma espécie. *S. albus* foi renomeado posteriormente como *S. epidermidis* e treze anos mais tarde Smith *et al.* (28) descreveram o envolvimento desses micro-organismos com três casos de septicemia. Vários anos mais tarde Pulverer e Halswick (29) relataram 128 casos de endocardites causadas por ECN e Pulverer (30) compartilhou sua frustração com a comunidade médica no *Fifth International Symposium on Staphylococci and Staphylococcal Infections*, da dificuldade de convencer os editores da publicação alemã que seus dados eram sérios quando tentou publicar seu trabalho em 1967. Wilson e Stuart (31) encontraram ECN em 53 culturas puras de 1.200 casos de infecções de feridas (4,4%). Pulverer e Pillich (32) também publicaram a incidência de ECN em infecções piogênicas na Alemanha apresentando dados de 1960, 1969 e 1970, encontrando ECN em cerca de 10% das lesões piogênicas. Pereira em 1962 (33) relatou que *S. saprophyticus* causava

infecções do trato urinário (ITU). Poucos anos depois Gallagher *et al*. (34) e Mabeck (35) também evidenciaram ITU causadas por *S. saprophyticus*.

Em muitos laboratórios *S. epidermidis* era usado como termo genérico e coletivo para todas as outras espécies de Estafilococos Coagulase-Negativa. Na década de 80 essa situação mudou radicalmente, quando o *National Nosocomial Infections Surveillance System* (NNIS) do *Centers for Disease Control and Prevention* (CDC) demonstrou um aumento significante de infecções hospitalares causadas por diferentes espécies de ECN (36).

Consideráveis progressos na classificação sistemática dos estafilococos e no desenvolvimento de métodos para a identificação do gênero, espécies e subespécies têm permitido aos clínicos se inteirarem da variedade de ECN presentes em amostras clínicas e, assim, os considerarem como agentes etiológicos de uma série de processos infecciosos (25). Atualmente, são reconhecidos como micro-organismos essencialmente oportunistas que se prevalecem de inúmeras situações orgânicas para produzirem graves infecções. Dados do SCOPE (*Surveillance and Control of Pathogens of Epidemiological Importance*) dos EUA referentes a um período de sete anos, de março de 1995 a setembro de 2002, indicaram os micro-organismos gram-positivos como os principais agentes de infecções da corrente sanguínea (65%) e os ECN como o agente mais frequente (31%), seguido por *S. aureus* (20%) (37). Dados recentes (junho de 2007 a março de 2010) de um estudo multicêntrico brasileiro utilizando a mesma metodologia do programa SCOPE dos Estados Unidos, SCOPE brasileiro, com o objetivo de estudar a epidemiologia e a microbiologia das infecções da corrente sanguínea nosocomial de pacientes provenientes de 16 hospitais brasileiros de vários tamanhos e

diferentes regiões revelaram *S. aureus* (14%) e ECN (12,6%) como os micro-organismos mais frequentemente isolados (38).

Dados reportados pelo *National Healthcare Safety Network* (NHSN) do CDC do período de janeiro de 2006 a outubro de 2007 ranquearam os ECN em 1° e *S. aureus* em 2° lugar na etiologia de IRAS (39). No Brasil, dados do Sistema de Vigilância de Infecção Hospitalar do Estado de São Paulo, Centro de Vigilância Epidemiológica (CVE) também relataram os ECN como os agentes mais associados com infecção da corrente sanguínea em 2010, mantendo a predominância de *Staphylococcus epidermidis* e outros ECN (30,1%), seguido por *S. aureus* (16,6%), taxas similares as encontradas em 2009 (40).

Os ECN mantêm uma relação simbiótica ou comensal com os seus hospedeiros, em algumas situações desenvolvendo mecanismos para a interferência do crescimento de outras bactérias patogênicas, sugerindo a coevolução entre o micro-organismo e o hospedeiro (41). *S. epidermidis* era descrito apenas como um micro-organismo comensal, mas sua importância como patógeno oportunista é reconhecida atualmente, principalmente por ser um dos principais agentes de infecções nosocomiais (42). As infecções causadas por *S. epidermidis* não são tão graves como as causadas por *S. aureus*, por não possuir muitos fatores de virulência, acometendo principalmente prematuros, pacientes imunossuprimidos e com implantes de próteses (43). O baixo nível de virulência mantido por *S. epidermidis* pode ser devido ao processo adaptativo entre patógeno e hospedeiro. A evolução favorece as espécies que oferecem pouco ou nenhum dano ao seu hospedeiro, e em micro-organismos menos virulentos, essas adaptações oferecem vantagens evolutivas através da duração

prolongada da infecção, que favorece o potencial de transmissão de um hospedeiro para o outro (44).

Massey *et al.* (44) desenvolveram um modelo matemático para compreender as vantagens de *S. epidermidis* manter um baixo nível de virulência em relação a *S. aureus*. Esse modelo matemático considerou a colonização de todo epitélio por *S. epidermidis* em todos os humanos, que difere de *S. aureus*, que coloniza quase que exclusivamente as narinas de algumas pessoas, e também considerou os fatores de regulação gênica envolvidos na colonização e interferência bacteriana. De acordo com esse modelo, *S. epidermidis* se dissemina mais facilmente do que *S. aureus* e a baixa virulência está relacionada ao potencial de secretar fatores que promovam a persistência de *S. epidermidis* no hospedeiro e não o ataque agressivo (42, 44).

Os ECN são a maior causa de bacteremia em pacientes mantidos em unidades de tratamento intensivo (UTI) e UTI neonatal (25), com destaque para recém-nascidos de baixo peso, os quais são imunologicamente imaturos e frequentemente requerem procedimentos invasivos para administração de substâncias nutritivas e medicamentosas (45). O aumento da incidência de bacteremia nosocomial por ECN em neonatos nos últimos 20 anos tem sido também associado ao aumento da sobrevivência de crianças prematuras com peso menor que 1.500g ao nascimento e à sua longa permanência no ambiente hospitalar (45, 46).

Contudo, a interpretação de hemoculturas positivas para os ECN é particularmente difícil devido ao fato desses micro-organismos colonizarem a pele e as membranas mucosas como comensais, e poderem contaminar as hemoculturas

durante a coleta de sangue (25, 47). A esse respeito, investigadores têm usado uma variedade de critérios clínicos e laboratoriais para distinguir contaminação de bacteremia. Assim, o diagnóstico de bacteremia tem sido feito com base nos dados clínicos dos pacientes e no isolamento de micro-organismos idênticos em duas ou mais hemoculturas. As culturas em que ocorre o crescimento de múltiplas linhagens ou de espécies de ECN em associação a outras espécies de micro-organismos são classificadas como contaminantes (25, 48-49). Entretanto, como o volume sanguíneo é pequeno em recém-nascidos (RNs) prematuros com baixo peso, somente uma hemocultura é geralmente realizada para se evitar a necessidade e os riscos de transfusões devido a venopunções constantes (25, 50). Assim, os neonatologistas têm se apoiado em critérios clínicos e laboratoriais, tais como, instabilidade térmica, bradicardia, apnéia, intolerância alimentar, piora do desconforto respiratório, intolerância à glicose, instabilidade hemodinâmica, hipoatividade/ letargia (48, 51).

D`Angio *et al*. (52) descreveram uma taxa de colonização por ECN entre 50% a 80% até 4 dias após a admissão dos RNs em unidade de tratamento intensivo neonatal (UTIN) e observaram que havia um aumento da resistência a múltiplos antibióticos de 32% para 82% no final de uma semana na UTIN. Não há dúvidas de que o isolamento de ECN de amostras de sangue, líquor e urina de um RN com sinais e sintomas de sepse é significativo, porém, com muita frequência pode representar uma contaminação no momento da coleta. De acordo com os critérios do CDC (49, 51) e da Agência Nacional de Vigilância Sanitária (ANVISA) no Brasil (48), os ECN devem ser isolados de pelo menos duas hemoculturas colhidas em dois locais diferentes, com intervalo máximo de 48 horas entre as coletas. Em caso de isolamento de ECN em

somente uma hemocultura, a evolução clínica deve ser valorizada, exames complementares (hemograma e Proteína C reativa) e crescimento do micro-organismo nas primeiras 48 horas de incubação. O crescimento após este período sugere contaminação, e se a amostra positiva tiver sido colhida somente de cateter vascular central (CVC), ECN não deve ser valorizado como agente etiológico da infecção.

Entre as 18 espécies encontradas no ser humano, *Staphylococcus epidermidis* é clinicamente o mais importante para os RNs. Essa bactéria, nos EUA, é responsável por cerca de 10% a 27% de todos os casos de sepse nas UTINs, com taxas de 55% em recém-nascidos de muito baixo peso (RNMBP = < 1.500 gramas) (53). As principais manifestações relatadas num estudo de sepse em prematuros foram apnéia e bradicardia (88%), necessidade de oxigênio (59%) e ventilação mecânica (69%), e os marcadores laboratoriais de fase aguda foram pouco sensíveis (54). O quadro clínico inclui sepse, meningite com ou sem alterações no líquor, enterocolite necrosante, pneumonia, onfalite, abscesso de tecido mole, endocardite, abscesso e osteomielite nos locais de venopunção. A letalidade é baixa, concordante com os nossos resultados (ANEXO 1) obtidos em estudo realizado com RNs da Unidade Neonatal do Hospital das Clínicas (HC) da Faculdade de Medicina de Botucatu (FMB) que mostraram uma letalidade de 13% relacionadas a infecções por esses micro-organismos.

Nesse estudo foram isoladas de 107 RNs, 117 amostras de ECN, dos quais 51% foram considerados patogênicos e 49% contaminantes. Entre os infectados, a maioria era RNs prematuros (80%), sendo a metade RNMBP, ou seja, com imaturidade imunológica. Além disso, estavam associados a dois ou mais procedimentos invasivos, sendo que 89% tinham um cateter venoso central, 65% nutrição parenteral e 61%

ventilação mecânica. A análise multivariada de regressão mostrou que um peso de nascimento < 1.500g aumentava a chance de infecção em 6 vezes, a presença de corpo estranho em 4,4 vezes, e o uso prévio de antibióticos aumentava em 5,4 vezes mais a chance de infecção. A espécie mais isolada foi *S. epidermidis* em 78% dos casos, sendo presente em 87% das infecções e em 65% das contaminações. Silbert *et al*. (55) em estudo para determinar a prevalência de infecção *versus* contaminação em pacientes com menos de 60 dias de vida, com hemoculturas positivas para ECN relataram também, no Brasil, que entre 41 RNs com hemocultura positiva para ECN, apenas 27% foram considerados infectados, sendo os demais 73% considerados como casos de contaminação ou casos duvidosos. Portanto, a maior dificuldade no diagnóstico de infecção por ECN é a contaminação no momento da coleta do material, sendo significativa para o sangue, e muito pior para corpo estranho e secreções, conforme se verificou em nosso estudo e no trabalho de Silbert *et al*. (55).

Outras espécies de ECN, incluindo duas amostras de *S. haemolyticus*, três de *S. lugdunensis*, uma amostra de *S. simulans*, uma de *S. warneri* e uma de *S. xylosus* também foram isoladas de crianças com evidência clínica de pneumonia, enterocolite necrosante e sepse. A identificação de espécies de ECN constitui um marcador útil de infecção, visto que *S. epidermidis* foi o agente etiológico mais frequentemente associado aos processos infecciosos (ANEXO 1). Silbert *et al*. (55), encontraram 91,1% dos isolados pertencentes à espécie *S. epidermidis*, 3 (6,7%) *S. hominis* e 1 (2,2%) *S. warneri*, sendo que no grupo de pacientes infectados somente *S. epidermidis* foi encontrado. Estes resultados confirmam os achados de Lowy e Hammer (56) que

acreditam na importância da identificação das espécies de ECN para diferenciação entre contaminação e infecção.

Estudos multicêntricos têm mostrado a significância desses micro-organsimos em infecções neonatais. Na Austrália, um estudo de 10 anos realizado com 18 unidades neonatais revelou o ECN como responsável por 1.281 casos de sepse, totalizando 57,1% de todos os episódios de sepse de início tardio, com incidência de 3,46/1000 nascidos vivos e a maioria (71%) era prematuros com 24–29 semanas de gestação (57). Recentemente, resultados semelhantes foram obtidos em estudo também multicêntrico realizado na Inglaterra por Vergnano *et al*. (58) envolvendo 12 unidades neonatais com 358 episódios de sepse causados por ECN em 321 crianças Os autores verificaram uma incidência total de infecções de 3/1000 nascidos vivos quando consideradas outras etiologias e um aumento para 8/1000 quando considerados os episódios causados por ECN. A maioria das infecções causadas por ECN ocorreu em crianças de ≤ 32 semanas de gestação (86%) e de extremo baixo peso ao nascer (65%).

Staphylococcus spp. também são considerados mundialmente os agentes etiológicos mais frequentes das peritonites em diálise peritoneal ambulatorial contínua (CAPD). A peritonite bacteriana se mantém como a complicação mais grave da diálise peritoneal (DP), sendo a causa mais frequente de saída do paciente do método dialítico (59), com importante impacto na mortalidade (60). O quadro clínico e a evolução dos episódios de peritonite são fortemente influenciados pelas características do agente causal. Os ECN são os agentes etiológicos mais frequentes (61), enquanto *S. aureus* se associa a infecções graves e com elevada letalidade.

A incidência de peritonite causada por *S. aureus* na Unidade de Diálise do HC da FMB diminuiu significantemente, de 0.13 em 1996-2000 para 0.04 episódios/paciente/ano em 2006 a 2010 (p = 0.03). Embora, não haja dados disponíveis que possam explicar a alteração da etiologia prevalente, pode-se sugerir que cuidados instituídos na rotina assistencial, destinados à profilaxia das infecções relacionadas ao cateter, bem como à erradicação de *S. aureus* de carreadores nasais, podem ter contribuído para a redução de infecções peritoneais por *S. aureus*. Entretanto, os episódios de peritonites causados por *S. aureus* cursam com infecção persistente, maior risco de hospitalização, remoção de cateter e morte (59, 60).

As peritonites causadas por ECN evoluem para cura, sem outras complicações, na maior parte dos casos (62), porém recidivas de infecções aparentemente curadas são frequentemente observadas. Estudos que compararam o curso clínico das infecções por essas espécies em anos recentes, entretanto, são encontrados em pequeno número de publicações (63), sendo pertinente avaliar se as taxas de resistência progressivamente crescentes entre os ECN, tiveram impacto na evolução e complicações das infecções por esses micro-organismos. Sendo assim, nosso grupo de pesquisa vem realizando vários estudos com pacientes em tratamento com diálise peritoneal ambulatorial contínua (CAPD), em pacientes da Unidade de Diálise do HC da FMB, com os objetivos de descrever as propriedades microbiológicas dos estafilococos causadores das peritonites, comparar as infecções por *S. aureus* com as causadas por ECN e estabelecer associações entre as características do agente e do hospedeiro na evolução dessas infecções.

A análise dos episódios de peritonite ocorridos entre janeiro de 1996 a dezembro de 2000 mostrou que a resolução das peritonites não foi influenciada por fatores do hospedeiro (idade, sexo, diabetes, uso de vancomicina, sistema de troca e tempo em diálise), enquanto a etiologia *S. aureus* foi um fator independentemente associado com a não resolução quando comparado com as peritonites por ECN (ANEXO 2). Fatores relacionados com as espécies e resistência antimicrobiana poderiam explicar esses resultados. Entretanto, não houve diferença na taxa de resolução entre as amostras resistentes a oxacilina e as sensíveis, e como a resistência a oxacilina é mais frequente entre os ECN do que para *S. aureus*, a contribuição da resistência aos antimicrobianos é inconsistente. Os resultados desse estudo indicaram que os fatores de virulência encontrados mais frequentemente em *S. aureus* poderiam ser responsáveis pela natureza mais agressiva de *S. aureus* e, portanto, pela pior evolução.

Estudo publicado mais recentemente pelo nosso grupo (ANEXO 3) com dois modelos de regressão logística corroborou nossa hipótese. No primeiro modelo quando não foram incluídos os fatores de virulência, a chance de resolução não foi influenciada por fatores do hospedeiro, mas foi maior para os episódios causados por *S. epidermidis* quando comparado com *S. aureus* (p= 0,0263). Entretanto, no segundo modelo quando foram incluídos os fatores de virulência não foi verificado diferença na probabilidade de resolução das peritonites causadas por *S. aureus* e S. *epidermidis*. Este achado pode ser explicado pelo fato de que a inclusão de enzimas e toxinas no modelo permitiu o controle do efeito desses fatores sobre as espécies, ou seja, o efeito das espécies observado no primeiro modelo para os episódios de

S. aureus pode ser devido ao efeito dos fatores de patogenicidade que são mais frequentes nessa espécie.

Além disso, no último modelo os episódios causados por *S. epidermidis* apresentaram mais baixa resolução quando comparado com os causados por outras espécies de ECN, independentemente dos fatores de virulência. Esses resultados reforçam a importância da identificação de espécies de ECN que realmente se comportam de forma diferente e não podem ser avaliadas como um grupo, mas sim como espécies distintas com características específicas. Estudos recentes descrevem *S. epidermidis* como um micro-organismo versátil, que pode viver entre o comensalismo e a patogenicidade. Emprega sofisticados mecanismos de regulação gênica para a adaptação rápida do seu metabolismo às mudanças nas condições externas, para a comunicação com outras células no nicho ecológico ou para escapar da resposta imune do hospedeiro (43).

A infecção do trato urinário (ITU) é uma das doenças infecciosas mais comuns na prática clínica, figurando como a segunda infecção mais frequente no ser humano, sendo sua incidência apenas inferior as do trato respiratório. Os agentes etiológicos, mais frequentemente envolvidos com ITU são as enterobactérias, não fermentadores, fungos, enterococos e os estafilococos. Dentre as espécies de estafilococos as mais comuns e importantes em relação às ITUs são *S. aureus* e *S. saprophyticus*, porém, outras espécies de ECN vem adquirindo importância nos últimos anos. Estudo realizado em nosso laboratório por Ferreira (64) mostrou a espécie *S. saprophyticus* como a mais frequente (56,4%) na etiologia de infecção urinária, seguida por *S. aureus* (16,9%), *S. epidermidis* (15,9%), *S. haemolyticus* (7,9%), *S. warneri* (1,9%) e *S.*

lugdunensis (1,0%). *S. aureus* é relativamente incomum em ITU na população em geral (65), todavia, em alguns pacientes causa colonização e infecção através da via ascendente. Outros fatores de risco como a instrumentação do trato urinário e a presença de cateterização aumentam o risco de ITU por este micro-organismo (66). Em nosso estudo *S. aureus* foi a segunda espécie mais encontrada em isolados de origem hospitalar (23,5%) sendo apenas menos frequente que *S. epidermidis* (41,1%). Também foi encontrada uma alta taxa de *S. aureus* (16,2%) em amostras não hospitalares (ambulatórios e centros de saúde).

Staphylococcus saprophyticus é o segundo agente etiológico mais frequente de ITU na comunidade e raramente é isolado de pacientes hospitalizados ou como contaminantes de cultura de urina (67). Embora em nosso estudo foram isolados 3 *S. saprophyticus* de pacientes internados e com infecção urinária, com duas pacientes internadas na enfermaria da obstetrícia, e uma na enfermaria da urologia do HC da FMB, após análise dos prontuários desses pacientes, as infecções não foram consideradas nosocomiais, devido a não estarem relacionadas a procedimentos hospitalares, e a manifestação clínica de infecção ter iniciado antes de 72 horas após a admissão.

Avaliando a idade e sexo dos pacientes com ITU por *S. saprophyticus*, 74 (73,2%) eram do sexo feminino, sendo que 56 (55,4%) das mulheres tinham entre 15 e 44 anos de idade. Braoios *et al*. (68) relataram em seu estudo que a maioria dos pacientes com ITU pertencia ao sexo feminino (69,1%) e tinham entre 20 a 49 anos (52,9%), deste modo nossos dados corroborram com os achados desses autores, demonstrando que, na vida adulta, a incidência de ITU por *S. saprophyticus* aumenta e ocorre um

predomínio no sexo feminino, com picos de maior acometimento no início da atividade sexual ou relacionado a esta.

ANEXO 1

CUNHA MLRS, LOPES CAM, RUGOLO LMSS, CHALITA LVS. Significância clínica de estafilococos coagulase-negativa isolados de recém-nascidos. J Pediatr (Rio J). 2002; 78(4): 279-88.

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ARTIGO ORIGINAL

Significância clínica de estafilococos coagulase-negativa isolados de recém-nascidos

Clinical significance of coagulase-negative staphylococci isolated from neonates

Maria de Lourdes R.S. Cunha[1], Carlos A.M. Lopes[2], Ligia M.S.S. Rugolo[3], Liciana V.A.S. Chalita[4]

Resumo

Objetivo: avaliar a significância clínica de estafilococos coagulase-negativa (ECN) isolados de processos infecciosos em recém-nascidos da unidade neonatal do Hospital das Clínicas da Faculdade de Medicina de Botucatu.

Método: as linhagens de ECN isoladas foram identificadas e classificadas em significativas e contaminantes, com base em uma série de dados clínicos e laboratoriais obtidos dos prontuários dos pacientes internados na unidade neonatal. Foram pesquisados os dados referentes a fatores perinatais de risco para infecção, evolução clínica, alterações do hemograma e/ou positividade de proteína C-reativa e antibioticoterapia.

Resultados: das 117 linhagens de ECN isoladas, 60 (51,3%) foram classificadas como significativas, e 57 (48,7%) como contaminantes. Das 54 crianças com infecção por ECN, 43 (79,6%) eram prematuras, e 27 (50,0%) tiveram peso ao nascimento < 1.500g. A maioria das crianças com infecção por ECN estava submetida a dois ou mais procedimentos invasivos (77,8%), incluindo o uso de cateter (88,9%), nutrição parenteral (64,8%) e ventilação mecânica (61,1%). O *S. epidermidis* foi a espécie mais freqüentemente isolada (77,8%), e mais associada com infecção (86,7%) do que com contaminação (68,4%). Outras espécies de ECN, incluindo duas linhagens de *S. haemolyticus*, três linhagens de *S. lugdunensis*, uma linhagem de *S. simulans*, uma de *S. warneri* e uma linhagem de *S. xylosus* também foram isoladas de crianças com evidência clínica de pneumonia, enterocolite necrosante e sepse.

Conclusão: a maioria dos recém-nascidos com infecção por ECN apresentou fatores predisponentes importantes para a instalação do processo infeccioso, incluindo o peso de nascimento <1.500g, a não remoção de corpo estranho e a antibioticoterapia prévia. A identificação de espécies de ECN constitui um marcador útil de infecção, visto que o *S. epidermidis* foi o agente etiológico mais freqüentemente associado aos processos infecciosos.

J Pediatr (Rio J) 2002; 78 (4): 279-88: recém-nascido, infecção, estafilococos coagulase-negativa, fatores de risco.

Abstract

Objective: to evaluate the clinical significance of coagulase-negative staphylococci (CNS) isolated from newborns' infections at Neonatal Unit of Hospital das Clínicas da Faculdade de Medicina de Botucatu.

Methods: the CNS strains isolated were identified and classified as clinically significant and contaminant, based on a series of clinical and laboratory data obtained from patients who stayed in the Neonatal Unit. The following data were analyzed: risk factors for infections, clinical evolution, abnormal blood cell counts and/or C-reactive protein e antibiotic therapy.

Results: among the 117 CNS strains isolated, 60 (51.3%) were classified as significant and 57 (48.7%) as contaminant. Among the 54 infants infected by CNS, 43 (79.6%) presented very low birthweight (< 1,500g). Most of the infants infected by CNS were submitted to two or more invasive procedures (77.8%), including use of catheter (88.9%), parenteral nutrition (64.8%) and mechanical ventilation (61.1%). *Staphylococcus epidermidis* was the most frequently isolated species (77.8%) and more often associated with infection (86.7%) than with contamination (68.4%). Other species of CNS, including two strains of *S. haemolyticus*, three strains of *S. lugdunensis*, one strain of *S. simulans*, one strain of *S. warneri* and one strain of *S. xylosus* were also isolated from infants with clinical evidence of pneumonia, necrotizing enterocolitis and sepsis.

Conclusions: most newborns infected by CNS presented important risk factors for infection onset, including birthweight < 1,500g, foreign body presence and previous use of antibiotics. The identification of CNS species constitutes a useful marker of infection, since *S. epidermidis* was the species more frequently associated with infection.

J Pediatr (Rio J) 2002; 78 (4): 279-88: neonates, infection, coagulase-negative staphylococci, risk factors.

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Introdução

No gênero *Staphylococcus*, a espécie *Staphylococcus aureus*, coagulase-positiva e produtora de uma série de outras enzimas e toxinas, é a mais conhecida, e freqüentemente implicada na etiologia de uma série de infecções e intoxicações no homem e nos animais, enquanto que os estafilococos coagulase-negativa (ECN) têm sido considerados saprófitas ou raramente patogênicos[1].

Contudo, durante a última década, considerável progresso na classificação sistemática dos estafilococos e no desenvolvimento de métodos para a identificação do gênero, espécies e subespécies, tem permitido aos clínicos se inteirarem da variedade de ECN presentes em amostras clínicas e, assim, os considerarem como agentes etiológicos de uma série de processos infecciosos[2]. Atualmente, são reconhecidos como microrganismos essencialmente oportunistas, que se prevalecem de inúmeras situações orgânicas para produzir graves infecções[3].

Esses microrganismos apresentam elevado risco potencial de bacteremia nosocomial entre recém-nascidos de baixo peso, os quais são imunologicamente imaturos, e freqüentemente requerem procedimentos invasivos para administração de substâncias nutritivas e medicamentosas[4,5]. O aumento da incidência de bacteremia nosocomial por ECN em neonatos, nos últimos 20 anos, tem sido também associado ao da sobrevivência de crianças prematuras com peso menor que 1.500g ao nascimento e à sua longa permanência no ambiente hospitalar[6,7].

Contudo, a interpretação de hemoculturas positivas para os ECN é particularmente difícil devido ao fato desses microrganismos colonizarem a pele e as membranas mucosas como comensais, e poder contaminar as hemoculturas durante a coleta de sangue[2]. A esse respeito, investigadores têm usado uma variedade de critérios clínicos e laboratoriais para distinguir contaminação de bacteremia. Assim, o diagnóstico de bacteremia tem sido feito com base nos dados clínicos dos pacientes e no isolamento de microrganismos idênticos em duas ou mais hemoculturas. As culturas em que ocorre o crescimento de múltiplas linhagens ou de espécies de ECN, em associação às outras espécies de microrganismos, são classificadas como contaminantes[2]. Entretanto, como o volume sangüíneo é pequeno em recém-nascidos prematuros com baixo peso, somente uma hemocultura é geralmente realizada para se evitar a necessidade e os riscos de transfusões devido a venopunções constantes[2]. Assim, os neonatologistas têm-se apoiado em critérios clínicos e laboratoriais, tais como letargia, intolerância alimentar, distensão abdominal, deterioração da função respiratória, instabilidade da temperatura corpórea, fatores de risco perinatais e dados hematológicos, dentre outros[8,9].

Os ECN podem ser facilmente diferenciados em espécies através de suas características bioquímicas[1], contudo, na maioria dos laboratórios de microbiologia clínica, tal identificação não é feita de rotina. Há divergências na literatura específica sobre o significado clínico da identificação de ECN; de acordo com os dados de alguns autores[10], esse procedimento não é clinicamente significativo, embora outros pesquisadores[11] acreditem que essa identificação seja importante na diferenciação entre contaminação e infecção. Segundo Archer[12], a identificação dos ECN é de grande importância para a associação de certas espécies com infecções específicas, tendo em vista que alguns dados sugerem que além de *S. epidermidis* e *S. saprophyticus*, que têm sido considerados patogênicos, algumas espécies como o *S. haemolyticus*, *S. lugdunensis* e o *S. schleiferi* estão mais associados às infecções do que outras espécies[13-15].

Embora a capacidade dos ECN de causar infecções seja bem documentada, esses microrganismos têm sido em muitos casos negligenciados quanto à sua importância clínica. Assim, decidimos avaliar essa questão em nosso âmbito, considerando como objetivos principais a avaliação da significância clínica das espécies e linhagens de ECN isoladas de processos infecciosos de recém-nascidos da unidade neonatal do Hospital das Clínicas da Faculdade de Medicina de Botucatu, UNESP.

Materiais e métodos

Linhagens

Neste estudo retrospectivo, foram estudadas 117 linhagens de ECN, isoladas de materiais clínicos provenientes de 107 recém-nascidos internados na unidade neonatal do Hospital das Clínicas da Faculdade de Medicina de Botucatu, no período de 1990 a 1996. Os procedimentos foram aprovados pelo Comitê de Ética em Pesquisa da Faculdade de Medicina.

Critérios de inclusão

Foram incluídas no estudo, linhagens de ECN isoladas de fluidos internos, incluindo sangue, urina e secreções, bem como de corpos estranhos, tais como cânulas, drenos e cateteres.

Nos casos de material biológico em que se encontrou uma microbiota normal, e os ECN estiveram em cultura associativa com outras bactérias, exigiu-se sempre sua nítida prevalência numérica.

Critérios de exclusão

Foram excluídas do estudo linhagens isoladas de recém-nascidos, cujo registro de dados clínicos e laboratoriais referentes a um período de uma semana anterior e posterior ao isolamento de ECN não foi localizado.

Identificação de estafilococos coagulase-negativa

Os isolados, obtidos a partir de espécimes clínicos, foram semeados em ágar sangue e corados pelo método de Gram, objetivando-se sua pureza e a observação de sua morfologia e coloração específica. Após a confirmação dessas características, as linhagens foram submetidas às provas de catalase e coagulase. O gênero *Staphylococcus*

foi diferenciado de *Micrococcus*, com base na prova de oxidação e fermentação da glicose[16] e pela resistência à bacitracina (0,04 U), indicada pela ausência de halo de inibição, ou formação de halo de até 9 mm, e pela sensibilidade à furazolidona (100 µg), caracterizada por halos de inibição de 15 a 35 mm de diâmetro[17].

Para a identificação dos estafilococos coagulase-negativa, foram seguidos os critérios propostos por Kloos e Schleifer[1], e Kloos e Bannerman[3], conforme esquema simplificado de provas bioquímicas, o qual estabelece a realização de testes de utilização de açúcares: xilose, arabinose, sacarose, trealose, manitol, maltose, lactose, xylitol, ribose e frutose, bem como da caracterização de hemolisinas, redução de nitrato, urease, ornitina decarboxilase e de resistência à novobiocina.

Relevância clínica

Os dados clínicos que apoiaram a definição de significância clínica foram obtidos a partir da análise de prontuários dos pacientes. Foram valorizados os dados referentes a fatores perinatais de risco para infecção, como ruptura prolongada de membrana (> 24 horas); idade gestacional (IG); peso ao nascimento; procedimentos invasivos, como a cateterização arterial ou venosa umbilical, cateterização venosa central ou periférica, intubação endotraqueal (ventilação mecânica), procedimentos cirúrgicos, diálise peritoneal, nutrição parenteral, drenagem de tórax e derivação ventrículo-peritoneal. Além disso, foi considerado se houve ou não a remoção de corpo estranho durante o episódio de infecção por ECN.

Foi analisada a evolução clínica dos recém-nascidos na semana que antecedeu e na semana que sucedeu o isolamento do ECN, valorizando-se os diagnósticos e o quadro clínico sugestivo de infecção por ECN, que se caracteriza por sinais e sintomas incidiosos e inespecíficos, incluindo, mais freqüentemente, o comprometimento do estado geral, a instabilidade térmica e a ocorrência de apnéias.

Associado à evolução clínica, foram valorizadas as alterações do hemograma e/ou positividade da proteína C-reativa (PCR) por ocasião do isolamento do agente. Os parâmetros hematológicos normais foram os propostos por Manroe et al.[18]

Quanto aos óbitos, estes foram atribuídos à infecção por ECN quando ocorreram nas primeiras 72 horas do isolamento deste agente, e à possível associação a ECN se ocorrido entre quatro e sete dias após o isolamento de ECN.

Outro aspecto investigado e que auxiliou na consideração de relevância clínica foi o uso de antibioticoterapia prévia, antibióticos adequados para ECN após o resultado do diagnóstico bacteriológico, bem como o uso de antibióticos específicos, ou seja, a vancomicina, a oxacilina ou a teicoplanina. A determinação de antibioticoterapia adequada foi sempre baseada nos resultados do antibiograma arquivado nos prontuários ou realizado por ocasião do estudo.

As linhagens de ECN incluídas no estudo foram classificadas em significativas e contaminantes, conforme o critério do CDC[19] modificado:

- ***significativas***: linhagens isoladas de recém-nascidos que apresentaram três ou mais dos seguintes critérios: fatores de risco para infecção, quadro clínico, alteração hematológica e antibioticoterapia adequada. Também foram consideradas significativas as linhagens isoladas de pacientes que apresentaram apenas dois dos critérios e não receberam antibioticoterapia adequada, mas foram a óbito;
- ***contaminantes***: linhagens isoladas de recém-nascidos que apresentaram somente fatores de risco para infecção e/ou apenas um dos demais critérios (quadro clínico ou alteração hematológica ou antibioticoterapia adequada). As linhagens isoladas de recém-nascidos que apresentaram três critérios, mas que tiveram evolução satisfatória do quadro infeccioso sem a administração de antibióticos adequados, também foram consideradas contaminantes. O isolamento de outro agente etiológico de fluidos internos e corpos estranhos, na mesma época do isolamento de ECN, também foi usado como critério para a classificação de contaminação.

Análise estatística

Os dados relativos à relevância clínica de linhagens de ECN foram analisadas pelo teste de qui-quadrado.

A prova não-paramétrica de Mann Whitney foi usada para análise do peso de nascimento e da idade dos recém-nascidos.

Os dados que na análise univariada apresentaram um valor de $p < 0,25$ foram posteriormente submetidos à análise multivariada, utilizando o modelo de regressão logística[20], com a finalidade de avaliar simultaneamente a influência das diversas variáveis na incidência de infecção por ECN.

O nível de significância para todos os testes foi fixado em $p < 0,05$[21].

Resultados

Linhagens

Um total de 117 linhagens de ECN foram isoladas de diferentes materiais clínicos provenientes de 107 recém-nascidos. A amostragem em referência constituiu-se de 60 isolados a partir de hemoculturas, 41 de corpos estranhos (30 de ponta de cateter, 10 de ponta de cânula, um de ponta de dreno torácico), 13 de secreções (dois de secreção de dreno, cinco de secreção gástrica, seis de secreção traqueal) e três de urina.

Identificação de estafilococos coagulase-negativa

Na Figura 1, acham-se expostos os resultados da distribuição das amostras de ECN isoladas, segundo a espécie. O

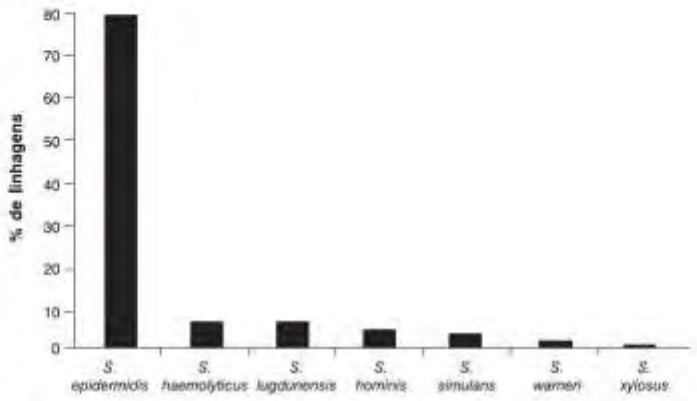

Figura 1 - Distribuição das espécies de ECN isoladas de recém-nascidos

S. epidermidis foi a espécie mais freqüentemente isolada, constituindo 77,8% da população estudada.

As Figuras 2 e 3 apresentam a distribuição das espécies de ECN classificadas como significativas e contaminantes, respectivamente. Das 117 linhagens incluídas no estudo, 60 (51,3%) foram classificadas como clinicamente significativas, e 57 (48,7%) como contaminantes.

Os resultados mostraram uma freqüência maior de *S. epidermidis* associado com infecção (86,7%) do que com contaminação (68,4%) ($p<0,05$). A análise estatística da distribuição das outras espécies não mostrou diferença significativa.

Dos 54 recém-nascidos com infecção por ECN, o *S. epidermidis* foi o agente etiológico isolado de 46 crianças (85,2%). O *S. haemolyticus* foi responsável pelo quadro infeccioso apresentado por duas crianças (3,7%), o *S. lugdunensis* por três (5,6%), e o *S. simulans* (1,8%), o *S. warneri* (1,8%) e o *S. xylosus* (1,8%) por um.

Relevância clínica

Dos 107 recém-nascidos, 54 foram considerados com infecção por ECN, e 53 sem infecção. Das 60 linhagens isoladas a partir de hemoculturas e analisadas quanto à significância clínica, 35 (58,3%) foram interpretadas como significativas, e 25 (41,7%) como contaminantes (Figuras 2 e 3). Das 41 linhagens isoladas de corpos estranhos, 21 (51,2%) foram interpretadas como significativas, sendo 14 isoladas de ponta de catéter (66,7%), seis de cânula (28,6%) e uma de ponta de dreno torácico (4,7%) (Figura 2). Das 13 linhagens isoladas de secreções, quatro (30,8%) foram consideradas significativas, sendo uma de secreção de dreno torácico, e três de secreção traqueal. As linhagens interpretadas como clinicamente significativas foram isoladas em maior proporção a partir do sangue do que de secreções ($p< 0,05$), porém sem diferença estatisticamente significativa quando comparado com corpo estranho. Por outro lado, as crianças classificadas como sem infecção apresentaram maior proporção de linhagens isoladas a partir de secreções do que de sangue ($p < 0,05$).

A distribuição percentual das crianças classificadas com infecção e sem infecção por ECN conforme a idade gestacional, peso ao nascimento e dados pessoais encontra-se na Tabela 1. Das 54 crianças com infecção por ECN, 43 (79,6%) eram prematuras, sendo 18 (33,3%) prematuras extremas (idade gestacional <31 semanas), comparado com 7 (13,2%) no grupo de crianças sem infecção. Entretanto, esta diferença no número de pacientes abaixo de 31 semanas de idade gestacional entre os dois grupos não foi estatisticamente significativa (Tabela 1). Nesta tabela, observa-se que 27 (50,0%) dos recém-nascidos com infecção por ECN apresentaram peso de nascimento <1.500g, com diferença significativa do grupo dos recém-nascidos sem infecção (20,8%). A mediana do peso ao nascimento também apresentou diferença estatisticamente significativa entre o grupo dos infectados por ECN (1.495g) e sem infecção (2.270g).

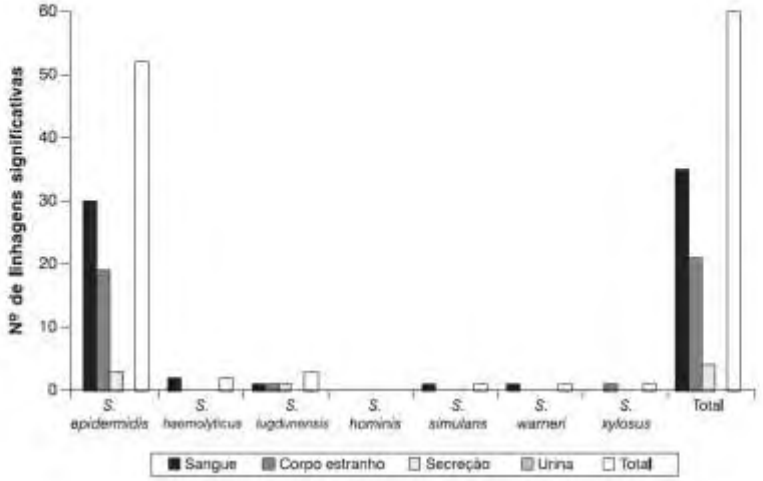

Figura 2 - Distribuição das espécies de ECN classificadas como clinicamente significativas, de acordo com o material clínico

A mediana da idade das crianças, por ocasião do isolamento de ECN, diferiu significantemente entre o grupo dos recém-nascidos com infecção (10 dias de vida) e crianças sem infecção (4 dias). Não houve diferença significativa quanto ao sexo e à procedência das crianças nos dois grupos.

A análise univariada dos dados clínicos dos pacientes, com relação aos fatores de risco perinatais para infecção, revelou diferença significativa entre o grupo das crianças com infecção e sem infecção, com respeito à internação em UTI, utilização de cateter, ventilação mecânica, nutrição parenteral, não remoção dos corpos estranhos e presença de dois ou mais corpos estranhos (Tabela 2).

Com referência aos dados hematológicos, foi observado uma maior proporção de recém-nascidos com neutrofilia

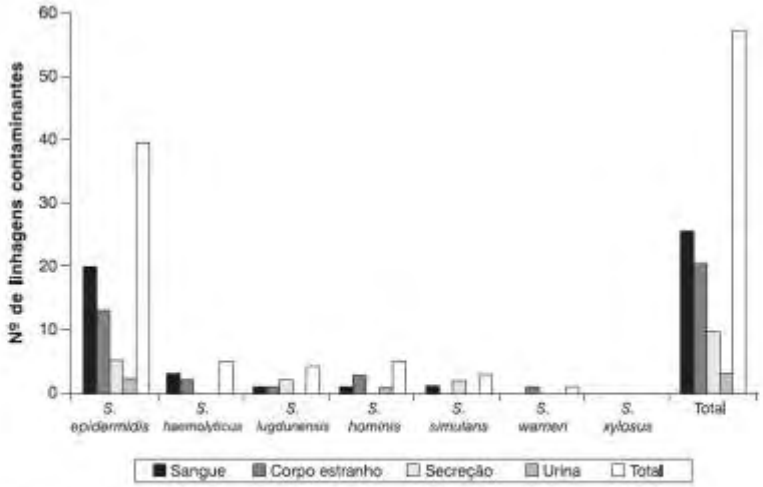

Figura 3 - Distribuição das espécies de ECN classificadas como contaminantes, de acordo com o material clínico

Tabela 1 - Dados pessoais dos recém-nascidos

Dados pessoais	Com infecção		Sem infecção		Total		Valor de p
	N	%	N	%	N	%	
IG <31	18	33,3	7	13,2	25	23,4	ns
IG 31-36	21	38,9	26	49,0	47	43,9	ns
IG 37	4	7,4	3	5,7	7	6,5	ns
IG ≥ 38	8	14,8	16	30,2	24	22,4	ns
PN < 1.500 g	27	50,0	11	20,8	39	36,4	0,0002
Mediana PN (g)	1.495		2.270		1.985		0,0011
Mediana idade (dias)	10		4		7		0,0042
Sexo M	24	44,4	27	50,9	51	47,7	ns
Nascido no HC/FMB	34	62,9	31	58,5	65	60,7	ns
Total	54	50,5	53	49,5	107	100	

IG: idade gestacional (semanas). Obs: 4 recém-nascidos com idade gestacional desconhecida.
ns: sem significância estatística (p>0,05). RN: recém-nascido, PN: peso ao nascimento, M: masculino, HC/FMB: Hospital das Clínicas da Faculdade de Medicina de Botucatu.

no grupo com infecção por ECN, quando comparados com o grupo sem infecção (Tabela 3).

A Tabela 4 apresenta os resultados da análise multivariada utilizando o modelo de regressão logística, apresentando como fatores de risco significativos na incidência de infeção por ECN, o muito baixo peso ao nascimento, a não remoção de corpo estranho e o uso de antibioticoterapia prévia. O cálculo do risco de ocorrência de infecção por ECN, sob a forma de razão de chances ou *Odds Ratios*, demonstrou que recém-nascidos com peso < 1.500g ao nascer apresentaram um risco de 5,98 vezes maior de infecção por ECN em relação ao recém-nascido com peso superior, enquanto a não remoção de corpo estranho elevou o risco de infecção em 4,40 vezes. Mostrou também que a antibioticoterapia prévia aumentou a chance de incidência de infecção em 5,38 vezes.

Durante a hospitalização, foi verificado um percentual de 37% de óbito no grupo de crianças com infecção por ECN, comparado com 17,0% no grupo dos recém-nascidos sem infecção. Entretanto, sete dos recém-nascidos que foram a óbito no primeiro grupo apresentaram infecção por gram-negativo, e quatro foram infectados com fungo, após

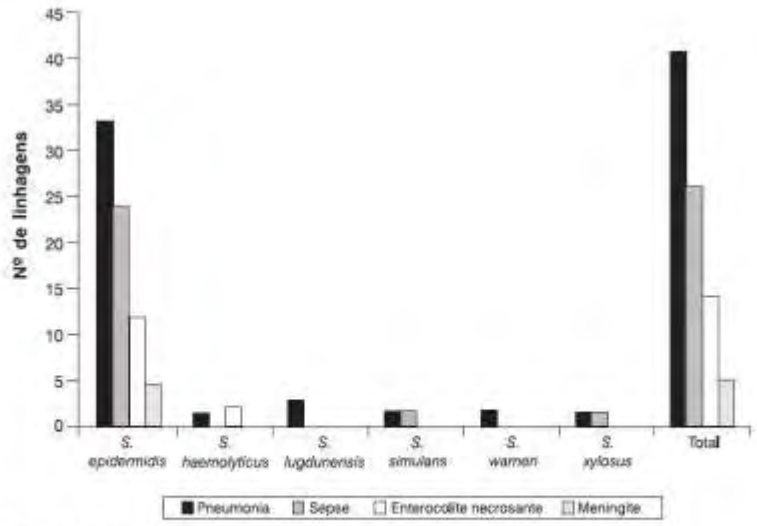

Figura 4 - Diagnóstico clínico dos recém-nascidos com infecção por ECN

Tabela 2 - Fatores perinatais de risco para infecção por ECN

Fatores de risco	Com infecção		Sem infecção		Total		Valor de p
	N	%	N	%	N	%	
Internação na UTI	53	98,1	33	62,3	86	80,4	< 0,0001
Cateter	48	88,9	33	62,3	81	75,7	0,0004
Complicação de cateter	4	7,4	1	1,9	5	4,7	ns
Dreno	10	18,5	5	9,4	15	14,0	ns
Ventilação mecânica	33	61,1	18	34,0	51	47,7	0,0138
Nutrição parenteral	35	64,8	22	41,5	57	53,3	0,0040
Derivação ventrículo-peritoneal	3	5,5	2	3,8	5	4,7	ns
Não remoção dos corpos estranhos	31	54,7	17	32,1	48	44,8	0,0010
Cirurgia	9	16,7	6	11,3	15	14,0	ns
Diálise	4	7,4	3	5,7	7	6,5	ns
Ruptura de membrana > 24 horas	18	33,3	17	32,1	35	32,7	ns
Dois ou mais corpos estranhos	42	77,8	20	37,7	62	57,9	0,0001
Total RN	54	50,5	53	49,5	107	100,0	

RN: recém-nascido, ns: sem significância estatística (p>0,05).

o episódio de infecção por ECN. Em adição, a mortalidade de dois recém-nascidos não foi associada com quadro infeccioso, e outros dois foram a óbito entre quatro a sete dias depois do isolamento de ECN. Sendo assim, os ECN contribuíram para a mortalidade de sete recém-nascidos (13,0%). Desses sete recém-nascidos, cinco estavam submetidos a corpos estranhos infectados com ECN, que foram removidos na data do óbito, e quatro crianças eram prematuras extremas com peso < 1.000g.

A Figura 4 apresenta o diagnóstico clínico apresentado pelas crianças na semana que antecedeu e na semana que sucedeu o isolamento de ECN. Embora os 54 recém-nascidos considerados com infecção por ECN tenham apresentado manifestações clínicas de sepse, a confirmação laboratorial foi observada em 26 (48,1%) dos recém-nascidos. Foram verificados 41(75,9%) episódios de infecção pulmonar confirmados radiologicamente, 14 (25,0%) de manifestações clínicas e radiológicas de enterocolite necrosante (NEC), e 5 (9,2%) de meningite. O *S. epidermidis* foi o agente etiológico predominante nesses processos infecciosos. Em adição, o *S. haemolyticus* foi associado com um episódio de pneumonia e dois de NEC, enquanto o *S. lugdunensis* foi o agente de três episódios de pneumonia, o *S. simulans* de um caso de pneumonia e um de sepse, o *S. warneri* de um caso de pneumonia, e o *S. xylosus* associado com um episódio de pneumonia e um de sepse.

Discussão

Nossas observações em 117 linhagens indicaram o *S. epidermidis* como a espécie mais freqüentemente isolada (77,8%), fato que corresponde ao relato de outros autores[5,8,22]. De acordo com os dados de Oren e Merzbach[10], a identificação de diferentes espécies de ECN não é clínica-

Tabela 3 - Dados laboratoriais dos recém-nascidos

Dados laboratoriais	Com infecção		Sem infecção		Total		Valor de p
	N	%	N	%	N	%	
Leucocitose	14	25,9	7	13,2	21	19,6	ns
Leucopenia	12	22,2	6	11,3	18	16,8	ns
Neutrofilia	24	44,4	9	17,0	33	30,8	0,0014
Desvio à esquerda	29	53,7	21	39,6	50	46,7	ns
Plaquetopenia	4	7,4	2	3,8	6	5,6	ns
PCR positiva	27	50,0	25	47,2	52	48,6	ns
Total RN	54	50,5	53	49,5	107	100,0	

PCR: proteína C-reativa, RN: recém-nascido, ns: sem significância estatística (p>0,05).

Tabela 4 - Ajuste do modelo de regressão logística

Dados dos recém-nascidos	Valor de p	*Odds Ratio*	Intervalo de confiança (95%)	
			LI	LS
Prematuridade	0,5409	1,669	0,323	8,620
Peso < 1.500 g	0,0085*	5,988	1,579	22,727
Internação na UTI	0,0666	12,04	0,843	166,66
Cateter	0,4630	2,159	0,276	16,854
Complicação de cateter	0,3393	6,535	0,138	333,33
Ventilação mecânica	0,1044	3,386	0,777	14,756
Nutrição parenteral	0,6691	1,475	0,248	8,752
Não remoção de corpos estranhos	0,0257*	4,405	1,197	16,393
Dois ou mais corpos estranhos	0,1612	5,524	0,506	58,823
Leucocitose	0,5396	1,650	0,333	8,182
Leucopenia	0,4019	2,020	0,389	10,526
Neutrofilia	0,0700	3,412	0,904	12,987
Desvio à esquerda	0,3316	1,926	0,513	7,246
Antibioticoterapia prévia	0,0096*	5,376	1,508	19,230

RN: recém-nascido * Significativo ao nível de 5% de significância.

mente significante. Entretanto, nossos resultados revelaram que as linhagens de *S. epidermidis* isoladas foram significativamente mais associadas com infecção (86,7%) do que com contaminação (68,4%). Silbert et al.[23], em estudo para determinar a prevalência de infecção *versus* contaminação em pacientes com menos de 60 dias de vida, com hemoculturas positivas para ECN, encontrou 91,1% dos isolados pertencentes à espécie *S. epidermidis*, 3 (6,7%) à *S. hominis* e 1 (2,2%) à *S. warneri*, sendo que no grupo de pacientes infectados, somente *S. epidermidis* foi encontrado. Esses resultados confirmam os achados de Lowy e Hammer[11], que acreditam na importância da identificação das espécies de ECN para diferenciação entre contaminação e infecção.

Embora o *S. epidermidis* seja a espécie mais caracterizada e envolvida etiologicamente, outras espécies de ECN patogênicas têm sido isoladas de uma variedade de fontes clínicas[2]. Em nosso estudo, outras espécies foram associadas com infecção, incluindo-se *S. haemolyticus*, *S. lugdunensis*, *S. simulans*, *S. warneri* e *S. xylosus*. Hall et al. [8] também isolaram linhagens de *S. haemolyticus* e *S. simulans* de crianças com evidência clínica e laboratorial de sepse, bem como de pneumonia.

Em nosso estudo, dos 54 recém-nascidos com infecção por ECN, a maioria (79,6%) era de prematuros, com 33,3% deles apresentando prematuridade extrema (IG < 31 semanas), e 50,0% com peso ao nascimento < 1.500 g, com mediana do peso igual a 1.495 g. Do ponto de vista comparativo, resultados similares têm sido obtidos por outros investigadores[4,8,24].

Vários são os fatores que contribuem para que o prematuro, especialmente o de muito baixo peso, seja mais susceptível à infecção. Colabora para a maior gravidade dos quadros infecciosos, a imaturidade do sistema imunológico, que se traduz na deficiência da fagocitose, opsonização por anticorpos e funções do complemento[25].

A mediana da idade dos 54 recém-nascidos com infecção por ECN foi de 10 dias, diferindo significativamente do grupo sem infecção (quatro dias). Esses dados denotam a característica peculiar desses microrganismos, ou seja, seu envolvimento, na maioria das vezes, na etiologia de quadros clínicos tardios de infecção hospitalar. Somente duas (4,4%) das crianças com infecção por ECN apresentaram idade inferior a dois dias de vida, e destas, uma apresentou ruptura de membrana > que 24 horas, como fator de risco. Resultados similares foram obtidos por Hensey et al.[26], que relataram a ocorrência de somente 12% das infecções por ECN nas primeiras 48 horas de vida, enquanto todas as infecções por *Streptococcus* do Grupo B, *Streptococcus viridans* e *Haemophilus influenzae* ocorreram nesse período e associadas com a rotura prolongada de membrana.

Dentre os fatores de risco para infecção, o uso de cateter foi significativamente mais freqüente nos recém-nascidos com infecção por ECN, e esse achado é concordante com os dados de outros pesquisadores que alertam para a importância desse procedimento[27]. Os cateteres vasculares são fontes de infecção, por serem facilmente colonizados por microrganismos da pele presentes ao longo do local de inserção, especialmente pelos ECN, que são os microrganismos prevalentes como flora normal da pele e mucosas.

No presente estudo, o uso de nutrição parenteral foi mais freqüente nos recém-nascidos com infecção. A importância da nutrição parenteral como fator de risco decorre não só da necessidade de utilização de cateteres vasculares

e da manipulação dos mesmos, mas também de sua composição. A administração de nutrição parenteral, e especialmente de lipídeos, através desses cateteres, pode servir como um meio de cultura que favorece rápida proliferação bacteriana[4].

A freqüência da intubação orotraqueal também foi significativamente maior no grupo dos recém-nascidos com infecção por ECN, semelhante ao observado por Stoll et al.[24] A presença da cânula na traquéia atua como corpo estranho, comprometendo a integridade das barreiras mucosas e propiciando a colonização por microrganismos.

Adicionalmente, a presença de dois ou mais corpos estranhos e sua não remoção foi significativamente mais elevada nos recém-nascidos com infecção, fato que vários autores têm alertado, e ressaltado a importância de sua rápida remoção, além da terapia com antimicrobianos[28]. Essa condição, quando não controlada, pode ser agravada pela produção de um exopolissacarídeo por alguns ECN associados ao processo infeccioso, que favorece a permanência desses microrganismos no corpo estranho pela interferência com a resposta imune do hospedeiro e redução da atividade de antimicrobianos[28]. Nesse sentido, nosso estudo confirma essa observação pelo fato de que três recém-nascidos com corpos estranhos colonizados com ECN foram a óbito, apesar da antibioticoterapia adequada.

Na avaliação do hemograma, foi observada uma freqüência significativamente maior de neutrofilia no grupo de crianças com infecção, quando comparado com o grupo dos recém-nascidos sem infecção. A proteína C-reativa (PCR) tem sido útil no diagnóstico e no acompanhamento da resposta à terapêutica dessas infecções. Em nosso estudo, não houve diferença significativa entre os recém-nascidos com infecção e os sem infecção. Essa condição pode ser explicada pelo fato de que o grupo de crianças sem infecção incluiu vários recém-nascidos com infecção por outros microrganismos que não os ECN.

Resultados obtidos por Schmidt et al.[29] mostraram que 64% das crianças com infecção por ECN apresentaram PCR positiva, e apenas 20% e 8% das crianças apresentaram a proteína nos grupos dos suspeitos e controle (sem infecção), respectivamente. Entretanto, 88% das crianças com sepse por outros patógenos também apresentaram resultado positivo.

Outro achado coerente com os dados da literatura[27] foi o uso de antibioticoterapia prévia nos recém-nascidos com infecção por ECN. O uso prévio de antibióticos pode suprimir a flora normal e selecionar microrganismos resistentes, aumentando não apenas o risco de infecção, mas também sua gravidade e a dificuldade de seu tratamento.

Os resultados obtidos na análise de regressão logística apontaram como fatores significativamente predisponentes para a infecção por ECN o peso de nascimento < 1.500g (OR= 5,98), a não remoção de corpo estranho (OR=4,40), e a antibioticoterapia prévia (OR=5,38), indicando um risco 4 a 5 vezes maior para a incidência de infecção por ECN, e a importância da valorização desses germes como agentes etiológicos significativos quando isolados de RN nessas condições.

A mortalidade dos recém-nascidos classificados com infecção por ECN em nosso estudo foi de 37,0%, e os ECN estiveram associados ao óbito de 13,0% dos recém-nascidos; em 3,7% houve possível associação com a infecção causada por ECN. A mortalidade associada a ECN aqui evidenciada é semelhante à encontrada por outros pesquisadores (7 a 14,3%) em países desenvolvidos[8]. A mortalidade durante a hospitalização (37,0%) foi elevada, porém compatível com a casuística estudada e com as cifras relatadas por outros autores[30]. Vários fatores interferem na mortalidade, alguns inerentes à população, como a limitada resposta imune do prematuro de baixo peso, que pode ser agravada por uma patologia prévia associada às dificuldades no diagnóstico precoce de infecção e à não remoção de corpos estranhos colonizados com esses microrganismos.

Nossos resultados mostraram uma freqüência de 26 episódios de sepse laboratorialmente confirmada, sendo que, em 15 (51,7%) desses episódios, os recém-nascidos tinham cateter ou cânula colonizados com esses microrganismos. Resultados similares foram verificados por Noel e Edelson[31] na UTI neonatal do Hospital de Nova Iorque, onde foi constatado que em 23 episódios envolvendo 38 pacientes, 57% destes ocorreram em crianças com cateteres vasculares contaminados ou suspeitos de contaminação.

A ocorrência de sepse associada a enterocolite necrosante (NEC) causada por ECN também tem sido descrita[31], e demonstra que esses microrganismos podem inicialmente colonizar o trato gastrintestinal através da intubação endotraqueal ou nasogástrica, e secundariamente causar sepse quando a integridade da barreira intestinal é comprometida pela isquemia[24]. Das 54 crianças com infecção por ECN, 14 (25,9%) apresentaram episódios de NEC, sendo seis (42,8%) desses, associados com sepse laboratorialmente confirmada. Em adição, quatro desses recém-nascidos com NEC e sepse estavam submetidos ao uso de cateter ou cânula, semelhante aos resultados relatados por Noel e Edelson[31].

Nossos resultados mostraram uma freqüência de 75,9% de infecções pulmonares associadas com ECN, superior à observada por outros autores[8]. Essa diferença pode ser devida a fatores próprios dos recém-nascidos prematuros que os predispõem às infecções pulmonares, freqüentemente por problemas respiratórios que prejudicam a oxigenação e trocas gasosas e pelo uso de ventilação mecânica, que lesa o órgão, tornando-o mais suscetível à infecção[24]. O *S. epidermidis* foi o agente etiológico associado com 82,9% dos episódios de pneumonia constatados, dados esses semelhantes aos encontrados por Hall et al.[8]

Em nosso estudo, das 117 linhagens de ECN isoladas, 60 foram consideradas de significância clínica, incluindo 35 isoladas a partir de hemoculturas, 21 de corpos estranhos e quatro de secreções, revelando diferença estatística em relação ao material biológico de origem, com uma maior frequência de linhagens clinicamente significativas isoladas de hemoculturas e de contaminantes isolados de secre-

ções. Entretanto, para as linhagens isoladas de corpo estranho, não foi verificada diferença estatisticamente significante.

Esses resultados demonstram que os ECN são importantes patógenos nosocomiais, e, portanto, quando do seu isolamento a partir de sangue e corpos estranhos de recém-nascidos prematuros de muito baixo peso (< 1.500g), não devem ser ignorados, e sim classificados como contaminantes, havendo a necessidade de um exame criterioso dos dados clínicos e laboratoriais do paciente para determinar a relevância clínica das linhagens isoladas.

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ANEXO 2

CUNHA MLRS, MONTELLI AC, FIORAVANTE AM, BATALHA JEN, CARAMORI JCT, BARRETTI P. Predictive factors of outcome following staphylococcal peritonitis in continuous ambulatory peritoneal dialysis. Clin Nephrol. 2005; 64(5): 378-82.

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Predictive factors of outcome following staphylococcal peritonitis in continuous ambulatory peritoneal dialysis

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Key words
peritonitis – staphylococci – CAPD – oxacillin resistance – predictive factors

Abstract. Background and aims: Staphylococcus epidermidis and other coagulase-negative staphylococci (CoNS) are the most common agents of continuous ambulatory peritoneal dialysis (CAPD) peritonitis. Episodes caused by Staphylococcus aureus evolve with a high method failure rate while CoNS peritonitis is generally benign. The purpose of this study was to compare episodes of peritonitis caused by CoNS species and S. aureus to evaluate the microbiological and host factors that affect outcome. Material and methods: Microbiological and clinical data were retrospectively studied from 86 new episodes of peritonitis caused by staphylococci species between January 1996 and December 2000 in a university dialysis center. The influence of microbiological and host factors (age, sex, diabetes, use of vancomycin, exchange system and treatment time on CAPD) was analyzed by logistic regression model. The clinical outcome was classified into two results (resolution and non-resolution). Results: The odds of peritonitis resolution were not influenced by host factors. Oxacillin susceptibility was present in 30 of 35 S. aureus lineages and 22 of 51 CoNS ($p = 0.001$). There were 32 of 52 (61.5%) episodes caused by oxacillin-susceptible and 20 of 34 (58.8%) by oxacillin-resistant lineages resolved ($p = 0.9713$). Of the 35 cases caused by S. aureus, 17 (48.6%) resolved and among 51 CoNS episodes 40 (78.4%) resolved. Resolution odds were 7.1 times higher for S. epidermidis than S. aureus ($p = 0.0278$), while other CoNS had 7.6 times higher odds resolution than S. epidermidis cases ($p = 0.052$). Episodes caused by S. haemolyticus had similar resolution odds to S. epidermidis ($p = 0.859$). Conclusions: S. aureus etiology is an independent factor associated with peritonitis non-resolution in CAPD, while S. epidermidis and S. haemolyticus have a lower resolution rate than other CoNS. Possibly the aggressive nature of these agents, particularly S. aureus, can be explained by their recognized pathogenic factors, more than antibiotic resistance.

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Introduction

In spite of technological advances and significant reductions in peritonitis, it is still the most important complication in continuous ambulatory peritoneal dialysis (CAPD). It is also the most frequent cause of therapy failure [Kavanagh et al. 2004] and strongly impacts patient's mortality [Fried et al. 1996].

Staphylococcus epidermidis and other coagulase-negative staphylococci (CoNS) are considered the most common etiological agents in CAPD peritonitis. They have been identified in approximately one third of all episodes worldwide [Kavanagh et al. 2004, Keane et al. 1996, Nakamoto et al. 2004].

CAPD peritonitis shows clinical improvement in the first 96 hours and resolves without complications in 54 – 65% of cases [Troidle et al. 1998]. However, clinical outcome is strongly influenced by the microbiological characteristics of the etiological agent. Episodes caused by Staphylococcus aureus and most cases caused by Gram-negative bacteria, particularly Pseudomonas aeruginosa, result in persistent infection and a high dialysis modality failure rate [Bunke et al. 1997, Szeto et al. 2001]. On the other hand, CoNS peritonitis is generally benign with few complications [Bunke et al. 1997].

However, there are cases of CoNS peritoneal infections with characteristics similar to S. aureus. Schnitzler et al. [1998] reported a severe case of peritonitis due to Staphylococcus lugdunensis, similar to S. aureus infections.

Gruer et al. [1984] studied 43 lineages of CoNS isolated during 41 CAPD episodes of peritonitis and observed that multiple bacterial resistances were common. Holley et al. [1990], comparing in vitro susceptibility of CoNS lineages isolated in 57 episodes between 1984 and 1986 and 32 diagnosed in 1987 and 1988, reported that methicillin-resistant lineage rates had increased from 5 to 28%.

Differences between species and drug susceptibility profiles can explain the different clinical outcome characteristics in infections caused by these microorganisms. The purpose of this study was to compare episodes of peritonitis caused by CoNS species and S. aureus to evaluate the microbiological and host factors that affect outcome. Literature is scarce on this type of analysis.

Material and methods

Microbiological and clinical data were retrospectively studied from all episodes of peritonitis caused by staphylococci species between January 1996 and December 2000 in a university dialysis center. We included only new episodes of peritonitis. These were the patients' first episodes or episodes diagnosed at least 14 days after completion of the last peritonitis treatment [Bunke et al. 1997, Peacock et al. 2000]. Cases associated with exit site or tunnel infections were excluded.

Peritonitis was defined as the presence of cloudy peritoneal fluid associated with one or more of the following symptoms: abdominal pain, fever, nausea, and vomiting. Occurrence of only one of these was considered peritonitis if the dialysate white cell count was greater than 100/µl, with at least 50% polymorph nuclear cells. Resolution was defined as disappearance of signs and symptoms within 96 hours of initiating antibiotic therapy, with negative results from peritoneal fluid culture at least 14 days after treatment completion. Relapse was defined as peritonitis recurrence with the same organism and susceptibility profile or no organism within 14 days of completing antibiotics [Bunke et al. 1997, Peacock et al. 2000]. Non-resolution was the generic term used for cases with initial non-resolution, relapse, peritoneal catheter removal or death.

For each case, information was collected on:

- episode: date, clinical findings, treatment, outcome (resolution, relapse, catheter removal or death),
- presence of diabetes mellitus,
- demographics data: age, sex, and race (Caucasian, non-Caucasian), treatment time on dialysis, and
- exchange system (standard or double bags).

All episodes were treated according to a local protocol adapted from the third report of The Ad Hoc Advisory Committee on Peritonitis Management [Keane et al. 1996] for staphylococcal episodes. All patients were treated within 24 hours of first clinical signs or symptoms. Antibiotic therapy was started with 500 or 750 mg/l i.p. cefazolin (patients with urinary volume more than 500 ml/24 h) and 250 mg/l i.p. amikacin as loading dose, followed by 500 or 750 mg/l cefazolin and 2 mg/kg amikacin each day in the last PD bag. As soon as culture results were available, therapy was evaluated. According to the National Committee for Clinical Laboratory Standards (NCCLS) [2002], for oxacillin-resistant S. aureus and CoNS, all penicillins, cephems (cephalosporins) and other β-lactams may appear active in vitro but are not effective clinically. Thus, for oxacillin-susceptible cocci the cefazolin was maintained, while for oxacillin-resistant cocci the cefazolin was replaced by 1 g/l i.p. vancomycin, repeated every five (in patients with urinary volume more than 500 ml/24 h) or seven days. In both situations, amikacin was discontinued. Vancomycin was also used for patients with no clinical improvement within the first 96 hours of antibiotic treatment, although microbiological tests revealed oxacillin susceptibility. Antibiotic therapy duration was 14 days for CoNS and 21 days for S. aureus episodes.

Microbiological methods

Microbiological materials were stored in a collection of cultures. Clinical specimen isolates were seeded on blood agar and Gram-stained to confirm purity and determine morphology and specific color. They were then submitted to catalase and coagulase tests. CoNS were identified by using the simplified biochemical test scheme proposed by Kloos and Schleifer [1975] and Kloos and Bannerman [1995].

Table 1. Breakpoints (mg/l) for susceptibility and resistance of Staphylococcus aureus and coagulase-negative staphylococci (CoNS) as per NCCLS [2002].

Antimicrobial agent	Interpretative standards	
	Susceptible	Resistant
Penicillin	≤ 0.12	≥ 0.25
Oxacillin for S. aureus	≤ 2.0	≥ 4.0
Oxacillin for CNS	≤ 0.25	≥ 0.5
Cephalothin	≤ 8.0	≥ 32
Levofloxacin	≤ 2.0	≥ 8.0

Table 2. Characteristics of 63 CAPD patients at first peritonitis episode by staphylococci.

Characteristic	Number (%)
Age (years)	
Birth – 20	4 (6.3)
21 – 40	12 (19.0)
41 – 59	22 (34.9)
60+	25 (39.7)
Sex (female)	43 (68.3)
Race (Caucasian)	39 (61.9)
Diabetics	28 (44.4)
Treatment time	
Less than 1 year	28 (44.4)
More than 1 year	35 (55.6)
Exchange system	
Standard	19 (30.2)
Double bag	44 (69.8)

Table 3. Causative microorganisms in 86 new episodes of peritonitis by S. aureus and coagulase-negative staphylococci (CoNS).

Microorganism	Lineages n (%)
S. aureus	35
CoNS	51 (100)
S. epidermidis	24 (47)
S. haemolyticus	11 (21.6)
S. warneri	5 (9.8)
S. hominis	5 (9.8)
S. xylosus	2 (3.9)
S. cohnii	2 (3.9)
S. simulans	1 (1.9)
S. lugdunensis	1 (1.9)

In vitro susceptibility was determined by minimal inhibited concentration (MIC) by the E-test (AB Biodisk, Solna, Sweden), a new quantitative method that uses a transparent strip of inert plastic with drug concentrations from 0.002 – 256 μg/ml. Sample susceptibility proportion to each drug was defined as per NCCLS breakpoints [2002] (Table 1). Samples with intermediate values were considered resistant.

Statistical analysis

In this observational study, several factors could have influenced the outcome: staphylococcus species, age, sex, race, presence of diabetes mellitus, exchange system, treatment time on CAPD, antibiotic susceptibility and use of vancomycin. The oxacillin susceptibility was not included in the model due to its association with the species (CoNS). Also, the susceptibility to cephalothin, penicillin, levofloxacin and ofloxacin was not included due to the association with oxacillin susceptibility. We, therefore, adopted a regression model that incorporates all factors, of interest and control, affecting infection outcome. Logistic regression model outcome was classified into two results, exhausted and mutually exclusive (resolution or non-resolution). The χ^2-test was used for frequency comparisons, $p < 0.05$ was considered statistically significant.

Results

There were 86 new peritonitis episodes diagnosed in 63 patients on regular CAPD. Demographic data at first episode are shown in Table 2. There were 35 episodes caused by S. aureus and 51 by CoNS. From CoNS, S. epidermidis was the most frequent species (24 cases, 47% of all CoNS episodes), followed by S. haemolyticus (11 cases, 21.6%). Other species were present in the remaining 16 cases (Table 3).

Oxacillin susceptibility was observed in 30 of 35 (85.7%) episodes due to S. aureus and in 22 of 51 (43.1%) due to CoNS ($p = 0.001$). Table 4 shows the antibiotic susceptibility profile in S. aureus, S. epidermidis, S. haemolyticus and other CoNS. The percentage of susceptible lineages was similar between CoNS species.

Overall, 57 episodes (66.3%) were resolved, 14 (16.3%) relapsed, 12 (13.9%) needed catheter removal and 3 (3.5%) died. Of the 35 S. aureus cases, 17 (48.6%) resolved, 8 (22.8%) relapsed, 7 (20%) needed catheter removal, and 3 (8.6%) resulted in death. For CoNS episodes, 40 (78.4%) resolved, 6 (11.8%) relapsed, and 5 (9.8%)

Table 4. Susceptibility profile for antibiotics in Staphylococci species isolated in 86 new episodes of peritonitis in CAPD.

Susceptible n (%)	S. aureus n = 35	S. epidermidis n = 24	S. haemolyticus n = 11	Other CoNS n = 16	p#
Oxacillin	30 (85.7)	10 (41.7)	6 (54.5)	6 (37.5)	0.001
Levofloxacin	31 (88.6)	20 (87.0)	9 (81.8)	13 (92.9)	0.892
Cephalothin	30 (85.7)	24 (100.0)	9 (81.8)	15 (93.7)	0.125
Penicillin	14 (40.0)	5 (20.8)	3 (27.3)	9 (56.2)	0.124

refers to comparison between S. aureus versus all CoNS together

Table 5. Comparison of odds by regression logistic analysis of resolution of peritonitis.

Factor	Log (Odds)	p	Odds ratio
Age (years) (birth \ to 20/60+)	0.5150	0.7450	
Age (years) (21 – 40/60+)	1.4076	0.1516	
Age (years) (41 – 59/60+)	0.7775	0.3701	
Sex (male/female)	0.2022	0.7921	
Race (Caucasian/non-Caucasian)	0.4956	0.5600	
Diabetes mellitus (no/yes)	–0.5555	0.3951	
System (standard/double bags)	0.5856	0.4861	
Treatment time on CAPD			
Less than one year	2.0445	0.2729	
More than one year	0.9547	0.2065	
Treatment with vancomycin (no/yes)	–0.2115	0.8839	
Etiological agent			
All CoNS/S. aureus	–2.2462	0.0051	9.4
S. epidermidis/S. aureus	–1.9629	0.0278	7.1
Other CoNS/S. epidermidis	2.0272	0.0526	
S. haemolyticus/S. epidermidis	0.1730	0.8592	

needed catheter removal. Of the 11 non-resolved episodes, 7 were due to S. epidermidis, 2 to S. haemolyticus and 2 to other CoNS. There were significantly more CoNS cases resolved than S. aureus episodes ($p < 0.001$).

There were 52 episodes involving oxacillin-susceptible lineages, of which 32 (61.5%) resolved, 10 (19.2%) relapsed and 10 (19.2%) had catheter losses; 34 infections were caused by oxacillin-resistant lineages, of which 20 (58.8%) resolved, 9 (26.5%) relapsed, and 5 (14.7%) needed catheter removal. The percentage of resolved cases was similar between susceptible and resistant oxacillin lineages ($p = 0.9713$); 2 of the 3 deaths associated with S. aureus were due to oxacillin-susceptible and one to oxacillin-resistant lineages.

Vancomycin was used in 56 episodes, 18 caused by S. aureus and 38 by CoNS. For S. aureus infections, vancomycin was prescribed for bacterial resistance in six cases, no improvement in clinical signs and symptoms in eight, and other unknown causes in two. For CoNS vancomycin was prescribed for bacterial resistance in 22 cases, no clinical improvement in 12, and other unknown causes in four. No cefazolin allergy was diagnosed. The time between diagnosis and the first vancomycin dose was 3.7 ± 1.7 days for S. aureus episodes and 3.9 ± 1.2 days for CoNS ($p = 0.793$).

Table 5 shows results adjusted to the regression model. Controlling for covariables, resolution odds were not influenced by host factors such as age, sex, diabetes, exchange system, treatment time on CAPD or vancomycin use.

Resolution odds were 7.1 times higher for S. epidermidis than for S. aureus ($p = 0.0278$), while other CoNS had similar odds resolution to S. epidermidis ($p = 0.0526$) and to S. haemolyticus cases ($p = 0.859$). For all CoNS episodes together, the resolution odds were 9.4 times higher than for S. aureus ($p = 0.0051$).

Discussion

The incidence of peritonitis due to CoNS has decreased significantly in recent years [Nakamoto et al. 2004]. However, CoNS still remains the most frequent etiologic group in CAPD peritonitis. In our study, S. epidermidis was the most frequently isolated lineage. This finding corresponds to other reports [Bunke et al. 1997, Kavanagh et al. 2004]. In addition to S. epidermidis, seven other CoNS species were identified, predominantly S.

haemolyticus (11 lineages). This result is important, since there is little literature on other CoNS, except when peritonitis is caused by uncommon agents.

The frequency of oxacillin-resistant agents was greater in CoNS than in S. aureus lineages. This agrees with other authors who have reported an increased resistance to methicillin in CoNS over the last two decades [Holley et al. 1990, Kim et al. 2004, Nakamoto et al. 2004].

Although some authors have shown the influence of demographic factors, it is only recently that the impact of clinical and laboratory parameters on peritonitis outcome was first analyzed. Krishnan et al. [2002] showed that CAPD duration and the number of days effluent cell count remained > 100/µl were the only factors that independently predicted episode outcome. Caucasians seemed to have a lower resolution rate than blacks, while other variables did not affect outcome.

In our study, variables such as age, race, sex, treatment time on CAPD, diabetes and exchange system did not influence clinical outcome. These results were similar to Krishnan et al. [2002] except for race and treatment time on dialysis. Troidle et al. [1999] also did not find differences in peritonitis outcome associated with dialysis therapy duration.

Peritonitis outcome from S. aureus was worse than from CoNS. Bunke et al. [1997] and Peacock et al. [2000] in prospective studies made similar observations. Factors related to species and antimicrobial resistance could explain this different outcome. Since oxacillin resistance rate was higher in CoNS than S. aureus, the contribution from drug resistance is inconsistent. Furthermore, there was no difference in episode resolution rate between oxacillin-susceptible and oxacillin-resistant lineages.

We can conclude that S. aureus etiology is an independent factor associated with peritonitis episode non-resolution in CAPD. Possibly, the aggressive nature of this agent can be explained by its recognized pathogenic factors, more than for antibiotic resistance.

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ANEXO 3

BARRETTI P, MONTELLI AC, BATALHA JEN, CARAMORI JCT, **CUNHA MLRS**. The role of virulence factors in the outcome of staphylococcal peritonitis in CAPD patients. BMC Infect Dis. 2009; 9: 212-9.

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The role of virulence factors in the outcome of staphylococcal peritonitis in CAPD patients

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Abstract

Background: Peritonitis continues to be the most frequent cause of peritoneal dialysis (PD) failure, with an important impact on patient mortality. Gram-positive cocci such as *Staphylococcus epidermidis*, other coagulase-negative staphylococci (CoNS), and *Staphylococcus aureus* are the most frequent etiological agents of PD-associated peritonitis worldwide. The objective of the present study was to compare peritonitis caused by *S. aureus* and CoNS and to evaluate the factors influencing outcome.

Methods: Records of 86 new episodes of staphylococcal peritonitis that occurred between 1996 and 2000 in the Dialysis unit of a single university hospital were studied (35 due to *S. aureus*, 24 to *S. epidermidis* and 27 to other CoNS). The production of slime, lipase, lecithinase, nuclease (DNAse), thermonuclease (TNAse), α- and β-hemolysin, enterotoxins (SEA, SEB, SEC, SED) and toxic shock syndrome toxin-1 (TSST-1) was studied in *S. aureus* and CoNS. Antimicrobial susceptibility was evaluated based on the minimal inhibitory concentration determined by the E-test. Outcome predictors were evaluated by two logistic regression models.

Results: The oxacillin susceptibility rate was 85.7% for *S. aureus*, 41.6% for *S. epidermidis*, and 51.8% for other CoNS ($p = 0.001$). Production of toxins and enzymes, except for enterotoxin A and α-hemolysin, was associated with *S. aureus* episodes ($p < 0.001$), whereas slime production was positive in 23.5% of CoNS and 8.6% of *S. aureus* strains ($p = 0.0047$). The first model did not include enzymes and toxins due to their association with *S. aureus*. The odds of resolution were 9.5 times higher for *S. epidermidis* than for *S. aureus* ($p = 0.02$) episodes, and were similar for *S. epidermidis* and other CoNS ($p = 0.8$). The resolution odds were 68 times higher for non-slime producers ($p = 0.001$) and were not influenced by oxacillin resistance among vancomycin-treated cases ($p = 0.89$). In the second model, the resolution rate was similar for *S. aureus* and *S. epidermidis* ($p = 0.70$), and slime ($p = 0.001$) and α-hemolysin ($p = 0.04$) production were independent predictors of non-resolution.

Conclusion: Bacterial species and virulence factors rather than antibiotic resistance influence the outcome of staphylococcal peritonitis.

Background

Peritonitis continues to be the most frequent cause of peritoneal dialysis (PD) failure [1], and has an important impact on patient mortality [2]. Gram-positive cocci such as *Staphylococcus epidermidis*, other coagulase-negative staphylococci (CoNS), and *Staphylococcus aureus* are the most frequent etiological agents of PD-associated peritonitis worldwide [3].

Previous studies comparing the outcome of peritonitis caused by *S. aureus* and CoNS have shown a lower resolution rate and a higher frequency of complications in the former [4-8]. Perez-Fontan et al [2] observed a mortality rate of 15.2% for *S. aureus* episodes and of only 0.5% for CoNS episodes. These findings are in contrast to the susceptibility profile observed for CoNS. According to Kim et al [9], the frequency of methicillin-resistance among CoNS increased from 18.4% in 1992-1993 to 41.7% in 2000-2001. Similar results have been reported by other investigators [10]. Previous data from our group showed that oxacillin resistance does not influence the outcome of staphylococcal peritonitis [8].

In addition to species and antibiotic resistance, other factors related to the causal agent may influence the prognosis of peritonitis. Recurrence of CoNS infections is frequently observed and has been suggested to be associated with the presence of a biofilm in the peritoneal catheter [11]. Biofilm formation is related to the production of an extracellular mucoid polysaccharide, called slime, which permits microorganisms to adhere to plastic surfaces [12]. Kristinsson et al [13] reported a higher recurrence rate of peritonitis for slime-positive strains compared to slime-negative ones, whereas Alexander and Rimland [14] did not observe a relationship between slime production and peritonitis outcome. In our unit, slime production was found to be an independent risk factor for the non-resolution of CoNS peritonitis [15].

The production of enzymes and toxins is a well-known fact in *Staphylococcus* species, particularly *S. aureus*. Proteases, lipases, nucleases, and collagenases convert tissue components into nutrients, facilitating bacterial growth and invasion [16], while toxic shock syndrome toxin (TSST-1) and enterotoxins have effects such as superantigenicity, pyrogenicity, toxicity and direct damage to endothelial [6].

Although these products are potential virulence factors in staphylococcal PD-associated peritonitis, their influence on the clinical outcome of these infections is unknown. The objective of the present study was to compare the capacity of traditional clinical and bacteriologic and selected virulence factors such as production of slime, enzymes and toxins to predict the outcome of new peritonitis episodes caused by *S. aureus*, *S. epidermidis* and other CoNS.

Methods

Data collection and definitions

The present study was approved by the institutional Ethics Committee. All episodes of continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis caused by staphylococcal species between January 1996 and December 2000 were reviewed. This period was chosen because a single antibiotic protocol based on the 1996 Update of the International Society for Peritoneal Dialysis [17] was used. After this period, new guidelines were proposed [18] and adopted in our unit. The diagnosis of peritonitis was made when at least two of the following criteria were present: (a) presence of a cloudy peritoneal effluent, (b) abdominal pain, (c) dialysate white cell count higher than 100/μL, with at least 50% polymorphonuclear cells, and (d) positive culture of peritoneal effluent [17].

