IDENTIFICAÇÃO DE MICRO-ORGANISMOS PRESENTES EM HEMOCULTURAS DE PACIENTES DE UNIDADES DE TERAPIA INTENSIVA E AVALIAÇÃO DOS *Staphylococcus* COAGULASE-NEGATIVA

Aydir Cecília Marinho Monteiro

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia de Parasitas e Micro-organismos*.

Maria de Lourdes Ribeiro de Souza da Cunha

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Por vezes sentimos que aquilo que fazemos

não é senão uma gota de água no mar. Mas

o mar seria menor se lhe faltasse uma gota.

Madre Tereza de Calcutá

Dedicatória

*Dedico este trabalho aos meus pais, **Cecília** (in memoriam) e **Jair**, que souberam amar incondicionalmente, se dedicaram integralmente à minha educação e formação e sempre apoiaram minhas decisões. Certamente vocês são a razão da minha vida e das minhas vitórias!*

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Resumo

A rápida e precisa identificação de micro-organismos causadores de infecções da corrente sanguínea é uma das funções mais importantes do laboratório de microbiologia clínica. Com o objetivo de diminuir o tempo gasto para se identificar esses micro-organismos, vários equipamentos foram desenvolvidos, entre eles o sistema VITEK® 2, usado na rotina de diversos laboratórios de microbiologia clínica para a identificação de isolados de amostras clínicas. Inúmeros micro-organismos são isolados de hemoculturas e as bactérias pertencentes ao grupo dos cocos Gram-positivos aeróbios são os principais agentes etiológicos das infecções de correntes sanguíneas, principalmente em pacientes mantidos em unidades de terapia intensiva (UTIs). Este trabalho comparou sistemas de identificação de espécies de micro-organismos isolados a partir de hemoculturas e acompanhou os pacientes com hemoculturas positivas para Estafilococos coagulase-negativa (ECN). A partir dessas hemoculturas positivas coletadas de 216 pacientes foram isolados e identificados 400 micro-organismos, dentre eles 5 bacilos Gram-positivos, 15 leveduras, 165 bacilos Gram-negativos e 215 cocos Gram-positivos. Essa identificação foi realizada pelo sistema VITEK® 2 e os resultados foram comparados com os obtidos em provas fenotípicas convencionais e nos métodos genotípicos. Os pacientes com hemoculturas positivas para ECN foram acompanhados durante sua permanência no hospital com a coleta de seus dados clínicos dos prontuários. O sistema automatizado VITEK® 2 identificou corretamente 94,7% das amostras, os cartões YST e GN apresentaram 100% de identificações corretas das leveduras e dos bacilos Gram-negativos, respectivamente, ao passo que o GP identificou corretamente 92,6% dos cocos Gram-positivos. A sensibilidade apresentada pelo sistema VITEK® 2 e a análise estatística permitem concluir que esse equipamento é uma opção viável na rotina de laboratórios de microbiologia clínica para a identificação de micro-organismos. A análise multivariada para o desfecho óbito no estudo de coorte dos pacientes que apresentaram hemocultura positiva para ECN revelou associações positivas com AIDS e com hemocultura positiva para *S. hominis*, enquanto o uso de meropenem mostrou associação negativa. Na análise do desfecho múltiplas hemoculturas positivas para ECN, foi verificada associação positiva com pacientes em choque que necessitavam de droga vasoativa. Isso sugere que a gravidade da sepse (choque) é um preditor da significância clínica de ECN com o isolamento em mais de uma hemocultura, um dos critérios mais utilizados para valorização clínica desses micro-organismos quando isolados da corrente sanguínea. A análise da curva de sobrevida dos pacientes incluídos na coorte revelou que pacientes com hemocultura positiva para a espécie *S. hominis* apresentaram menor sobrevida quando comparados com pacientes com

hemoculturas para outras espécies de ECN, indicando a importância dessa espécie na gravidade das infecções da corrente sanguínea.