Only cases considered to be new episodes, i.e., a patient's first peritonitis or an episode diagnosed at least 28 days after completion of the last peritonitis treatment, were included in the study [17]. Thus, 86 of 122 diagnosed staphylococcal peritonitis episodes were analyzed. Exclusion criteria were staphylococcal peritonitis within 28 days prior to presentation, presence of concomitant exit site or tunnel infections, incomplete clinical data, concomitant antibiotic use for other indications, and use of an empirical antibiotic protocol other than the combination of cefazolin and amikacin.

Resolution was defined as the disappearance of signs and symptoms within 96 h after the beginning of antibiotic therapy and a negative peritoneal fluid culture at least 28 days after treatment completion [7]. Relapse was defined as an episode with the same organism or a negative culture result that occurs within 28 days of completion of antibiotic therapy for a prior episode [17]. Non-resolution was the term used for cases presenting initial non-resolution, relapse, peritoneal catheter removal, or death.

The following information was recorded for each case: (1) episode: date, clinical findings, treatment, outcome (resolution, relapse, catheter removal, or death); (2) presence of diabetes mellitus; (3) demographic data: age, gender, and race (Caucasian, non-Caucasian), dialysis treatment time; (4) exchange system (standard or double bag).

Clinical management

All episodes were treated according to the local protocol adapted from the third report of the Ad Hoc Advisory Committee on Peritonitis Management for staphylococcal episodes [17]. Patients were treated within 24 h of the onset of the first clinical signs or symptoms and antibiotic

therapy was started with 500 or 750 mg/L cefazolin, ip (patients with urine volume >500 mL/24 h) and 250 mg/L amikacin, ip, as loading dose, followed by 500 or 750 mg/L cefazolin and 2 mg/kg amikacin per day in the last PD bag. Therapy was evaluated as soon as the culture results were available. For oxacillin-susceptible cocci, cefazolin was maintained, whereas for oxacillin-resistant cocci cefazolin was replaced with 1 g/L vancomycin, ip, administered at intervals of 5 (patients with urine volume >500 mL/24 h) or 7 days. Amikacin was discontinued in both cases. Vancomycin was also administered to patients who presented no clinical improvement within the first 96 h of antibiotic treatment, although the microbiological tests revealed oxacillin susceptibility. The duration of antibiotic therapy was 14 days for CoNS episodes and 21 days for *S. aureus* episodes.

Microbiological tests

Microbiological samples were stored in a culture collection. The isolates obtained from clinical specimens were seeded onto blood agar and gram-stained to confirm purity and to determine morphology and specific color. The isolates were then submitted to catalase and coagulase tests. CoNS were identified using the simplified biochemical test scheme proposed by Kloos and Schleifer [19] and Kloos and Bannerman [20].

In vitro susceptibility was evaluated based on the minimal inhibitory concentration determined by the E-test (AB Biodisk, Solna, Sweden), a quantitative method that uses a transparent strip of inert plastic containing drug concentrations ranging from 0.002 to 256 μg/mL. The proportion of strains susceptible to each drug was defined based on the 2005 CLSI breakpoints [21]. Strains presenting intermediate values were considered to be resistant.

Determination of the production of pathogenic factors

Slime

Slime production was evaluated according to Christensen et al [22]. Colonies of CoNS isolated on blood agar were inoculated into tubes measuring 12.0 × 75.0 mm and containing 2.0 mL trypticase soy broth (TSB) and incubated for 48 h at 37°C. Next, 1.0 mL 0.4% trypan blue or toluidine blue O solution was added to the tubes. After gentle shaking to guarantee staining of the material adhered to the inner surface of the tubes, the dye was discarded. A positive result was defined as the presence of a layer of stained material adhered to the inner wall of the tubes. The presence of a colored ring only at the liquid-air surface was classified as negative.

Alpha- and beta-hemolysin

Production of α- and β-hemolysin or cytolytic toxins was determined on plates containing blood agar base supplemented with 5% rabbit blood and 5% sheep blood, respectively. The plates were incubated for 24 h at 37°C. The formation of hemolysis zones around the isolated colonies indicated a positive result.

Lipase and lecithinase

Lipolytic activity was determined on plates containing blood agar base enriched with 0.01% $CaCl_2{:}2H_2O$ and 1% Tween 80. A positive result was defined as the formation of opacity around the colony after incubation for 18 h at 37°C, followed by incubation at room temperature for 24 h [23]. The production of lecithinase was evaluated using Baird-Parker medium. The formation of an opaque halo around the colony indicated a positive result [24].

DNAse and TNAse

Nuclease (DNAse) and thermonuclease (TNAse) were determined by the metachromatic toluidine blue O agar diffusion-DNA technique according to Lachica et al [25]. For the detection of DNAse, supernatants were obtained from CoNS cultures in BHI broth previously incubated for 24 h at 37°C and centrifuged at 8000 *g* for 10 min at 4°C. The culture supernatant was first heated in a water bath for 20 min and then placed in the wells for the detection of TNAse. Nuclease (DNAse and TNAse) activity was determined by measuring the diameter of pink halos (mm) formed on the medium. Positive results were interpreted by comparison of the halos with those obtained for a standard DNAse- and TNAse-positive *S. aureus* strain (ATCC 25923). Culture supernatants obtained by the sac culture method of Donnelly et al [26], as described below, were also tested for DNAse and TNAse production.

Enterotoxins and toxic shock syndrome toxin-1

The sac culture method for toxin production [26] was used to determine the toxigenic profile of the strains. Dialysis sacs filled with 50 mL double-concentrated BHI broth were placed in U-shaped Erlenmeyer flasks and autoclaved for 15 min at 121°C. A loopful of organisms was added to 18 mL sterile 0.2 M phosphate buffer in 0.9% NaCl, pH 7.4. After incubation for 24 h at 37°C on a shaker at 200 rpm, the cultures were centrifuged at 8000 *g* for 10 min at 4°C and the supernatants obtained were stored at -20°C until the time of use. The extracellular products were detected by reverse passive latex agglutination (RPLA) according to Shingaki et al [27], using the SET-RPLA-T900 and TST-RPLA-TD940 kits (Oxoid Diagnostic Reagents) for the detection of enterotoxins A (SEA), B (SEB), C (SEC) and D (SED) and TSST-1, respectively. Culture supernatants were first treated with 5% (v/v) normal rabbit serum or 5% purified rabbit IgG to block nonspecific reactions. Samples that presented nonspecific reactions even after this treatment were filtered through a Millipore membrane (8.0 μm) and, if necessary, diluted 1:10 with 0.02 M phosphate buffer in 0.9% NaCl, pH 7.4. A positive reaction was classified as (+), (++) and (+++)

according to the agglutination pattern described by the manufacturer of the kit. The formation of a rose button was interpreted as a negative result.

Statistical analysis

Continuous variables were compared using the unpaired t-test or nonparametric Mann-Whitney U-test. Binary variables were compared by the chi-square or Fisher's exact test. Multivariate analysis by logistic regression was used to test for factors that independently predicted the outcome of a peritonitis episode, with outcome being classified into two mutually exhausted and exclusive results (resolution or non-resolution). A model was adopted that would incorporate the effect of all factors-interest and control - on the outcome of infection. All baseline demographic, clinical, and microbiological variables, including age, gender, diabetic status, dialysis duration, exchange system, use of vancomycin, *Staphylococcus* species, oxacillin susceptibility and pathogenic factor production, were included in the model. A p value less than 0.05 was considered to be significant.

Results

Eighty-six new CAPD-associated staphylococcal peritonitis episodes occurred in 63 patients between 1996 and 2000. Forty-three of the patients were females, 39 were Caucasians, and 28 had diabetes. The distribution of patients according to age was as follows: birth to 20 years (n = 4), 21 to 40 years (n = 12), 41 to 59 years (n = 22), and 60 years or older (n = 22). Treatment time on CAPD was less than one year in 28 patients and longer than one year in 35. Forty-four patients used a double bag system and 19 used a standard bag exchange system.

Microbiological investigation

Thirty-five episodes were caused by *S. aureus* and 51 by CoNS. Among CoNS, *S. epidermidis* was the most frequent species (24 cases), followed by *S. haemolyticus* (11 cases) and other species (16 cases) (Table 1).

Table 1: Causative agent of the 86 new episodes of peritonitis caused by *S. aureus* and coagulase-negative staphylococci.

Microorganism	No. of strains (%)
S. aureus	35
CoNS	51 (100)
S. epidermidis	24 (47)
S. haemolyticus	11 (21.5)
S. warneri	5 (9.8)
S. hominis	5 (9.8)
S. xylosus	2 (3.9)
S. cohnii	2 (3.9)
S. simulans	1 (1.9)
S. lugdunensis	1 (1.9)

CoNS: coagulase-negative staphylococci.

Oxacillin susceptibility was observed in 30 (85.7%) of the 35 episodes due to *S. aureus*, in 10 (41.7%) of the 24 episodes due to *S. epidermidis*, and in 12 (44.4%) of the 27 episodes due to other CoNS ($p = 0.0002$). Five cases of intermediate susceptibility were detected among CoNS and one among *S. aureus* strains.

Positive slime production was observed in three cases due to *S. aureus* (8.6%) and in nine (17.6%) due to CoNS ($p = 0.345$), including *S. epidermidis* in four, *S. haemolyticus* in two, *S. warneri* in two, and *S. lugdunensis* in one.

With respect to toxigenic profile, 25 (71.4%) of the 35 *S. aureus* strains were toxin producers, whereas only seven (13.7%) of the 51 CoNS strains produced some type of toxin ($p < 0.00001$). The rate of enzyme production was higher in *S. aureus* strains than in *S. epidermidis* or other CoNS, except for α-hemolysin whose production was similar in all strains. The rates of toxin and enzyme production by *S. aureus*, *S. epidermidis* and other CoNS species are shown in Table 2.

Clinical outcome

Overall, 57 (66.3%) episodes were resolved, 14 (16.3%) relapsed, 12 (13.9%) required removal of the catheter, and three (3.5%) progressed to death. Among the 35 *S. aureus* cases, 17 (48.6%) were resolved, eight (22.8%) relapsed, seven (20%) required catheter removal, and three (8.6%) progressed to death. Regarding CoNS episodes, 40 (78.4%) were resolved, six (11.8%) relapsed, and five (9.8%) required catheter removal. Among these 11 non-resolved episodes, seven were due to *S. epidermidis*, two to *S. haemolyticus*, and two to other CoNS. There were significantly more CoNS cases resolved than *S. aureus* episodes ($p < 0.001$).

There were 52 episodes involving oxacillin-susceptible strains, 32 (61.5%) of them were resolved, 10 (19.2%) relapsed, and 10 (19.2%) required catheter removal. Thirty-four infections were caused by oxacillin-resistant strains, 20 (58.8%) of them were resolved, nine (26.5%) relapsed, and five (14.7%) required catheter removal. The resolution rate was similar for oxacillin-susceptible and -resistant strains ($p = 0.9713$).

Vancomycin was used in 56 episodes, 18 caused by *S. aureus* and 38 by CoNS. This antibiotic was prescribed because of bacterial resistance in 28 cases, lack of improvement in 20, and other undefined causes in eight. The time between diagnosis and the first vancomycin dose was 3.7 ± 1.7 days for *S. aureus* episodes and 3.9 ± 1.2 days for CoNS episodes ($p = 0.793$).

Two regression models were constructed. In Model 1 slime production was the only pathogenic factor, whereas

Table 2: Rates of toxin and enzyme production by *S. aureus*, *S. epidermidis* and other coagulase-negative staphylococci strains isolated from 86 new episodes of peritonitis.

	S. aureus	*S. epidermidis*	Other CoNS	p
Toxins				
SEA	4 (11.4)	1 (4.1)	- (-)	0.15
SEB	12 (34.3)	- (-)	2 (7.4)	0.0006
SEC	8 (22.8)	- (-)	6 (22.2)	0.04*
SED	- (-)	- (-)	- (-)	-
TSST-1	12 (34.3)	1 (4.1)	- (-)	0.0008
Enzymes				
α-Hemolysin	17 (48.6)	8 (33.3)	8 (29.8)	0.26
β-Hemolysin	29 (82.3)	6 (25)	7 (25.9)	< 0.00001
Lipase	34 (97.1)	4 (16.7)	5 (18.5)	< 0.00001
Lecithinase	34 (97.1)	2 (8.3)	6 (22.2)	< 0.00001
DNAse	34 (97.1)	- (-)	4 (14.8)	< 0.00001
TNAse	34 (97.1)	- (-)	4 (14.8)	< 0.00001

CoNS: coagulase-negative staphylococci; SEA, SEB, SEC, and SED: enterotoxins A, B, C and D, respectively; TSST-1: toxic shock syndrome toxin; * *S. aureus* vs. *S. epidermidis* and other CoNS vs. *S. epidermidis*.

in Model 2 the production of toxins and enzymes was included. Only lecithinase, α-hemolysin and TNAse were included since associations were observed between lecithinase and lipase, α-hemolysin and β-hemolysin, and TNAse and DNAse (p = 0.001). All toxins were included, except for toxin D which was not produced by any of the strains. Since an interaction effect was observed between *S. aureus* species and oxacillin susceptibility (p = 0.01), the influence of oxacillin susceptibility was evaluated at each vancomycin treatment co-variable level in both models.

In Model 1 (Table 3), controlling for co-variables, the odds of resolution was not influenced by host factors such as age, gender, diabetes, exchange system, or CAPD treatment time. The odds of resolution were 9.5 times higher for *S. epidermidis* than for *S. aureus* episodes ($p = 0.0263$), whereas similar resolution odds were observed for *S. epidermidis* and the other CoNS ($p = 0.085$). Among strains isolated from infections treated with vancomycin, no significant difference was observed between oxacillin-susceptible and -resistant strains (p = 0.89). In contrast, among strains isolated from infections not treated with vancomycin, there was a significant difference between strains susceptible and resistant to oxacillin (p = 0.0113). In this case, the chance of cure of infections caused by oxacillin-susceptible strains was 137 times higher than that of

Table 3: Odds comparison of peritonitis resolution by logistic regression analysis (Model 1).

Factor	Log (Odds)	p	Odds ratio (95% CI)
Age (birth to 20 years/>60 years)	-0.0510	0.96	
Age (21 to 40 years/>60 years)	1.7097	0.09	
Age (41 to 59 years/>60 years)	0.7511	0.84	
Gender (male/female)	1.2523	0.19	
Race (Caucasian/non-Caucasian)	-0.0818	0.92	
Diabetes mellitus (no/yes)	-0.6709	0.12	
System (standard/double bag)	1.2585	0.22	
Treatment time on CAPD			
<1 year	0.2074	0.89	
>1 year	0.9547	0.21	
Treatment with vancomycin			
Oxacillin susceptible/oxacillin resistant	-0.1070	0.89	
No treatment with vancomycin			
Oxacillin susceptible/oxacillin resistant	1.9440	0.01	137 (3.0; 6,202.4)
Etiological agent			
S. epidermidis/*S. aureus*	2.2548	0.02	9.5 (1.3; 69.6)
Other CoNS/*S. epidermidis*	-1.6113	0.08	
Slime production (no/yes)	4.2139	0.001	68 (5.0; 914.8)

CoNS: coagulase-negative staphylococci; CI: 95% Confidence Interval for the true Odds Ratio.

infections caused by resistant strains. With respect to slime production, the chance of cure of infections caused by non-producers was estimated to be up to 68 times higher than that of infections caused by slime producers (p = 0.0015).

As observed for the first model, in Model 2 (Table 4) resolution odds were not influenced by host factors. Among strains isolated from infections treated with vancomycin, no significant difference was observed between those susceptible and resistant to oxacillin (p = 0.1523), whereas there was a significant difference among strains not treated with vancomycin (p = 0.0039). With respect to slime production, the chance of cure of infections caused by non-producers was estimated to be 184 times higher than that of infections caused by producers (p = 0.0012). In contrast to Model 1, *S. aureus* did not differ from *S. epidermidis* in terms of the probability of peritonitis resolution (p = 0.7014), whereas the chance of cure of infections caused by other CoNS species was estimated to be 46 times higher than that of infections caused by *S. epidermidis* (p = 0.0175). Alpha-hemolysin production was an independent predictor of resolution odds, with episodes caused by non-producers presenting an 8.2 times higher chance of resolution than those caused by producers (p = 0.0423). No significant effects on the probability of peritonitis resolution were observed for the remaining enzymes and toxins.

Discussion

In the present study we investigated new episodes of staphylococcal peritonitis in PD patients and compared episodes caused by *S. aureus*, *S. epidermidis* and other CoNS, focusing on the role of virulence factors in peritonitis outcome. CoNS were the most frequent etiological agent, in agreement with other studies. In addition to *S. epidermidis*, seven other CoNS species were identified, the most predominant being *S. haemolyticus*. This is an important finding since studies regarding other CoNS are scarce in the literature.

The resolution rate of episodes caused by *S. aureus* was lower than that of infections caused by CoNS. Similar findings have been reported in the prospective studies of Bunke et al [5] and Peacock et al [6], and in the recent retrospective study of Davenport [1]. Since the rate of oxacillin resistance was higher among CoNS strains than among *S. aureus* strains, a contribution of drug resistance is unlikely. In fact, our results showed no difference in reso-

Table 4: Odds comparison of peritonitis resolution by logistic regression analysis (Model 2).

Factor	Log (Odds)	p	Odds ratio (95% CI)
Age (birth to 20 years/>60 years)	0.0602	0.97	
Age (21 to 40 years/>60 years)	4.2565	0.06	
Age (41 to 59 years/>60 years)	1.9474	0.09	
Gender (male/female)	1.2461	0.37	
Race (Caucasian/non-Caucasian)	-1.3322	0.21	
Diabetes mellitus (no/yes)	-0.6711	0.47	
System (standard/double bags)	2.3397	0.12	
Treatment time on CAPD			
<1 year	0.0531	0.98	
>1 year	-0.3045	0.77	
Treatment with vancomycin			
Oxacillin susceptible/oxacillin resistant	1.6316	0.15	
No treatment with vancomycin			
Oxacillin susceptible/oxacillin resistant	10.0189	0.004	23,906 (25.4; exp{16.9274})
Etiological agent			
S. epidermidis S. aureus	-1.6481	0.70	
Other CoNS/*S. epidermidis*	3.8238	0.017	46 (2.0; 1,069.4)
Slime production (no/yes)	5.2149	0.001	184 (7.8; 4,354.2)
Enzyme production (no/yes)			
α-Hemolysin	2.1092	0.04	8.2 (1.1; 63.1)
TNAse	2.0500	0.28	
Lecithinase	3.5545	0.06	
Toxin production (no/yes)			
Enterotoxin A	2.3914	0.26	
Enterotoxin B	0.5669	0.13	
Enterotoxin C	4.0501	0.06	
TSST-1	1.3694	0.48	

lution rates between episodes caused by oxacillin-susceptible and oxacillin-resistant strains.

All patients received an intermittent regimen of cefazolin plus amikacin as initial treatment. Some investigators [28,29] argue that the continuous addition of beta-lactams to the PD fluid is more effective for the treatment of CoNS infections by overcoming moderate bacterial resistance. Since a higher resolution rate was observed for CoNS episodes compared to *S. aureus* episodes and the number of strains presenting moderate resistance to oxacillin was low, it is unlikely that the antibiotic regimen has influenced the results.

Regression analysis using the two models showed no influence of age, race, gender, CAPD treatment time, diabetes, or exchange system on the progression of peritonitis. Similar results have been reported by Krishnan et al [7], except for the influence of race and dialysis treatment time.

Slime production was independently associated with non-resolution of peritonitis. This finding agrees with data on CoNS infections previously published by our group [15] and with the results of Kristinsson et al [13]. The latter authors proposed that slime production may promote bacterial adherence to catheters, facilitating colonization and peritonitis relapse. Thus, slime production might be a virulence factor and simultaneously worsens the response to infection, protecting bacterial cells from the host's natural defense mechanisms and from the action of antibiotics.

In contrast to Model 1, no difference in the probability of peritonitis resolution was observed between *S. aureus* and *S. epidermidis* when Model 2 was used. This finding might be explained by the fact that inclusion of enzymes and toxins in the model permitted the control for the effect of species factor on these bacterial products. In other words, the species effect observed in the first model for *S. aureus* episodes was not separated from the pathogenicity factors included in the second model. In addition, whereas the chance of cure of *S. epidermidis* episodes compared to other CoNS infections tended to be lower in the first model, the second model showed a significantly lower probability of cure of *S. epidermidis* episodes, an independent outcome not influenced by toxin or enzyme production.

Among the virulence factors studied, only α-hemolysin production was significantly and independently associated with a higher probability of non-resolution. Recent data published by Haslinger-Löffler et al [30] suggest that α-hemolysin plays a specific role in the pathogenesis of peritonitis. Using cultured human peritoneal mesothelial cells, these authors showed that the *S. aureus* subgroup characterized as invasive and α-hemolysin producing induced caspase-independent cell death. Unlike *S. aureus*, no cytotoxic effects were triggered by any of the *S. epidermidis* strains which were noninvasive and did not produce α-hemolysin. These findings, together with our results, suggest that this enzyme plays a pathogenic role in PD-associated peritonitis. Since mesothelial cells participate in the early host defense against infections [31], damage caused by α-hemolysin may contribute to the poor course of *S. aureus* peritonitis.

This study has some limitations, particularly the small number of cases, which reduce its statistical power and prevented the separate analysis of *S. aureus* and CoNS peritonitis episodes. Further studies with bigger number of cases are necessary to overcome this limitation and to confirm the present results.

Conclusions

In conclusion, host factors, as well as dialysis treatment time and exchange system, probably have little or no influence on the response to PD-associated peritonitis treatment. However, the prognosis of these infections is strongly influenced by characteristics of the causative agent such as species and virulence factors. Although these factors may act in concert, our data suggest that slime and α-hemolysin production independently contribute to poor peritonitis outcome.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PB participated in the design and coordination of the study and in the collection of the clinical data, analyzed the data, and wrote and revised the manuscript. ACM participated in the design of the study and performed the E-test. JENB performed the microbiological tests. JCTC participated in the collection of the clinical data and contributed to the design of the study. MLRSC participated in the coordination of the study, supervised the laboratory work, reviewed and approved the analyses, and contributed to the writing and revision of the paper. All authors read and approved the final manuscript.

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4. Infecções relacionadas a cateteres

Com o advento dos centros de terapia intensiva (CTI) ocorreu um avanço no tratamento do paciente crítico, promovendo maior sobrevida mesmo na população de maior risco, como na sepse, nos imunodeprimidos, nos pacientes oncológicos e nos recém-nascidos prematuros de muito baixo peso. No entanto, a evolução do arsenal terapêutico, utilizando-se de técnicas cada vez mais invasivas, resultou em mecanismos de quebras de barreiras e exposição de tecidos previamente íntegros, tornando-os suscetíveis à infecção. A infecção relacionada ao cateter (IRC) é um exemplo desta realidade, ela ocorre quando há invasão da corrente sanguínea por um germe através do cateter vascular. A infecção associada ao uso de dispositivos intravasculares representa de 10 a 20% de todas as infecções relacionadas à assistência à saúde (IRAS) e é uma das causas mais frequentes de morbidade e mortalidade, representando uma fonte de bacteremia e sepse em pacientes hospitalizados, aumentando o tempo de permanência hospitalar, os custos de internação e o índice de mortalidade. Estudo brasileiro publicado recentemente utilizando a metodologia SCOPE revelou a presença do cateter venoso central como o fator de risco principal para a ocorrência de infecções da corrente sanguínea nosocomial, sendo encontrado em 70,3% dos pacientes (38).

Aproximadamente 65% dessas infecções resultam da migração de micro-organismos da microbiota da pele a partir do sítio de inserção do cateter, 30% da contaminação é intraluminal e 5% por outras vias, como infusão de fluidos contaminados e focos infecciosos à distância (69).

Vale ressaltar que mais de 150 milhões de cateteres vasculares são utilizados anualmente em Hospitais e Clínicas nos Estados Unidos. A maioria desses dispositivos são cateteres venosos periféricos, porém um número expressivo destes, mais de 5 milhões, são cateteres venosos centrais (CVC) inseridos em vasos profundos ou centrais (70). Mais de 200.000 infecções da corrente sanguínea ocorrem nos Estados Unidos, a maioria relacionada com CVC não tunelizado. A finalidade desses cateteres é variada e envolve a administração de medicamentos, sangue e derivados, nutrição parenteral, monitoramento da condição hemodinâmica do paciente e acesso vascular para realização de hemodiálise (71).

Em Unidades de Cuidados Intensivos Neonatais a taxa de infecção é inversamente proporcional ao peso de nascimento do recém-nascido variando de 9,1 por 1000 cateteres dias em crianças com peso ao nascimento <1000 g a 3,5 por 1000 cateteres dias em crianças com peso de nascimento >2500 g (69). No Brasil, a infecção da corrente sanguínea relacionada com cateter (ICSRC) é a principal infecção em UTI neonatal. Segundo dados de Pessoa-Silva *et al*. (72), a incidência de ICSRC variou de 17,3 /1000 CVC-dia em RN entre 1501g a 2500g até 34,9/1000 CVC-dia em RN < 1000g.

A maioria das ICSRC é causada pelos Estafilococos coagulase-negativa (ECN). De acordo com dados de Perlman *et al*. (73), estes micro-organismos foram responsáveis por 55,5% das ICSRC em neonatos, seguido por *Staphylococcus aureus* (13,2%) e *Enterococcus* (9,2%). Os micro-organismos Gram-negativos como as enterobactérias *Escherichia coli, Enterobacter*, *Klebsiella pneumoniae* e Bacilos Gram-negativos não fermentadores, *Pseudomonas aeruginosa* e *Acinetobacter baumannii* foram isolados em cerca de 20,2% dessas infecções. *Staphylococcus epidermidis* foi isolado de cerca

de 39,8% das ICSRC, *S. warneri* (6,9%) e outras espécies de ECN (8,7%), sendo a maioria das ICSRC causada pelos micro-organismos gram-positivos (79,8%). Dados reportados pelo NHSN do CDC (39) referente ao período de janeiro de 2006 a outubro de 2007 também ranquearam os ECN em 1° na etiologia das ICSRC (34,1%) seguido por espécies de *Enterococcus* (16%), espécies de *Candida* (11,8%) e *S. aureus* em 4° lugar (9,9%). Os micro-organismos Gram-negativos incluindo as enterobactérias *Escherichia coli, Enterobacter*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* foram responsáveis por 12,4% dessas infecções e os Bacilos Gram-negativos não fermentadores *Pseudomonas aeruginosa* e *Acinetobacter baumannii* por 5,3%.

A patogênese dessas infecções é complexa e parcialmente conhecida, especula-se estar diretamente relacionada à aderência dos micro-organismos, que se tornam capazes de colonizar o dispositivo. Há quatro fontes potenciais para colonização do cateter e a ocorrência de ICSRC, o sitio de inserção do cateter na pele, o canhão, a via hematogênica e a contaminação do infundido. A pele é a principal fonte para colonização e infecção de cateter de curta duração. As bactérias que estão na pele do paciente migram ao longo de sua superfície, colonizando a extremidade distal, resultando em infecção (74). O canhão (*hub*) é outra fonte para colonização e os micro-organismos podem ser introduzidos pelas mãos da equipe de saúde, contaminando o canhão, migrando ao longo da superfície interna do cateter, podendo causar uma infecção da corrente sanguínea. Quando há uso prolongado do canhão do cateter (acima de 30 dias), pode ser esperada maior colonização da superfície interna (75). A contaminação hematogênica do CVC, a partir de um foco infeccioso à distância, como por exemplo, pneumonia, infecção gastro-intestinal, ou do trato urinário, foi

sugerida, mas não é uma causa importante de colonização do cateter, e raramente é comprovada (76). As soluções de nutrição parenteral e emulsões lipídicas promovem o crescimento de muitas bactérias e fungos, como *Candida parapsilopsis* e *Malassezia furfur* (77). Embora muitas epidemias de bacteremia hospitalar foram causadas pelo infundido contaminado, é muito baixa a contribuição desta fonte para as bacteremias primárias hospitalares endêmicas.

Todas essas fontes de contaminação são importantes, mas nenhuma compete com a contaminação por micro-organismos do próprio paciente em áreas próximas à inserção dos cateteres. Isso explica porque os ECN são os micro-organismos frequentemente mais associados com essas infecções, já que são os mais encontrados na pele.

A aderência de micro-organismos na superfície de cateter é dependente da interação de três fatores: hospedeiro, micro-organismo e o material do cateter. O hospedeiro reage contra o cateter, considerado corpo estranho, e forma ao seu redor uma cobertura de fibrina e fibronectina. Esses componentes protéicos do hospedeiro que estão recobrindo a superfície do cateter permitem a aderência de *S. aureus.* Esses micro-organismos são produtores de coagulase, promovendo a trombogênese, além de várias outras proteínas que estão em sua superfície denominadas MSCRAMMs (*Microbial Surface Components Recognizing Adhesive Matrix Molecules*), adesinas que possuem receptores para proteínas liberadas pelo hospedeiro, como fibrinogênio e fibronectina que permitem a aderência e colonização do cateter (78-79).

Os estafilococos coagulase-negativa podem colonizar a superfície nativa do cateter, assim como superfícies condicionadas por essas proteínas do hospedeiro. Uma vez aderidas, essas bactérias proliferam, formando múltiplas camadas e produzem um polissacarídeo extracelular, formando o biofilme, que potencializa sua patogenicidade. O biofilme não só favorece a aderência dos micro-organismos como também sua manutenção, atuando como barreira ao ataque dos antibióticos, neutrófilos, fagócitos, macrófagos e anticorpos. A concentração de antibiótico requerida para destruir bactérias em um biofilme é 100 a 1000 vezes maior do que a necessária para destruir as mesmas espécies em suspensão, dificultando o tratamento e aumentando a possibilidade de infecções recorrentes, uma vez que as bactérias ficam protegidas do sistema imune do hospedeiro (80).

A *Candida* também figura como micro-organismo preocupante uma vez que há estudos mostrando ser responsável por cerca de 80% das infecções fúngicas no ambiente hospitalar. Em adição, algumas espécies, na presença de soluções contendo glicose, podem produzir o biofilme similar ao bacteriano, o que explica o aumento da proporção de ICSRC devido à patógenos fúngicos especialmente em pacientes recebendo nutrição parenteral (81).

O terceiro fator importante para a aderência microbiana é o material do cateter. Estudos *in vitro* demonstram que cateteres de cloreto de polivinil ou polietileno são menos resistentes à aderência de microrganismos do que cateteres feitos de teflon, silicone ou poliuretano (82).

Em relação ao tempo de permanência na instituição, índice de mortalidade e custos de internação dos pacientes com ICSRC, todos esses aspectos aumentam significativamente. A ocorrência dessa infecção prolonga a internação de 6,5 a 22 dias e acrescenta um custo de U$29.000 a U$56.000 por episódio de infecção. Estima-se que pacientes nessas condições tenham uma mortalidade de 13% a 28% maior em relação a pacientes da mesma gravidade sem essa complicação (83,84).

A sepse relacionada a cateter (SRC) em recém-nascidos também tem se constituído em séria complicação, pois os cateteres intravasculares são amplamente utilizados para inúmeros procedimentos em UTI neonatal, possibilitando o rápido acesso intravenoso para a administração de medicamentos e nutrição parenteral, entre outros (85). Embora a utilização de cateteres seja procedimento fundamental para a sobrevida dos RN em UTI neonatal, também atua como importante fator de risco para o desenvolvimento de infecção e contribui para o aumento da incidência de infecções da corrente sanguínea e consequente aumento nas taxas de morbidade e do tempo de hospitalização (82).

Assim como nos indivíduos adultos, cerca de 60% das infecções da corrente sanguínea relacionadas com cateteres em RNs são causadas por bactérias gram-positivas, dentre as quais se destacam as espécies de estafilococos coagulase-negativa, já que são também os principais componentes da microbiota da pele e mucosas, sendo reconhecidos como os agentes etiológicos mais frequentemente envolvidos com infecções em RNs, principalmente os com muito baixo peso ao nascimento (< 1500g) (69).

Até pouco tempo atrás não havia consenso entre os estudiosos em relação ao diagnóstico de infecções relacionadas a cateteres. Em 2002 o CDC padronizou esses conceitos, diferenciando a colonização significativa do cateter e a sepse relacionada com cateter. De acordo com o CDC (82), na colonização significativa do cateter há uma forte correlação entre inflamação local do cateter e o isolamento de mais de 15 Unidades Formadoras de Colônias (UFC) de um micro-organismo do segmento do cateter. Por isso é frequentemente empregado o termo "infecção local" nessa eventualidade. Outros pesquisadores usam o termo "colonização" para descrever esse evento em oposição ao termo "contaminação" quando menos de 15 UFC forem isoladas. Porém uma cultura positiva de um segmento do cateter na ausência de hemocultura positiva não deve ser considerada uma bacteremia ou fungemia relacionada com o dispositivo.

O termo sepse ou ICSRC é o termo empregado no caso de além de uma cultura semiquantitativa com crescimento ≥ 15 UFC ou cultura quantitativa com ≥ 1000 UFC do segmento do cateter, for isolado o mesmo micro-organismo (espécie e antibiograma) na hemocultura, além da presença de sinais clínicos de sepse (febre, hipotermia, apnéias), sem fonte aparente de infecção, exceto seu cateter. Portanto, o diagnóstico definitivo é estabelecido quando o cateter é significativamente colonizado com o mesmo micro-organismo encontrado na hemocultura (82). Vale acrescentar que a falta de padronização de critérios clínicos, diagnósticos e a diversidade de conceitos de infecção, comprometem os sistema de vigilância epidemiológica das infecções, bem como dificulta a generalização dos resultados de pesquisas realizadas.

Neste contexto, antes do desenvolvimento da técnica de cultura semiquantitativa, a maioria dos laboratórios de Microbiologia Clínica utilizava a cultura em caldo das pontas de cateteres, para avaliação qualitativa. Esta técnica resultava em dados poucos confiáveis e sem especificidade, não permitindo distinção entre colonização e infecção. Em estudo realizado em nosso laboratório (ANEXO 4) comparando a cultura qualitativa com a cultura semiquantitativa no diagnóstico de ICSRC em recém-nascidos da Unidade Neonatal do HC da FMB foi verificado que embora a cultura semiquantitativa tenha apresentado menor sensibilidade (90%), apresentou uma maior especificidade (71%) em comparação à sensibilidade de 100% e especificidade de 60% encontradas pela técnica qualitativa, permitindo aos autores concluir que o método de cultura semiquantitativa de cateter apresentou vantagens para o diagnóstico de ICSRC em RNs quando comparado com o método qualitativo tradicional.

A confiabilidade da cultura depende da técnica empregada. A cultura semiquantitativa proposta por Maki *et al*. (86), que é o método de rolar o cateter em placa de ágar sangue, é o mais utilizado para determinar as taxas de infecção da corrente sanguínea relacionada ao cateter, existindo vários estudos comprovando sua importância. Em seu trabalho Maki *et al*. (86) demonstraram que uma cultura semiquantitativa da ponta de cateter com ≥ 15 UFC correlacionou melhor com a presença de infecção, entretanto, esses autores encontraram somente quatro casos de sepse relacionada a cateter e todos relacionados com números de micro-organismos com crescimento confluente. Analisando esses resultados os autores discutiram que o ponto de corte ≥ 15 UFC considerado resultado positivo, necessitava de outras

avaliações para determinação do número de UFC que melhor se correlaciona com a presença de ICSRC, sem reduzir a sensibilidade do teste.

O diagnóstico de ICSRC em Unidades Neonatais tem sido realizado usando métodos que são similares aos empregados para o diagnóstico dessas infecções em pacientes adultos, conforme o critério proposto por Maki *et al*. (86). Neste contexto foi desenvolvido um estudo pelo nosso grupo para determinar o ponto de corte da cultura semiquantitativa que melhor correlaciona com a presença de ICSRC em recém-nascidos (ANEXO 5). Foram estudadas 85 pontas de cateteres provenientes de 63 RN, sendo que os micro-organismos isolados de cateteres e hemoculturas periféricas foram identificados e submetidos ao teste de sensibilidade às drogas pelo método de difusão da droga com disco. O ponto de corte ótimo foi determinado pela curva de operação resposta (Curva ROC) e o padrão ouro correspondeu ao diagnóstico de certeza de ICSRC, com o isolamento do mesmo micro-organismo (espécie e perfil de sensibilidade às drogas) na cultura de cateter e em hemocultura periférica, na ausência de outro foco aparente de infecção, exceto seu cateter. Dos 11 episódios de infecção diagnosticados, 8 (72,7%) foram associados aos estafilococos coagulase-negativa, dos quais 6 pertenciam a espécie *S. epidermidis*. Pela curva ROC, o ponto de corte ótimo para o diagnóstico de infecção relacionada a cateter foi ≥ 122 UFC, com 91,0% de sensibilidade e 81,1% de especificidade, comparado com 91,0% de sensibilidade e 71,6% de especificidade utilizando o ponto de corte ≥ 15 UFC. O ponto de corte ≥122 UFC revelou maior especificidade e maior VPP, sem perda da sensibilidade quando comparado com ≥15 UFC.

Em estudo realizado por Collignon *et al*. (87) com pacientes adultos, os autores sugerem que o melhor ponto de corte para a detecção de ICSRC é de ≥5 UFC, já que apresentou maiores taxas de sensibilidade (92%) e a mesma taxa de especificidade (83%) encontrada para culturas com crescimento ≥15 UFC. Para determinar se a cultura semiquantitativa de pontas de cateteres é útil para o diagnóstico de ICSRC, um teste com alta especificidade e valor preditivo positivo é necessário e desejado, entretanto, Collignon *et al*. (87) encontraram um valor preditivo positivo de apenas 8,8%, com 124 cateteres apresentando resultados falsos-positivos. Por outro lado, para o ponto de corte de ≥100 UFC a especificidade seria de 94%, com redução dos falsos-positivos para 46. Segundo Brun-Buisson *et al*. (88), os autores deveriam escolher como melhor ponto de corte o crescimento de ≥100 UFC, ao invés de ≥5 UFC, pois um teste de diagnóstico com VPP menor que 10% não pode ser considerado adequado para uso no diagnóstico clínico.

Em nosso estudo apesar da maioria dos casos de ICSRC apresentarem crescimento superior a 122 UFC, foi observado um caso de ICSRC por *S. aureus* cuja cultura apresentou crescimento de apenas 8 UFC. Outros autores também têm verificado ICSRC e culturas semiquantitativas com crescimento <15 UFC (87). Esses resultados podem ser explicados pelo uso de antibióticos antes da cultura ou então pela contaminação intraluminal do cateter, que constitui o fator limitante da cultura semiquantitativa, a qual detecta somente os micro-organismos aderidos na superfície externa do dispositivo (89). Esta técnica é limitada principalmente em cateteres de longa permanência, nos quais a superfície interna é a fonte de colonização predominante. Essa desvantagem não é verificada nos métodos quantitativos tais

como o método por vórtex ou sonicação. No método por vórtex descrito por Brun-Buisson *et al*. (88), é feito um "flush" interno com água destilada estéril no segmento do cateter que descola micro-organismos da superfície interna, seguido por diluição seriada e a semeadura em placa de ágar sangue. O crescimento igual ou superior a 1000 UFC/ml é indicativo de ICSRC.

Baseado nos aspectos descritos acima, e tendo em vista que na cultura semiquantitativa determina-se a presença de micro-organismos somente da superfície externa do cateter e na cultura quantitativa além da superfície externa isolam-se também micro-organismos presentes no seu lúmen, outro estudo, com o objetivo principal de estudar comparativamente a técnica semiquantitativa preconizada por Maki *et al.* (1977)(86) e a técnica quantitativa descrita por Brun-Bruisson *et al*. (88) está sendo desenvolvido atualmente em nosso laboratório.

Uma desvantagem dessas metodologias de cultura semiquantitativa e quantitativa é a necessidade de retirada do cateter para a realização da cultura, de forma que outras metodologias de diagnóstico de ICSRC que dispensam a retirada do dispositivo devem ser consideradas. Os métodos mais promissores são aqueles que permitem a manutenção do acesso. Esta vantagem é particularmente marcante em pacientes críticos, com dificuldade de acesso, e naqueles com cateteres de longa permanência. Isso tem levado ao uso de novas técnicas para o diagnóstico de ICSRC sem remoção do cateter. Entre essas novas técnicas que sugerem infecção sem remoção do cateter está o Método Diferencial de Tempo de Positividade (DTP) com hemoculturas qualitativas coletadas do CVC e de veia periférica, utilizando monitorização contínua do crescimento de micro-organismos, onde o intervalo do

tempo de positividade da cultura no sangue do CVC e hemocultura periférica maior que duas horas, indica positividade de ICSRC. Basta a comparação dos registros do tempo de crescimento das hemoculturas periféricas e do cateter, quando for feito por método automatizado, se a cultura do cateter positivou duas horas ou mais antes da periférica, o exame é considerado positivo. A outra técnica é a hemocultura pareada quantitativa colhida de cateter e veia periférica, onde o crescimento de micro-organismos pelo menos cinco vezes maior no sangue colhido do cateter do que na veia periférica, indica resultado positivo (90).

A técnica DTP é uma técnica mais simples que a hemocultura quantitativa e amplamente disponível já que muitos laboratórios de microbiologia clínica têm adotado o uso de sistemas automatizados no monitoramento de hemoculturas. Assim, essa técnica, pode ser utilizada para grandes estudos prospectivos clínicos, constituindo em um método fácil de ser adotado e não muito caro para o diagnóstico de ICSRC e que dispensa a remoção do cateter (91). Em trabalho realizado por Blot *et al*. (91), foi possível fazer o diagnóstico em 16 dos 17 pacientes que tiveram um resultado positivo da hemocultura colhida do CVC pelo menos 2 horas antes da hemocultura periférica, com 91% de sensibilidade e 94% de especificidade.

Para a elucidação das ICSRC é fundamental determinar a similaridade das linhagens de micro-organismos isoladas das pontas de cateteres e das hemoculturas. Os testes bioquímicos podem determinar o gênero e a espécie do micro-organismo em questão, e o antibiograma pode determinar a similaridade das amostras isoladas de cateter e hemocultura a partir do perfil de resistência da bactéria presente nas amostras, auxiliando não só no tratamento das ICSRC, mas também no diagnóstico

dessas infecções (**Figura 1**). Em estudo comparativo entre técnicas moleculares e métodos microbiológicos na determinação das fontes de infecções nosocomiais Martín-Lozano *et al*. (92) avaliaram a utilidade do antibiograma para estudos epidemiológicos e concluíram que o diagnóstico da fonte de bacteremia utilizando-se de critérios clínicos convencionais e/ou microbiológicos, incluindo antibiograma, nem sempre são precisos e suficientes. Vários dos problemas são resultantes da expressão variável de características fenotípicas que são utilizadas como parâmetros.

Por essas razões, significantes esforços têm sido realizados no sentido de desenvolver métodos alternativos que combinam facilidade, confiabilidade e baixo custo. Surgiram então as técnicas de tipagem molecular que podem representar um potencial discriminatório adicional, principalmente porque não dependem da expressão de genes para avaliação. Alguns desses métodos são baseados nos princípios da reação em cadeia da polimerase (PCR), tais como o RAPD-PCR (*Random Amplified Polymorphic DNA Based PCR*) e o REP-PCR (*Repetitive Extragenic Palindromic Sequence Based PCR*). O RAPD-PCR é basicamente uma variação do protocolo de PCR, com duas características distintas: utiliza um “primer” único ao invés de um par de “primers” e o primer único tem sequência arbitrária e, portanto sua sequência alvo é desconhecida (93). O REP-PCR faz uso de primers complementares àqueles de ocorrência natural, altamente conservado, com sequências repetitivas e não codificadoras (geralmente 30 a 500pb) (94). Esses métodos apresentam a vantagem de um custo mais baixo e uma relativa facilidade metodológica, quando comparada com outras técnicas.

Buscando-se metodologias com especificidade e sensibilidade mais refinadas e que sejam capazes de estabelecer relações genéticas entre os isolados obtidos de

cateteres e hemoculturas, foi desenvolvido em nosso laboratório um estudo por Pazzini (95) que analisou por meio de técnicas de tipagem molecular o perfil genômico de micro-organismos isolados de cateteres e hemoculturas através de técnicas baseadas em PCR e comparou através do agrupamento por coeficientes de similaridade as amostras isoladas de cateteres e hemoculturas para o diagnóstico de ICSRC em recém-nascidos.

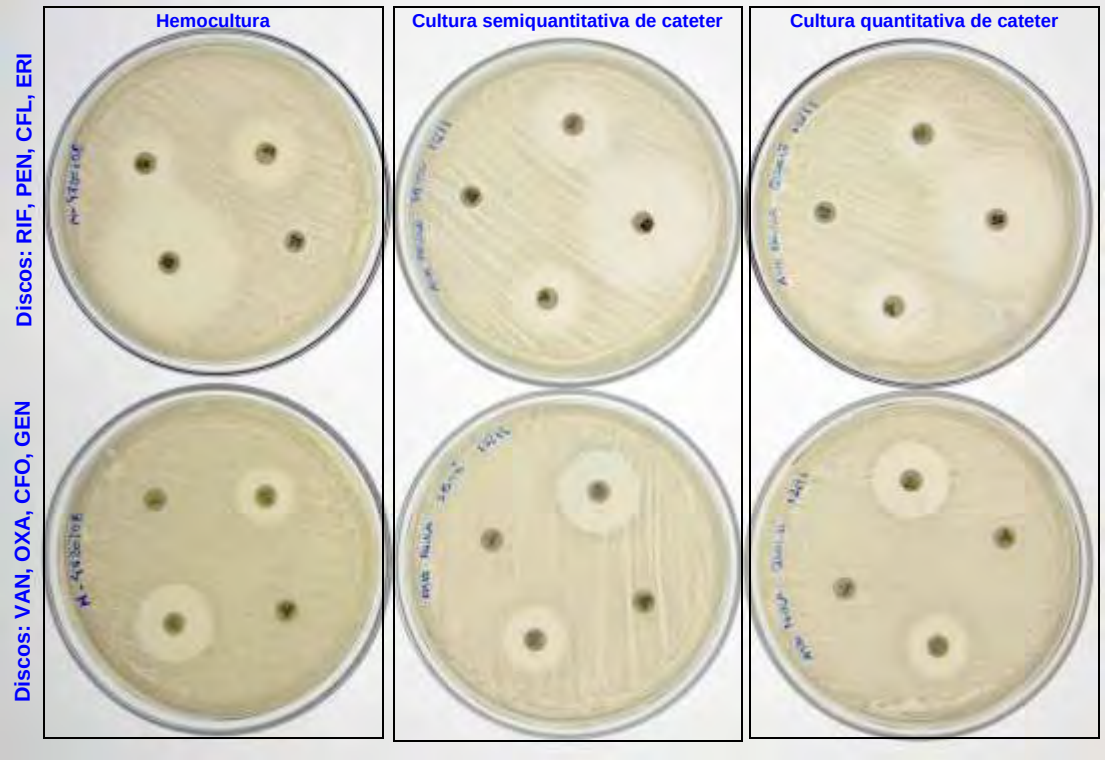

Figura 1: Teste de sensibilidade às drogas antimicrobianas – técnica de disco difusão em ágar para determinação da similaridade de estafilococos coagulase-negativa isolados de cateter e hemocultura. Vancomicina (VAN), Oxacilina (OXA), Cefoxitina (CFO), Gentamicina (GEN), Rifampicina (RIF), Penicilina (PEN), Cefalotina (CFL) e Eritromicina (ERI).

Foram testados primers aleatórios da Operon Technology Inc. (OPT13, OPR18, OPR13, OPK18 e OPERON21) e da Invitrogen (RAPD1, RAPD7, M13, 1026, Random

primer, ERIC1 e ERIC2) para padronização das reações de RAPD. Foram selecionados para o estudo os primers mais reprodutíveis, com maior capacidade de diferenciação e com bandas mais bem definidas e fortes para cada grupo de micro-organismos (**Figura 2**). Para a avaliação da capacidade de diferenciação dos primers, foram utilizadas linhagens de padrão internacional *American Type Culture Collection* (ATCC) e amostras da mesma espécie das estudadas, mas não relacionadas, como por exemplo, amostras provenientes de outro hospital. Procurou-se selecionar primers que apresentassem no mínimo seis bandas bem definidas e para melhor reprodutibilidade da técnica de RAPD-PCR foram selecionados dois primers para cada gênero ou espécie do micro-organismo estudado.

Todas as 21 amostras positivas para ICSRC no teste fenotípico (mesmo perfil de sensibilidade entre os isolados de cateter e hemocultura pelo método de disco difusão) foram também positivas para ICSRC pelos métodos genotípicos. Entretanto, 10 amostras foram positivas nos testes genotípicos e negativas no teste fenotípico, sendo que dessas, 7 foram positivas na técnica de RAPD com os dois primers (RAPD 1 e M13) e na técnica de REP-PCR, e três foram positivas na técnica de RAPD com o primer RAPD 1, e somente uma com o primer M13. Não foi verificada diferença estatística significativa entre os resultados obtidos no teste de disco difusão e na técnica de RAPD com os primers estudados.

Os resultados mostraram uma detecção significativa de bactérias do gênero *Staphylococcus* em todos os métodos utilizados para avaliação de ICSRC ($p< 0,0001$). Em relação às espécies de *Staphylococcus*, a análise dos resultados revelou uma frequência maior de *S. epidermidis* associada a essas infecções ($p< 0,0001$).

Segundo Tenover *et al*. (96), a combinação de dois ou mais métodos de tipagem proporciona maior possibilidade de discriminação dos padrões das estirpes estudadas. Neste sentido, observou-se que apesar das diferenças encontradas nas técnicas, a realização das técnicas de RAPD-PCR e de REP-PCR foi importante para a segurança na confirmação das ICSRC, além disso, devido à baixa reprodutibilidade do

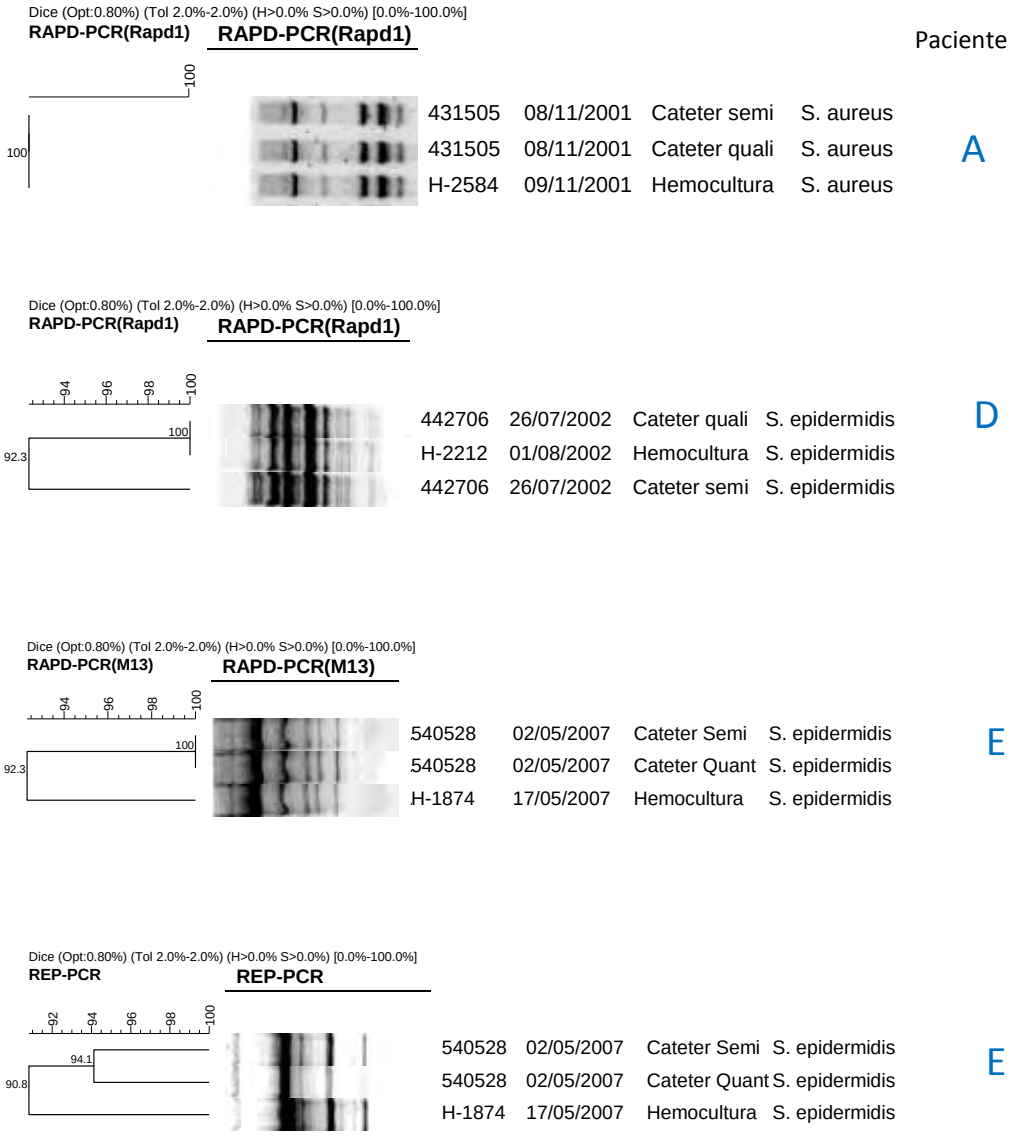

Figura 2: Dendogramas obtidos pela técnica de *Random Amplified Polymorfic DNA Based PCR* (RAPD-PCR) e *Repetitive Extragenic Palindromic Sequence Based*

PCR (REP-PCR) em amostras de *Staphylococcus* spp. isoladas de cateteres e hemoculturas para determinação da similaridade. *S. aureus* com o primer RAPD1 (paciente A), com coeficiente de similaridade 100% e para *S. epidermidis* com o primer RAPD 1 (paciente D), RAPD-PCR com o primer M13 (paciente E) e REP-PCR (paciente E), com coeficiente de similaridade entre 90,8 e 94,1%.

método de RAPD-PCR, a realização de duas reações RAPD-PCR com diferentes primers auxilia em atenuar esse problema.

A determinação do perfil clonal das amostras de *S. epidermidis* isoladas de hemoculturas pela técnica de RAPD-PCR e REP-PCR demonstrou clones de *S. epidermidis* persistentes de 1 a 7 anos na UTI neonatal da Faculdade de Medicina de Botucatu. Todos os clusters majoritários apresentaram isolados que foram associados com ICSRC e, em algumas situações clusters menores também apresentaram isolados associados com ICSRC.

Trabalhos realizados por Huebner *et al*.(97), Neumeister *et al*. (98) e Villari *et al*. (99) também revelaram clones persistentes de *S. epidermidis* em ambiente hospitalar. Villari *et al*. (99) sugerem que uma parcela significativa de infecções por *S. epidermidis* pode ser atribuída à transmissão entre pacientes e que certas cepas podem se tornar endêmicas durante longos períodos.

Estes resultados confirmam a importância da infecção cruzada dos ECN na UTI Neonatal. O sucesso dos clones predominantes nestes estudos pode estar relacionado a fatores ainda não caracterizados que fornecem aos micro-organismos, vantagens na

colonização ou na sua capacidade de infectar pacientes, sendo que possivelmente entre esses fatores estão a formação de biofilme e a resistência a antimicrobianos.

Tenover *et al*. (96) em estudo para comparação de métodos moleculares para a tipagem de isolados de *S. aureus* apresentaram alguns padrões para validade de boas técnicas moleculares, como reprodutibilidade, poder discriminatório, facilidade de uso e a facilidade de interpretação das técnicas, além de, uma intensidade maior das bandas que pode auxiliar na interpretação dos resultados (100). No nosso estudo as técnicas RAPD-PCR e REP-PCR apresentaram um bom poder discriminatório, pois diferenciaram as amostras estudadas das cepas ATCC e de isolados não relacionados com um valor de coeficiente de similaridade ≤ 70. Entretanto, o método de RAPD-PCR com os primers RAPD1 e M13 para amostras de *Staphylococcus* spp. apresentou melhores padrões da bandas quando comparados com o REP-PCR, facilitando assim a interpretação dos resultados. Também foi possível verificar na determinação do perfil clonal das amostras de *S. epidermidis* isoladas de hemoculturas um maior agrupamento dos isolados associados com ICSRC, além da maior facilidade de aplicação do método de RAPD-PCR quando comparado com método de REP-PCR. Apesar de relatos de baixa reprodutibilidade na técnica de RAPD-PCR (101) não foi verificada dificuldade referente a esse aspecto nesse estudo.

As técnicas de tipagem molecular apresentaram um poder discriminatório adicional, principalmente nas infecções causadas por ECN, sendo esse resultado de grande importância, uma vez que esses micro-organismos fazem parte da microbiota normal, e o entendimento das relações entre esses micro-organismos é fundamental para a elucidação das ICSRC.

ANEXO 4

MARCONI C, **CUNHA MLRS**, LYRA JC, BENTLIN MR, BATALHA JEN, SUGIZAKI MF, RUGOLO LMSS. Comparison between qualitatitive and semiquantitative catheter-tip cultures: laboratory diagnosis of catheter-related infection in newborns. Braz J Microbiol. 2008; 39: 262-7.

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COMPARISON BETWEEN QUALITATIVE AND SEMIQUANTITATIVE CATHETER-TIP CULTURES: LABORATORY DIAGNOSIS OF CATHETER-RELATED INFECTION IN NEWBORNS

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ABSTRACT

This prospective study evaluated semiquantitative and qualitative catheter-culture methods for diagnosis of catheter-related infection (CRI) in newborns. Catheter tips from newborns admitted to the Neonatal Unit of the University Hospital of the Botucatu Medical School, UNESP were included in the study. Catheter cultures were performed with both semiquantitative and qualitative techniques. For CRI diagnosis, microorganisms isolated from catheter cultures and from peripheral blood cultures were identified and submitted to agent susceptibility test. The gold standard was the certain CRI diagnosis when same microorganism (specie and profile of susceptibility to agents) was isolated from both catheter tips and peripheral blood culture. A total of 85 catheters from 63 newborns were included in the study. The semiquantitative culture method, despite presenting lower sensitivity (90%), showed higher specificity (71%) when compared to 100% of sensitivity and 60% of specificity in the qualitative method. The identification of the microorganisms obtained from the catheter cultures showed a prevalence of coagulase-negative staphylococci (CNS) species. The specie *Staphylococcus epidermidis* (77.5%) was the prevalent in the catheters with positive semiquantitative cultures. Among 11 episodes with CRI diagnosis, 8 (72.7%) were associated with CNS species, of which 6 were *S. epidermidis*. Two episodes of CRI by *S. aureus* and one by *Candida parapsilosis* were also detected. The semiquantitative catheter-culture method showed advantages for CRI diagnosis in newborns when compared to the conservative qualitative method.

Key-words: Catheter-related infection, catheter culture, semiquantitative culture, newborns, coagulase-negative staphylococci.

INTRODUCTION

The medical advances achieved in neonatal intensive care units (Neonatal ICU) in the last few decades have enabled a significant increase in preterm newborns' survival rates, particularly in those with low birth weight (17,19). Numerous resources have been routinely used in these units, such as mechanical ventilation, parenteral nutrition and the insertion of umbilical catheters as well as the extensive use of antibiotics and long periods in neonatal ICU. These factors, despite contributing to lives of newborns (NB) preservation, act as predisposing factors to the development of nosocomial neonatal infections (14,19,25,32,34,35).

Among all the used resources, intravascular catheters are distinguished. They are frequently used in numerous procedures in neonatal ICU, since they allow rapid intravenous access for the medication administration, parenteral nutrition and others (31). Although the use of catheters is a fundamental

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procedure for NB's survival in neonatal ICU, it is also considered an important risk factor for infection acquisition. Additionally, it contributes to increase the incidence of bloodstream infections and, therefore, increases morbidity rates and hospitalization periods (3).

Similarly to what occurs in adults, nearly 60% of the catheter-related bloodstream infections in NB are caused by gram-positive bacteria, among which coagulase-negative staphylococci (CNS) species are distinguished as they are associated with 37.7% of such infections (5). There are some important catheter contamination sources, however the most frequent is the NB's flora on the catheter insertion site (31). This explains why CNS are the most frequent microorganisms associated with such infections. Since they are the major component of NB skin and mucosal flora, they are recognized as the most frequent etiologic agents in NB infections, mainly in those with low birth weight (< 1,500g) (9,16,20).

Catheter-related infections (CRI) are diagnosed when identical microorganisms are isolated from catheter and blood cultures in absence of other apparent source of infection, except the catheter (4). The culture's reliability depends on the adopted technique. The major catheter-culture methods utilized for CRI diagnosis are the qualitative and semiquantitative methods. The qualitative or conservative broth method is the simplest and the most commonly used. However, the semiquantitative method proposed by Maki *et al.* (24) is recommended for CRI diagnosis by the Centers for Disease Control and Prevention (4). According to Maki's proposal, semiquantitative catheter-tip culture is considered positive in the presence of 15 or more Colony-Forming Units (CFU) growth. Hence, this study aimed to compare two methods for CRI diagnosis in NB.

MATERIAL AND METHODS

Sampling

Eighty-five catheter tips from 63 newborns admitted to the Neonatal Unit of the University Hospital of the Botucatu Medical School, UNESP, between September 2001 and June 2003, were included in this prospective study.

Catheter tips from patients who had presented one or more blood cultures collected close to the date of catheter removal were included. Catheters from NB whose clinical data and laboratory records referring to a one-week period prior to the device's removal date were not available were excluded of the study. The procedures were approved by Medical School Research Ethics Committee.

Catheter culture

The catheter-tip cultures were performed by Maki's semiquantitative method (24). The catheters were aseptically removed by the medical staff and the approximately 5 cm distal tips were collected, placed in dry sterile vials and immediately transported to the laboratory for processing. The segments were rolled on the surface of Blood Agar plates and incubated at 37ºC for 72 hours. The plates were examined daily and counted as soon as growth was detected, the result was expressed in CFU. The catheter tips were also cultured with Qualitative or Conservative method that consisted in immersing the catheter tips in Brain Heart Infusion (BHI) with subsequent incubation at 37ºC for 72 hours. The broths were examined daily and when cloudy, a subculture was performed in Blood Agar.

Blood culture

The blood cultures were collected and cultivated by the Bactec Automated System, according to Koneman *et al.* guidelines (23).

Microorganism identification

The microorganisms were submitted to Gram staining for purity assessment and morphology and specific stain examination. After confirmation of these characteristics, identification tests were performed as recommended by Koneman *et al.* (23).

Identification of coagulase-negative Staphylococci

Identification was performed according Kloos and Bannerman (21), Kloos and Schleifer (22) and Cunha *et al.* (10) with a simplified scheme of biochemical tests which includes the performance of catalase and coagulase tests, as well as of sugar utilization tests: xylose, arabinose, saccharose, trehalose, mannitol, maltose, lactose, xylitol, ribose and fructose, in addition to the characterization of hemolysins, nitrate reduction, urease production, ornithine descarboxylase and Novobiocin resistance (5 µg).

Susceptibility test

The susceptibility test was performed using the technique of agent diffusion from impregnated discs on agar, according National Committee for Clinical Laboratory Standards-NCCLS guidelines (27). For inoculum preparing, BHI cultures of the microorganisms obtained from catheter tips and blood cultures were previously incubated for 4 to 6 hours and adjusted to 0,5 McFarland density scale before plating. The following discs were utilized: Penicillin G (10U), Oxacillin (1µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Cephalothin (30 µg), Netilmicin (30 µg), Gentamicin (30 µg), Novobiocin (5 µg), Cefotaxime (30 µg), Cefaclor (30 µg), Levofloxacin (5 µg), Ofloxacin (5 µg), Rifampin (5 µg), Vancomycin (30 µg) and Teicoplanin (30 µg). Following the incubation at 37ºC for 24 hours, the halos were measured (mm), and the results obtained were compared between the microorganisms isolated from the same NB (catheter and blood cultures) in order to observe the similarity between samples.

Catheter-related infection (CRI) diagnosis

CRI was diagnosed according to CDC guidelines (4) by the presence of two or more of the following signs or symptoms: fever (≥ 38ºC), hypothermia (<36ºC), apnea, bradycardia or shock signs, in addition to the presence of one or more positive blood cultures in patients whose catheter semiquantitative culture was positive, if the same microorganism (specie and agent susceptibility) had been isolated from the catheter and the peripheral blood culture without another apparent source of infection focus except the catheter.

Statistics

Culture accuracy was obtained by determination of sensitivity (S) and specificity (SP), and CRI was calculated from the positive predictive value (PPV) and the negative predictive value (NPV).

RESULTS

Eighty-five catheters from 63 NB admitted to the Neonatal Unit of the University Hospital of the Botucatu Medical School, UNESP were studied. The mean time of catheter use was 10 days, ranging from 1 to 33 days. Among the catheters, 36.6% were the umbilical vascular type, 34.1% umbilical arterial, 17,1% central vascular and 12.2% peripheral.

Of all the catheters included in the study, 54 (63.5%) had negative cultures by Maki's semiquantitative method, of which 13 (15.3%) showed growth with less than 15 CFU, whereas the remaining 41 (48.2%) catheters did not present growth of any colony. The semiquantitative culture was positive, by showing growth equal or superior to 15 CFU, in 31 (36.5%) catheter tips (Table 1).

In relation to the qualitative method, it was observed that 45 (52.9%) catheter tips had negative cultures, whereas the remaining 40 (47.1%) had positive culture by this method, by presenting culture medium clouding (Table 1).

The catheter-related infection diagnosis was observed in 11 episodes. In six (54.5%) of them, it was observed a confluent growth on Blood Agar plates (Fig. 1); other 4 (36.4%) CRI episodes presented culture growth ≥100 CFU, and, in the remaining episode it was verified an 8 CFU growth. Besides this last episode did not present a culture growth superior to 15 CFU, it consisted in a certain CRI diagnosis since it presented infection by the same specie, *Staphylococcus aureus*, with similar agent susceptibility in both catheter and blood culture in this patient.

The results regarding the identification of the microorganisms obtained from the catheter semiquantitative cultures revealed a predominance of CNS species (Table 2) either in cultures with growth <15 CFU (76.9%) or in those presenting

Table 1. Results from the catheter cultures by the semiquantitative and qualitative methods.

Catheter	Catheter culture				
	Semiquantitative			Qualitative	
	No growth	<15 CFU	≥15 CFU	Negative	Positive
N	41	13	31	45	40
%	48.2	15.3	36.5	52.9	47.1

N= Total number of catheters. CFU= Colony-Forming Units.

Table 2. Microorganisms isolated from catheter tips by the semiquantitative culture.

Microorganisms no. (%)	<15 CFU	≥15 CFU	CRI
S. epidermidis	9 (69.2)	24 (77.5)	6 (54.5)
S. warneri	-	1 (3.2)	1 (9.1)
S. simulans	-	1 (3.2)	1 (9.1)
S. haemolyticus	1 (7.7)	-	-
Total CNS	10 (76.9)	26 (83.9)	8 (72.7)
S. aureus	1 (7.7)	1 (3.2)	2 (18.2)
Acinetobacter baumannii		1 (3.2)	
Candida parapsilosis	1 (7.7)	3 (9.7)	1 (9.1)
Gram Positive Rods	1 (7.7)		
Total	13	31	11

Figure 1. Catheter-tip semiquantitative culture with confluent growth.

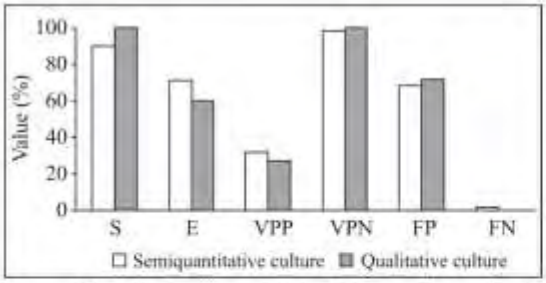

Figure 2. Sensitivity (S), specificity (SP), positive predictive (PPV), negative predictive (NPV), false-negative (FN) and false-positive (FP) values for the semiquantitative and qualitative catheter-culture methods.

growth ≥15 CFU (83.9%). *S. epidermidis* was the prevalent specie (77.5%) related to catheter colonization (≥ 15 CFU). Among the 11 CRI episodes, 8 (72.7%) were associated with CNS species, of which 6 were *S. epidermidis*.

The comparison between the two techniques for CRI diagnosis (Fig. 2) showed 100% sensitivity for the conservative broth culture or qualitative method. For this method, it was also find specificity and positive predictive value of, respectively, 60% and 27%. Even though Maki's semiquantitative method presented lower sensitivity (90%), the rates related to specificity (71%) and positive predictive value (32%) were higher when compared to the qualitative method.

DISCUSSION

The advances achieved in neonatology in the last few decades have enabled significant increase in the survival of preterm newborns as well as in that of newborns with low weight at birth; nevertheless, a progressive increase in the diagnosis of nosocomial infection in neonatal ICU (17) have also been verified as a result. The use of invasive procedures in such units is an important risk factor for the development of nosocomial infections (33), among which catheter-related infections (CRI) are distinguished (3). In this prospective study, 85 catheters from 63 NB admitted to the ICU and submitted to peripheral, umbilical venous, umbilical arterial or central venous catheterization with a mean duration of 10 days were analyzed. Similarly to findings from studies in the literature (7,26,32), no correlation between the catheterization type used and the presence or absence of CRI was found.

CRI is diagnosed when identical microorganisms are detected in both catheter and blood cultures (4), and the comparison of microorganisms through the susceptibility test, although less specific than genotyping methods, is considered to be efficient and relatively inexpensive (28).

In this study, all catheters from NB with confirmed CRI diagnosis presented positive culture by the conservative catheter-tip culture method, or qualitative method. In relation to Maki's semiquantitative culture method (24), it is considered one of the most frequently used techniques for CRI diagnosis by presenting a ≥15 CFU growth, and various studies show its importance (8,28). However, in this study, a catheter tip of a patient with diagnosed *Staphylococcus aureus* CRI presented negative semiquantitative culture, with a growth of only 8 CFU. Other authors also verified the association between CRI and semiquantitative cultures with growth inferior to 15 CFU (8). These results can be explained by either use of antibiotics prior to the culture collection or intraluminal contamination of the catheter, which is a limiting factor to the semiquantitative culture, since it only detects the microorganisms adhered to the external surface of the device (18). On the other hand, some authors point out the contamination of the catheter's external surface as a predominant infection route in short-duration catheters (<30 days), whereas intraluminal contamination is important in prolonged catheterization with a mean duration of 23.4 to 26.5 days (1,18).

Coagulase-negative staphylococci (CNS), which are the major components of NB's skin and mucosa flora (11), are the etiologic agents most frequently involved with CRI in NB (5,20). In our study, the frequencies of microorganisms associated with CRI occurrence are in agreement with data in the literature, since CNS species were related to most of the cases observed (72.7%). Also, these are microorganisms that are knowingly related to most CRI episodes in NB (5,7,19). In this study, two cases of CRI by *S. aureus* and one case by *Candida parapsilosis* were also detected and both microorganisms are cited in the literature as frequent, although not prevalent, etiologic agents in CRI cases in NB (7,29). From 1991 to 1999, CNS were responsible for 37.7% of CRI in pediatric ICUs (5), which was followed by *S. aureus* (12.6%), whereas *Candida* spp. represented 8% to 9% of the findings. Various preceding studies on adult patients also showed that the three major agents causing CRI are coagulase-negative staphylococci, *S. aureus* and *Candida* spp. (2,12,15). The two former agents mainly originate from the skin during catheter insertion or from the health care staff's hands. *Candida albicans*, followed by *C. parapsilosis* are the main representatives of this gender in catheter-related infections (6,13,15).

Among the 8 CRI cases associated with CNS species, *Staphylococcus epidermidis* was present in 6 (54.5%) cases. Such higher frequency of *S. epidermidis* in this disease is expected, since, according to D'Angio (11), this species is predominant in NB's flora around the fourth day of life. Such predominance in the colonization of individuals and the greater pathogenicity of some strains can explain the fact that *S. epidermidis* is the species most commonly associated with infectious processes in NB as reported in a study performed by

Cunha *et al.* (9). CNS were also the prevalent organisms in catheter colonization, that is, catheters with growth ≥ 15 CFU.

The comparison between the qualitative and semiquantitative catheter-culture techniques showed agreement with data in the literature. In a metaanalysis by Safdar *et al.* (30), the sensitivity mean found for the qualitative culture was of 90%, while specificity was 72%. Nevertheless, although the semiquantitative culture showed lower sensitivity (85%), it exhibited higher specificity (82%). In this study, although the sensitivity found in the qualitative-culture method was 100% in contrast to 90% shown by the semiquantitative method, specificity was lower for the qualitative method, where 60% was found in contrast to 71%. The semiquantitative culture also showed larger positive predictive value (PPV), in addition to being a more rapid technique, since it is capable of detecting positive results in up to 24 hours.

It was concluded that the semiquantitative culture is a rapid and efficient technique for diagnosing catheter-related infection in NB. However, it requires careful interpretation, and its result must be part of a set of factors that can indicate diagnosis and a specific treatment.

RESUMO

Comparação entre culturas qualitativa e semiquantitativa de ponta de cateter: Diagnóstico laboratorial de infecção relacionada a cateter em recém-nascidos

Este estudo prospectivo avaliou os métodos semiquantitativo e qualitativo de cultura de cateter para o diagnóstico de infecção relacionada a cateter (IRC) em recém-nascidos (RN). Foram incluídas pontas de cateteres provenientes de recém-nascidos internados na Unidade Neonatal do Hospital das Clínicas da Faculdade de Medicina de Botucatu, UNESP. Foram utilizadas as técnicas semiquantitativa e qualitativa de cultura de cateter. Para o diagnóstico de IRC, os microrganismos isolados das culturas de cateteres e de hemoculturas periféricas foram identificados e submetidos ao teste de sensibilidade a antimicrobianos. O padrão ouro correspondeu ao diagnóstico de certeza de IRC, com o isolamento do mesmo microrganismo (espécie e perfil de sensibilidade a antimicrobianos) isolado em hemocultura periférica. Foram estudados 85 cateteres provenientes de 63 RN. A cultura semiquantitativa, embora tenha apresentado menor sensibilidade (90%), apresentou uma maior especificidade (71%) em comparação à sensibilidade de 100% e especificidade de 60% encontradas na cultura qualitativa. Através da identificação dos microrganismos obtidos nas culturas de cateteres, observou-se uma predominância de espécies de Estafilococos coagulase-negativa (ECN). A espécie *Staphylococcus epidermidis* foi a prevalente (77,5%) nos cateteres com culturas semiquantitativas positivas. Dos 11 episódios de IRC diagnosticados, 8 (72,7%) foram associados a espécies de ECN, dos quais 6 eram da espécie *S. epidermidis*. Também foram detectados dois casos de IRC por *S. aureus* e um caso por *Candida parapsilosis*. O método de cultura semiquantitativo cateter apresentou vantagens para o diagnóstico de IRC em RN quando comparado com o método qualitativo tradicional.

Palavras-chave: Infecção relacionada a cateter, cultura de cateter, cultura semiquantitativa, recém-nascidos, Estafilococos coagulase-negativa.

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ANEXO 5

MARCONI C, **CUNHA MLRS**, LYRA JC, BENTLIN MR, BATALHA JEN, SUGIZAKI MF, CORRENTE JE, RUGOLO LMSS. Usefulness of catheter tip culture in the diagnosis of neonatal infections. J Pediatr (Rio J.) 2009; 85: 80-3.

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Usefulness of catheter tip culture in the diagnosis of neonatal infections

Utilidade da cultura da ponta de cateter no diagnóstico de infecção neonatal

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Resumo

Objetivo: Determinar o número de unidades formadoras de colônias que melhor correlaciona com a infecção relacionada a cateter em recém-nascidos.

Métodos: Este foi um estudo prospectivo de culturas semiquantitativas de pontas de cateteres de recém-nascidos da unidade neonatal da Faculdade de Medicina de Botucatu. Os microrganismos isolados de cateteres e hemoculturas periféricas foram identificados e submetidos ao teste de sensibilidade a drogas. O ponto de corte ótimo foi determinado pela curva *receiver operating characteristic* (ROC).

Resultados: Foram estudados 85 cateteres de 63 recém-nascidos. A espécie *Staphylococcus epidermidis* foi prevalente (75%) nos cateteres. Dos 11 episódios de infecção diagnosticados, oito (72,7%) foram associados aos estafilococos coagulase-negativa, dos quais seis pertenciam à espécie *S. epidermidis*. Pela curva ROC, o ponto de corte ótimo para o diagnóstico de infecção relacionada a cateter foi 122 unidades formadoras de colônias.

Conclusão: O ponto de corte 122 unidades formadoras de colônias melhor se correlacionou com o diagnóstico de infecção relacionada a cateter em recém-nascidos.

J Pediatr (Rio J). 2009;85(1):80-83: Infecção relacionada a cateter, cultura de cateter, cultura semiquantitativa, recém-nascidos, estafilococos coagulase-negativa.

Abstract

Objective: To determine the number of colony-forming units (CFU) that best correlates with catheter-related infections (CRI) in newborns.

Methods: This was a prospective study of semiquantitative cultures of catheter tips obtained from newborns in the neonatal unit at Faculdade de Medicina de Botucatu, state of São Paulo, Brazil. The microorganisms isolated from catheter and peripheral blood cultures were identified and submitted to a drug susceptibility test. The optimal cutoff point was determined by the receiver operating characteristic (ROC) curve.

Results: A total of 85 catheters obtained from 63 newborns were studied. *Staphylococcus epidermidis* was the predominant species in the catheters (75%). Eight of 11 (72.7%) CRI episodes were associated with coagulase-negative staphylococci, six of which were of the *S. epidermidis* type. ROC curve analysis indicated that the optimal cutoff point for the diagnosis of CRI was 122 CFU.

Conclusion: The cutoff point of 122 CFU correlated best with the diagnosis of CRI in newborns.

J Pediatr (Rio J). 2009;85(1):80-83: Catheter-related infection, catheter culture, semiquantitative culture, newborn, coagulase-negative *Staphylococcus*.

Introdução

Observa-se um aumento progressivo na sobrevida dos recém-nascidos prematuros e com baixo peso ao nascimento. A utilização de procedimentos invasivos, como os cateteres intravasculares, constitui importante fator de risco para o desenvolvimento de infecções nosocomiais, dentre as quais se destacam as infecções relacionadas a cateter (IRC)[1,2].

As IRC são diagnosticadas quando microrganismos idênticos são isolados de culturas de cateteres e das hemoculturas sem fonte aparente de infecção, exceto seu cateter[3]. O

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principal método de cultura utilizado com esta finalidade é o semiquantitativo, proposto por Maki et al.[4] e recomendado para o diagnóstico de IRC pelo Centers for Disease Control and Prevention[3]. Segundo critérios de Maki et al.[4], são consideradas culturas positivas de cateter aquelas que apresentam crescimento ≥ 15 unidades formadoras de colônias (UFC), já que este número de UFC melhor correlaciona com a presença de infecção quando comparado com o método qualitativo de cultura de cateter, onde a infecção e contaminação são indistinguíveis. Embora outros autores já tenham realizado estudos para avaliação de pontos de corte em pacientes adultos[4,5], não foram encontrados estudos que avaliam o melhor ponto de corte da cultura semiquantitativa para o diagnóstico de IRC em recém-nascidos. Sendo assim, este estudo objetivou determinar o número de UFC que melhor se correlaciona com a presença de IRC em recém-nascidos.

Métodos

Neste estudo prospectivo, foram incluídas 85 pontas de cateteres provenientes de 63 recém-nascidos internados na unidade neonatal do Hospital das Clínicas da Faculdade de Medicina de Botucatu (UNESP), SP, no período fixado de 2 anos, de setembro de 2001 a agosto de 2003. Os procedimentos foram aprovados pelo Comitê de Ética em Pesquisa da Faculdade de Medicina.

Foram incluídos neste estudo microrganismos isolados a partir de pontas de cateteres e de hemoculturas de pacientes que possuíam hemoculturas coletadas próximas à data de remoção dos cateteres. Foram excluídas as amostras isoladas de recém-nascidos cujos dados clínicos e laboratoriais referentes a um período de 1 semana anterior e 1 semana posterior à data de remoção do cateter não foram localizados.

As culturas de pontas de cateteres foram realizadas pelo método semiquantitativo de Maki et al.[4], e as hemoculturas foram colhidas e cultivadas pelo sistema automatizado Bactec, conforme as normas descritas por Koneman et al.[6]. A identificação dos microrganismos foi realizada conforme preconizado por Koneman et al.[6]. e, para a identificação das espécies de estafilococos coagulase-negativa (ECN), foram utilizados os critérios propostos por Kloos & Scheifeir[7] e Kloos & Bannerman[8], utilizando um esquema simplificado de provas bioquímicas. O teste de sensibilidade às drogas antimicrobianas foi realizado pela técnica da difusão da droga em ágar a partir de discos impregnados, conforme critérios recomendados pelo National Committee for Clinical Laboratory Standards (NCCLS)[9]. Os halos de inibição foram medidos (mm), e os resultados foram comparados entre os germes isolados do mesmo recém-nascido (cateter e hemocultura) para verificar a similaridade entre as amostras.

O diagnóstico de certeza de IRC foi definido segundo critérios propostos pelo Centers for Disease Control (CDC)[3], pela presença de dois ou mais dos seguintes sinais e sintomas: febre (≥ 38 ºC), hipotermia (< 36 ºC), apnéia, bradicardia ou sinais de choque, além da presença de pelo menos uma hemocultura positiva em paciente cujo cateter vascular apresentou cultura semiquantitativa positiva. O mesmo microrganismo (espécie e perfil de sensibilidade às drogas) foi isolado a partir do cateter e da hemocultura periférica, sem fonte aparente de outro foco de infecção, exceto seu cateter.

Para a avaliação dos pontos de corte da cultura semiquantitativa no diagnótico de IRC, foram calculadas a sensibilidade e especificidade para os números de UFC das culturas. O padrão-ouro correspondeu ao diagnóstico de certeza de IRC, com o isolamento do mesmo microrganismo (espécie e antibiograma) nas culturas de cateter e nas hemoculturas.

O ponto de corte ótimo foi determinado pela curva *receiver operating characteristic* (ROC), através da representação da taxa de verdadeiros positivos (sensibilidade) no eixo y, contra a taxa de falsos positivos (1 - especificidade) no eixo x. Foram analisadas todas as contagens obtidas nas culturas dos cateteres: 1, 2, 7, 8, 9, 11, 12, 17, 22, 23, 30, 36, 60, 73, 122, 125, 130, 150, 193 e 300 UFC.

Resultados

Foram estudadas 85 pontas de cateteres provenientes de 63 recém-nascidos internados na UNESP. A identificação dos microrganismos das culturas de cateteres revelou uma predominância de espécies de ECN (81,8%) (Tabela 1), dentre as quais o *Staphylococcus epidermidis* foi a espécie mais frequente (75%).

A análise do perfil de sensibilidade a drogas dos isolados provenientes de 17 pacientes cujas culturas de cateter e sangue provenientes foram positivas para a mesma espécie de microrganismo permitiu o diagnóstico de IRC em 11 do total de casos. Com relação aos microrganismos associados à etiologia dos episódios de IRC diagnosticados, oito (72,7%) foram associados a espécies de ECN, dos quais seis (54,5%) pertenciam à espécie *Staphylococcus epidermidis*. Também foram detectados dois casos de IRC por *S. aureus* e um por *Candida parapsilosis*.

Através da análise da curva ROC, o ponto de corte ótimo correspondeu a 122 UFC, já que apresentou maior sensibilidade (91%), maior especificidade (81,1%) e maiores valores preditivos positivo (41,7%) e negativo (98,4%) quando comparado aos outros pontos de corte avaliados. O cálculo do comprimento do intervalo de confiança a 95% (IC95%), mostrou que o tamanho amostral foi adequado para a determinação dos pontos de corte estudados, e a área abaixo da curva correspondeu a 0,860, indicando bom ajuste da curva ROC. Do total de 11 casos diagnosticados de IRC, 10 (90,9%) apresentaram crescimento ≥122 UFC e em apenas um caso verificou-se o crescimento de oito UFC.

Discussão

O diagnóstico das IRC nas unidades neonatais tem sido realizado conforme o recomendado pelo CDC[3], sendo similares aos empregados para o diagnóstico dessa infecção em

Tabela 1 - Espécies de microrganismos isolados a partir das culturas semiquantitativas de cateter

Microrganismos	Crescimento positivo n (%)	IRC n (%)
Staphylococcus epidermidis	33 (75)	6 (54,5)
Staphylococcus warneri	1 (2,3)	1 (9,1)
Staphylococcus simulans	1 (2,3)	1 (9,1)
Staphylococcus haemolyticus	1 (2,3)	-
Total ECN	36 (81,8)	8 (72,7)
Staphylococcus aureus	2 (4,5)	2 (18,2)
Acinetobacter baumannii	1 (2,3)	-
Candida parapsilosis	4 (9,1)	1 (9,1)
Bacilo gram-positivo	1 (2,3)	-
Total	44	11

ECN = estafilococos coagulase-negativa; IRC = infecção relacionada a cateter.

pacientes adultos. Devido à inexistência na literatura de trabalhos específicos para o diagnóstico de IRC em recém-nascidos, este estudo prospectivo foi conduzido.

As espécies envolvidas na etiologia dos casos de IRC neste estudo estão de acordo com a literatura, já que os ECN foram associados a 81,8% dos casos de IRC e esses são os microrganismos mais frequentemente envolvidos com a IRC em recém-nascidos[10,11]. Também foram observados episódios de IRC por *S. aureus* e *Candida parapsilosis*, sendo que ambos microrganismos são considerados frequentes, embora não predominantes, agentes etiológicos de IRC em recém-nascidos[11,12].

Dentre os casos de IRC associados a espécies de ECN, a espécie *S. epidermidis* foi a mais encontrada. A maior frequência do *S. epidermidis* nesta intercorrência é esperada, já que é a espécie predominante na flora do recém-nascido. Esta predominância na colonização dos indivíduos e a maior patogenicidade de algumas cepas podem explicar o fato de o *S. epidermidis* ser a espécie mais comumente associada aos processos infecciosos em recém-nascidos conforme o relatado em estudo realizado por Cunha et al.[13].

A análise da curva ROC demonstrou que o ponto de corte de 122 UFC revelou maior especificidade, sem perda da sensibilidade quando comparado com 15 UFC, ponto de corte recomendado pelo CDC[3] cujos valores de sensibilidade, especificidade e valor preditivo positivo (VPP) foram, respectivamente, 91,0, 71,6 e 32,3% em nossos resultados. Em estudo realizado por Collignon et al.[5],com pacientes adultos, foi encontrado que o melhor ponto de corte para a detecção de IRC é de ≥ 5 UFC. Considerando que a cultura semiquantitativa de pontas de cateteres é útil para o diagnóstico de IRC, um teste com alta especificidade e VPP é necessário e desejado; entretanto, o VPP encontrado por Collignon et al.[5] foi de apenas 8,8%. Segundo Brun et al.[14], esses autores deveriam escolher como melhor ponto de corte o crescimento de ≥ 100 UFC, ao invés de ≥ 5 UFC, pois um teste de diagnóstico com VPP menor que 10% não pode ser considerado adequado para uso no diagnóstico clínico. Em nosso estudo, o ponto de corte de ≥ 122 UFC correspondeu a menor número de falsos-positivos e maior especificidade e VPP quando comparado com os outros valores estudados.

Apesar da maioria dos casos de IRC apresentarem crescimento superior a 122 UFC, foi observado um caso de IRC por *S. aureus* cuja cultura apresentou crescimento de apenas oito UFC. Outros autores também têm verificado IRC e culturas semiquantitativas com crescimento < 15 UFC[5]. Esses resultados podem ser explicados pelo uso de antibióticos antes da cultura ou então pela contaminação intraluminal do cateter, que constitui o fator limitante da cultura semiquantitativa, a qual detecta somente os microrganismos aderidos na superfície externa do dispositivo[15]. Outra desvantagem dessa metodologia é a necessidade de retirada do cateter para a realização da cultura, de forma que outras metodologias de diagnóstico de IRC devem ser consideradas, como a utilização de hemoculturas pareadas de veia periférica e cateter que dispensam a retirada do dispositivo.

Concluiu-se que a cultura semiquantitativa com crescimento ≥ 122 UFC melhor se correlaciona com a presença de infecção relacionada a cateter em recém-nascidos quando comparado com o crescimento de ≥ 15 UFC, que é o valor considerado até hoje na literatura. Entretanto, sua interpretação deve ser cautelosa, devendo esse resultado fazer parte de um conjunto de fatores que indicam o diagnóstico e um tratamento específico.

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5. Identificação de *Staphylococcus* spp.

Apesar do aumento na significância clínica de espécies de ECN, na maioria dos laboratórios de rotina, os estafilococos são identificados somente com base em aspectos morfológicos das colônias, coloração de gram, produção de catalase e coagulase, que permitem apenas a classificação de estafilococos isolados de amotras clínicas somente em *S. aureus* e não-*S. aureus*, com o último simplesmente sendo classificado como ECN.

No entanto, com o aumento na ocorrência de infecções causadas por diferentes espécies de ECN, torna-se cada vez mais importante aprender mais sobre a epidemiologia, resistência aos antimicrobianos e o potencial patogênico das espécies individualmente. Isto pode ser particularmente importante em relação a isolados de hemocultura, uma vez que muitas vezes é difícil determinar a significancia clínica do ECN isolado.

Kloos e Schleifer (9) delinearam uma chave a partir da qual os ECN podem ser facilmente diferenciados em espécies através de suas características bioquímicas. O esquema proposto por esses autores e modificado por Bannerman (5) é o método usado convencionalmente. No entanto, este método é relativamente trabalhoso para ser utilizado na rotina dos laboratórios de microbiologia clínica devido ao grande número de testes bioquímicos.

A identificação precisa dos ECN é importante para fazer uma previsão do potencial patogênico de cada espécie e do perfil de susceptibilidade a antibióticos, permitindo assim uma conduta mais adequada na avaliação da significancia clínica de

cada espécie. Há divergências na literatura específica sobre o significado clínico da identificação de ECN. De acordo com os dados de alguns autores (102), esse procedimento não é clinicamente significativo, embora outros pesquisadores (56) acreditem que essa identificação seja importante na diferenciação entre contaminação e infecção. A identificação dos ECN é de grande importância para a associação de certas espécies com infecções específicas (103), tendo em vista que alguns dados sugerem que além de *S. epidermidis* e *S. saprophyticus*, que têm sido considerados patogênicos, algumas espécies como *S. haemolyticus*, *S. lugdunensis* e *S. schleiferi* estão mais associados às infecções do que outras espécies (104-105). Os isolados repetidos de ECN de pacientes com doenças invasivas devem ser identificados para permitir uma comparação das cepas. Em adição, a identificação de espécies é um pré-requisito antes de iniciar os procedimentos para realização de estudos epidemiológicos.

O desenvolvimento de métodos para a identificação de espécies e subespécies de estafilococos permite obter informações sobre a variedade de ECN presentes em amostras clínicas e considerá-los como agentes etiológicos de processos infecciosos. Nos últimos anos, vários sistemas comerciais para a rápida identificação de estafilococos foram desenvolvidos como uma alternativa para os protocolos de identificação clássica (5). No entanto, estes sistemas de diagnóstico apresentam problemas, tais como custo e tempo de incubação, e muitas vezes fornecendo resultados não confiáveis (106-107). Além disso, muitos desses kits foram projetados para o identificação de todas as espécies conhecidas de ECN (provenientes de amostras clínicas, veterinárias, e de alimentos) e, portanto, não são muito

específicos. Com base nas considerações acima e tendo em vista a necessidade de métodos rápidos, simples e confiáveis, foi desenvolvido um estudo com o objetivo de comparar quatro técnicas para a identificação de ECN, ou seja, um método de referência (5, 9), o comercial API Staph miniaturizado e dois métodos modificados em nosso laboratório, além da proposição de uma chave simplificada com a finalidade de desenvolver métodos alternativos de identificação que combinam confiabilidade, simplicidade e baixo custo, especialmente para locais com recursos limitados (ANEXO 6).

Dois métodos de identificação modificados em nosso laboratório foram usados (método simplificado e o método de disco). O método simplificado foi dividido em duas etapas. Durante a primeira etapa, a fermentação de xilose, sacarose, trealose, maltose e manitol, produção de hemolisina e crescimento anaeróbio em tioglicolato foram testados. Os testes utilizados na segunda etapa variaram de acordo com os resultados obtidos na primeira etapa após 72 h de incubação a 37^{O}C. Os 100 isolados de amostras clínicas de estafilococos foram testados pelo quatro métodos propostos simultaneamente. Linhagens de referência internacionais (ATCC) de *S. epidermidis*, *S. simulans*, *S. saprophyticus* e *S. xylosus* foram corretamente identificadas pelos quatro métodos. Das linhagens de referência somente o *S. warneri* ATCC 10209 foi erroneamente identificado pelo sistema API Staph, com a caracterização desta estirpe como S. *saprophyticus* (ID% = 58,7%), como *S. hominis* (ID% = 19,5%), ou como *S. warneri* (% ID = 15,8%). O método simplificado realizado em duas etapas não diferiu do método de referência em termos de identificação de espécies de ECN. Identificação imprecisa pelo sistema API Staph foi observado para *S. epidermidis* (2,2%), *S. warneri*

(47,1%), *S. hominis* (25%) e *S. haemolyticus* (37,5%). A sensibilidade e especificidade do método simplificado foi de 100% para todas as espécies estudadas. O método de disco mostrou uma sensibilidade de 93,8% para o identificação de *S. hominis* devido à não-fermentação da sacarose no disco, levando à classificação desta estirpe como *S. caprae* e 100% de sensibilidade para a outras espécies, enquanto a especificidade foi de 98,8% para *S. hominis*, 98,9% para *S. caprae*, e 100% para as outras espécies.

O método simplificado usando o esquema de identificação proposto levou à identificação de *S. epidermidis*, *S. hominis*, *S. xylosus*, *S. capitis* e *S. simulans* em uma única etapa, com um total de sete testes bioquímicos, um número inferior ao empregado no método de referência (16 testes). Desde que *S. epidermidis* é a espécie mais frequentemente isolada, 70 a 90% das amostras isoladas no laboratório clínico podem ser identificadas utilizando um número reduzido de testes.

O método comercial API Staph kit apresentou a menor precisão na identificação de ECN entre os métodos estudados (84% de concordância), resultados concordantes com os obtidos por Bannerman *et al*. (108) e Renneberg *et al*. (109). As espécies *S. warneri* e *S. hominis* foram as mais difíceis de identificar. Bannerman *et al*. (108) também relataram uma menor precisão na identificação destas espécies. Em estudo de Ieven *et al*. (110), *S*. *hominis* foi identificado com o mínimo de precisão pelo sistema API Staph ID 32. Este achado pode ser explicado pela falta de testes complementares, como resistência a novobiocina, crescimento anaeróbio em tioglicolato e produção de hemolisina. Três (3%) das 100 cepas analisadas pela API Staph foram identificadas erroneamente como *S. aureus*, fato também relatado por Renneberg *et al*. (109). O kit demonstrou ser ineficiente nestes casos, uma vez que não solicita o resultado do teste

fundamental e mais amplamente aceito para a identificação de *S. aureus*, ou seja, o teste de coagulase (23).

Em diversos laboratórios de rotina a identificação de *S. saprophyticus* é realizada principalmente com base na resistência a novobiocina (5µg), ausência de hemólise, e teste negativo para coagulase e/ou DNAse. Entretanto, tem sido reconhecido que outras espécies de ECN, incluindo *S. cohnii*, *S*. *sciuri*, *S. xylosus* e *S. hominis* subesp. *novobiosepticus* são também resistentes à novobiocina nessa concentração (23, 111). Esses resultados sugerem que provas adicionais, incluindo fermentação de carboidratos e outros testes, devam ser usadas em conjunto com a prova de sensibilidade à novobiocina para a correta identificação de espécies de ECN (112).

Nos últimos anos, diversos sistemas comerciais para identificação de estafilococos foram desenvolvidos como uma alternativa para o protocolo de identificação clássico de Bannerman (5), que é muito trabalhoso e demorado para ser utilizado na rotina laboratorial. Os sistemas automatizados e kits comerciais, com base em testes bioquímicos miniaturizados, são amplamente utilizados atualmente, tanto em laboratórios de rotina como de pesquisa. No entanto, estes sistemas de diagnóstico apresentam problemas, tais como custo, tempo de incubação, e o mais importante, ainda não são capazes de fazer uma diferenciação confiável entre as diferentes espécies de ECN devido à expressão variável das características fenotípicas. Além disso, muitos destes sistemas automatizados e kits são baseados em resultados colorimétricos e a subjetividade na sua interpretação pode levar à ambiguidade (113).

Bannerman *et al*. (108) avaliaram a base atualizada do cartão de identificação GPI (Identificação de Gram-Positivo) usado com o sistema automatizado de identificação bacteriana Vitek I (Biomérieux) em 500 isolados clínicos. A concordância global entre o cartão GPI e os métodos convencionais foi de 89%. O cartão identificou 92% dos isolados de *S. epidermidis*, 95% de *S. haemolyticus*, 88% de *S. capitis* subesp. *capitis* e 100% de *S. saprophyticus* estudados. Os micro-organismos não incluídos na base de dados, como *S. lugdunensis*, ou foram identificados erroneamente ou não foram identificados pelo cartão GPI.

Na avaliação realizada por Perl *et al*. (107), o cartão GPI identificou corretamente apenas 67% de 185 isolados. Esses pesquisadores ressaltaram que o baixo rendimento do cartão GPI em seu estudo pode ter sido devido à preponderância de estafilococos "não *S. epidermidis*" entre os 227 isolados avaliados.

Outra técnica utilizada para identificação de estafilococos é a técnica da PCR que permite a identificação genotípica de várias espécies de estafilococos com alta sensibilidade e especificidade. A técnica de ITS-PCR permite a análise dos espaços intergênicos transcritos ou "intergenic transcribed spacers" (ITS) entre os loci gênicos 16S e 23S do RNAr, técnica usada frequentemente em PCR "fingerprint" para identificação e discriminação de linhagens bacterianas em nível de espécie e subespécie (114). Em gel de poliacrilamida ou agarose, as regiões amplificadas formam um padrão de bandas específico para cada espécie. Utilizando os respectivos padrões ATCC, pode-se comparar o padrão de bandas das amostras de referência com as amostras investigadas, não restando dúvidas quanto à identificação precisa das diferentes espécies de estafilococos. Esse método foi originalmente descrito por Barry

et al. (115), sendo a identificação de *Staphylococcus* também estudada por Jensen *et al.* (116), que aplicaram a técnica com sucesso na diferenciação de linhagens de quatro espécies de estafilococos: *S. aureus*, *S. epidermidis*, *S. saprophyticus*, e *S. warneri.*

Nos últimos dez anos, a técnica de ITS-PCR tem sido muito utilizada na tipagem de linhagens bacterianas, pois as regiões ITS possuem grande polimorfismo (116-117). Esse polimorfismo é devido à presença de genes de RNAt, responsáveis pelo comprimento e sequência das regiões entre operons, semelhantes nas mesmas espécies (116,118). O estudo da taxa de polimorfismos das regiões intergênicas transcritas (16S e 23S do RNAr) mostrou-se útil, tanto epidemiologicamente quanto taxonomicamente amplificando as regiões 16S e 23S do RNAr utilizando os primers G1 e L1 descritos por Jensen *et al*. (116).

Couto *et al*. (113) aplicaram a técnica de ITS-PCR para identificar 600 amostras de estafilococos originárias de diferentes hospitais, usando como controle positivo 29 linhagens de referência (ATCC) das espécies reconhecidas no gênero *Staphylococcus*. As 29 espécies estafilocócicas apresentaram padrão singular de ITS-PCR, provando ser um método rápido e seguro para identificação de estafilococos de amostras clínicas, fornecendo alta confiabilidade, reprodutibilidade e rapidez.

Análises de DNA tem sido o método de preferência na identificação de micro-organismos, pois possuem maior especificidade e sensibilidade. Amostras de ECN não identificadas em nível de espécie ou identificadas erroneamente por testes fenotípicos convencionais podem ser corretamente identificadas através de técnicas genotípicas. Apesar da diminuição dos preços das técnicas moleculares nos últimos anos, esses

métodos ainda continuam apresentando um custo muito elevado para serem utilizados na rotina em laboratórios de microbiologia clínica. Assim, esforços significativos tem sido feitos no intuito de desenvolver métodos alternativos de identificação combinando velocidade, confiabilidade e baixo custo. Estudo desenvolvido em nosso laboratório (ANEXO 7) objetivou comparar três métodos de identificação fenotípica para estafilococos isolados de infecções urinárias, incluindo o esquema simplificado de provas bioquímicas proposto em estudo anterior, a utilização da prova de sensibilidade ao disco de novobiocina e o sistema automatizado de identificação bacteriana Vitek I, utilizando como método de referência a identificação genotípica através da técnica de ITS-PCR.

Entre as 101 amostras estudadas, todos os 17 *S. aureus* foram positivos para coagulase, DNAse e gene *coa*, entretanto, dos 84 isolados de ECN dois apresentaram DNAse positiva, porém foram negativos para coagulase e gene *coa*, sendo que as 82 amostras restantes foram negativas para coagulase, DNAse e gene *coa*. A prova da DNAse apresentou uma sensibilidade de 100,0% e especificidade de 97,6% e a coagulase obteve valores iguais ao método genotípico de referência (gene *coa*) com sensibilidade e especificidade de 100,0% e taxas de concordância de 98,0% para DNAse e 100,0% para coagulase.

Em relação à comparação da sensibilidade e especificidade entre os métodos de identificação para todas as espécies estudadas, o disco de novobiocina obteve sensibilidade de 89,1% e especificidade de 89,1%, sendo que para Vitek I a sensibilidade e especificidade foram de 81,2% e 92,0% respectivamente, e para o método simplificado de provas bioquímicas a sensibilidade e especificidade foram de

98,0%. Em relação à identificação de *S. saprophyticus*, o método simplificado de provas bioquímicas e a prova de sensibilidade a novobiocina tiveram sensibilidade e especificidade de 100,0%, sendo que o Vitek I apresentou 84,2% de sensibilidade e 100,0% de especificidade. Apesar da rápida identificação (2 a 15 horas) e do grande número de provas bioquímicas (29 provas), o Vitek I falhou em identificar as espécies de ECN mais encontradas em amostras de urina (*S. saprophyticus*), devido principalmente a não detecção da resistência a novobiocina em alguns isolados (6/9)*;* e *S. epidermidis*, o segundo ECN mais frequente em amostras urinárias e o primeiro em outros materiais clínicos, pela falha na prova da sacarose (5/5). Em relação às duas espécies identificadas erroneamente pelo método simplificado de provas bioquímicas, *S. haemolyticus* apresentou resultado incorreto para prova de urease, sendo que o mesmo isolado também foi urease positiva no Vitek I, sugerindo que raras espécies de *S. haemolyticus* possam apresentar urease positiva, e *S. epidermidis* apresentou prova da trealose positiva, podendo ter ocorrido uma possível contaminação do açúcar, visto que o mesmo isolado foi trealose negativa no Vitek I.

Kim *et al*. (119), compararam os resultados de 120 amostras clínicas de ECN identificadas pelo Vitek 2 e pelo Microseq 500 system (*Applied Biosystems*), um sistema comercial para análise do gene 16S RNAr. O Vitek 2 identificou corretamente 105 (87,5%) dos isolados, e incorretamente 6 (5,0%), e quando o resultado com baixo nível de discriminação e a correta identificação foram consideradas em conjunto, o índice de concordância foi de 95,0% (114/120). Portanto, mesmo quando foi utilizado um aparelho mais atual e um software atualizado, as taxas de concordância entre o método genotípico de identificação e a automação, foram inferiores as taxas de

concordância entre o método simplificado de provas bioquímicas (98,0%) e o método genotípico, encontrados em nosso estudo, confirmando que, os sistemas automatizados apesar de mais rápidos, ainda não são capazes de fazer uma diferenciação confiável entre as diferentes espécies de ECN.

ANEXO 6

CUNHA MLRS, SINZATO YK, SILVEIRA LVA. Comparison of methods for the identification of coagulase-negative staphylococci. Mem Inst Oswaldo Cruz. 2004; 99(8): 855-60.

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Comparison of Methods for the Identification of Coagulase-negative Staphylococci

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Coagulase-negative staphylococci (CNS) species identification is still difficult for most clinical laboratories. The scheme proposed by Kloos and Schleifer and modified by Bannerman is the reference method used for the identification of staphylococcal species and subspecies; however, this method is relatively laborious for routine use since it requires the utilization of a large number of biochemical tests. The objective of the present study was to compare four methods, i.e., the reference method, the API Staph system (bioMérieux) and two methods modified from the reference method in our laboratory (simplified method and disk method), in the identification of 100 CNS strains. Compared to the reference method, the simplified method and disk method correctly identified 100 and 99% of the CNS species, respectively, while this rate was 84% for the API Staph system. Inaccurate identification by the API Staph method was observed for Staphylococcus epidermidis *(2.2%),* S. hominis *(25%),* S. haemolyticus *(37.5%), and* S. warneri *(47.1%). The simplified method using the simple identification scheme proposed in the present study was found to be efficient for all strains tested, with 100% sensitivity and specificity and proved to be available alternative for the identification of staphylococci, offering, higher reliability and lower cost than the currently available commercial systems. This method would be very useful in clinical microbiology laboratory, especially in places with limited resources.*

Key words: coagulase-negative staphylococci - methods - identification - API Staph

Forty species of the genus *Staphylococcus* have been identified thus far (Trülzsch et al. 2002, Bannerman 2003, Kwok & Chow 2003, Spergser et al. 2003). *S. aureus*, a coagulase-positive species which produces a series of other enzymes and toxins, is the best known and has been frequently implicated in the etiology of a series of infections and intoxications in animals and humans, whereas coagulase-negative staphylococci (CNS), representing the majority of species, have been considered to be saprophytic or rarely pathogenic (Kloos & Schleifer 1975). Over the last decade, however, CNS have been recognized as the etiological agents of a series of infectious processes, representing the microorganisms most commonly isolated from blood cultures (Huebner & Goldmann 1999).

About half of CNS species naturally colonize humans, and at present they are considered essentially opportunistic etiological agents, that prevail in numerous organic situations to produce severe infections (Bannerman 2003). The emergence of CNS as pathogens of different infections can be the result of the increasing use of invasive procedures such as intravascular catheters and prostheses in patients undergoing intensive treatment, immunocompromised patients, premature children, patients with neoplasias, and transplant patients (Kloos & Bannerman 1994).

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The species that most frequently cause diseases in humans are *S. epidermidis* (bacteremia, infections due to implanted medical devices such as prostheses and catheters, infection of surgical wounds, peritonitis in patients on continuous peritoneal dialysis, osteomyelitis, endophthalmitis etc.), *S. haemolyticus* (endocarditis, peritonitis, septicemia, and infections of the urinary tract, wounds, bone, and joints), and *S. saprophyticus* (urinary infections and septicemic processes). Other significant opportunistic pathogens include *S. hominis*, *S. warneri*, *S. capitis*, *S. simulans*, *S. cohnii*, *S. xylosus*, and *S. saccharolyticus* (Bannerman 2003). *S. lugdunensis* seems to be associated with endocarditis after implantation of prosthetic valves, with peritonitis, with soft tissue infection, and with vertebral osteomyelitis (Osmon et al. 2000).

In view of the known pathogenic potential of CNS within the hospital environment, interest regarding the variety of species related to infection and their toxigenic potential and virulence has increased over the last decade and has led to the publication of various studies on these aspects. However, despite the growing characterization of CNS infections, these microorganisms are not identified in clinical microbiology laboratories. The scheme proposed by Kloos and Schleifer (1975) and modified by Bannerman (2003) is the method conventionally used; however, this method is relatively laborious for routine use since a large number of biochemical tests are required. In most laboratories of routine, staphylococci are identified based on morphological aspects of the colonies, gram staining and catalase and coagulase production, which only permit the classification of staphylococci into *S. aureus* and non-*S. aureus* isolates, with the latter simply being classified as CNS.

The development of methods for the identification of staphylococcal species and subspecies permits clinicians to obtain information about the variety of CNS present in clinical specimens and to consider them as etiological agents of infectious processes. Accurate identification of CNS is needed in order to have an early prediction of the potential pathogenicity or antibiotic susceptibility of each clinical isolate and to clarify the clinical significance of each species. Repeat CNS isolates from patients with invasive diseases should be identified to allow a comparison of the strains. On the other hand, species identification is a prerequisite before typing procedures for epidemiological studies are undertaken.

In recent years, several commercial systems for the rapid identification of staphylococci have been developed as an alternative to the classical identification protocols (Bannerman 2003). However, these diagnostic systems present problems such as cost and incubation time, often provide unreliable results (Grant et al. 1994, Perl et al. 1994). Additionally, many of these kits were designed for the identification of all known CNS species (i.e., clinical, veterinary, and alimentary isolates) and thus are not very specific. Based on the above considerations and in view of the need for rapid, simple, and reliable methods, the objective of the present study was to compare four techniques for the identification of CNS, i.e., a reference method (Kloos & Schleifer 1975, Bannerman 2003), the commercial API Staph system, and two methods modified from the reference method in our laboratory to develop alternative identification methods combining simplicity, reliability, and low cost, especially to places with limited resources.

MATERIALS AND METHODS

Isolates - One-hundred CNS isolates obtained from clinical specimens of patients hospitalized at the University Hospital of the Faculty of Medicine, Universidade Estadual Paulista (Unesp), Botucatu Campus, were studied. Strains were isolated as described by Koneman et al. (1997).

Identification of CNS - The isolates obtained from clinical specimens were plated onto blood agar and gram stained in order to guarantee their purity and the preservation of their morphology and specific staining. After confirmation of these characteristics, the isolates were submitted to the catalase and coagulase tests. *Staphylococcus* was differentiated from *Micrococcus* species on the basis of the oxidation and fermentation of glucose, resistance to bacitracin (0.04 U) indicated by absence of an inhibition halo or presence of an inhibition halo measuring up to 9 mm in diameter, and susceptibility to furazolidone (100 µg) characterized by inhibition zones measuring 15 to 35 mm in diameter (Baker 1984).

The four methods described below were used for the identification of CNS. The following international reference CNS strains were used as controls: *S. epidermidis* (ATCC 12228), *S. simulans* (ATCC 27851), *S. warneri* (ATCC 10209), *S. xylosus* (ATCC 29979), and *S. saprophyticus* (ATCC 15305).

Reference method proposed by Kloos and Schleifer (1975) and Bannerman (2003) - This method consists of a set of biochemical tests that determine the utilization of the sugars xylose, arabinose, sucrose, trehalose, maltose, mannitol, lactose, xylitol, ribose, fructose, and mannose, production of hemolysin, nitrate reduction, presence of urease and ornithine decarboxylase, and resistance to novobiocin characterized by an inhibition halo of up to 16 mm. Readings of the tests were obtained after 24, 48, and 72 h of incubation at 37°C in an air incubator.

API Staph - The API Staph system (bioMérieux) is a ready-to-use test battery consisting of 20 biochemical tests to which an homogenous bacterial suspension at 0.5 McFarland turbidity is added. After 24 h of incubation at 37°C and addition of the VP (VP1 and VP2), NIT (NIT1 and NIT2), and PAL (ZYM A and ZYM B) reagents accompanying the kit, the reactions were interpreted and microorganisms were identified using the analytical catalog. Identification is based on a numerical system consisting of seven digits which provides percent identification (%ID), with a value ≥ 80% being acceptable.

Modified methods - Two identification methods modified in our laboratory were used (simplified method and disk method). The simplified method was divided into two steps. During the first step, fermentation of xylose, sucrose, trehalose, maltose, and mannitol, production of hemolysin, and anaerobic growth in thioglycolate were tested (Table I). The tests used in the second step varied according to the results obtained in the first identification step after 72-h incubation at 37°C. The complementary tests used during the second step (when necessary) are specified in Table II.

The disk method consisted of the following tests: fermentation of arabinose, sucrose, trehalose, maltose, mannitol and lactose, nitrate reduction, production of hemolysin, tests for urease and ornithine decarboxylase, and resistance to novobiocin. For the sugar fermentation test, commercially available disks specific for each sugar were placed in tubes containing 2.5 ml Purple Broth Base medium. Bacterial suspensions were inoculated as described by Kloos and Schleifer (1975). Readings for the two methods were obtained after 24, 48, and 72 h of incubation at 37°C, and CNS species were identified according to the identification scheme proposed in the Figure.

Statistical analysis - To determine the degree of agreement between the methods used for the identification of CNS (simplified method, disk method and API Staph) and the reference method (Kloos & Schleifer 1975, Bannerman 2003), the sensitivity and specificity of the tests (Sox 1986) were assessed as follows.

Sensitivity: proportion of CNS strains that tested positive for a certain species by the reference method and which were identified as the same species by the method analyzed (simplified method, disk or API Staph).

Specificity: proportion of CNS strains that tested negative for a certain species by the reference method and which were also negative for the same species when tested by the method analyzed (simplified method, disk or API Staph).

RESULTS

The 100 staphylococcal isolates were tested by the four proposed methods. The results obtained with the reference method (Kloos & Schleifer 1975, Bannerman

TABLE I

Summary of the tests used in the first step of the simplified method for the identification of human *Staphylococcus* species

Species	Coagulase	D-Xylose	Sucrose	D-Trehalose	Maltose	Manitol	Anaerobic growth thioglycolate	Hemolysis
S. aureus [a]	+	–	+	+	+	+	+	+
S. schleiferi subsp. *coagulans* [a]	+	–	+, –	–	–	+, –	+	+
S. schleiferi subsp. *schleiferi* [b]	–	–	–	+, –	–	–	+	+
S. epidermidis [a]	–	–	+	–	+	–	+	+, –
S. haemolyticus [b]	–	–	+	+	+	+, –	+	+
S. saprophyticus [b]	–	–	+	+	+	+, –	+	–
S. warneri [b]	–	–	+	+	+	+, –	+	+, –
S. hominis subsp. *hominis* [a]	–	–	+	+, –	+	–	–	–
S. hominis subsp. *novobiosepticus* [b]	–	–	+	–	+	–	–	–
S. simulans [a]	–	–	+	+, –	±	+	+	+, –
S. lugdunensis [b]	–	–	+	+	+	–	+	+
S. capitis subsp. *capitis* [a]	–	–	+	–	–	+	+	+, –
S. capitis subsp. *urealyticus* [a]	–	–	+	–	+	+	+	+, –
S. cohnii subsp. *cohnii* [b]	–	–	–	+	+, –	+, –	+	+, –
S. cohnii subsp. *urealyticum* [b]	–	–	–	+	+	+	+	+, –
S. xylosus [a]	–	+, ±	+	+	+	+	+, –	–
S. caprae [b]	–	–	–	+	+, –	+, –	+	+, –

+: positive reaction; –: negative reaction; +, –: positive or negative; *a*: species identified in a single step; *b*: species identified in two steps.

TABLE II

Biochemical tests used in the second identification step of the simplified method for the identification of human *Staphylococcus* species

Result of 1st step: sucrose (–)	Nitrate reduction	Urease production
S. cohnii subsp. *cohnii*	–	–
S. cohnii subsp. *urealyticum*	–	+
S. caprae	+	+
S. schleiferi subsp. *schleiferi*	+	–

Result of 1st step: sucrose (+) mannitol (–) hemolysis (+)	β-D-fructose	Urease production	Ornithine decarboxylase
S. warneri	+	+	–
S. haemolyticus	+, –	–	–
S. lugdunensis	+	+, –	+

Result of 1st step: sucrose (+) mannitol (–) hemolysis (–)	Resistance to novobiocin
S. warneri	–
S. saprophyticus	±

Result of 1st step: sucrose (+) mannitol (+)	β-D-fructose	Urease production	Resistance to novobiocin
S. saprophyticus	+	+	+
S. warneri	+	+	–
S. haemolyticus	+, –	–	–

+: positive reaction; –: negative reaction; +, –: positive or negative

2003) were compared to those obtained with the modified methods and the API Staph system.

Table III shows the agreement in identification between the analyzed assays and the reference method. The simplified method and the disk method showed 100 and 99% positivity when compared to the reference method, while this percentage was 84% for the API Staph system. Of the 16 isolates with disagreement of identification by the API Staph method, 10 (62.5%) showed correct identification but with a %ID of 7.1 to 31%, lower than the acceptable value.

The international reference strains (ATCC) of *S. epidermidis*, *S. simulans*, *S. saprophyticus*, and *S. xylosus* were correctly identified by the four methods. Only the *S. warneri* reference strain (ATCC 10209) was inaccurately identified by the API Staph system, with characterization of this strain as *S. saprophyticus* (%ID = 58.7%), as *S. hominis* (%ID = 19.5%), or as *S. warneri* (%ID = 15.8%).

The simplified method carried out in two steps did not differ from the reference method in terms of the identification of CNS species. In contrast to the other methods, the disk method showed inaccurate identification and erroneously identified an *S. hominis* strain (6.5%) due to the non-fermentation of sucrose on the disk, leading to the classification of this strain as *S. caprae*. No incongruence was observed for the other species.

Inaccurate identification by the API Staph system was observed for *S. epidermidis* (2.2%), *S. warneri* (47.1%), *S. hominis* (25%), and *S. haemolyticus* (37.5%), while agreement with the reference method was 100% for *S. lugdunensis*, *S. simulans*, *S. xylosus*, *S. saprophyticus*, *S. caprae*, and *S. cohnii* subspecies *urealyticum*.

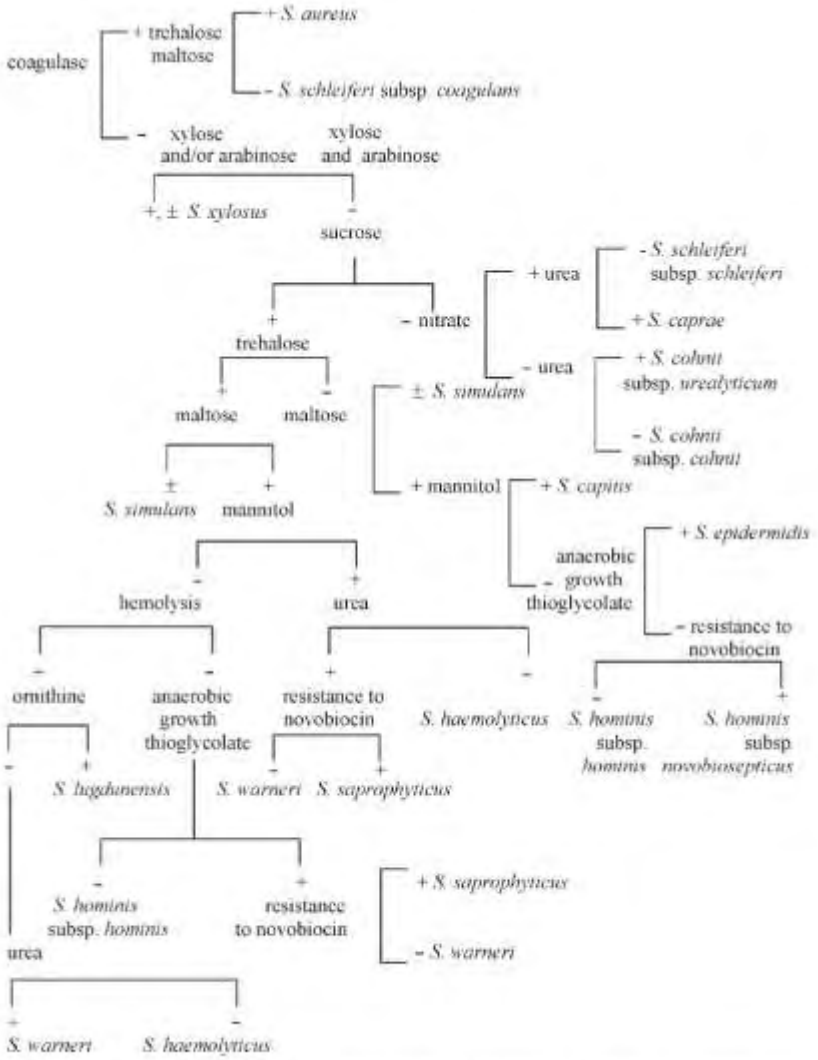
The largest discrepancy was observed between the reference method and the API Staph system, with the latter method not accurately identifying 1 *S. epidermidis*

strain (identified as *S. lugdunensis*), 3 *S. haemolyticus* strains (2 identified as *S. aureus* and 1 as *S. hominis*), 4 *S. hominis* strains (2 identified as *S. lugdunensis*, 1 as *S. haemolyticus* and 1 as *S. aureus*), and 8 *S. warneri* strain (3 identified as *S. lugdunensis*, 2 as *S. haemolyticus*, 2 as *S. hominis* and 1 as *S. saprophyticus*) (Table III).

As shown in Table IV, the sensitivity and specificity of the simplified method were 100% for all species studied. The disk method showed 93.8% sensitivity for the identification of *S. hominis* and 100% sensitivity for the other species, while specificity was 98.8% for *S. hominis*, 98.9% for *S. caprae*, and 100% for the other species. The sensitivity of the API Staph was 100% for *S. lugdunensis*, *S. simulans*, *S. xylosus*, *S. saprophyticus*, *S. caprae*, and *S. cohnii* subspecies *urealyticum*, 97.8% for *S. epidermidis*, 52.9% for *S. warneri*, 75% for *S. hominis*, and 62.5% for *S. haemolyticus*. The specificity of this test ranged from 94.8 to 98.9% for *S. warneri*, *S. hominis*, *S. haemolyticus*, *S. lugdunensis*, and *S. saprophyticus* strains, and was 100% for the other species.

DISCUSSION

CNS are the microorganisms most commonly isolated from blood cultures, representing a serious health problem in many developing countries and also developed (Renneberg et al. 1995). Some studies have suggested an association between *S. epidermidis* and nosocomial infections (Vuong & Otto 2002) with this species being identified in 74 to 92% of patients with bacteremias caused by CNS (Martin et al. 1989). However, other studies have reported a series of infections caused by other CNS species (Herwaldt et al. 1996), mainly *S. haemolyticus* which



Simple scheme for the identification of human *Staphylococcus* species; +: positive; –: negative; +, –: positive or negative; ±: weak

TABLE III

Identification agreement between the modified methods and the API Staph system compared to the reference method

Species	Nr of isolates tested	Methods					
		Simplified		Disk		API Staph	
	N	N	%	N	%	N	%
S. epidermidis	45	45	100	45	100	44	97,8
S. warneri	17	17	100	17	100	9	52,9
S. hominis	16	16	100	15	93,8	12	75
S. haemolyticus	8	8	100	8	100	5	62,5
S. lugdunensis	4	4	100	4	100	4	100
S. simulans	3	3	100	3	100	3	100
S. xylosus	2	2	100	2	100	2	100
S. saprophyticus	2	2	100	2	100	2	100
S. caprae	2	2	100	2	100	2	100
S. cohnii	1	1	100	1	100	1	100
Totals	100	100	100	99	99	84	84

S: Staphylococcus

TABLE IV

Sensitivity and specificity of the simplified method, disk method, and API Staph system for the identification of human *Staphylococcus* species compared to the reference method [a]

Species	Sensitivity (%)			Specificity (%)		
	Simplified	Disk	API Staph	Simplified	Disk	API Staph
S. epidermidis	100	100	97,8	100	100	100
S. warneri	100	100	52,9	100	100	98,8
S. hominis	100	93,8	75	100	98,8	97,6
S. haemolyticus	100	100	62,5	100	100	96,7
S. lugdunensis	100	100	100	100	100	94,8
S. simulans	100	100	100	100	100	100
S. xylosus	100	100	100	100	100	100
S. saprophyticus	100	100	100	100	100	98,9
S. caprae	100	100	100	100	98,9	100
S. cohnii	100	100	100	100	100	100

a: Gold Standard (Kloos & Schleifer 1975, Bannerman 2003)

is the second most frequently detected species (Bannerman 2003). Since CNS are the etiological agents of a series of infectious processes, identification of these microorganisms is important for the determination of their physiopathological characteristics and clinical importance and for epidemiological studies, and has led to the publication of various studies analyzing identification methods for these bacteria (Knapp & Washington 1989, Bannerman et al. 1993, Piccolomini et al. 1994, Renneberg et al. 1995, Ieven 1995, De Paulis et al. 2003).

In the present study, the methods modified in our laboratory yielded good results in terms of the correct classification of CNS species compared to the reference method, with 100% agreement being observed for the simplified modified method and 99% agreement for the disk method.

The simplified method using the identification scheme proposed here (Figure) led to the identification of *S. epidermidis*, *S. hominis*, *S. xylosus*, *S. capitis*, and *S. simulans* in a single step, using a total of seven biochemical tests, a number lower than that employed in the reference method (16 tests). Since *S. epidermidis* is the most frequently isolated species, 70 to 90% of the strains (Bannerman 2003) isolated in the clinical laboratory can be identified using a reduced number of tests.

With respect to incubation time, the results showed that 91% of the strains analyzed in the study fermented the species-specific sugar within 48 h of incubation at 37°C. The other strains (9%) tested positive for the fermentation of given sugars after 72 h of incubation, demonstrating the importance of an incubation of the sugar fermentation tests of at least 72 h in order to correctly identify these microorganism.

The identification of *S. cohnii*, *S. schleiferi* subspecies *schleiferi*, *S. caprae*, *S. warneri*, *S. haemolyticus*, *S. saprophyticus*, and *S. lugdunensis* required the execution of two or three additional biochemical tests, referred to as the second step, which varied according to the result obtained in the first step of simplified method. However, 20 (37,7%) strains that required the second step for their identification fermented trehalose within 24 h, thus permitting prior continuation of the additional tests. A longer time was needed to identify *S. cohnii*, *S. schleiferi* subspecies *schleiferi*, and *S. caprae*, since the second step required for the identification of these species in-

cluded the nitrate reduction test whose result is only available after 48 h. However, this fact does not actually result in a delay in the diagnosis of CNS since the frequency of these species in clinical samples is low.

The disk method was also found to be highly efficient and practical since it does not require previous preparation of sugars, thus preventing the loss of culture media, in addition to reaching high agreement in the identification of CNS with the reference method.

The commercial API Staph kit showed the lowest accuracy in the identification of CNS among the methods studied (84% agreement), in agreement with the studies of (Bannerman et al. 1993, Renneberg et al. 1995).

S. warneri and *S. hominis* were the most difficult species to identify. Bannerman et al. (1993) also reported a lower accuracy in the identification of these species. In the study of Ieven et al. (1995), *S. hominis* was identified with the least accuracy by the API ID 32 Staph system. This finding might be explained by the lack of complementary tests such as novobiocin resistance, anaerobic growth in thioglycolate and hemolysin production.

In the case of *S. haemolyticus*, incorrect identification by the API Staph system might be explained by the fact that the kit does not suggest hemolysin production as a complementary test, which would be essential for the identification of *S. haemolyticus* strains.

Three (3%) of the 100 strains analyzed by the API Staph system were identified as *S. aureus*, a fact also reported by Renneberg et al. (1995). The kit was found to be inefficient in these cases since it did not request the result of the fundamental and most widely accepted test for the identification of *S. aureus*, i.e., the coagulase test (Koneman et al. 1997).

According to Piccolomini et al. (1994), the low agreement between the API Staph and the traditional biochemical test for identification of CNS can be explained by the use of different incubation times, substrate concentrations and/or sensitivity markers.

In conclusion, the two methods modified in our laboratory were found to be highly efficient for routine use due to their high sensitivity and specificity compared to the reference method, in addition to requiring fewer tests and thus being more economical and faster than the standard method. Despite requiring a shorter incubation time (18 h), the API Staph system showed a lower sensitivity in the identification of some species. Undoubtedly, CNS species identification will be facilited and encouraged by the availability of a simple, inexpensive, and accurate procedure, especially in places with limited resources.

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ANEXO 7

Ferreira AM, Bonesso MF, Mondelli AL, **Cunha MLRS**. Comparison of phenotypic and genotypic methods for identification of *Staphylococcus* spp. isolated from patients with urinary tract infection. (Em preparação para submissão).

Running title: Methods for identification

Title: Comparison of phenotypic and genotypic methods for identification of *Staphylococcus* spp. isolated from patients with urinary tract infection

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Abstract

Urinary tract infection (UTI) is one of the most frequent infectious diseases in clinical practice, and it is the second most common infection in human. *S. saprophyticus* is the second most frequent agent of community-acquired UTI, and it is mainly isolated from the urine of sexually active young women. This study aimed at comparing three phenotypic methods for identifying *Staphylococcus* spp. isolated from patients with UTI by using the genotypic ITS-PCR test as reference. The 101 staphylococci studied were identified by novobiocin disk testing, Vitek I, the simplified method of biochemical tests and ITS-PCR. The simplified method of biochemical tests obtained agreement rates of 98.0% in relation to ITS-PCR, while Vitek I showed 81.2% and the novobiocin disks 89.1%. No other novobiocin-resistant non-*S. saprophyticus* was found. Hence, the novobiocin disks showed to be a feasible alternative for *S. saprophyticus* identification in urinary samples in laboratories with limited resources. ITS-PCR and the simplified method of biochemical tests showed greater reliability than the commercial systems presently available. This study confirms that the automated systems are not yet able to perform a correct differentiation of species of CNS and that simple, reliable and inexpensive methods can be used for identification routine.

Key words: *Staphylococcus*, urinary tract infection, methods for identification, ITS-PCR, automated systems.

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Introduction

Urinary tract infection (UTI) is one of the most common diseases in internal medicine (Stamm & Hooton 1993, Warren et al. 1999, Lopes & Tavares 2005, Moura et al. 2009), and it is the second most common infection in human beings (Valiquette 2001), as it is only less prevalent than that in the respiratory tract (Hörner 2006).

The etiological agents most frequently involved with community-acquired UTI, in order of frequency, are: *Escherichia coli*, *Staphylococcus saprophyticus* and species of *Proteus* spp., *Klebsiella* spp. and *Enterococcus faecalis*. *E. coli* alone is accountable for 70% to 85% of community-acquired urinary tract infections (Bishara et al. 1997, Hooton & Stamm 1997, Gupta et al. 2001). However, when UTI is acquired in hospitals, etiological agents are diversified. Enterobacteria predominate with a reduction in the frequency of *E. coli* (although it usually remains as the primary cause) and an increase in *Proteus* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., *Enterobacter* spp., *Enterococcus faecalis* (Carton et al. 1989, Bishara et al. 1997, Hooton & Stamm 1997) and fungi, with distinction for species of the *Candida* genus.

Staphylococcus saprophyticus is the second most frequent acute UTI agent in the community, following *E. coli* (Henry et al. 1998, Gupta et al. 1999), and it is mainly isolated from the urine of sexually active young women (Jordan et al. 1980, Abrahamsson et al. 1993, Svanborg & Godaly 1997), showing undistinguishable symptoms from those caused by *Escherichia coli*. Reports of septicemia and pyelonephritis by this organism have also been recorded (Colledge 1989, Lee et al. 1987).

Other coagulase-negative staphylococci (CNS) are usually considered to show uncertain pathogenicity for the urinary tract, nevertheless, various researchers have evaluated the presence of some CNS species (*S. haemolyticus*, *S. epidermidis*, *S. simulans*, *S. sciuri*, *S.*

capitis, *S. xylosus*, *S. warneri*, *S. cohnii*, *S. lentus* and *S. hominis*) with clinical significance in urinary tract infections (Guirguitzova et al. 2002, Alcaráz et al. 2003).

The emergence of *Staphylococcus* spp. not only as human pathogens, but also as reservoirs of antibiotic-resistance determinants, requires the development of rapid and reliable identification methods. In several routine laboratories, *S. saprophyticus* identification is performed based on novobiocin (5µg) resistance, hemolysis absence and negative testing for coagulase and/or DNAse. However, it has been acknowledged that other CNS species, including *S. cohnii*, *S. sciuri*, *S. xylosus* and *S. hominis*, are also resistant to novobiocin in this concentration (Hussain et al. 1986, Konemann et al. 2001). These results suggest that additional tests, including carbohydrate fermentation and others, must be used conjointly with the novobiocin sensitivity test for accurate identification of CNS species (Cunha & Lopes 2002).

In the last few years, various commercial systems for staphylococcus identification have been developed as an alternative for the classic identification protocols by Bannerman et al. (2003), which are labored and slow to be used in laboratory routine. Automated processes and commercial kits, based on miniaturized biochemical tests, are widely used nowadays both in routine laboratories and in research, however, these diagnostic systems pose problems, such as high cost, incubation time and, most importantly, they are still not capable of reliably differentiating CNS species due to the variable expression of phenotypic characteristics.

Additionally, many of such automated systems and kits are based on colorimetric results, and subjectivity in their interpretation may lead to ambiguity (Couto et al. 2001). In 2004 a new identification key was organized by Cunha et al. (2004). The method, which became known as the simplified method of biochemical tests, is divided into two phases. This method was compared with the reference method proposed by Kloos & Schleifer (1975) and Bannerman (2003) and showed 100% agreement in accurate species identification, thus being

considered highly efficient for routine use due to its sensitivity and specificity, in addition to using few tests and consequently being more economical and faster than the reference method by providing results in 72 hours.

Another technique used for staphylococcus identification is the ITS-PCR technique, which has been widely used for typing bacterial lineages, since the ITS regions have great polymorphism (Jensen et al. 1993, Gürtler & Stanisich 1996). DNA analyses have been the preferential method for microorganism identification as they show greater specificity and sensitivity. CNS samples that are not identified in species level or erroneously identified by conventional phenotypic tests can be correctly identified by means of genotypic techniques (Maes et al. 1997, Kawamura et al. 1998). CNS identification is highly important for associating certain species with specific infections (Rupp & Archer 1994), considering the indication that, in addition to *S. epidermidis* and *S. saprophyticus*, which have been found to be pathogenic, some species, such as *S. haemolyticus*, *S. lugdunensis* and *S. schleiferi*, are more frequently associated with infections than other species (Herchline & Ayers 1991, Low et al. 1992).

A lot of effort has been put into developing alternative identification methods combining speed, reliability and low cost. For these reasons, this study aimed at comparing three phenotypic identification methods for staphylococci found in urine samples: simplified scheme of biochemical tests, using tests of sensitivity to novobiocin disks, and the automated bacterial identification system Vitek I, utilizing genotypic identification as reference through the ITS-PCR technique.

Materials an methods

Samples

One hundred and one samples of *Staphylococcus* spp. isolated from the urine of different patients were used in the study. The samples were sent to the Laboratory of Microbiology of the Botucatu School of Medicine University Hospital - UNESP - Univ. Estadual Paulista, Botucatu, SP, Brazil, from March 10 to November 14, 2008 after being obtained from different hospital wards, outpatient units, emergency rooms and several health care centers in Botucatu and region.

The research project was approved by the Research Ethics Committee of the Botucatu School of Medicine - UNESP (Of.-416/08-CEP).

Inclusion criteria

Male and female individuals of all ages were included. They showed positive uroculture for *Staphylococcus* spp. which were compatible with UTI and a colony count that was equal to or higher than 100,000 colony-forming units per milliliter of urine ($\geq 10^5$ CFU/ml), according to the evaluation criteria by Kass (1956).

Exclusion criteria

Samples collected from vesical catheters, suprapubic punctures, positive urocultures with colony count lower than 100,000 colony-forming units per milliliter of urine ($<10^5$ CFU/ml) were excluded.

Sample size estimation

Sample size was calculated by the Fisher & Belle (1993) formula, using a 95% confidence interval and 5% accuracy for the expected prevalence of patients with UTI, using as a base the proportion of patients with UTI caused by *Staphylococcus* spp., which was of 5% in a

study conducted in the Laboratory of Microbiology of the Botucatu School of Medicine University Hospital.

Although the sample size estimation indicated 73 samples as the minimum number, all the *Staphylococcus* spp. samples isolated from March 10 to November 14, 2008 which met the inclusion and exclusion criteria were used, thus totaling 101 samples.

Collection

The patients were instructed to collect medium-jet urine, with previous hygiene of the genital region. The first jet was discarded, and the medium jet was collected into an appropriate sterile bottle. The remaining urination was discarded. In children and newborns, the sample was obtained by using an aseptically placed collection bag. The collector was replaced every 45 minutes to 1 hour, and hygiene was repeated in order to prevent fecal contamination. The samples collected at the School of Medicine University Hospital were transported in ambient temperature (20 to 25ºC) and processed within up to 1 hour. The samples collected at the health care centers were transported in thermal boxes under refrigeration and processed as soon as they reached the laboratory.

Isolation and counting of the number of colonies

Urine was homogenized and seeded by using a disposable loop (0.001 ml or 1 µl), without centrifugation. The loop was vertically immersed in urine, and seeding was performed by the depletion technique for quantification. Results were reported in colony-forming units by milliliter of urine (CFU/ml).

The samples were seeded onto dishes containing Cled (Cysteine Lactose Electrolyte-Deficient Medium) agar medium, incubated for 18 to 24 hours at a temperature of 35°C, and the number of colonies was counted.

Phenotypic identification of Staphylococcus spp.

The isolates were seeded in blood agar with 5% of sheep blood and stained by the Gram method aiming at its purity and observance of its morphology and specific coloration. After these characteristics were confirmed, the lineages were submitted to tube catalase, DNAse and coagulase testing (gold standard) in order to differentiate *Staphylococcus aureus* and coagulase-negative staphylococci species as recommended by Koneman et al. (2001). The *Staphylococcus* genus was differentiated from *Micrococcus* based on the bacitracin-resistance (0.04 U) test indicated by the absence of an inhibition halo or formation of a halo of up to 9 mm, and by sensitivity to furazolidone (100 μg), characterized by inhibition halos >15 mm in diameter (Baker 1984, Vasconcelos et al. 2011).

Phenotypic identification of Staphylococcus aureus

The coagulase-positive staphylococci, in addition to the previously described tests, were submitted to the trehalose fermentation test in order to differentiate *Staphylococcus aureus* species from *Staphylococcus schleiferi* subsp. *coagulans* (Cunha et al. 2004).

For identification using the automated device Vitek I (Biomérieux), the results of the catalase and coagulase tests were informed by external marks made on the identification cards.

Phenotypic identification of coagulase-negative staphylococci

The coagulase-negative staphylococci were identified by the simplified method of biochemical tests described by Cunha et al. (2004), which is divided into two phases. During the first phase, the fermentation tests of xylose, saccharose, trehalose, maltose, mannitol, hemolysin production and anaerobic growth in thioglycollate were performed. The tests performed in the second phase vary according to the results obtained in the first phase and

include urease production, nitrate reduction, β-D-fructose fermentation, ornithine decarboxylation and novobiocitin resistance.

In addition to identification by the simplified method, the samples were identified by the automation device Vitek I, using the GPI (Gram-Positive Identification) card, according to the manufacturer's instructions.

A presumptive identification of *Staphylococcus saprophyticus* was performed based on novobiocin (5μg) resistance and hemolysis absence. The result was considered to be resistant when observing halos of up to 12 mm or an absence of halos, and sensitive when observing halos >16 mm (Konemann et al. 2001). Resistant samples were identified as *Staphylococcus saprophyticus*, and the sensitive samples were considered to be *Staphylococcus epidermidis* species (Trabulsi et al. 1989).

Nucleic acid extraction

Total nucleic acid was extracted from *Staphylococcus* lineages cultured in blood agar and individually inoculated in BHI broth at 37°C for 24 hours.

Extraction was performed by the Illustra Kit (GE Healthcare), according to the manufacturer's instructions, and the extracted DNA was stored under refrigeration at -20°C.

Detection of the coa gene by the polymerase chain reaction (PCR) technique

For the amplification reaction, 1 μl of the DNA sample was added in 49 μl of the reaction mixture containing $MgCl_2$ 4 mmol l^{-1} M, 200 μmol l^{-1} of each dNTP, 15 pmol l^{-1} of each primer (*coa*1: 5 GTA GAT TGG GCA ATT ACA TTT TGG AGG 3 and *coa*2: 5 CGC ATC AGC TTT GTT ATC CCA TGT A 3) and 1 U of Taq DNA polymerase.

Amplification was performed by a thermocycler TC-100™, using the parameters described by Kearns et al. (1999). The positive result was indicated by observing the presence

of 117-pb DNA fragments. Reference lineages for positive (*S. aureus* ATCC 29213) and negative (*S. epidermidis* ATCC 35983) *coa* were used.

The efficiency of amplifications was monitored by electrophoresis of the 3% Ultrapure™ agarose gel reaction prepared in 0.5X Tris-Borate-EDTA buffer. A marker of 100-pb molecular weight was used as standard. DNA was stained with SYBR® Safe and later photographed under UV transillumination.

Genotypic identification of Staphylococcus spp.

The *Staphylococcus* spp. samples were submitted to genotypic identification using primers of conserved sequences adjacent to genes 16S and 23S. This method, described by Barry et al. (1991) and Couto et al. (2001), is known as ITS-PCR (Internal Transcribed Spacer-PCR).

The technique was performed as described by Couto et al. (2001) using primers G1 (5'-GAAGTCGTAACAAGG) and L1 (5'-CAAGGCATCCACCGT). The efficiency of amplifications was monitored by electrophoresis of 3% metagen agarose gel reaction prepared in 1.0 X TBE buffer and stained with SYBR® Safe. The size of the amplified products was compared with the 100 pb standard and later photographed under UV transillumination.

Statistical analysis

In order to evaluate the accuracy of the coagulase and DNAse tests and of the phenotypic identification methods for *Staphylococcus* spp. (novobiocin disk, simplified methods of biochemical tests and Vitek I system) tests were applied for sensitivity and specificity evaluation according to Fletcher et al. (1991) by using the search for the *coa* gene and ITS-PCR as gold standard, respectively.

Results

Comparison between the DNAse test, tube coagulase test and coagulase gene (coa gene)

Among the 101 studied samples, all the 17 *S. aureus* were positive for coagulase, DNAse and the *coa* gene (Fig. 1); however, of the 84 CNS, two showed positive DNAse, but were negative for coagulase and the *coa* gene, and the 82 remaining samples were negative for coagulase, DNAse and the *coa* gene. The DNAse test showed 100% sensitivity and 97.6% specificity, and coagulase showed similar values to those by the reference genotypic method (*coa* gene) with 100% sensitivity and specificity and agreement rates of 98.0% for DNAse and 100.0% for coagulase (Table I).

Phenotypic and genotypic identification of Staphylococcus spp.

As to the identification by using the novobiocin disk, 57 *S. saprophyticus* and 27 *S. epidermidis* were identified. The simplified method of biochemical tests identified 57 isolates as *S. saprophyticus*, 15 *S. epidermidis*, seven *S. haemolyticus*, four *S. warneri* and one *S. lugdunensis*, while Vitek I identified 48 *S. saprophyticus*, 12 *S. epidermidis*, nine *S. haemolyticus* and four *S. warneri*. By ITS-PCR (Fig. 2) 57 *S. saprophyticus*, 16 *S. epidermidis*, eight *S. haemolyticus*, two *S. warneri* and one *S. lugdunensis* were identified; however, six samples of *S. auricularis*, four of *S. simulans* and one of *S. xylosus* were only identified by Vitek I. The 17 *S. aureus* found were identified by all the methods (Table II).

The main failure in *S. saprophyticus* identification by Vitek I was the non-detection of novobiocin resistance, which occurred in six of the nine isolates, followed by the positive arginine test (five isolates), and four isolates showed both tests in disagreement. The five *S. epidermidis* identified as *S. auricularis* by Vitek I were saccharose negative, and the two *S. warneri* showed negative urease testing. In relation to the two species erroneously identified

by the simplified method of biochemical tests, *S. haemolyticus* showed incorrect results for the urease test and *S. epidermidis* for the trehalose test (Table III).

Regarding the sensitivity and specificity comparison between the identification methods for all the studied species, the novobiocin disk showed 89.1% sensitivity and 89.1% specificity, and as to Vitek I, sensitivity and specificity were of 81.2% and 92.0% respectively, and as to the simplified method of biochemical tests, sensitivity and specificity were of 98.0% (Table IV).

Discussion

The comparison of the coagulase test with DNAse production showed that all the samples identified as *S. aureus* by the tube coagulase test were also DNAse positive. Rao et al. (2002) reported four MRSA isolates with negative results for DNAse which were confirmed as *S. aureus* by the latex agglutination test for detection of the Cumpling factor, tube coagulase, identification by the Rapid ID32 Staph kit and typed by PFGE (pulsed-field gel electrophoresis), identified as variant sub-types of EMRSA-15 (epidemic methicillin-resistant *S. aureus*) and still not previously described. However, in our study, no DNAse-negative sample was identified as *S. aureus*.

Kateete et al. (2010) also evaluated the sensitivity and specificity of the DNAse test, where they observed 75.0% sensitivity and 96.0% specificity. These are lower values than those obtained in this study, which were of 100.0% sensitivity and 97.6% specificity. Such results are also similar to those found by Bello & Qahtani (2005), according to which sensitivity and specificity were of 93.0% and 96.0% respectively. The same authors found, among the 180 *S. aureus* analyzed, 7 (3.9%) isolates producing fibrinolysins that lysed the clot within four hours, thus showing the importance of reading this test after 1, 4 and 24 hours in order to prevent identification errors. However, despite the less expressive number of samples

evaluated in this study, clot lysis was not observed in any of the *S. aureus* isolates, thus suggesting that none of the samples produced fibrinolysin.

Cunha et al. (2006) found clinically significant 59 CNS samples and seven DNAse-producing samples, of which three were *S. epidermidis*, three *S. lugdunensis* and one *S. haemolyticus*, reporting that enzyme concentration is important for its detection and that it can be improved by the dialysis bag culture method. In this study, the enzyme concentration methodology was not used since the DNAse test was one of the tests used with the purpose to distinguish the *S. aureus* species from CNS. Even without using the concentration, two (2.3%) positive samples to the DNAse test were found among the 84 CNS samples, thus confirming the production of such enzyme by CNS. Therefore, this test must be cautiously used for that purpose, since it may identify CNS samples as *S. aureus* or *S. aureus* as CNS, as reported in other studies (Rao et al. 2002, Bello & Qahtani 2005, Kateete et al. 2010).

Our results showed that most of the isolated species were of *S. saprophyticus* (56.4%) although other species were less frequently found: *S. aureus* (16.9%); *S. epidermidis* (15.9%); *S. haemolyticus* (7.9%); *S. warneri* (1.9%) and one *S. lugdunensis* (1.0%).

Antibiotic resistance is usually codified by plasmids; hence, novobiocin resistance genes can be transferred inter and between species (D'Azevedo et al. 2007). In this study, no other novobiocin-resistant non-*S. saprophyticus* species was found; however, several authors have reported novobiocin resistance in other CNS species besides *S. saprophyticus* (Large et al. 1989, Cunha & Lopes 2002, D'Azevedo et al. 2007), including samples isolated from patients with UTI (Mctaggart & Elliott 1989, Higashide et al. 2008).

Trabulsi et al. (1989) suggested that the three main species of clinical interest could be identified by the coagulase test and novobiocin sensitivity (*S. aureus*: coagulase positive and novobiocin sensitive; *S. epidermidis*: coagulase negative and novobiocin sensitive and *S.*

saprophyticus: coagulase negative and novobiocin resistant). In our evaluation, all *S. aureus* and *S. saprophyticus* were correctly identified by using only the results from the coagulase test and novobiocin resistance; however, among the 27 novobiocin-sensitive samples identified as *S. epidermidis*, other species were found, and 11 samples were incorrectly identified by only using these parameters; hence, the performance of additional tests is necessary for correct identification.

In our study, agreement between ITS-PCR and Vitek I was of 81.2%. Vitek I identified 100% of *S. aureus*, 84.2% of *S. saprophyticus*, 62.5% of *S. epidermidis* and 87.5% of *S. haemolyticus*; however, it did not identify the isolates of *S. warneri* or *S. lugdunensis.* Bannerman et al. (1993) compared Vitek I (Biomérieux) with these conventional biochemical tests in 500 clinical isolates, obtaining 89.0% agreement. Vitek I correctly identified 92.0% of the isolates of *S. epidermidis*, 95.0% of *S. haemolyticus*, 88.0% of *S. capitis* subesp. *capitis* and 100.0% of *S. saprophyticus.* They also found three isolates of *S. warneri*, which were identified as *S. epidermidis*, *S. simulans*, and *S. conhii*; one *S. simulans* strain was erroneously identified as *S. warneri* or *S. hominis*. Of the 37 isolates of *S. hominis*, seven were incorrectly identified, and three (43.0%) as *S. epidermidis*, three (43.0%) as *S. saprophyticus* and one (14.0%) as *S. warneri*.

In our evaluation, Vitek I incorrectly identified six samples of *S. epidermidis*, of which five were identified as *S. auricularis* and one as *S. simulans*; the two *S. warneri* found by ITS-PCR were incorrectly identified by Vitek I, one as *S. haemolyticus* and the other as *S. auricularis*, and no agreement between the methods occurred. Of the eight *S. haemolyticus*, one was identified as *S. epidermidis*; of the 57 *S. saprophyticus*, Vitek I incorrectly identified nine isolates, four *S. warneri*, three *S. simulans*, one *S. haemolyticus* and one *S. xylosus*.

Caierão et al. (2006) compared 94 CNS isolates by the conventional biochemical method (Bannerman et al. 1993) with the Vitek I system and found that 20 isolates were incorrectly identified, of which 11 were *S. hominis*. The automated Vitek I system was able to correctly characterize 74 of 94 isolates (78.7%). These were similar results to those obtained in this study, where Vitek I correctly identified 82 (81.1%) of the 101 isolates; however, that was slightly lower than the results reported by Bannerman et al. (1993), who correctly identified 454 of 500 isolates (90.8%).

The simplified method of biochemical tests described by Cunha et al. (2004) showed 98.0% agreement with ITS-PCR, with rates of 93.7% for *S. epidermidis*, 87.5% for *S. haemolyticus* and 100% for *S. aureus*, *S. warneri*, *S. lugdunensis* and *S. saprophyticus*. One of the great challenges in CNS identification is related to the expression of genes, which may often not be expressed, thus hindering the identification of certain strains within the same species. Additionally, isolates from patients submitted to long therapy with antimicrobials may change their typical biochemical characteristics, and ITS-PCR can then be a tool for identifying rare phenotypes and aberrant species.

As regards accuracy, the simplified method of biochemical tests and novobiocin-sensitivity test showed 100.0% sensitivity and specificity for *S. saprophyticus* identification, and Vitek I showed 84.2% sensitivity and 100.0% specificity. Despite rapid identification (2 to 15 hours) and the large number of biochemical tests (29 tests), Vitek I failed to identify the CNS species most frequently found in urine samples (*S. saprophyticus*) mainly due to the non-detection of novobiocin resistance in some isolates (6/9), and *S. epidermidis*, the second CNS most frequently found in urine samples and the first in other clinical materials, due to failure in the saccharose test (5/5).

In relation to the two species erroneously identified by the simplified method of biochemical tests, *S. haemolyticus* showed incorrect results for the urease test, and the same isolate was also urease positive by Vitek I, thus suggesting that rare species of *S. haemolyticus* may show positive urease. *S. epidermidis* showed positive trehalose test results, and sugar contamination may have occurred, considering that the same isolate was trehalose negative by Vitek I.

The simplified method showed superiority in identification due to the fact that Vitek I did not perform essential tests for identifying certain species and to the need for a longer incubation period for some sugar fermentation tests (72 hours), as observed by Cunha et al. (2004). The most difficult species to identify were *S. warneri* and *S. haemolyticus*, and such difficulty was also reported by other authors (Bannerman et al. 1993, Ieven et al. 1995, Cunha et al. 2004). This finding can be explained by the lack of complementary and essential tests, such as hemolysin production.

Kim et al. (2008) compared the results of 120 CNS clinical samples identified by Vitek 2 and by the Microseq 500 system (Applied Biosystems), a commercial system for analysis of gene 16S rRNA. Vitek 2 correctly identified 105 (87.5%) of the isolates and 6 (5.0%) incorrectly. When the result with low level of discrimination and the correct identification were conjointly considered, the agreement rate was of 95.0% (114/120). Therefore, even when a more updated device and software were used, the agreement rates between the genotypic identification method and automation were lower than the agreement rates between the simplified method of biochemical tests (98.0%) and the genotypic method in this study, thus confirming that the automated systems, despite being faster, are still not capable of reliably differentiating different CNS species.

The simplified method of biochemical tests showed agreement rates of 98.0% in relation to ITS-PCR, while Vitek showed 81.2%, and the novobiocin disk presented 89.1% of agreement with ITS. It is noteworthy that the low agreement of Vitek I is mainly due to the identification of nine *S. saprophyticus* identified as being of other species, while the best correlation of the novobiocin disk with ITS occurred exactly because it identified all *S. saprophyticus* and because no other novobiocin-resistant CNS species were isolated among the studied species in addition to *S. saprophyticus*.

It is important to note that, in this method, all the novobiocin-sensitive species were identified as *S. epidermidis*; hence, when agreement with ITS-PCR was analyzed, there was a false impression that it would be a good method for *S. epidermidis* identification (100.0% agreement); however, other novobiocin-sensitive species (eight *S. haemolyticus*, two *S. warneri* and one *S. lugdunensis*) would have been identified as *S. epidermidis* if only this test had been used, with specificity of the novobiocin disk for this species of 87.0%.

Hence, in laboratories that do not have automation or where biochemical tests are not performed for species identification, the best recommendation would be to release such results as CNS, thus preventing identification errors.

The DNAse test showed good correlation with the coagulase test and with the *coa* search gene for *Staphylococcus aureus* identification; however, such test, must be cautiously used for that purpose since it may identify CNS samples as *S. aureus* or *S. aureus* as CNS.

The novobiocin disk showed to be a feasible alternative to identify *S. saprophyticus* in urinary samples in laboratories with limited resources, and ITS-PCR and the simplified, method of biochemical tests showed greater reliability than the automated system for identifying staphylococci. Despite the reduction in prices of molecular techniques in the last few years, this method still shows high cost to be used in the routine of clinical microbiology laboratories;

however, the simplified biochemical method proposed by Cunha et al. (2004) is a feasible alternative as it offers lower cost, can be easily performed and provides reliable results. Also, the time for identification can be reduced by performing the urease and novobiocin tests in the first phase for *Staphylococcus* spp. identification in urine samples.

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Illustrations

Fig. 1: Electrophoresis in agarose gel for the *coa* gene (117 pb) search by the PCR technique. Lane 1: molecular weight marker (100 pb); 3, 6, 7 and 16: positive samples; 2, 4, 5 and 8 to 15: negative samples; 17: positive control; *S. aureus* ATCC 29213; 18: negative control; *S. epidermidis* ATCC 35983; 19: H_2O.

Fig. 2: ITS-PCR (Internal Transcribed Spacer-PCR) for genotypic identification of *Staphylococcus* spp species. Lane 1: molecular weight marker (100 pb); 2: *S. saprophyticus* ATCC 15305; 3 to 12: *S. saprophyticus* isolates; 13: negative control (H_2O).

TABLE I

Agreement and accuracy test for the DNAse and coagulase methods

Accuracy Test	Methods		
	DNAse	Coagulase	*coa* gene
Sensitivity (%)	100.0	100.0	100.0
Specificity (%)	97.6	100.0	100.0
PPV (%)	89.4	100.0	100.0
NPV (%)	100.0	100.0	100.0
Agreement (%)	98.0	100.0	100.0

PPV: positive predictive value; NPV: negative predictive value.

TABLE II

Comparison of identification methods

Species	Identification Methods							
	Novobiocin		Vitek I		Simplified		ITS-PCR	
	N	%	N	%	N	%	N	%
S. saprophyticus	57	56.4	48	47.6	57	56.4	57	56.4
S. aureus	17	16.9	17	16.9	17	16.9	17	16.9
S. epidermidis	27	26.7	12	11.9	15	14.9	16	15.9
S. haemolyticus	0	0	9	8.9	7	6.9	8	7.9
S. auricularis	0	0	6	5.9	0	0	0	0
S. simulans	0	0	4	3.9	0	0	0	0
S. warneri	0	0	4	3.9	4	3.9	2	1.9
S. lugdunensis	0	0	0	0	1	1.0	1	1.0
S. xylosus	0	0	1	1.0	0	0	0	0
Total	101	100	101	100	101	100	101	100

TABLE III

Evaluation of biochemical tests that failed to identify species

	ITS-PCR	Simplified Method		Vitek I System	
No.	Identification	Identification	Incor.Test	Identification	Incor. Test
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. warneri*	Arg +; Rib +
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. warneri*	Arg +; Nov S
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. warneri*	Rib +
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. warneri*	Nov S
2	*S. saprophyticus*	*S. saprophyticus*	-	*S. simulans*	Arg +; Nov S
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. simulans*	Nov S
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. xylosus*	Xyl +
1	*S. saprophyticus*	*S. saprophyticus*	-	*S. haemolyticus*	Arg +; Nov S; Ara +
4	*S. epidermidis*	*S. epidermidis*	-	*S. auricularis*	Sac -
1	*S. epidermidis*	*Staph.epidermidis*	-	*S. simulans*	Man +; Tre +
1	*S. epidermidis*	*S. warneri*	Tre +	*S. auricularis*	Sac -
1	*S. warneri*	*S. warneri*	-	*S. haemolyticus*	Ure -
1	*S. warneri*	*S. warneri*	-	*S. auricularis*	Ure -
1	*S. haemolyticus*	*S. warneri*	Ure +	*S. epidermidis*	Ure +; Arg -; Tre -
1	*S. lugdunensis*	*S. lugdunensis*	-	*S. epidermidis*	Lac -; Tre -

+ : positive; - : negative; S: sensitive; Incor. Test.: incorrect tests; Arg: arginine; Rib: ribose; Nov: novobiocin; Xyl: xylose; Ara: arabinose; Sac: saccharose; Man: mannitol; Tre: trehalose; Ure: urease; Lac: lactose. Biochemical test results were compared to the identification tables described by Konemann et al. 2001.

TABLE IV

Comparison of sensitivity and specificity between the methods with ITS-PCR

Species	Sensitivity (%)			Specificity (%)		
	Novobiocin	Vitek I	Simplified	Novobiocin	Vitek I	Simplified
S. aureus	100.0	100.0	100.0	100.0	100.0	100.0
S. epidermidis	100.0	62.5	93.7	87.0	97.6	100.0
S. warneri	0.0	0.0	100.0	0.0	95.9	97.9
S. haemolyticus	0.0	87.5	87.5	0.0	97.8	100.0
S. lugdunensis	0.0	0.0	100.0	0.0	100.0	100.0
S. saprophyticus	100.0	84.2	100.0	100.0	100.0	100.0
Total (%)	89.1	81.2	98.0	89.1	92.0	98.0

6. Biofilme Estafilocócico

Os fatores de virulência produzidos pelos ECN e como eles contribuem na patogenicidade das infecções associadas com corpos estranhos estão em investigação. Evidências indicam que a patogenicidade pode estar relacionada com a produção de um polissacarídeo extracelular que permite a aderência desse micro-organismo à superfície lisa, colonizando cateteres, válvulas cardíacas, marcapassos e próteses articulares, formando o biofilme (120). Este confere proteção aos micro-organismos contra os mecanismos de defesa do sistema imune do hospedeiro e de agentes antimicrobianos, sendo diretamente pelo bloqueio da penetração desses na célula bacteriana, ou indiretamente por mantê-la em um inativo estado de repouso. Por estas razões, sua formação é considerada o principal fator de virulência dos ECN e as mais importantes infecções causadas por estes micro-organismos são as que envolvem corpos estranhos (121).

Muitos autores definem biofilme como associações de micro-organismos e de seus produtos extracelulares, que se encontram aderidos a superfícies bióticas ou abióticas. São complexos ecossistemas microbianos que podem ser formados por populações desenvolvidas a partir de uma única ou de múltiplas espécies. Na maioria dos ambientes naturais, o biofilme consiste de múltiplas espécies, em contraste, em infecções associadas a biomateriais, aproximadamente 80% das células são *S. epidermidis.* Esse fenômeno pode ser explicado pelo fácil acesso deste habitante da pele a cateteres e implantes (122).

A adesão de *S. epidermidis* depende de propriedades físico-químicas das superfícies polimérica e bacteriana. Como as superfícies plásticas são hidrofóbicas, e o principal parâmetro que determina a adesão é a hidrofobicidade da superfície bacteriana, a adesão primária de *S. epidermidis* tem sido similar em muitos biomateriais investigados (123).

Quando um determinado material é implantado em um indivíduo, os fluídos corpóreos como as proteínas do soro e plaquetas passam a recobrí-lo, o que modifica as propriedades das superfícies facilitando a aderência bacteriana (124-125). *S. epidermidis* e *S. aureus* expressam dezenas de proteínas na superfície bacteriana, chamadas MSCRAMMs (*Microbial Surface Components Recognizing Adhesive Matrix Molecules*), que se ligam especificamente a proteínas da matriz extracelular do hospedeiro, tal como fibrinogênio, colágeno, fibronectina e vitronectina. Dessas, a mais estudada é a Fbe, também conhecida como SdrG, uma proteína que facilita a interação desses micro-organismos ao fibrinogênio (126).

Também foi demonstrado que amostras produtoras de biofilme, dependendo das condições de crescimento, contêm consideráveis quantidades de ácidos teicóicos extracelulares (ECTA) (127), que aumentam a adesão a superfícies revestidas com fibronectina, sugerindo um provável papel na virulência de *S. epidermidis* (128).

No segundo estágio da formação do biofilme, ocorre a multiplicação dos ECN na monocamada de células fixadas ao plástico ou ao hospedeiro. Para formar as camadas bacterianas, as células vão se ligando umas as outras através do PIA

(polissacarídeo de adesão intercelular), uma molécula polissacarídica que favorece a adesão entre as células, também chamada de poli-N-acetilglicosamina (PNAG) (129-130). O PIA está localizado na superfície celular, é um homopolímero linear de até 130 resíduos de β-1-6-N-acetilglicosamina, composto por duas frações polissacarídicas: polissacarídeo I (>80%) com 15 a 20% dos resíduos deacetilados e, portanto carregados positivamente e polissacarídeo II (< 20%) estruturalmente relacionado ao polissacarídeo I, mas com baixa quantidade de resíduos D-glicosamina não N-acetilados e contém fosfato e éster ligado a succinato, sendo aniônico (122,130-131).