Abstract

The rapid and accurate identification of microorganisms which cause bloodstream infections is one of the major roles of the clinical microbiology laboratory. Aiming at reducing the time spent in identifying such microorganisms, several devices have been developed, including the VITEK® 2 system, which is routinely used in a number of clinical microbiology laboratories for identifying isolates from clinical specimens. A lot of microorganisms are isolated from blood cultures; bacteria belonging to the group of aerobic Gram-positive cocci are the main etiological agents of bloodstream infections, especially in patients kept in intensive care units (ICUs). The objectives of this study were to compare the performance of (manual and automated) species identification systems for microorganisms isolated from blood cultures by evaluating the performance of VITEK® 2 system and also to conduct a cohort study of patients who had blood cultures positive for Coagulase-negative staphylococci (CoNS) in order to address two outcomes: isolation of CoNS in more than one blood culture as opposed to a single blood culture and the factors associated with patient prognosis. Four hundred microorganisms isolated from blood cultures were identified: 5 Gram-positive bacilli, 15 yeasts, 165 Gram-negative bacilli, and 215 Gram-positive cocci. This identification was carried out by the VITEK® 2 system and the results were compared with those obtained from conventional phenotypic tests and genotypic methods. Patients with blood cultures positive for CoNS were followed during their stay in hospital with collecting the clinical data from their medical records. The automated VITEK® 2 system accurately identified 94.7% of the specimens. The YST and GN ID cards had 100% accurate identifications of the yeasts and the Gram-negative bacilli respectively, whereas the GP ID card accurately identified 92.6% of the Gram-positive cocci. The susceptibility of the VITEK® 2 system and the statistical analysis allow the conclusion that this instrument is a viable option in the routine of clinical microbiology laboratories for microorganism identification. The multivariate analysis for death as the outcome in the cohort study of patients with CoNS positive blood culture revealed positive associations with AIDS and with blood culture positive for *S. hominis*, while the use of meropenem showed a negative association. In analyzing the outcome of multiple blood cultures positive for CoNS, a positive association was observed in patients with shock (requiring vasoactive drugs). It suggests that the sepsis (shock) severity is a predictor of CoNS clinical significance with the isolation in more than one blood culture, which is one of the most commonly used criteria for clinical recovery of the microorganisms when isolated from the bloodstream. The analysis of the survival curve of patients included in the cohort

revealed that patients with blood cultures positive for *S. hominis* species showed lower survival compared to patients with blood cultures for other species of CoNS, which indicates the importance of this species in the severity of bloodstream infections.

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1. Introdução

Infecções de corrente sanguínea ocorrem pela presença de micro-organismos viáveis circulantes no sangue e comprovados laboratorialmente[1]. Esses micro-organismos são provenientes da microbiota do paciente ou da contaminação decorrente do manuseio inapropriado desse paciente pelos profissionais de saúde[2]. A sepse é definida como a resposta inflamatória sistêmica ao agente causador da infecção e resulta da complexa interação entre o sistema imune e o micro-organismo infectante que se multiplica a uma taxa que excede sua remoção do organismo[3]. É uma síndrome clínica caracterizada por febre, calafrio, mal-estar, taquicardia, hiperventilação, toxidade ou prostração. Os seus sintomas são produzidos por toxinas microbianas e/ou citocinas produzidas por células inflamatórias, podendo manifestar desde quadros leves até o óbito[4].

Essas infecções são consideradas um problema de saúde pública por elevarem as taxas de morbidade e mortalidade dos pacientes, o tempo de hospitalização e os custos no tratamento[5], além de serem a principal causa da mortalidade de pacientes em estado crítico[6]. Nos EUA, entre os anos 2003 e 2007, foi registrado um aumento nos casos de sepse de 415.000 para 700.000 por ano, com um aumento nos custos associados de 15,4 bilhões de dólares para 24,3 bilhões nesse período[7]. Há uma estimativa de que ocorram 17 milhões de casos anualmente em todo o mundo[8]. Os números dos casos de sepse brasileiros não são conhecidos, mas os dados disponíveis indicam elevada mortalidade nos hospitais públicos que atendem ao SUS (Sistema Único de Saúde)[9].