A produção do PIA (**Figura 3**) é mediada por produtos do gene cromossomal *ica (intercelular adhesion)*, organizados em uma estrutura *operon*. Este operon contém os genes *icaADBC* (biossíntese), mais o *icaR*, que tem a função regulatória e é transcrito no sentido oposto. Assim que ocorre a ativação deste operon, são codificadas quatros proteínas necessárias para a síntese do PIA que são IcaA, IcaD, IcaB e IcaC (130). O PIA é sintetizado a partir de UDP-N-acetilglicosamina por uma enzima N-acetilglicosaminatransferase, a qual é codificada pelo lócus de adesão intercelular (*ica*), em particular pelo *icaA.* A expressão única deste gene induz uma baixa atividade enzimática produzindo baixa quantidade do polissacarídeo. Entretanto, a expressão simultânea do i*caA* e do *icaD* promovem um aumento significativo da N-acetilglicosaminatransferase, que atua formando oligômeros com 10-20 resíduos aproximadamente de β-1,6-N-acetilglicosamina (130, 132). Foi demonstrado que *icaD* sozinho não induz atividade enzimática de transferase,

enquanto que o *icaA* isolado induz pouca atividade. Entretanto, quando *icaA* e *icaD* são expressos conjuntamente, a atividade enzimática da transferase é aumentada,

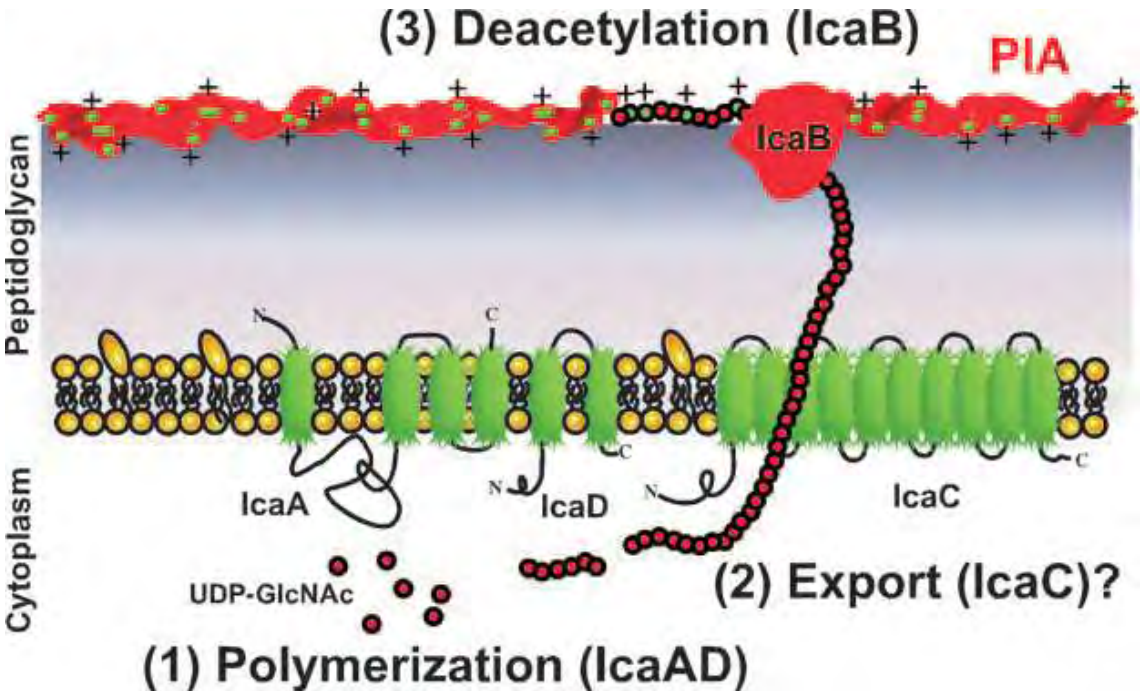

Figura 3: Modelo da biossíntese do Polissacarídeo de Adesão Intercelular (PIA). Fonte: Vuong *et al*. (134).

formando maior quantidade de PIA. Outra possibilidade proposta é que a proteína transcrita por *icaA,* uma proteína transmembrana que contém aproximadamente 412 aminoácidos (133), necessita de icaD para obter uma conformação ativa. Neste mesmo trabalho foi mostrado que o produto do gene *icaC*, uma proteína hidrofóbica integral de membrana com aproximadamente 355 aminoácidos (133), é necessária para a síntese de oligômeros de N-acetilglucosamina para reagir com anticorpos do PIA. O gene *icaC* quando expresso concomitantemente com o *icaA* e *icaD*, induz a síntese de oligômeros mais longos com 130 resíduos, e

presumivelmente também tem a função de exportar a cadeia nascente de PIA. Após a exportação o PIA é desacetilado pela proteína IcaB que introduz cargas positivas, que são cruciais para sua localização superficial e função biológica. A produção de PIA e a sua desacetilação têm sido reconhecidas como fatores chave na virulência de *S. epidermidis* (134-135) e são os mecanismos predominantes no acúmulo em biofilme (130).

Vários autores têm enfatizado a produção de biofilme como um marcador epidemiológico da infecção (120, 136-137). Por outro lado, outros investigadores não encontraram nenhuma associação entre cepas produtoras de biofilme e a ocorrência de infecções causadas por esses micro-organismos (138-140). Com base nas considerações acima, decidimos avaliar a virulência das amostras de ECN isoladas de recém-nascidos internados na Unidade Neonatal do HC da FMB (ANEXO 8). O presente estudo mostrou que uma pequena proporção dos isolados, que produziram biofilme (22,2%) estiveram associados a processos infecciosos, porém foi verificado uma frequência significativamente maior de linhagens de *S. epidermidis* produtoras de biofilme isoladas de hemoculturas e de corpos estranhos do que de secreções. As amostras de ECN produtoras de biofilme têm sido isoladas mais frequentemente de pacientes com sepse do que de pacientes sem doença invasiva, indicando o risco de doença invasiva em pacientes portadores de ECN produtores de biofilme (137).

Em outro estudo, realizado para verificar os fatores de risco associados a não cura de peritonites em pacientes submetidos à diálise peritoneal ambulatorial contínua (CAPD), foi verificado que a produção do biofilme foi um fator

independente associado com a não resolução de casos de peritonites causados por ECN (ANEXO 9), sendo que as peritonites causadas por ECN não produtores de biofilme apresentaram chance de cura 27 vezes maior do que nos episódios causados pelas amostras de ECN biofilme positivas.

Em relação à detecção da produção de biofilmes são utilizados principalmente os métodos qualitativos, como o método de aderência em tubo de borossilicato (TM) proposto por Christensen *et al.* (136), o método do Ágar Vermelho Congo (CRA) descrito por Freemam *et al.* (141) e o método quantitativo de aderência em placa de poliestireno (TCP) descrito por Christensen *et al*. (142). Ainda em complemento a esses métodos são utilizados os moleculares como a reação em cadeia da polimerase (PCR), que amplificam genes envolvidos na produção do biofilme. Uma coleção de 80 amostras de estafilococos coagulase-negativa (ECN) isoladas de materiais clínicos, provenientes de recém-nascidos (RN) da Unidade Neonatal do HC da FMB e 20 de fossas nasais obtidas de indivíduos sadios foram estudadas em nosso laboratório quanto à produção de biofilme, objetivando avaliar três métodos fenotípicos de detecção de biofilmes em ECN e a pesquisa dos genes *icaA*, *icaD* e *icaC* utilizando a técnica de PCR (ANEXO 10). Entre os métodos fenotípicos foram utilizados os métodos de aderência em placa de poliestireno (TCP), aderência em tubo de borossilicato (TM) e o método do Ágar Vermelho Congo (CRA).

Na pesquisa da produção de biofilme pela técnica de aderência em tubo de borossilicato (**Figura 4**), foi verificado que das 100 amostras estudadas, 82 foram produtoras de biofilme, sendo 44 amostras isoladas de ponta de cateteres, 23 de

hemocultura e 15 de fossas nasais. Com relação à análise de cada espécie, observou-se que 70 (85,4%) amostras de *S. epidermidis* foram positivas. A produção de biofilme também foi verificada em *S. warneri* (4), *S. cohnni* (3), *S. xylosus* (2), *S. saprophyticus* (2) e *S. lugdunensis* (1). Os resultados mostraram que o teste de aderência ao tubo de borossilicato apresentou 100% de sensibilidade e 100% de especificidade, quando comparado com a técnica de PCR (presença concomitante dos genes *icaA* e *D* ou *icaACD*).

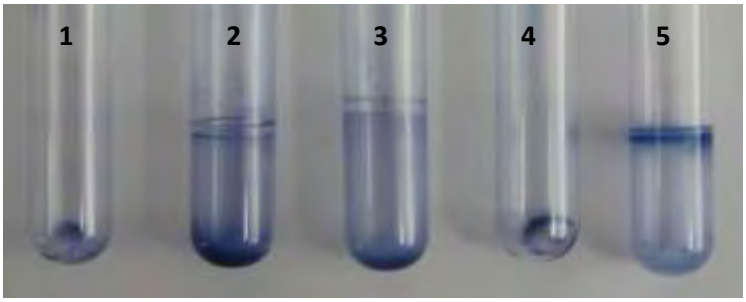

Figura 4: Pesquisa da produção de biofilme pelo método de aderência em tubo (TM) em amostras de Estafilococos coagulase-negativa. 1: amostra não produtora de biofilme, 2 e 3: amostras produtoras de biofilme, 4: *S. epidermidis* ATCC 12228 - (controle negativo), 5: *S. xylosus* ATCC - 29979 (controle positivo).

O teste de aderência a placa de poliestireno (**Figura 5**) com leitura no filtro de 540nm, apresentou 96% de sensibilidade e 94% de especificidade para a presença dos genes *icaA* e *D* ou *icaACD* concomitantes, quando comparado com a PCR.

Em relação à utilização do teste CRA, de acordo com o proposto por Freeman *et al*. (141), espécies produtoras de biofilme formam colônias negras secas no CRA, enquanto que as não produtoras formam colônias vermelhas. Das 100 amostras de ECN estudadas, 76 formaram colônias negras no Ágar Vermelho Congo (**Figura 6**), com 44 amostras revelando coloração preta brilhante e 32 amostras com coloração preta e seca. Os resultados mostraram 24 amostras com as colorações variando entre rosa (2%), vermelha (5%) e bordô (17%). Assim, foi necessário adotar uma escala de cinco cores comparando os resultados das placas do CRA com a técnica de PCR com a finalidade de analisar a variação da coloração das colônias com a presença dos genes *ica*. Dessa forma, foram classificadas as amostras positivas em preto brilhante (PB) e preto seca (PS) e as negativas em vermelha (V), rosa (R) e bordô (B). Em estudos realizados por outros pesquisadores utilizando o CRA, também foram adotadas escalas de cores para se obter um melhor diagnóstico, porém com algumas variações nas tonalidades em relação à escala proposta nesse estudo. Estudos realizados por Vogel *et al*. (120) e Arciola *et al*. (79) tem classificado as amostras positivas com as colorações muito negro (vb), negro (b) e quase negro (ab) e as negativas como bordô (brd), vermelho (r) e muito vermelho (vr).

O método do Ágar Vermelho Congo apresentou sensibilidade de 89% e especificidade de 100% em comparação com a presença concomitante dos genes *icaA* e *icaD* ou *icaACD*.

Os dados obtidos nesse estudo não revelaram diferença em relação à frequência da produção de biofilme entre os isolados de amostras clínicas e de

fossas nasais de portadores saudáveis em todos os testes utilizados para a pesquisa de biofilme. A produção de biofilme similar em amostras de ECN isoladas de diferentes origens, incluindo espécimes clínicos, ambiente e da microbiota de pessoas saudáveis, também foi relatada por outros pesquisadores (143-144).

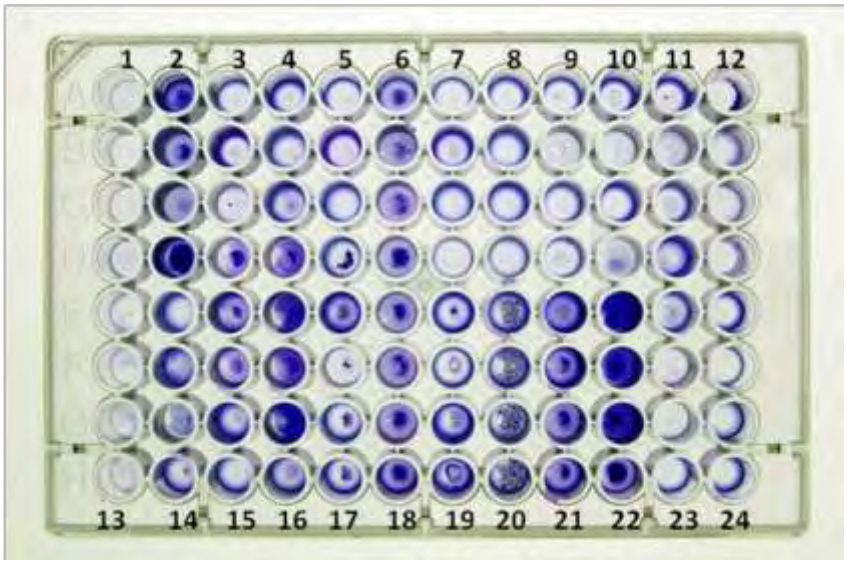

Figura 5: Pesquisa da produção de biofilme pelo método de aderência em placa de poliestireno (TCP) em amostras de Estafilococos coagulase-negativa. Coluna 1, Linhas A, B, C, D (TSB estéril); Coluna 13, Linhas E, F, G, H *S. epidermidis* ATCC 12228 (Controle Negativo) – Coluna 2, Linhas A, B, C, D *S. xylosus* ATCC 35984 (Controle positivo); Colunas – 3, 7, 8, 10, 11, 17 amostras classificadas como fraco aderente – 4, 6, 14, 15,16, 18, 19, 20, 21, 22 amostras classificadas como fortes aderentes – 5, 9, 12, 23 e 24 amostras não aderentes.

Os resultados mostraram uma amostra positiva pelo método do teste de aderência a placa de poliestireno e negativa para a presença dos genes *ica*, indicando a formação de biofilme PIA-independente. Nos casos da formação de biofilme independente de PIA, proteínas adesivas podem substituir o polissacarídeo, sendo que a proteína mais importante envolvida na formação de biofilme é a Aap- proteína associada ao acúmulo (145).

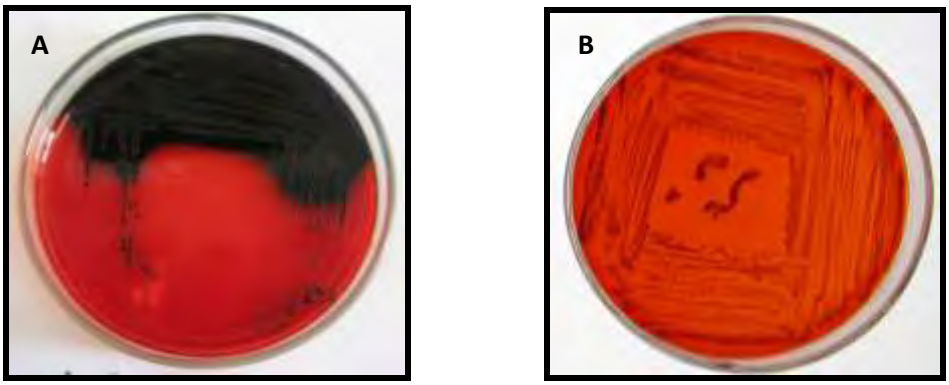

Figura 6: Pesquisa da produção de biofilme pelo método de Ágar vermelho Congo (CRA) em amostras de Estafilococos coagulase-negativa. **A** *S. simulans* ATCC 27851, controle positivo, com coloração preto brilhante no Ágar Vermelho Congo. **B** Amostra negativa para produção de biofilme no Ágar Vermelho Congo, *S. cohnni* (coloração vermelha).

Em estudo desenvolvido por Rohde *et al*. (146), 27% das linhagens produtoras de biofilme isoladas de infecções de proteses ortopédicas formaram biofilme independentes de PIA, sendo que na maioria dos casos a formação de biofilme parecia ser mediada pela Aap. Aap é uma proteína de 220 kD que precisa se

proteoliticamente clivada a uma forma menor de 140 kD para induzir a formação de biofilme (147).

Também tem sido sugerido que a expressão de Bap (Proteína associada ao biofilme) leva à formação de biofilme, mesmo na ausência do operon *icaADBC*, ressaltando a importância dessa proteína no estabelecimento de biofilmes. Há evidências da significância de Bap durante mastite causada por *S. aureus* em bovinos (148). Um gene homólogo de *bap,* chamado *bhp*, ocorre em *S. epidermidis* de uma maneira similar a *bap* em amostras isoladas de animais. No entanto, o mecanismo pelo qual Bap e, eventualmente Bhp contribuem para a formação de biofilme ainda não está estabelecido (148-149).

Recentemente também tem sido demonstrado a importância do DNA extracelular na formação de biofilmes em *S. epidermidis* (150). A importância do DNA genômico extracelular (eDNA) como um componente estrutural do biofilme foi demonstrada pela primeira vez em *Pseudomonas aeruginosa* (151), mas foi posteriormente demonstrado em uma variedade de espécies bacterianas. Vários estudos sugerem que a liberação de DNA extracelular de *S. epidermidis* é principalmente causado pela atividade da autolisina Atle (gene *atle*) (152), sendo que o eDNA é requerido para adesão inicial nas superfícies, bem como na etapa subsequente durante a maturação do biofilme. O tratamento das células de *S. epidermidis* com DNAse I inibiu a formação de biofilme no tempo inicial, sugerindo que a liberação de DNA contribui para a fixação dos *S. epidermidis* nas superfícies. Em *S. aureus* a lise celular e a liberação de eDNA tem sido associadas à presença do gene de autolisina (mureína hidrolase) gene *cidA,* enquanto a produção de

termonuclease tem mostrado papel na degradação do eDNA e dispersão do biofilme (153). Estudos em desenvolvimento em nosso laboratório tem como objetivos detectar a presença dos genes *Aap, bap, bhp e atle* em *S. aureus* e Estafilococos coagulase-negativa isolados de leite de ovinos com mastite.

O biofilme garante o estabelecimento de um sistema de comunicação que coordena as atividades metabólicas em benefício mútuo, assim como a produção de fatores de virulência que facilitam a disseminação desses microrganismos no hospedeiro. Um fator que contribui para a sobrevivência das bactérias dentro do hospedeiro permitindo que estas controlem de maneira eficiente os seus mecanismos de virulência, inclusive a formação do biofilme, ocorre através de um processo conhecido como *quorum-sensing* (QS), no qual a comunicação bactéria-bactéria baseia-se em moléculas sinalizadoras, chamadas auto-indutores, através das quais podem regular seu comportamento de acordo com a densidade populacional. Com isso, as bactérias podem perceber o momento em que estão em alta densidade celular para expressar fatores de virulência e garantir que o ataque conjunto ao hospedeiro seja eficiente. Quando um pequeno número de bactérias libera auto-indutor no ambiente, sua concentração é muito baixa para ser detectada; porém, quando estão em alta densidade populacional, a concentração de auto-indutores alcança um nível suficiente para fazer com que as células respondam aos estímulos, ativando ou reprimindo genes alvo. Dessa maneira, este sistema permite que as bactérias coordenem seu comportamento de acordo com as condições do ambiente, adaptando-se de acordo com a disponibilidade de

alimento, temperatura, pH, osmolaridade, bem como a expressão de fatores de virulência, produção de antibióticos, formação de biofilmes e outros (130).

S. aureus regula seus fatores de virulência através de dois sistemas *quorum-sensing* (QS), que regulam um ao outro (154-155). O QS1 consiste na ativação do RNAIII através da RAP (proteína ativadora de RNA), uma proteína de 21-KDa que induz a fosforilação da proteína alvo de RAP (TRAP) (155-156). Conforme as bactérias vão se multiplicando, a RAP atinge um limiar de concentração (em meio à fase de crescimento exponencial) induzindo a fosforilação da sua molécula alvo, a TRAP (157). A fosforilação da TRAP ativa a síntese do QCII, que é composta pelos produtos do sistema *agr* (gene regulador acessório). No locus *agr*, dois promotores com orientações opostas, P2 e P3, produzem dois transcritos, RNAII e RNAIII, respectivamente. O RNAIII é formado por 510 nucleotídeos responsáveis pela transcrição de genes de muitos fatores de virulência, como toxinas extracelulares, enzimas e proteínas de superfície celular presentes em *S. aureus* (158). O RNAII é formado por quatro genes, *agrA*, *agrB, agrC* e *agrD*, arranjados em um operon (*agr*), e juntos trabalham para induzir a síntese do RNAIII. Os produtos dos genes *agrB* e *agrD*, respectivamente as proteínas agrB e agrD se juntam formando um polipeptídeo autoindutor (AIP). A AgrC é uma proteína transmembrana com receptores para AIP e quando AIP se liga à proteína AgrC, este fosforila a proteína AgrA ativando-a. A proteína AgrA fosforilada age como um indutor dos promotores P2 e P3. A síntese do RNAIII é induzida quando a concentração do AIP, específico no meio, atinge determinados níveis que geralmente são detectados na passagem da fase exponencial de crescimento para a fase estacionária. O produto final da

cascata do locus *agr* é o RNAIII, um RNAm que funciona como indutor ou repressor dos genes acessórios (155-156, 158).

Os sistemas QS1 e QS2 (**Figura 7**) se interagem, pois, a interação entre a fosforilação da TRAP e AgrC através de seus respectivos auto-indutores, RAP ou AIP, regula a expressão de moléculas de adesão ou toxinas, ativando a expressão de exotoxinas na presença de alta densidade celular e a supressão de adesinas, com consequente disseminação dos micro-organismos pelo tecido do hospedeiro. Sendo assim, a TRAP demonstra ser uma molécula chave na regulação da patogênese, pois quando sua expressão é inibida por mutagênese ou por peptídeos inibitórios, as bactérias não formam biofilmes, não produzem toxinas e não causam doenças (159-160).

Um peptídeo (YSPWTNF-NH2), conhecido como RIP (peptídeo inibidor de RNA) pode inibir a atividade do RNAIII, impedindo a fosforilação da TRAP, interferindo no sistema *quorum sensing*. O RIP tem se mostrado muito eficaz no tratamento de infecções estafilocócicas associadas a dispositivos médicos, incluindo aquelas causadas por cepas de *S. aureus* e *S. epidermidis* resistentes a múltiplas drogas (157, 161). Estudos com implantes em animais para observação da produção de biofilme, e a utilização do peptídeo inibidor de RNA (RIP), mostraram que a injeção do peptídeo (RIP) nos animais inibiu o RNAIII, impedindo a fosforilação da proteína (TRAP), interferindo no sistema *quorum-sensing*, e como resultado houve uma baixa produção de biofilme e infecções (162). Outros estudos mostraram que o RIP ao interferir no sistema *quorum-sensing*, além de ser muito eficaz na terapia de prevenção de infecções estafilocócicas associadas a

dispositivos, também reduz a carga bacteriana e pode ser útil para o tratamento de feridas infectadas, podendo representar uma alternativa interessante para o futuro em relação aos antibióticos convencionais (163).

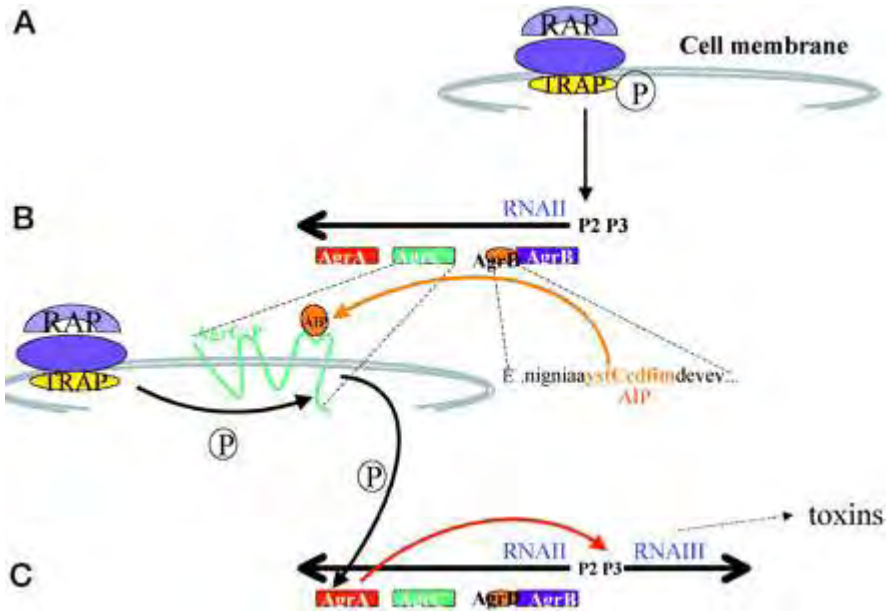

Figura 7: Interação de TRAP e Sistema Agr (Fonte: Balaban et al. 2001)

Em relação aos antibióticos, as bactérias nos biofilmes, geralmente apresentam um aumento na concentração inibitória mínima (CIM), resultando em redução da suscetibilidade. Vários fatores contribuem para o aumento dessa resistência aos antimicrobianos, por exemplo, o modo de crescimento do biofilme permite que as bactérias sobrevivam à exposição a vários antimicrobianos em concentrações até 1000 vezes maiores em relação às bactérias planctônicas (164).

Estudos mostraram que a relativa resistência aos antimicrobianos pode ser devido a penetração limitada destes, pois algumas partes do biofilme são difíceis de atingir ou a atividade metabólica é lenta. Alguns antimicrobianos reduziram a

atividade em ambientes privados de oxigênio, o que também contribui para a resistência do biofilme, devido à disponibilidade de oxigênio ser reduzida nos níveis mais profundos (165). Outro fator pode ser a expressão de certos genes dentro do biofilme que conferem aumento da resistência aos antimicrobianos entre as espécies gram-positivas, principalmente os estafilococos, revelando preocupações quanto ao uso de antibióticos e a necessidade de novos agentes (164, 166-167).

Devido aos Estafilococos coagulase-negativa serem uma das principais causas de infecções relacionadas a dispositivos médicos, pela sua capacidade de aderência e a consequente formação do biofilme, nos quais as bactérias apresentam resistência a múltiplas drogas antimicrobianas, tornando o tratamento de infecções do biofilme um esforço difícil e caro, outro estudo em desenvolvimento em nosso laboratório objetiva o estudo da estrutura do biofilme bacteriano em diferentes espécies de ECN por microscopia eletrônica de varredura, avaliação da antibioticoterapia utilizada para tratamento dessas infecções em biofilmes e células livres e a busca de alternativas para prevenção da formação de biofilme estafilocócico utilizando o RIP em modelos experimentais *in vitro*.

ANEXO 8

CUNHA MLRS, RUGOLO LMSS, LOPES CAM. Study of virulence factors in coagulase-negative staphylococci isolated from newborns. Mem Inst Oswaldo Cruz. 2006; 101: 661-8.

Study of virulence factors in coagulase-negative staphylococci isolated from newborns

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Coagulase-negative staphylococci (CNS) have been identified as the etiological agent in various infections and are currently the microorganisms most frequently isolated in nosocomial infections. However, little is known about the virulence factors produced by CNS that contribute to the pathogenesis of infections caused by these microorganisms. The study of CNS isolated from infectious processes of newborns hospitalized in the Neonatal Unit of the Hospital of the Botucatu Medical School, Unesp, indicated Staphylococcus epidermidis *as the most frequently isolated species (77.8%), which was also associated with clinically significant situations. The analysis of virulence factors revealed the production of slime in 20 (17.1%) of all CNS samples isolated and the synthesis of a broad spectrum of enzymes and toxins, including hemolysins (19.6%), lipase (17.1%), lecithinase (3.4%), DNAse (15.4%), thermonuclease (7.7%), and enterotoxin A, B or C (37.6%). Taking into consideration that the etiological importance of CNS has often been neglected, the present investigation confirmed that these microorganisms should not be ignored or classified as mere contaminants.*

Key words: coagulase-negative staphylococci - virulence factors - slime - enzymes - toxins

Few reports of infections with coagulase-negative staphylococci (CNS) were published before the 1970s; clinicians and microbiologists considered them to be contaminants of clinical samples, with *Staphylococcus aureus* being the only pathogenic species within the genus *Staphylococcus* (Kloos & Bannerman 1995). This distinction, which has been widely used for clinical diagnosis, represents a challenge in relation to the role of these microorganisms in infectious processes.

At present, CNS are considered to be basically opportunistic microorganisms that prevail in numerous organic conditions, producing serious infections (Kloos & Bannerman 1995, Lark et al. 2000). The recognition of CNS as etiological agents may also be due to the valorization of this group of organisms as opportunistic pathogens and to the increasing use of invasive procedures such as intravascular catheters and to prosthetic interference. Data obtained between 1995 and 1998 by the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) program have demonstrated that CNS are the etiologic agents most frequently found in nosocomial bacteremias in the United States (Edmond et al. 1999).

The virulence factors produced by CNS and how they contribute to the pathogenicity of infections associated with foreign bodies are currently under investigation. Evidence indicates that pathogenicity might be related to the production of an extracellular polysaccharide, known as slime, that permits these microorganisms to adhere to smooth plastic surfaces, colonizing catheters, prosthetic heart valves, pacemakers, and joint prostheses (Vogel et al. 2000).

Differentiation between virulent and non-virulent strains has been difficult since the virulence factors of these microorganisms are still not well defined (Gemmell 1987). According to this author and to Koneman et al. (1997), CNS produce other virulence factors, such as hemolysins, lipases, proteases, and toxins.

Based on the above considerations, we decided to evaluate this question in our institution, with the main objectives being the identification of CNS species isolated from clinical cases of newborns hospitalized in the Neonatal Unit of the Hospital of the Botucatu Medical School, Unesp, Botucatu, Brazil, and the determination of the production of slime, enzymes, and toxins by the different isolates.

MATERIALS AND METHODS

Organisms - CNS isolates were obtained from 107 newborns hospitalized in the Neonatal Unit of the University Teaching Hospital, Unesp, Botucatu, between 1990 and 1996. The procedures used were approved by the Ethics Committee of the School.

Identification of CNS - CNS were isolated on blood agar. Bacterial colonies were stained by the Gram method and submitted to catalase and coagulase tests (Koneman et al. 1997). The genus *Staphylococcus* was differentiated from *Micrococcus* by the glucose oxidation and fermentation test and by resistance to bacitracin (0.04 U) and sensitivity to furazolidone (100 µg) as described by Koneman et al. (1997).

CNS species were identified as described by Kloos and Schleifer (1975), Kloos and Bannerman (1995), and Cunha et al. (2004). The following tests were used: utiliza-

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tion of xylose, arabinose, sucrose, trehalose, mannitol, maltose, lactose, xylitol, ribose and fructose, characterization of hemolysis, nitrate reduction, urease, decarboxylase ornithine, and resistance to novobiocin.

The following international CNS reference strains were used as controls: *S. epidermidis* (ATCC 12228), *S. simulans* (ATCC 27851), *S. warneri* (ATCC 10209), *S. xylosus* (ATCC 29979), and *S. saprophyticus* (ATCC 15305).

Clinical relevance - Data were obtained by analysis of the patients' medical records. Data regarding perinatal infection risk factors were reviewed, including prolonged membrane rupture (> 24 h), gestational age, birthweight, and invasive procedures such as arterial or venous umbilical catheterization, central venous or peripheral catheterization, mechanical ventilation, surgical procedures, peritoneal dialysis, parenteral nutrition, chest drainage, and ventriculoperitoneal shunts. The possibility that foreign bodies had been removed during CNS infection was also examined.

The progression of the clinical situation of the newborn during the week before and after CNS isolation was analyzed, with emphasis on diagnosis and clinical charts suggestive of CNS infection, characterized by insidious and non-specific symptoms, more frequently affecting general health, thermal instability, and apnea (Hall et al. 1987).

With progression of the clinical situation, alterations in the blood count and/or positivity for C-reactive protein were determined at the time of CNS isolation. Normal hematological parameters were those proposed by Manroe et al. (1979). The deaths observed were attributed to CNS infection occurring within the first 72 h after agent isolation, and possible association with CNS between days 4 and 7 after CNS isolation.

Another aspect investigated and considered to be of clinical relevance was the previous use of antibiotic therapy, including antibiotics adequate for CNS after bacteriological diagnosis, as well as the use of specific antibiotics such as vancomycin, oxacillin, or teicoplanin.

The CNS isolated in the present study were classified as "significant" and "contaminant" according to modified CDC criteria (Garner et al. 1996), as follows:

"Significant" - CNS isolated from newborns who presented three or more of the following features: risk factors for infection, clinical or hematological alterations, and adequate antibiotic therapy. Isolates were also considered to be significant in patients who presented only two of these features and who died without receiving adequate antibiotic therapy.

"Contaminant" - CNS isolated from newborns who presented only risk factors for infection and/or only one of the other features (clinical or hematological alterations, or adequate antibiotic therapy). Isolates from newborns who presented all three features, but showed a satisfactory course of the infection without the administration of adequate antibiotics were also considered to be contaminants. The isolation of another etiological agent from internal fluids and foreign bodies at the time of CNS isolation was also used as a criterion for the classification of contamination.

Study of slime production - Slime production was analyzed as described by Christensen et al. (1982).

Determination of hemolysin production - The production of hemolysins and cytolytic toxins was determined on plates containing blood agar base consisting of 5% rabbit blood and 5% sheep blood incubated at 37°C for 24 h. A positive result was indicated by the formation of hemolysis zones around the isolated colonies.

Determination of lipase and lecithinase - Lipolytic activity (Jessen et al. 1959) was determined on plates containing blood agar base enriched with 0.01% $CaCl_2.2H_2O$ and 1% Tween 80. A positive result was defined as the formation of opacity around the colonies after incubation at 37°C for 18 h, followed by incubation for 24 h at room temperature. The production of lecithinase (Owens 1974) was studied in Baird-Parker medium. A positive result was indicated by the formation of an opaque halo around the colonies.

Determination of DNAse and TNAse - Nuclease (DNAse) and thermonuclease (TNAse) were determined by the metachromatic Toluidine blue O-DNA agar diffusion technique according to Lachica et al. (1971). Positive results were interpreted by comparing the halos obtained with the *S. aureus* reference strain (ATCC 25923), DNAse, and positive TNAse.

Culture supernatants obtained by the sac culture method (Donnelly et al. 1967), as described below, were also tested for DNAse and TNAse production.

Production of toxins - The toxigenic profile of the isolates was determined using the sac culture method of toxin production (Donnelly et al. 1967). Culture supernatants obtained were stored at –20°C until the time of use.

Detection of enterotoxins and TSST-1 - Enterotoxins and toxic shock syndrome toxin 1 (TSST-1) were detected by the reversed passive latex agglutination (RPLA) assay as described by Shingaki et al. (1981). The SET-RPLA-T900 and TST-RPLA-TD940 (Oxoid Diagnostic Reagents) kits were used for the detection of enterotoxin A (SEA), enterotoxin B (SEB), enterotoxin C (SEC) and enterotoxin D (SED), and of TSST-1, respectively. The culture supernatant was previously treated with 5% (v/v) normal rabbit serum or 5% purified rabbit IgG to prevent the occurrence of nonspecific reactions (Pereira et al. 1997). Samples that presented nonspecific reactions even after these procedures were filtered through a Millipore membrane (8 µm) and, if necessary, diluted 1:10 with 0.02 M phosphate buffer in 0.9% NaCl, pH 7.4.

The following international CNS reference strains were used as controls: *S. aureus* (ATCC 13565, SEA producer), *S. aureus* (ATCC 14458, SEB producer), *S. aureus* (ATCC 19095, SEC producer), and *S. aureus* (ATCC 23235, SED producer).

Statistical analysis - The data were analyzed by the χ^2 test or Fisher's exact test, with $n < 20$. The nonparametric Mann-Whitney test was used for the analysis of newborn birthweight and age. The level of significance was set at $p < 0.05$ for all tests.

RESULTS

Organisms - A total of 117 CNS were isolated from different materials collected from 107 newborns. Sixty isolates were obtained from blood cultures collected between 1990 and 1996, 41 isolates were from foreign bodies (30 from catheter tips, 10 from cannula tips, 1 from a chest drain tip), 13 from secretions (2 drain secretions, 5 gastric secretions, 6 tracheal secretions), and three from urine, all obtained between 1994 and June 1996.

Identification of CNS - *S. epidermidis* was the CNS species most frequently isolated, corresponding to 91 (77.8%) isolates. The remaining species were distributed among *S. haemolyticus* (7 isolates, 6%), *S. lugdunensis* (7 isolates, 6%), *S. hominis* (5 isolates, 4.3%), *S. simulans* (4 isolates, 3.4%), *S. warneri* (2 isolates, 1.7%), and *S. xylosus* (1 isolate, 0.8%).

Clinical relevance - Of the 60 blood culture isolates, 35 (58.3%) were interpreted to be significant and 25 (41.7%) to be contaminants. Of the 41 CNS isolated from foreign bodies, 21 (51.2%) were considered to be significant, including 14 isolates from catheter tips (66.7%), six from cannula tips (28.6%), and one from a chest drain tip (4.7%). Of the 13 isolates from secretions, four were considered to be significant, including one from chest drain secretion and three from tracheal secretion.

Of the 107 newborns, 54 had CNS infection and 53 were infection-free. Table I shows that 27 (50%) of the infected newborns presented a birthweight < 1500 g, significantly different from infection-free newborns (20.8%). Median birthweight also showed a significant difference between the CNS-infected group (1495 g) and the infection-free group (2270 g). Median age at CNS isolation differed significantly between the infection group (10 days old) and the infection-free group (4 days old). Most CNS-infected newborns (42, 77.8%) were submitted to two or more invasive procedures, including the use of a catheter in 48 (88.9%), parenteral nutrition in 35 (64.8%), and mechanical ventilation in 33 (61.1%).

The results showed a higher frequency of *S. epidermidis* associated with infection (86.7%) than with contamination (68.4%) ($p < 0.05$). No significant differences were observed for the other species.

Clinically significant CNS were isolated at a higher proportion from blood than secretions ($p < 0.05$), but there was no statistically significant difference when compared to foreign bodies.

Slime production - Fig. 1 shows that 20 (17.1%) of the 117 CNS isolates were positive for slime production, including 18.7% of *S. epidermidis* strains, 28.6% of *S. lugdunensis* strains, 20% of *S. hominis* strains. *S. haemolyticus*, *S. simulans*, *S. warneri* or *S. xylosus* strains were negative for slime production.

Table II shows that 12 (23.1%) of the 52 significant *S. epidermidis* isolates were slime producers compared to five (12.8%) of the 39 contaminant isolates, with no significant difference between groups. Slime production was not observed in any of the other significant species. Among contaminant samples, two of the four *S. lugdunensis* samples and one of the five *S. hominis* samples produced slime.

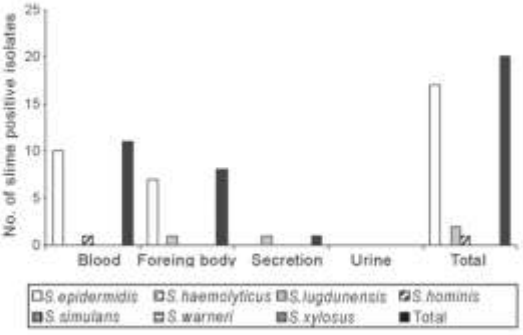

Fig. 1: production of slime by coagulase-negative staphylococci according to species and clinical material. *S: Staphylococcus.*

TABLE I

Perinatal risk factors of coagulase-negative staphylococci infection

Risk factors	With infection		Infection free		Total		
	N	%	N	%	N	%	p value
Median BW (g)	1495		2270		1985		0.0011
BW < 1500 g	27	50	11	20.8	39	36.4	0.0002
Median age (days)	10		4		7		0.0042
Catheter	48	88.9	33	62.3	81	75.7	0.0004
Chest drainage	10	18.5	5	9.4	15	14	ns
Mechanical ventilation	33	61.1	18	34	51	47.7	0.0138
Parenteral nutrition	35	64.8	22	41.5	57	53.3	0.0040
Ventriculoperitoneal shunt	3	5.5	2	3.8	5	4.7	ns
Non-removal of foreign body	31	54.7	17	32.1	48	44.8	0.0010
Membrane rupture > 24 h	18	33.3	17	32.1	35	32.7	ns
Two or more foreign bodies	42	77.8	20	37.7	62	57.9	0.0001
Total of neonates	54	50.5	53	49.5	107	100	

BW: birthweight; ns: $P > 0.05$ (values did not differ significantly at the 5% level of significance).

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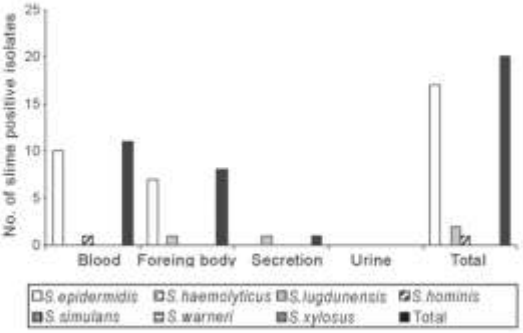

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TABLE II

Frequency of enzyme- and slime-producing coagulase-negative staphylococci isolates according to species and clinical relevance

	Species											
	S. epidermidis		*S. haemolyticus*		*S. lugdunensis*		*S. hominis*		*S. simulans*		*S. warneri*	
	S^{ns}	C^{ns}	S	C	S	C	S	C	S	C	S	C
Enzymes	52^{a}	39	2	5	3	4	0	5	1	3	1	1
Hemolysin	7	4	2	3	1	4	0	0	0	0	1	0
Lipase	6	8	1	1	0	1	0	0	0	2	0	1
Lecithinase	1	1	0	1	0	0	0	0	0	0	1	0
DNAse	3	6	1	1	3	2	0	0	0	2	0	0
TNAse	2	3	0	0	1	2	0	0	0	1	0	0
Slime	12	5	0	0	0	2	0	1	0	0	0	0

S: Staphylococcus; a: total number of strains; S: clinically significant strains ; C: contaminant strains; ns: $P > 0.05$ (values did not differ significantly at the 5% level of significance).

Enzyme production - The distribution of significant *S. epidermidis* isolates according to enzyme production is shown in Table II. Enzyme concentration is important for the identification of CNS producers of DNAse and TNAse. Direct detection of DNAse and TNAse in supernatants from overnight cultures in BHI broth only revealed production by one *S. lugdunensis* strain. However, when the culture supernatants were concentrated by the sac culture method (Donnelly et al. 1967), production was observed in various species.

Of the 52 clinically significant *S. epidermidis* isolates, seven produced hemolysin, six lipase, three DNAse, and two TNAse. No significant difference in enzyme production was observed between the *S. epidermidis* contaminant isolates and the clinically significant isolates.

The two clinically significant enzyme-producing *S. haemolyticus* isolates produced hemolysin, one produced lipase, and one produced DNAse (Table II). Among the three clinically significant *S. lugdunensis* isolates, one produced hemolysin, all three produced DNAse, and one produced TNAse (Table II).

Production of enterotoxins and TSST-1 - Fig. 2 shows that 44 (37.6%) isolates produced one or a combination of two or more enterotoxins. The distribution of CNS toxin producers according to clinical relevance is shown in Table III. Of the 52 clinically significant *S. epidermidis* isolates, 18 (34.6%) produced enterotoxins, 14 of them producing only SEC, one concomitantly producing SEA and SEB, two producing SEB and SEC, and one simultaneously producing SEA, SEB, and SEC. There was no significant difference in enterotoxin production between clinically significant and contaminant *S. epidermidis* isolates.

Simultaneous production of SEA and SEB and SEB and SEC was also observed in one clinically significant *S. lugdunensis* strain (Table III). Isolated production of SEC was observed in one isolate each of contaminant *S. haemolyticus*, *S. lugdunensis*, *S. hominis*, and *S. simulans* (Table III). The only *S. simulans* strain considered to be significant also produced SEC, whereas *S. warneri* and *S. xylosus* produced no enterotoxins. None of the 117 CNS studied produced SED or TSST-1.

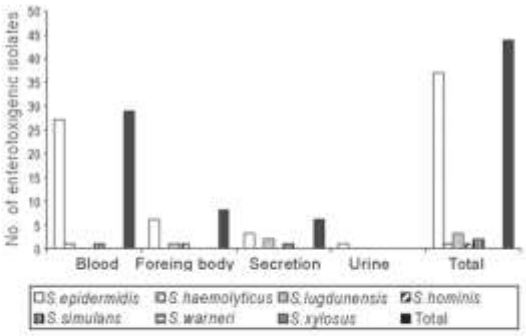

Fig. 2: production of enterotoxins by coagulase-negative staphylococci according to species and clinical material. *S: Staphylococcus.*

DISCUSSION

CNS are important microorganisms indigenous to humans and have emerged over recent years as etiologic agents in a series of infections (Hall et al. 1987, Kloos & Bannerman 1995). In a study carried out by the Centers for Disease Control and Prevention (Atlanta, GA) between 1986 and 1994, involving 99 hospitals, 13,179 cases of infection in newborns were confirmed, with CNS being the agents most frequently isolated in hospital infections (Gaynes et al. 1996). In the 1990s, CNS appeared as the main cause of sepsis in neonatal intensive care units, with an incidence of 33 to 55% among very low birthweight infants (< 1500 g) (Kacica et al. 1994). The occurrence of CNS in neonatal intensive care units has been attributed to the higher survival rates of premature low birthweight newborns and to procedures such as the use of vascular catheters, parenteral nutrition, mechanical ventilation, and prolonged hospital stays (Fleer & Verhoef 1984).

In the present study, *S. epidermidis* was the most frequently isolated species (77.8%), in agreement with other investigators (Hall et al. 1987, Neumeister et al. 1995). It was also the most frequently isolated species in clinically significant situations, such as newborns with infection (88.9%). The predominance of *S. epidermidis* among CNS

TABLE III

Frequency of toxin-producing coagulase-negative staphylococci strains according to species and clinical relevance

	Species									
	S. epidermidis		*S. haemolyticus*		*S. lugdunensis*		*S. hominis*		*S. simulans*	
	S^{ns}	C^{ns}	S	C	S	C	S	C	S	C
Toxins	52^{a}	39	2	5	3	4	0	5	1	3
SEA	0	0	0	0	0	0	0	0	0	0
SEB	0	0	0	0	0	0	0	0	0	0
SEC	14	17	0	1	0	1	0	1	1	1
SED	0	0	0	0	0	0	0	0	0	0
TSST-1	0	0	0	0	0	0	0	0	0	0
SEA+SEB	1	0	0	0	1	0	0	0	0	0
SEB+SEC	2	2	0	0	1	0	0	0	0	0
SEA+SEB+SEC	1	0	0	0	0	0	0	0	0	0
Total	18^{ns}	19^{ns}	0	1	2	1	0	1	1	1

S: *Staphylococcus*; *a*: total number of strains; S: clinically significant strains; C: contaminant strains; SEA: enterotoxin A; SEB: enterotoxin B; SEC: enterotoxin C; SED: enterotoxin D; TSST-1: toxic shock syndrome toxin-1; ns: $P > 0.05$ (values did not differ significantly at the 5% level of significance).

species causing infection in newborns has been recognized by other investigators, with its frequency ranging from 60 to 90% (Hall et al. 1987, Neumeister et al. 1995).

Although *S. epidermidis* is the most frequent etiological agent, other pathogenic CNS species have been isolated from various clinical sources (Kloos & Bannerman 1995). In the present study, other species were found to be associated with infection, including two *S. haemolyticus* strains, three *S. lugdunensis* strains, and one strain each of *S. simulans*, *S. warneri*, and *S. xylosus*. Hall et al. (1987) isolated three *S. haemolyticus* strains, two *S. hominis* strains, two *S. warneri* strains, and one *S. simulans* strain from children with clinical and laboratory evidence of sepsis and also pneumonia.

There are few reports of infection with *S. lugdunensis* in newborns, probably because this species has only been described recently (Freney et al. 1988). However, studies on adult patients have shown that this species is an opportunistic and significant pathogen (Fleurette et al. 1989, Lambe et al. 1990).

The mechanisms by which CNS provoke infections have not been completely elucidated. However, in opportunistic situations, these microorganisms cross protection barriers such as the skin and mucosa and colonize sites adjacent to the normal flora. Several authors have emphasized the production of exopolysaccharide or slime as an epidemiological marker of infection (Christensen et al. 1982, Hall et al. 1987, Vogel 2000). On the other hand, other investigators have found no association between slime-producing strains and the occurrence of infections caused by these microorganisms (Christensen et al. 1983, Riley & Schneider 1992).

The present study shows that a small proportion of the isolates which produce this exopolysaccharide (22.2%) are associated with infectious processes. Other authors also found no evidence of slime being a virulence factor (Christensen et al. 1982). Riley and Schneider (1992) also suggested that slime production does not seem to be an important virulence factor of *S. saprophyticus* isolated from women with urinary tract infection.

Various experiments using animal models have been carried out to determine the importance of slime as a virulence factor. Baddour et al. (1984), in an experimental study on catheter-induced endocarditis in rats, observed a difference in virulence between *S. epidermidis* and *S. hominis*. All animals inoculated with *S. epidermidis* developed endocarditis compared to only 12.5% of animals inoculated with *S. hominis* ($p < 0.001$). However, the authors found no association between slime production and the development of endocarditis, suggesting that this polysaccharide is not a critical determinant of virulence. Additionally, Patrick et al. (1992), using mice with subcutaneous implants, observed that slime-producing samples did not increase the risk of infection. The authors suggested that traumatized tissue, associated with the presence of catheters, might be a sufficient condition for the development of infections caused by CNS, and that factors other than slime-mediated colonization determine the pathogenicity of these microorganisms.

However, if slime production promotes adherence to prostheses, thus acting as a virulence factor, infection control becomes more difficult since it protects CNS cells from antimicrobial agents and the host's natural defense mechanisms. Gray et al. (1984) reported that the mucous substance produced by CNS can interfere with the cell-mediated immune response. Davenport et al. (1986) showed that only 32% of infections caused by slime-producing CNS were cured by antibiotics, whereas a 100% success rate was achieved for non-producing strains. These results suggest that the control of infections caused by slime-producing CNS requires the removal of the prosthesis, as well as conventional antibiotic therapy.

Slime production can also vary among different species. According to Christensen et al. (1983), this characteristic is more frequent in strains of *S. capitis*, *S. epidermidis*, *S. hominis*, and *S. saprophyticus*. Among

the CNS species isolated in the present study, slime production was observed in *S. epidermidis*, *S. lugdunensis*, and *S. hominis*. Slime production by *S. lugdunensis* has also been reported by Fleurette et al. (1989).

The study of CNS pathogenicity has also shown that various metabolites are produced by these microorganisms, including enzymes and toxins which may play a role in the pathogenicity of these microorganisms (Gemmell 1987). Other authors have observed the production of hemolysins or cytolytic toxins by CNS. In the present study, hemolysins were produced by isolates of *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, and *S. warneri*, but not by strains of *S. hominis*, *S. simulans*, and *S. xylosus*. Similar results have been reported by Kloos and Schleifer (1975), Fleurette et al. (1989), Lambe et al. (1990), and Cunha et al. (2004).

The production of lipase, DNAse, and TNAse by these organisms has also been reported. Lambe et al. (1990) verified that most *S. epidermidis*, *S. warneri*, and *S. hominis* strains included in their study produced lipase and DNAse. In the present investigation, except for *S. hominis* and *S. xylosus*, all species produced lipase. With regard to DNAse and TNAse production, production of these enzymes was observed in strains of *S. epidermidis*, *S. lugdunensis*, and *S. simulans*, and production of DNAse by *S. haemolyticus*. Production of TNAse by *S. lugdunensis* has also been demonstrated by Fleurette et al. (1989). Gramoli and Wilkinson (1978) detected TNAse production in some strains of *S. xylosus*, *S. simulans*, *S. capitis*, and *S. sciuri*.

Among the species included in the present study, *S. epidermidis* was the only one that produced all of these exoenzymes, but when the isolates involved in the etiology of the infections were compared with the contaminant isolates, no significant difference was observed. These findings are similar to those reported by Nataro et al. (1994), and suggest that the infections caused by these microorganisms do not only depend on virulence factors but also on the conditions that predispose the host to infection, including factors innate to newborns and the use of invasive procedures. Analysis of risk factors in newborns indicated that a birthweight < 1500 g, the presence of foreign bodies – catheters, mechanical ventilation, parenteral nutrition etc. – and non-removal of foreign bodies were factors significantly predisposing to CNS infection.

In the present study, concomitant production of SEA + SEB and SEB + SEC and isolated production of SEC were observed in these organisms. Production of SEC was also detected in one *S. haemolyticus* strain, one *S. hominis* strain, and one *S. simulans* strain, but production of SED and TSST-1 was not observed in any of the CNS studied.

Valle et al. (1991) found a toxigenic capacity in 45 (16.5%) CNS isolated from goats, including *S. epidermidis*, *S. haemolyticus*, *S. warneri*, and *S. xylosus*, which simultaneously produced TSST-1 and TSST-1 + SEC. Crass and Bergdoll (1986) reported the isolated production of SEA and SEC, or in combination with TSST-1, by CNS isolated from patients with toxic shock syndrome or other infections. However, production of TSST-1 by CNS has been questioned by other investigators who did not confirm these findings (Parsonnet et al. 1987, Kreiswirth et al. 1987).

Although much controversy about the production of these toxins by CNS still exists, the present results and those reported by other authors demonstrate that their toxic ability cannot be ignored. According to Bergdoll and Chesney (1991), these microorganisms have been associated with the etiology of serious staphylococci infections, and there is no reason not to consider them as toxic.

The exact role of extracellular staphylococci products in the pathogenicity of a systemic infection is still unclear. Enterotoxins and TSST-1 have received renewed attention by researchers due to their "superantigen" properties. As "superantigens", enterotoxins bind directly to the class II major histocompatibility complex, without the typical process of normal antigens, resulting in the stimulation of many T cells and, therefore, an overproduction of cytokines such as interleukin-1 (IL-1), IL-2, gamma interferon, and tumor necrosis factor alpha (Marrack & Kappler 1990). Current evidence indicates that the physiological events that occur in neonatal sepsis are mediated by activated cytokines in response to the presence of bacterial components (Kilpatrick & Harris 1998). The fact that these toxins are superantigens and that the release of immunological mediators increases the inflammatory response observed during the pathogenesis of sepsis and neonatal shock suggests that enterotoxins and TSST-1 may play an important role in the progression of infections caused by toxigenic CNS strains.

The existing divergences regarding the toxigenicity of CNS emphasize the need for further studies using sensitive and reliable genotypic techniques to confirm the ability of these staphylococci to produce toxins.

Our study revealed the presence of one or more virulence factors in 77.8% of the CNS strains isolated, suggesting that CNS virulence factors provide a selective advantage for skin colonization of hospitalized newborns. Very low birthweight newborns submitted to invasive procedures show a higher risk of subsequent infection with these strains.

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ANEXO 9

CUNHA MLRS, CARAMORI JCT, FIORAVANTE AM, BATALHA JEN, MONTELLI AC, BARRETTI P. The significance of slime as virulence factor in coagulase-negative staphylococci in peritonitis. Perit Dial Intern. 2004; 24:191-3.

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SHORT REPORTS

Significance of Slime as Virulence Factor in Coagulase-Negative Staphylococcus Peritonitis in CAPD

Staphylococcus epidermidis and other coagulase-negative staphylococci (CoNS) are the most common etiological agents of peritonitis in continuous ambulatory peritoneal dialysis (CAPD) (1). The pathogenicity of CoNS infections can be related to the production of slime, an extracellular mucoid polysaccharide that allows bacterial adherence to plastic and smooth surfaces, colonizing catheters (2). Few studies have evaluated slime as a virulence factor in peritonitis. Kristinsson *et al.* (3) suggested that recurrences were associated with the presence of biofilm made by slime in the catheter. On the contrary, Alexander and Rimland (4) did not observe correlation between slime production and CoNS pathogenicity in peritonitis. The objectives of the present study were to identify the CoNS species and its slime production, as well as to determine if slime influences the outcome of an episode of peritonitis in CAPD.

MATERIALS AND METHODS

Microbiological and clinical data from all episodes of peritonitis caused by CoNS that occurred from January 1996 to December 2000 were retrospectively studied. We included only new episodes, characterized as the patient's first CoNS peritonitis or an episode diagnosed 14 days after the patient's last peritonitis treatment.

Peritonitis was defined as the presence of cloudy peritoneal fluid associated with one or more of these findings: abdominal pain, fever, nausea, and vomiting. The occurrence of only one of these symptoms (signals) was considered peritonitis if there was a dialysate white cell count greater than 100/μL, with at least 50% polymorphonuclear cells. Resolution was defined as the disappearance of the symptoms and signs, with negative culture of peritoneal fluid at least 14 days after the last antibiotic therapy. Relapse was diagnosed when the reappearance of clinical findings occurred within 14 days after treatment of a previous episode, that had had a negative culture, with the same micro-organism identified and presenting the same sensitivity profile. Nonresolution was the generic denomination for cases with initial nonresolution, relapse, peritoneal catheter removal, or death.

For each case, protocols were filled with information about (1) episode: date, clinical findings, treatment, outcome (resolution, relapse, catheter removal, or death); (2) presence of diabetes mellitus; (3) demographic data: age, sex, race (Caucasian, non-Caucasian), treatment time on dialysis; and (4) exchange system (standard or double bags). All episodes were treated according to the protocol proposed by the third report of The Ad Hoc Advisory Committee on Peritonitis Management (1).

Microbiological materials were stored in a collection of cultures. CoNS was identified using the simplified biochemical test scheme proposed by Kloos and Schleifer (5) and Kloos and Bannerman (6).

Evaluation of slime production was as described by Christensen *et al.* (7). First, isolated colonies from blood agar were inoculated in tubes (12.0 × 75.0 mm) containing 2.0 mL trypticase soy broth and incubated at 37°C for 48 hours. Second, 1.0 mL 0.4% trypan blue or toluidine blue O solution was added to the contents; after that, a gentle shaking was done to guarantee coloring of material adhering to the inner surface of the tubes and the colorant was discarded. Finally, a positive result was indicated by the presence of a layer of stained material adhering to the internal wall of the tubes; the presence of a colored ring only at the liquid–air surface was not considered positive.

In vitro sensitivity of CoNS was determined by the minimal inhibited concentration using E-test (AB Biodisk, Solna, Sweden), a new quantitative method that uses a transparent strip of inert plastic with concentrations of drugs from 0.002 to 256 μg/mL. The proportion of samples sensitive to each drug was defined according to the NCCLS, 1998 (8).

In the present study, several factors could have had an effect on outcome: lineage of CoNS, slime production, age, sex, race, presence of diabetes mellitus, exchange system, treatment time on CAPD, oxacillin-sensitivity, and use of vancomycin. Thus, we adopted a model that incorporates the effect of all factors — of interest and of control — of the infection's outcome. Therefore, using a logistic regression model, outcome was classified into one of two results, exhausted and mutually exclusive: resolution or nonresolution. For comparison between frequencies,

the chi-square test or Fisher's exact test was used. A p value < 0.05 was considered statistically significant.

RESULTS

Fifty-one new episodes of peritonitis caused by CoNS were diagnosed in 38 patients. Demographic data are shown in Table 1.

Staphylococcus epidermidis was the most frequently identified species, constituting 47% (24 of all CoNS), followed by *S. haemolyticus*, identified in 11 cases (21.6%). Other species were present in the 16 remaining cases (31.4%) (Table 2). Oxacillin resistance was present in 5 of 24 *S. epidermidis* and 6 of 27 other CoNS ($p = 0.81$). Slime production was positive in 9 cases (17.6%). Among them, there were 4 of *S. epidermidis*, 2 *S. haemolyticus*, 2 *S. warneri*, and 1 *S. lugdunensis* lineage (Table 2). Comparing all CoNS, we found no significant statistical difference in slime production ($p = 0.38$).

Among the 51 peritonitis episodes, 35 resolved, 10 relapsed, and 6 needed catheter removal. No deaths occurred. Of the 9 episodes involving slime producers, only 2 (22.2%) resolved; of the other 7, there were 4 (44.4%) relapses and 3 (33.3%) catheter losses. Among the 42 involving nonproducers of slime, 33 (78.6%) resolved, 6 (14.3%) relapsed, and in 3 (7.1%) the catheter was removed. Controlling for covariables, the odds of resolution of peritonitis involving slime nonproducers were 27 times higher than episodes caused by slime producers ($p = 0.0086$) (Table 3).

DISCUSSION

In the present series, *S. epidermidis* was the most frequently isolated lineage, corresponding with other reports (1). Episodes caused by this agent tended to have a lower frequency of resolution. Since the slime production for *S. epidermidis* and other CoNS was not different, we could suspect that other virulence factors are involved. However, we should be careful about this, since the number of slime producers was small. The percent of oxacillin-resistant lineages was similar between *S. epidermidis* and other CoNS, suggesting that outcome was not influenced by the sensitivity profile. Furthermore, according to the logistic regression model, there was no difference in resolution rate between oxacillin-sensitive and oxacillin-resistant lineages. This finding could be due to the efficacy of vancomycin, used in episodes caused by resistant germs. Again, we should be careful, since the number of oxacillin-resistant lineages was small.

Our results showed that the unresolved episodes were significantly and independently associated with slime producer lineages. Although patient and sample numbers were small, the statistical method used was chosen because it is able to accommodate these deficits. Kristinsson *et al.* (3) attributes the severity of CoNS peritonitis to slime production contributing to the relapse. The present study brings out the fact that, of the 6 episodes of peritonitis with evolution toward catheter removal, 3 were caused by slime producer lineages. Gray *et al.* (9) reported that slime could interfere with cell-mediated immune response. Davenport *et al.* (2) brought out the fact that only 32% of infections caused by CoNS slime producers resolved with the use of antibiotics, whereas 100% of infections caused by nonproducer lineages resolved.

In the present study, all patients were treated according to an international valid protocol, suggesting that slime production had a negative influence on outcome, possibly impairing the adequacy of antibacterial action.

In conclusion, slime production is an independent factor associated with nonresolution of an episode of

TABLE 1
Characteristics of 38 CAPD Patients with Coagulase-Negative Staphylococcus Peritonitis

Characteristic	*N* (%)
Age (years)	
Birth to 20	2 (5.2)
21–40	6 (15.8)
41–59	11 (28.9)
60+	19 (50)
Sex (female)	28 (73.7)
Race (Caucasian)	26 (68.4)
Diabetic	17 (44.7)
Treatment time	
Less than 1 year	24 (61.2)
More than 1 year	14 (36.8)
Exchange system	
Standard	13 (34.2)
Double bag	25 (65.8)

TABLE 2
Causative Micro-Organisms in 51 New Episodes of Peritonitis by Coagulase-Negative Staphylococci (CoNS) and Frequency of Slime Producers

Causative micro-organism	Strain [*n* (%)]	Slime producer [*n* (%)]
S. epidermidis	24 (47.0)	4 (16.7)
S. haemolyticus	11 (21.6)	2 (18.2)
S. warneri	5 (9.8)	2 (40.0)
S. hominis	5 (9.8)	0
S. xylosus	2 (3.9)	0
S. cohnii	2 (3.9)	0
S. simulans	1 (1.9)	0
S. lugdunensis	1 (1.9)	1 (100)
Total CoNS	51 (100)	9 (17.6)

TABLE 3
Comparison of Odds by Regression Logistic Analysis of Resolution of Peritonitis

Factor	Log (odds)	*p* Value	Odds ratio
Species (others/*S. epidermidis*)	2.2121	0.0744	
Species (others/*S. haemolyticus*)	0.6406	0.5742	
Age (years)			
Birth to 20/60+	2.6174	0.4380	
21 to 40/60+	1.7522	0.2553	
41 to 59/60+	1.6077	0.1703	
Gender (male/female)	0.3763	0.7622	
Race (Caucasian/non-Caucasian)	0.0036	0.9976	
Diabetes mellitus (no/yes)	0.1193	0.8994	
System ("standard"/double bags)	1.3051	0.2660	
Duration of dialysis (<1 year/> 1 year)	0.5422	0.6622	
Slime producer (no/yes)	3.2962	0.0086	27.00
Sensitivity to oxacillin (sensitive/resistant)	2.0204	0.1610	
Treatment with vancomycin (no/yes)	1.9915	0.1654	

peritonitis caused by CoNS during CAPD. Prospective studies with a higher number of samples and using fresh cultures would be of interest in confirming this finding.

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ANEXO 10

Oliveira A, **Cunha MLRS**. Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC Res Notes. 2010; 3: 260.

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BMC Research Notes

PROJECT NOTE Open Access

Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci

Adilson Oliveira, Maria de Lourdes RS Cunha*

Abstract

Background: The ability of biofilm formation seems to play an essential role in the virulence of coagulase-negative staphylococci (CNS). The most clearly characterized component of staphylococcal biofilms is the polysaccharide intercellular adhesin (PIA) encoded by the *icaADBC* operon. Biofilm production was studied in 80 coagulase-negative staphylococci (CNS) strains isolated from clinical specimens of newborns with infection hospitalized at the Neonatal Unit of the University Hospital, Faculty of Medicine of Botucatu, and in 20 isolates obtained from the nares of healthy individuals without signs of infection. The objective was to compare three phenotypic methods with the detection of the *icaA*, *icaD* and *icaC* genes by PCR.

Findings: Among the 100 CNS isolates studied, 82% tested positive by PCR, 82% by the tube test, 81% by the TCP assay, and 73% by the CRA method. Using PCR as a reference, the tube test showed the best correlation with detection of the *ica* genes, presenting high sensitivity and specificity.

Conclusions: The tube adherence test can be indicated for the routine detection of biofilm production in CNS because of its easy application and low cost and because it guarantees reliable results with excellent sensitivity and specificity.

Background

Coagulase-negative staphylococci (CNS) are the microorganisms most frequently involved in nosocomial infections among neonates. These infections are generally associated with the use of catheters and other medical devices [1]. The capacity to adhere to polymer surfaces and consequent biofilm production are the main virulence factors of CNS, especially *S. epidermidis*, the most frequently isolated species.

The biofilm protects CNS against the action of antibiotics administered for the treatment of these infections and also against the patient's immune system. Removal of the foreign body is often necessary for cure [2,3]. In this respect, CNS infections seem to be related to the health condition of the patient and to the production of this extracellular polysaccharide [4,5].

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The biofilm consists of layers of cell clusters embedded in a matrix of extracellular polysaccharide, called polysaccharide intercellular adhesin (PIA), which consists of β-1,6-N-acetylglycosamine and is synthesized by N-acetylglucosaminyltransferase [6]. PIA is involved in cell-cell adhesion and is essential for biofilm production by CNS, which is observed in most clinical strains of *S. epidermidis* [7,8].

The synthesis of PIA is mediated by the products of the chromosomal *ica* gene (intercellular adhesion), which are organized in an operon structure. This operon contains the *icaADBC* genes, in addition to the *icaR* gene which exerts a regulatory function and is transcribed in the opposite direction. Once this operon is activated, four proteins are transcribed, IcaA, IcaD, IcaB and IcaC, which are necessary for the synthesis of PIA [9-11]. PIA is synthesized from UDP-N-acetylglucosamine by N-acetylglucosaminyltransferase, which is encoded by the *ica* locus, particularly *icaA*. The expression of this gene alone induces low enzymatic activity and the production of low amounts of polysaccharide.

However, the simultaneous expression of *icaA* and *icaD* promotes a significant increase in N-acetylglucosaminyltransferase, with a consequent increase in the amount of polysaccharide, forming oligomers of 10-20 β-1,6-N-acetylglucosamine residues [12,13]. The *icaC* gene, when expressed concomitantly with *icaA* and *icaD*, induces the synthesis of longer oligomers that contain up to 130 residues. The *icaB* gene probably encodes a secretory protein that does not seem to be involved in the biosynthesis of PIA [14,15].

Recent studies have shown that additional components, such as accumulation-associated protein (Aap), DNA and RNA independently or in cooperation with the *ica* operon, have also been suggested to be important in CNS biofilms [16-18]. Bap [biofilm-associated protein] has been shown to be involved in the initial attachment, intercellular adhesion and biofilm formation of *S. aureus* [19]. Interestingly, Bap homologue protein (Bhp) is found in human *S. epidermidis* strains and induces an alternative mechanism of biofilm formation that does not depend on PIA [20].

Various methods are currently used in medical areas for the detection of biofilm production, including visual assessment by electron microscopy using different types of microscopes. The most versatile and effective nondestructive approach for studying biofilms is confocal laser scanning microscopy (CLSM). CLSM markedly reduces the need for pretreatments such as disruption and fixation, which reduce or eliminate the evidence of microbial relationships, complex structures and biofilm organization, without the limitations encountered with scanning electron microscopes [21-23]. Bridier *et al.* [24] proposed CLSM combined with the use of 96-well microtiter plates compatible with high resolution imaging for the study of biofilm formation and structure. The authors reported that the combined use of microplates and confocal imaging proved to be a good alternative to other high throughput methods commonly used since it permits the direct, *in situ* qualitative and quantitative characterization of biofilm architecture.

However, qualitative methods, such as the tube adherence test described by Christensen *et al.* [25] and the Congo red agar (CRA) method described by Freeman *et al.* [26], and quantitative methods such as the tissue culture plate (TCP) assay described by Christensen *et al.* [27] are used in routine laboratories. Molecular techniques such as the polymerase chain reaction (PCR), which amplifies the genes involved in biofilm production, complement these methods.

The objective of the present study was to investigate biofilm production in CNS strains isolated from clinical specimens of newborns hospitalized at the Neonatal Unit of the University Hospital, Faculty of Medicine of Botucatu, and in isolates obtained from the nares of healthy individuals by three phenotypic methods and by PCR for detection of the *icaA*, *icaD* and *icaC* genes.

Materials and methods

Strains

A total of 100 CNS isolates were studied, including 80 isolated from clinical specimens obtained from newborns hospitalized at the Neonatal Unit of the University Hospital, Faculty of Medicine, Universidade Estadual Paulista (UNESP), Botucatu Campus, and 20 obtained from the nares of healthy subjects. The following international reference strains were used as controls: the non-biofilm producers *S. epidermidis* ATCC 12228 and *S. warneri* ATCC 10209 (negative control), and the biofilm producers *S. epidermidis* ATCC 35983, *S. simulans* ATCC 27851 and *S. xylosus* ATCC 29979 (positive control).

Identification of coagulase-negative staphylococci

The isolates obtained from the clinical specimens were seeded onto blood agar and stained by the Gram method for the determination of purity, morphology and specific staining. After confirmation of these characteristics, the strains were submitted to catalase and coagulase tests. The genus *Staphylococcus* was differentiated from *Micrococcus* according to the method described by Baker *et al.* [28].

The simplified scheme proposed by Cunha *et al.* [29] was used for the identification of CNS based on sugar utilization tests. After species confirmation, the isolates were stored in nutrient broth with glycerol in a freezer at -70°C.

Study of biofilm production

Investigation of biofilm production by adherence to borosilicate test tubes (Christensen et al. 1982)

Biofilm production was investigated by the tube adherence test proposed by Christensen *et al.* [25]. A positive result was defined as the presence of a layer of stained material adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid-air interface was not considered to be positive.

Investigation of biofilm production by adherence to polystyrene plates (modified method of Christensen et al. 1985)

The quantitative method of adherence to polystyrene plates (TCP) proposed by Christensen *et al.* [27] was also used in the present study, with modifications. These modifications included an increase in the glucose concentration of TSB from 0.25% to 2%, a longer incubation period (24 h instead of 18 h), and determination of optical density in dry plates and plates washed with 95% ethanol using filters of 492 and 540 nm. Analysis of the sensitivity and specificity of the TCP method using

PCR as a parameter showed that the use of dry plates and of the 540-nm filter provided the best sensitivity (97.6%) and specificity (94.4%) when compared to ethanol-containing plates and the 492-nm filter, and was therefore chosen for analysis of the results.

The isolates were classified into three categories: non-adherent, optical density equal to or lower than 0.111; weakly adherent, optical density higher than 0.111 or equal to or lower than 0.222; strongly adherent, optical density higher than 0.222.

When the cut-off corresponded to non-adherent the isolates were classified as negative, and as positive when the cut-off corresponded to weakly or strongly adherent. Sensitivity and specificity were calculated for each situation in relation to the concomitant presence of the *icaA* and *icaD* or *icaADC* genes using PCR as the reference method.

Investigation of biofilm production by the Congo red agar method proposed by Freeman et al. (1989)

Phenotypic characterization of biofilm production was performed by culture of the CNS isolates on CRA plates as proposed by Freeman *et al.* [26]. According to the authors, biofilm producers form black colonies on CRA, whereas non-producers form red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes [30].

A five-color reference scale was used to accurately determine all color variations shown by the colonies. Isolates presenting two tones of black, bright black (BB) and dry-opaque black (OB), were classified as biofilm producers, whereas red, pink and bordeaux colonies were classified as negative. In some cases, red and bordeaux subcolonies arose in the center of black colonies (BB) after 48 h of culture. These colonies were removed and subcultured for 24 h on CRA to obtain pure isolates of the producer and non-producer variants. These isolates were also submitted to the phenotypic tests (tube adherence test and TCP assay) and to detection of the *ica* genes by PCR.

Detection of the icaA, icaC and icaD genes specific for biofilm production

The procedure used for detection of the *ica* genes involved the following steps: nucleic acid extraction using the Illustra kit (GE Healthcare), PCR amplification according to the parameters described by Arciola *et al.* [31], and visualization of the amplified products by gel electrophoresis.

Statistical analysis

Sensitivity and specificity [32] were evaluated by comparing the phenotypic methods used for the detection of biofilm production and PCR for the detection of the genes involved in biofilm synthesis. All isolates carrying at least two of the genes studied were considered to be positive for biofilm production. Sensitivity and specificity were evaluated as follows: sensitivity - proportion of PCR-positive isolates (concomitant presence of the *icaA* and *icaD* or *icaADC* genes) that tested positive by the other phenotypic methods; specificity - proportion of PCR-negative isolates (no detection of the *ica* gene) that tested negative by the phenotypic methods.

Agreement between the tests and the presence of the *ica* genes was evaluated using the kappa index [33,34].

Results

Isolates

A total of 100 CNS isolates were studied, including 80 isolated from clinical specimens obtained from newborns hospitalized at the Neonatal Unit of the University Hospital, Faculty of Medicine, Universidade Estadual Paulista (UNESP), Botucatu, Brazil, and 20 obtained from the nares of healthy subjects. Among the 80 isolates obtained from clinical specimens, 50 were isolated from catheter tips and 30 by blood culture.

Identification of coagulase-negative staphylococci

S. epidermidis was the most frequently detected species (81%), followed by *S. cohnii* (4%), *S. saprophyticus* (4%), *S. warneri* (4%), *S. haemolyticus* (2%), *S. xylosus* (2%), *S. capitis* (2%), and *S. lugdunensis* (1%).

Table 1 shows the frequency of CNS species according to clinical specimen. *S. epidermidis* was the CNS species most frequently isolated from clinical specimens (catheter and blood culture) and from nares specimens of healthy subjects. *S. haemolyticus*, *S. xylosus*, *S. lugdunensis* and *S. capitis* were also isolated from clinical specimens, whereas *S. cohnii* and *S. saprophyticus* were only isolated from the nares of healthy subjects. *S. warneri* was isolated from blood culture and nares specimens.

Study of biofilm production

Detection of the icaA, icaC and icaD genes specific for biofilm production

The presence of the *icaA* (103 bp), *icaC* (400 bp) and *icaD* (198 bp) genes in the CNS isolates was

Table 1 Frequency of coagulase-negative staphylococci isolated from different clinical specimens

Species	Catheter tip	Blood culture	Nasal fossa	Overall frequency	Percentage (%)
S. epidermidis	45	27	9	81	81
S. cohnii	0	0	4	4	4
S. haemolyticus	1	1	0	2	2
S. saprophyticus	0	0	4	4	4
S. warneri	0	1	3	4	4
S. xylosus	2	0	0	2	2
S. lugdunensis	0	1	0	1	1
S. capitis	2	0	0	2	2
Total	50	30	20	100	100

demonstrated by the amplification of the corresponding fragments (Figure 1). The *icaA* and *icaD* genes were detected concomitantly in 40 (40%) of the 100 CNS isolates, and the *icaA*, *icaC* and *icaD* genes were detected concomitantly in 42 (42%). None of the genes studied could be identified in 18 (18%) isolates. Except for *S. haemolyticus* and *S. capitis*, all other CNS species were positive for the genes studied.

Study of biofilm production by the tube adherence test (Christensen *et al.* 1982)

Investigation of biofilm production by the tube adherence test showed that 82 of the 100 isolates were biofilm producers, including 44 strains isolated from catheter tips, 23 isolated by blood culture, and 15 isolated from nares specimens. With respect to species, 70 (85.4%) *S. epidermidis* isolates were positive. Biofilm production was also observed in *S. warneri* (n = 4), *S. cohnii* (n = 3), *S. xylosus* (n = 2), *S. saprophyticus* (n = 2), and *S. lugdunensis* (n = 1).

The sensitivity and specificity of the tube adherence test were calculated using the presence of the *ica* genes as a parameter. The results showed 100% sensitivity and 100% specificity of the tube test [24] when compared to PCR (concomitant presence of the *icaA* and *icaD* or *icaACD* genes).

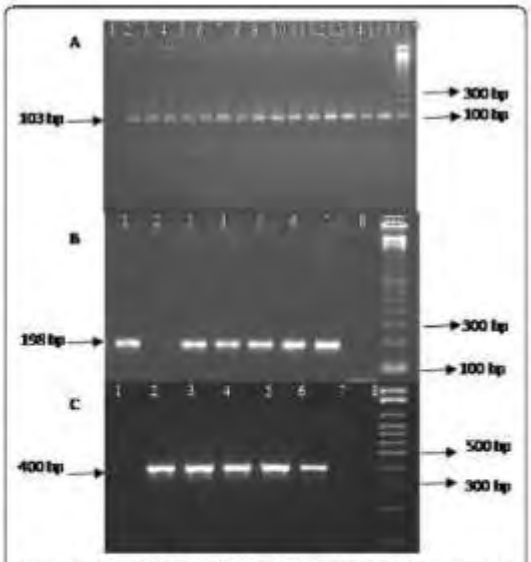

Figure 1 Agarose gel electrophoresis of PCR products stained with Syber Safe. A → *icaA* gene (103 bp), 1: negative control, 2-15: positive samples, 16: positive control and 17: molecular weight (100 bp), B → *icaD* gene (198 bp), 1: positive sample, 2: negative sample, 3-6: positive samples, 7: positive control, 8: negative control and 9: molecular weight (100 bp), C → *icaC* gene (400 bp), 1: negative sample, 2-5: positive samples, 6: positive control, 7: negative sample, 8: negative control, 9: molecular weight (100 bp).

Study of biofilm production by the tissue culture plate method (Christensen *et al.* 1985)

Among the 100 isolates studied, 35 (35%) were classified as weakly adherent and 46 (46%) as strongly adherent, for a total of 81 (81%) positive isolates and 19 (19%) negative isolates. Forty-four (54.3%) of the 81 positive isolates were obtained from catheter tips, 21 (25.9%) from blood culture, and 16 (19.8%) from nares specimens. Among the 19 negative isolates, 6 (31.6%) were obtained from catheter tips, 9 (47.4%) from blood culture, and 4 (21.0%) from nares specimens.

The TCP assay using a 540-nm filter presented 97.6% sensitivity and 94.4% specificity when compared to PCR recognizing the concomitant presence of the *icaA* and *icaD* or *icaACD* genes. One nares specimen tested false-positive, i.e., it was classified as strongly adherent but did not carry any of the *ica* genes. Two blood culture samples tested false-negative, i.e., they were classified as non-adherent but tested positive for the *icaACD* genes.

Analysis of agreement showed moderate agreement (kappa = 0.44) between the TCP assay and the concomitant presence of the *icaA* and *icaD* or *icaACD* genes.

To determine the relationship between the amount of biofilm and the concomitant presence of the *icaACD* genes, the positive results classified as strongly adherent obtained by spectrophotometric analysis of dry plates using a 540-nm filter were compared to those obtained by PCR. The results showed that 26 (56.5%) of the 46 strongly adherent isolates carried the *icaACD* genes. Moderate agreement (kappa = 0.44) was observed between strongly adherent isolates and the concomitant presence of the *icaACD* genes.

Study of biofilm production by the Congo red agar method proposed by Arciola *et al.* (2001)

The international reference strains *S. simulans* ATCC 27851 and *S. xylosus* ATCC 29979, used as positive control, exhibited a bright black color, whereas *S. epidermidis* ATCC 12228 and *S. warneri* ATCC 10209 (negative controls) formed bordeaux and bordeaux metallic colonies, respectively.

Among the 100 CNS isolates studied, 73 formed black colonies on CRA, including bright black colonies in 41 and dry black colonies in 32. The results revealed 27 non-biofilm producers whose colony color ranged from pink (2%) to red (7%) and bordeaux (18%). Two variants were observed among black colonies (BB), one with a bordeaux spot in the center and the other with a red spot. The center of these colonies was removed and subcultured for 24 h on CRA to obtain pure isolates.

After isolation, the two subcolonies were found to be negative, presenting a red and bordeaux color, respectively. After reisolation from the center, these isolates also tested negative by the tube test and TCP assay and the *icaADC* genes were not detected by PCR.

Nine isolates formed bordeaux colonies and were therefore considered to be negative. However, these isolates tested positive for the concomitant presence of the *icaA* and *icaD* genes or *icaACD* genes and were classified as false-negative when compared to PCR. Two of these isolates were obtained by blood culture and seven were isolated from nares specimens.

The CRA method presented 89% sensitivity and 100% specificity when compared to PCR recognizing the concomitant presence of the *icaA* and *icaD* or *icaACD* genes.

Comparison of biofilm detection methods in CNS

Table 2 shows the comparison between the phenotypic methods for the detection of biofilm production and the presence of the *icaA* and *icaD* genes or *icaACD* genes. The tube adherence test was the phenotypic method showing the best sensitivity (100%) and specificity (100%) in biofilm detection when compared to PCR.

Discussion

In view of the large number of infections caused by biofilm-producing bacterial, a reliable method for their diagnosis is necessary. In the present study, 100 CNS strains isolated from clinical specimens of newborns and from the nares of healthy subjects were analyzed. The results revealed *S. epidermidis* as the most frequently isolated species in both clinical and nares specimens, corresponding to 81% of all strains isolated. Other CNS species were also identified, including *S. cohnii*, *S. saprophyticus*, *S. warneri*, *S. haemolyticus*, *S. xylosus*, *S. capitis*, and *S. lugdunensis*. Similar results have been reported in other studies [35,36]. The finding of *S. epidermidis* as the most frequently isolated species in most studies might be due to the fact that this microorganism possesses certain mechanisms that favor its adaptation to some sites, with this species being the most prevalent bacterium in human skin and mucosa [37].

The present results showed no difference in the frequency of biofilm production between isolates obtained from clinical samples and from nares specimens of healthy subjects, irrespective of the detection method used. Similar biofilm production by CNS strains isolated from different sources, including clinical specimens, environment and microbiota of healthy individuals, has also been reported by other investigators [35,38].

The *icaA* and *icaD* genes were detected concomitantly in 40% of the 100 CNS isolates studied and the *icaACD* genes in 42%. These results differ from those reported by Cafiso *et al.* [9] who also investigated the presence of genes involved in biofilm production. In that study, 35% of the isolates were positive for the *icaA* and *icaD* genes and only 4 isolates carried the *icaACD* genes. Some isolates only carried the *icaD* gene. In the present study, PCR was found to be an efficient method for detection of the *ica* operon. Other investigators also reported PCR to be an important tool for the identification of *ica* genes since the technique is simple, rapid and reliable and only requires minimal amounts of DNA [9,39]. PCR was used in this study as a reference for the phenotypic method based on several studies [9,39-41] that demonstrated the efficiency of this technique in detecting the genes of the *ica* operon. In addition, these genes are important virulence markers of clinical CNS isolates since their expression is associated with the production of PIA, the most clearly characterized component of staphylococcal biofilms.

With respect to the phenotypic methods, the tube adherence test presented 100% sensitivity and 100% specificity when compared to PCR recognizing the concomitant presence of the *icaA* and *icaD* or *icaACD* genes. Ruzicka *et al.* [40] also reported good sensitivity and specificity for the tube test and PCR when analyzing isolates obtained from infections. According to Morales *et al.* [10] and Cunha *et al.* [42], the test provides

Table 2 Sensitivity and specificity of phenotypic methods for the detection of biofilm production in coagulase-negative staphylococci

Method		*ica* Positive*	*ica* negative**	Sensitivity %	Specificity %
Tube test	Adherent	82	0	100	100
	Non-adherent	0	18		
Plate test	Adherent	80	1	97,6	94,4
	Non-adherent	2	17		
CRA test	Adherent	73	0	89	100
	Non-adherent	9	18		

* *ica* positive: Positive for *icaA* and *icaD* or *icaACD*.

** *ica* negative: Negative for *icaA* and *icaD* or *icaACD*.

CRA: Congo red agar.

reliable results for biofilm detection in CNS and is adequate for routine use.

According to the present study and reports in the literature [43], the expression of the *ica* genes is highly variable and can be induced by variations in the culture conditions, such as an increase in the concentration of sugars or other substances that induce stress. Mathur *et al.* [44] also obtained better results when the glucose concentration of TSB was increased to 1% and the period of incubation was prolonged to 24 h. The addition of large amounts of sugar to a medium colonized with CNS induces a stress condition which, in turn, stimulates fermentation, thus increasing the production of PIA and consequent biofilm production [5].

In the present study, 81% of the isolates were positive in the TCP test, with the observation of moderate agreement (0.44) between this test and PCR recognizing the concomitant presence of the *icaA* and *icaD* or *icaACD* genes. Similar results have been reported by Arciola *et al.* [45] who used the TCP assay for the detection of biofilm-producing isolates, with 81.2% of the strains testing positive.

According to Gerke *et al.* [14], when the *icaC* gene is expressed concomitantly with the *icaA* and *icaD* genes, oligomers of up to 130 UDP-N-acetylglucosamine residues are synthesized and the production of PIA thus increases. To confirm the results reported by these authors and to determine the reliability of the TCP method in terms of the quantification of biofilm production, the relationship between strongly adherent isolates and the concomitant presence of the three *ica* genes (*icaA*, *icaC* and *icaD*) detected by PCR was evaluated. The results showed that 56.5% of strongly adherent isolates carried the three genes. Moderate agreement (0.44) was observed between strongly adherent isolates and the concomitant presence of the *icaACD* genes.

In the present study, the colonies grown on CRA presented diverse colors, thus, a five-color scale was adopted for comparison of the results of the CRA test and PCR in order to correlate the variation in colony color with the presence of the *ica* genes. Colonies presenting a bright black and dry black color were classified as positive and those presenting a red, pink or bordeaux color as negative. Color scales were also adopted in other studies using the CRA test for better diagnostic performance, but color tones different from those proposed in this study were used. Very black, black and almost black colonies were classified as positive and bordeaux, red and very red colonies as negative [31,46]. In the present study, two black color variants were observed, one with a bordeaux dot in the center and the other with a red dot. These colonies were removed and subcultured for 24 in CRA to obtain pure isolates. All isolated subcolonies were again plated onto CRA plates and were found to be negative, showing a bordeaux and red color, respectively, and no *icaADC* genes were detected by PCR, similar to the findings of Arciola *et al.* [46].

In the present study, 73% of the 100 isolates tested by the CRA method were positive. Similar results have been reported in the study of Cafiso *et al.* [9], in which 83% of CNS strains isolated from catheter-associated infections were positive in the CRA test. However, lower positivity rates in this test have been reported in other studies. In investigations on the detection of slime production by *S. epidermidis* strains isolated from catheter [46] and prosthesis-associated [31] infections, 49% and 57% positive isolates were identified by the CRA method, respectively. Using the CRA method, Silva *et al.* [36] observed biofilm production in only 25% of CNS strains isolated from clinical specimens of newborns in a neonatal intensive care unit.

Comparison between the CRA test and the results obtained by PCR revealed nine CNS isolates forming bordeaux colonies which tested positive for the concomitant presence of the *icaA* and *icaD* genes or *icaACD* genes, with these results thus being false-negative when compared to PCR. Arciola *et al.* [31] identified eight isolates whose colony color ranged from red to bordeaux and that were classified as negative; however, the *ica* genes were detected in all of them. Cafiso *et al.* [9] observed three CNS isolates that were negative for biofilm production in the CRA test but contained the complete *ica* operon. In another study, six isolates that carried the *icaA* and *icaD* genes detected by PCR were negative in the CRA test [46]. Although PCR detects the presence of genes irrespective of their expression, PCR-positive isolates should be considered to be potential biofilm producers.

The CRA test is easier and faster to perform than other phenotypic tests. However, it is slightly imprecise in the identification of positive isolates when compared to molecular analysis of the genes involved in biofilm production, a fact also observed by Fitzpatrick *et al.* [47].

The present results demonstrated the presence of intercellular adhesion genes (*icaACD*) and consequent biofilm production in most CNS isolates. The proportion of biofilm-positive/*ica*-positive versus biofilm-positive/*ica*-negative strains was 82:0%, 80:1% and 73:0% by the tube test, TCP assay and CRA, respectively. In the study of Jiang *et al.* [17], the proportion of biofilm-positive/*ica*-positive versus biofilm-positive/*ica*-negative strains was 22:2%. In another study investigating *S. epidermidis* clinical strains isolated from blood cultures, this proportion was 85:2% [48]. Qin *et al.* [49] studied the two biofilm-positive/*ica*-negative strains SE1 and SE4 identified among 101 clinical isolates of *S.*

epidermidis collected at Ruijin Hospital, Shanghai, China [17]. The authors investigated the *aap* and *bhp* genes that induce an alternative PIA-independent mechanism of biofilm formation. However, the two genes were not detected in the SE1 and SE4 strains, but were present in the RP62A reference strain (biofilm-positive/*ica*-positive). These authors suggested a novel molecular mechanism mediating biofilm formation in SE1 and SE4 clinical isolates. This mechanism needs to be further investigated.

In the present study, the tube adherence test showed the best correlation with the PCR results and can be indicated for the routine detection of biofilm production in CNS.

Conclusion

The present results demonstrated the presence of intercellular adhesion genes (*icaACD*) and consequent biofilm production in most CNS isolates. The presence of this biofilm, in turn, facilitates the development of infections by compromising the immune system of the patient and contributing to the failure of antibiotic therapy, which may result in recurrent infections and the emergence of multiresistant pathogens. In addition, the results suggest the use of the tube adherence test for the routine detection of biofilm production in CNS because of its easy application and low cost and because it guarantees reliable results with excellent sensitivity and specificity.

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Authors' contributions

AO: Responsible for conceiving the idea of the study, performed the microbiological tests, and wrote the article. MLRSC: Responsible for conceiving the idea, coordinating laboratory work, data analysis, and manuscript writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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7. Toxinas Estafilocócicas

Vários fatores de virulência são responsáveis pelos sintomas e gravidade das infecções causadas por *Staphylococcus* spp. Esses fatores incluem as toxinas α, β, γ e δ, a leucocidina Panton-Valentine e um grupo de superantígenos tóxicos pirogênicos (23).

As toxinas estafilocócicas podem ser enquadradas, de forma geral, em dois grupos: agentes ativos em membranas, que compreendem as toxinas alfa, beta, delta e gama, e a leucocidina; e as toxinas com atividades de superantígenos (SAgs), que incluem a família das toxinas pirogênicas (PTs) que são as enterotoxinas estafilocócicas (SEs), a Toxina 1 da Síndrome do Choque Tóxico (TSST-1) e a família das toxinas esfoliativas (ETs) (168). Alguns dos genes dessas toxinas podem ser frequentemente transportados por elementos genéticos móveis, tais como fagos e ilhas de patogenicidade (SaPI), as quais são segmentos de DNA de variados tamanhos, potencialmente móveis, codificadores de genes associados à virulência, sendo transferidos horizontalmente entre as cepas. Como exemplo, pode-se citar genes que codificam enterotoxina B e C, e a toxina da síndrome do choque tóxico (169).

Segundo Peacock *et al*. (170), o número de genes associados à virulência carreados por uma cepa bacteriana é o produto da interação entre as taxas de aquisição do gene, o custo para manutenção biológica e a taxa de falência da cepa causadora da doença. Como a vasta maioria das infecções graves por *Staphylococcus* spp. não pode ser explicada pela ação de somente um determinado fator de virulência, é imperativa a ação de vários desses fatores durante o processo infeccioso.

Além do biofilme, *Staphylococcus* spp. produzem outros fatores de virulência tais como, hemolisinas, lipases, proteases e toxinas que também podem ter um papel ainda não determinado na patogênese de sérias infecções. No mínimo três toxinas citolíticas ou lesivas à membrana são produzidas por estafilococos, incluindo-se a toxina alfa (α), beta (β) e a delta (δ) (171-174). Estas toxinas também foram descritas como hemolisinas, com base em sua capacidade de lisar hemácias, mas como sua atividade biológica não é restrita às hemácias, introduziu-se o termo toxina citolítica (175).

Destas, a hemolisina ou toxina delta vem merecendo atenção de pesquisadores pelo seu envolvimento com a enterocolite necrosante (NEC), que afeta recém-nascidos (172-174, 176). Vários autores têm descrito a ocorrência de enterocolite necrosante em associação a ECN (177-178). A toxina delta pode danificar uma variedade de células como resultado de sua ação tipo detergente nas membranas celulares e, tem sido diferenciada de outras hemolisinas pela sua termoestabilidade e neutralização pela lecitina (179). Estudos indicam ainda que a toxina delta possa ter um papel importante na patogenia de doenças intestinais, podendo variar desde uma diarréia aguda a uma grave enterite (176, 179).

A produção de hemolisina alfa também está associada à patogenicidade de *Staphylococcus* spp., pelo fato de ser conhecida sua capacidade de danificar o tecido após o estabelecimento do foco de infecção (180), e de sua ação dermonecrótica e letal para animais experimentais (181). Stephen e Pietrowski (179) observaram variação na suscetibilidade de hemácias de diferentes espécies à

toxina alfa e, dentre elas, as de coelho revelaram ser 1.000 vezes mais suscetíveis do que as humanas.

Estudo realizado pelo nosso grupo para comparar a evolução das peritonites causadas por *S. aureus* e espécies de ECN em pacientes em diálise peritoneal da Unidade de Diálise do HC da FMB (ANEXO 3) revelou que a produção de hemolisina alfa foi relacionada com a pior evolução das peritonites, com uma taxa de resolução de 8,2 vezes maior para as peritonites causadas por *Staphylococcus* spp. não produtores dessa toxina. Esse resultados corroboram com o dados publicados pela Haslinger-Löffler *et al*. (182) que sugerem que α-hemolisina desempenha um papel específico na patogênese da peritonite. Esses autores mostraram que somente *S. aureus* invasivos e produtores de α-hemolisina induziam morte de células mesoteliais caspase-independente. Esses resultados indicam que a α-hemolisina representa um importante mecanismo de *S. aureus* de causar danos persistentes no peritônio durante as peritonites. Ao contrário de *S. aureus*, não foram verificados efeitos citotóxicos pelas linhagens de *S. epidermidis* testadas que não eram invasivas e não produziam a α-hemolisina.

Recentemente outro estudo realizado na mesma Unidade de Diálise, porém analisando somente os episódios de peritonites causados por *S. aureus* num período de quinze anos (1996 a 2010), indicou a produção da β-hemolisina como fator independentemente associado a não resolução das peritonites (ANEXO 11). A toxina beta, também chamada de esfingomielinase C, é uma proteína termolábil tóxica para várias células, incluindo hemácias, leucócitos e macrófagos (179). Esta enzima catalisa a hidrólise de fosfolipídios da membrana de células suscetíveis,

revelando ser a intensidade de lise proporcional à concentração de esfingomielina presente na membrana citoplasmática exposta na superfície celular (180). A incubação de pequenas quantidades de toxina beta com hemácias de carneiro à 37^{o}C, resulta em pouca ou nenhuma lise. Entretanto, sua propriedade hemolítica é aumentada pela subsequente exposição das hemácias à temperatura de 4^{o}C, fenômeno esse conhecido como lise "hot-cold". A β-Hemolisina não lisa a maioria dos tipos de células do hospedeiro, mas as deixa vulneráveis a uma série de outros agentes líticos, como α-hemolisina e a leucocidina Panton-Valentine (183).

Em um modelo de lesão pulmonar em ratos, Hayashida *et al*. (183) relataram que nos animais infectados com *S. aureus* deficientes em β-hemolisina as lesões foram significativamente atenuadas em comparação com os animais infectados com *S. aureus* capaz de expressar essa toxina. Esta doença experimental foi caracterizada por intensa inflamação neutrofílica e redução da expressão de syndecan-1 em células epiteliais alveolares e pôde ser reproduzida pela administração de β-hemolisina recombinante, mas não por mutantes para β-hemolisina com deficiência na atividade de esfingomielinase.

Recentemente Huseby *et al*. (184) mostraram que a β-hemolisina forma ligações cruzadas com DNA extracelular, independentemente da atividade da esfingomielinase, produzindo uma nucleoproteína insolúvel *in vitro* e contribuindo para a formação de biofilme. Usando um modelo de endocardite infecciosa em coelho, os autores observaram que essa toxina estimula a formação de biofilme *in vivo*.

Outra importante citotoxina que vem merecendo a atenção de vários pesquisadores é a Leucocidina Panton-Valentine (PVL) codificada pelos genes *lukS-PV* e *lukF-PV*, e atualmente encontrada principalmente em amostras de *S. aureus* resistentes a meticilina adquiridos na comunidade (CA-MRSA). A Leucocidina Panton-Valentine (PVL) é uma toxina associada com extensa necrose tecidual, responsável por infecções de pele graves e pneumonias necrotizantes. A leucocidina foi associada à infecção de pele e de partes moles por dois estudiosos, Panton e Valentine, no ano de 1932. Os genes *lukS-PV* e *lukF-PV* que codificam a PVL são carreados por um bacteriófago específico (phiSLT) que infecta e lisogeniza células de *S. aureus* integrando os genes da PVL no cromossomo desses micro-organismos. Quando as proteínas LukS-PV e a LukF-PV são transcritas e secretadas, formam um heptâmero causando lise na membrana de leucócitos polimorfonucleares (PMNs). Dependendo da concentração da toxina, os PMNs podem sofrer lise ou apoptose. Devido a essas evidências, provavelmente a ação da PVL não esteja diretamente associada à necrose tecidual, mas sim relacionada aos grânulos lisossomais citotóxicos liberados pela lise dos PMNs, ao oxigênio reativo liberado dos granulócitos ou, ainda, à cascata inflamatória (185).

Estudo realizado em nosso laboratório com amostras isoladas de culturas clínicas e de vigilância de pacientes do Hospital Estadual Bauru (186) revelou 18,1% das amostras de *S. aureus* resistentes a meticilina (MRSA) positivas para o gene *pvl* comparado com 7,5% em amostras de *S. aureus* sensíveis a meticilina (MSSA) ($p<0,009$). Embora vários autores relatem que a produção dessa toxina é mais frequente em *S. aureus* resistente a meticilina, resultados obtidos em outro estudo

desenvolvido em nosso laboratório com amostras de *S. aureus* isoladas de infecções de pele mostraram que todas as amostras de *S. aureus* que apresentaram o gene da PVL foram sensíveis à oxacilina e nenhuma das amostras de CA-MRSA carreou o gene da PVL (187). Esses resultados estão de acordo com o modelo de Boyle-Vavra e Daum (185) que sugerem que primeiro *S. aureus* sensível a oxacilina é infectado e lisogenizado pelo fago que carreia os genes da PVL para depois adquirem por transferência horizontal o gene *mecA*.

Além das citotoxinas os estafilococos também podem produzir toxinas superantigênicas e entre essas estão as enterotoxinas que são exoproteínas hidrossolúveis, com peso molecular de 26.000 a 29.000 Da, ricas em lisina, ácido aspártico e glutâmico, com duas cisteínas formando ponte de dissulfeto (188). Apresentam-se relativamente resistentes ao calor e às enzimas proteolíticas tripsina, pepsina, renina e papaína o que permite a sua passagem pelo trato gastrintestinal sem perda de atividade (189).

A purificação e a caracterização das enterotoxinas estafilocócicas tiveram início em 1959 (190) e, atualmente, são 23 as enterotoxinas sorologicamente distintas e designadas pelas letras SEA a SElV (191,192). Todas mostram atividade superantigênica, enquanto, apenas algumas (SEA a SEI, SER, SES e SET) têm provado ser eméticas (193-194). De acordo com o Comitê International de Nomenclatura dos Superantígenos Estafilocócicos, somente os superantígenos que induzem emese após a administração oral em um modelo experimental de primatas devem ser designadas como enterotoxinas estafilocócicas. O Comitê também recomenda que outras toxinas similares que não apresentam

propriedades eméticas em modelos animais primatas ou que ainda não foram testadas devem ser designadas como enterotoxinas-like estafilocócicas (SEl) do tipo X (195-196).

Uma toxina envolvida na Síndrome do Choque Tóxico foi inicialmente designada como enterotoxina estafilocócica F (SEF) (188). No entanto, não mostrou a atividade emética *in vivo* após administração oral que é característica de uma enterotoxina verdadeira, e portanto foi mais tarde designada como TSST-1 (197). Todos os genes que codificam toxinas estão localizados em elementos genéticos móveis, incluindo bacteriófagos, ilhas de patogenicidade (SaPI) e plasmídeos (198-200). Os genes de enterotoxinas SEB, SEC, SEG, SEI, SElM, SElN, SElO, SElK, , SElQ e TSST-1 estão localizados em ilhas de patogenicidade (201-205). Os genes das SEA, SEE e SElP estão localizados em profagos (203, 206-207), ao passo que, aqueles de SED, SElJ e SER são encontrados no plasmídeo pIB485 (208-210). Dentre as quatro ilhas de patogenicidade descritas em *S. aureus*, SaPI3 tem grande importância por reunir genes de SE em um cluster denominado *enterotoxin gene cluster* ou *cluster egc*, o qual agrupa os genes codificadores de SEG (*seg*), SEI (*sei*), SElM (*selm*), SElN (*seln*) e SElO (*selo*), além de dois pseudogenes (*ϕent1* e *ϕent2*), ainda sem função biológica determinada (202, 211). A transferência horizontal de genes de enterotoxinas entre linhagens de estafilococos constituem um papel importante na evolução de *S. aureus* e de ECN como patógenos. Para uma melhor compreensão da patogenicidade ou virulência dos estafilococos, é importante saber sobre a real extensão da diversidade de superantígenos estafilocócicos (210).

A detecção de enterotoxina estafilocócica é decisiva para confirmar um surto de intoxicação alimentar e determinar a enterotoxigenicidade de linhagens (212). Os métodos de imunodifusão em ágar foram os primeiros desenvolvidos, aplicando-se a difusão simples unidimensional, difusão dupla, técnica de microlâmina e difusão em tubo capilar (212). Uma questão tem sido levantada a respeito da sensibilidade dos métodos de difusão em gel para detecção de enterotoxinas. Porcentagens significantes de linhagens toxigênicas produzem concentrações de 1 ng/ml ou menos, estando portanto, abaixo dos níveis detectáveis pelos métodos de difusão (213).

Neste sentido, desenvolveram-se os métodos de hemaglutinação reversa passiva (RPHA), aglutinação de látex (RPLA), radioimunoensaio (RIA) e ensaio imunoenzimático (ELISA), que apresentam sensibilidade de 1,0 a 0,1 ng/ml (212, 214). Porém, a detecção de ng pode ser dificultada por reações falso-positivas decorrentes da interferência de componentes alimentares, antígenos microbianos e metabólitos extracelulares, o que pode comprometer as metodologias já desenvolvidas (212, 214-215).

Além disso, o anti-soro é comercialmente disponível apenas para SEA, SEB, SEC, SED e SEE. Testes experimentais têm sido desenvolvidos para algumas das novas toxinas (SEG, SEH e SEI), mas não são comercializados devido a dificuldades na purificação e preparação de anticorpos específicos (216). Uma grande variedade de métodos baseados no ELISA têm sido descritos para detecção de SE (217), incluindo o sistema de triagem automatizado VIDAS Staph Enterotoxin II - SET2 (BioMerieux), kit Tecra™ (3M) e Kit Elisa Transia ^{TM} (Transia - Diffchamb S.A.

Lyon, France). No Brasil, o kit de aglutinação reversa e passiva de látex (RPLA, Oxoid) tem sido usado com bastante frequência para a detecção de enterotoxinas em alimentos e sobrenadantes de cultura (174, 215, 218). Esses Kits são muito utilizados pela sua simplicidade e sensibilidade, mas podem apresentar resultados falsos positivos devido à reação entre os antígenos e a ocorrência de reações inespecíficas.

Com o intuito de resolver o problema desenvolveram-se técnicas de amplificação como a Reação em Cadeia da Polimerase (PCR) que permite a identificação de genes responsáveis pela produção de enterotoxinas e TSST-1 com alta sensibilidade e especificidade. Johnson *et al*. (219) desenvolveram um protocolo para a detecção de genes para enterotoxinas A a E e TSST-1, utilizando oligonucleotídeos sintetizados a partir das análise computadorizada das sequências dos genes de enterotoxinas estafilocócicas A a E (*sea*, *seb*, *sec-1, sed*, *see*) e *tst*, publicadas anteriormente.

Apesar da pouca atenção que tem sido dada ao perfil toxigênico dos ECN, alguns pesquisadores enfatizam que esses micro-organismos podem produzir a TSST-1 e/ou uma das enterotoxinas estafilocócicas, isolada ou concomitantemente, não podendo assim, serem ignorados quanto à sua importância clínica e capacidade toxigênica (220-222).

No estudo de Crass e Bergdoll (220), as linhagens que produziram somente TSST-1 ou TSST-1 e enterotoxina A, concomitantemente, foram isoladas em sete (50%) de 14 mulheres com síndrome do choque tóxico (TSS). Os ECN foram

também isolados de quatro pacientes com TSS que tinham cultura positiva para *S. aureus,* sendo contudo observado que somente as linhagens de ECN eram produtoras de toxina. Linhagens de ECN produtoras de TSST-1 e de enterotoxinas A e C também foram isoladas de pacientes com outras infecções e de alimento implicado em um caso de intoxicação alimentar. Resultados similares foram obtidos por Kahler *et al*. (221) em um caso de Síndrome do Choque Tóxico (TSS) em que a estirpe produtora de TSST-1 foi isolada da vagina da paciente, enquanto que nenhum *S. aureus* foi encontrado. Valle *et al*. (223), estudando a produção de TSST-1 por linhagens de estafilococos isoladas de cabras, verificaram que 16,5% das linhagens de ECN isoladas eram produtoras dessa toxina.

Vários trabalhos que utilizaram a reação em cadeia da polimerase (PCR) para a confirmação da enterotoxigenicidade de estafilococos mostraram divergência entre os resultados obtidos com ensaios imunológicos e a PCR (219, 224), indicando a possibilidade de erro devido a ocorrência de resultados falso positivos. Nesse sentido várias pesquisas foram desenvolvidas em nosso laboratório com o objetivo de comprovar a capacidade enterotoxigênica dos ECN. O primeiro estudo (ANEXO 12) teve como objetivo principal a detecção de genes de enterotoxinas em estafilococos coagulase-negativa isolados de alimentos. Das vinte amostras de ECN isoladas, três apresentaram o gene *sea* e uma amostra apresentou o gene *sec-1.* O gene *sea* foi detectado em uma amostra de *S. epidermidis,* em uma amostra de *S. xylosus* e em uma de *S. hominis*, enquanto que o gene *sec-1* foi detectado em uma amostra de *S. xylosus*, porém essas toxinas não foram detectadas pelo método RPLA.

Udo *et al*. (225), investigando manipuladores de alimentos encontraram amostras de ECN e *S. aureus* produtores de enterotoxinas e TSST-1 pelo método RPLA. Do total de ECN investigados, 14,1% foram produtores de enterotoxinas ou TSST-1, sendo que amostras das espécies *S. hominis, S. warneri, S. saprophyticus, S. epidermidis, S. xylosus, S. haemolyticus e S. schleiferi* foram positivas para as toxinas SEA, SEB, SEC e/ou TSST-1. Marín *et al*. (226) descreveram estudo realizado com estafilococos produtores de enterotoxinas em amostras de presunto pelo método RPLA. Dos 135 estafilococos isolados, dois pertenciam a espécie *S. epidermidis* e um foi produtor da SEC.

Johnson *et al*. (219) observaram que em 2 das 88 linhagens de *S. aureus* o fenótipo determinado pelo RPLA divergiu do genótipo observado na PCR. Essas linhagens apresentavam genes *sec* e *tst* e não produziam as toxinas quando detectadas pelo RPLA. Schmitz *et al*. (227) avaliaram 50 isolados de *S. aureus* quanto à presença de genes de s*ec*, *seb* e *tst,* e observaram duas linhagens que apresentavam o gene *sec*, mas não produziam SEC pelo RPLA.

Em um segundo estudo desenvolvido pelo nosso grupo (ANEXO 13) a técnica de PCR foi utilizada para a detecção dos genes responsáveis pela produção de enterotoxinas e TSST-1 em 120 linhagens de *S. aureus* e 120 linhagens de ECN isoladas de recém-nascidos e os resultados obtidos foram comparados com a detecção de enterotoxinas pelo método RPLA. Do total de 120 isolados de *S. aureus*, 38,3% foram produtores de enterotoxinas pelo RPLA, enquanto que a PCR detectou genes em 46,6% das amostras. Resultados similares foram verificados em linhagens de ECN, com uma frequência superior de positividade para a detecção de

genes específicos pela PCR (40,0%) quando comparado com a produção de enterotoxina pelo método RPLA (26,7%).

Entretanto, outros autores não obtiveram resultados positivos para a presença de genes codificadores de enterotoxinas em amostras de ECN de isolados clínicos, como em estudo realizado por Becker *et al*. (228) utilizando a técnica de PCR multiplex. Kreiswirth *et al*. (229) utilizando a técnica de hibridização de DNA avaliaram várias espécies de ECN quanto à presença de genes responsáveis pela expressão de TSST-1. Esses autores incluíram no estudo algumas amostras relatadas por Crass e Bergdoll (220) como amostras produtoras de TSST-1. Em nenhuma das amostras de ECN foi confirmada a presença do gene *tst,* além de duas amostras TSST-1 positivas, identificadas anteriormente como ECN, apresentarem-se coagulase positivas, indicando um erro de identificação.

Outros autores também questionam a capacidade dos ECN de serem produtores de enterotoxinas e TSST-1 e sugerem que essas amostras possam ser *S. aureus* mutantes os quais não expressam a enzima coagulase (230-231). Com o intuito de descartar essa possibilidade, em nosso laboratório todas as amostras de ECN que apresentam genes que codificam toxinas são submetidas a uma identificação genotípica para a confirmação da espécie. A técnica ITS-PCR não deixa dúvidas quanto ao padrão de bandas característico referente a cada espécie investigada quando comparada com espécies de referência internacional (ATCC), além de excluir possíveis erros que possam ocorrer na identificação fenotípica. Embora tenham ocorrido algumas discrepâncias entre esses métodos, em relação à

diferenciação da espécie, todas as amostras identificadas como ECN pelo método fenotípico confirmaram ser ECN pela técnica genotípica.

Eliminando-se possíveis erros de identificação, a divergência nos resultados obtidos em relação aos ECN serem capazes de produzir ou não toxinas pode estar relacionada à escolha da técnica adequada para extração do DNA e detecção de genes responsáveis pela expressão dessas enterotoxinas. A padronização das técnicas que vai desde o emprego de meios de cultura, pH, temperatura e concentrações de nutrientes ideais, bem como o uso de controles de extração, parâmetros utilizados nas reações de amplificação e enzimas, é fundamental para o bom desempenho da pesquisa. Sabe-se que uma das desvantagens da PCR multiplex é que ela destaca a presença dos genes que estão em maior frequência, mascarando ou não detectando os que aparecem em menor frequência, diferente da técnica PCR que utiliza um par de *primers* para cada reação, a qual é feita isoladamente para cada toxina investigada.

A técnica de PCR detecta os genes de interesse independentemente de sua expressão, assim, os genes responsáveis pela produção das enterotoxinas podem estar presentes e não estarem ativos. O desenvolvimento das técnicas moleculares possibilita a detecção do RNA mensageiro (RNAm) através da técnica de Reação em Cadeia da Polimerase - Transcriptase Reversa (RT-PCR). Por essa técnica o RNAm é convertido a cDNA (DNA complementar) pela enzima transcriptase reversa, sendo a expressão do gene proporcional ao número de cópias de RNAm do gene de interesse. O cDNA é amplificado pela técnica de PCR com a utilização dos primers específicos para confirmar a expressão, o que não deixa dúvidas quanto ao

potencial toxigênico do micro-organismo. Em estudo posterior, com a utilização da técnica de RT-PCR para a detecção de SEA, SEB, SEC, SED e TSST-1 em amostras de *S. aureus* e ECN (ANEXO 14) foi verificado a expressão de RNAm que codifica as toxinas estafilocócicas em 43 (39,8%) das 108 amostras positivas pela técnica de PCR. A expressão de toxinas pelos ECN foi significativamente inferior quando comparada a dos *S. aureus*, correspondendo a 13,9% do total de amostras com potencial toxigênico. O *S. epidermidis* foi a espécie mais toxigênica entre os ECN, sendo que cinco amostras foram positivas para a expressão de RNAm que codifica as SEA e SEC. Entre as outras espécies de ECN, somente *S. lugdunensis* apresentou resultado positivo pela técnica de RT-PCR, para a SEC.

A técnica de RT-PCR também foi utilizada para a detecção de RNAm que codifica as enterotoxinas SEE, SEG, SEH e SEI em 90 amostras de *S. aureus* e 90 amostras de ECN (ANEXO 15) revelando a expressão de RNAm em 42 (50,6%) das 83 amostras positivas pela técnica de PCR. O RNAm detectado correspondeu às toxinas das classes SEG, SEH e SEI. A expressão de toxinas pelos ECN foi inferior quando comparada a de *S. aureus*, correspondendo a 34,5% do total de amostras produtoras, sendo que a expressão das toxinas pelas amostras de *S. aureus* correspondeu a 59,3%. *S. epidermidis* foi a espécie mais toxigênica entre os ECN, sendo que sete amostras foram positivas para a expressão de RNAm que codifica as enterotoxinas SEG, SEH e SEI. Entre as outras espécies de ECN, somente as espécies *S. warneri* e *S. lugdunensis* apresentaram resultado positivo pela técnica de RT-PCR, sendo que a primeira foi positiva para SEI e a segunda para SEG e SEI.

Algumas associações encontradas pela técnica de PCR chamam atenção por existirem em várias amostras como a presença concomitante dos genes *seg* e *sei* em 16 amostras de *S. aureus* e em 10 de ECN. A coexistência dos genes *seg* e *sei* pode ser devida à sua localização cromossômica, ambos pertencem a um operon do *egc* (*enterotoxin gene cluster*), o qual contém cinco genes de enterotoxinas (*seg, sei, selm, seln* e *selo*) e está localizado em ilhas de patogenicidade de *S. aureus* (SaPIs). Maiques *et al*. (232) reportaram que uma SaPI encontrada em um linhagem de *S. aureus* foi capaz de ser transferida por um bacteriófago para outra espécie de estafilococos, tal como *S. epidermidis*.

Finalmente, nossos resultados sobre a capacidade de linhagens de ECN expressarem superantígenos estafilocócicos foram corroborados pelo estudo publicado recentemente por Madhusoodanan *et al*. (233) que comprovaram a primeira evidência de uma ilha de patogenicidade em um isolado clínico de *S. epidermidis* (SePI). O genoma dessa cepa foi analisado e confirmou-se a presença de uma SePI-1 que codifica os genes das SEC-3 e SElL, sendo também confirmado a expressão desses superantígenos por RT-PCR quantitativo (qRT-PCR) e por western immunoblotting.

O método de RT-PCR mostrou ser uma técnica rápida e eficaz, deixando evidente a capacidade dos ECN de expressarem os RNAm que codificam essas enterotoxinas, porém, como depende da ativação dos genes, uma maior investigação de fatores do meio e esclarecimento dos mecanismos regulatórios que possam estar interferindo em sua expressão devem ser avaliados.

A expressão dos genes codificadores de fatores de virulência de *S. aureus* é coordenada por reguladores globais. Esses reguladores ajudam a bactéria a se adaptar a um ambiente hostil pela produção de fatores que as capacitam sobreviver e subsequentemente causar infecção no momento apropriado. Vários dos reguladores de virulência globais, como o sistema *agr* (gene regulador acessório), *sar* (regulador acessório estafilocócico) e *sae* (elemento acessório estafilocócico), tem sido bem caracterizados (234). Outros sistemas, tais como *arl* (lócus relacionado a autolisina)*, sar* homólogos (*rot*-repressor de toxinas, *mgrA* – regulador global múltiplo*, sarS, saR, sarT, sarU, sarV, sarX, sarZ* e *tcaR* – associado a teicoplanina), o sistema *srr* (resposta respiratória estafilocócica) e *trap* (alvo da proteína ativadora de RNA III) requerem estudos futuros para determinar seus exatos papéis na regulação da virulência (234). De todos esses sistemas regulatórios existentes, o mais caracterizado é o *agr*. Novick e Jiang (235) descreveram a expressão do locus *agr* como um importante regulador em muitos dos fatores de virulência de *S. aureus*.

O locus *agr* (gene regulador acessório) é um agrupamento de genes com atividade *quorum sensing,* o mecanismo pelo qual a bactéria consegue "perceber" a densidade celular do meio em que se encontra através da comunicação entre as células presentes, permitindo uma reação fenotípica de acordo com a fase de crescimento em que a cultura se encontra. O *quorum sensing* é importante em *Staphylococcus* porque certas proteínas, tais como os fatores de virulência, só devem ser expressas em determinada fase de crescimento (235). Esse sistema coordena a expressão dos genes que estão ligados às necessidades biológicas de

Staphylococcus, permitindo a adesão às células e ao tecido do hospedeiro, a disseminação no organismo, e a degradação de células e tecidos, tanto para a sua nutrição quanto para a proteção contra a defesa do hospedeiro (236).

A expressão do sistema *agr* contribui com a patogênese estafilocócica em diferentes momentos da infecção. Considerando-se a curva de crescimento de *Staphylococcus* observa-se, com exceção da enterotoxina A que é produzida durante todas as fases de crescimento, que todas as exoproteínas são secretadas na fase pós-exponencial. Por outro lado, as proteínas associadas à membrana e as adesinas são produzidas durante a fase exponencial e não na fase pós-exponencial (237).

São descritos quatro polimorfismos do locus *agr* em *Staphylococcus,* sendo denominados *agr* I, II, III e IV (238). Nesses grupos há variação nos genes *agrB*, *agrC* e a*grD*, e consequentemente no AIP e na proteína AgrC, sendo a ligação da AIP com seu receptor específico para cada grupo alélico. Quando ocorre ligação de um AIP de um grupo alélico no receptor AgrC de outro grupo, o AIP não produz fator intrínseco e não produz o sinal através da proteína AgrC, funcionando portanto, como um antagonista. Um AIP só é agonista para seu próprio grupo alélico, de forma que bactérias de grupos *agr* diferentes interferem na regulação de proteínas acessórias umas das outras (239).

Novick *et al*. (235) descreveram a expressão do locus *agr* como um importante regulador de muitos fatores de virulência de *S. aureus*. Embora vários autores contestem o potencial toxigênico de ECN (230-231), o operon *agr* que

possui importante papel na regulação da expressão de toxinas estafilocócicas, têm sido encontrado também em outras espécies de estafilococos, tais como *S. intermedius* (240), *S. lugdunensis* (241) e *S. epidermidis* (242). A predominância de *S. epidermidis* e a detecção de *S. lugdunensis* entre as espécies de ECN toxigênicas em nosso estudo podem estar associadas a esses sistemas regulatórios estafilocócicos também estarem presentes nessas espécies. Nesse sentido, estudos em desenvolvimento em nosso laboratório tem justamente como objetivo principal detectar o sistema *agr* em diferentes espécies de ECN e analisar a relação com a presença e expressão de superantígenos estafilocócicos.

Verifica-se, então, que os estafilococos podem usar combinações de diversos fatores de virulência, os quais podem resultar em síndrome clínica e possivelmente contribuir para a dispersão bacteriana no organismo do hospedeiro. Os superantígenos têm a propriedade de estimular a atividade dos linfócitos T, levando à produção de altos níveis de citocinas, que podem levar à anergia, inflamação, citotoxicidade, deleção de células T e auto-imunidade (191, 243) o que facilita a colonização do indivíduo.

Espécies de *Staphylococcus*, comensais ou patogênicas, possuem uma gama extensa de fatores de virulência que podem ser expressos ou não, dependendo do momento da vida do micro-organismo, de seu ambiente e dos sistemas regulatórios presentes que podem afetar diretamente a produção de toxinas. Esses fatores, responsáveis pela invasão tissular direta ou pela toxigenicidade devido à ação de toxinas liberadas, são muito importantes na alta prevalência e sucesso desses micro-organismos em infecções tanto na comunidade quanto em hospitais.

As bases moleculares da patogenicidade dos estafilococos são multifatoriais, dependendo da presença e também expressão de vários genes acessórios. Dessa forma, a presença de genes toxigênicos nas amostras de estafilococos sem transcrição de RNAm do gene não exclui a possibilidade de produção de toxinas em determinado momento, seja a produção *in vivo* ou em meios de cultura sob condições ótimas.

ANEXO 11

Barretti P, Moraes TMC, Camargo CH, Caramori JCT, Mondelli AL, Montelli AC, **Cunha MLRS.** Peritoneal Dialysis-Related Peritonitis Due to *Staphylococcus aureus*: A Single-Center Experience over 15 Years. (Submetido para publicação).

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Peritoneal Dialysis-Related Peritonitis Due to Staphylococcus aureus: A Single-Center Experience over 15 Years

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Corresponding Author:	Maria de Lourdes Ribeiro de Souza da Cunha UNESP Botucatu, São Paulo BRAZIL
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Abstract:	Peritonitis caused by Staphylococcus aureus is a serious complication of peritoneal dialysis(PD). We reviewed the records of 62 S. aureus peritonitis episodes that occurred between 1996 and 2010 in the dialysis unit of a single university hospital. Peritonitis incidence was calculated for three subsequent 5-year periods and compared using a Poisson regression model. The production of biofilm, enzymes, and toxins was evaluated. Oxacillin resistance was evaluated based on minimum inhibitory concentration and presence of the mecA gene. Logistic regression was used for the analysis of demographic, clinical, and microbiological factors influencing peritonitis outcome. Resolution and death rates were compared with 117 contemporary coagulase-negative staphylococcus(CoNS)episodes. The incidence of S. aureus peritonitis declined significantly over time from 0.13 in 1996-2000 to 0.04 episodes/patient/year in 2006-2010 (p = 0.03). The oxacillin resistance rate was 11.3%. Toxin and enzyme production was expressive, except for enterotoxin D. Biofilm production was positive in 88.7% of strains. The presence of the mecA gene was associated with a higher frequency of fever and abdominal pain. The logistic regression model showed that diabetes mellitus (p = 0.009) and β-hemolysin production (p = 0.006) were independent predictors of non-resolution of infection. The probability of resolution was higher among patients aged 41 to 60 years than among those >60 years (p = 0.02). A trend to higher death rate was observed for S. aureus episodes (9.7%) compared to CoNS episodes (2.5%), (p =0.08), whereas resolution rates were similar. Despite the decline in incidence, S. aureus peritonitis remains a serious complication of PD which is associated with a high death rate. The outcome of this infection is negatively influenced by host factors such as age and diabetes mellitus. In addition, β-hemolysin production is predictive of non-resolution of infection, suggesting a pathogenic role of this factor in PD-related S. aureus peritonitis.
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Clinical and Microbiological Outcome Predictors in Peritoneal Dialysis-Related Peritonitis Due to *Staphylococcus aureus*: A Single-Center Experience over 15 Years

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Short title: Peritoneal dialysis peritonitis due to *S. aureus*

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Abstract

Peritonitis caused by *Staphylococcus aureus* is a serious complication of peritoneal dialysis (PD), which is associated with poor outcome and high PD failure rates. We reviewed the records of 62 *S. aureus* peritonitis episodes that occurred between 1996 and 2010 in the dialysis unit of a single university hospital and evaluated the host and bacterial factors influencing peritonitis outcome. Peritonitis incidence was calculated for three subsequent 5-year periods and compared using a Poisson regression model. The production of biofilm, enzymes, and toxins was evaluated. Oxacillin resistance was evaluated based on minimum inhibitory concentration and presence of the *mec*A gene. Logistic regression was used for the analysis of demographic, clinical, and microbiological factors influencing peritonitis outcome. Resolution and death rates were compared with 117 contemporary coagulase-negative staphylococcus (CoNS) episodes. The incidence of *S. aureus* peritonitis declined significantly over time from 0.13 in 1996-2000 to 0.04 episodes/patient/year in 2006-2010 ($p = 0.03$). The oxacillin resistance rate was 11.3%. Toxin and enzyme production was expressive, except for enterotoxin D. Biofilm production was positive in 88.7% of strains. The presence of the *mec*A gene was associated with a higher frequency of fever and abdominal pain. The logistic regression model showed that diabetes mellitus ($p = 0.009$) and β-hemolysin production ($p = 0.006$) were independent predictors of non-resolution of infection. The probability of resolution was higher among patients aged 41 to 60 years than among those >60 years ($p = 0.02$). A trend to higher death rate was observed for *S. aureus* episodes (9.7%) compared to CoNS episodes (2.5%), ($p = 0.08$), whereas resolution rates were similar. Despite the decline in incidence, *S. aureus* peritonitis remains a serious complication of PD which is associated with a high death rate. The outcome of this infection is negatively influenced by host factors such as age and diabetes mellitus. In addition, β-hemolysin production is predictive of non-resolution of infection, suggesting a pathogenic role of this factor in PD-related *S. aureus* peritonitis.

Author Summary

Gram-positive cocci are the main etiological agents of peritoneal dialysis (PD)-related peritonitis. *Staphylococcus aureus* is associated with more severe episodes of peritonitis, a higher risk of hospitalization, catheter removal, and death. In this first Latin American study, we analyzed a series of *S. aureus* peritonitis cases seen over a period of 15 years. We reviewed clinical and microbiological characteristics of *S. aureus* peritonitis in PD patients, including virulence factors (biofilm, enterotoxins, toxic shock syndrome toxin-1, α- and β-hemolysins, lipase, lecithinase, nuclease, and thermonuclease) and oxacillin resistance determined based on the presence of the *mec*A gene and minimum inhibitory concentration. The incidence of peritonitis due to *S. aureus* declined significantly over time, but the infection was associated with poor outcome. The outcome of PD-related peritonitis is negatively influenced by host factors such as age and diabetes mellitus. Beta-hemolysin production is the only factor predictive of non-resolution, suggesting a pathogenic role of this factor in PD-related *S. aureus* peritonitis. The role of β-hemolysin in the pathogenesis of *S. aureus* infections continues to be poorly understood and has not been previously reported in PD-related peritonitis.

Introduction

Peritonitis is a serious complication of peritoneal dialysis (PD) and is responsible for a high rate of technique failure and death in PD patients [1]. Gram-positive cocci are the main etiological agents of peritonitis in the world, with coagulase-negative staphylococci (CoNS) being the most common microbial agents, whereas *Staphylococcus aureus* is associated with more severe episodes, a higher risk of hospitalization, catheter removal, and death [1,2]. Although *S. aureus* is responsible for a small proportion of peritonitis episodes in most countries, it continues to be the leading cause of this infection in some Latin American countries, particularly in Brazil [3].

A poor prognosis of PD-related *S. aureus* peritonitis has been frequently reported [2,4,5], but there are only two reports [6,7] that specifically describe the clinical outcome and predictors of treatment response in this infection. In the largest series, Govindarajulu et al. [6] showed that methicillin-resistant *S. aureus* (MRSA) peritonitis was independently predictive of an increased risk of permanent hemodialysis transfer and tended to be associated with a high risk of hospitalization. Szeto et al. [7] reported a lower primary response rate and complete cure rate for episodes caused by MRSA compared to episodes due to other *S. aureus*. In both cases the clinical outcome of *S. aureus* peritonitis was not encouraging. The rates of relapse, catheter removal and hospitalization were 20%, 23% and 67%, respectively, in the study of Govindarajulu et al. [6]. In the series of Szeto et al. [7], only 51% of patients with methicillin-susceptible *S. aureus* peritonitis and 46% with MRSA peritonitis presented complete cure without relapse, recurrent or repeat episodes, or need for catheter removal.

In addition to antibiotic resistance, the severity of *S. aureus* infections is associated with virulence factors produced by this bacterium, such as enzymes (coagulase, lipase, and nucleases) and multiple toxins with diverse activities. One family of protein toxins are the staphylococcal enterotoxins and the related toxic shock

syndrome toxin-1 (TSST-1) that act as superantigens [8]. The biofilm produced by most *S. aureus* strains facilitates bacterial adhesion to catheters and colonization and simultaneously worsens the response to infection, protecting bacterial cells from the host's natural defense mechanisms and from the action of antibiotics [8,9]. Although these products may influence clinical outcome, their role in PD-related *S. aureus* peritonitis is still not fully defined. Data published by Haslinger-Löffler et al. [10] suggest that α-hemolysin plays a specific role in the pathogenesis of peritonitis. Using cultured human peritoneal mesothelial cells, these authors showed that α-hemolysin produced by *S. aureus* was able to induce caspase-independent cell death. In a recent study, our group demonstrated that biofilm and α-hemolysin production were the only independent predictors of non-resolution of staphylococcal peritonitis [11]. However, the small number of *S. aureus* episodes analyzed was an important limitation of that study.

For the last 15 years we have monitored clinical and microbiological characteristics of *S. aureus* peritonitis in PD patients, including virulence factors produced by this pathogen and presence of the *mec*A gene that confers resistance to methicillin/oxacillin. The objective of the present study was to describe the experience of a single Brazilian center with PD-related *S. aureus* peritonitis, focusing on host and bacterial factors that influence peritonitis outcome.

Results

A total of 682 peritonitis episodes were diagnosed in our unit between 1996 and 2010. The overall peritonitis rate was 0.96 episodes per patient per year. Seventy-three (10.7%) episodes were caused by *S. aureus*. After application of the exclusion criteria, 62 episodes that occurred in 56 patients were analyzed. The demographic and baseline clinical data of the patients are summarized in Table 1. The clinical findings in

peritonitis episodes were expressed in Table 2. The incidence of *S. aureus* peritonitis declined significantly over time and was 0.13 episodes per patient per year in 1996-2000, 0.10 in 2001-2005, and 0.04 in 2006-2010 (p = 0.03). The annual *S. aureus* peritonitis rate is presented in figure 1; a strong decline of the incidence was observed after 2003. Vancomycin was used in 35 (56.5%) episodes. Overall, 32 (51.6%) episodes were resolved; among cases that were not resolved one (0.16%) relapsed, 18 (29%) required removal of the catheter due to refractory peritonitis, five (8%) were resolved with a second antibiotic regimen, and six (9.7%) progressed to death. Of 117 contemporary CoNS peritonitis episodes, 63 (53.8%) were resolved and three (2.5%) progressed to death. The death rate tended to be lower among episodes caused by CoNS than among *S. aureus* episodes (p = 0.08), whereas resolution rates were similar (p = 0.16).

All strains were susceptible to vancomycin (MIC ≤ 3 µg/ml) and seven (11.3%) were resistant to oxacillin (MIC ≥ 4 µg/ml). The vancomycin MIC or proportion of oxacillin-resistant isolates did not change significantly over time (Figure 2). The *mec*A gene was detected in seven (11.3%) strains.

The rates of toxin and enzyme production by *S. aureus* are shown in Table 3. No associations were observed between the production of virulence factors and the frequency of initial clinical findings. However, fever was observed in 83.3% of episodes caused by bacteria expressing the *mec*A gene, whereas this clinical symptom was present in only 24% of episodes due to *mec*A gene-negative strains (p = 0.03). In addition, there was a trend towards a higher rate of abdominal pain (100%) among strains expressing the *mec*A gene compared to *mec*A gene-negative isolates (64.8%) (p = 0.08). The production of virulence factors and presence of the *mec*A gene were not associated with catheter removal, hospitalization, or death rate.

Gender, age, vancomycin use, presence of diabetes, production of virulence factors (β-hemolysin, lecithinase, deoxyribonuclease, SEC, and TSST-1), presence of the *mec*A gene, and dialysis vintage were associated with a higher chance of non-resolution in univariate analysis (Table 4), and were therefore included in the multivariate logistic regression model. Multivariate analysis showed that the presence of diabetes and β-hemolysin production were factors independently associated with a higher odds ratio of non-resolution of peritonitis episodes. In contrast, age of 41-60 years was associated with a lower chance of non-resolution when compared to age >60 years. No significant association with peritonitis outcome was observed for the other variables (Table 4).

Discussion

The present results showed a marked decline in the incidence and proportion of peritonitis episodes caused by *S. aureus* over the past 15 years, in agreement with other studies [3,12]. The introduction of safer connection systems and the routine use of prophylactic antibiotics at the catheter exit site possibly contributed to the reduction of the incidence of *S. aureus* peritonitis; however the strong decline in the incidence observed after the introduction of the prophylaxis with mupirocin reinforces the role of this strategy on *S. aureus* peritonitis prevention. In addition, in the present series we observed a higher death rate among *S. aureus* episodes compared to episodes caused by CoNS as previously reported [1].

There are few studies reporting the influence of demographic and clinical factors on the prognosis of *S. aureus* peritonitis episodes. Szeto et al. [7] observed an association between adjuvant rifampicin treatment and a significantly lower risk of relapse, whereas the complete cure rate was similar for cephalosporin and vancomycin empiric treatment protocols. Govindarajulu et al. [6] found that the presence of

peripheral vascular disease and the use of vancomycin compared to cephalosporins were significantly associated with an increased risk of relapse of *S. aureus* peritonitis. According to these authors, female gender and middle tertile of age were independent predictors of a lower risk of relapse. Similarly, we observed that patient age of 41 to 60 years was associated with a higher chance of peritonitis resolution when compared to older patients. In addition, the presence of diabetes was an independent predictor of non-resolution of peritonitis. It is known that the immune response is dysregulated in diabetic patients, increasing the risk of developing infection. In addition, advanced glycation end-products act on peritoneal mesothelial cells, with a potentially negative impact on the local immune response [13]. Although diabetes has been reported to be a risk factor for peritonitis [14], to our knowledge, there are no studies showing diabetes to be a predictor of poor outcome after a peritonitis episode. In the present series, vancomycin use was not an independent predictor of outcome, in agreement with the study of Szeto et al. [7]. We observed no influence of other demographic or clinical factors on resolution rate. Similar results have been reported by Krishnan et al. [15] in a retrospective series of peritonitis episodes of different causes.

Little is known about the influence of specific virulence factors on peritonitis caused by *S. aureus*. MRSA peritonitis has been associated with poor outcome in the two largest series of *S. aureus* peritonitis [6,7]. In the present series, we found a low oxacillin resistance rate, which was confirmed by determination of the *mecA* gene. On the other hand, the *S. aureus* strains studied presented expressive enzyme, toxin, and biofilm production. In contrast to previous studies we found no association between oxacillin resistance and resolution rate; however, in the present series only seven peritonitis episodes were caused by oxacillin-resistant *S. aureus*, a fact that may have influenced the results. Episodes caused by *mecA*-positive *S. aureus* isolates were associated with more severe initial clinical symptoms. Studies investigating the role of the *mecA* gene in the virulence of *S. aureus* are scarce in the literature. Fowler Jr et al.

[16] found an increasing proportion of MRSA among strains isolated from nasal carriage, uncomplicated bacteremia, and bacteremia with hematogenous complications. Analyzing the same sample later, Gill et al. [17] confirmed a higher frequency of the *mecA* gene among *S. aureus* strains isolated from severe infections. In our laboratory [18] analyzing 336 MRSA and 107 MSSA strains, we observed a significantly higher proportion of strains expressing SEA, SEB, SEC and TSST-1 genes among MRSA. Taken together, these findings show that, although the number of strains expressing the *mec*A gene was small in this series, a role of the *mec*A gene in *S. aureus* virulence cannot be ruled out.

Among bacterial factors studied, β-hemolysin production was significantly and independently associated with lower resolution odds. The role of β-hemolysin in the pathogenesis of *S. aureus* infections has not been previously reported in PD-related peritonitis. Nevertheless, some pathways may be suggested based on the findings of experimental models. β-Hemolysin is one of the toxins produced by *S. aureus* which acts as a sphingomyelinase, degrading sphingomyelin in the outer layer of cell membranes [19]. Deletion of the catalase and β-toxin genes in *S. aureus* strains has been shown to cause strong attenuation of virulence in intramammary and subcutaneous experimental infections of ewes and lambs and in a murine skin abscess model [19]. Using a mouse model of lung injury, Hayashida et al. [20] found that animals infected with β-hemolysin-deficient *S. aureus* presented significantly attenuated lesions compared to those infected with *S. aureus* expressing this toxin. This experimental disease was characterized by intense neutrophilic inflammation and reduced expression of syndecan-1 in alveolar epithelial cells and could be reproduced by administration of recombinant β-hemolysin, but not of mutant β-hemolysin deficient in sphingomyelinase activity.

Extracellular DNA is a major structural component in the biofilms of pathogenic *S. aureus*. Huseby et al. [21] showed that β-hemolysin forms covalent cross-links to

itself in the presence of DNA, irrespective of sphingomyelinase activity, producing an insoluble nucleoprotein matrix in vitro. Using an infectious endocarditis rabbit model, the authors observed that this toxin stimulates biofilm formation in vivo. β-Hemolysin does not lyse most types of host cells but leaves them vulnerable to a number of other lytic agents, such as α-hemolysin and Panton-Valentine leukocidin [20]. We recently demonstrated that α-hemolysin production predicts poor outcome in peritonitis episodes caused by *S. aureus* and CoNS [11].

A reservoir of phospholipids exists on the peritoneal surface and the main constituents of peritoneal phospholipids are phosphatidylcholine and sphingomyelin [22]. Indeed, evidence suggests that the phospholipids present on the peritoneal surface are derived from peritoneal mesothelial cells. In this respect, β-hemolysin, a sphingomyelinase, may participate directly in biofilm formation, contributing to a poorer outcome of peritonitis episodes, or may render host peritoneal cells susceptible to other pathogenic factors.

Surprisingly, biofilm production was not a predictor of peritonitis resolution. However, the percentages of non-producers was low, a fact impairing the comparison with biofilm producers; therefore, a role of biofilm production in peritonitis outcome cannot be ruled out. Finally, other *S. aureus* virulence factors that act in a synergistic and coordinated fashion may play a pathogenic role.

The present study has several limitations, the most important of them is the absence of more accurate tests to assess production of β-hemolysin such as mRNA levels using quantitative real time-PCR or specific detection such as by ELISA or Western Blot. Also, the small number of cases analyzed that may reduce its statistical power, and since it is a single-center study its results cannot be extrapolated. Nevertheless, it is the first Latin American study analyzing a series of *S. aureus* peritonitis cases. In this respect, *S. aureus* remains the most frequent PD-related

etiology in several Latin American countries, including Brazil [3]. Furthermore, this study focused on the role of virulence factors on the outcome of this infection.

In conclusion, despite a strong reduction in the incidence of *S. aureus* peritonitis, our results showed a poorer outcome of episodes caused by this bacterium when compared to episodes due to CoNS, particularly a higher death rate. Among demographic factors, older age and diabetes were predictors of a lower resolution rate. These findings highlight the importance of peritonitis as a serious complication of PD, particularly among elderly and diabetic patients. ß-Hemolysin production was the only virulence factor that negatively influenced peritonitis outcome; however further studies using specific tests to detect the presence of ß-hemolysin are necessary to confirm this result.

Materials and Methods

All episodes of ambulatory PD-related peritonitis caused by *Staphylococcus aureus* between January 1996 and December 2010 were reviewed. The diagnosis of peritonitis was made when at least two of the following criteria were present: 1) presence of a cloudy peritoneal effluent; 2) abdominal pain; 3) dialysate containing more than 100 white blood cells per µl (at least 50% polymorphonuclear cells), and 4) positive culture of peritoneal effluent [23,24]. Exclusion criteria were episodes of relapsing *S. aureus* peritonitis, presence of concomitant exit site or tunnel infections, and incomplete clinical data. Resolution was defined as the disappearance of signs and symptoms within 96 h after the beginning of antibiotic therapy and a negative peritoneal fluid culture at least 28 days after treatment completion [23,24]. Relapse was defined as an episode due to the same organism, or a negative culture result that occurs within 28 days of completion of antibiotic therapy for a prior *S. aureus* episode [23,24]. Death related to peritonitis was defined as death of a patient with active peritonitis, or admitted with peritonitis, or within 2 weeks of a peritonitis episode [23,24]. Non-resolution was the term used for cases presenting initial non-resolution, relapse, peritoneal catheter removal due to refractory peritonitis, need for a second antibiotic regimen, or death.

The following information was recorded for each case: 1) episode: date, clinical findings, treatment, and outcome (resolution, relapse, catheter removal, or death); 2) presence of diabetes mellitus; 3) demographic data: age, gender and race (Caucasian, non-Caucasian), and dialysis treatment time; 4) dialysis modality (continuous ambulatory peritoneal dialysis or automated peritoneal dialysis); 5) educational level (illiterate, elementary, secondary, and higher).

The study was approved by the Research Ethics Committee of the Faculty of Medicine of Botucatu, Brazil (OF. 028/08-CEP). This study was exempted from the requirement to obtain written informed consent from the participants and/or their legal

guardians because the *Staphylococcus* strains included in the study had already been isolated and stored in the Culture Collection of the Department of Microbiology and Immunology, UNESP, Botucatu, São Paulo, Brazil.

Patients were treated within 24 h of the onset of the first clinical signs or symptoms using contemporary empiric antibiotic recommendations [23-25]. From 1996 to 2000 (period 1) empiric antibiotic therapy consisted of intraperitoneal (i.p.) cefazolin plus amikacin. Two protocols were used from 2000 to 2005 (period 2): the first consisted of i.p. cefazolin plus amikacin and the second of i.p. cefazolin plus ceftazidime. After 2005 (period 3) all episodes were first treated with i.p. vancomycin plus amikacin. Therapy was evaluated and adjusted as soon as the culture results were available. The duration of antibiotic therapy was 21 days.

In the period 1 no antibiotic prophylaxis at exit site was prescribed and two connection systems were used: the Y set system and the twin bag system, which was introduced in 1999; automated PD (APD) was used from 1998. In the period 2 no antibiotic prophylaxis at exit site was prescribed until 2003, when daily mupirocin cream application at exit site began to be recommended; the twin bag system or APD were prescribed for all patients. In the period 3 until December 2006 all patients were oriented to daily mupirocin cream application, and from January 2007 daily gentamicin cream application at exit site was prescribed to all incident patients; the twin bag system or APD were prescribed for all patients.

The incidence of *S. aureus* peritonitis was calculated for the three subsequent periods of 5 years and is expressed as episodes per patient per year.

The initial cultures were performed with the Bactec® Automated System (Becton Dickinson Company, Sparks, Maryland, USA) and then seeded onto blood agar. The isolates were Gram stained to confirm purity and to determine morphology and specific color. After confirmation of these characteristics, tests for identification of

the isolates were performed as recommended by Koneman et al. [26]. The isolates were stored in a culture collection.

The in vitro susceptibility of *S. aureus* to oxacillin and vancomycin was evaluated based on the minimum inhibitory concentration determined by the E-test (AB Biodisk, Solna, Sweden). This quantitative method uses a transparent strip of inert plastic that contains drug concentrations ranging from 0.002 to 256 µg/ml. The proportion of strains susceptible to each drug was defined based on the 2011 CLSI breakpoints [27]. Strains presenting intermediate values were considered to be resistant.

Whole nucleic acids were extracted from *S. aureus* strains cultured on blood agar, individually inoculated into brain heart infusion (BHI) broth, and incubated at 37ºC for 24 h. Nucleic acids were extracted using the illustra blood genomic Prep Mini Spin kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer instructions. Staphylococcal cells were first digested with lysozyme (10 mg/ml) and proteinase K (20 mg/ml). Next, 500 µl extraction solution was added to the mixture. After centrifugation at 5000 *g* for 1 min, the supernatant was transferred to a GFX column and centrifuged at 5000 *g* for 1 min. The supernatant was discarded and 500 µl extraction solution was added to the column. After centrifugation and disposal of the supernatant, 500 µl wash solution was added to the column. The column was then centrifuged at 14,000 rpm for 3 min and transferred to a 1.5-ml tube. Milli-Q water (200 µl) preheated to 70ºC was used for elution. The isolates were centrifuged at 5000 *g* for 1 min and the GFX column was discarded. Extracted DNA was stored under refrigeration at 4ºC.

PCR amplification was performed in 0.5-ml microcentrifuge tubes containing 10 pmol of each primer, 2.0 U Taq DNA polymerase, 100 µM deoxyribonucleotide triphosphates, 10 mM Tris-HCl (pH 8.4), 0.75 mM $MgCl_2$, and 3 µl nucleic acid in a total volume of 25 µl. *mecA* gene amplification was carried out in an appropriate thermal

cycler using the mecA1 (AAA ATC GAT GGT AAA GGT TGG) and mecA2 (AGT TCT GCA GTA CCG GAT TTG) primers as described by Murakami et al. [28]: 40 cycles of denaturation at 94ºC for 30 s, annealing of primers at 55.5ºC for 30 s, and extension at 72ºC for 1 min. After completion of the 40 cycles, the tubes were incubated at 72ºC for 5 min and then cooled to 4ºC. The *S. aureus* ATCC 33591 and ATCC 25923 references strains were included in all reactions as positive and negative controls, respectively.

The efficiency of amplification was monitored by electrophoresis on 1.5% agarose gel prepared in 1X TBE buffer and stained with ethidium bromide. The size of the amplified products was compared with a 100-bp standard and the gels were photographed under UV transillumination.

Biofilm production was evaluated according to Christensen et al. [29]. Colonies isolated on blood agar were inoculated into tubes measuring 12.0 x 75.0 mm and containing 2.0 ml trypticase soy broth and incubated at 37ºC for 48 h. Next, 1.0 ml 0.4% trypan blue or Toluidine blue O solution was added to the tubes. After gentle shaking to guarantee staining of the material adhered to the inner surface of the tubes, the dye was discarded. A positive result was defined as the presence of a layer of stained material adhered to the inner wall of the tube. The presence of a colored ring only at the liquid-air surface was classified as a negative result.

Production of α- and β-hemolysin were determined on plates containing blood agar supplemented with 5% rabbit blood and 5% sheep blood, respectively. The plates were incubated at 37ºC for 24 h. The formation of hemolysis zones around the isolated colonies indicated a positive result.

Lipolytic activity was evaluated on plates containing blood agar enriched with 0.01% $CaCl_2$:$2H_2O$ and 1% Tween 80. A positive result was defined as the formation of opacity around the colony after incubation at 37ºC for 18 h, followed by incubation at room temperature for 24 h [30]. The production of lecithinase was evaluated using

Baird-Parker medium. The formation of an opaque halo around the colony indicated a positive result [31].

Nuclease (DNAse) and thermonuclease (TNAse) were determined by the metachromatic Toluidine blue O agar diffusion-DNA technique according to Lachica et al. [32]. Supernatants obtained by the sac culture method of Donnelly et al. [33] as described below were transferred to the wells of plates containing metachromatic Toluidine blue O agar. The culture supernatant was first heated in a water bath for 20 min for the detection of TNAse. Nuclease (DNAse and TNAse) activity was evaluated by measuring the diameter of pink halos (mm) formed on the medium. Positive results were interpreted by comparing the halos with those obtained for a standard DNAse- and TNAse-positive *S. aureus* strain (ATCC 25923).

For the evaluation of the production of enterotoxins and TSST-1, the sac culture method [33] was used to determine the toxigenic profile of the strains. Dialysis sacs filled with 50 ml double-concentrated BHI broth were placed in U-shaped Erlenmeyer flasks and autoclaved at 121ºC for 15 min. A loopful of organisms was added to 18 ml sterile 0.2 M phosphate buffer in 0.9% NaCl, pH 7.4. After incubation at 37ºC for 24 h on a shaker at 200 rpm, the cultures were centrifuged at 8000 *g* for 10 min at 4ºC and the supernatants obtained were stored at -20ºC until the time of use. The extracellular products were detected by reverse passive latex agglutination (RPLA) using the SET-RPLA-T900 and TST-RPLA-TD940 kits (Oxoid Diagnostic Reagents, Cambridge, UK) for the detection of enterotoxins A (SEA), B (SEB), C (SEC) and D (SED) and TSST-1, respectively, according to manufacturer instructions. Samples that presented nonspecific reactions after this treatment were filtered through a Millipore membrane (0.22 µm) and, if necessary, diluted 1:10 with 0.02 M phosphate buffer in 0.9% NaCl, pH 7.4. A positive reaction was classified as (+), (++) and (+++) according to the agglutination pattern described by the manufacturer of the kit. The formation of a rose button was interpreted as a negative result.

Statistical analysis.

Peritonitis incidences were compared using the Poisson regression model. The association between microbiological characteristics (oxacillin resistance, presence of *mec*A gene and production of pathogenic factors) and the frequency of clinical findings at initial presentation (abdominal pain, fever, nausea or vomiting, and arterial hypotension) was analyzed by the chi-square or Fisher's exact test. These tests were also used to compare resolution and death rates between *S. aureus* peritonitis episodes and 117 contemporary CoNS cases. Multivariate analysis by logistic regression was used to test baseline demographic, clinical, and microbiological factors that independently predicted the outcome of a peritonitis episode. Outcome was classified as two mutually exhausted and exclusive results (resolution or non-resolution). For this purpose, univariate analysis using the chi-square or Fisher's exact test (binary variables) or logistic regression (continuous variables) was first performed to select the variables that would enter the final model, with $p > 0.20$ being used as an elimination criterion. A p value less than 0.05 was considered to be significant. All statistical analyses were performed using the SPSS 16.0 software (SPSS®, Inc.).

Author Contributions

PB participated in the design and coordination of the study and in the collection of the clinical data, analyzed the data, and wrote and revised the manuscript. TMCM performed the microbiological tests. CHC contributed to the microbiological tests, data analysis, and writing of the paper. JCTC participated in the collection of the clinical data and contributed to the design of the study. ALM contributed to the microbiological tests. ACM participated in the design of the study and performed the E-test. MLRSC participated in the coordination of the study, supervised the laboratory work, and

contributed to the writing and revision of the paper. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Annual Rate (episodes/patient/year) of *Staphylococcus aureus* Peritonitis from January 1996 to December 2010.

Figure 2. Vancomycin Minimum Inhibitory Concentration (MIC50) and Proportion of Oxacillin-Resistant *Staphylococcus aureus* Strains Isolated from Peritoneal Dialysis Patients with Peritonitis between 1996 and 2010.

Table 1. Summary of Patient Characteristics at Baseline (*n* = 56)

	Frequency	**%**
Age (years)		
≤ 20	4	7.2
21-40	12	21.4
41-60	20	35.7
>60	20	35.7
Male gender	23	41.1
Presence of diabetes	28	50
Educational Level		
Elementary	30	53.6
Secondary	8	14.3
Higher	6	10.7
Illiterate	7	12.5
Unknown	5	8.9
PD modality		
APD	9	16.6
CAPD	47	83.3

PD, peritoneal dialysis; APD, automated peritoneal dialysis; CAPD, continuous ambulatory peritoneal dialysis.

Table 2. Clinical findings in *S. aureus* peritonitis episodes

Sign or symptom	**N**	**%**
Cloudy Dialysis Effluent	60	96.8
Abdominal pain	42	67.7
Nausea or vomiting	26	41.9
Fever	18	29.0
Hypotension	12	19.3

Table 3. Production Rates of Pathogenic Factors by *S. aureus* strains Isolated from 62 Peritonitis Episodes

	N	**%**
Enzymes		
α-Hemolysin	27	43.5
β-Hemolysin	24	38.7
Lipase	52	83.9
Lecithinase	57	91.9
Deoxyribonuclease	58	93.5
Thermonuclease	56	90.3
Toxins		
SEA	7	11.3
SEB	17	27.4
SEC	12	19.4
TSST-1	17	27.4
Biofilm	55	88.7

SEA, SEB, SEC, enterotoxins A, B and C, respectively; TSST-1, toxic shock syndrome toxin-1.

Table 4. Odds Comparison of Peritonitis Resolution by Logistic Regression Analysis

Factor	*p* value (univariate)	*p* value (multivariate)	OR	95% CI
Gender (female)	0.109	0.143	4.551	0.599-34.611
Caucasian race	0.642			
Age (years)	0.042			
≤ 20		0.999	Exp(3.849)	0.000-
21- 40		0.871	0.845	0.111-6.466
41- 60		0.020	0.091	0.012-0.684
> 60 (reference)				
Educational level	0.934			
Elementary				
Secondary				
Higher				
Illiteracy				
Vancomycin use	0.037	0.242	0.325	0.049-2.140
Presence of diabetes	0.042	0.009	14.682	1.960-112.676
Enzyme production				
α-Hemolysin	0.632			
β-Hemolysin	0.077	0.006	16.597	2.246-122.615
Lipase	0.204			
Lecithinase	0.185	0.697	2.248	0.038-131.697
Deoxyribonuclease	0.033	0.999	Exp(6.296)	0.000-
Thermonuclease	0.934			
Presence of *mec*A gene	0.195	0.838	1.430	0.046-44.342
Biofilm production	0.623			
Toxin production				
SEA	0.265			
SEB	0.312			
SEC	0.071	0.217	5.621	0.363-87.052
TSST-1	0.205	0.399	0.365	0.035-3.805
Dialysis vintage (months)	0.07	0.092	0.943	0.880-1.010
Dialysis modality (APD vs CAPD)	0.477			

SEA, SEB, SEC, enterotoxins A, B and C, respectively; TSST-1: toxic shock syndrome toxin-1; APD, automated peritoneal dialysis; CAPD, continuous ambulatory peritoneal dialysis.

Figure 1

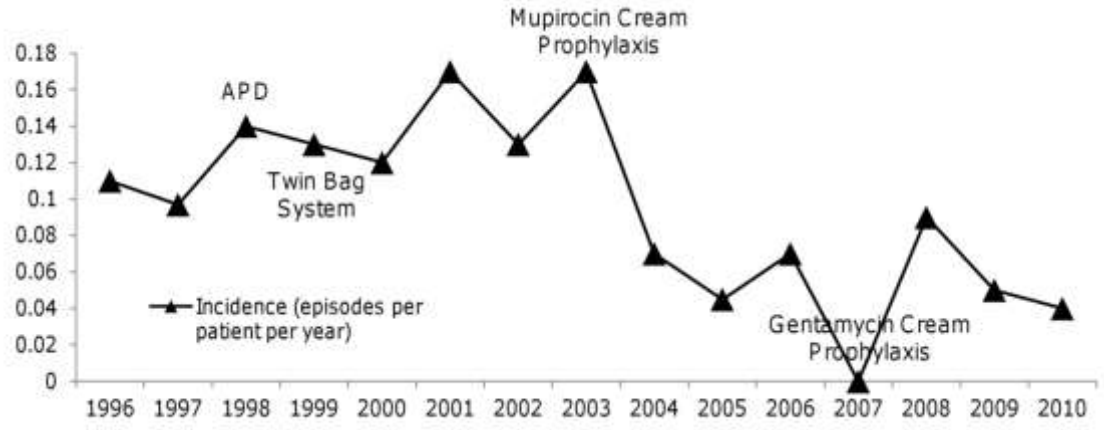
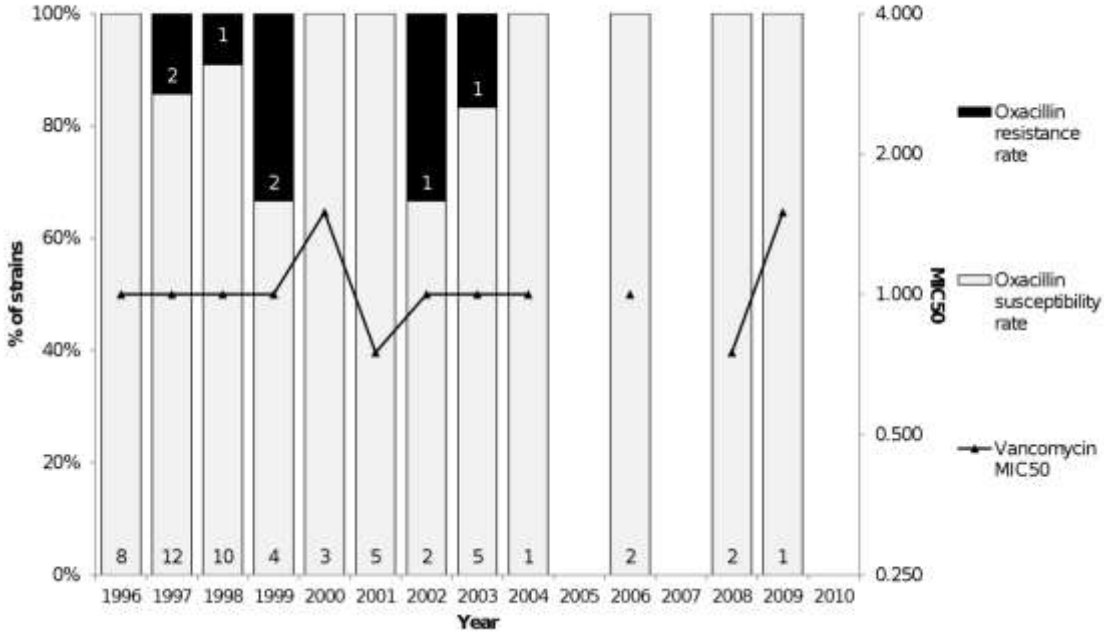


Figure 2



ANEXO 12

CUNHA MLRS, PERESI E, CALSOLARI RA, ARAÚJO JÚNIOR JP. Detection of enterotoxins genes in coagulase-negative staphylococci isolated from foods. Braz J Microbiol. 2006; 37(1): 70-4.

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DETECTION OF ENTEROTOXINS GENES IN COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM FOODS

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ABSTRACT

Staphylococcal food poisoning is caused by ingestion of enterotoxins preformed in the food contaminated essentially through human manipulation or raw material obtained from animals. Although coagulase-positive *Staphylococcus aureus* is the main agent responsible for food intoxication, some researches emphasise that coagulase-negative staphylococci (CNS) are able to produce staphylococcal enterotoxins and may be a potential cause of food poisoning. In the present study CNS were isolated from foods and the toxigenic capacity of the strains determined. A total of 88 food samples were analysed and 22.7% were positive for CNS strains. Staphylococcal counts ranged from 3.0×10^2 to 1.4×10^6 CFU/g or mL of food examined. *S. epidermidis* predominated among the isolates (40%). Further isolates included *S. xylosus* (20%), *S. warneri* (20%), *S. saccharolyticus* (15%), and *S. hominis* (5%). Four isolates were positive for enterotoxin genes, as detected by polymerase chain reaction, with *sea* being the predominant gene. Although no enterotoxin production was detected by the reverse passive latex agglutination method, the data showed that the toxigenic capacity of CNS should not be ignored, requiring investigation of this group of microorganisms in food.

Key words: coagulase-negative staphylococci, PCR, enterotoxins

INTRODUCTION

Staphylococcus aureus enterotoxins are the most frequent causes of food poisoning, with outbreaks caused by mishandling of foods after heat treatment (21,23). The heat destroys the vegetative bacterial microbiota in food, and the non competitive staphylococci, introduced by inadequate handling process, may grow.

Although enterotoxins are produced mainly by coagulase-positive staphylococci, some coagulase-negative staphylococci (CNS), involved in a variety of human and animal infections (11), have also raised interest. Very little is known about the growth of CNS in foods. These strains have rarely been implicated in food poisoning because they do not grow rapidly in foods. Nevertheless CNS can contaminate foods because humans are common carriers of these microorganisms and some may be related to specific human infections (4).

Since immunoassays require a detectable amount of toxin, molecular techniques can complement the assay through detection of staphylococcal enterotoxin genes as important tool in the microbiology laboratory. Taking into account that the toxigenic potential of CNS is often neglected, in the present study enterotoxin genes in CNS strains isolated from foods were analysed by PCR and the results correlated with latex agglutination assay data.

MATERIALS AND METHODS

Food samples

A total of 88 food samples were analysed, including bakery goods (n=38), milk (n=18), snacks (n=11), white cheese (n=10), sandwich (n=8,) and pork meat (n=3), purchased from local markets or delicatessens in Botucatu, SP (Brazil) for a period of twelve months (2003). The interval between the sampling and the analysis was less than one hour.

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Isolation of staphylococci

Twenty-five grams of each sample was suspended in 225 mL peptone water, and 0.1 mL of 10^{-1} to 10^{-6} dilutions was spread on the surface of Baird-Parker agar. The plates were incubated for 48 h at 37ºC and typical colonies (black to dark grey, smooth, convex, well-defined contours, surrounded or no by a dull halo) were counted and the number expressed in CFU/g or mL.

Identification of coagulase-negative staphylococci

Representatives of each colony type were checked by Gram method for purity and morphology, and submitted to catalase and coagulase tests. The genus *Staphylococcus* was differentiated from *Micrococcus* by glucose oxidation and fermentation tests, resistance to bacitracin (0.04 U, absence of an inhibition halo or formation of a halo of up to 9 mm), and sensitivity to furazolidone (100 mg, inhibition halo of 15 to 35 mm) (2).

CNS were identified by simplified biochemical test scheme proposed by Kloos and Schleifer (10) and Kloos and Bannerman (11), which is based on the utilization of xylose, arabinose, sucrose, trehalose, mannitol, maltose, lactose, xylitol, ribose, and fructose, characterization of hemolysis, reduction of nitrate, presence of urease and ornithine decarboxylase, and resistance to novobiocin.

Toxin production

Staphylococcal enterotoxin was produced using the dialysis bag method described by Donnelly *et al.* (8). A 30 to 40 cm dialysis bag (Cut-Off 12,000-16,000 MW, Inlab) was previously washed with distilled water, closed at one end, filled with 50 mL of double-concentrated brain heart infusion broth (BHI), and the other end closed. The bag, bended to a "U" shape, was placed in a 250 mL Erlenmeyer flask and autoclaved for 15 min at 121ºC. The flask was added of 18 mL of 0.02 M phosphate buffer pH 7.4 in 0.9% NaCl, inoculated with staphylococci previously cultured in 5 mL BHI at 37ºC/18 h. After incubation at 37ºC, 200 rpm for 24 h, the culture was centrifuged at 8000 *g*/ 10 min at 4ºC and the supernatants stored at -20ºC and in liquid nitrogen until use.

Detection of enterotoxins

For the detection of toxins by reverse passive latex agglutination (RPLA) method, the supernatants concentrated in dialysis bags (8) were filtered through an 8 µm Millipore membrane to avoid nonspecific reactions. Extracellular enterotoxins were detected by RPLA method as described by Shingaki *et al.* (20) using the SET-RPLA-T900 kit for the detection of SEA, SEB, SEC and SED (Oxoid Diagnostic Reagents). Briefly, microplate wells with a V-shaped bottom were inoculated with 25 µL of the supernatant and 25 µL latex sensitised with anti-enterotoxins. Standard toxins (provided by manufacturer, Oxoid Diagnostic Reagents) were used as positive controls and the occurrence of nonspecific reactions was tested by addition of 25 µL of the supernatant to 25 µL of control latex. The plates were covered with cellophane and homogenised in a micromixer for 3 min. After incubation for 20 to 24 h at environmental temperature, the results were recorded according to the agglutination pattern described by the manufacturer. Positive reactions were classified as (+), (++), and (+++), while formation of a pink bud was interpreted as a negative result.

Detection of enterotoxin genes

DNA extraction

Total DNA was extracted from CNS strains cultured on blood agar, inoculated individually into BHI broth and incubated at 37ºC/ 24h. The GFX kit (Amersham Biosciences) was used for DNA extraction by manufacturer's protocol, which consists of initial digestion of the staphylococcal cells with lysozyme (10 mg/mL) and proteinase K (20 mg/mL). Then 500 µL of the extraction solution was added, and the mixture was centrifuged (5000 *g*/ 1 min). The supernatant was then transferred to a GFX column and centrifuged (5000 *g*/ 1 min). The eluent was discarded, and 500 µL of extraction solution was again added to the column. After centrifugation and disposal of the collected eluent, 500 µL of the wash solution was added to the column and the column was centrifuged at 20800 *g*/ 3 min. The column was then transferred to a 1.5-mL tube and 200 µL Milli-Q water heated at 70ºC was used for elution under centrifugation (5000 *g*/ 1 min).

PCR

PCR was carried out in 0.5-mL microcentrifuge tubes added of 20 pmol of each primer (Table 1), 2.5 U Taq DNA polymerase, 200 µM dNTPs, 20 mM Tris-HCl, pH 8.4, 0.75 mM $MgCl_2$, and 5 µL DNA (total volume of 50 µL). A negative control in which DNA was replaced with water was run in parallel in all reactions. Amplification was performed with an MJ Research PTC-100 thermocycler as described by Johnson *et al.* (9) with some modification, which consisted of a first cycle at 94ºC for 4 min, denaturation at 94ºC for 2 min, annealing at 55ºC for 1 min and 30 sec, and extension at 72ºC for 1 min and 30 sec, followed by a second cycle of denaturation at 94ºC for 2 min, annealing at 53ºC for 1 min and 30 sec, and extension at 72ºC for 1 min and 30 sec. In the third cycle, the annealing temperature was reduced to 51ºC, followed by additional 37 cycles at 94ºC for 2 min, 51ºC for 1 min and 30 sec and 72ºC for 1 min and 30 sec. At the end of the 40 cycles, the tubes were incubated at 72ºC for 7 min and stored at 4ºC.

Analysis of the amplified products

Amplification efficiency was determined on 2% agarose gels in 1X TBE buffer stained with ethidium bromide. The size of the amplified products was compared with 50- and 100-kb standards and the gels were photographed under UV transillumination.