A sepse é associada com taxas de mortalidade que variam de 20% a 50%[10]. O diagnóstico de um paciente séptico é de fundamental importância já que a terapia antimicrobiana depende desse diagnóstico. Tal importância evidencia-se nos casos de pacientes que receberam antibioticoterapia adequada ao perfil de sensibilidade do agente infeccioso isolado em cultura e tiveram menor mortalidade que os pacientes que receberam antibioticoterapia inadequada e, posteriormente, esse tratamento foi ajustado com o diagnóstico obtido na cultura[4]. O diagnóstico precoce seguido pelo tratamento adequado melhora o prognóstico do paciente séptico[11-13]. Cada hora de atraso na administração do antibiótico correto está associada com uma diminuição média de 8% na taxa de sobrevivência a um choque séptico[14].

As infecções de corrente sanguínea apresentam uma maior incidência desde o levantamento do *National Nosocomial Infections Surveillance* (NNIS) em 1991[15]. Esse aumento é justificado pelo avanço nos cuidados de saúde e pelo uso de dispositivos invasivos, principalmente o cateter intravenoso no tratamento e monitoramento dos pacientes graves em

UTIs[16-18]. A sepse é considerada a segunda causa de morte em UTI não coronariana nos Estados Unidos[19] e a principal causa em países latino-americanos[20].

Inúmeros micro-organismos são isolados da corrente sanguínea. Entre as bactérias, os bacilos Gram-negativos apresentam maior mortalidade associada quando comparados aos cocos Gram-positivos, porém a sua frequência nas infecções de corrente sanguínea diminuiu desde a década de 1990[21,22]. O *National Healthcare Safety Network* (NHSN) realizou um levantamento de dados entre 2006 e 2007 que apontou os Estafilococos coagulase-negativa (ECN) como os principais agentes causadores de infecções[23]. Tal fato se repetiu nos dados apresentados em 2013 do levantamento realizado entre 2009 e 2010[24].

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 cocos Gram-positivos como os principais agentes de infecções da corrente sanguínea (65%) e os ECN como os agentes mais frequentes (31%), seguidos por *S. aureus* (20%)[17]. Dados brasileiros coletados entre junho de 2007 e março de 2010 de um estudo multicêntrico utilizando a mesma metodologia do programa SCOPE americano, o SCOPE brasileiro, com o objetivo de estudar a epidemiologia e a microbiologia das infecções da corrente sanguínea nosocomiais de pacientes provenientes de 16 hospitais brasileiros de diversos tamanhos e diferentes regiões, revelaram o *Staphylococcus aureus* (14%) e os ECN (12,6%) como os micro-organismos mais isolados[25]. Os dados do Sistema de Vigilância de Infecção Hospitalar do Estado de São Paulo, o 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%)[26]. Essa prevalência de ECN é repetida em resultados apresentados em estudos entre os anos 2006 e 2013 realizados na Ásia e no Brasil[27-30].

O gênero *Staphylococcus* é composto por 52 espécies[31] classificadas em dois grupos de acordo com a capacidade de produção da enzima coagulase. O primeiro grupo, conhecido como Estafilococos coagulase positiva, tem como principal representante o *S. aureus*[32]. No segundo grupo, conhecido como Estafilococos coagulase-negativa (ECN), as principais espécies envolvidas nas infecções humanas são *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. cohnii*, *S. xylosus*, *S. capitis*, *S. warneri*, *S. hominis*, *S. simulans* e *S. lugdunensis*[33, 34].

Os ECN, apesar de anteriormente terem sido considerados não patogênicos, atualmente estão implicados em várias infecções, tanto em hospedeiros imunodebilitados quanto em indivíduos saudáveis[35]. Esse grupo de bactérias é integrante da microbiota humana,

ou seja, são habitantes naturais da pele e das mucosas dos seres humanos e vivem de forma simbiótica com seu hospedeiro. Em casos de traumas da barreira cutânea, inoculação através de agulhas, ou implante de dispositivos médicos (cateteres, próteses, entre outros), esses micro-organismos adquirem potencial patogênico e podem causar sérias infecções. Estes são os micro-organismos com maior frequência de isolamento em materiais clínicos[36,37].

Há algumas décadas os ECN são considerados importantes patógenos, principalmente em infecções que acometem pacientes imunocomprometidos, prematuros e aqueles com implantes. Atualmente, eles são reconhecidos como micro-organismos essencialmente oportunistas que se prevalecem de inúmeras situações orgânicas para produzirem graves infecções[38,39]. No ambiente hospitalar, os ECN são os maiores causadores de bacteremia, maioritariamente em pacientes mantidos em UTIs[36].

A confirmação da presença de micro-organismos em hemoculturas está entre as funções mais importantes do laboratório de microbiologia clínica[40], pois o exame de hemocultura, considerado o padrão ouro no diagnóstico da sepse, é capaz de elucidar a etiologia e orientar o tratamento antibiótico adequado a fim de melhorar o prognóstico do paciente em sepse, e assim reduzir a morbidade e mortalidade[41,42]. Um grande número de sistemas comerciais de cultura de sangue está disponível, incluindo uma cultura convencional do sangue em caldo, o sistema de lise-centrifugação e sistemas automatizados[43]. Entre os sistemas automatizados, o BACTEC™ da empresa BD (Becton, Dickinson and Company) é o mais utilizado nos laboratórios clínicos hospitalares, uma vez que ele capta o crescimento do micro-organismo em tempo significativamente menor que os meios convencionais[44]. Há um dispositivo nesse equipamento que detecta o crescimento do micro-organismo nos frascos de hemocultivo através da liberação de CO_2 proveniente do metabolismo bacteriano durante sua fase de crescimento. Cada frasco colocado no aparelho é incubado a 37ºC e continuamente homogeneizado por rotação. A cada dez minutos, por todos os frascos passam feixes de luz que são filtrados e refletidos em um sensor sensível à presença de CO_2. Caso ocorra o acúmulo de CO_2 no fundo do frasco, o sensor é ativado e o aparelho gera um alerta sonoro de positividade[45].

O uso de sistemas automatizados para realização da hemocultura diminuiu consideravelmente o tempo desse exame no laboratório, mas o passo seguinte de identificação e testes de susceptibilidade antimicrobiana (TSA) ainda demora de 48 a 72 horas[46]. Isso ocorre porque a identificação bacteriana no laboratório de microbiologia clínica é tradicionalmente realizada pelas etapas de isolamento do micro-organismo, análise fenotípica das suas características através de coloração de Gram, análise das estruturas morfológicas,

exigência nutricional para crescimento e reações bioquímicas[47]. Embora métodos convencionais fenotípicos sejam relativamente baratos e permitam a identificação dos micro-organismos mais comuns, certos grupos de bactérias são de difícil identificação, tornando equipamentos específicos muitas vezes necessários[48]. Essa demora nos processos posteriores à positividade da hemocultura pede o desenvolvimento de métodos mais rápidos e eficientes para a identificação dos micro-organismos isolados e para seus TSA, principalmente quando se trata da urgência inerente aos casos de sepse[49]. Os avanços tecnológicos responsáveis por uma identificação rápida dos isolados clínicos junto aos TSA caracterizam um benefício clínico e financeiro[50].

A automação na microbiologia clínica ocorreu como uma resposta ao aumento do número de isolados clínicos processados na rotina dos laboratórios de microbiologia e ela foi facilitada pela interface entre os sistemas de informática dos laboratórios e dos hospitais[51,52]. Nesse cenário, vários sistemas automatizados foram produzidos para a identificação de micro-organismos e para a realização dos TSA. A identificação das espécies ocorre com base na interpretação automática dos resultados obtidos em testes bioquímicos ou a partir de bandejas de microdiluição após incubação de 18 horas com determinação do crescimento através de fotometria[53-55].

O sistema VITEK (BioMérieux) surgiu na década de 1970 como um sistema automatizado de identificação de micro-organismos e determinação de susceptibilidade a antimicrobianos. Posteriormente, ele evoluiu para o sistema VITEK® 2, um equipamento capaz de realizar automaticamente as etapas necessárias para a identificação de micro-organismos e determinação de susceptibilidade antimicrobiana com um inóculo primário preparado a partir de um cultivo em meio sólido padronizado pelo próprio fabricante do equipamento. Esse sistema realiza uma análise cinética da cultura inoculada com leituras de cada teste em intervalos de 15 minutos. O sistema óptico do equipamento combina um fluorímetro multicanal e um fotômetro de leitura para gravar dados de turbidez, fluorescência e sinais colorimétricos[55]. O equipamento trabalha com cartelas para identificação de amostras denominadas de Reagent Card, sendo que cada cartela contém 64 poços com substratos individuais capazes de analisar diferentes atividades metabólicas, tais como: acidez, basicidade, hidrólise e crescimento ou não na presença de inibidores. Para garantir o crescimento bacteriano, a tecnologia usada no sistema VITEK® 2 mantém os níveis de oxigenação através de um filme que recobre os dois lados do cartão, e ao mesmo tempo, esse filme evita a contaminação dos cartões. Os cartões são produzidos para séries específicas de micro-organismos: bacilos Gram-negativos (GN card), cocos Gram-positivos (GP card),