RESULTS

Table 2 shows the distribution of CNS according to species and food analysed, slowing that twenty food samples were positive for CNS species. Of the 20 CNS isolates, 12 were isolated from bakery goods, 2 from milk, 1 from cheese, 2 from sandwiches, and 3 from pork meat.

CNS counts in the foods ranged from 3.0×10^2 to 1.4×10^6 CFU/g or mL, except for snacks, where no staphylococci were isolated (Table 3).

S. epidermidis was the most predominant specie, accounting for 40% of all CNS, followed by *S. xylosus* (20%), *S. warneri* (20%), *S. saccharolyticus* (15%), and *S. hominis* (5%).

The gene specific analysis by PCR for enterotoxin production revealed *sea* gene in three CNS isolates, while *sec*-1 gene was detected in only one isolate (Table 4). Despite potentially toxigenic strains of CNS were detected by molecular tool, the analysis of enterotoxin by RPLA method showed negative results.

The *sea* gene was detected in one *S. epidermidis* isolated from chocolate cream-filled puffs, in one *S. xylosus* isolate from cream-filled puffs and in one *S. hominis* from apple pie. The *sec*-1 gene was detected in only one *S. xylosus* isolated from fruit pie.

DISCUSSION

As shown in Table 2, *S. epidermidis* was the most frequently isolated species (40%). This species is a common inhabitant of human skin and mucous membranes of individuals manipulating food and animals, and is able to contaminate raw products and processed foods (4).

Both *S. warneri* and *S. xylosus* were the second most frequent species in our study (20%). In a study carried out by Udo *et al.* (24) with restaurant workers, the prevalent CNS species on the hands of food handlers were *S. hominis* (23.6%), *S. warneri* (20.6%) and *S. epidermidis* (3.4%). Since human nares and fingers are the main sources of *S. aureus* (16,22), and because CNS inhabit the human skin and mucous membranes, these microorganisms can contaminate food if these are not handled properly. Therefore, enterotoxigenic CNS strains may contribute to staphylococcal food poisoning (4).

In the present study, staphylococcal enterotoxin genes were detected in one *S. epidermidis* isolate, two *S. xylosus* isolates and one *S. hominis* isolate (Table 4).

Valle *et al.* (25) determined the enterotoxigenic character of staphylococci isolated from the skin, nasal mucosa and milk of

Table 1. Primers used for the detection of staphylococcal enterotoxin genes.

Primers	5' to 3' nucleotide sequence	Target	Amplicon size (bp)
sea1	TTG GAA ACG GTT AAA ACG AA	Enterotoxin A	120
sea2	GAA CCT TCC CAT CAA AAA CA	Enterotoxin A	
seb1	TCG CAT CAA ACT GAC AAA CG	Enterotoxin B	478
seb2	GCA GGT ACT CTA TAA GTG CC	Enterotoxin B	
sec1	GAC ATA AAA GCT AGG AAT TT	Enterotoxin C	257
sec2	AAA TCG GAT TAA CAT TAT CC	Enterotoxin C	
sed1	CTA GTT TGG TAA TAT CTC CT	Enterotoxin D	317
sed2	TAA TGC TAT ATC TTA TAG GG	Enterotoxin D	

Source: Johnson *et al.*, 1991 (9).

Table 2. Frequency of coagulase-negative staphylococci (CNS) isolated from foods.

Species	Isolates n=20	Bakery goods n=38	Snack n=11	Milk n=18	White cheese n=10	Sandwich n=8	Pork meat n=3
S. epidermidis	8(40.0)	4(20.0)	-	2(10.0)	-	2(10.0)	-
S. xylosus	4(20.0)	4(20.0)	-	-	-	-	-
S. hominis	1 (5.0)	1(5.0)	-	-	-	-	-
S. warneri	4(20.0)	3(15.0)	-	-	1(5.0)	-	-
S. saccharolyticus	3(15.0)	-	-	-	-	-	3(15.0)
Total	20(100.0)	12(60.0)	-	2(10.0)	1(5.0)	2(10.0)	3(15.0)

N= Number of isolates CNS of the 88 samples analysed; n = Total number of samples analysed.

Table 3. Number of coagulase-negative staphylococci (CNS) in foods.

Food	Isolates N (%)	Species	Average Count CFU/g or mL
Bakery goods (n=38)	4 (20.0)	*S. epidermidis*	1.3×10^1
	4 (20.0)	*S. xylosus*	6.0×10^4
	1 (5.0)	*S. hominis*	5.5×10^4
	3 (15.0)	*S. warneri*	3.2×10^4
Milk (n=18)	2 (10.0)	*S. epidermidis*	3.2×10^4
White cheese (n=10)	1 (5.0)	*S. warneri*	1.4×10^6
Sandwich (n=8)	2 (10.0)	*S. epidermidis*	7.2×10^4
Pork meat (n=3)	3 (15.0)	*S. saccharolyticus*	3.0×10^2

n = Total number of samples analysed.

Table 4. Detection of toxin genes by PCR in CNS species isolated from foods.

Species	*sea*	*seb*	*sec*-1	*sed*
S. epidermidis	1	-	-	-
S. xylosus	1	-	-	-
S. xylosus	-	-	1	-
S. hominis	1	-	-	-
S. warneri	-	-	-	-
S. saccharolyticus	-	-	-	-
Total	3	-	1	-

sea, *seb*, *sec*-1, and *sed*: enterotoxins genes A, B, C and D, respectively.

133 healthy goats. Twenty-two percent of CNS strains produced enterotoxins, including *S. caprae*, *S. xylosus*, *S. warneri* and *S. epidermidis*.

Analysis of the toxigenic profile of CNS isolated in our study by PCR revealed the presence of the *sea* gene in one *S. xylosus* isolate, one *S. hominis* isolate and one *S. epidermidis* isolate (Table 4). It has been widely accepted that most staphylococcal food intoxications are caused by the ingestion of enterotoxin A (17,18,26). The presence of the *sec*-1 gene was observed in one *S. xylosus* isolate. All theses toxigenic CNS were isolated from bakery goods. Bakery goods covered and filled with cream are leading causes of food poisoning worldwide (1,5,7).

The enterotoxigenicity of CNS has been described by several investigators (6,14,25), and questioned by others (3), but few studies have been conducted to determine the enterotoxigenic capacity of CNS in foods. In this study, a small number of CNS harboured enterotoxin genes but, their detection was important because confirms that CNS isolated from foods can produce enterotoxins. These results confirm the findings reported by Crass and Bergdoll (6) who isolated enterotoxin- producing CNS from food implicated in a food poisoning outbreak.

Comparing the PCR and RPLA data, the isolates were shown to be PCR-positive for enterotoxin gene did not evidence detectable production of enterotoxin by RPLA method. Similar findings have been reported by others (9,19) and might be due to toxin production below the detection limit of the RPLA method or to the non-expression of genes. According to Schmitz *et al.* (19), in clinical practice, staphylococcal strains carrying a toxin gene should be considered potential producers of this toxin since toxin production *in vivo* cannot be excluded. Many factors affect *S. aureus* growth and enterotoxin production in foodstuffs, such as the presence of specific amino acids that vary with the strains (15), glucose, pH, acetic acid, lactic acid, sodium chloride and competing microorganisms (12). Thus considerable research effort is still required for better understanding of the interactions between CNS and the food matrix, and the mechanisms of enterotoxin production in foodstuffs.

PCR was found to be a rapid and reliable method for the identification of genes responsible for the production of staphylococcal toxins. The determination of the enterotoxigenicity of strains is mainly based on immunological methods, which depend on the concentration of the toxin produced and, therefore, might not detect low-producing strains.

The importance of our results lies not only in scientific aspects considering the scarcity of available data in this respect, but in providing practical information about food safety, which is of great importance since some CNS species, such as *S. xylosus*, are used as a starter culture in fermented meat products (13). In addition, the present results confirm the need for further studies in order to better characterize the enterotoxigenic potential of CNS in foods.

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RESUMO

Detecção de genes de enterotoxinas em estafilococos coagulase-negativa isolados de alimentos

A intoxicação alimentar estafilocócica ocorre devido à ingestão de alimentos contaminados com enterotoxinas. Essa contaminação tem sido oriunda, principalmente, da manipulação humana, ou de matérias-primas procedentes de animais portadores. Embora *Staphylococcus aureus* coagulase positiva, seja o principal agente de intoxicação alimentar, alguns

pesquisadores enfatizam que os estafilococos coagulase-negativa (ECN) podem produzir as enterotoxinas estafilocócicas, podendo contribuir para a intoxicação alimentar. Este estudo teve como objetivos isolar os ECN de alimentos e verificar a capacidade enterotoxigênica dessas linhagens. Foram estudadas 88 amostras de alimentos, sendo que 22,7% foram positivas para ECN com crescimento entre 10^2 e 10^6 UFC/g or mL. A espécie predominante dentre as linhagens isoladas foi *S. epidermidis* (40%), seguido por *S. warneri* (20%), *S. xylosus* (20%), *S. saccharolyticus* (15%) e *S. hominis* (5%). Entre as linhagens isoladas, quatro apresentaram genes para produção de enterotoxinas pelo método de Reação da Polimerase em Cadeia (PCR), com predominância do gene *sea*. Não se detectou a produção de enterotoxina pelo método de aglutinação em látex (RPLA). Através dos resultados obtidos, observou-se que os ECN isolados de alimentos não devem ser ignorados quanto à sua capacidade toxigênica, necessitando de maior estudo e atenção para melhor caracterização desse grupo de microrganismos em alimentos.

Palavras-chave: estafilococos coagulase-negativa, PCR, enterotoxinas

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ANEXO 13

CUNHA MLRS, CALSOLARI RA, ARAÚJO JÚNIOR JP. Detection of enterotoxin and toxic shock syndrome toxin 1 genes in *Staphylococcus*, with emphasis on coagulase-negative staphylococci. Microbiol Immunol. 2007; 51: 381-90.

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Detection of Enterotoxin and Toxic Shock Syndrome Toxin 1 Genes in *Staphylococcus*, with Emphasis on Coagulase-Negative Staphylococci

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***Abstract*: The detection of staphylococcal enterotoxins is decisive for the confirmation of an outbreak and for the determination of the enterotoxigenicity of strains. Since the recognition of their antigenicity, a large number of serological methods for the detection of enterotoxins in food and culture media have been proposed. Since immunological methods require detectable amounts of toxin, molecular biology techniques represent important tools in the microbiology laboratory. In the present study, polymerase chain reaction (PCR) was used to identify genes responsible for the production of enterotoxins and toxic shock syndrome toxin 1 (TSST-1) in *S. aureus* and coagulase-negative staphylococci (CNS) isolated from patients and the results were compared with those obtained by the reverse passive latex agglutination (RPLA) assay. PCR detection of toxin genes revealed a higher percentage of toxigenic *S. aureus* strains (46.7%) than the RPLA method (38.3%). Analysis of the toxigenic profile of CNS strains showed that 26.7% of the isolates produced some type of toxin, and one or more toxin-specific genes were detected in 40% of the isolates. These results suggests the need for further studies in order to better characterize the pathogenic potential of CNS and indicate that attention should be paid to the toxigenic capacity of this group of microorganisms.**

***Key words*: PCR, Coagulase-negative staphylococci, Enterotoxins, TSST-1**

Within the genus *Staphylococcus*, *S. aureus*, a coagulase-positive species which produces a series of other enzymes and toxins, is the most widely known and has frequently been implicated in the etiology of a series of infections and intoxications in man and animals, while coagulase-negative staphylococci (CNS) have been considered to be saprophytic or rarely pathogenic (21).

Before the 1970s, reports of infections caused by CNS were rare, with these bacteria being recognized by clinicians and microbiologists as contaminants of clinical samples (22). However, this detection, widely used for clinical diagnosis, has posed a challenge regarding the role that these microorganisms play in infectious processes.

CNS are currently known to be essentially opportunistic microorganisms that take advantage of numerous organic situations to produce severe infections (22–26). The emergence of CNS as etiological agents might also be the result of the recognition of this group as opportunistic pathogens and of the increasing use of invasive procedures such as intravascular catheters and prostheses in immunocompromised patients, and in intensive treatment, premature children, patients with neoplasia and transplant patients (22).

Several virulence factors are responsible for the symptoms and severity of infections caused by *S. aureus*. These factors include hemolysins α, β, γ and δ, leukocidin, exfoliative toxins A and B, and a group of

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Abbreviations: ATCC, American Type Culture Collection; BHI, brain heart infusion; CNS, coagulase-negative staphylococci; ELISA, enzyme linked immuno sorbent assay; IL-1, interleukin 1; IL-2, interleukin 2; PCR, polymerase chain reaction; RPLA, reverse passive latex agglutination; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC_1, staphylococcal enterotoxin C_1; SEC_2, staphylococcal enterotoxin C_2; SEC_3, staphylococcal enterotoxin C_3; SED, staphylococcal enterotoxin D; SEE, staphylococcal enterotoxin E; SEG, staphylococcal enterotoxin G; SEH, staphylococcal enterotoxin H; SEI, staphylococcal enterotoxin I; SEJ, staphylococcal enterotoxin J; SEK, staphylococcal enterotoxin K; SEL, staphylococcal enterotoxin L; SEM, staphylococcal enterotoxin M; TSST-1, toxic shock syndrome toxin 1.

pyrogenic toxic superantigens consisting of toxic shock syndrome toxin 1 (TSST-1) and staphylococcal enterotoxins that cause staphylococcal food poisoning (24). Due to their superantigen nature, enterotoxins and TSST-1 directly bind to the major histocompatibility complex class II molecule without undergoing the typical processing of normal antigens, a fact resulting in increased T cell stimulation and, consequently, excessive production of cytokines such as interleukin 1 (IL-1), IL-2, interferon gamma and tumor necrosis factor alpha (29). Current evidence indicates that the physiological effects of neonatal sepsis are mediated by cytokines activated in response to the presence of bacterial components inside the cell (20).

Staphylococcal enterotoxins are water-soluble exoproteins with a molecular weight ranging from 26 to 29 kDa and are characterized by a disulfide loop close to the center of the molecule (5, 16). The purification and characterization of staphylococcal enterotoxins started in 1959 (3), and today 14 serologically distinct enterotoxins designated by different letters are known: SEA, SEB, SEC_1, SEC_2, SEC_3, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, and SEM (1, 3, 4, 8–10, 17, 30, 33, 39, 40).

Although little attention has been paid to the toxigenic profile of CNS, some investigators have emphasized that these microorganisms can produce TSST-1 alone or in combination with a staphylococcal enterotoxin, and therefore their clinical importance and toxigenic capacity cannot be ignored (11, 19, 37).

The detection of staphylococcal enterotoxins is decisive for the confirmation of an outbreak and for the determination of the enterotoxigenicity of strains (6). A large number of serological methods for the detection of enterotoxins in food and culture media have been proposed (6). However, the immunological methods used for toxin detection are time consuming and do not always identify toxin-producing strains because toxin production depends on the growth conditions which differ from *in vivo* conditions (17). In addition, immunological methods can yield false-positive results because of cross-reactions between antigens and the occurrence of nonspecific reactions (27, 28, 38).

Since detectable amounts of toxin are required when immunological methods are used, the development of molecular biology techniques for the detection of staphylococcal enterotoxins represent an important tool in the microbiology laboratory. Based on these aspects and taking into account that the pathogenic potential of CNS is often neglected, the objective of the present study was to identify enterotoxin and TSST-1 genes by PCR in strains of CNS and *S. aureus* isolated from patients and to compare the results with those of a latex agglutination assay.

Materials and Methods

Isolates. A total of 240 staphylococcal strains, including 120 CNS and 120 *S. aureus* strains, isolated from clinical samples obtained from newborns hospitalized at the Neonatal Unit, University Hospital, Botucatu Medical School, SP, Brazil were studied.

The following toxigenic *S. aureus* international reference strains were used as positive control: SEA-producing *S. aureus* ATCC 13565, SEB-producing *S. aureus* ATCC 14458, SEC-producing *S. aureus* ATCC 19095, and SED-producing *S. aureus* ATCC 23235.

Identification of S. aureus *and coagulase-negative staphylococci.* The isolates obtained from clinical specimens were seeded onto blood agar and Gram-stained in order to confirm their purity and to determine their morphology and specific color. After confirmation of these characteristics, the isolates were submitted to catalase and coagulase tests. The genus *Staphylococcus* was differentiated from *Micrococcus* species on the basis of oxidation and glucose fermentation tests, resistance/susceptibility to bacitracin (0.04 U) indicated by the absence or presence of an up to 9 mm inhibition halo, and susceptibility to furazolidone (100 μg) characterized by inhibition halos measuring 15 to 35 mm in diameter (2).

CNS were identified according to the criteria proposed by Kloos and Schleifer (21) and Kloos and Bannerman (23) using a simple biochemical test scheme based on the utilization of the sugars xylose, arabinose, sucrose, trehalose, mannitol, maltose, lactose, xylitol, ribose and fructose, a coagulase test, hemolysin characterization, nitrate reduction, the presence of urease and ornithine decarboxylase, and susceptibility to novobiocin.

The following CNS international reference strains were used as control: *S. epidermidis* (ATCC 12228), *S. simulans* (ATCC 27851), *S. warneri* (ATCC 10209), and *S. xylosus* (ATCC 29979).

After species identification, the isolates were stored in liquid nitrogen.

Toxin production. For the detection of toxins by the reverse passive latex agglutination (RPLA) method, supernatants were concentrated using dialysis bags (12). For this purpose, 30 to 40 cm dialysis bags were previously washed with distilled water, tied at one end, then filled with 50 ml double-concentrated brain heart infusion (BHI) broth and tied at the other end. The bags thus prepared were placed in 250 ml Erlenmeyer flasks in such a way as to form a "U" at the bottom of the flask. The flasks were autoclaved for 15 min at 121

C, and volumes of 18 ml 0.02 M phosphate buffer, pH 7.4, in 0.9% NaCl were inoculated with a loopful of staphylococcal samples previously cultured in 5 ml BHI at 37 C for 18 hr. These mixtures were then transferred to the Erlenmeyer flasks containing the bags and the flasks were incubated at 37 C for 24 hr under shaking at 200 rpm. After incubation, the cultures were centrifuged at 8,000×*g* at 4 C for 10 min. The supernatants were stored at −20 C and in liquid nitrogen until use.

Detection of enterotoxins and TSST-1. Extracellular enterotoxins and TSST-1 were identified by the RPLA method as described by Shingaki et al. (35) using the SET-RPLA-T900 kit for the detection of SEA, SEB, SEC and SED, and the TST-RPLA-TD940 kit (both from Oxoid Diagnostic Reagents) for the detection of TSST-1. Briefly, microplate wells with a V-shaped bottom were inoculated with 25 µl of the supernatant obtained in the above section and filtered through an 8 µm Millipore membrane to prevent the occurrence of nonspecific reactions. Then, 25 µl latex sensitized with anti-enterotoxins and anti-TSST-1 was added. Standard toxins were used as positive controls and the occurrence of nonspecific reactions was tested by the addition of 25 µl of the supernatant to 25 µl of control latex. The plates were covered with cellophane and the reagents homogenized in a micromixer for 3 min. After incubation for 20 to 24 hr at ambient temperature, the results were recorded using a magnifying glass and illumination above a dark bottom. A positive reaction was classified as (+), (++), or (+++) according to the agglutination pattern described by the manufacturer. The formation of a pink bud was interpreted as a negative result.

Detection of enterotoxin and TSST-1 genes. Standardization of the DNA extraction procedure: Total DNA was extracted from *Staphylococcus* strains cultured on blood agar, individually inoculated into BHI broth and incubated at 37 C for 24 hr. In order to standardize a simple and rapid DNA extraction method that shows good reproducibility, extraction kits instead of the classical phenol-chloroform extraction procedure were used. Two commercially available DNA extraction kits were tested: DNAzol (Invitrogen) and GFX (Amersham Biosciences). DNAzol extraction consisted of the centrifugation of 200 µl culture at 1,000×*g* for 1 min and resuspending of the pellet in 500 µl DNAzol. The mixture was centrifuged at 4,000×*g* for 2 min at 4 C and the DNA was precipitated with 250 µl 100% ethanol. The DNA was then washed once with 95% ethanol, dried at 66 C and resuspended in 200 µl 8 mM NaOH, and the pH was neutralized with 1 M HEPES.

In parallel, the DNAzol extraction procedure was modified to eliminate interferences. The optimized extraction method consisted of the addition of 10 mg/ml lysozyme and 20 mg/ml proteinase K to the staphylococcal culture pellet before performing the extraction described above.

In the case of the GFX extraction kit, staphylococcal cells were also first digested with 10 mg/ml lysozyme and 20 mg/ml proteinase K. Then, 500 µl of the extraction solution was added and the mixture was centrifuged at 10,000×*g* for 4 min. The supernatant was transferred to the GFX column and centrifuged at 5,000 ×*g* for 1 min. The collected eluent was discarded and 500 µl of the extraction solution was again added to the column. After centrifugation and discarding of the collected eluent, 500 µl of the wash solution was added to the column and the column was centrifuged at 20,000×*g* for 3 min. The column was then transferred to a 1.5 ml tube and 200 µl Milli Q water heated to 70 C was used for elution.

Absorbance at 260 and 280 nm was measured in a 1:100 dilution of the extracted DNA to determine the concentration and purity of the sample according to the following formula: concentration (µg/ml)=A_{260}× 50×dilution factor.

Standardization of PCR: PCR was carried out in 0.5 ml microcentrifuge tubes in a total volume of 50 µl containing 20 pmol of each primer (Table 1), 2.5 U *Taq* DNA polymerase, 200 µM dNTPs, 20 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, and 5 µl DNA. A negative control in which DNA was replaced with water was run in parallel in all reactions. Amplification was performed in a PTC-100 thermocycler (MJ Research) initially using the parameters described by Johnson et al. (18): one cycle at 94 C for 4 min, followed by 30 cycles of denaturation at 94 C for 2 min, annealing of the primers at 55 C for 2 min and extension at 72 C for 2 min. At the end of the 30 cycles, the tubes were incubated at 72 C for 7 min and stored frozen at 4 C.

Subsequently, other parameters were tested in order to improve the amplification reactions, which consisted of a first cycle at 94 C for 4 min, denaturation at 94 C for 2 min, annealing at 55 C for 1 min and 30 sec and extension at 72 C for 1 min and 30 sec, followed by a second cycle of denaturation at 94 C for 2 min, annealing at 53 C for 1 min and 30 sec and extension at 72 C for 1 min and 30 sec. In the third cycle, the annealing temperature was reduced to 51 C, followed by additional 37 cycles at 94 C for 2 min, 51 C for 1 min and 30 sec and 72 C for 1 min and 30 sec. At the end of the 40 cycles, the tubes were incubated at 72 C for 7 min and stored at 4 C.

Visualization of the amplified products: Amplification efficiency was determined on 2% agarose gels in

Table 1. Primers for the detection of staphylococcal enterotoxin genes

Gene	5' to 3' nucleotide sequence	Target
sea1	TTG GAA ACG GTT AAA ACG AA	Enterotoxin A
sea2	GAA CCT TCC CAT CAA AAA CA	Enterotoxin A
seb1	TCG CAT CAA ACT GAC AAA CG	Enterotoxin B
seb2	GCA GGT ACT CTA TAA GTG CC	Enterotoxin B
sec1	GAC ATA AAA GCT AGG AAT TT	Enterotoxin C
sec2	AAA TCG GAT TAA CAT TAT CC	Enterotoxin C
sed1	CTA GTT TGG TAA TAT CTC CT	Enterotoxin D
sed2	TAA TGC TAT ATC TTA TAG GG	Enterotoxin D
tst1	ATG GCA GCA TCA GCT TGA TA	Toxic shock syndrome toxin 1
tst2	TTT CCA ATA ACC ACC CGT TT	Toxic shock syndrome toxin 1

Source: Johnson et al. (18).

‹ TBE buffer stained with ethidium bromide. The ›e of the amplified products was compared with 50 d 100 bp standards and the gels were photographed der UV transillumination.

Statistical analysis. The results were compared by › chi-square test for independent samples or by the sher exact test when less than 20 isolates were charac-›ized. The level of significance was set at $P<0.05$ for tests.

Results

Isolates

Among the 120 CNS strains originating from 111 newborns, 64 CNS were isolated from blood cultures, 41 from foreign bodies (30 from catheter tips, 10 from cannula tips, and one from the tip of a chest drain), and 15 from secretions. Of the 120 *S. aureus* strains originating from 107 newborns, 25 were isolated from blood cultures, 44 from foreign bodies (25 from catheter tips, 16 from cannula tips, and three from the tip of chest drains), and 51 from secretions.

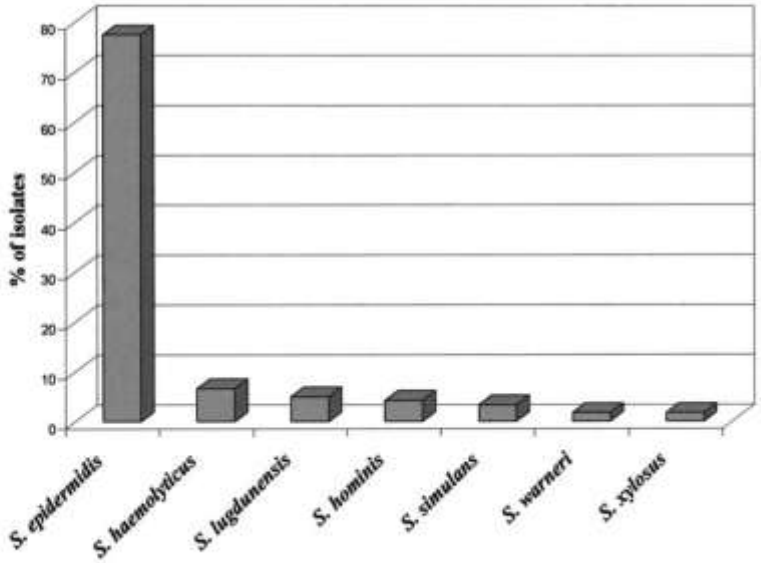

Fig. 1. Distribution of coagulase-negative staphylococci species isolated from newborns.

Identification of Coagulase-Negative Staphylococci

Figure 1 shows the distribution of the isolated CNS samples according to species. *S. epidermidis* was the most frequently isolated species, accounting for 77.5% of the population studied. The remaining isolates were distributed among *S. haemolyticus* (6.6%), *S. lugdunensis* (5.0%), *S. hominis* (4.2%), *S. simulans* (3.3%), *S. warneri* (1.7%), and *S. xylosus* (1.7%).

Optimization of DNA Extraction and PCR Conditions

DNA extraction. A better amplification efficiency was observed when the DNA was extracted with the GFX (Amersham Biosciences) kit and the DNAzol (Invitrogen) kit preceded by cell lysis with lysozyme and proteinase K.

Although the DNAzol kit yielded good results, it is possible that DNA is lost during aspiration of the supernatants, which does not occur when using the GFX columns. In view of these aspects and because extraction with the GFX kit is easy and rapid and shows good reproducibility, we used this procedure for extracting DNA from the staphylococcal isolates studied.

PCR. Amplification was initially performed using the parameters described by Johnson et al. (18) (Fig. 2, A). However, better PCR results (Fig. 2, B) were obtained with the modified protocol described in "Standardization of PCR," which was thus used in the present study to increase the efficiency of detection.

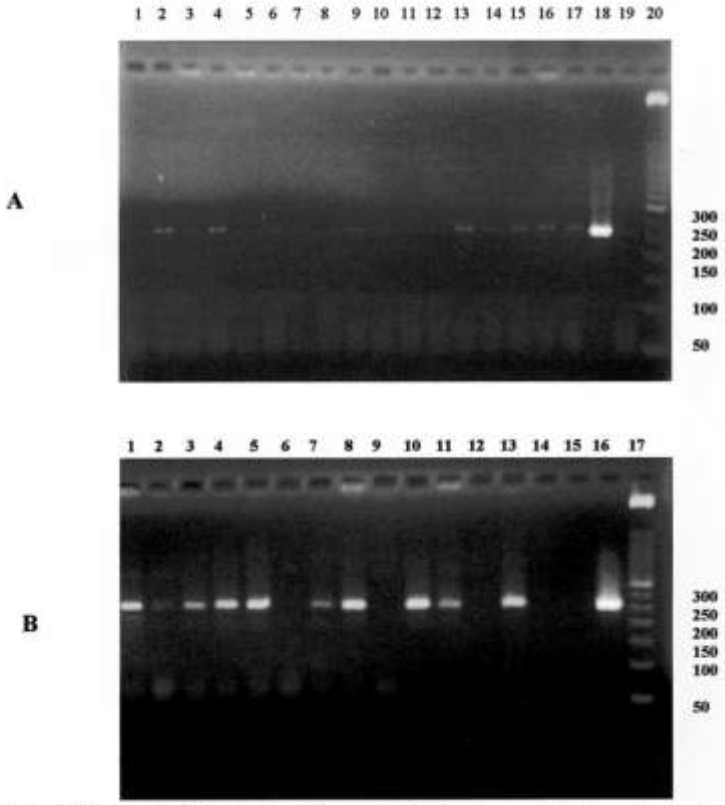

Fig. 2. The PCR products of the enterotoxin C genes in CNS isolates, amplified according to the parameters by Johnson et al. (18) (A) and by our parameters described in "Materials and Methods" (B). (A) Lane 5: CNS isolate negative for *sec1*; lanes 1–4 and 6–17: CNS isolates positive for the *sec1* gene; lane 18: *S. aureus* ATCC 19095 (*sec1*); lane 19: negative control; lane 20: 50-bp molecular weight marker. (B) Lanes 6, 9, 12, and 14: CNS isolates negative for *sec1*; lanes 1, 2, 3, 4, 5, 7, 8, 10, 11, and 13: CNS isolates positive for the *sec1* gene; lane 15: negative control; lane 16: *S. aureus* ATCC 19095 (*sec1*); lane 17: 50 bp molecular weight marker.

Toxigenicity of the Staphylococcal Isolates

Table 2 and Fig. 3 show the distribution of *S. aureus* and CNS samples according to toxin production and presence of toxin genes. Of the 120 *S. aureus* isolates, 46 (38.3%) produced SEA, SEB, SEC, SED or TSST-1 alone or in combination and the toxin-specific genes were detected in 56 (46.7%), compared to 32 (26.7%) and 48 (40.0%) CNS samples, respectively.

The distribution of toxigenic *S. aureus* strains according to clinical material (blood, foreign body and secretion) did not differ significantly at the 5% level, while a significant difference was observed among CNS strains, with a higher percentage of toxin-producing strains being observed in samples isolated from blood cultures. On the other hand, detection of toxigenic genes did not differ between isolates from blood and foreign bodies, but showed a significant differences between samples isolated from blood culture and secretions.

Most of the isolates that produced toxins detected by the RPLA assay were also positive for the detection of the corresponding genes by PCR. However, two CNS isolates that tested positive by RPLA for the production of TSST-1 and one strain that tested positive for the production of SEB+SEC were negative for the genes by PCR. With respect to *S. aureus*, only the *sea* gene

Table 2. Distribution of staphylococci isolated from newborns according to toxigenicity of the strains, detection method and clinical material

	S. aureus								CNS							
	Blood N=25		Foreign body N=44		Secretion N=51		Total N=120		Blood N=64		Foreign body N=41		Secretion N=15		Total N=120	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
RPLA*	7[a]	28.0	15[a]	34.1	24[a]	47.0	46	38.3	27[a]	42.2	3[b]	7.3	2[b]	13.3	32	26.7
PCR**	8[a]	32.0	20[a]	45.4	28[a]	54.9	56	46.7	32[a]	50.0	14[a]	34.1	2[b]	13.3	48	40.0

* Detection of toxin production by the agglutination assay.

** Detection of toxin genes by PCR.

[a,b] Values followed by different letters indicate a statistically significant difference ($P < 0.05$).

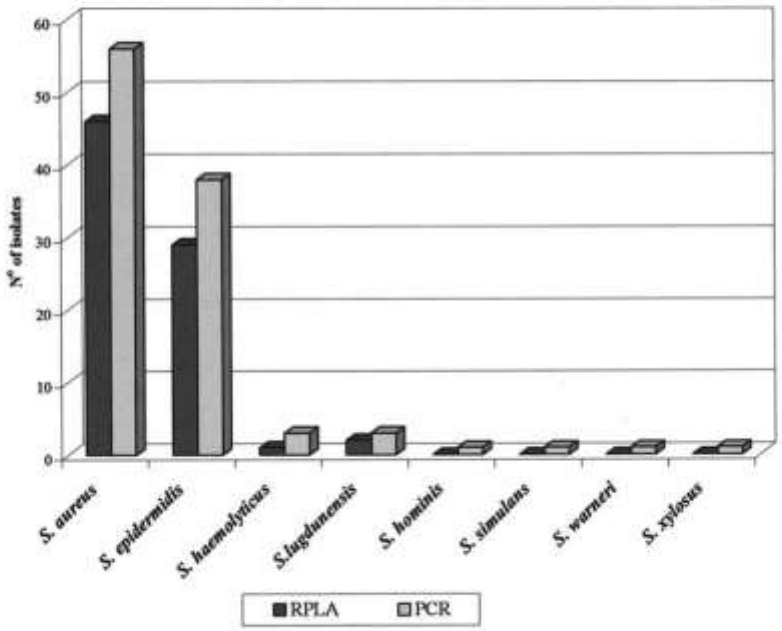

Fig. 3. Detection of toxin production and toxin genes in coagulase-negative staphylococci isolates.

was detected in one SEA+SEC producing isolate, while in one SEA+SEB producing isolate only the *seb* gene was identified (Table 3).

Similar results were obtained for the various CNS species studied, with a higher positivity being observed for the detection of the corresponding genes by PCR compared to the determination of enterotoxin production by the RPLA method (Table 4). Of the 93 *S. epidermidis* strains, 29 (31.2%) produced toxins and genes for one, two or more toxins were identified in 38 (40.9%).

Analysis of the distribution of the CNS samples according to species and clinical material revealed a significantly higher frequency of isolation of toxigenic *S. epidermidis* from blood cultures than from foreign bodies and secretions, while no difference was observed for the other species.

Toxin production and toxin genes were identified in *S. haemolyticus* and *S. lugdunensis* (Table 5). The *sea* gene was detected in only one of the five *S. hominis* strains and in one of the two *S. warneri* isolates (Table

Table 3. Distribution of staphylococci isolated from newborns accorging to toxigenicity of the strains and detection method

Toxin	Toxin production RPLA		Toxin genes PCR	
	S. aureus	CNS	*S. aureus*	CNS
SEA	8	0	9	7
SEB	15[a]	0[b]	17[a]	1[b]
SEC	7[b]	18[a]	15[b]	26[a]
SED	1	0	2	0
TSST-1	6[a]	7[a]	4[a]	5[a]
SEA+SEB[1]	5	0	4	0
SEA+SEC[2]	2	0	1	2
SEA+TSST-1[3]	0	0	1	1
SEB+SEC[4]	0	1	0	0
SEC+TSST-1[5]	2	5	3	5
SEA+SEC + TSST-1[6]	0	1	0	1
Total	46	32	56	48

SEA, SEB, SEC, and SED: enterotoxins A, B, C and D, respectively. TSST-1: toxic shock syndrome toxin 1. [1] Concomitant production of enterotoxins A and B. [2] Concomitant production of enterotoxins A and C. [3] Concomitant production of enterotoxin A and TSST-1. [4] Concomitant production of enterotoxins B and C. [5] Concomitant production of enterotoxin C and TSST-1. [6] Concomitant production of enterotoxins A , C and TSST-1. [a, b] Values followed by different letters indicate a statistically significant difference ($P<0.05$).

Table 4. Distribution of coagulase-negative staphylococci isolated from newborns according to toxigenicity of the strains, detection method and clinical material

Species	No. of isolates	RPLA* No. of isolates / No. total				PCR** No. of isolates / No. total			
		Blood	Foreign body	Secretion	Total *N* (%)	Blood	Foreign body	Secretion	Total *N*(%)
S. epidermidis	93	25/50[a]	3/32[b]	1/11[b]	29(31.2)	28/50[a]	9/32[b]	1/11[b]	38(40.9)
S. haemolyticus	8	1/6	0/2	0/0	1(12.5)	2/6	1/2	0/0	3 (37.5)
S. lugdunensis	6	1/3	0/1	1/2	2/6	2/3	0/1	1/2	3/6
S. hominis	5	0/1	0/3	0/1	0/5	0/1	1/3	0/1	1/5
S. simulans	4	0/2	0/1	0/1	0/4	0/2	1/1	0/1	1/4
S. warneri	2	0/1	0/1	0/0	0/2	0/1	1/1	0/0	1/2
S. xylosus	2	0/1	0/1	0/0	0/2	0/1	1/1	0/0	1/2
Total	120	27/64	3/41	2/15	32(26.7)	32/64	14/41	2/15	48(40.0)

* Detection of toxin production by the latex agglutination assay. ** Detection of toxin genes by PCR. [a, b] Values followed by different letters indicate a statistically significant difference ($P<0.05$).

Table 5. Frequency of toxigenic coagulase-negative staphylococci (CNS) isolates according to species and detection method

Toxin	CNS species													
	S. epidermidis N=93		*S. haemolyticus* N=8		*S. lugdunensis* N=6		*S. hominis* N=5		*S. simulans* N=4		*S. warneri* N=2		*S. xylosus* N=2	
	RPLA*	PCR**	RPLA	PCR	RPLA	PCR	RPLA	PCR	RPLA	PCR	RPLA	PCR	RPLA	PCR
SEA	0	4	0	1	0	0	0	1	0	0	0	1	0	0
SEB	0	1	0	0	0	0	0	0	0	0	0	0	0	0
SEC	15	20	1	2	2	3	0	0	0	1	0	0	0	0
SED	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TSST-1	7	5	0	0	0	0	0	0	0	0	0	0	0	0
SEA+SEB[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SEA+SEC[2]	0	2	0	0	0	0	0	0	0	0	0	0	0	0
SEA+TSST-1[3]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SEB+SEC[4]	1	0	0	0	0	0	0	0	0	0	0	0	0	0
SEC+TSST-1[5]	5	5	0	0	0	0	0	0	0	0	0	0	0	0
SEA+SEC+TSST-1[6]	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Total	29	38	1	3	2	3	0	1	0	1	0	1	0	1

* Detection of toxin production by the latex agglutination assay. ** Detection of toxin genes by PCR. SEA, SEB, SEC, and SED: enterotoxins A, B, C and D, respectively. TSST-1: toxic shock syndrome toxin 1. [1] Concomitant production of enterotoxins A and B. [2] Concomitant production of enterotoxins A and C. [3] Concomitant production of enterotoxin A and TSST-1. [4] Concomitant production of enterotoxins B and C. [5] Concomitant production of enterotoxin C and TSST-1. [6] Concomitant production of enterotoxins A , C and TSST-1.

5). The *sec1* gene was identified in one of the four *S. simulans* isolates, while one of the two *S. xylosus* strains presented a combination of the *sea+tst* genes (Table 5). Comparison of the CNS species regarding their capacity to produce detectable amounts of toxin revealed a significant difference between *S. epidermidis* (31.2%) and the other species (11.1%). However, no difference in the presence of toxin genes was observed between species.

Furthermore, no difference in toxin production and the presence of the corresponding genes was observed between *S. aureus* and *S. epidermidis*, or between *S. aureus* and the CNS isolates as a whole.

Comparison of the type of toxin produced by CNS and *S. aureus* revealed a higher frequency of the *sec1* gene among CNS isolates and of the *seb* gene in *S. aureus*, as well as a higher frequency of production of these specific toxins.

Discussion

The ability of CNS to cause infections has been well documented (13); however in many cases CNS isolated from laboratory cultures are still considered to be contaminants of little clinical importance.

The progress made in the classification of staphylococci and in the development of detection methods does not only permit the clinician to come in contact with a variety of species present in clinical samples but also to support the notion that CNS are real etiological agents.

The mechanisms whereby CNS cause infections have not been completely established. On the skin, CNS generally show a benign relationship with the host. However, if the skin barrier is damaged by trauma or implantation of a foreign body, these microorganisms can enter the circulation and, depending on their ability to adhere and escape the immune system, might multiply and produce substances toxic to the host.

Studies of the pathogenesis of CNS have shown that these microorganisms produce various metabolites, including enzymes and toxins (14). CNS have been isolated from infections associated with the development of toxic shock syndrome (7, 11, 19). However, the production of TSST-1 by CNS has been questioned by other investigators who did not confirm these findings (25, 32).

The determination of the production of staphylococcal toxins is still mainly based on immunological methods, which depend on the concentration of the toxin produced and, therefore, might not detect low-producing strains. The classical methods used for the detection of enterotoxins and TSST-1 in culture supernatants are immunodiffusion, RPLA and ELISA. However, these methods are time consuming and do not always identify toxin-producing strains because toxin production depends on the growth conditions which differ from *in vivo* conditions (15). In addition, immunological methods can yield false-positive results due to cross-reactions between antigens and the occurrence of nonspecific reactions (27, 28, 38).

Analysis of the toxigenic profile of CNS strains showed that 26.7% of the isolates produced some type of toxin and one or more toxin-specific genes were detected in 40%. These results confirm the findings of Crass and Bergdoll (11) and Kahler et al. (19) who isolated enterotoxin- and TSST-1-producing CNS from patients with toxic shock syndrome and other infections, and from food implicated in a food poisoning outbreak.

In the present study, *S. epidermidis* strains were found to produce SEA, SEB, SEC and TSST-1 alone or in combination. SEC production was observed in one *S. haemolyticus* isolate and two *S. lugdunensis* isolates. SEC and TSST-1 production by *S. epidermidis*, *S. haemolyticus*, *S. warneri* and *S. xylosus* has also been reported by Valle et al. (37) for strains isolated from goats.

Our results showed a higher frequency of CNS strains containing the *sec-1* gene alone or in combination with other genes. This finding differed significantly from those obtained for *S. aureus* where the *seb* gene predominated. According to Niskanen and Koiranen (31), SEB-producing *S. aureus* strains have more frequently been identified among staphylococci isolated from human clinical samples, while SEC-producing strains are commonly of animal origin.

Comparison of the results obtained by PCR and RPLA revealed that some PCR-positive isolates did not show detectable production upon RPLA. Similar findings have been reported by others (18, 34) and might be due to toxin production below the detection limit of the RPLA assay or to the non-expression of genes. According to Schmitz et al. (34), in clinical practice, staphylococcal strains carrying a toxin gene should be considered potential producers of this toxin since toxin production *in vivo* cannot be excluded.

On the other hand, in the present study production of TSST-1 and SEB+SEC was detected in *S. epidermidis* and production of the SEA+SEB and SEA+SEC combinations was observed in *S. aureus*, while the corresponding genes were not identified by PCR. Tsen and Chen (36) also found *S. aureus* strains positive for SEA by RPLA and negative by PCR. These findings might be the result of the occurrence of cross-reactions and nonspecific reactions in the latex agglutination assay (27, 28, 38).

The study of the toxigenic potential of CNS is important for the determination of the etiological significance of these species. We observed a higher frequency of *S. epidermidis* isolates producing detectable amounts of toxin compared to other species, as well as a predominance of toxigenic *S. epidermidis* strains isolated from blood cultures. These results demonstrate that these microorganisms are important nosocomial pathogens, and should therefore not be ignored or considered to be contaminants when isolated from blood. In these cases, microbiological results and the clinical signs present should be analyzed together in order to obtain a better assessment.

PCR was found to be a rapid and reliable method for the identification of genes responsible for the production of staphylococcal toxins, with further perspectives for its widespread use in other situations. In addition, the present results suggest the need for further studies in order to better characterize the pathogenic potential of CNS and indicate that attention should be paid to the toxigenic capacity of this group of microorganisms.

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ANEXO 14

Calsolari RAO; Pereira VC, ARAÚJO JÚNIOR JP, **CUNHA MLRS**. Determination of toxigenic capacity by reverse transcription polymerase chain reaction in coagulase-negative staphylococci and *Staphylococcus aureus* isolated from newborns in Brazil. Microbiol Immunol. 2011; 55: 394-407.

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ORIGINAL ARTICLE

Determination of toxigenic capacity by reverse transcription polymerase chain reaction in coagulase-negative staphylococci and *Staphylococcus aureus* isolated from newborns in Brazil

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ABSTRACT

Staphylococcus* spp. are frequently found in hospital environments and are associated with a wide variety of infections. Various virulence factors are responsible for the pathogenicity of staphylococci, among which staphylococcal enterotoxins and TSST-1 (toxic-shock syndrome) are noteworthy. In this study, 90 samples of *Staphylococcus aureus* and 90 samples of coagulase-negative staphylococci (CNS) isolated from different clinical materials were investigated by polymerase chain reaction (PCR) in order to study the genes encoding staphylococcal toxins A (*sea*), B (*seb*), C (*sec-1*), D (*sed*) and TSST-1 (*tst*). The samples shown to be positive for the presence of one or more genes were tested for their capacity to express mRNA encoding the respective toxins by reverse transcription-PCR (RT-PCR). As regards the CNS species, *S. epidermidis* was the most frequently isolated, corresponding to 71.1% of the total number of samples of CNS investigated. One hundred and eight samples were positive according to PCR, of which 59 (54.6%) were *S. aureus* and 49 (45.4%) were CNS. *S. aureus* showed toxigenic genes for all classes of toxins investigated whereas CNS showed all genes except for that of toxin D. Assessment of mRNA expression by RT-PCR showed 43 positive samples, 37 (86.0%) *S. aureus* samples producing SEA, SEB, SEC, SED and/or TSST-1 and six (14.0%) CNS samples producing SEA and SEC. RT-PCR and sequencing of PCR products confirmed the toxigenic capacity of *S. epidermidis* and *S. lugdunensis*, indicating the need for greater attention to such microorganisms when they are isolated from infectious processes.

Key words coagulase-negative staphylococci, RT-PCR, *Staphylococcus aureus*, toxins.

Members of the *Staphylococcus* genus are the most commonly found pathogens in hospital environments and cause a wide variety of infections. Nosocomial infections, a major cause of morbidity and mortality, are frequently acute and pyrogenic and, if not treated, may lead to bacteremia involving various organs. In the *Staphylococcus* genus, *S. aureus* have always been the major species implicated in nosocomial infections, and they remain the main

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List of Abbreviations: BHI, brain heart infusion; BLAST, Basic Local Alignment Search Tool; CNS, coagulase-negative staphylococci; DEPC, diethylpyrocarbonate; dNTP, deoxynucleotide-triphosphate; IFN, interferon; IL, interleukin; ITS-PCR, internal transcribed spacer-PCR; MHC, major histocompatibility complex; mRNA, messenger ribonucleic acid; RPLA, reverse passive latex agglutination; *S., Staphylococcus*; SE, staphylococcal enterotoxin; SEA, staphylococcal enterotoxin A; *sea*, gene of staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; *seb*, gene of staphylococcal enterotoxin B; SEC1, staphylococcal enterotoxin C1; *sec-1*, gene of staphylococcal enterotoxin C; SEC2, staphylococcal enterotoxin C2; SEC3, staphylococcal enterotoxin C3; SED, staphylococcal enterotoxin D; *sed*, gene of staphylococcal enterotoxin D; TNF, tumor necrosis factor; TSS, toxic-shock syndrome; TSST-1, toxic-shock syndrome toxin-1; *tst*, gene of staphylococcal toxin TSST-1.

cause of staphylococcal community-acquired infections in most countries (1). However, in the last few decades, CNS have been identified as causing serious infections due to the increasing use of invasive procedures, such as intravascular catheters and prostheses in immunocompromised and intensively-treated patients, pre-term infants, patients with neoplasia and transplanted individuals (2).

Various virulence factors are responsible for the symptoms and severity of infections caused by *S. aureus*, among which SEs and TSST-1 are noteworthy. Together, they form the group of superantigens (3).

Staphylococcal toxins were firstly described in 1959 by Bergdoll *et al.* (4). Currently, there are 23 serologically distinct enterotoxins, including SEA to SEIV (5, 6). All share superantigenic activity, whereas only a few of them (SEA to SEI, SER, SES, and SET) have been proved to be emetic (7, 8). The International Nomenclature Committee for Staphylococcal Superantigens proposed that only staphylococcal superantigens that induce emesis after oral administration in an experimental model of primates should be designated as staphylococcal enterotoxins. The committee also recommended that other similar toxins that do not exhibit emetic properties in primate animal models, or that have not yet been tested, should be designated as staphylococcal enterotoxin-like toxins type X (9, 10). One toxin involved in the TSS was initially designated as SEF (11). However, it did not show the *in-vivo* biological activity which is characteristic of a true enterotoxin and was later designated as TSST-1 (12). The TSST-1 encoding gene has little gene sequence homology with the genes encoding staphylococcal enterotoxins and with those of streptococcal pyrogenic exotoxins (13). Despite this fact, these toxins are structurally and functionally similar (14).

Staphylococcal intoxication occurs after ingestion of food contaminated with the enterotoxins produced by bacteria of the *Staphylococcus* genus, and *S. aureus* is its major agent. However, some authors have reported the isolation of other enterotoxigenic coagulase-positive species, such as *S. intermedius* and *S. hyicus* (15, 16).

Enterotoxigenicity of other CNS, including *S. cohnii*, *S. epidermidis*, *S. saprophyticus*, *S. sciuri*, *S. warneri*, *S. chromogenes* and *S. lentus* has also been reported (17–21).

In addition to their important role in staphylococcal food intoxication, enterotoxins are also capable of activating nonspecific T-cell proliferation and are therefore referred to as superantigens. Bacterial superantigens can nonspecifically activate T cells because they bind externally to the Vβ domain of the T-cell receptor and α chain of the class-II MHC molecule present in antigen-presenting cells, without having been previously processed. Such binding produces a signal which induces proliferation and polyclonal activation of approximately 10 to 30% of the T-cell repertoire, CD4 T cells comprising the predominant responsive population. Hence, there is substantial production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2 by T cells, and such as IL-1 and TNF-α by monocytes. The massive production of pro-inflammatory cytokines triggers an intense inflammatory reaction, leading to damage to the host's tissues (22).

Various methods for detecting the presence of enterotoxins have been developed, immunoassays being the most commonly used due to their simplicity and sensitivity (23). However, such methods do not always detect toxin-producing strains as their production depends on growth patterns that do not represent *in-vivo* conditions (24).

With the development of molecular biology, some techniques for detecting the presence of these enterotoxins have been proposed. Currently, PCR is one of most frequently used methods because it enables identification of the genes responsible for enterotoxin production with high sensitivity and specificity (25, 26).

Although toxigenic genes have been reported in CNS species (27, 28), many authors still question their toxigenic potential and suggest the possibility of errors in species identification (29, 30). By using ITS-PCR, Couto *et al.* identified different CNS species (31), thus amplifying an extremely conserved region in the genus. Hence, genotypic identification confirms phenotypic identification and excludes the possibility of error. The advancement of genotypic methods also provides the possibility of detecting the mRNA sequence responsible for the expression of the target enterotoxin by using RT-PCR. Confirmation of the presence of the sequence of mRNA which encodes synthesis of the toxin eliminates doubts concerning the microorganism's toxic potential.

Disagreement in relation to the enterotoxigenicity of CNS and their capacity to cause food intoxication and/or other associated diseases indicates a need for further investigation using reliable techniques that can confirm the capacity of such staphylococci to produce toxins. Hence, this study aimed to: (i) identify samples of *S. aureus* and CNS isolated from clinical materials obtained from newborns; (ii) confirm phenotypic identification by ITS-PCR; (iii) detect the presence of genes which encode enterotoxins and TSST-1 and; (iv) detect the expression of mRNA responsible for the production of such toxins.

MATERIAL AND METHODS

Ethics statement

This research was approved by the Research Ethics Committee of the Faculty of Medicine of Botucatu, Brazil (OF. 26/2005-CEP). This study was exempted from the requirement to obtain written informed consent from study

participants and/or their legal guardians because the samples of *Staphylococcus* included in the study had already been isolated and stored in the Collection of Cultures of the Department of Microbiology and Immunology, Botucatu, SP, UNESP, Brazil.

Microorganisms

One hundred and eighty staphylococci samples were studied. They included 90 samples of CNS and 90 samples of *S. aureus* from biological material collected from newborns hospitalized at the University Hospital of the Botucatu School of Medicine.

Toxigenic international-reference *S. aureus* strains were used as positive controls, including SEA-producing *S. aureus* ATCC 13565, SEB-producing *S. aureus* ATCC 14458, SEC-producing *S. aureus* ATCC 19095 and SED-producing *S. aureus* ATCC 23235.

Phenotypic identification of *Staphylococcus aureus* and coagulase-negative staphylococci

The organisms in the samples were identified by catalase and coagulase tests, glucose fermentation and oxidation test, bacitracin resistance (0.04U) and furazolidone sensitivity (100 μg) (32). The criteria proposed by Kloos and Schleifer (33), Kloos and Bannerman (34) and Cunha *et al.* (35) were followed for identification of CNS according to a simplified biochemical testing scheme.

Genotypic identification of coagulase-negative staphylococci

DNA extraction

DNA was extracted from *Staphylococcus* strains cultivated in blood agar, individually inoculated in BHI broth and incubated at 37°C for 24 hr.

For extraction, the Illustra Kit (GE Healthcare, Little Chalfont, UK) was used. First, staphylococci cells were digested with lysozyme (10 mg/mL) and proteinase K (20 mg/mL). Next, 500 μL of the extraction solution was added to the mixture, which was centrifuged at 10,000 × g for 4 min. Then, the supernatant was transferred to a GFX column and centrifuged at 5000 × g for 1 min. The collected fluid was discarded, and 500 μL of extraction solution was again added to the column. After centrifugation and discarding of the collected fluid, 500 μL of the washing solution was added to the column and centrifuged at 20,000 × g for 3 min. Next, the column was transferred to a 1.5-mL tube, and 200 μL of Milli-Q water heated to 70°C was used for elution.

Nucleic acid amplification (polymerase chain reaction)

The genotypic technique used for identifying CNS was ITS-PCR, which enables the identification of different *Staphylococcus* species through amplification of an extremely conserved region using primers G1 (5′-GAAGTCGTAACAAGG-3′) and L1 (5′-AAGGCATCCACCGT-3′) obtained from the conserved sequences adjacent to genes 16S and 23 S (31). For *S. aureus* detection, primers Sa442-1 e Sa-422-2, which amplify a conserved fragment of 108 bp specific for *S. aureus* according to Martineau *et al.* (36) and the primers for detection of the *coa* gene described by Kearns *et al.* (37) were chosen.

Internal transcribed spacer-PCR and PCR using primers specific for *S. aureus* and the *coa* gene were used to identify CNS positive for the production of toxins, or genes encoding such toxins, and therefore confirm that these samples were actually CNS species and not mutant *S. aureus* samples that did not produce coagulase. For controls, international-reference ATTC strains of each *Staphylococcus* species were used.

The PCR reactions were performed in 0.5-mL microcentrifuge tubes in total volumes of 50 μL containing 20 pmol of each primer, 2.5U of Taq DNA polymerase, 200 μM of deoxyribonucleotide triphosphates, 20 mM of Tris-HCl (pH 8.4), 1.5 mM of $MgCl_2$, and 5 μL of the sample. Negative controls were provided for all reactions by substituting water for the nucleic acid. Incubation was performed in a PTC-100 thermocycler (MJ Research, Waltham, MA, USA) as follows: denaturation at 94°C for 4 min, followed by 25 cycles consisting of denaturation at 94°C for 1 min, ramp-primer annealing up to 55°C for 2 min followed by 7 more min at 55°C, ramp extension up to 72°C for 2 min followed by 7 more min at 72°C. After completing the 25 cycles, the tubes were incubated at 72°C for 7 min before cooling to 4°C.

The parameters of amplification using primers specific for *S. aureus* and the *coa* gene were the same as those described by Martineau *et al.* (36) and Kearns *et al.* (37).

For test control results, the following international-reference strains were used: *S. epidermidis* (ATCC 12228), *S. simulans* (ATCC 27851), *S. warneri* (ATCC 10209), *S. xylosus* (ATCC 29979), *S. saprophyticus* (ATCC 15305), *S. hominis* (ATCC 27844), *S. aureus* (ATCC 13565), *S. aureus* (ATCC 14458), *S. aureus* (ATCC 19095), and *S. aureus* (ATCC 23235).

In order to visualize the products amplified by ITS-PCR, 1% polyacrylamide gel electrophoresis was performed according to the protocol described by Sambrook and Russell (38) and later stained with 0.2% silver nitrate.

Detection of enterotoxins and toxic-shock syndrome toxin-1 genes

Nucleic acid amplification (polymerase chain reaction)

The PCR reactions were performed in 0.5-mL microcentrifuge tubes in total volumes of 50 μL containing 20 pmol

of each primer (Table 1), 2.5U of Taq DNA polymerase, 200 μM of deoxyribonucleotide triphosphate, 20 mM of Tris-HCl (pH8.4), 1.5 mM of $MgCl_2$ and 5 μL of the sample. Negative controls were provided for all reactions by substituting of nucleic acid by water. Incubation was performed in a PTC-100 thermocycler (MJ Research) as follows: first cycle at 94°C for 4 min, denaturation at 94°C for 2 min, primer annealing at 55°C for 2 min and extension to 72°C for one min and 30 s, followed by a second denaturation cycle at 94°C for 2 min, primer annealing at 53°C for 2 min and extension to 72°C for one min and 30 s. In the third cycle, the annealing temperature was reduced to 51°C for 2 min followed by 37 more denaturation cycles at 94°C for 2 min, primer annealing at 51°C and extension to 72°C for one min and 30 s. After completing 40 cycles, the tubes were incubated at 72°C for 7 min before cooling to 4°C.

Detection of enterotoxin and toxic-shock syndrome toxin-1 mRNA expression by polymerase chain reaction

RNA extraction

Total RNA was extracted from *Staphylococcus* spp. samples cultivated in blood agar, individually inoculated into BHI broth and incubated at 37°C for 24 hr. Extraction was performed by Trizol (Invitrogen, Carlsbad, CA, USA). The cells were lysed by 1.0 mL of Trizol and the homogenate transferred to 1.5-mL tubes to which 0.2 mL of chloroformium was added, and again homogenized for 3 min at 15–30°C. Following this process, the homogenate was centrifuged at 12,000 × g for 15 min at 5°C, and then the aqueous phase containing total RNA was collected and transferred to another tube. RNA precipitation was performed by adding 250 μL of isopropyl alcohol with incubation at ambient temperature for 10 min and centrifugation at 12,000 × g for 10 min at 5°C, and then total RNA washing was performed by 1 mL 70% ethanol to which was added 0.1% of DEPC.

Twenty-five μL of Milli-Q water with DEPC and RNA-guard (Amersham Bioscience, Piscataway, NJ, USA), at a concentration of 1/10, was added to the total RNA extracted, and this mixture incubated for 10 min at 56°C. From the mixture of 25 μL, 11 μL were removed for untreated cDNA acquisition. In order to control total RNA extraction by eliminating all DNA that may have been extracted together with total RNA, 1 μL of 1U/μL RNase-Free Dnase (Promega, Madison WI, USA) + 1 μL of DNAse buffer (10× reaction buffer) were added to the remaining RNA (14 μL). The mixture was incubated for 30 min at 37°C, and then 1 μL of DNAse stop solution (Promega, Madison WI USA) was added and the mixture again incubated for 10 min at 65°C. cDNA was not obtained for a part (6 μL) of such DNAse-treated RNA. This was used for enzyme-action control, and the remaining 11 μL were used for cDNA acquisition.

cDNA acquisition

One μL of random primer at 75 ng/μL was added to a 0.5-mL microtube (RNAse/DNAse free) with 11 μL of the sample (2.5 μg of total RNA). Next, the mixture was heated to 70°C for 10 min for RNA denaturation and rapidly cooled to 4°C. Later, 4 μL of the reverse transcriptase buffer (first strand buffer 5×), 2 μL of dithiothreitol 0.1 M and 1 μL of nucleotides (dNTP mix 20 mM) were added to the mixture. The reaction was then heated to 25°C for 2 min for primer binding. After heating, 1 μL of reverse transcriptase, Superscript RT III (Invitrogen) was added to the reaction, and the mixture taken to a PTC-100 MJ Research Thermocycler for cDNA acquisition.

For cDNA acquisition, cycles of 25°C for 5 min, 42°C for 50 min, 70°C for 15 min, and then cooling to 4°C were used. The cDNA obtained was stored at −20°C until use.

Table 1. Primers for detection of staphylococcal enterotoxin genes

Primer	5′ to 3′ nucleotide sequence	Target
sea1	TTGGAAACGGTTAAAACGAA	Enterotoxin A
sea2	GAACCTTCCCATCAAAAACA	Enterotoxin A
seb1	TCGCATCAAACTGACAAACG	Enterotoxin B
seb2	GCAGGTACTCTATAAGTGCC	Enterotoxin B
sec1	GACATAAAAGCTAGGAATTT	Enterotoxin C
sec2	AAATCGGATTAACATTATCC	Enterotoxin C
sed1	CTAGTTTGGTAATATCTCCT	Enterotoxin D
sed2	TAATGCTATATCTTATAGGG	Enterotoxin D
tst1	ATGGCAGCATCAGCTTGATA	Toxic shock syndrome toxin 1
tst2	TTTCCAATAACCACCCGTTT	Toxic shock syndrome toxin 1
rRNA 16S 1	CCTATAAGACTGGGATAACTTCGGG	rRNA 16S
rRNA16S 2	CTTTGAGTTTCAACCTTGCGGTCG	rRNA 16S

Source: Johnson *et al.* (25) Mason *et al.* (37).

The protocol described under the heading 'Nucleic acid amplification (polymerase chain reaction)' in Materials and Methods was used for cDNA amplification.

For internal control of RNA extraction, 16S ribosomal RNA was searched by using primers 16S1 and 16S2 (Table 1), which correspond to the regions of the rRNA gene that are conserved among staphylococci and specific to the genus (39).

Visualization of amplified products

The efficacy of amplifications was monitored by submitting the reaction to electrophoresis in 2% agarose gel prepared in 1.0× TBE buffer and staining with ethidium bromide. The size of the amplified products was compared with standards of 50 and 100 bp and later photographed under ultraviolet transillumination.

Toxin assay using a commercially available kit

To confirm toxigenic capacity in CNS strains positive for RT-PCR, the sac culture method of toxin production was used (40) and the extracellular products detected by RPLA as described by Shingaki *et al.* (41). For this, 30 to 40 cm dialysis tubes, previously washed in distilled water, were tied closed at one end, immediately filled with 50 mL of BHI broth in double concentration, and tied closed at the other end. These sacs were placed in U-shaped 250-mL Erlenmeyer flasks and the flasks autoclaved for 15 min at 121°C. A loopful of organisms previously cultivated in 5.0 mL of BHI broth at 37°C for 18 hr were added to 18.0 mL of sterile phosphate buffer 0.2 M, pH 7.4, in 0.9% NaCl. These mixtures were then transferred to the Erlenmeyer flasks containing the sacs and incubated at 37°C for 24 hr on a shaker at 200 rpm. After incubation, the cultures were centrifuged at 8000 × g/4°C for 10 min and the culture supernatants obtained were tested by RPLA.

For these tests a SET-RPLA-T900 kit (Oxoid Diagnostic Reagents, Cambridge, UK) was used for detecting SEA and SEC. A 96-well microplate, with V-shaped bottom, was filled with 25 μL samples of the above culture supernatant filtered through a Millipore membrane (8.0 μm) to avoid the occurrence of nonspecific reactions. Then 25 μL of sensitized latex was added with the anti-enterotoxins. Standard toxins were used as positive controls, and the nonspecific reactions analyzed by adding 25 μL of the culture supernatant and 25 μL of the control latex. These prepared microplates were covered with cellophane and the reagents homogenized in a micro-mixer for 3 min. After incubation for 20 to 24 hr at ambient temperature, the results were registered with the aid of a light on a dark background. Positive reactions were classified as +, ++, or +++, according to the pattern of agglutination described by the kit manufacturer. The formation of a rose button was interpreted as being a negative result.

Toxin gene sequencing

To confirm the detection of toxins genes in CNS, the PCR products of each species positive for toxin genes were assessed by DNA sequencing.

The basic procedure used was that described by Sanger *et al.* (42). After the PCR procedure, residual oligonucleotide primers, dNTPs and enzyme were removed using S400 HR Columns (GE Healthcare), as per the manufacturer's protocol. Amplicon samples were then analyzed and quantified on 2% agarose gels stained with ethidium bromide (0.5 mg/mL) and compared using the Low DNA Mass Ladder (Invitrogen). The samples were submitted to cycle sequencing using the kit ABI Big Dye Terminator (v3.1, Perkin Elmer, Applied Biosystems, Foster, CA, USA) and primers sense or antisense to each toxin genes (Table 1), as recommended by the manufacturer. The data were obtained in an automated DNA sequencer model the ABI Prism model 377 DNA Sequencer (Applied Biosystems, Foster, CA, USA). The Clustal X program was used to align nucleotide sequences. The obtained sequences were submitted for analysis to GenBank by using BLAST to confirm the toxin gene (http://www.ncbi.nih.gov/BLAST).

Statistical analysis

The obtained data were analyzed by the χ^2 test when no expected values < 5 were found, and by the likelihood ratio test for differences of proportions with χ^2 approximate distribution when expected values <5 were observed. Fisher's Exact test was used for 2 × 2 tables.

The results of CNS identification by the phenotypic and genotypic methods were compared by calculating Pearson's correlation coefficient.

RESULTS

Identification of coagulase-negative staphylococci

Phenotypic identification (Fig. 1) was distributed as follows: 68 (75.6%) samples of *S.epidermidis*, five (5.6%) samples of *S.lugdunensis*, two (2.2%) samples of *S. warneri*, seven (7.8%) samples of *S. haemolyticus*, four (4.4%) samples of *S. hominis*, three (3.3%) samples of *S. simulans* and one sample (1.1%) of *S. xylosus*. In addition to phenotypic identification, 60 CNS samples were submitted to genotypic identification by ITS-PCR (Fig. 1),

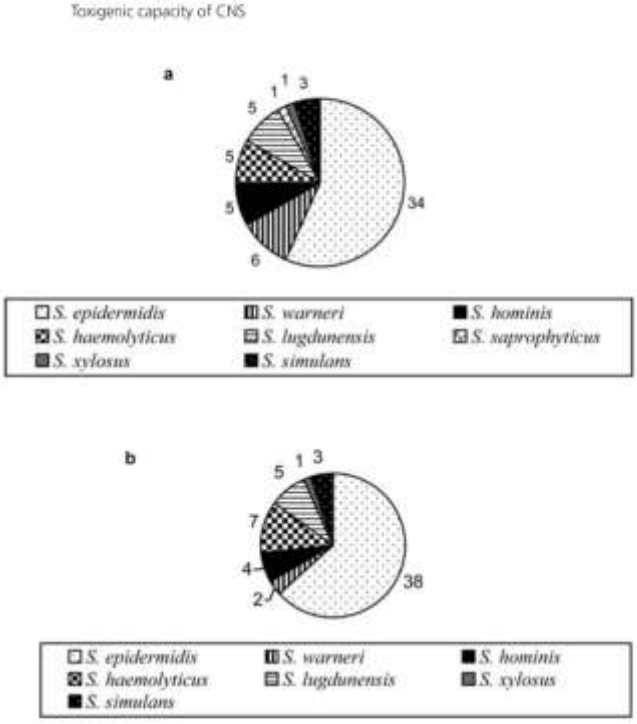

Fig. 1. (a) Genotypic and (b) phenotypic identification of CNS species.

including 38 samples of *S. epidermidis* which were positive for the presence of genes encoding staphylococcal toxins. All samples referring to the other CNS species were identified by the genotypic method ($n = 22$).

Comparison of the two techniques for identifying these samples showed nine (15.0%) discrepancies between the methods (Fig. 1). With the new technique, the identification of some species changed. Five *S. epidermidis* samples identified by biochemical testing were not confirmed by the genotypic technique. Three such samples were re-identified as *S. warneri* and two as *S. hominis*. Two samples identified as *S. haemolyticus* by the phenotypic method were also re-identified as *S. warneri*. In addition, one sample of *S. hominis* was re-identified as *S. saprophyticus*, and one sample of *S. warneri* identified by ITS-PCR as *S. epidermidis*. No strain of CNS included in the study amplified with primers of *coa* gene and primers specific for *S. aureus*, thus confirming them all to be CNS.

Figures 2 and 3 show a picture of polyacrylamide gel for CNS identification by the ITS-PCR and gel electrophoresis using primers specific for *S. aureus* and the *coa* gene.

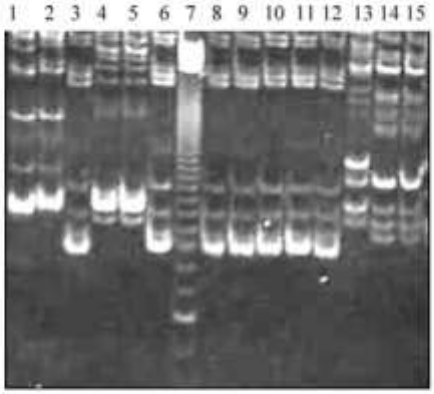

Fig. 2. Polyacrylamide gel electrophoresis for CNS identification by ITS-PCR. Columns: 1: Sample of *S. xylosus*, 2: *S. xylosus* ATCC 29979, 3: Sample identified as *S. warneri* by the phenotypic method, but with a genotypic pattern of *S. epidermidis*, 4: Sample of *S. warneri*, 5: *S. warneri* ATCC 10209, 6: Sample of *S. epidermidis*, 7: Molecular-weight marker (100 bp), 8, 9, 10, 11: Samples of *S. epidermidis*, 12: *S. epidermidis* ATCC 12228, 13: *S. aureus* ATCC 13565, 14, 15: *S. aureus* ATCC 19095.

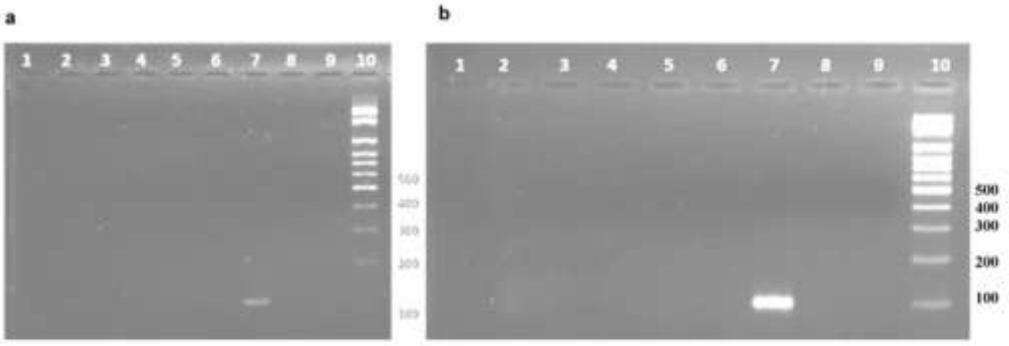

Fig. 3. (a). Agarose gel electrophoresis for detection of the gene *coa* (117 bp) in coagulase-negative *Staphylococcus* strains by PCR. Lanes: 1, *S. lugdunensis*; 2–6 *S. epidermidis*, negative strains; lane 7, positive control (*S. aureus* ATCC 29213); lane 8, negative control (*S. epidermidis* ATCC 12228); lane 9, water; lane 10, molecular weight marker (100 bp). (b). Agarose gel electrophoresis for detection of the gene Sa442 (108 bp) in coagulase-negative *Staphylococcus* strains by PCR. Lanes: 1, *S. lugdunensis*; 2–6, *S. epidermidis*, negative strains; lane 7, positive control (*S. aureus* ATCC 29213); lane 8, negative control (*S. epidermidis* ATCC 12228); lane 9, water; lane 10, molecular weight marker (100 bp).

Toxigenic profile determination by polymerase chain reaction

PCR showed that, of the 90 samples isolated from *S. aureus*, 59 (65.6%) were positive for the genes encoding enterotoxins A, B, C, D and/or TSST-1, whereas of 90 CNS samples, 49 (54.4%) were positive for the presence of some of these genes. Of the total number of 180 staphylococci samples, 108 (60.0%) showed genes responsible for the production of enterotoxins A, B, C, D or of TSST-1.

As illustrated in Table 2, gene *sec-1* was the most frequently detected in *S. aureus* samples, being found alone or associated with other toxin genes in 26 (44.1%) of the toxigenic samples. Gene *sea* occurred in 19 (32.2%); gene *seb* in 16 (27.1%), and gene *tst* in 8 (13.6%) of the toxigenic samples.

Of the 49 toxigenic CNS samples, gene *sec-1* was also the most prevalent, being found in 33 (67.4%) of the tested samples, whereas gene *sea* was observed in 14 (28.6%) of the toxigenic samples, gene *seb* in only one and gene *tst* in 13 (26.5%) (Fig. 4).

Analysis of the presence of different toxin genes in *S. aureus* species, when compared to that in the *S. epidermidis* species and other CNS, showed the presence of gene *seb* particularly in *S. aureus*, the difference being statistically significant ($P < 0.00020$, see Table 2).

When the CNS species were evaluated for toxigenicity, 33 (51.6%) of the 64 samples isolated from *S. epidermidis* were found to be positive. Of the six *S. warneri* samples investigated, all showed toxigenic genes whereas, of the five *S. hominis* species evaluated, three were shown to be toxigenic. A similar result was found for the five *S. lugdunensis* samples, with three toxigenic samples. Of the five *S. haemolyticus* samples isolated, two were positive for the presence of toxigenic genes. Species *S. saprophyticus* and *S. xylosus*, with one sample each, were also shown to be toxigenic. On the other hand, the three *S. simulans* samples did not show the genes under investigation.

Table 2. Toxigenic profile of staphylococci isolated from newborns by the PCR technique

Toxin genes PCR	Species			Total	*P* value
	S. aureus	*S. epidermidis*	Others		
sea	11	5	4	20	0.5683
seb	12	1	0	13	0.0020
sec-1	20	15	8	43	0.7308
sed	1	0	0	1	0.4986
tst	4	5	0	9	0.1489
*sea+seb**	4	0	0	4	0.0597
*sea+sec-1***	3	2	1	6	0.9920
*sea+tst****	1	0	1	2	0.2961
*sec-1+tst*****	3	4	2	9	0.5754
*sea+sec-1+tst******	0	1	0	1	0.3482
Total	59	33	16	108	0.3052

*, concomitant presence of genes of enterotoxins A and B; **, concomitant presence of genes of enterotoxins A and C; ***, concomitant presence of genes of enterotoxins A and TSST-1; ****, concomitant presence of genes of enterotoxins C and TSST-1; *****, concomitant presence of genes of enterotoxins A, C and TSST-1.

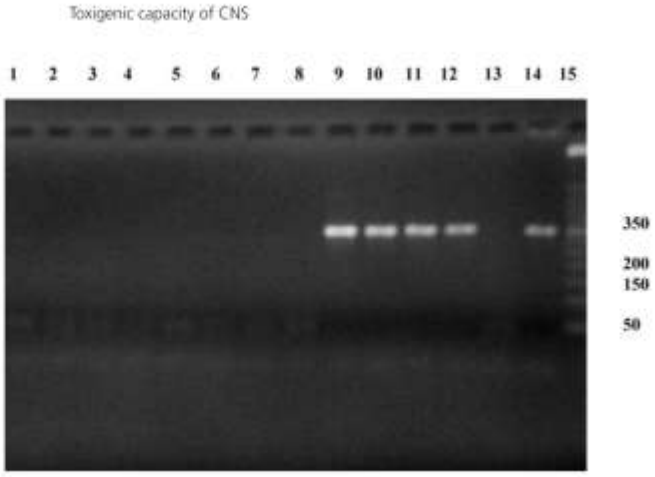

Fig. 4. Agarose gel electrophoresis showing the products amplified by PCR for the gene of toxic shock toxin 1, TSST-1 (350 bp). Lines: 1, 2, 3, 4, 5, 6, 7, 8, CNS strains negative for gene *tst*; 9, 10, 11, 12, CNS strains positive for gene *tst*; 13, negative control; 14, positive control (*S. aureus* ATCC 51650); 15, Molecular-weight marker (50 bp).

Toxigenic profile determination by the reverse transcription polymerase chain reaction technique

The samples of *S. aureus* ($n = 59$) and CNS ($n = 49$) which were shown to be toxigenic by PCR were submitted to RNA extraction, in order to investigate toxin expression by RT-PCR. The internal control of RNA extraction through the 16S ribosomal RNA search showed a positive result for all samples.

Of the 108 toxigenic samples identified by PCR, 43 (39.8%) were shown to be positive for toxin expression when submitted to the RT-PCR technique. Its expression corresponded to 37 (34.3%) *S. aureus* samples and 6 (5.6%) CNS samples (Table 3).

In relation to the total number of toxigenic samples, there was a statistically significant difference ($P < 0.001$) between *S. aureus* ($n = 37$) and CNS samples ($n = 6$) in positivity for toxin expression (Table 4).

The *S. aureus* samples expressed mRNA for all tested toxin classes, that is, SEA, SEB, SEC, SED and TSST-1, whereas the CNS samples only expressed mRNA for SEA and SEC (Table 4). Of the 16 *S. aureus* samples presenting toxigenic genes for SEB, their expression was confirmed by RT-PCR in 13, corresponding to 81.3% of the total *S. aureus* genes for that toxin. SEA expression was also confirmed in 12 (63.2%) of the 19 samples with the *sea* gene (Fig. 5). As to SEC, expression was observed in 13 (50.0%) of the 26 samples presenting gene *sec-1*. TSST-1 was found in three of the eight samples presenting the gene responsible for its production. The only sample presenting the SED gene confirmed its expression when evaluated by RT-PCR (Table 5).

Of the 14 CNS samples presenting genes for SEA, expression of that toxin was confirmed in three whereas, of the 33 samples with genes for SEC by PCR, expression was confirmed in only three (Fig. 6). None of the 13 samples presenting genes for TSST-1 showed toxin expression when investigated by RT-PCR (Table 5).

Table 3. Distribution of staphylococci isolated from newborns, according to toxigenicity of samples by the PCR and RT-PCR methods.

	Species					
	S.aureus		CNS		Total	
	N	%	N	%	N	%
PCR	59	65.5	49	54.4	180	60.0
RT-PCR	37	34.3	6	5.6	108	39.8

Table 4. Toxigenic profile determination in *Staphylococcus* spp. by the RT-PCR technique.

	Species				
Toxins	*S. aureus*	*S. epidermidis*	Others	Total	*P* value
SEA	9	3	0	12	0.0627
SEB	11	0	0	11	0.0003
SEC	10	2	1	13	0.1173
SED	1	0	0	1	0.4986
TSST-1	1	0	0	1	0.4986
SEA+SEB	2	0	0	2	0.2472
SEA+SEC	1	0	0	1	0.4986
SEC+TSST-1	2	0	0	2	0.2472
Total	37	5	1	43	<0.001

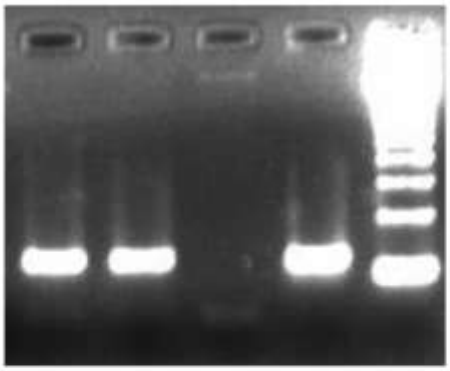

Fig. 5. Agarose gel electrophoresis for SEA investigation (120 bp) in *S. aureus* samples by RT-PCR e. Lanes: 1, SEA-producing sample without RNA treatment with DNAse; 2, SEA-producing sample previously treated with DNAse prior to cDNA acquisition; 3, RNA without cDNA acquisition (enzyme control); 4, *S. aureus* ATCC 13565; 5, Molecular-weight marker (100 bp).

Table 5. Comparison of the PCR and RT-PCR techniques in *S. aureus* and CNS samples

	PCR		RT-PCR	
Toxin/enterotoxin genes	*S. aureus*	CNS	*S. aureus*	CNS
sea/SEA	11	9	9	3
seb/SEB	12	1	11	0
sec-1/SEC	20	23	10	3
sed/ SED	1	0	1	0
tst /TSST-1	4	5	1	0
sea+*seb**	4	0	2	0
sea+*sec-1***	3	3	1	0
sea+*tst****	1	1	0	0
sec-1+*tst*****	3	6	2	0
sea+*sec-1*+*tst******	0	1	0	0
Total	59	49	37	6

*, concomitant presence of genes of enterotoxins A and B; **, concomitant presence of genes of enterotoxins A and C; ***, concomitant presence of genes of enterotoxins A and TSST-1; ****, concomitant presence of genes of enterotoxins C and TSST-1; *****, concomitant presence of genes of enterotoxins A, C and TSST-1.

Toxin assay using commercially available kit

Toxin detection by the RPLA method confirmed enterotoxin production in CNS strains positive by RT-PCR. The five samples of *S. epidermidis* positive for expression of mRNA encoding SEA and SEC, and the only sample of *S. lugdunensis* with a positive result by RT-PCR for SEC were all determined by RPLA to indeed produce these enterotoxins.

Toxin gene sequencing

Amplicons from three samples of *S. epidermidis* were sequenced, one harboring the *sec-1* gene only, one positive for both the *sec-1* and *tst* genes and one positive for the *sea* gene. PCR products from other CNS species harboring genes of staphylococcal toxins were confirmed by DNA sequencing, including one sample of *S. warneri* positive for both the *sec-1* and *sea* genes, one *S. lugdunensis* isolate positive for the *sec-1* + *tst* genes and one *S. hominis* isolate also positive for both the *sec-1* and *tst* genes. Amplicons from *S. aureus* ATCC 13565 positive for *sea*, *S. aureus* ATCC 19095 positive for *sec-1* and *S. aureus* positive for *tst* were also analyzed by DNA sequencing.

After BLAST analysis, the sequences of *sea*, *sec-1* and *tst* amplicons from CNS species were 100% identical to the published sequences of *S. aureus* (GenBank accession n° STATOXAA, SAENTC1 and AY074881.1, respectively).

DISCUSSION

Staphylococci are microorganisms frequently found in hospital environments and are associated with a wide variety of infections. Various virulence factors are responsible for the pathogenicity of staphylococci, among which superantigenic toxins, enterotoxins and the TSST-1 toxin are noteworthy.

Among toxigenic species, *S. aureus* is the best known and most thoroughly described in published reports (43–45); however, some studies have also reported toxin production by CNS (18–21, 46).