leveduras (YST card) e bactérias anaeróbias (ANC card). Esse sistema identifica mais de 330 espécies de micro-organismos, ou seja, mais de 95% das espécies isoladas na rotina dos laboratórios de microbiologia clínica[56].

O sistema VITEK® 2 é aprimorado e avaliado há mais de vinte anos. A mudança dos cartões de identificações ID-GPC e ID-GNB para os cartões GP e GN tornou o sistema mais eficiente[57] e os estudos sobre a sensibilidade do sistema na identificação de micro-organismos isolados de hemoculturas relataram taxas corretas de identificação acima dos 90%[58-60].O desempenho do sistema VITEK® 2 na identificação dos bacilos Gram-negativos é satisfatório desde os primeiros estudos, apresentando taxas de identificação corretas próximas a 100%[60-63].

A identificação de cocos Gram-positivos pelo sistema automatizado VITEK® 2 é estudada há mais de 15 anos e apresenta taxas de identificação corretas entre 94% e 98%[59,64], o que representam desempenhos acima dos 90% exigidos dos equipamentos comerciais no campo da microbiologia clínica. Ao longo dos anos, esses estudos demonstraram que, entre os cocos Gram-positivos, os ECN são as espécies com maior taxa de resultados incorretos[65-69]. Esses resultados insatisfatórios ocorrem porque os sistemas automatizados de identificação não são capazes de fazer uma diferenciação totalmente confiável entre as distintas espécies de ECN devido à expressão variável das características fenotípicas desses micro-organismos[70]. Um fato importante observado desde 1997 é que o lento metabolismo de determinadas espécies leva a um resultado ambíguo na identificação das mesmas[66].

Os métodos de biologia molecular para a identificação de micro-organismos, como a reação em cadeia da polimerase (PCR) e sequenciamento do DNA de região do gene 16S do rRNA, são alternativas para se identificar bactérias em nível de espécie ou são como complemento aos métodos fenotípicos tradicionais[71,72]. Há relatos antigos da utilização dessas técnicas na identificação de espécies bacterianas para resolução de problemas de identificação em laboratório de microbiologia clínica[73]. No entanto, os processos de amplificação e sequenciamento do DNA são métodos trabalhosos, caros e dependentes de mão de obra qualificada, o que os torna inviáveis para a rotina dos laboratórios de microbiologia clínica[47].

Uma identificação rápida e precisa dos isolados em amostras clínicas de hemocultura se faz necessária, pois ela é capaz de elucidar as etiologias de doenças infecciosas e de orientar o tratamento antibiótico adequado a fim de melhorar o prognóstico do paciente em sepse, reduzindo, assim, a morbidade e mortalidade. A utilização de equipamentos automatizados, como o sistema VITEK® 2, é uma solução devido a sua prontidão, acurácia e sensibilidade na obtenção dos resultados. Além da rapidez nos resultados de hemoculturas

positivas, outro aspecto observado nos últimos anos é o aumento das infecções causadas por ECN. Portanto, o estudo da sua epidemiologia é de extrema importância para o melhor conhecimento do potencial patogênico desses micro-organismos visando à profilaxia e ao tratamento adequado dessas infecções.