In this study, the toxigenic potential of 90 *S. aureus* samples and 90 CNS samples was evaluated by PCR for the detection of genes which encode staphylococcal enterotoxins and TSST-1 and by RT-PCR for the capacity of such samples to express those genes. The samples were submitted to phenotypic identification, and all CNS samples presenting toxin genes were subjected to genotypic confirmation of the identity of their species by ITS-PCR. Comparison of the results of species identification showed good correlation between the methods. *S. epidermidis* species was found to be the most frequent, corresponding to 64 (71.0%) of the 90 CNS samples investigated. *S. warneri*, *S. haemolyticus*, *S. lugdunensis*, *S. hominis*, *S. simulans*, *S. xylosus* and *S. saprophyticus* were also identified. These results are similar to those described by Udo *et al.* (19) and Kloos and Bannerman (2), who also found a higher frequency for *S. epidermidis*.

Of the 180 studied samples, when investigated by PCR 108 (60.0%) were positive, for genes decoding staphylococcal toxins. Of these, 59 (54.6%) corresponded to *S. aureus* samples and 49 (45.4%) to CNS samples. The toxigenic profile study of CNS showed 54.4% of samples

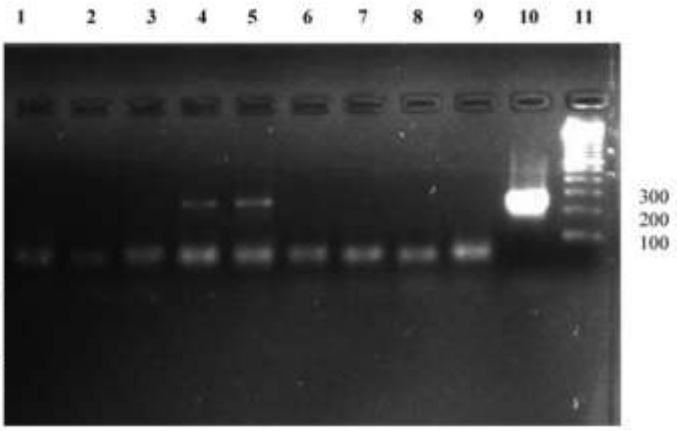

Fig. 6. Agarose gel electrophoresis for detection of SEC (257 bp) in CNS by RT-PCR. Lanes: 1, 2, 3, negative strains; 4, SEC-producing strain in the absence of RNA treatment with DNAse; 5, SEA-producing strain treated with DNAse before cDNA amplification; 6, RNA of the same strain without cDNA amplification; 7, 8, 9, SEC-negative strains; 10, *S. aureus* ATCC 19095; 11, molecular weight marker (100 bp).

with one or more genes, *S. epidermidis* being the species presenting the highest toxigenic potential. Of the 64 *S. epidermidis* samples investigated, 51.6% showed isolated or associated genes for toxins A (*sea*), C (*sec-1*) and TSST-1 (*tst*). However, other CNS species isolated were also positive by PCR. Among them, *S. warneri*, in which all six isolated samples were shown to be toxigenic for the genes *sea* and/or *sec*-1, is noteworthy. Three *S. lugdunensis* samples showed the genes *sec-1*; in one of them this was associated with the gene *tst*. Three samples of *S. hominis*, two of *S. haemolyticus* and one each of *S. saprophyticus* and *S. xylosus* also showed the genes *sea*, *sec-1* and/or *tst*. The only species that did not show toxin genes was *S. simulans*, with three negative samples.

Various environmental factors and regulatory systems within microorganisms can directly affect toxin production. Hence, the presence of toxin genes in the staphylococci samples does not guarantee the occurrence of toxin production at any particular moment, whether *in vivo* or in culture media under optimal conditions.

Some authors, such as Cunha *et al.*, have also reported the presence of toxigenic genes in CNS (27). They reported identifying enterotoxigenic CNS among CNS isolated from food. Of the twenty CNS samples isolated, three showed the gene *sea*, and one presented the gene *sec-1*. The gene *sea* was detected in one sample of *S. epidermidis*, one of *S. xylosus* and one of *S. hominis*, whereas the gene *sec-1* was detected in a sample of *S. xylosus*. When evaluating CNS for the presence of toxin genes, Schmitz *et al.* also found two positive samples for the gene *sec-1* in 50 investigated samples (46). However other authors, such as Becker *et al.*, failed to identify toxin genes in CNS samples from clinical isolates using the multiplex PCR technique (47). Kreiswirth and Novick evaluated various CNS for the presence of genes responsible for TSST-1 expression by utilizing the DNA hybridization technique (48). These authors included some samples that had been reported by Crass and Bergdoll to be TSST-1-producing samples in their study (49). The presence of the gene *tst* was not confirmed in the CNS samples studied. In addition, two TSST-1 positive samples previously identified as CNS were shown to be coagulase positive, indicating an identification error.

Other authors have also questioned the capacity of CNS to produce enterotoxins and TSST-1 and suggested that such samples may be mutant *S. aureus* which do not express the coagulase enzyme (29, 30, 50). With the purpose of pointing out his possibility that all CNS samples can present toxin genes, genotypic identification was performed for species confirmation in this study. The ITS-PCR technique left no doubt about the identity of each investigated sample because of the characteristic band patterns found and compared to international-reference species (ATCC); in addition this technique corrected any errors that may have occurred in phenotypic identification. Although some discrepancies between these methods were found in relation to species differentiation, all samples identified as CNS by the phenotypic method were confirmed to be CNS by the ITS-PCR technique. In addition, PCR using primers specific for *S. aureus* and detection of the *coa* gene confirmed that all CNS samples studied were indeed CNS and did not amplify with these primers.

Disagreement in findings regarding whether or not CNS are able to produce toxins can be reduced or eliminated by choosing an adequate technique for DNA extraction and detection of the genes responsible for the expression of such enterotoxins, thus eliminating possible

identification errors. Standardization of technique, ranging from the growth media, pH, temperature and ideal nutrient concentrations as well as the use of extraction controls, and variables used in amplification and enzyme reactions, is fundamental for the good performance of a study. It is known that one of the disadvantages of multiplex PCR is that it emphasizes the presence of more frequently found genes, thus masking or not detecting those appearing less frequently. In contrast, PCR uses a different pair of primers for each reaction performed and each toxin investigated.

Our results show a predominance of the gene *sec-1* in samples of CNS, either isolated alone, or associated with other genes. However, the gene *seb* predominated in the analysis of toxin gene type in relation to clinical material, having mainly been found in *S. aureus* samples isolated from foreign bodies and secretions. This result can be explained by the fact that the *S. aureus* samples included in this study were most frequently obtained from such clinical materials, 77.7% of them having been isolated from foreign bodies and secretions and 22.3% from blood.

However, PCR detects genes contained in the strains regardless of their expression, for even if the gene is present in the microorganism, it may not be active. RT-PCR detects the mRNA sequence responsible for expression of the target enterotoxin, thus eliminating doubts concerning the microorganism's toxic potential.

Our studies showed expression of the mRNA which encodes staphylococcal toxins in 43 (39.8%) of the 108 positive samples by the PCR technique. The mRNA detected corresponded to toxins of classes SEA, SEB, SEC and/or TSST-1. Significantly fewer CNS than *S. aureus* expressed toxin, CNS comprising 13.9% of the total number of producing samples. *S. epidermidis* was the most toxigenic species among the CNS, five samples being positive for expression of mRNA encoding SEA and SEC. Among the other CNS species, only *S. lugdunensis* showed a positive result by the RT-PCR technique for SEC.

Some authors using the RPLA method have also reported enterotoxin and TSST-1 production by *S. epidermidis, S. haemolyticus* and *S. warneri* (51). When investigating food handlers by the RPLA method, Udo *et al.* also found samples of CNS and *S. aureus* which produced enterotoxins and TSST-1 (19). Of the total CNS investigated, 14.1% produced enterotoxins or TSST-1, samples of *S. hominis, S. warneri, S. saprophyticus, S. epidermidis, S. xylosus, S. haemolyticus* and *S. schleiferi* being positive for toxins SEA, SEB, SEC and/or TSST-1. Marin *et al.* have described a study conducted on enterotoxin-producing staphylococci in ham samples by the RPLA method (52). Of the 135 staphylococci isolated, two belonged to the *S. epidermidis* species, and one produced SEC.

When studying *S. aureus* samples with one or more specific genes for expression of staphylococcal toxins, Cunha *et al.* found 46.7% of positive samples by the PCR technique, whereas only 38.3% of the samples were shown to produce the respective toxins by the RPLA method (28). Also, in the same study, the RPLA method confirmed production of toxins in 26.7% of the 40% of the CNS samples with one or more specific genes for toxin expression. In a study aiming at evaluating the virulence factors of CNS isolated from newborns, by using the RPLA method the authors found that 37.6% of CNS produced SEA, SEB or SEC (53).

The *S. aureus* toxigenic samples investigated in this study showed more production of SEB (68.8%) and SEA (38.5%) when evaluated by the RT-PCR technique. According to Niskanem and Koiranem, SEB-producing *S. aureus* strains are more frequently observed among staphylococci isolated from clinical samples from humans, whereas SEC-producing strains are usually of animal origin (54).

Various studies have described staphylococcal regulatory systems, such as systems *agr* (55), *sar* (56), *rot* (57), which can directly affect staphylococcal enterotoxin production. Novick described expression of locus *agr* as an important regulator in many *S. aureus* virulence factors (58). Although several authors have questioned the toxigenic potential of CNS (29–30), operon *agr*, which plays an important role in regulating staphylococcal toxin expression, has also been found in other *Staphylococcus* species, such as *S. intermedius* (59), *S. lugdunensis* (60) and *S. epidermidis* (61). Locus *agr* of *S. epidermidis* shows 68% homology when compared with an *agr* locus of *S. aureus* (61), in addition to presenting genes *agrA, B, C* and *D*, which are involved in regulation of toxin production.

In addition to these regulatory systems within the microorganisms, some external factors may act directly on enterotoxin production. It is known that enterotoxin production is optimal at neutral pH and that it decreases in acid pH. Bergdoll reported that glucose in the culture medium has an important inhibitory effect on enterotoxin production, particularly for enterotoxins SEB and SEC (62). Such inhibition is due to a pH decrease resulting from glucose metabolism. According to Regassa *et al.* (63) and Novick (58), glucose and pH decrease may be associated with *agr* expression. Pereira *et al.* have analyzed SEA and SEB production by CNS at different temperatures, pH and NaCl concentrations (64). The greatest production occurred at a temperature of 39.4°C and at pH 7.0. Therefore, enterotoxin synthesis may differ depending on laboratory and natural conditions.

The exact mechanisms by which such systems are activated or inhibited have not yet been fully clarified. The presence of an *agr* locus in the *S. epidermidis* species, and their capacity to produce delta toxin associated with the *agr* system, makes it evident that the system is active and neither excludes nor guarantees the occurrence of enterotoxin production at any particular moment (61). Hence, the predominance of *S. epidermidis* among the toxigenic CNS species may be associated with the particular staphylococcal regulatory systems that are present in that species.

Various immunologic methods have been described for detecting staphylococcal enterotoxins and TSST-1, including immunodiffusion, reverse and passive latex agglutination (RPLA), and ELISA tests and kits that are commercially available. However, those methods do not always detect toxin-production, because some strains produce such a small amount of toxin that it is beneath the detection limit of the method used (27). Other disadvantages of immunologic tests are nonspecific reactions and cross-reactions between antigens that can produce false-positive results (65).

When we evaluated the toxigenic potential of our samples by detecting genes producing enterotoxins and TSST-1, we eliminated all the disadvantages associated with immunologic methods, because if the specific gene for production of a certain toxin is present in the sample, the possibility of toxin production cannot be disregarded (46, 63).

In this study, we found PCR to be rapid and reliable for determining the presence of specific genes for staphylococcal toxins, and also confirmed CNS identification by ITS-PCR, thus excluding the possibility that the samples were mutant *S. aureus*. We also showed that RT-PCR is a rapid and effective technique for demonstrating the capacity of CNS to express the mRNA encoding these enterotoxins; however, as expression depends on gene activation, further investigation of environmental factors and clarification of the regulatory mechanisms that may interfere with such expression must be evaluated.

The results obtained identify CNS as important pathogens with significant toxigenic potential, emphasizing that greater attention must be given to such microorganisms, which have often been dismissed as merely contaminants.

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ANEXO 15

Vasconcelos NG, Pereira VC, Araújo Júnior JP, **CUNHA MLRS**. Molecular detection of enterotoxins E, G, H and I in *Staphylococcus aureus* and coagulase-negative staphylococci isolated from clinical samples of newborns in Brazil. J Appl Microbiol. 2011; 111: 749-62.

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Molecular detection of enterotoxins E, G, H and I in *Staphylococcus aureus* and coagulase-negative staphylococci isolated from clinical samples of newborns in Brazil

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Abstract

Aims: The objective of this study was to investigate the detection of SEE, SEG, SEH and SEI in strains of *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) using RT-PCR.

Methods and Results: In this study, 90 *Staph. aureus* strains and 90 CNS strains were analysed by PCR for the detection of genes encoding staphylococcal enterotoxins (SE) E, G, H and I. One or more genes were detected in 54 (60%) *Staph. aureus* isolates and in 29 (32·2%) CNS isolates. *Staphylococcus epidermidis* was the most frequently isolated CNS species ($n = 64$, 71·1%), followed by *Staphylococcus warneri* ($n = 8$, 8·9%) and other species (*Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus simulans*, *Staphylococcus saprophyticus* and *Staphylococcus xylosus*: $n = 18$, 20%). The genes studied were detected in *Staph. epidermidis*, *Staph. warneri*, *Staph. haemolyticus*, *Staph. hominis*, *Staph. simulans* and *Staph. lugdunensis*. The highest frequency of genes was observed in *Staph. epidermidis* and *Staph. warneri*, a finding indicating differences in the pathogenic potential between CNS species and highlighting the importance of the correct identification of these micro-organisms. RT-PCR used for the detection of mRNA revealed the expression of SEG, SEH and/or SEI in 32 (59·3%) of the 90 *Staph. aureus* isolates, whereas expression of some of these genes was observed in 10 (34·5%) of the 90 CNS isolates.

Conclusions: *Staphylococcus epidermidis* was the most toxigenic CNS species. Among the other species, only *Staph. warneri* and *Staph. lugdunensis* presented a positive RT-PCR result. PCR was efficient in confirming the toxigenic capacity of *Staph. aureus* and CNS.

Significance and Impact of the Study: This study permitted to confirm the toxigenic capacity of CNS to better characterize the pathogenic potential of this group of micro-organisms. In addition, it permitted the detection of SEG, SEH and SEI, enterotoxins that cannot be detected by commercially available immunological methods.

Introduction

Members of the genus *Staphylococcus* are the most common pathogens found in hospitals and are the aetiological agents of a wide variety of infections. *Staphylococcus aureus* is the most important species associated with nosocomial and community-acquired staphylococcal infections in many countries (Cookson and Phillips 1988; Marques *et al.* 1989; Wey *et al.* 1990; Wenzel *et al.* 1991). Few reports regarding infections caused by coagulase-negative staphylococci (CNS) were available before the 1970s, with these micro-organisms being recognized as

contaminants of clinical samples by clinicians and microbiologists (Kloos and Bannerman 1994). However, although widely used for clinical diagnosis, this distinction represents a challenge regarding the role of these micro-organisms in infectious processes.

Various virulence factors are responsible for the symptoms and severity of infections caused by *Staph. aureus*. Staphylococcal enterotoxins (SEs), which cause staphylococcal food poisoning, are a group of superantigens (Koneman *et al.* 1997). The exact role of these extracellular products in the pathogenesis of a systemic infection is still unclear. At present, 23 serologically distinct enterotoxins, including SEA to SElV, have been identified (Bergdoll *et al.* 1959, 1965; Avena and Bergdoll 1967; Casman *et al.* 1967; Reiser *et al.* 1984; Yu-Cheng and Wong 1995; Munson *et al.* 1998; Zhang *et al.* 1998; Jarraud *et al.* 2001; Letertre *et al.* 2003; Omoe *et al.* 2005; Thomas *et al.* 2006; Schlievert and Case 2007; Ono *et al.* 2008; Larkin *et al.* 2009). All share superantigenic activity, whereas only few of them (SEA to SEI, SER, SES and SET) have been proved to be emetic (Lina *et al.* 2004). The International Nomenclature Committee for Staphylococcal Superantigens proposed that only staphylococcal superantigens inducing emesis after oral administration in an experimental model of primates should be designated as SEs. The committee also recommends that other similar toxins that do not exhibit emetic properties in primate animal models or that have not yet been tested should be designated as SE-like toxins (SEl) type X (Lina *et al.* 2004).

The detection of SEs is decisive to confirm an outbreak of food poisoning and to determine the enterotoxigenicity of the strains (Bergdoll 1990). Immunological methods were first developed for this purpose, but their sensitivity and practicality are questionable. In addition, these methods are time-consuming and not always detect toxin-producing strains. In an attempt to solve this problem, amplification techniques such as the polymerase chain reaction (PCR) were developed, which permit the identification of enterotoxin genes with high sensitivity and specificity. Johnson *et al.* (1991) developed a protocol for the detection of SEA to SEE and TSST-1 genes using oligonucleotides synthesized based on the computer analysis of the *sea*, *scb*, *sec*, *sed*, *see* and *tst* gene sequences published previously. Jarraud *et al.* (1999), investigating the role of enterotoxins in staphylococcal toxic shock syndrome, included the SEG and SEI genes in their study.

Although the presence of toxigenic CNS species has been reported in the literature (Orden *et al.* 1992; Vernozy *et al.* 1996; Udo *et al.* 1999; Cunha *et al.* 2006, 2007), many investigators question the toxigenic potential of these micro-organisms.

Furthermore, PCR is a highly sensitive and specific method for the genotypic identification of different staphylococcal species. Couto *et al.* (2001), using intergenic transcribed spacer (ITS)-PCR, identified different CNS species by amplifying a region that is highly conserved in the genus. This technique permits the analysis of ITS between the 16S and 23S rRNA gene loci and is frequently used in fingerprint PCR for the identification and discrimination of bacterial strains at the species and subspecies level (Daffonchio *et al.* 2003).

DNA analysis is the preferred method for the identification of micro-organisms because of its higher specificity and sensitivity. CNS isolates not identified to the species level or erroneously identified by conventional phenotypic tests may be correctly identified by genotypic methods, such as DNA–DNA hybridization (Kawamura *et al.* 1998) and ITS-PCR (Maes *et al.* 1997). The latter technique determines the rate of polymorphisms in transcribed intergenic regions (16S and 23S rRNA) to genetically distinguish intra- and interspecies strains (Daffonchio *et al.* 2003) and has been found to be useful in epidemiological (Kostman *et al.* 1992; Gürtler 1993) and taxonomic studies (Barry *et al.* 1991; Jensen *et al.* 1993). Mendoza *et al.* (1998) amplified the 16S and 23S rRNA regions in 220 *Staphylococcus* isolates using primers G1 and L1 described by Jensen *et al.* (1993) and showed that this technique is an important tool for the identification of these strains. Various studies have reported the usefulness of ITS-PCR for the identification of staphylococci (Mendoza *et al.* 1998; Shittu *et al.* 2004; Fujita *et al.* 2005). According to Couto *et al.* (2001), the method is simple and permits the rapid identification of a large number of isolates with a high degree of reliability.

The divergences regarding the enterotoxigenicity of CNS and their potential to cause food poisoning highlight the need for further studies using reliable techniques to demonstrate the toxin-producing capacity of these staphylococci. Therefore, the objective of this study was to investigate the toxigenicity of *Staph. aureus* and CNS strains using RT-PCR in view of the lack of international and Brazilian studies applying this technique to the evaluation of toxin production in CNS. This study will permit to confirm the toxigenic capacity of CNS to better characterize the pathogenic potential of this group of micro-organisms. In addition, it permits the detection of SEG, SEH and SEI, enterotoxins that cannot be detected by commercially available immunological methods.

Materials and methods

Micro-organisms

A total of 180 staphylococcal strains, including 90 CNS and 90 *Staph. aureus* strains, isolated from clinical samples (blood, cannula, catheter tip, chest drain and

different secretions) were analysed. The samples were obtained from newborns hospitalized at the Neonatal Unit of the University Hospital, Botucatu Medical School, between 1990 and 2005.

Identification of *Staphylococcus aureus*

The isolates obtained from the clinical specimens were seeded onto blood agar and stained by the Gram method for the determination of purity and observation of morphology and specific staining. After confirmation of these characteristics, the strains were submitted to catalase and coagulase tests. The genus *Staphylococcus* was differentiated from *Micrococcus* by testing the oxidation and fermentation of glucose, resistance to bacitracin (0·04 U) indicated by the absence of an inhibition halo or the formation of a halo of <9 mm and susceptibility to furazolidone (100 μg) characterized by inhibition halos measuring 15–35 mm in diameter (Baker 1984). After species confirmation, the isolates were stored in nutrient broth containing glycerol at −70°C.

For *Staph. aureus* detection, primers Sa442-1 e Sa-422-2 that amplify a conserved fragment of 108 bp specific for *Staph. aureus* according to Martineau *et al.* (1998) and the primers for detection of the *coa* gene described by Kearns *et al.* (1999) were chosen.

Genotypic identification of coagulase-negative staphylococci

All CNS isolates were submitted to genotypic identification using primers targeting conserved sequences adjacent to the 16S and 23S genes. This method described by Barry *et al.* (1991) and Couto *et al.* (2001) is known as ITS-PCR. This technique was used to reidentify CNS strains previously characterized by phenotypic methods as described in section Identification of *Staphylococcus aureus*, to permit the safe and reliable identification of strains positive for the production of toxins or genes encoding these toxins and, thus, to confirm that these strains are indeed CNS species and not *Staph. aureus* mutants that do not produce coagulase. International ATCC reference strains were used as control for each *Staphylococcus* species.

DNA was extracted as described in section DNA extraction, according to the method of Couto *et al.* (2001) using primers G1 (5′-GAAGTCGTAACAAGG) and L1 (5′-CAAGGCATCCACCGT). Amplification consisted of a denaturation step at 94°C for 4 min, followed by 25 cycles of amplification for 1 min at 94°C, ramping to 55°C for 2 min, 7 min at 55°C, ramping to 72°C for 2 min, 2 min at 72°C and an additional extension step at 72°C for 7 min (Couto *et al.* 2001). The following international reference strains were used as control for the test results: *Staph. aureus* (ATCC 13565), *Staph. aureus* (ATCC 14458), *Staph. aureus* (ATCC 19095), *Staph. aureus* (ATCC 43300), *Staph. aureus* (ATCC 23235), *Staphylococcus auricularis* (ATCC 33753), *Staphylococcus capitis* ssp. *capitis* (ATCC 27843), *Staphylococcus capitis* ssp. *ureolyticus* (ATCC 49325), *Staphylococcus caprae* (ATCC 35538), *Staphylococcus cohnii* (ATCC 49330), *Staphylococcus cohnii* ssp. *cohnii* (ATCC 29974), *Staphylococcus epidermidis* (ATCC 12228), *Staph. epidermidis* (ATCC 35983), *Staphylococcus haemolyticus* (ATCC 29970), *Staphylococcus hominis* (ATCC 27844), *Staphylococcus hominis* ssp. *novobiosepticus* (ATCC 700237), *Staphylococcus lentus* (ATCC 700403), *Staphylococcus lugdunensis* (ATCC 700328), *Staphylococcus saprophyticus* (ATCC 15305), *Staphylococcus schleiferi* ssp. *schleiferi* (ATCC 43808), *Staphylococcus sciuri* ssp. *sciuri* (ATCC 29062), *Staph. simulans* (ATCC 27851), *Staphylococcus xylosus* (ATCC 29979) and *Staphylococcus warneri* (ATCC 10209).

The efficiency of the amplification reactions was monitored by electrophoresis on 3% MetaPhor agarose gel in 1× TBE buffer. The gel was stained with ethidium bromide or Syber Safe (Invitrogen, Carlsbad, CA). The size of the amplified products was compared with a 100-bp molecular standard, and the gels were photographed under UV transillumination.

The results were analysed by the chi-square test when there were no expected values less than five and by the likelihood ratio test for the differences of proportions with an approximate chi-square distribution when the expected values were less than five. Fisher's exact test was used for 2×2 contingency tables. The Tukey's test for the comparison of proportions was used to determine significant differences in the presence of genes and species between the types of clinical material.

Detection of enterotoxin genes

DNA extraction

Total DNA was extracted from *Staphylococcus* strains cultured on blood agar and then inoculated individually into brain–heart infusion broth and incubated for 24 h at 37°C. The Illustra kit (GE Healthcare, Little Chalfont, UK) was used for DNA extraction. First, staphylococcal cells were digested with 10 mg ml^{-1} lysozyme and 20 mg ml^{-1} proteinase K. Next, 500 μl extraction solution was added, and the mixture was centrifuged at 10 000 ***g*** for 4 min. The supernatant was then transferred to a GFX column and centrifuged at 5000 ***g*** for 1 min. The collected fluid was discarded, and 500 μl extraction solution was again added to the column. After centrifugation and discarding of the collected fluid, 500 μl washing solution was added and the column was centrifuged at 20 000 ***g***

for 3 min. The column was then transferred to a 1·5-ml tube and eluted with 200 μl Milli-Q water heated to 70°C.

The extracted DNA was diluted 1 : 100, and absorbance was read at 260 and 280 nm for the determination of concentration and purity of the sample. DNA concentration was calculated as follows: concentration (μg ml^{-1}) = A_{260} × 50 × dilution factor.

Amplification of nucleic acids (PCR)

PCR was carried out in 0·5-ml centrifuge tubes containing 10 pmol of each primer (Johnson *et al.* 1991; Jarraud *et al.* 1999; Mason *et al.* 2001), 2·5 U Taq DNA polymerase, 200 μmol l^{-1} deoxyribonucleotide triphosphates, 20 mmol l^{-1} Tris–HCl, pH 8·4, 0·75 mmol l^{-1} $MgCl_2$ and 3 μl of the sample in a final volume of 25 μl. A negative control in which the nucleic acid was replaced with water was included in all reactions. Amplification was carried out in an MJ Research PTC-100 thermocycler (MJ Research, Waltham, MA) using the following parameters: one cycle at 94°C for 4 min, denaturation at 94°C for 2 min, primer annealing at 55°C and extension at 72°C for 1 min 30 s, followed by a second cycle of denaturation at 94°C for 2 min, annealing at 53°C and extension at 72°C for 1 min 30 s. In the third cycle, the annealing temperature was reduced to 51°C, and 37 cycles were carried out using the last parameters. After completing 40 cycles, the tubes were incubated at 72°C for 7 min and then cooled to 4°C.

Detection of enterotoxins by RT-PCR

Extraction of RNA

Total RNA was extracted from *Staphylococcus* strains cultured on blood agar and then inoculated individually into brain–heart infusion broth and incubated for 24 h at 37°C. RNA was extracted with the Trizol reagent (Invitrogen). Briefly, the cells were lysed with 1·0 ml Trizol, and the homogenate was transferred to 1·5-ml tubes containing 0·2 ml chloroform and again homogenized for 3 min at 15–30°C. Next, the homogenate was centrifuged at 12 000 ***g*** for 15 min at 5°C, and the aqueous phase containing total RNA was collected and transferred to another tube. For RNA precipitation, 250 μl isopropyl alcohol was added and the mixture was incubated at room temperature for 10 min and then centrifuged at 12 000 ***g*** for 10 min at 5°C. The total RNA extracted was washed with 1·0 ml 70% ethanol containing 0·1% diethylpyrocarbonate (DEPC). Purified total RNA was treated with DNase to degrade contaminating genomic DNA (Omoe *et al.* 2002).

The extracted nucleic acid was diluted 1 : 100, and absorbance was read at 260 and 280 nm for determination of the concentration and purity of the sample. RNA concentration was calculated as follows: concentration (μg ml^{-1}) = A_{260} × 40 × dilution factor.

Synthesis of cDNA

In a 0·5-ml microtube (RNase/DNase free), 5 μl of random primers (75 ng μl^{-1}) was mixed with 10 μl RNAguard (20 U; GE Healthcare), 5–45 μl of the sample (2·5 μg total RNA) and 60 μl (qsp) Milli-Q water plus DEPC. The mixture was heated to 70°C for 10 min for RNA denaturation and cooled quickly to 4°C. Next, 20 μl 5× first-strand buffer, 10 μl 0·1 mol l^{-1} dithiothreitol and 5 μl 20 mmol l^{-1} dNTP mix were added, and the reaction was heated to 25°C for 2 min for primer ligation. After heating, 5 μl 20 U Superscript™ reverse transcriptase II (Invitrogen) was added and cDNA was synthesized in an MJ Research PTC-100 thermocycler. The final reaction volume was 100 μl.

The following parameters were used for cDNA synthesis: 25°C for 5 min, 42°C for 50 min and 70°C for 15 min. The reaction was then cooled to 4°C. The cDNA obtained was amplified by PCR, and the amplified products were visualized by electrophoresis as described in section Amplification of nucleic acids (PCR).

Statistical analysis

The results were analysed by the comparison of proportions regarding the presence of genes or mRNA encoding SEs. Tukey's test was used for the comparison of three proportions and a comparison test for proportions when only two groups were analysed. The level of significance was set at $P < 0·05$ for all tests. The results of genotypic and phenotypic identification were compared by Spearman's correlation test.

Results

Identification of *Staphylococcus aureus* and coagulase-negative staphylococci

The distribution of the 180 *Staphylococcus* spp. isolates and of the 90 CNS isolates according to clinical material is shown in Table 1. Genotypic identification showed that *Staph. epidermidis* was the most frequently isolated CNS species among the 90 strains analysed (n = 64, 71·1%), followed by *Staph. warneri* (n = 8, 8·9%) and other species such as *Staph. lugdunensis*, *Staph. haemolyticus*, *Staph. hominis*, *Staph. simulans*, *Staph. xylosus* and *Staph. saprophyticus* (n = 26, 28·9%). No strain of CNS included in the study amplified with primers of *coa* gene and primers specific for *Staph. aureus*, thus confirming them all to be CNS (Fig. 1).

Table 1 Distribution of toxigenic *Staphylococcus* spp. according to clinical material

	Enterotoxin genes			
	Staphylococcus aureus (*n* = 90)	*Staphylococcus epidermidis* (*n* = 64)	Other CNS (*n* = 26)	Total (*n* = 180)
Clinical sample	*N*/Total (%)	*N*/Total (%)	*N*/Total (%)	*N*/Total (%)
Blood	10/24 (41·7)	13/47 (63·8)	9/16 (68·7)	32/87 (36·8)
Catheter	10/20 (50·0)	4/7 (71·4)	1/6	15/33 (45·5)
Cannula	8/12	0/5	1/2	9/19 (47·4)
Chest drain	6/6	1/1	0/0	7/7 (100·0)
Secretion	20/28 (71·4)	0/4	0/2	20/34 (58·8)
Total	54/90 (60·0)	18/64 (28·1)	11/26 (42·3)	83/180 (46·1)

CNS, coagulase-negative staphylococci; *N*/Total, number of toxigenic strains/total number of strains.

Figure 1 (a) Agarose gel electrophoresis for the detection of the gene coa (117 bp) in coagulase-negative *Staphylococcus* strains (CNS) by PCR. Lanes 1–16: negative CNS strains for gene coa; lane 17: negative control (*Staphylococcus epidermidis* ATCC 12228), lane 18: positive control (*Staphylococcus aureus* ATCC 29213); lane 19: water; lane 20: molecular weight marker (100 bp). (b) Agarose gel electrophoresis for the detection of the gene Sa442 (108 bp) in coagulase-negative *Staphylococcus* strains by PCR. Lanes 1–16: negative CNS strains; lane 17: negative control (*Staph. epidermidis* ATCC 12228), lane 18: positive control (*Staph. aureus* ATCC 29213); lane 19: water; lane 20: molecular weight marker (100 bp).

Detection of enterotoxin genes

PCR for the detection of enterotoxin genes revealed the presence of the *see*, *seg*, *seh* and/or *sei* genes in 83 (46·1%) of the 180 staphylococcal strains studied. One or more genes were detected in 54 (60·0%) of the 90 *Staph. aureus* isolates and in 29 (32·2%) of the 90 CNS isolates (Tables 2 and 3; Figs 2 and 3).

Variable combinations of these toxin genes were observed, as well as a high prevalence of strains carrying these genes alone or in combination. For example, the concomitant presence of the *seg* and *sei* genes was observed in 16 *Staph. aureus* isolates and in 10 CNS isolates. In addition, the combination of three genes was detected in two *Staph. aureus* isolates (*see*, *seg* and *sei*) and in one CNS isolate (*see*, *seh* and *sei*). The *seg*/*sei* gene combination predominated in CNS species (Table 3).

Comparison of toxigenicity between the *Staph. aureus* and CNS isolates showed no significant differences between strains. However, a significant difference in toxigenicity was observed between *Staph. aureus* and *Staph. epidermidis* isolates ($P = 0{\cdot}002$), with the former being more toxigenic. No significant differences in toxigenicity were observed between *Staph. epidermidis* and the

Table 2 Frequency of *Staphylococcus aureus* and coagulase-negative staphylococcal species carrying enterotoxin genes

Gene	*Staph. aureus*	CNS	Total
see	–	5	5
seg	20	1	21
seh	8	3	11
sei	1	8	9
see/seg	3	–	3
see/seh	1	–	1
see/sei	–	1	1
seg/seh	2	–	2
seg/sei	16	10	26
see/seg/sei	2	–	2
see/seh/sei	–	1	1
seg/seh/sei	1	–	1
Total	54	29	83

CNS, coagulase-negative staphylococci; *see*, presence of the enterotoxin E gene; *seg*, presence of the enterotoxin G gene; *seh*, presence of the enterotoxin H gene; *sei*, presence of the enterotoxin I gene.

other CNS strains ($P = 0{\cdot}244$) or between the *Staph. aureus* isolates and CNS species other than *Staph. epidermidis* ($P = 0{\cdot}494$).

Statistical analysis of the frequency of each gene revealed some differences. The frequency of the *see* gene did not differ significantly between species ($P > 0{\cdot}05$). In contrast, the *seg* gene was more frequent in *Staph. aureus* when compared with the group of CNS as a whole, *Staph. epidermidis* and other CNS species ($P < 0{\cdot}05$), but no difference was observed among CNS species. No difference in the frequency of the *seh* gene was observed between *Staph. aureus* and *Staph. epidermidis*, but this gene was more frequent in *Staph. aureus* and *Staph. epidermidis* when compared with other CNS species. Similarly, the *sei* gene was more frequent in *Staph. aureus* compared with CNS species, except for *Staph. epidermidis*, but no difference in the frequency of this gene was observed between *Staph. epidermidis* and the other CNS species (Table 3).

The distribution of toxigenic *Staph. aureus* strains according to biological material (blood, foreign body and secretion) is shown in Table 1, and the distribution of toxigenic CNS strains is shown in Tables 1, 2 and 3.

For the analysis of the number of toxigenic *Staphylococcus* spp. in clinical samples, catheter, cannula and chest drain samples were pooled in a group called foreign bodies. This group was compared with blood and secretion samples, and no significant difference ($P > 0{\cdot}05$) in the frequency of enterotoxin genes was observed between strains isolated from these different types of clinical material. The genes studied were detected in *Staph. epidermidis*, *Staph. warneri*, *Staph. haemolyticus*, *Staph. lugdunensis*, *Staph. hominis* and *Staph. simulans*. No enterotoxin genes were found in *Staph. xylosus* and *Staph. saprophyticus*. *Staphylococcus epidermidis* and *Staph. warneri* presented the highest frequency of genes (Table 3).

Determination of the toxigenic profile of the isolates by RT-PCR

RNA was extracted from the *Staph. aureus* ($n = 54$) and CNS ($n = 29$) isolates found to be toxigenic by PCR as described in section Extraction of RNA for the investigation of toxin gene expression by RT-PCR. The internal control of RNA extraction (16S rRNA) was detected in all strains.

RT-PCR used for the detection of mRNA of the genes responsible for the production of SEE, SEG, SEH and SEI revealed the expression of the *seg*, *seg* and/or *sei* genes in 32 (59·3%) of the 54 toxigenic *Staph. aureus* strains,

Table 3 Frequency of coagulase-negative staphylococcal species carrying the genes encoding enterotoxins SEE, SEG, SEH and SEI

Species	Total number of strains	Number of toxigenic strains	Enterotoxin genes						
			see	*see/sei*	*seg*	*seh*	*sei*	*seg/sei*	*see + seh + sei*
Staphylococcus epidermidis	64	18	1	–	1	3	5	7	1
Staphylococcus warneri	8	5	4	1	–	–	–	–	–
Staphylococcus haemolyticus	4	1	–	–	–	–	–	1	–
Staphylococcus lugdunensis	3	3	–	–	–	–	2	1	–
Staphylococcus hominis	6	1	–	–	–	–	1	–	–
Staphylococcus simulans	2	1	–	–	–	–	–	1	–
Staphylococcus saprophyticus	2	–	–	–	–	–	–	–	–
Staphylococcus xylosus	1	–	–	–	–	–	–	–	–
Total	90	29	5	1	1	3	8	10	1

see, presence of the enterotoxin E gene; *seg*, presence of the enterotoxin G gene; *seh*, presence of the enterotoxin H gene; *sei*, presence of the enterotoxin I gene; *seg + sei*, concomitant presence of the enterotoxin G and I genes; *see + seh + sei*, concomitant presence of the enterotoxin E, H and I genes.

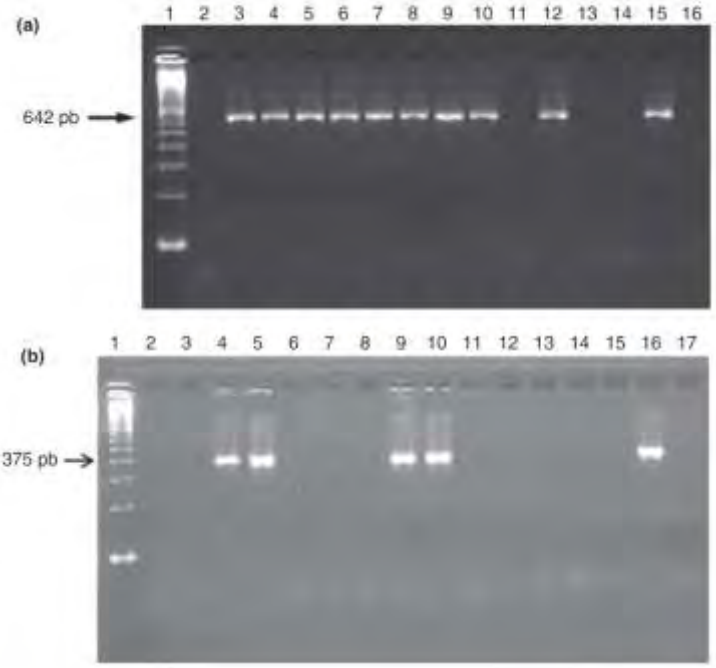

Figure 2 (a) Agarose gel electrophoresis for the detection of *seg* gene (642 bp) in *Staphylococcus aureus* strains by PCR. Note the presence of the gene in isolates 3–10 and 12. Lane 15: positive control; lane 16: negative control. A 100-bp ladder was used as molecular size marker (Lane 1). (b) Agarose gel electrophoresis for the detection of *seh* gene (375 bp) in *Staph. aureus* strains by PCR. Note the presence of the gene in isolates 4, 5, 9 and 10. Lane 16: positive control; lane 17: negative control. A 100-bp ladder was used as molecular size marker (Lane 1).

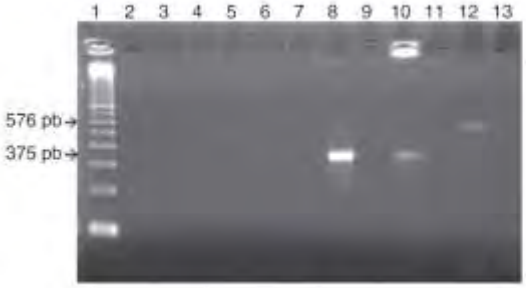

Figure 3 Agarose gel electrophoresis for the detection of the *seh* gene (375 bp) in *Staphylococcus aureus* strains and of the *sei* gene (576 bp) in CNS strains by PCR. Note the presence of the *seh* gene in *Staph. aureus* isolates 8 and 10 and of the *sei* gene in CNS isolate 12. Lane 13: negative control. A 100-bp ladder was used as molecular size marker (Lane 1).

whereas the expression of some of these genes (*seg*, *seh*, *sei* and *seg* + *sei*) was observed in 10 (34·5%) of the 29 CNS isolates. Expression of the *seg*, *seh* and/or *sei* genes was observed in 42 (23·3%) of the 180 staphylococcal isolates studied (Table 4).

Table 5 and Fig. 4 show the strains in which expression of enterotoxin genes was confirmed by RT-PCR. Interestingly, the combination of SEG/SEI was detected in four *Staph. aureus* isolates. None of the 13 *Staph. aureus* and CNS strains carrying the *see* gene showed expression of this toxin when investigated by RT-PCR.

Comparison of toxin expression showed a significantly higher proportion of positive *Staph. aureus* strains when compared with *Staph. epidermidis* ($P < 0·001$) and other CNS ($P = 0·003$). No significant difference in toxin expression was observed between *Staph. epidermidis* and the other CNS species ($P = 0·749$).

When analysing whether a certain toxin was more prevalent in the groups of species, a statistically relevant difference was observed between *Staph. aureus*, *Staph. epidermidis* and the other CNS species, with SEG and SEH being more frequently expressed in *Staph. aureus* than in *Staph. epidermidis* and in the other CNS species; however, there was no difference between *Staph. epidermidis* and the other CNS. On the other hand, expression of SEI did not differ between *Staph. aureus* and *Staph. epidermidis* or between *Staph. epidermidis* and the other CNS, but was more frequent in *Staph. aureus* when compared with the other CNS species, except for *Staph. epidermidis*.

Analysis of the presence of enterotoxin mRNA according to clinical material showed a higher proportion of positive strains among staphylococcal isolates obtained from blood compared with isolates obtained from foreign bodies and secretion ($P < 0·05$).

Table 4 Distribution of staphylococci isolated from newborns according to strain toxigenicity demonstrated by PCR and RT-PCR

	Species					
	Staphylococcus aureus		CNS		Total	
	n	%	*n*	%	n	%
PCR	54	60	29	32·2	83	46·1
RT-PCR	32	59·3	10	34·5	42	50·6

CNS, coagulase-negative staphylococci.

Table 5 Comparison of PCR and RT-PCR between *Staphylococcus aureus* and CNS strains

	PCR		RT-PCR	
Toxin gene/enterotoxin	*Staph. aureus*	CNS	*Staph. aureus*	CNS
see/SEE	–	5	–	–
seg/SEG	20	1	10	–
seh/SEH	8	3	4	1
sei/SEI	1	8	–	3
see + *seg*/SEG	3	–	2	–
see + *seh*	1	–	–	–
see + *sei*/SEI	–	1	–	1
seg + *seh*/SEG + SEH	2	–	1	–
seg + *sei*				
SEG	16	10	2	2
SEI			7	1
SEG + SEI			3	1
see + *seg* + *sei*				
SEI	2	–	1	–
SEG + SEI			1	–
see + *seh* + *sei*/SEI	–	1	–	1
seg + *seh* + *sei*/SEH	1	–	1	–
Total	54	29	32	10

CNS, coagulase-negative staphylococci; *see*, presence of the enterotoxin E gene; *seg*, presence of the enterotoxin G gene; *seh*, presence of the enterotoxin H gene; *sei*, presence of the enterotoxin I gene; SEG, staphylococcal enterotoxin G; SEH, staphylococcal enterotoxin H; SEI, staphylococcal enterotoxin I.

Discussion

Most CNS are natural inhabitants of the human skin and mucosae (Kloos and Musselwhite 1975; Kloos 1980). In view of the growing importance of CNS in nosocomial and community-acquired infections, the identification of these micro-organisms to the species level is fundamental to better understand the pathogenic mechanisms of different CNS species (Heikens *et al.* 2005).

The present study evaluated the toxigenic potential of 90 *Staph. aureus* and 90 CNS strains by the detection of genes encoding SEE, SEG, SEH and SEI using PCR and

Figure 4 Agarose gel electrophoresis for the detection of the expression of SEI (576 bp) in coagulase-negative staphylococci by RT-PCR. Lane 2: RNA without cDNA synthesis (enzyme control); lane 3: isolate producing SEI treated with DNase before cDNA synthesis; lane 4: isolate producing SEI whose RNA was not treated with DNase; lane 5: *Staphylococcus aureus* carrying the *sei* gene used as positive control; lane 6: negative control. A 100-bp ladder was used as molecular size marker (Lane 1).

the capacity of the isolates to express these genes using RT-PCR. Genotypic identification showed that *Staph. epidermidis* was the most frequent species, corresponding to 64 (71·1%) of the 90 CNS strains investigated, followed by *Staph. warneri* (8·9%), in addition to *Staph. haemolyticus*, *Staph. lugdunensis*, *Staph. hominis*, *Staph. simulans*, *Staph. xylosus* and *Staph. saprophyticus*. Similar results have been reported by Udo (1999) and Kloos and Bannerman (1994), who also found a higher frequency of *Staph. epidermidis*. Studies correlating the identification of CNS species with clinical significance have always reported a predominance of *Staph. epidermidis* (>75%) (Gahrn-Hansen 1987; Powell and Sanderson 1987; Couto *et al.* 2001). This predominance might be explained by the prevalence of *Staph. epidermidis* in skin and the fact that this micro-organism possesses virulence factors not present in other CNS species (Baddour *et al.* 1986; Gemmell 1986).

PCR used in the present study was an efficient method for the detection of staphylococcal toxin genes in *Staph. aureus* and CNS, demonstrating the toxigenic capacity of these micro-organisms. Staphylococcal toxins are important virulence factors, and each bacterial strain may or may not carry one or more genes encoding these toxins. Staphylococcal toxin genes were detected by PCR in 83 (46·1%) of the 180 isolates studied, including 54 (60·0%) *Staph. aureus* isolates and 29 (32·2%) CNS isolates.

Among the 64 *Staph. epidermidis* strains investigated, 28·1% carried the *see* (SEE), *seg* (SEG), *seh* (SEH) and *sei* (SEI) genes alone or in combination. However, other CNS species also tested positive by PCR. For example, the *see* gene was detected alone or in combination in five of the eight *Staph. warneri* isolates. Three *Staph. lugdunensis* isolates were also toxigenic, with predominance of the *sei* gene alone or in combination. One *Staph. hominis* isolate was positive for the *sei* gene, and the *seg* and *sei* genes were detected in one *Staph. haemolyticus* isolate. Only one *Staph. simulans* isolate carried the *seg* and *sei* gene combination. None of the genes studied was detected in *Staph. saprophyticus* or *Staph. xylosus* isolates. Predominance of CNS strains carrying the *sei* gene alone or in combination with other genes was observed in the present study.

Some gene combinations detected by PCR call attention because they were observed in various strains, such as the combination of the *seg* and *sei* genes detected in 26 *Staph. aureus* and CNS isolates. Luz (2008) analysed *Staph. aureus* strains isolated from milk and cottage cheese regarding the presence of the *seg*, *seh*, *sei* and *sej* genes and also found a prevalence of strains (25%) carrying the combination of the *seg* and *sei* genes. Other studies also reported a predominance of the *seg/sei* gene combination in *Staph. aureus* strains isolated from clinical and nasal samples (Becker *et al.* 2003; Nashev *et al.* 2004). Similar results regarding the presence of this gene combination were reported by Jarraud *et al.* (1999, 2002) and Omoe *et al.* (2002). Jarraud *et al.* (1999) isolated 12 *Staph. aureus* strains from patients with toxic shock syndrome and staphylococcal scarlet fever that only carried the *seg* and *sei* genes. Jarraud *et al.* (1999, 2002) analysed the distribution of the *seg* and *sei* genes in 230 *Staph. aureus* strains isolated from different clinical materials and demonstrated a prevalence of these genes in strains isolated from nasal carriers and from patients with toxaemia. The coexistence of the *seg* and *sei* genes might be due to their chromosome location as both genes are found in a tandem orientation and belong to an operon, called the enterotoxin gene cluster (*egc*), which contains five enterotoxin genes (*seg*, *sei*, *sem*, *sen* and *seo*) and two pseudogenes (φ *ent1* and φ *ent2*) (Jarraud *et al.* 2001).

The presence of this gene combination indicates a high toxigenic potential of CNS strains that are often overlooked and only considered to be contaminants. This fact emphasizes the importance of this gene combination for the pathogenicity of the staphylococcal strains isolated from clinical samples collected at the University Hospital of the Botucatu Medical School. Furthermore, a combination of three genes was also detected in three *Staph. aureus* isolates (*see*, *seg* and *sei* in two and *seg*, *seh* and *sei* in one) and in one CNS isolate (*see*, *seh* and *sei*), demonstrating a high pathogenic potential of the strain.

Other enterotoxin genes frequently detected in the strains studied were the *seg* and *seh* genes in *Staph. aureus* and the *sei* and *see* genes in CNS. Other enterotoxin genes were also present but at a lower frequency, a fact that does not rule out the pathogenicity of the gene products when expressed. In contrast, other investigators did not detect toxin genes in clinical CNS isolates, for example, Becker *et al.* (2001) using multiplex PCR. These differences in the results might be explained by the different methods used for detection of the genes. Various PCR methods are available for the typing of staphylococcal toxins (Johnson *et al.* 1991; Schmitz *et al.* 1998). Most methods require various steps for the distinction between different enterotoxin (*se*) genes. More recently, a multiplex PCR method was described for the typing of *Staph. aureus* toxins (Becker *et al.* 1998; Monday and Bohach 1999; Mehrotra *et al.* 2000). This technique is based on the combinations of specific primers targeting the *se* gene or the combinations of universal forward primers and specific reverse primers (Sharma *et al.* 2000). One of the disadvantages of multiplex PCR is low sensibility compared with unique PCR, generating a need for a higher initial concentration of the target sequences. This factor is associated with competition between primers for target sequences and reaction components. Because of that and polymorphisms of sequences, preliminary assays are necessary for optimization and determination of more adequate concentrations of reagents and sample's DNA to amplificate all wanted sequences equally (Perry *et al.* 2007). The PCR in the present study was performed separately for each toxin investigated, thus facilitating the detection of isolated genes.

PCR has been shown to be a useful, rapid and reliable tool for the detection of enterotoxin genes (Johnson *et al.* 1991; Becker *et al.* 1998; Tamaparu *et al.* 2001). However, PCR is only able to demonstrate the presence or absence of a certain gene in the strain but does not indicate the production of enterotoxins. Demonstration of the capacity of a strain to produce sufficient amounts of SE to induce disease requires the development of biological assays or immunological methods for the detection of these toxins (Omoe *et al.* 2002). In this respect, RT-PCR permits the detection of messenger RNA sequences responsible for the production of enterotoxins, thus demonstrating gene activity. The presence of toxin mRNA sequences leaves no doubt regarding the toxigenic potential of the micro-organism.

To complement the present study, RT-PCR was used to detect mRNA sequences responsible for the production of the target enterotoxin in staphylococcal strains positive for the presence of some enterotoxin gene. Despite reports demonstrating the presence of toxin genes in CNS species (Cunha *et al.* 2006, 2007), many investigators still

question their toxigenic potential, with the use of this genotypic technique being important for the confirmation of the toxigenicity of CNS species.

The present study revealed the expression of staphylococcal toxin mRNA in 42 (50·6%) of the 83 PCR-positive strains. The mRNA detected corresponded to toxins of the SEG, SEH and SEI classes. Toxin expression was lower in CNS (34·5% of all producing strains) than in *Staph. aureus* isolates (59·3%). *Staphylococcus epidermidis* was the most toxigenic CNS species, with seven isolates expressing mRNA for SEG, SEH and SEI. Among the other CNS species, only *Staph. warneri* and *Staph. lugdunensis* presented a positive RT-PCR result, with the observation of expression of SEI mRNA in the former and of SEG and SEI mRNA in the latter. Udo (1999), investigating food handlers, identified enterotoxin-producing CNS and *Staph. aureus* strains using reverse and passive latex agglutination (RPLA). Toxin production was observed in 34·5% of CNS isolates that carried enterotoxin genes, whereas *Staph. hominis*, *Staph. saprophyticus*, *Staph. xylosus*, *Staph. haemolyticus* or *Staph. simulans* did not produce any of the enterotoxins studied. Marin *et al.* (1992) studied enterotoxin-producing staphylococci in ham samples by RPLA. Among the 135 staphylococcal isolates, two belonged to *Staph. epidermidis* and one was a producer of the classical enterotoxin SEC. In the study of Cunha *et al.* (2007) investigating *Staph. aureus* isolates carrying one or more staphylococcal toxin genes, 46·7% of the isolates tested positive by PCR, whereas production of the respective toxins was only observed in 38·3% of the strains when analysed by RPLA. In the same study, 40% of the CNS strains carried one or more toxin-specific genes and toxin production was confirmed in 26·7% of these isolates by RPLA. In another study investigating virulence factors of CNS isolated from newborns, Cunha *et al.* (2006) demonstrated by RPLA that 37·6% of the strains were producers of the classical enterotoxins SEA, SEB and SEC. Similar results have been reported by Crass and Bergdoll (1986) and Kahler *et al.* (1986) who isolated CNS-producing enterotoxins and TSST-1 from patients with toxic shock syndrome and other infections, as well as from foods related to outbreaks of food poisoning.

The predominance of certain enterotoxin genes alone or in combination observed in some species such as *Staph. epidermidis*, *Staph. warneri* and *Staph. lugdunensis* points to differences in the pathogenic potential of CNS species and demonstrates the importance of correct identification of these micro-organisms. One important step of the present study was the genotypic identification of the CNS isolates by ITS-PCR using conserved primer sequences targeting regions adjacent to the 16S and 23S genes. This method was described by Barry *et al.* (1991) and Couto *et al.* (2001) to rule out the possibility that toxigenic isolates are mutant *Staph. aureus* strains that do not produce coagulase and to confirm the toxigenic capacity of CNS. Other investigators also questioned the capacity of CNS to produce enterotoxins and suggested that these strains might be *Staph. aureus* mutants that do no express the coagulase enzyme (Victor *et al.* 1969; Lotter and Genigeorgis 1975; Fox *et al.* 1996). Therefore, to rule out this possibility, all CNS isolates were submitted to genotypic identification for species confirmation. ITS-PCR produces a characteristic band pattern for each species when compared with the international references species (ATCC). In addition, this method permits to rule out possible errors that may occur in phenotypic identification. Excluding possible identification errors, the divergence in the results obtained regarding the ability of the CNS isolates to produce toxins might be related to the choice of the adequate technique for DNA extraction and detection of genes responsible for the expression of these enterotoxins. Standardization of the techniques includes the use of ideal growth media, pH, temperature and nutrient concentrations, as well as the use of extraction controls, parameters used in the amplification reactions and enzymes that are fundamental for the good performance of the investigation.

Staphylococcus aureus produces a wide variety of virulence factors, including adhesion molecules, peptidoglycan, enzymes and extracellular toxins (Archer 1998). The importance of these micro-organisms as pathogens resides in the combination of toxin-mediated virulence, invasive potential and resistance profile to antimicrobial agents (Le Loir *et al.* 2003). Genetic variation between *Staph. aureus* strains is associated with the pathogenic potential of these strains (Bohach *et al.* 1990; Day *et al.* 2001) and is observed in constitutive and accessory genes (Lan and Reeves 2000). A large number of toxigenic *Staph. aureus* and CNS strains were identified in the present study, with most of these isolates carrying more than one toxin gene, thus demonstrating the toxigenic potential not only of *Staph. aureus* but also of CNS.

In the present study, concomitant expression of various enterotoxin genes was observed especially in *Staph. aureus* isolates. According to Peacock *et al.* (2002), the number of virulence-associated genes carried per bacterial strain is the product of the interaction between rates of gene acquisition, cost for biological maintenance and the rate of decay of the strain causing disease. Because most severe infections caused by *Staph. aureus* cannot be explained by the action of a single virulence factor, it is possible that various factors act together during the infectious process. A significant expression of SEG and SEH by *Staph. aureus* strains was observed in the present study. Similar results have been reported by Luz (2008) who observed the expression of toxin genes in 20% of *Staph. aureus* isolates, with four of

12 isolates expressing only the *seg* gene, four expressing the *seh* gene, one expressing the *sei* gene, one expressing the *seg/seh* combination, one expressing the *seg/sej* combination and one expressing the *seg/sei/sej* combination.

The present results showed the concomitant production of toxins by CNS species, although at a lower proportion when compared with *Staph. aureus*. According to Lisa (2004), at least 34 different extracellular proteins are produced by pathogenic staphylococcal strains, with several of these proteins already possessing a defined role in the pathogenesis of staphylococcal disease. Some of the genes encoding these factors are frequently located on mobile genetic elements such as phages and pathogenicity islands. These elements are potentially mobile DNA segments of variable size that carry virulence-associated genes (Kapper and Kacker 1999) and are transferred horizontally between strains (Betley and Mekalanos 1985; Lindsay *et al.* 1998; Yamaguchi *et al.* 2000; Yoshizawa *et al.* 2000). Mechanisms that facilitate the escape of micro-organisms from the host immune system favour bacterial survival and colonization. As a consequence, strains that present more effective escape mechanisms are more prevalent. The ability of *Staph. aureus* to escape the host defence system is related, at least in part, to its enterotoxin-producing capacity (Lan and Reeves 2000).

The present results highlight CNS as important pathogens and demonstrate their toxigenic potential. Thus, greater attention should be paid to these micro-organisms that are often considered to be mere sample contaminants, especially in the case of samples obtained from neonatal ICU patients whose results should be considered with greater care. Despite the lack of statistical significance, a higher frequency of *Staph. epidermidis* isolates producing detectable amounts of toxins was observed when compared with other CNS species. In addition, most *Staphylococcus* spp. strains expressing these genes were isolated from blood samples, a finding demonstrating that these micro-organisms are important nosocomial pathogens that should not be ignored and considered as contaminants when isolated from blood cultures. In these cases, the laboratory microbiological results need to be analysed together with clinical signs and symptoms of the patient. Sepsis is one of the most common causes of neonatal mortality in hospitals (Koneman *et al.* 1997). Therapeutic procedures such as catheter insertion, infusion techniques and contamination of electrolyte solutions such as parenteral nutrition are frequent sources of bloodstream infection (Le Loir *et al.* 2003).

Molecular methods contribute to the understanding of the complex genetics of staphylococcal virulence mechanisms and permit to determine the pathogenic potential of bacterial isolates by evaluating the presence of genes encoding virulence factors as well as their expression. PCR was found to be a rapid and reliable method for the detection of specific staphylococcal toxin genes. In addition, ITS-PCR permitted to confirm the identification of CNS, thus excluding the possibility that these isolates were *Staph. aureus* mutants. RT-PCR was also found to be a rapid and efficient method that was able to demonstrate the capacity of CNS to express enterotoxin mRNA.

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8. Resistência aos Antimicrobianos

A aquisição de resistência a vários antimicrobianos tem sido o principal problema para o tratamento de infecções causadas por *Staphylococcus* spp. em hospitais e na comunidade.

Na era pré-antibiótica, o prognóstico de tratamento para as infecções estafilocócicas severas era extremamente pobre. A introdução da penicilina na terapêutica em 1944 resolveu temporariamente o problema dessas infecções. No entanto, em 1946 surgiram as primeiras amostras resistentes, com 6% de *S. aureus* produtores de penicilinase. Em 1948, mais de 50% das amostras de *S. aureus* do ambiente hospitalar eram resistentes à penicilina (244). Esta proporção cresceu subsequentemente para cerca de 80 a 90% (245). A disseminação progressiva dessas amostras reduziu drasticamente o valor terapêutico desse antimicrobiano, sendo que atualmente, apenas um pequeno percentual de amostras de *S. aureus* se apresenta sensível. Dados similares são verificados no Brasil (246) e de acordo com os dados de estudo local realizado com amostras de *S. aureus* isoladas de hemoculturas de pacientes do HC da FMB, 93% das amostras de *S. aureus* são resistentes à essa droga (ANEXO 16).

Os ECN também demonstram elevada resistência à penicilina, cerca de 70% segundo estudo realizado por Tavares (247), e > 70% segundo estudo realizado com 745 linhagens de ECN isolados de um hospital universitário na China entre 2004 e 2009 (248). Segundo estudo realizado por Cunha *et al*. (249) a produção da enzima β-

lactamase foi verificada em 89,3% das amostras de ECN isoladas de hemoculturas. A resistência à penicilina é atribuida à produção de enzimas capazes de inibir a ação da droga, conhecidas como penicilinases ou mais genericamente β-lactamases, capazes de hidrolisar o anel β-lactâmico das penicilinas (20). A produção de penicilinase pode ser mediada por plasmídio, mas a integração cromossomal desse gene é frequente (250).

O emprego da meticilina e outras penicilinas semi-sintéticas, tais como a oxacilina e a meticilina resistentes à ação de penicilinases, iniciado em 1959, representou uma etapa significativa na terapia antiestafilocócica. Contudo, a resistência a essas drogas foi detectada cerca de dois anos após (251). As amostras resistentes a meticilina se espalharam rapidamente e sua frequência tem aumentado em diversas regiões geográficas, sendo responsáveis, particularmente por surtos de infecções hospitalares (250). Quando a resistência foi descrita em 1961, a meticilina era utilizada nos testes de sensibilidade e no tratamento das infecções causadas por *S. aureus*. Entretanto, no início de 1990 a oxacilina que é da mesma classe de drogas, foi selecionada como o agente de escolha para tratamento e testes de sensibilidade, sendo a sigla MRSA (*Staphylococcus aureus* resistente à meticilina) ainda utilizada para descrever a resistência devido ao papel histórico (245).

A resistência intrínseca à oxacilina em *S. aureus* é mediada pela produção de uma proteína ligadora de penicilina (PBP) suplementar (PBP 2' ou PBP 2a), que apresenta baixa afinidade às penicilinas semi-sintéticas, sendo o determinante genético desta proteína de natureza cromossômica, o gene *mecA*. Para destruir as bactérias, muitos antibióticos se ligam às PBP de forma a torná-las inativas. Essas

proteínas estão envolvidas na construção da parede celular dos micro-organismos e sem a parede corretamente montada, as bactérias não conseguem manter sua integridade e morrem. Enquanto normalmente as amostras de estafilococos empregam três proteínas ligadoras de penicilina, PBPs 1, 2 e 3 na síntese da parede celular, os estafilococos resistentes à meticilina ou oxacilina (MRSA) possuem um PBP suplementar, PBP 2' ou PBP 2a. Portanto, quando o gene *mecA* está presente, a célula é capaz de crescer na presença de oxacilina e de outros β-lactâmicos (250,252).

Esse gene é carreado em um elemento genético móvel identificado como Cassete Cromossômico Estafilocócico *mec* (SCC*mec*), integrado em um local específico do cromossomo chamado *orfx* e composto pelo complexo do gene *mec*, pelo complexo do gene *ccr* e pelas regiões J. O complexo *mec* é composto do gene *mecA* e de seus genes regulatórios *mecI* e *mecRI,* o complexo do gene *ccr* é responsável pela integração e excisão do SCC*mec* no cromossomo. Já as regiões J não são essenciais ao cassete cromossômico, mas podem carrear genes que codificam resistência a agentes antimicrobianos não β-lactâmicos e a metais pesados (253). Até o momento foram descritos onze tipos de SCC*mec* (**Tabela 1**), que são definidos pela combinação dos tipos de complexo do gene *ccr* e a classe do complexo do gene *mec*, sendo os subtipos definidos por polimorfismos da região J em uma mesma combinação dos complexos *mec* e *ccr* (253).

Embora a resistência mediada pelo gene *mecA* esteja presente em todas as células da população com resistência intrínseca, esta pode ser expressa somente por uma pequena percentagem delas, levando ao que se chama de resistência heterogênea. A expressão de resistência nas linhagens com resistência intrínseca tem

sido categorizadas em quatro classes de expressão fenotípica, classes 1 a 4, com a classe 1 sendo a mais heterogênea e a 4 a homogênea (254).

Na cultura de linhagens com resistência heterôgenea de classe 1 a grande maioria das células (99,9 ou 99,99%) apresentam concentração inibitória mínima (CIM) de 1,5 a 3 μg/ml, mas essa cultura também contém um baixo número de bactérias (10^{-7} a 10^{-8}) que poderiam formar colônias mesmo na presença de 25 μg/ml ou mais de oxacilina. Nas culturas de linhagens de classe 2, a maioria das células (≥ 99,9%) apresentam CIM de 6 a 12 μg/ml, e nessas culturas a frequência de células altamente resistentes (capazes de crescer na presença de 25 μg/ml) é maior (10^{-5}) do que nas linhagens de classe 1 (254). As culturas de linhagens de classe 3 são compostas de bactérias (99 a 99,9%) que apresentam altos níveis de resistência à oxacilina (CIM= 50 a 200 μg/ml), mas geralmente possuem uma subpopulação (10^{-3}) de células altamente resistentes, capazes de formar colônias mesmo na presença de 300 a 400 μg de oxacilina/ml. As culturas de classe 4 são compostas de células com resistência homogênea, com todas as células apresentando altos níveis de resistência, com CIM de 400 a 1000 μg/ml (254).

Tabela 1: Tipos de SCC*mec* identificados em *Staphylococcus aureus*

Tipo SCC*mec*	Complexo gene *mec*	Complexo gene *ccr*	Elementos genéticos Adicionais
I	B(IS*431-mecA-ΔmecR1-ψIS1272*)	1 (A1B1)	
II	A(*IS431-mecA*-ψ*mecR1-mec*I)	2 (A2B2)	pUB110, Tn554
III	A(IS*431- mecA*-ψ*mecR1-mec*I)	3 (A3B3)	SCC_{Hg}, pI258,Tn554, pT181, ψTn554
IV	B(IS*431-mecA-ΔmecR1-ψIS1272*)	2 (A2B2)	
V	C2(IS*431-mecA-ΔmecR1*-IS*431*) *	5 (C1)	
VI	B(IS*431-mecA-ΔmecR1-ψIS1272*)	4 (A4B4)	
VII	C1(IS*431-mecA-ΔmecR1*-IS431)**	5 (C1)	
VIII	A(*IS431-mecA*-Δ*mecR1-mec*I)	4 (A4B4)	Tn554
IX	C2 (IS*431-mecA-ΔmecR1*-IS*431*)	1 (A1B1)	
X	C1(IS*431-mecA-ΔmecR1*-IS431)	7 (A1B6)	
XI	E(blaZ-*mec*A_{LGA}*251-mec*R1*LGA251-mec*I*LGA251*)	8 (A1B3)	

** Duas IS*431* arranjadas na mesma direção.

** Duas IS*431* arranjadas em direções opostas.

pUB110: carreia o gene *ant(4')* que codifica resistência a vários aminoglicosídeos, kanamicina, tobramicina e bleomicina.

Tn554: carreia o gene *ermA* gene, codifica resistência constitutiva e induzível a macrolídeos, lincosamina e estreptogramina. (MLS).

SCC_{Hg}: cassete cromossômico estafilocócico de resistência a metais pesados.

pI258: codifica resistência as penicilinas e metais pesados (mercúrio).

pT181: codifica resistência a tetraciclina.

ψTn554: codifca resistência ao cádmio.

Outras modalidades de resistência também têm também sido descritas em linhagens que não apresentam o gene *mecA* e são denominadas *borderline*. A resistência *borderline* pode ser devido a dois mecanismos, o primeiro seria a inativação de oxacilina mediada pela hiperprodução de β-lactamase (255) e o segundo seria a resistência modificada, chamada de MOD-SA, mediada pela alteração das PBPs intrínsecas com afinidade alterada para a oxacilina (254). Essas modalidades de resistência apresentam baixo nível de resistência, CIM de 8 μg/ml (252).

A expressão fenotípica codificada pelo gene *mecA* é afetada por vários fatores, incluindo pH, temperatura e osmolaridade (256). Quando condições apropriadas são utilizadas para a detecção laboratorial de MRSA, incluindo a suplementação do Ágar Mueller-Hinton com NaCl e a temperatura e o tempo adequados, conforme preconizado pelo *Clinical and Laboratory Standards Institute* (CLSI) (257), a detecção é obtida sem muitas dificuldades. Entretanto, para as linhagens mais heterogêneas, a detecção pode ser mais difícil, mesmo com os métodos de referência (252).

A detecção adequada de resistência à oxacilina mediada pelo gene *mecA* é importante para o laboratório clínico. Embora os métodos preconizados detectem a maioria das linhagens oxacilina resistentes, há duas situações que requerem etapas adicionais para confirmar essa sensibilidade ou resistência. A primeira é a ocorrência de linhagens extremamente heterogêneas e que são determinadas como sensíveis pelos métodos de referência. A segunda é a ocorrência de resistência *borderline* (CIM próximo do *breakpoint* para sensibilidade) que deve ser diferenciada da resistência mediada pelo gene *mecA*, visto que a significância clínica da resistência determinada pelo gene *mecA* é muito maior. Estudos experimentais com animais e alguns dados

clínicos mostraram que o uso de antibióticos β-lactâmicos foram efetivos nas infecções causadas por linhagens sem o gene *mecA* e com baixos níveis de resistência (*borderline*) (258-259). Entretanto, infecções causadas por isolados com o gene *mecA* requerem tratamento com vancomicina (260).

Os métodos de referência preconizados pelo CLSI para a detecção de resistência à oxacilina em *S. aureus* incluem a determinação da CIM pelos métodos de diluição da droga em ágar ou em caldo, o método de difusão com disco, o teste de triagem com ágar Mueller Hinton (MH) adicionado de 4% de NaCl e 6μg de oxacilina e o teste de difusão com disco de cefoxitina (257, 261).

Nos métodos de diluição, a sensibilidade de detectar isolados com resistência mediada pelo gene *mecA* varia de 98 a 100% (262). Embora poucos relatos documentem o grau de heterogenicidade das amostras testadas, é suposto que as linhagens resistentes não detectadas pelos métodos de diluição seriam linhagens mais heterogêneas (252).

Estudos que avaliaram o desempenho da difusão em disco para a detecção de MRSA mostraram que este método é menos confiável para linhagens heterogêneas. Os estudos que usaram linhagens confirmadas com heterogêneas, relataram uma sensibilidade de 61% de um total de 80 linhagens *mecA* positivas, sendo muitas heterogêneas. Felten *et al*. (263) relataram uma sensibilidade de 88,5% para linhagens de classe 1 (extremamente heterogêneas) e de 96,4% para linhagens de classe 2 (263). Velasco *et al*. (264) verificaram que os métodos baseados na difusão em ágar com disco, E-test e microdiluição frequentemente não foram completamente confiáveis na

detecção de linhagens com o gene *mecA.* Do total de 51 amostras positivas para o gene *mecA*, três foram reportadas como falso negativas por esses métodos. Segundo estes mesmos autores o disco de cefoxitina (30 μg) como método de triagem apresentou melhores resultados, com 100% de sensibilidade e 98,0% de especificidade. Resultados similares foram encontrados recentemente por nosso grupo com *S. aureus* isolados de pacientes das Unidades de Pediatria da Faculdade de Medicina de Botucatu, sendo que a detecção pelo método de disco difusão com cefoxitina apresentou 100% de sensibilidade e 98% de especificidade comparado com 94,4% e 98,8% de sensibilidade e especificidade, respectivamente, para o disco de oxacilina (ANEXO 17).

Entretanto estudo realizado pelo mesmo grupo com amostras de *S. aureus* isoladas de hemoculturas de pacientes adultos do mesmo hospital mostrou uma menor sensibilidade para os métodos de disco difusão, com 86,9% e 91,3% de sensibilidade para os discos de oxacilina e cefoxitina, respectivamente e a mesma especificidade (91,3%). O método de triagem apresentou sensibilidade similar (91,3%) ao disco de cefoxitina e a mesma especificidade. Os melhores resultados foram obtidos com o E-test com 97,8% de sensibilidade e a mesma especificidade observada para os outros métodos (ANEXO 16).

Estudo realizado por Cunha *et al*. (249), em isolados de ECN provenientes de pacientes do Hospital das Clínicas da Faculdade de Medicina de Botucatu, mostrou uma taxa de 82,5% de resistência à oxacilina, sendo que a sensibilidade obtida no teste de difusão com discos de oxacilina e cefoxitina foi a mesma (95,3%). O método de E-

test foi o que apresentou maior sensibilidade (98,8%), com a mesma especificidade (83,3%) encontrada nos outros métodos.

Considerando que os métodos fenotípicos para detecção de MRSA podem algumas vezes fornecer resultados questionáveis, tem sido proposto testes moleculares para a detecção do gene *mecA* ou de seu produto PBP 2a. A pesquisa do gene *mecA* usando a técnica da Reação em Cadeia da Polimerase (PCR) é considerada o padrão ouro para detecção de MRSA (261). Segundo o CLSI (261) os testes para detecção do gene *mecA* ou da proteína codificada por esse gene (PBP 2a), são os testes mais adequados para a determinação de resistência à oxacilina e podem ser utilizados para confirmar os resultados obtidos pelos testes com disco nas infecções mais graves.

As amostras MRSA são normalmente resistentes aos demais β-lactâmicos, macrolídeos, aminoglicosídeos, cloranfenicol, quinolonas e tetraciclina (265-266). Com base em observações *in vitro*, o CLSI (261) preconiza que *S. aureus* resistentes à oxacilina, devem ser reportados como resistentes a todos os β-lactâmicos, incluindo cefalosporinas e carbapenens. As infecções causadas por MRSA são então tratadas com um antibiótico glicopeptideo, a vancomicina, introduzida em 1968 e ainda eficaz para o tratamento dessas infecções.

Com o desenvolvimento de *Enterococcus* resistentes à vancomicina em 1988 e o potencial de transferência dessa resistência para *S. aureus*, a vigilância de resistência à vancomicina tem sido objeto de grande interesse científico por todo o mundo. Em 1996, o primeiro isolado clínico de *S. aureus* com sensibilidade reduzida à vancomicina, valor de CIM na faixa intermediária (CIM = 8 μg/ml), chamado de *S. aureus*

intermediário à vancomicina (VISA) foi relatado no Japão (267). Em adição, em junho de 2002, oito pacientes com infecções causadas por *S. aureus* com sensibilidade reduzida à vancomicina, foram confirmados nos Estados Unidos (268). Um mês depois o CDC relatou o primeiro relato de *S. aureus* resistente à vancomicina (VRSA, com CIM = ou $\geq$ 32 μg/ml) em um paciente em Michigan, Estados Unidos. A amostra isolada do paciente continha o gene *vanA* e também o gene *mecA* de resistência à oxacilina. A presença do gene *vanA* neste VRSA sugere que a resistência pode de ter sido adquirida através da passagem de material genético de enterococos resistentes à vancomicina para *S. aureus.* Em outubro do mesmo ano (268) foi documentando o segundo isolado clínico de VRSA em um paciente na Pennsylvania. O isolado VRSA também continha o gene *vanA* e o gene *mecA*. A presença do gene *vanA* também sugere que o determinante de resistência foi adquirido de *Enterococcus* resistente à vancomicina isolado do mesmo paciente. Em abril de 2004, foi relatado o terceiro VRSA isolado de um paciente em Nova York. O isolado também continha os genes *mecA* e *vanA* de resistência à oxacilina e vancomicina, respectivamente. Segundo o CDC os três isolados VRSA não eram epidemiologicamente relacionados (268-269).

O CDC (270) confirmou recentemente o décimo primeiro caso de *S. aureus* resistente à vancomicina (VRSA) nos Estados Unidos (**Tabela 2**). Esses dados reforçam a importância do papel dos laboratórios clínicos no diagnóstico de casos de VRSA para assegurar o pronto reconhecimento, isolamento e controle da infecção.

Tabela 2: Histórico dos casos de *Staphylococcus aureus* Resistentes à Vancomicina (VRSA) nos Estados Unidos e informação geográfica.

Caso	Estado	Ano	Idade	Origem	Diagnóstico	Doenças de base
1	Michigan	2002	40	Úlcera plantar e cateter	Infecção de tecido mole	Diabetes, diálise
2	Pennsylvania	2002	70	Úlcera plantar	Osteomielite	Obesidade
3	New York	2004	63	Urina de tubo de nefrostomia	Sem infecção	Esclerose Múltipla Diabetes, Cálculos renais
4	Michigan	2005	78	Ferida no dedo do pé	Gangrena	Diabetes, doença vascular
5	Michigan	2005	58	Ferida cirúrgica após paniculectomia	Infecção de sítio cirúrgico	Obesidade
6	Michigan	2005	48	Úlcera plantar	Osteomielite	Úlcera Crônica
7	Michigan	2006	43	Ferida no Tríceps	Fasciíte Necrotizante	Diabetes, diálise, úlcera crônica
8	Michigan	2007	48	Ferida no dedo do pé	Osteomielite	Diabetes, obesidade, úlcera crônica
9	Michigan	2007	54	Ferida de sítio cirúrgico após amputação do pé	Osteomielite	Diabetes, encefalopatia hepática
10	Michigan	2009	53	Ferida plantar	Infecção de tecido mole	Diabetes, obesidade, lupus, artrite reumatóide
11	Delaware	2010	64	Drenagem de ferida	Infecção protética	Diabetes, doença renal, diálise

Fonte: CDC (270)

A prescrição do antimicrobiano apropriado por profissionais de saúde e a adesão às diretrizes para controle e vigilância de MRSA e enterococos resistentes à vancomicina (VRE) são necessárias para evitar o surgimento de mais cepas VRSA.

No Brasil, cepas VISA foram descritas por Oliveira *et al*. (271), sendo quatro cepas isoladas de pacientes da Unidade de Queimados e uma cepa de paciente da Unidade de Ortopedia, com todos os pacientes submetidos ao tratamento com vancomicina por mais de 30 dias. O desenvolvimento da sensibilidade intermediária à vancomicina pode estar relacionado com o contato prolongado do micro-organismo com esse antimicrobiano, despertando uma preocupação epidemiológica para a detecção e controle dessa resistência nos hospitais brasileiros. A resistência intermediária a vancomicina pode envolver alterações metabólicas com espessamento da parede bacteriana, devido à aceleração na síntese de peptidoglicano. Assim, mais sítios D-alanil-D-alanina estão disponíveis para a ligação da vancomicina, que acaba se esgotando e não inibe a síntese do peptidoglicano, levando ao espessamento da parede (272).

Assim como *S. aureus*, quando os ECN são multiresistentes aos antibióticos comumente usados, o glicopeptídeo vancomicina tem sido considerado como o antibiótico de escolha (25). Entretanto, Veach *et al*. (273) isolaram linhagens de *S. haemolyticus* resistentes à vancomicina de pacientes submetidos à terapia prolongada com esse antimicrobiano. As linhagens de *S. haemolyticus* isoladas dos pacientes que estavam em tratamento com vancomicina mostraram uma diminuição na suscetibilidade a essa droga quando comparadas com as linhagens isoladas antes da antibioticoterapia (273). Embora sejam raras, essas linhagens podem ser o sinal do

início da resistência a um importante antibiótico usado no tratamento das infecções estafilocócicas.

No ano de 2006, foi isolado de uma criança de cinco anos no Hospital San Jerônimo de Monteria na Colômbia, uma cepa de *S. cohnii* proveniente de líquido pleural com resistência a vancomicina. Este paciente se apresentava em terapia prolongada com vancomicina e a concentração inibitória mínima (CIM) pelo método de E-test foi de 64 μg/ml. O mesmo isolado apresentou resistência a oxacilina, teicoplamina, ceftazidima e trimetropim-sulfametoxazol (274).

Outro relato, desta vez em amostras de ECN provenientes de pacientes saudáveis foi descrito por Palazzo *et al*. (275) no Brasil. Um total de quatro amostras de *Staphylococcus* spp. (1 *S. epidermidis*, 1 *S. haemolyticus* e 2 *S. capitis*) resistentes a vancomicina foram isoladas de trabalhadores de uma escola particular e de um hospital localizado na região de Ribeirão Preto. A concentração inibitória mínima que estas amostras apresentaram quando testadas pela técnica de E-test variou entre 16 μg/mL e ≥256 μg/mL. Nos estudos realizados por Palazzo *et al*. (275) o gene *van* não foi o mecanismo responsável pela resistência de suas amostras, pois os genes *vanA* e *vanB* não foram detectados, sugerindo que a espessura da parede bacteriana pode ter contribuído para a resistência à vancomicina.

A escolha de um método laboratorial adequado influencia na habilidade da vigilância da resistência aos glicopeptídeos e faz com que a prevalência mundial efetiva dos isolados seja ainda desconhecida (245). Apesar do teste de disco difusão ser o mais utilizado na rotina laboratorial, não é o teste recomendado para a detecção de VISA ou

VRSA (261, 270). Os métodos recomendados pelo CDC (270) e CLSI (261) são a determinação da CIM ou o teste de triagem em ágar BHI contendo 6 μg/mL de vancomicina. Todos *Staphylococcus* spp. que apresentarem crescimento na triagem devem ser confirmados em relação à presença de cultura pura e testados por um método para determinação da CIM da vancomicina. Assim como MRSA, o VISA expressa resistência de maneira heterogênea. As colônias que expressam a resistência à vancomicina possuem crescimento mais lento, dificultando a detecção por métodos rotineiros usados para testes de sensibilidade na maioria dos laboratórios clínicos (disco difusão). Por essa razão, *S. aureus* com CIM entre 2 e 4 μg/mL devem ser criteriosamente analisados (245). *Staphylococcus* spp. com sensibilidade diminuída à vancomicina apresentam opções de tratamento limitadas e requerem precauções do controle da infecção para diminuir a transmissão e minimizar a instalação de possíveis surtos.

Outras classes mais recentes de antibióticos, como estreptograminas (quinupristina/dalfopristina), oxazolidona (linezolida), glicilciclina (tigeciclina) e lipopeptídeos (daptomicina), podem ser opções no tratamento de VISA ou VRSA. Entretanto, nos estafilococos já foram selecionados alguns mecanismos específicos de resistir aos novos antibióticos. Embora estes isolados resistentes sejam raros, sob um ponto de vista pessimista pode-se dizer que uma vez que já existam cepas resistentes, a vida útil destes novos antibióticos já está ameaçada. Sob um ponto de vista mais otimista, pode-se dizer que a disponibilidade de várias alternativas antimicrobianas contra os micro-organismos multiresistentes, pode atrasar a disseminação da

resistência (276). O uso criterioso dessas novas classes é, no entanto, imperativo para que possamos preservar essas opções terapêuticas.