2. Objetivos

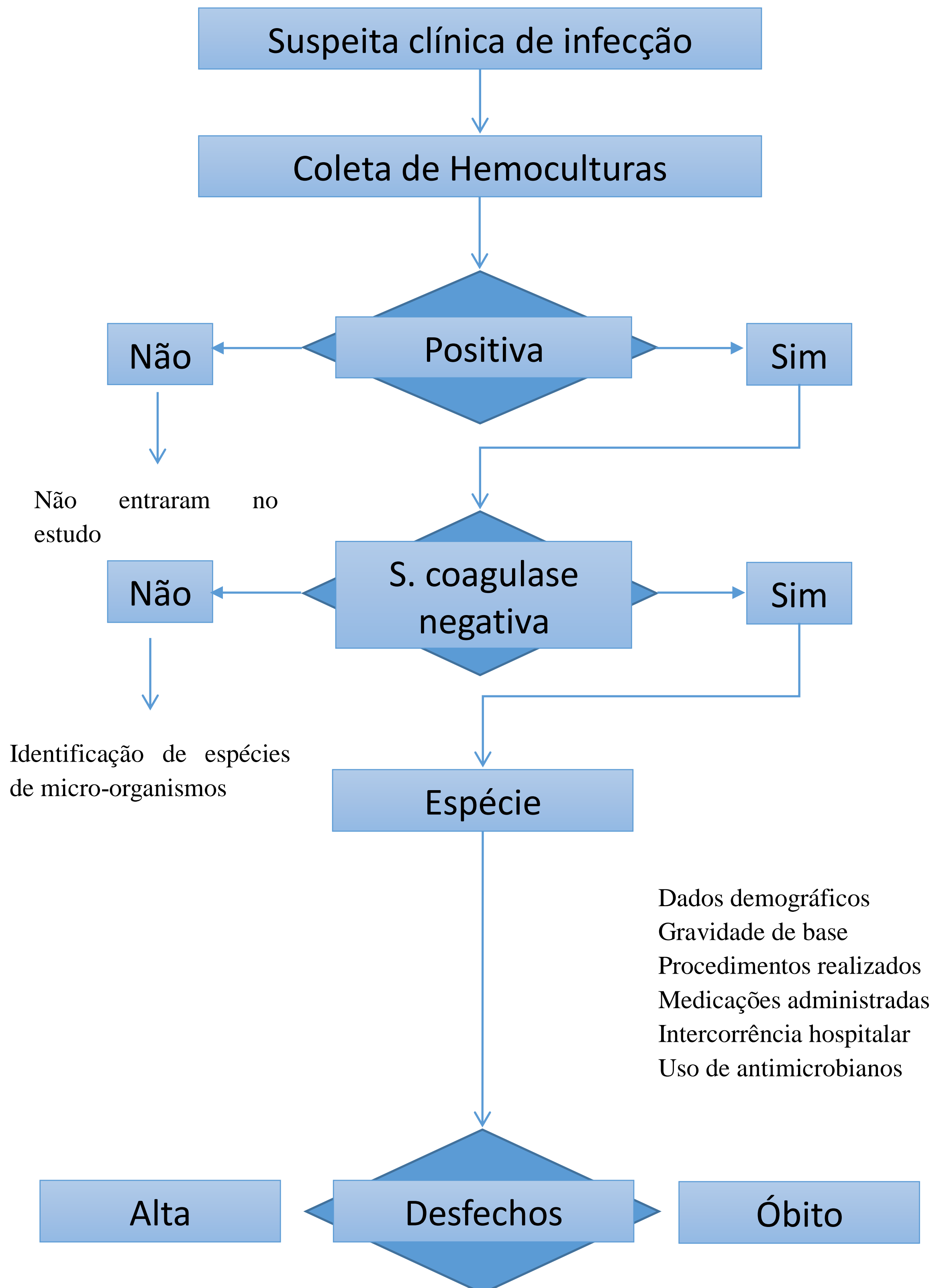
2.1 Objetivos gerais

Comparar o desempenho de sistemas (manual e automatizado) de identificação de espécies de micro-organismos isolados a partir de hemoculturas e realizar um estudo de coorte com os pacientes que apresentaram hemoculturas positivas para Estafilococos coagulase-negativa (ECN) com a finalidade de abordar dois desfechos de interesse: o isolamento de ECN em mais de uma hemocultura em oposição à cultura única e os fatores associados ao prognóstico desses pacientes.

2.2 Objetivos específicos

- Isolar e identificar através de métodos convencionais os micro-organismos presentes nas hemoculturas positivas de pacientes internados na UTI do Hospital das Clínicas da Faculdade de Medicina de Botucatu.
- Identificar por métodos genotípicos os micro-organismos presentes nos frascos de hemocultura, diretamente do sangue cultivado, através da técnica de PCR ou sequenciamento de regiões específicas do RNA r16S.
- Comparar os resultados da identificação dos micro-organismos isolados pelo método fenotípico convencional com os resultados liberados pelo equipamento VITEK® 2, utilizando o método genotípico como padrão ouro.
- Acompanhar pacientes com hemoculturas positivas para Estafilococos coagulase-negativa (ECN) para analisar os fatores associados ao isolamento de ECN em mais de uma hemocultura em oposição à positividade em hemocultura única.
- Avaliar as diferentes espécies de ECN e os dados clínicos dos pacientes com hemoculturas positivas para ECN para identificar fatores de risco associados ao desfecho óbito.

3. Delineamento do estudo



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5. Apresentação da Tese

Os resultados obtidos e a discussão desses resultados encontram-se apresentados na forma de artigos científicos.

5.1 Comparison of methods for the identification of microorganisms isolated from blood cultures. Publicado na revista *Annals of Clinical Microbiology and Antimicrobials* (2016).

5.2 Evaluation of the outcome of patients with blood culture positive for coagulase-negative staphylococci: death and hospital discharge.

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RESEARCH

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Comparison of methods for the identification of microorganisms isolated from blood cultures

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Abstract

Background: Bloodstream infections are responsible for thousands of deaths each year. The rapid identification of the microorganisms causing these infections permits correct therapeutic management that will improve the prognosis of the patient. In an attempt to reduce the time spent on this step, microorganism identification devices have been developed, including the VITEK® 2 system, which is currently used in routine clinical microbiology laboratories.

Methods: This study evaluated the accuracy of the VITEK® 2 system in the identification of 400 microorganisms isolated from blood cultures and compared the results to those obtained with conventional phenotypic and genotypic methods. In parallel to the phenotypic identification methods, the DNA of these microorganisms was extracted directly from the blood culture bottles for genotypic identification by the polymerase chain reaction (PCR) and DNA sequencing.

Results: The automated VITEK® 2 system correctly identified 94.7 % (379/400) of the isolates. The YST and GN cards resulted in 100 % correct identifications of yeasts (15/15) and Gram-negative bacilli (165/165), respectively. The GP card correctly identified 92.6 % (199/215) of Gram-positive cocci, while the ANC card was unable to correctly identify any Gram-positive bacilli (0/5).

Conclusions: The performance of the VITEK® 2 system was considered acceptable and statistical analysis showed that the system is a suitable option for routine clinical microbiology laboratories to identify different microorganisms.

Keywords: Blood culture, Phenotypic identification, Genotypic identification, Automated VITEK® 2 system

Background

Sepsis is a global health problem and an estimated 17 million cases of sepsis occur each year in the world [1]. The early initiation of appropriate antibiotic therapy is determinant for the prognosis and survival of patients with bloodstream infections [2]. Patients receiving antibiotic therapy that is adapted based on the susceptibility profile of the infectious agent isolated from blood cultures exhibit lower mortality than those treated initially with inadequate antibiotic therapy [3]. In addition, technological advances that permit the rapid and reliable identification of most pathogens involved in infectious diseases have long been recognized to have clinical benefits, including shorter hospital stays and lower mortality, as well as financial benefits by reducing healthcare costs [4].

The objective of this study was to evaluate the sensitivity of the VITEK® 2 system, a system that automatically performs the processes required for microorganism identification and for the determination of antimicrobial susceptibility using a standard primary inoculum isolated from subcultures of positive

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blood cultures. Although classical identification methods are still considered the gold standard, these methods are slow, time consuming and prone to subjective interpretations. On the other hand, the VITEK® 2 system reduces the time necessary for identification and permits the standardization of inter- and intra-laboratory results, the storage of results, issuing rapid epidemiological reports, and simultaneous identification and antimicrobial susceptibility testing; however, the system is poorly efficient in identifying certain species of Gram-positive cocci [42].