ANEXO 16

MARTINS A, PEREIRA VC, CUNHA MLRS. Oxacillin Resistance of *Staphylococcus aureus* Isolated from the University Hospital of Botucatu Medical School in Brazil. Chemotherapy.2010; 56: 112-9.

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Oxacillin Resistance of *Staphylococcus aureus* Isolated from the University Hospital of Botucatu Medical School in Brazil

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Key Words
Cefoxitin · E-test · *mecA* · MRSA · Oxacillin · *Staphylococcus aureus*

Abstract
Background: Oxacillin is the main drug used for the treatment of *Staphylococcus aureus* infections. However, resistance of *S. aureus* to oxacillin has become a major problem over recent decades. The aim of this study was to determine oxacillin resistance in *S. aureus* isolates obtained from the University Hospital of the Botucatu Medical School, Universidade Estadual Paulista, Brazil. ***Materials and Methods:*** A total of 102 isolates collected between 2002 and 2006 were analyzed by detection of the *mecA* gene, cefoxitin and oxacillin disk diffusion methods, screening test on Mueller-Hinton agar and E-test. ***Results:*** Forty-six (45.1%) isolates were *mecA* positive. The oxacillin disk diffusion method showed 86.9% sensitivity and 91.1% specificity. The screening method and cefoxitin disk diffusion presented a similar sensitivity (91.3%) and the same specificity. The E-test showed 97.8% sensitivity and the same specificity as observed for the other methods. ***Conclusions:*** The E-test yielded the best results compared to the other methods.

Introduction

Among the wide variety of bacterial infections, those caused by the genus *Staphylococcus* have a marked impact on human health. The main species, *Staphylococcus aureus*, causes many types of infections ranging from skin infections, such as impetigo and folliculitis, to systemic manifestations, including pneumonia, bacteremia and osteomyelitis. The drug of choice for the treatment of infections caused by *S. aureus* is oxacillin. Penicillinase-resistant drugs introduced in 1959 represented a significant step in anti-staphylococcal treatment. The first report of oxacillin resistance was published in 1961, shortly after the introduction of the drug [1]. The first case of oxacillin resistance was reported in the United Kingdom in 1962, followed by reports in several European hospitals and eventually in hospitals worldwide [2].

Today, methicillin-resistant *Staphylococcus aureus* (MRSA) is a global problem, and studies conducted throughout Europe, Africa, America and Asia have shown MRSA prevalence compared to methicillin-susceptible isolates [1, 3]. Resistance of *S. aureus* to oxacillin is mainly associated with the presence of the *mecA* gene; however, other types of resistance, called borderline resistance, have also been identified in strains that do not car-

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ry this gene. Borderline resistance is the result of two mechanisms: inactivation of oxacillin mediated by the hyperproduction of β-lactamase [4, 5], or a modified resistance, called MOD-SA, which is mediated by the production of modified penicillin-binding proteins with altered affinity for oxacillin [4].

Oxacillin resistance of *S. aureus* can be detected by genotypic and phenotypic methods. The former present high sensitivity and specificity, whereas the latter are of low cost and easy to perform; however, phenotypic methods present lower sensitivity and specificity compared to molecular tests [1].

The standard methods adopted by the Clinical and Laboratory Standards Institute (CLSI) for the detection of oxacillin resistance in *S. aureus* include the determination of the minimum inhibitory concentration (MIC) by agar or broth dilution, disk diffusion and screening test on Mueller-Hinton agar supplemented with 6 μg/ml oxacillin and 4% NaCl. Recently, the cefoxitin disk diffusion method has also been included [6–8]. Although the E-test has not been standardized by the CLSI, this method currently presents an alternative for MIC determination. In the study of Tveten et al. [9], the agar dilution test showed 97.6% sensitivity and 95.4% specificity, whereas the E-test presented 100% sensitivity and 95.4% specificity, thus demonstrating that the E-test is a good phenotypic test for determining *S. aureus* resistance.

Antimicrobial treatment of *S. aureus* infections has become increasingly more restricted due to the increase in oxacillin resistance over the past few years. The aim of the present study was to identify oxacillin resistance in *S. aureus* by disk diffusion methods, screening test, E-test and detection of the *mecA* gene by PCR. *S. aureus* isolates were collected from blood cultures of patients hospitalized in different wards of the University Hospital of Botucatu Medical School between 2002 and 2006.

Materials and Methods

Sampling

A total of 102 *Staphylococcus* spp. isolates were included in this study. The isolates were collected from blood cultures of patients hospitalized in different wards of the University Hospital of Botucatu Medical School between 2002 and 2006 and stored in the culture collection of the Department of Microbiology, Institute of Biosciences, Botucatu. Strains were isolated according to Koneman et al. [10].

Identification of S. aureus

The isolates obtained from the clinical specimens were seeded onto blood agar and stained by the Gram method in order to guarantee their purity and to observe their morphology and specific staining. After confirmation of these characteristics, the strains were submitted to catalase and coagulase tests. The genus *Staphylococcus* was differentiated from the genus *Micrococcus* by the oxidation-fermentation test and by bacitracin resistance (0.04 U). Bacitracin resistance was identified by a growth inhibition zone of up to 9 mm in diameter or the absence of such a zone. In addition, the isolates were tested for susceptibility to furazolidone (100 mg), characterized by a growth inhibition zone ranging from 15 to 35 mm in diameter [11]. The coagulase test was performed in order to differentiate *S. aureus* from coagulase-negative staphylococci [10]. Fermentation of maltose, trehalose and mannitol and resistance to polymyxin B were tested to differentiate *S. aureus* from other coagulase-positive staphylococci such as *S. intermedius*, *S. schleiferi* subsp. *coagulans* and *S. hyicus*. After species confirmation, the isolates were stored at –70°C in nutrient broth containing glycerol. Isolates whose phenotypic identification was difficult were submitted to PCR for amplification of the *Sa 442* gene according to the protocol described by Martineau et al. [12], using the primers *Sa442-1* (5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3') and *Sa442-2* (5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3').

Agar Dilution and Cefoxitin and Oxacillin Disk Diffusion Methods

Susceptibility to oxacillin was determined by the agar dilution method using impregnated disks according to the criteria established by the CLSI [6, 13]. Inocula were prepared by incubation in brain heart infusion broth for 24 h and then adjusted to 0.5 McFarland turbidity standard. Oxacillin (1 μg) and cefoxitin (30 μg) disks were used. *S. aureus* reference strains American Type Culture Collection (ATCC) 25923 (oxacillin susceptible and *mecA* negative) and ATCC 33591 (oxacillin resistant and *mecA* positive) were used as controls in all experiments.

Screening Test on Mueller-Hinton Agar Supplemented with 6 μg/ml Oxacillin and 4% NaCl

The screening test using Mueller-Hinton agar supplemented with 6 μg/ml oxacillin and 4% NaCl was performed in order to determine the resistance of *S. aureus* to oxacillin [14]. Inocula were prepared by incubation in brain heart infusion broth for 24 h and then adjusted to 0.5 McFarland turbidity standard. After preparation of the inoculum, the strains were spot inoculated onto the agar surface with a sterile swab. The plates were incubated at 35°C for 24 h. The presence of MRSA was confirmed by the growth of at least one colony on the agar surface.

DNA Extraction

Genomic DNA was extracted from *S. aureus* strains cultured on blood agar and individually inoculated into brain heart infusion broth and incubated at 37°C for 24 h. Extraction was performed using the illustra blood genomicPrep Mini Spin Kit (GE Healthcare) according to the manufacturer's instructions.

DNA Amplification

PCR was performed in 0.5-ml microcentrifuge tubes containing 10 pmol of each primer, 2.0 U Taq DNA polymerase, 100 μM triphosphate deoxyribonucleotides, 10 mM Tris-HCl, pH 8.4, 0.75 mM $MgCl_2$ and 3 μl DNA in a total volume of 25 μl. The following primers were used: *mecA1* (AAA ATC GAT GGT AAA GGT

Table 1. Determination of oxacillin sensitivity in *S. aureus* samples by phenotypic and genotypic methods

PCR*	Phenotypic methods, n (%)							
	disk diffusion							
	oxacillin		cefoxitin		screening		E-test	
	S	R	S	R	S	R	S	R
mecA+ (n = 46)	6 (5.9)	40 (39.2)	4 (3.9)	42 (41.2)	4 (3.9)	42 (41.2)	1 (1.0)	45 (44.1)
mecA– (n = 56)	51 (50)	5 (4.9)	51 (50)	5 (4.9)	51 (50)	5 (4.9)	51 (50)	5 (4.9)
Total (n = 102)	57 (55.9)	45 (44.1)	55 (53.9)	47 (46.1)	55 (53.9)	47 (46.1)	52 (51.0)	50 (49.0)

Screening: Mueller-Hinton agar supplemented with 6 μg/ml oxacillin + 4% NaCl, oxacillin S (1 μg): ≥13 mm – sensitive, oxacillin R (1 μg): ≤12 mm – resistant, cefoxitin S (30 μg): ≤21 mm – resistant, cefoxitin R (30 μg): ≥22 mm – sensitive. S = Oxacillin-sensitive strain; R = oxacillin-resistant strain.

TGG) and *mecA2* (AGT TCT GCA GTA CCG GAT TTG), as described by Murakami et al. [15]. The reactions were carried out in an appropriate thermal cycler. Reference strains (ATCC) were used in all reactions, with *S. aureus* ATCC 33591 serving as positive control and *S. aureus* ATCC 25923 as negative control.

Determination of MICs of Drugs by the E-Test

The *S. aureus* isolates were tested for in vitro sensitivity against the following drugs: oxacillin, netilmicin, erythromycin, sulfamethoxazole-trimethoprim and vancomycin. For this purpose, the MICs of these drugs were determined by the E-test. The concentrations of the different drugs used in the E-test ranged from 0.016 to 256 μg/ml for oxacillin, erythromycin, netilmicin and vancomycin, and from 0.002 to 32 μg/ml for trimethoprim-sulfamethoxazole (proportion of 1/19). Isolates were considered to be sensitive and resistant based on the breakpoints (μg/ml) indicated by the CLSI in 2008 (netilmicin ≤12 sensitive, ≥32 resistant; vancomycin ≤4 sensitive, ≥32 resistant; oxacillin ≤2 sensitive, ≥4 resistant; erythromycin ≤5 sensitive, ≥8 resistant, and trimethoprim-sulfamethoxazole ≤2/38 sensitive, ≥8/152 resistant). For the analysis of the results, isolates presenting intermediate levels were considered to be resistant. The results of the study of the MICs of the various drugs are expressed as MIC_{50} (concentration of the drug necessary for 50% inhibition of the bacterial population tested) and MIC_{90} (concentration necessary for 90% inhibition of the bacterial population). The range of MICs and proportion of isolates sensitive to each drug were determined according to the definition of the CLSI [15].

Determination of β-Lactamase Production and Hyperproduction

The production of β-lactamase by *S. aureus* isolates was determined using disks impregnated with nitrocefin (chromogenic cephalosporin-cefinase BBL) and placed on the *S. aureus* colonies previously incubated at 35°C for 24 h on a Mueller-Hinton agar plate containing an oxacillin E-test strip. A positive reaction was defined as the development of red staining and a negative reaction as the lack of alteration in color. International reference strains were used as positive (*S. aureus* ATCC 33591 and ATCC 25923) and negative controls (*Staphylococcus xylosus* ATCC 29979). Hyperproduction of β-lactamase was determined by the amoxicillin (20 μg)-clavulanic acid (10 μg) disk test. The breakpoint for sensitivity consisted of the formation of an inhibition halo of ≥20 mm after 24 h of incubation at 35°C [16].

Statistical Analysis

Sensitivity and specificity tests [17, 18] were performed to compare the agar dilution method, cefoxitin and oxacillin disk diffusion methods and screening tests with PCR. The last method is considered to be the gold standard for the detection of intrinsic oxacillin resistance (detection of the *mecA* gene). Sensitivity was defined as the proportion of PCR-positive *S. aureus* isolates (detection of the *mecA* gene) that were oxacillin resistant by the phenotypic methods. Specificity was defined as the proportion of PCR-negative *S. aureus* isolates (lack of detection of the *mecA* gene) that were oxacillin resistant by the phenotypic methods.

Results

Isolates

A total of 102 isolates were identified as *S. aureus* based on morphological, biochemical and genotypic characteristics. Forty-seven of the 102 isolates included in the present study were not resistant to polymyxin B, one of the criteria used for the differentiation of *S. aureus* from other coagulase-positive staphylococci. These isolates were tested by PCR for the detection of the *Sa442* gene and all of them were positive.

Oxacillin and Cefoxitin Disk Diffusion Methods

Forty-five (44.1%) of the 102 *S. aureus* isolates were resistant to oxacillin and 57 (55.9%) were susceptible by the oxacillin disk diffusion method (table 1). Using the cefoxitin disk diffusion method, 47 (46.1%) isolates were resistant to oxacillin and 55 (53.9%) were sensitive (table 1).

Table 4. Multidrug resistance in MRSA and MSSA isolates

Resistance, n	MRSA, n (%)	MSSA, n (%)	p value
0	5 (10.9)	44 (78.6)	<0.001
1	1 (2.2)	5 (8.9)	0.346
2	2 (4.3)	1 (1.8)	0.880
3	38 (82.6)	6 (10.7)	<0.001
Total	46 (100.0)	56 (100.0)	

MRSA: Oxacillin-resistant *S. aureus* by PCR (*mecA*+); MSSA: oxacillin-sensitive *S. aureus* by PCR (*mecA*–).

Table 5. Comparison between the detection of the *mecA* gene and the oxacillin and cefoxitin disk diffusion tests, screening method and E-test

Phenotypic test	*mecA*		Sensitivity %	Specificity %
	positive (n = 46)	negative (n = 56)		
Cefoxitin disk, 30 µg	42	51	91.3	91.1
Oxacillin disk, 1 µg	40	51	86.9	91.1
E-test	45	51	97.8	91.1
Screening*	42	51	91.3	91.1

* Mueller-Hinton agar supplemented with 6 µg/ml oxacillin and 4% NaCl.

(78.5%) of the MSSA isolates were sensitive to all drugs tested.

Determination of β-Lactamase Production and Hyperproduction

Ninety-five (93%) of the 102 *S. aureus* isolates included in the present study were producers of β-lactamase. Five isolates were negative for the gene *mecA*, but were resistant to oxacillin by the phenotypic methods used, being sensitive to the disk of amoxicillin-clavulanic acid, confirming resistance to oxacillin mediated by hyperproduction of β-lactamase.

Distribution of MRSA Isolates among the Wards of the University Hospital of Botucatu Medical School

Forty-six oxacillin-resistant *S. aureus* isolates were detected by PCR in the following hospital units: 7 (15.2%) in the intensive care unit, 6 (13%) in the surgery ward, 5 (10.9%) in the cardiology ward, 5 (10.9%) in the general medicine ward and 5 (10.9%) in the emergency room. MRSA percentages ranged from 2.2 to 6.5% in the remaining wards.

Evolution of Oxacillin Resistance at the University Hospital of Botucatu Medical School between 2002 and 2006

Among the 102 *S. aureus* strains isolated between 2002 and 2006, a gradual increase in oxacillin-resistant isolates was observed from 2002 (42.1%) to 2004 (55.0%). However, the following reductions in MRSA isolates were found between 2004 and 2006: 55% in 2004, 45% in 2005 and 34.6% in 2006.

Statistical Analysis

The sensitivity and specificity of the phenotypic methods for the detection of oxacillin resistance were calculated using *mecA* gene detection as the gold standard (table 5). The oxacillin disk diffusion method showed a sensitivity of 86.9% and specificity of 91.1%, whereas the cefoxitin disk diffusion method presented a higher sensitivity (91.3%) and the same specificity (91.1%). The sensitivity and specificity of the screening test were the same as those of the cefoxitin test. The E-test presented the highest sensitivity (97.8%) and the same specificity (91.1%) as the other methods (table 5).

Discussion

The prevalence of MRSA has been growing considerably in hospitals worldwide. In the present study, 46 (45.1%) oxacillin-resistant isolates were detected by PCR. These results agree with Wey et al. [4] who reported an oxacillin resistance rate of about 50% in university hospitals. Intrinsic oxacillin resistance in *S. aureus* is mediated by the production of a supplemental penicillin-binding protein (PBP2′ or PBP2a), which is encoded by the chromosomal *mecA* gene and shows low affinity for semisynthetic penicillin. This gene is identical in all staphylococcal strains and is therefore considered to be a useful molecular marker of oxacillin resistance [19].

Screening tests play an important role in the detection of isolates that are susceptible or resistant to oxacillin since resistance to vancomycin has already been reported. Therefore, few antibiotics remain for prophylaxis of these infections. Oxacillin resistance has been a major problem in the treatment of *Staphylococcus* infection, mainly because of the heterogeneous expression of oxacillin resistance [9].

The present results showed low sensitivity (86.9%) of the disk diffusion method for the detection of oxacillin resistance. Swenson [20] reported low sensitivity of disk diffusion tests, ranging from 61 to 88.5%. Studies evaluating the performance of disk diffusion tests for the detection of MRSA showed that this method is less reliable in the case of heterogeneous strains [21].

In the present study, the specificity of the oxacillin disk diffusion method was 91.1%. Higher specificity rates have been reported by Boutiba-Ben Boubaker et al. [22] (99.1%) and by Zhu et al. [23] (98.3%).

Cefoxitin is considered to be an excellent inducer of *mecA* gene expression [24]. Recent studies evaluating the cefoxitin disk diffusion method for the detection of methicillin resistance showed good performance of the test, with sensitivity and specificity of ~100% [24–26]. However, in the present study, the specificity of the cefoxitin disk diffusion method (91.1%) was similar to that of the oxacillin disk. Some investigators reported better results with the cefoxitin disk compared to the oxacillin disk [24, 27, 28]. The quality of the impregnated cefoxitin disks is a relevant factor for the detection of oxacillin resistance by oxacillin and cefoxitin disk diffusion methods. Souza Antunes et al. [29] reported differences in the detection of oxacillin resistance between different brands of oxacillin and cefoxitin disks.

Zhu et al. [23] observed a higher sensitivity (96.6%) and specificity (94.9%) of the cefoxitin disk diffusion method than those found in the present study. However, the rates obtained by these authors are lower than those reported in most studies showing a sensitivity of ~100%. A possible explanation for this difference in sensitivity and specificity might be related to heterogeneous resistance among isolates. Although few studies regarding heterogeneity among isolates are available, one may suppose that the resistant strains not detected by diffusion methods are more heterogeneous.

The sensitivity and specificity of the screening test were 91.3 and 91.1%, respectively. The performance of the screening test using Mueller-Hinton agar supplemented with 6 μg/ml oxacillin and 4% NaCl also depends on the level of heterogeneity among the strains tested. Sensitivity was lower (<95%) in studies including a larger number of heteroresistant strains, although sensitivity >97% was reported in other studies [20, 21]. Perez et al. [8], investigating isolates from hospitals located in the southern region of Brazil, obtained a higher sensitivity (98.5%) and specificity (100%) with the screening test compared to *mecA* gene detection. In a comparative study, Swenson [20] evaluated different methods for the identification of MRSA isolates, and the screening test presented 90% sensitivity and 92% specificity, in agreement with the present results. Laboratory detection of MRSA is usually reliable when suitable conditions are used, including the supplementation of Mueller-Hinton agar with NaCl, and temperature and incubation periods as recommended by the CLSI. However, detection of some heterogeneous strains might be more difficult, even when standard methods are used [20].

The recent introduction of molecular techniques for the detection of oxacillin resistance has shown excellent results. These techniques are based on the detection of the *mecA* gene, which encodes the major mechanism of oxacillin resistance. In the present study, 46 (45.1%) of the 102 isolates were *mecA* positive. A similar rate (41.3%) of *mecA*-positive *S. aureus* isolates was reported by Perez et al. [8] in a study conducted at hospitals located in the southern region of Brazil.

Although 100% sensitivity of phenotypic methods using cefoxitin has been reported in the literature, a lower sensitivity was observed for the phenotypic methods tested here. These findings emphasize the importance of molecular techniques for the confirmation of oxacillin resistance in *S. aureus* isolated from serious infections in order to provide adequate treatment and to prevent MRSA transmission in hospital settings.

The E-test, an alternative to the microdilution assay, presented higher sensitivity (97.8%) in the detection of oxacillin resistance than the other phenotypic methods and the same specificity (91.1%). Although the E-test is not the standard method for the detection of oxacillin resistance in *S. aureus*, several investigators who compared this method with broth microdilution and agar dilution obtained similar or better results with the E-test [9, 30, 31]. MIC determination methods are time-consuming and therefore cannot be routinely used for surveillance of MRSA. In this respect, the E-test is a good phenotypic test which is easier to perform.

With respect to multidrug resistance of the MRSA and MSSA isolates, the percentages of MRSA isolates resistant to erythromycin and trimethoprim-sulfamethoxazole were similar to those reported by Chi et al. [32] (89.1% resistant to erythromycin and 86.9% resistant to trimethoprim-sulfamethoxazole). The MIC_{50} and MIC_{90} of trimethoprim-sulfamethoxazole and netilmicin were higher in MRSA isolates, and a difference in the MIC_{50} of erythromycin was observed between MRSA and MSSA isolates. The finding of a higher MIC of these drugs in MRSA isolates might be related to the prevalence of the *SCCmec* cassette type present in each hospital, since the

cassettes found in nosocomial isolates normally carry resistance to erythromycin and trimethoprim-sulfamethoxazole [33, 34].

Although no association exists between the chromosomal cassettes that induce resistance to oxacillin in *Staphylococcus* and resistance to netilmicin, the frequency of resistance to the latter was also high. Anupurba et al. [35] reported resistance to netilmicin in 47.5% of MRSA isolates, a rate lower than that found in the present study (78.3%).

The tests used for the detection of oxacillin resistance in the hospital of the present study revealed a higher prevalence of MRSA in the intensive care unit, in agreement with Cavalcanti et al. [36], who also reported a high prevalence of MRSA isolates in intensive care units.

Other modalities of resistance, called borderline resistance, have been reported for strains that do not carry the *mecA* gene (MIC value close to the susceptible breakpoint). In the present study, 5 isolates were resistant to oxacillin by the phenotypic methods, did not carry the *mecA* gene and presented hyperproduction of β-lactamase (borderline). Experimental animal studies and some clinical data have shown that β-lactam antibiotics were effective against infections caused by strains that did not carry the *mecA* gene and presented low resistance levels (borderline) [37, 38]. On the other hand, infections caused by *mecA*-positive isolates require vancomycin treatment [34].

A reduction in the frequency of MRSA was observed over the last 2 years (2005 and 2006) in the hospital where the study was carried out, in contrast to the gradual increase in the percentage of MRSA during the first 3 years. The reduction in MRSA was particularly sharp in 2006, a finding that might be related to improvements in hospital infection control practices and in the rational use of antibiotic therapy.

An accurate detection of *mecA*-mediated oxacillin resistance is important for clinical laboratories. Although standard methods are able to identify most oxacillin-resistant strains, there are two situations in which additional procedures are required to confirm susceptibility or resistance. The first situation refers to the occurrence of extremely heterogeneous strains identified as sensitive by standard methods. The second situation refers to the borderline resistance of strains, which must be differentiated from *mecA* resistance, since the clinical significance of the latter is much higher. In the present study, β-lactamase hyperproduction and heterogeneous MRSA populations seemed to be the major causes of false-positive and false-negative results in the detection of oxacillin resistance by phenotypic methods, respectively. This fact highlights the importance of molecular techniques for the detection of oxacillin resistance in *S. aureus* to prevent unnecessary administration of vancomycin, an antimicrobial agent that is associated with therapeutic complications and may lead to the selection of resistant isolates.

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ANEXO 17

PEREIRA VC, MARTINS A, RUGOLO LMSS, **CUNHA MLRS**. Detection of Oxacillin Resistance in *Staphylococcus aureus* isolated from the neonatal and Pediatric Units of a Brazilian Teaching Hospital. Clin Med Pediatr. 2009; 3: 23-31.

Detection of Oxacillin Resistance in *Staphylococcus aureus* Isolated from the Neonatal and Pediatric Units of a Brazilian Teaching Hospital

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Abstract

Objective: To determine, by phenotypic and genotypic methods, oxacillin susceptibility in *Staphylococcus aureus* strains isolated from pediatric and neonatal intensive care unit patients seen at the University Hospital of the Botucatu School of Medicine.

Methods: A total of 100 *S. aureus* strains isolated from the following materials were studied: 25 blood cultures, 21 secretions, 12 catheters, 3 cannulae and one chest drain from 62 patients in the neonatal unit, and 36 blood cultures, one pleural fluid sample and one peritoneal fluid sample from 38 patients in the pediatric unit. Resistance of the *S. aureus* isolates to oxacillin was evaluated by the disk diffusion method with oxacillin (1 µg) and cefoxitin (30 µg), agar screening test using Mueller-Hinton agar supplemented with 6 µg/ml oxacillin and 4% NaCl, and detection of the *mecA* gene by PCR. In addition, the isolates were tested for β-lactamase production using disks impregnated with Nitrocefin and hyperproduction of β-lactamase using amoxicillin (20 µg) and clavulanic acid (10 µg) disks.

Results: Among the 100 *S. aureus* strains included in the study, 18.0% were resistant to oxacillin, with 16.1% MRSA being detected in the neonatal unit and 21.0% in the pediatric unit. The oxacillin (1 µg) and cefoxitin (30 µg) disk diffusion methods presented 94.4% and 100% sensitivity, respectively, and 98.8% specificity. The screening test showed 100% sensitivity and 98.8% specificity. All isolates produced β-lactamase and one of these strains was considered to be a hyperproducer.

Conclusions: The 30 µg cefoxitin disk diffusion method presented the best result when compared to the 1 µg oxacillin disk. The sensitivity of the agar screening test was similar to that of the cefoxitin disk diffusion method and higher than that of the oxacillin disk diffusion method. We observed variations in the percentage of oxacillin-resistant isolates during the study period, with a decline over the last years which might be related to improved nosocomial infection control and the rational use of antibiotics.

Keywords: *Staphylococcus aureus*, oxacillin, *mecA*, Pediatrics, NICU

Introduction

The genus *Staphylococcus* comprises about 40 species, with *Staphylococcus aureus* being the main representative and the causative agent of a wide variety of infections. Nosocomial infections are the main cause of morbidity and mortality in pediatric (PICU) and neonatal intensive care units (NICU).

Antimicrobial resistance is easily acquired by these microorganisms because of the high risk of plasmid transfer between strains in the hospital environment and the abusive use of antimicrobial drugs, which represent important factors for the transfer of resistance genes and the selection of multiresistant strains.[1,2] The prevalence of oxacillin-resistant *S. aureus* (MRSA) in hospitals has increased in most countries. The intrinsic resistance of *S. aureus* to oxacillin is due to the production of a supplemental penicillin-binding protein (PBP 2' or PBP 2a), which presents low affinity for semi-synthetic penicillins and is encoded by the chromosomal gene *mecA*. Although the resistance of *mecA* gene is present in all the cells of the population with intrinsic resistance, this could be expressed by a small percentage of them, leading to what has been called heterogeneous resistance. Other resistance mechanisms have

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been described in strains that do not possess the *mecA* gene, with these strains being called borderline resistant. Borderline resistance is caused by two mechanisms: inactivation of oxacillin due to β-lactamase hyperproduction[3] and modified resistance, known as MOD-SA, due to the production of modified intrinsic PBPs with affinity for altered oxacillin.[4]

The reference methods for the detection of oxacillin resistance in *S. aureus* recommended by the Clinical and Laboratory Standards Institute (CLSI)[5] include the determination of the minimum inhibitory concentration (MIC) of the drug by the agar or broth dilution method, disk diffusion method and, more recently, the cefoxitin disk diffusion test.[5,6]

Since phenotypic methods for the detection of MRSA may yield questionable results, molecular tests for the detection of the *mecA* gene or its product PBP 2a have been proposed. Detection of the *mecA* gene by the polymerase chain reaction (PCR) is considered to be the gold standard for the diagnosis of MRSA. The determination of oxacillin resistance in *Staphylococcus* is important to guide therapy and to prevent unnecessary treatment of the patient with vancomycin, an antibiotic associated with therapeutic complications and whose use may lead to the selection of resistant strains. The prevalence of MRSA presents varies widely, particularly as a function of the size and type of the health institution. Thus, the objective of the present study was to determine oxacillin susceptibility in *S. aureus* strains isolated from patients hospitalized in the PICU and NICU of the University Hospital of the Botucatu School of Medicine (HC-FMB) using the oxacillin and cefoxitin disk diffusion methods. These methods were compared to *mecA* gene detection by PCR.

Materials and Methods

Strains

A total of 100 *S. aureus* strain isolated from the following materials were studied: 25 blood cultures, 21 secretion, 12 catheters, 3 cannulae and one chest drain from 62 patients hospitalized in the NICU of HC-FMB, and 36 blood cultures, one pleural fluid sample and one peritoneal fluid sample from 38 patients hospitalized in the PICU between 1991 and 2007. The strains were isolated as described by Koneman et al.[7]

Detection of oxacillin resistance by the 1 μg oxacillin and 30 μg cefoxitin disk diffusion method and by the agar screening test using Mueller-Hinton agar supplemented with 4% NaCl and 6 μg/mL oxacillin

Oxacillin sensitivity was tested by the agar disk diffusion method according to the criteria of the CLSI.[5] The inocula were prepared in brain-heart infusion (BHI) broth and incubated for 4 to 6 h and turbidity was set to 0.5 McFarland standard. The following disks were used: 1 μg oxacillin and 30 μg cefoxitin. Once the density was adjusted, the inoculum was spread with a sterile swab on Mueller-Hinton agar and the disks impregnated with the drug were applied. The plates were incubated for 24 h at 35 °C and antimicrobial activity was evaluated by determining the diameter of the inhibition zone as recommended by the CLSI.[5] The *S. aureus* ATCC 25923 (negative control) and ATCC 33591 (positive control) reference strains were used in all experiments.

For the detection of MRSA, Mueller-Hinton agar plates containing 6 μg/ml oxacillin and 4% NaCl were used.[8] The inoculum was prepared by previous incubation in BHI broth for 24 h and turbidity was set to 0.5 McFarland standard. After preparation of the inoculum, the strains were seeded in spots on the agar surface with a sterile swab. The plates were incubated for 24 h at 35 °C and the presence of MRSA was defined as the growth of at least one colony on the agar surface.

Detection of the *mecA* gene by PCR

Total nucleic acids were extracted from *S. aureus* strains cultured on blood agar, individually inoculated into BHI broth and incubated for 24 h at 37 °C. DNA was extracted using the GFX kit (Amersham Pharmacia Biotech). Briefly, staphylococcal cells were first digested with 10 μg/ml lysozyme and 20 μg/ml proteinase K. Next, 500 μl of the extraction solution was added and the mixture was centrifuged at 5000 × g for 1 min. The supernatant was then transferred to a GFX column and centrifuged at 5000 × g for 1 min. The collected fluid was discarded and 500 μl of the extraction solution was again added to the column. After centrifugation and discarding the collected fluid, 500 μl of the washing solution was added to the column which was centrifuged at 14,000 × rpm for

3 min. The column was then transferred to a 1.5-ml tube and 200 µl Milli-Q water heated to 70 °C was used for elution. The isolates were centrifuged at 5000 × g for 1 min and the GFX column was discarded. The extracted DNA was stored in a refrigerator at 4 °C.

PCR was carried out in 0.5-ml microcentrifuge tubes in a total volume of 25 µl containing 1 µM of each primer, 2.0 U Taq polymerase, 100 µM deoxyribonucleotide triphosphates, and 150 µg nucleic acid. The following primers were used: *mecA1* (forward)—5' AAA ATC GAT GGT AAA GGT TGG 3', and *mecA2* (reverse) – 5' AGT TCT GCA GTA CCG GAT TTG 3'. The size of the amplified product is 533 bp. PCR was carried out in an appropriate thermocycler using the following parameters as described by Murakami et al.[9] 40 cycles of denaturation at 94 °C for 30 s, annealing at 55.5 °C for 30 s and extension at 72 °C for 1 min. After the 40 cycles, the tubes were incubated at 72 °C for 5 min before cooling to 4 °C. International reference strains were used as positive (*S. aureus* ATCC 33591) and negative controls (*S. aureus* ATCC 25923) in all tests.

The efficiency of the amplification reactions was evaluated by electrophoresis on 2% agarose gel prepared in 0.5X TBE buffer and stained with ethidium bromide. The size of the amplified products was compared with the 100-bp standard and the gels were photographed under UV illumination.

Determination of β-lactamase production and β-lactamase hyperproduction

The production of β-lactamase by the *S. aureus* isolates was tested using disks impregnated with Nitrocefin (chromogenic cephalosporin, cefinase, BBL). The disk was moistened with one or two drops of sterile distilled water and placed on the *S. aureus* colonies previously incubated for 24 h at 35 °C on the Mueller-Hinton plates used for the disk diffusion test with oxacillin (1 µg) and cefoxitin (30 µg), because these drugs stimulate β-lactamase production in *Staphylococcus*.

A positive reaction was indicated by the development of a red color, whereas the absence of a change in color indicated a negative reaction. For β-lactamase-negative strains, the reaction was reexamined after 1 h according to manufacturer recommendations. For correct analysis of the results, the tested disks were compared with positive (*S. aureus* ATCC 29213) and negative (*S. xylosus* ATCC 29979) control strains.

The isolate that was negative for the *mecA* gene and presented resistance to oxacillin by the phenotypic methods was tested to determine whether it was a hyperproducer of β-lactamase. This strain was tested using disks containing amoxicillin (20 µg) and clavulanic acid (10 µg). The sensitivity breakpoint was the formation of an inhibition zone ≥20 mm after incubation for 24 h at 35 °C.[10]

Statistical analysis

Sensitivity and specificity tests[11] were performed to compare the agar screening method, disk diffusion test with cefoxitin and oxacillin and PCR. The last method is considered to be the gold standard for the detection of intrinsic oxacillin resistance (detection of the *mecA* gene).

Sensitivity was defined as the proportion of PCR-positive *S. aureus* isolates (detection of the *mecA* gene) that were resistant to oxacillin using the following phenotypic methods: disk diffusion with cefoxitin and oxacillin, and agar screening test (Mueller-Hinton agar supplemented with 6 µg/mL oxacillin and 4% NaCl).

Specificity was defined as the proportion of PCR-negative *S. aureus* isolates (no detection of the *mecA* gene) that were sensitive to oxacillin using the following phenotypic methods: disk diffusion with cefoxitin and oxacillin, and agar screening test (Mueller-Hinton agar supplemented with 6 µg/mL oxacillin and 4% NaCl).

Results

Detection of oxacillin resistance by the 1 µg oxacillin and 30 µg cefoxitin disk diffusion method and by the agar screening test using Mueller-Hinton agar supplemented with 4% NaCl and 6 µg/mL oxacillin

Among the 100 *S. aureus* strains isolated from PICU and NICU patients of HC-FMB, 11 (11%) were found to be resistant to oxacillin in the 1 µg oxacillin disk diffusion test. In seven (7%) isolates, growth of colonies was observed inside the inhibition zone, suggesting heterogenous

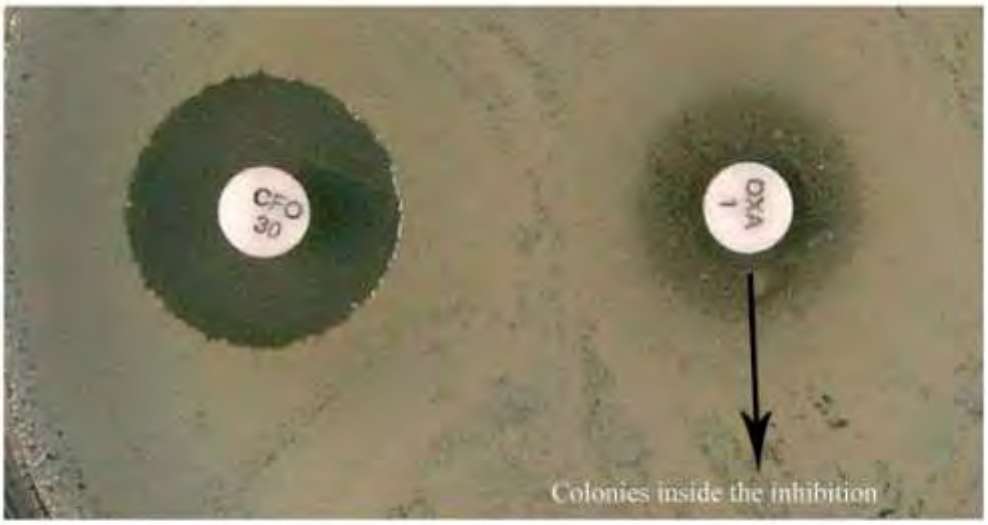

Figure 1. Strain resistant to cefoxitin and growth of colonies inside the inhibition zone, suggesting heterogenous resistance.

resistance. Using 30 µg cefoxitin disks, oxacillin resistance was observed in 18 (18%) isolates and one (1%) presented growth of colonies inside the inhibition zone, suggesting heterogeneous resistance (Fig. 1). Nineteen (19%) isolates were found to be resistant by the agar screening method using *S. aureus* ATCC 33591 and ATCC 25923 as control strains (Fig. 2).

Detection of the *mecA* gene by PCR

PCR revealed the presence of the *mecA* gene in 18 (18%) isolates. Eleven of these isolates were

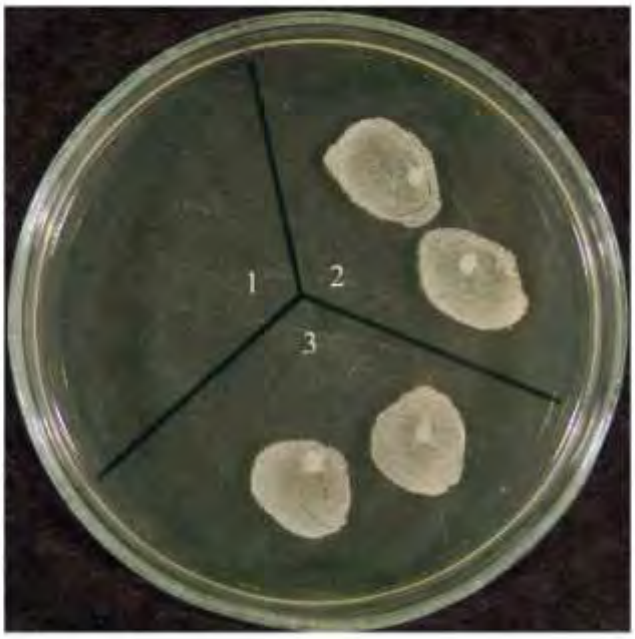

Figure 2. *Staphylococcus aureus* strains sensitive to oxacillin (1) and resistant to oxacillin (2 and 3) by the agar screening method (Mueller-Hinton agar containing 6 µg/mL oxacillin + 4% NaCl).

resistant in the 1 µg oxacillin and 30 µg cefoxitin disk diffusion tests and one isolate only showed resistance in the 30 µg cefoxitin disk diffusion test. Among the other six PCR-positive isolates, five presented heterogenous resistance by the oxacillin disk diffusion method and resistance by the cefoxitin disk diffusion method. One isolate showed heterogenous resistance with both disk diffusion methods. All 18 *mecA*-positive isolates were found to be resistant in the agar screening test (Fig. 3 and Table 1).

Determination of β-lactamase production and β-lactamase hyperproduction

The production of β-lactamase was determined using disks impregnated with Nitrocefin. All *S. aureus* isolates were confirmed to be producers of β-lactamase.

The isolate that was negative for the *mecA* gene, was resistant by the 30 µg cefoxitin disk diffusion and screening methods, and showed heterogenous resistance in the 1 µg oxacillin disk diffusion test was studied to determine whether it was a hyperproducer of β-lactamase. This was tested with a disk of amoxicillin (20 µg) and clavulanic acid (1 µg) and was sensitive to this drug, then confirming the hyperproduction of β-lactamase, that was inhibited by the presence of clavulanic acid.

Statistical analysis

Statistical analysis showed 94.4% sensitivity and 98.8% specificity of the oxacillin disk diffusion method and 100% sensitivity and 98.8% specificity of the cefoxitin disk diffusion method. The sensitivity and specificity of the screening test was 100% and 98.8%, respectively (Table 2).

Evolution of oxacillin resistance in *S. aureus* strains isolated from patients seen at HC-FMB

Sixty-two samples from NICU patients and 38 from PICU patients were studied. Among the strains isolated from the neonatal unit, 10 (16.1%) were resistant to oxacillin, three of them isolated from blood cultures, 4 from secretions and 3 from catheters. Eight (21%) of the strains isolated from the PICU were MRSA, with 7 strains being isolated from blood cultures and one strain isolated from pleural fluid (Table 3).

The strains of the present study were isolated between 1991 and 2007. Twenty-eight strains were isolated between 1991 and 1994, and seven (25%) were found to be resistant to oxacillin. Twenty-five strains were isolated between 1995 and 2000, and two (8%) were MRSA. Of the 24 strains isolated between 2001 and 2004, seven (29.2%) were MRSA. Twenty-three strains were isolated between 2005 and 2007, and two (8.7%) were resistant to oxacillin (Fig. 4).

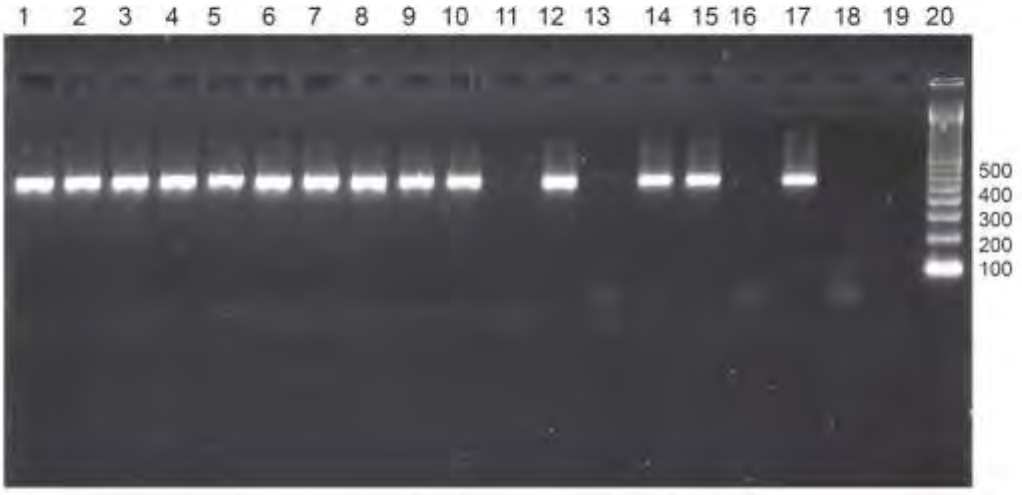

Figure 3. Agarose gel electrophoresis for the detection of the *mecA* gene (533 bp) in *Staphylococcus aureus* strains by PCR. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 15: positive strains; lanes 11, 13, and 16: negative strains; lane 17: positive control (*S. aureus* ATCC 33591); lane 18: negative control (*S. aureus* ATCC 25923); lane 19: water; lane 20: molecular weight marker (100 bp).

Table 1. Presence or absence of the *mecA* gene in strains tested by the oxacillin and cefoxitin disk diffusion method and the agar screening method.

PCR[a]	Phenotypic test					
	Disk diffusion				Screening method[b]	
	Oxacillin (1 µg)		Cefoxitin (30 µg)			
	S	R	S	R	S	R
mecA + (N = 18)	1	17	0	18	0	18
mecA – (N = 82)	81	1	81	1	81	1
Total (N = 100)	82	18	81	19	81	19

[a]Polymerase chain reaction.
[b]Mueller-Hinton agar containing 6 µg/mL oxacillin + 4% NaCl.
[S]Oxacillin-sensitive sample.
[R]Oxacillin-resistant sample.

The seven (25%) MRSA strains isolated between 1991 and 1994 and the two (8%) MRSA strains isolated between 1995 and 2000 originated from the neonatal unit. In contrast, the seven (29.2%) MRSA strains isolated between 2001 and 2004 were from children of the pediatric unit. Of the two oxacillin-resistant strains isolated between 2004 and 2007, one (4.4%) was from the neonatal unit and the other (4.4%) was from the pediatric unit (Table 4 and Fig. 4).

Discussion

The prevalence of oxacillin-resistant *S. aureus* in hospitals has increased in most countries. Prevalence rates of MRSA may vary, particularly as a function of the size and type of the medical institution. In the present study, 100 *S. aureus* strains isolated from inpatients of the PICU and NICU of HC-FMB were tested for MRSA.

Although high frequencies of oxacillin-resistant *S. aureus* have been reported especially in large hospitals and university hospitals, in the present study the percentage of MRSA isolated from inpatients of the PICU and NICU of HC-FMB between 1991 and 2007 was only 18%. Similar results have been reported for PICU and NICU patients with bacteremia in the United Kingdom, with a percentage of MRSA of 15.5%.[12] Furthermore, the prevalence of MRSA observed here was lower than that reported in studies conducted in other countries. In the United States, a prevalence of MRSA infection of 47% was reported in a hospital in Texas in 2003.[13] A study conducted in India found a prevalence of oxacillin-resistant *S. aureus* of 66% among NICU inpatients.[14] Similar rates have been reported in studies conducted in Japan and Israel, with MRSA infection rates of 52.5% and 60%, respectively, among inpatients of an NICU.[15,16]

In the present study, phenotypic tests such as the disk diffusion method and agar screening test were compared with a genotypic method (*mecA* gene detection by PCR). The cefoxitin disk diffusion method presented 100% sensitivity and 98.8% specificity and was superior to the oxacillin disk diffusion method (94.4% sensitivity and

Table 2. Determination of the sensitivity and specificity of phenotypic and genotypic methods for the detection of oxacillin resistance in *S. aureus* strains.

Phenotypic test	*MecA*		Sensitivity	Specificity
	Positive (N = 18)	Negative (N = 82)	%	%
Oxacillin disk (1 µg)	17	81	94.4	98.8
Cefoxitin disk (30 µg)	18	81	100	98.8
Screening method[a]	18	81	100	98.8

[a]Mueller-Hinton agar containing 6 µg/mL oxacillin + 4% NaCl.

Table 3. Determination of oxacillin resistance in *Staphylococcus aureus* strains according to hospital unit and clinical material.

	Neonatal unit			Pediatric unit		
	N NICU	N MRSA	%	N PICU	N MRSA	%
Blood culture (N = 61)	25	3	12	36	7	19.5
Secretion (N = 21)	21	4	19	0	0	0
Peritoneal fluid (N = 1)	0	0	0	1	0	0
Pleural fluid (N = 1)	0	0	0	1	1	100
Catheter (N = 12)	12	3	25	0	0	0
Cannula (N = 3)	3	0	0	0	0	0
Chest drain (N = 1)	1	0	0	0	0	0

N, total number of strains.
N MRSA, number of oxacillin-resistant *S. aureus* strains.
N NICU, number of strains originating from the neonatal unit.
N PICU, number of strains originating from the pediatric unit.

98.8% specificity) in terms of the detection of oxacillin-resistant *S. aureus*. Similar results have been reported by Velasco et al.[17] According to these authors, the 30 µg cefoxitin disk diffusion method, recently recommended by the NCCLS[6] and CLSI[5] as a screening test, was the best method showing 100% sensitivity and 98% specificity. Recent studies evaluating cefoxitin disks for the detection of MRSA also obtained good results, with a sensitivity of about 100% and specificity of 99%.[18–21] Cauwelier et al.[20] evaluated methicillin resistance in 155 clinical MRSA isolates by different methods including oxacillin and cefoxitin disks, latex agglutination and an agar screening test. The cefoxitin disk diffusion method presented 100% sensitivity and 99% specificity, whereas sensitivity fell to 91.7% in the oxacillin disk diffusion test. According to these authors, compared to the gold standard (*mecA* gene detection), the disk diffusion method with 30 µg cefoxitin is preferable to the 1 µg oxacillin disk diffusion method for the detection of MRSA.

The *mecA* gene was detected in all isolates that were resistant in the Mueller-Hinton agar screening test. The sensitivity of the method was 100% and specificity was 98.8%. The performance of the screening method using Mueller-Hinton agar supplemented with 4% NaCl and 6 µg oxacillin depends on the degree of heterogeneity of the isolates tested, with lower sensitivity (≤95%) being reported in studies including a larger number of heteroresistant strains and values >97% in other studies.[22–24] In this investigation, the sensitivity of the agar screening test for the detection of MRSA was similar to that of cefoxitin disks and higher than that of the oxacillin disk diffusion method. The high sensitivity of the agar screening method might be explained by the small number of heteroresistant strains. Different results have been reported by Cauwelier et al.[20] who observed 91.7% sensitivity and 100% specificity. The authors attributed the low sensitivity in the detection of heterogeneous populations to the high variability in the expression of PBP 2a.

Other resistance mechanisms have been described in strains that do not possess the *mecA* gene, with these strains being called borderline resistant. There are two mechanisms of borderline resistance, one is the inactivation of oxacillin due to hyperproduction of β-lactamase[3] and the other is modified resistance, called MOD-SA, due to the production of modified intrinsic PBPs with affinity for altered oxacillin.[4] However, these mechanisms are characterized by low levels of resistance (MIC of 8 µg/ml).[24] In the present study, one isolate presented resistance to oxacillin in all phenotypic

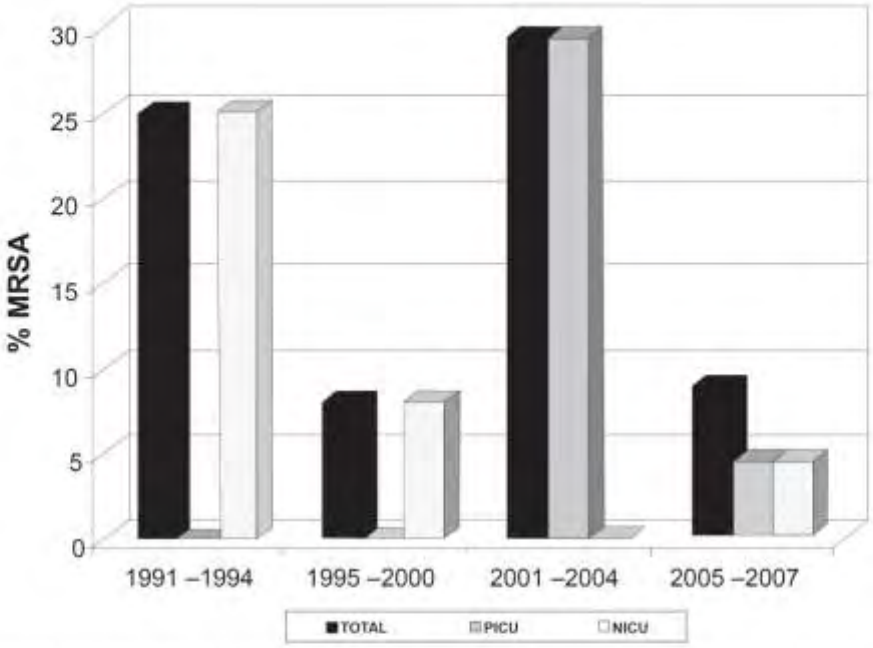

Figure 4. Evolution of oxacillin resistance in *Staphylococcus aureus* isolated at the University Hospital of the Botucatu School of Medicine between 1991 and 2007. PICU: pediatric intensive care unit; NICU: neonatal intensive care unit.

tests despite the absence of the *mecA* gene and was sensitive to the amoxicillin-clavulanic acid disk. This strain was therefore considered to be a hyperproducer of β-lactamase, suggesting borderline resistance.

The MRSA strains detected during the first two study periods (1991 to 2000) were isolated from samples originating from the neonatal unit. This finding can be explained by the fact that all strains studied until 1998 exclusively originated from the neonatal unit and strains from the pediatric unit were only included after 1999. Between 2001 and 2004, all oxacillin-resistant strains originated from the pediatric unit, with a decline in the number of MRSA originating from the NICU. In contrast, in the last period the proportion of MRSA originating from the NICU and PICU was the same. Overall analysis of our data showed variations in the percentage of oxacillin-resistant *S. aureus* during the study period. The percentage of resistant isolates declined between 1995 and 2000, followed by an increase from 2001 to 2004, and then declined

Table 4. Number of oxacillin-resistant and sensitive *Staphylococcus aureus* strains according to the period studied.

	MRSA		MSSA		Total
	NICU	PICU	NICU	PICU	
1991–1994	7	0	21	0	28
1995–2000	2	0	20	3	25
2001–2004	0	7	4	13	24
2005–2007	1	1	7	14	23
Total	10	8	52	30	**100**

MRSA, oxacillin-resistant *S. aureus*.
MSSA, oxacillin-sensitive *S. aureus*.
NICU, neonatal intensive care unit.
PICU, pediatric intensive care unit.

again over the following years. These reductions might be related to improved nosocomial infection control and the rational use of antibiotics.

Acknowledgment

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Disclosure

The authors report no conflicts of interest.

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9. Epidemiologia de *Staphylococcus* spp. Resistentes à Meticilina

Linhagens endêmicas de MRSA carreando determinantes de resistência múltipla tornaram-se um problema mundial a partir do início da década de 80 (234). Recentes avanços científicos considerando a origem genética da resistência a meticilina em *S. aureus* têm alavancado um maior entendimento da epidemiologia do MRSA. Análises genéticas detalhadas de cepas de MRSA de diversas partes do mundo revelaram que a transferência do gene *mecA* do MRSA para *S. aureus* suscetível à meticilina (MSSA) ocorreu poucas vezes, assim sendo, a crescente incidência do MRSA resulta da disseminação mundial de apenas alguns clones, e não da emergência de novos clones de MRSA a partir de cepas sensíveis (277-278). Esses resultados sugerem que todos os pacientes que apresentam infecções ou colonização por MRSA adquiriram suas cepas de uma fonte externa. A transmissão de clones de uma cidade a outra, de um país a outro e até mesmo de um continente a outro foi determinada pela transferência de pacientes infectados ou colonizados com MRSA (279).

Utilizando-se a combinação de várias técnicas de tipagem molecular foram identificados seis clones de MRSA amplamente disseminados por todo o mundo. Esses clones denominados de Ibérico, Brasileiro, Húngaro, Nova Iorque/Japão, Pediátrico e EMRSA-16 são responsáveis por 68% dos MRSA e representam linhagens bem sucedidas em termos de habilidade de causar infecção, persistência e capacidade de disseminação de uma área geográfica para outra e até mesmo entre continentes (280). Esses clones foram nomeados de acordo com a área geográfica onde foram primeiramente descritos (clone epidêmico brasileiro, ibérico, clone Nova Iorque

/Japão), pelas características epidemiológicas dos pacientes em que foram descritos (clone pediátrico), pelo número baseado em seu padrão de PFGE (USA 100, USA 800) ou pelo tipo fágico (EMRSA-15, EMRSA-16)(281).

O clone epidêmico Brasileiro (CEB) foi descrito inicialmente em 1993 (282), em um estudo realizado na cidade de São Paulo, e encontra-se amplamente disseminado por várias regiões do mundo, incluindo países da América Latina e Europa (283-284). No Brasil, este clone pôde ser identificado em diferentes hospitais, de todas as regiões do país, frequentemente associado a surtos epidêmicos (246, 285).

Estudos epidemiológicos moleculares destacam a contínua evolução global/propagação dos clones de MRSA, com crescente resistência a antibióticos e virulência. Temos uma compreensão apenas parcial dos fatores contributivos para a propagação dos clones de MRSA, mas é provável que alguns desses fatores sejam: migração de populações humanas, métodos ineficazes de controle da transmissão de MRSA por pacientes infectados e estratégias de tratamento pouco eficientes, inclusive o uso/escolha inadequados de antibióticos. Em hospitais, pacientes já portadores de MRSA no momento da internação tem maior risco de sofrer uma infecção derivada das bactérias colonizadas, ou de transmitir MRSA para outros pacientes (286).

Taxas de colonização ou infecção com MRSA podem variar de acordo com a localização geográfica, tipo de serviço hospitalar e população específica. Pacientes colonizados contaminam o ambiente com suas cepas facilitando a transmissão cruzada. Esse fato ressalta a importância de medidas de precaução e isolamento de pacientes colonizados ou infectados por MRSA, complementadas por protocolos eficazes de higiene, limpeza e manutenção dos ambientes hospitalares (287).

As infecções por MRSA são associadas com considerável morbidade e mortalidade, sendo também mais dispendiosas de se manejar quando comparadas a outras infecções (288). Esse aumento considerável nos gastos relativos ao manejo dessas infecções deve-se à hospitalização prolongada, ao aumento dos cuidados em isolamento, além de cuidados médicos adicionais e da sobrecarga financeira em terapia secundária.

Alguns estudos sugerem que a triagem de pacientes de alto risco para colonização por MRSA é uma medida custo efetiva para limitar a disseminação desses organismos nos hospitais (289). O sucesso no controle de MRSA tem sido grande em países que aderiram a rigorosas políticas de controle de infecção hospitalar baseadas na transmissão e na restrição do uso de antibióticos. Portanto a rápida e confiável detecção de pacientes carreadores de MRSA é crucial na estratégia de controle de infecção. Wernitz *et al*. (290) mostraram que a triagem extensiva de MRSA na admissão hospitalar, apesar do custo, mostrou um importante impacto positivo na redução das taxas de infecção por MRSA.

A disseminação de MRSA em centros de assistência a saúde é difícil de controlar e vários *guidelines* internacionais recomendam medidas que incluem culturas ativas de vigilância para identificação de pacientes colonizados ou infectados com MRSA, rígidas medidas de precaução e isolamento, e a quarentena para pacientes recém admitidos no serviço hospitalar até que se afaste a possibilidade de colonização por MRSA (291). A Holanda é um exemplo de país onde há uma rigorosa política de "busca e destruição" (*search and destroy*) de MRSA e um baixo nível endêmico desses micro-organismos. Todos os pacientes e profissionais da saúde são considerados

colonizados até que provem o contrário e sujeitos a intensos e dispendiosos procedimentos de controle de infecção. Neste caso uma rápida triagem de MRSA é importante para identificar os não carreadores que podem ter esses procedimentos relaxados.

A tipagem dos tipos de cassete SCC*mec* é útil como ferramenta epidemiológica (292), uma vez que diferentes tipos são mais prevalentes em ambientes hospitalar ou comunitário. O MRSA adquirido na comunidade (CA-MRSA) é um patógeno potencialmente emergente que vem apresentando frequência crescente de isolados (293). Os pacientes acometidos pelo CA-MRSA são caracterizados por não terem sido internados em hospitais no ano anterior à infecção, nem sido submetidos a procedimentos médicos como diálise, cirurgia ou cateter, fatos muito comuns em infecção por MRSA de origem hospitalar (HA-MRSA). Enquanto o MRSA hospitalar (HA-MRSA) se caracteriza por uma ampla resistência a diversos antibióticos, as cepas CA-MRSA mostram uma sensibilidade (entre 85% e 100%) a drogas, como clindamicina, gentamicina, ciprofloxacina, sulfametaxazol/trimetoprim e vancomicina, mostrando-se resistente apenas à oxacilina e a outros betalactâmicos (281).

A diferença entre os perfis de resistência das cepas de HA-MRSA e CA-MRSA parece ser explicado pelo tamanho e distribuição dos cassetes cromossomais (SCC*mec*) que possuem o determinante de resistência à oxacilina. Entre os principais tipos de SCC*mec* (I, II, III, IV e V), somente os tipos I, II e III são encontrados em cepas HA-MRSA, enquanto que os tipos IV e V podem ser observados em cepas CA-MRSA, nas quais o tipo IV tem um menor tamanho e custo metabólico, tornando este um elemento

seletivamente favorecido para a transferência entre as linhagens de *Staphylococcus* (294).

Embora as infecções por CA-MRSA sejam geralmente cutâneas, doenças invasivas como bacteremias, endocardites, osteomelites e pneumonias já foram descritas, bem como surtos de infecções hospitalares (295-296). Os CA-MRSA Panton-Valentine positivos são facilmente transmitidos entre familiares, e também em grande escala na comunidade, como prisões, escolas e times de esportes. O contato pele-pele envolvendo abrasões e contato indireto com objetos contaminados como toalhas, lençóis, equipamentos de esportes parecem representar um modo de transmissão (281). Estudo atual em desenvolvimento em nosso laboratório tem como objetivo a detecção de CA-MRSA em presidiários da cidade de Avaré.

É possível que alguns clones de MRSA sejam mais propensos que outros a determinar doença invasiva, isto pode se dever à presença de fatores de virulência, que aumentam suas chances de alcançar sítios normalmente estéreis, e sobreviver, proliferar e disseminar no hospedeiro. Esses fatores podem se relacionar à secreção de exotoxinas, hemolisinas, leucocidinas e também à produção de biofilme (297). Embora muitos fatores de virulência de *S. aureus* tenham sido identificados em seu genoma, as diferenças de potencial patogênico e invasivo entre as cepas disseminadas no ambiente ainda é desconhecida. Nosso grupo recentemente estudou a presença de genes de fatores de virulência e resistência à meticilina entre as cepas de *S. aureus* de culturas de vigilância (colonizadoras) e invasivas (culturas clínicas) de pacientes de hospital de ensino de pequeno porte. A presença de SCC*mec* tipos III e IV, bem como dos genes que codificam as toxinas esfoliativa B e Leucocidina Panton Valentine foram

independentemente associadas com a invasão (ANEXO 18). A detecção desses genes em cepas de *S. aureus* entre os pacientes colonizados pode ser utilizada com indicativo de pacientes que precisam de maior acompanhamento e medidas intensivas de controle de infecção.

A compreensão da epidemiologia das infecções por MRSA tem implicações importantes para as medidas de controle. Para isso, torna-se necessário documentar a disseminação de clones e identificar os fatores individuais relacionados a sua aquisição. A eletroforese em gel de Pulsed-Field (PFGE) é ainda o método mais amplamente utilizado para estudos de micro-epidemiologia (surtos locais). O método baseia-se na digestão direta do DNA genômico por uma enzima de restrição (tipicamente *SmaI*), seguido pela separação dos fragmentos em gel de eletroforese em campo elétrico alternado. Resultados obtidos pelo nosso grupo em estudo com pacientes de um hospital universitário para determinação do perfil clonal de amostras de MRSA isoladas de pacientes queimados pela técnica de PFGE revelaram dois clones majoritários com similaridade maior que 80%. Em adição, 15 isolados que foram agrupados nos dois clones majoritários apresentaram SCC*mec* Tipo III ou IIIA e apenas um isolado foi positivo para o gene *pvl*. Esses dados estão de acordo com os resultados observados por Bartels *et al*. (298) em estudo com 32 MRSA isolados de hospitais da República da Georgia em que todos isolados apresentaram SCC*mec* Tipo IIIA e foram PVL negativos. A presença de dois clones majoritários pode indicar a presença de clones MRSA endêmicos na unidade de queimados diferentes do clone epidêmico brasileiro (CEB), encontrado frequentemente nos hospitais brasileiros. Em um estudo realizado no Hospital São Paulo (HSP), pertencente à UNIFESP, que analisou amostras

de 2002 a 2005, observou-se a persistência do clone epidêmico brasileiro dentro do ambiente hospitalar, além da emergência de novos clones, diferentes do CEB, nos últimos anos avaliados, o que poderia indicar a evolução temporal deste clone nos ambientes nosocomiais (299).

Estudo desenvolvido na Unidade Pediátrica do Hospital das Clínicas da Faculdade de Medicina de Botucatu revelou a maioria dos isolados de MRSA com SCC*mec* tipo III (60%) e relacionados ao Clone Epidêmico Brasileiro (CEB-HU 25) mostrando que esse clone ainda prevalente em infecções hospitalares no Brasil também está presente em nosso âmbito. Contudo, com o passar dos anos, observou-se aumento de MRSA com SCC*mec* tipo IV e diminuição do SCC*mec* tipo III (300), confirmando a tendência mundial de aumento da presença de SCC*mec* tipo IV no ambiente hospitalar e diminuição da prevalência de clones hospitalares (301,302).

Dada a complexa epidemiologia, a presença de CA-MRSA em hospitais e a circulação de cepas HA-MRSA na comunidade, o estabelecimento de uma delimitação clara entre CA-MRSA e HA-MRSA não é possível. Pesquisadores do CDC têm utilizado uma terceira categoria de MRSA hospitalar de início na comunidade (HACO-MRSA). Esta categoria inclui os casos que seriam HA-MRSA pela história de exposição a assistência a saúde, mas que a aquisição tem início na comunidade. Esse esquema de classificação, HA, CA, e HACO-MRSA, ainda tem limitações, porque uma história de exposição a um ambiente de assistência a saúde não exclui a possibilidade de aquisição de MRSA na comunidade (303-304). Portanto, a revisão da nomenclatura é necessária para refletir melhor a epidemiologia contemporânea de MRSA.

Estudo realizado em nosso laboratório com pacientes com infecções de pele atendidos na triagem da Dermatologia do Hospital das Clínicas da Faculdade de Medicina de Botucatu revelou *S. aureus* como o principal agente causador de infecções de pele nos pacientes estudados da comunidade, sendo 10,6% MRSA, com a maioria apresentando SCC*mec* tipo IV e II. Evidências anteriores apontam esses tipos de SCC*mec* como os mais encontrados colonizando indivíduos na comunidade, prevalecendo o tipo IV (305). Apesar da menor frequência com que foram isolados das infecções de pele, os ECN apresentaram maior número de amostras resistentes e com o gene *mecA* (38%), com maior frequência do SCC*mec* tipo IV. A resistência a outras classes de antimicrobianos foi encontrada tanto em cepas que carrearam o gene *mecA* quanto em amostras negativas para este. Amostras de ambas as espécies carrearam cassetes característicos de ambientes hospitalares, porém não houve diferença em relação à susceptibilidade aos antimicrobianos entre o SCC*mec* do tipo IV e os outros tipos de SCC*mec.* No presente estudo não foi encontrado MRSA carreando o gene da toxina PVL. As amostras que carrearam os genes *luk*S-PV e *luk*F-PV eram MSSA e responsáveis tanto por infecções primárias (furúnculo e impetigo) quanto secundárias (187).

Esses resultados são similares ao estudo desenvolvido pelo mesmo grupo, porém em pacientes com feridas crônicas atendidos em Unidades Básicas de Saúde (UBS) da cidade de Botucatu. A prevalência de MRSA isolados de feridas de 107 pacientes atendidos em 18 Unidades Básicas da cidade de Botucatu foi de 14,3% de MRSA e houve a presença de SCC*mec* somente do tipo II e IV com ausência do gene da PVL nas amostras estudadas. Esses dados divergem dos encontrados na literatura que

relatam esses micro-organismos como resistentes a poucos antibióticos não beta-lactâmicos e por carrear frequentemente os genes da PVL (306). Dados interessantes obtidos no estudo descrito acima foi o isolamento de MRSA de pacientes de UBS distantes com o mesmo perfil antimicrobiano e multiresistente. Esse projeto continua em desenvolvimento, agora com o objetivo de estudar a diversidade genética e a disseminação por várias técnicas de tipagem molecular, incluindo o PFGE, MLST (*multilocus sequence typing*) e *spa* typing.

Apesar da PFGE ser uma técnica adequada para estudar surtos, não é suficiente para estudos de longo prazo ou para estudos de epidemiologia global. Além das limitações dos critérios de interpretação relacionadas com o curto período de tempo, e com áreas geográficas restritas, a PFGE apresenta problemas de reprodutibilidade. Diferentes resultados podem ser obtidos quando a técnica é realizada em laboratórios diferentes, mesmo quando condições padronizadas são usadas (307). Trabalhos publicados a respeito da clonalidade de *S. aureus* são frequentemente complementados pela técnica denominada *multilocus sequence typing* (sequenciamento multilocus – MLST). A técnica baseia-se na análise de sequências de sete genes conservados (*housekeeping* genes) do micro-organismo, no qual diferentes sequências correspondem a diferentes alelos de cada gene e a um determinado tipo de sequência denominado ST (*sequence type*). Os resultados de MLST são inseridos em uma base de dados digital no endereço http://www.mlst.net, o que permite comparações entre sequências de *S. aureus* descritas em diferentes partes do mundo.

A respeito dos clones mundiais, foram classificados pelo MLST como ST-239 (clone epidêmico brasileiro - CEB), ST-5 (clones pediátrico e Nova Iorque/Japão), e ST-

247 (Ibérico). Em um estudo realizado no Hospital São Paulo (HSP), UNIFESP, a tipagem de *S. aureus* SCC*mec* IV por *Multilocus Sequence Typing* (MLST) demonstrou pertencerem ao complexo clonal (CC5), mostrando um novo alelo, resultando em um novo (ST) que pode estar emergindo em infecções comunitárias e nosocomiais no Brasil (308).

Em 2002, Enright *et al*. (19) analisaram uma coleção internacional de *S. aureus* isolados de hospitais e adquiridos na comunidade tipados por SCC*mec* e MLST. A análise revelou novas informações sobre grupos clonais de *S. aureus*. Diversos complexos clonais (CC), compostos por isolados de *S. aureus* com a mesma ST ou ST relacionadas (apresentando pelo menos cinco loci em comum), foram identificados. Cinco grandes CCs de MRSA foram incluídos: CC8, CC5, CC30, CC45, CC22. Curiosamente, a comparação das sequências de nucleotídeos de MRSA e MSSA incluída no CC8 permitiu o estabelecimento de um ancestral comum de MSSA. Diferentes isolados MRSA teriam se originado a partir desse ancestral MSSA por eventos distintos de aquisição de SCC*mec*.

A origem de SCC*mec* não é conhecida, entretanto Wu *et al*. (309) sugeriram que *Staphylococcus sciuri* abrigou o ancestral da PBP2a, desde que foi demonstrado que uma PBP presente em *S. sciuri* apresentava uma similaridade de 87,8% na sequência de aminoácidos em comparação com a PBP2a. Essas cepas de *S. sciuri* eram susceptíveis à meticilina, mas na presença de meticilina essas cepas se tornaram resistentes devido ao aumento na taxa de transcrição do gene *mecA* homólogo. Portanto, quando o gene *mecA* foi introduzido no MSSA, este se tornou resistente à meticilina, e assim foi classificado como MRSA (309). Além disso, Wielders *et al*. (310)

isolaram de uma epidemia um MSSA e, depois, uma cepa MRSA isogênica de um recém-nascido, que não tinha estado em contato com MRSA anteriormente. O gene *mecA* era idêntico ao gene *mecA* de uma cepa de *S. epidermidis* isolada do recém-nascido. Foi sugerido que o MRSA tinha se originado *in vivo* através da transferência horizontal do gene *mecA* entre as duas espécies de estafilococos (310). Essa observações reforçam a idéia de transmissão horizontal do gene *mecA* e a importância dos ECN como reservatórios de genes de resistência que podem ser transferidos para *S. aureus* que são mais patogênicos.

Outro método de tipagem muito utilizado é o *spa* typing desenvolvido por Frénay *et al*. (311) que determina a variação da sequência da região polimórfica X da proteína A de *S. aureus*. Nesse método o gene da proteína A (*spa*) é amplificado por PCR e submetido ao sequenciamento para análise da região polimórfica X ou sequência curta de repetições (SSR). A região SSR do gene *spa* está sujeita a mutações espontâneas, bem como a perda e ao ganho de repetições. A essas repetições são atribuídos códigos alfa-numéricos e o tipo de *spa* é deduzido a partir da ordem de repetições específicas (312). Dado que o *spa typing* envolve o sequenciamento de apenas um gene, tem vantagens significativas em termos de velocidade, facilidade de uso, padronização, interpretação, comparação interlaboratorial e reprodutibilidade em relação ao MLST e outras técnicas como o PFGE (313). Tem sido demonstrado que o *spa* typing pode ser usado para estudar tanto a evolução molecular bem como surtos de MRSA em hospital. A nomenclatura universal e o acesso público aos dados da tipagem de *spa*, são garantidos pela iniciativa SeqNet.org (www.seqnet.org), que dirige

a central *spa* do servidor (http://spaserver.ridom.de) ao qual os dados do *spa typing* são sincronizados (314).

Embora seja relativamente pequeno o número de estudos tratando da epidemiologia molecular dos clones de MRSA em nossa região, ficou claro que diversos clones estão circulando em nosso âmbito, e que esses clones diferem em sua virulência e perfil de resistência antimicrobiana. A caracterização desses clones é importante para que sejam formuladas estratégias terapêuticas locais adequadas. Exemplificando, pode-se utilizar um conhecimento mais completo dos clones circulantes em determinada região para avaliar a relação entre tipos clonais, sintomas e sinais da doença, escolha dos antimicrobianos e resultados clínicos. Ressalte-se ainda que clones com maior virulência estão começando a surgir mais frequentemente, tanto nos hospitais como na comunidade, e há evidências que fatores de virulência podem ser transferidos entre clones nosocomiais e clones associados à comunidade por meio de recombinação. Esses padrões variáveis têm implicações significativas para a prática clínica. Assim, há necessidade de programas regionais de epidemiologia molecular para que se tenha conhecimento de identificações e caracterizações precisas dos clones de MRSA circulantes na nossa região, para auxiliar na escolha da terapia antimicrobiana empírica mais apropriada.

ANEXO 18

Pimenta Rodrigues MV, Fortaleza CMCB, Souza CSM, Teixeira NB, **Cunha MLRS**. Genetic determinants of methicillin resistance and virulence among *Staphylococcus aureus* isolates recovered from clinical and surveillance cultures in a Brazilian teaching hospital (Submetido para publicação).

Title page

Genetic determinants of methicillin resistance and virulence among *Staphylococcus aureus* isolates recovered from clinical and surveillance cultures in a Brazilian teaching hospital.

Running title: *S. aureus*: SCC*mec* and virulence genes.

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ABSTRACT

We studied the presence of virulence and methicillin-resistance genes among colonizing and invasive *Staphylococcus aureus* strains from a small teaching hospital. The presence of SCC*mec* types III and IV, as well as of genes coding for exfoliative toxin B and Panton Valentine Leukocidin, was independently associated with invasiveness.

Key words: *Staphylococcus aureus* - SCC*mec* - virulence factors - invasiveness

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TEXT

A comprehensive approach to *Staphylococcus aureus* epidemiology within healthcare settings should include the identification of reservoirs, transmission dynamics and invasiveness. The latter issue is of major interest. While many patients are asymptomatically colonized with methicillin-susceptible (MSSA) or resistant (MRSA) *S. aureus*, only a few develop infection (Jarvis, 1996). However, previous studies have shown that colonization is a major risk factor - or even a preceding stage - for infection (Safdar & Bradley, 2008).

We attempted to assess determinants of infection by comparing the proportion of isolates recovered from surveillance and clinical cultures that harbored genes coding for virulence or methicillin resistance. The rational of our study was that isolates from clinical cultures, which are collected upon the diagnosis of an infectious syndrome, may be representative of “invasive” strains.

The study was performed with isolates of *S. aureus* collected from October 2006 to March 2009 from hospitalized patients in the Hospital Estadual Bauru (HEB), one of the teaching hospitals from Botucatu School of Medicine. The hospital has 285 active beds, distributed among four intensive care units (ICUs), one unit for burn patients and several medical, surgical and pediatric wards. Surveillance cultures (nasopharyngeal swabs) were routinely obtained from all patients upon admission. In ICUs and burn units, those cultures were also performed weekly thereafter. For burn patients, besides nasopharyngeal swabs, specimens were obtained from burn wound and other body sites. Clinical cultures were collected upon medical indication.

We studied isolates provided by HEB´s microbiology laboratory. Whenever one patient had more than one surveillance or clinical culture positive for *S. aureus*, only the first was included in the analysis. However, if that patient presented positivity both surveillance and clinical cultures, both isolates were analyzed. Several PCR-based methods were applied to identify virulence genes (Table I) (Johnson et al. 1991, Lina et al. 1999, Arciola et al. 2001, Jarraud et al. 2002). Methicillin resistance was assessed by molecular identification and typing of SCC*mec* (Oliveira & Lencastre, 2002). Isolates were also submitted to strain typing through Pulsed-Field Gel Electrophoresis (PFGE) (Chung et al, 2000). Band patterns were digitalized and analyzed with Bionumerix (Applied Maths, Belgium). Clones were defined on the basis of a similarity (Dice coefficient) greater than 0.8.

The proportion of isolates harboring specific genes was compared through univariate statistical tests: Chi-square or Fisher´s Exact test. Later, all results were simultaneously introduced in a single-step multivariable model (logistic regression). A p-value of 0.05 was set as significance limit.

A total of 309 isolates were included in the study, 225 (72.8%) from surveillance cultures and 84 (27.2%) from clinical specimens. The most frequent sites of surveillance cultures were: nasopharynx (59.6%), burn wound (22.7%) and oropharynx (10.2%). Among clinical specimens, blood (46.4%), wound secretions (33.3%) and tissue fragments (14.3%) predominated.

PFGE patterns revealed a polyclonal picture. Colonizing isolates growth from surveillance cultures were grouped in 48 clones. The most disseminated clone comprised ten isolates. Forty-one clones were identified among invasive

isolates (i.e., those recovered from clinical cultures). The major clone grouped eight isolates. It is worth noting that in both cases the major clones were detected all through the study period.

Other results are summarized in Table II. Briefly, we found an independent relation between presence of SCC*mec* types III and IV and invasiveness. Also, invasive strains were more likely to harbor genes for exfoliative toxin b (*etb*) and the Panton Valentine leukocidin (*pvl*).

The finding of greater invasiveness among MRSA is not surprising. This may be partly due to direct and indirect ("populational") ecological pressure of antimicrobial use (Lipsitch & Samore 2002). Also, other clinical factors, such as greater severity-of-illness and length-of-stay in the hospital are both risk factors for healthcare-acquired infection and for MRSA acquisition.

On the other hand, *pvl* lysis of leukocytes may act as a mechanism for evasion from immune response, facilitating *S. aureus* survival and tissue invasion (Boyle-Vavra & Daum 2007). Of note, we found *pvl* genes in 33 out of 203 *SCCmec* III and in 3 out of 9 SCC*mec* IV-harboring isolates. This difference did not reach statistical significance. None of the MSSA strains was positive for *pvl*.

The finding of a relation between *etb* and invasiveness was rather puzzling. This gene was equally distributed among MSSA (3.1%) and MRSA (4.1%) isolates. It was found in 3 out of 40 isolates from blood cultures and in 4 out of 28 isolates from wound secretions. We can only hypothesize that

superatingenic action may improve invasiveness. Of note, *etb* surpasses *eta* in pyrogenic activity (Bukowski et al. 2010).

Of course, our results could be improved by confronting molecular results with clinical and epidemiological data. Tough it was not the main objective of our study, we revised all MRSA cases in order to identify community-acquired strains. We found out that 15 (out of 220) MRSA-positive patients had positive cultures upon admission and did not refer any previous contact with healthcare settings in the last year. Among the isolates, 14 harboured SCC*mec* III and only one SCC*mec* IV. This is a starting point for further research. Still, our results point out to a role of both virulence factors and methicillin resistance in the transition from colonization to infection. The early identification of those genes among colonized patients may delimitate a group of patient deserving more intensive application of infection control measures.

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TABLE I

List of virulence factors assessed in the study, alongside with the genes tested and the methods references

Virulence factors	Genes	Reference
Enterotoxins	*sea*	Johnson et al (1991)
	seb	
	sec-1	
Staphylococcal Toxic Shock Syndrome Toxin 1	*tst*	
Panton-Valentine Leukocidin	*LukPV*	Lina et al (1999)
Biofilm production	*icaA*	Arciola et al (2001)
	icaD	
Exfoliative toxins	*eta*	Johnson et al (1991)
	etb	
	etd	
Hemolysins	*hla*	Jarraud et al (2002)
	hld	

Table II

Results of univariate and multivariable analysis of molecular predictors for invasiveness among *S. aureus* strains.

	Univariate				Multivariable	
Genes	**Clinical Cultures (84)**	**Surveillance cultures (225)**	**OR (95%CI)**	**P**	**OR (95%CI)**	**P**
SCC*mec*						
Absence (reference)	14 (16.7)	75 (33.3)	reference	...	reference	...
type II	4 (4.8)	1 (0.4)	**21.43 (2.22-206.26)**	**0.004**	10.79 (0.95-122.14)	0.06
type III	59 (70.2)	44 (19.6)	**2.19 (1.15-4.18)**	**0.02**	**2.19 (1.08-4.45)**	**0.03**
type IV	7 (8.3)	5 (2.2)	**7.50 (2.08-27.02)**	**0.003**	**5.28 (1.35-20.63)**	**0.02**
Virulence Genes						
tst	19 (22.6)	28 (12.4)	**2.06 (1.08-3.93)**	**0.03**	1.36 (0.65-2.84)	0.41
sea	20 (23.8)	60 (26.7)	0.86 (0.38-1.54)	0.61	1.16 (0.57-2.33)	0.69
seb	22 (26.2)	43 (19.1)	1.50 (0.83-2.71)	0.17	1.64 (0.85-3.16)	0.14
sec1	20 (23.8)	75 (33.3)	0.63 (0.34-1.11)	0.11	0.55 (0.27-1.10)	0.09
eta	2 (2.4)	1 (0.4)	5.46 (0.49-61.06)	0.18	3.57 (1.48-27.46)	0.32
etb	8 (9.5)	4 (1.8)	**5.82 (1.70-19.86)**	**0.002**	**6.38 (1.48-27.46)**	**0.01**
etd	2 (2.4)	0 (0.0)	undefined	0.07	undefined*	0.99
pvl	21 (25.0)	25 (11.1)	**2.67 (1.40-5.09)**	**0.002**	**2.38 (1.16-4.86)**	**0.02**
ica A	80 (95.2)	220 (97.8)	0.46 (0.12-1.74)	0.26	0.71 (0.14-3.71)	0.69
ica D	84 (100.0)	216 (96.0)	undefined	0.12	undefined*	0.99
hla	84 (100.0)	223 (99.1)	undefined	1.00	undefined*	0.99
hld	84 (100.0)	225 (100.0)	undefined	1.00	not included**	not included

Note. Cases are in number(%). Significant results are presented in boldface.
All variables were dichotomic except sccMec (which was analyzed as a dummy variable, with absence as reference category)
OR, Odds Ratio. CI, Confidence Interval.

* CI ranging from zero to infinite. **Not included due to presence in all analyzed isolates.

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