Studies using direct inoculation of VITEK® 2 cards from blood culture bottles have been conducted in an attempt to further reduce the time of identification of microorganisms that cause bloodstream infections, but the results were acceptable only for Gram-negative bacilli and were inaccurate for Gram-positive cocci [5, 6]. For this reason, the present study used inocula of microorganisms cultured previously on solid media for 24 h.

The difference of this study was the prospective evaluation of the VITEK® 2 system during the routine work of a clinical microbiology laboratory in a university hospital using 400 microorganisms isolated from blood cultures collected during the hospitalization period of patients rather than to conduct a retrospective study of samples stored for years.

Methods

Isolates studied

Four hundred microorganisms isolated from positive blood cultures of patients hospitalized in intensive care units of the Botucatu University Hospital between August 2012 and February 2014 were identified. The blood samples were inoculated into blood culture bottles and incubated in the BACTEC™ 9050 apparatus.

Identification of microorganisms in positive blood cultures

Automated phenotypic identification

Samples exhibiting microbial growth were submitted to Gram staining and cultured on solid media directly from the blood culture bottles. After subculture on blood and MacConkey agar, the isolates were inoculated into the following specific identification cards of the automated VITEK® 2 system using the standard protocol: Gram-positive cocci (GP), Gram-positive bacilli (GN), Gram-negative bacilli (ANC), and yeasts (YST). Gram-positive cocci, Gram-positive bacilli and yeast were inoculated into the cards from colonies grown on blood agar and Gram-negative bacilli from colonies grown on MacConkey agar, all diluted in saline (0.9 % NaCl) to a 0.5 McFarland standard.

Phenotypic identification by conventional methods

Phenotypic identification consisted of Gram staining for the observation of morphology and specific staining, followed by a series of biochemical tests specific for each group of microorganisms. Gram-positive cocci were submitted to the catalase test for differentiation between *Staphylococcus* and *Enterococcus*. The following biochemical test battery was used for the identification of species of the genus *Staphylococcus*: coagulase, sugar fermentation (sucrose, maltose, trehalose, xylose, and mannitol), anaerobic growth on semi-solid sodium thioglycolate medium and, if necessary, ornithine and urease production and novobiocin susceptibility [7]. Isolates previously identified as Gram-positive, catalase-negative, bile esculin-positive, NaCl-positive (growth in brain heart infusion broth with 6.5 % NaCl) and pyrrolidonyl-aminopeptidase test-positive cocci were submitted to biochemical tests of fermentation of mannitol, arabinose, arginine and sorbitol, motility, and presence or absence of a pigment on sheep blood agar. Gram-negative bacilli were first tested for glucose fermentation. Glucose-fermenting bacilli were submitted to manual biochemical tests known as EPM/MILi/Citrate, an identification system based on the following tests: production of H_2S, urease and L-tryptophan desaminase; motility; indol production; lysine decarboxylase production, and the ability to use citrate as a single carbon source. Non-glucose-fermenting Gram-negative bacilli were identified based on motility, growth at a temperature of 42 °C, and production of DNAse. Yeasts were isolated on Sabouraud agar, replated on CHROMagar, and identified based on the color, texture and shape of their colonies [8].

DNA extraction from the isolates

Extraction of bacterial DNA Bacterial DNA was extracted directly from the blood sample of the blood culture bottle using the Illustra Kit (GE Healthcare) according to the protocol of the manufacturer, with modifications in the first centrifugation [9] and the addition of 800 μL benzyl alcohol [10]. For sample collection, the lid of the blood culture bottle was first disinfected with cotton soaked in 70 % alcohol. Next, 1.5 mL of the culture was aspirated with a sterile needle and syringe and transferred to sterile microtubes. The microtubes were centrifuged at 850*g* for 2 min and the supernatant was removed by aspiration with a micropipette and sterile tips and the supernatant stored (directly in a DNA-free microtube) at −20 °C until the time of extraction.

For DNA extraction, the sample was centrifuged at 10,000*g* for 1 min, the supernatant was discarded, and 500 μL lysozyme was added to the sediment. The mixture was vortexed, 800 μL benzyl alcohol was added, and

the mixture was again shaken and centrifuged at 7000*g* for 5 min. Next, 300 μL of the supernatant was carefully removed and transferred to a new sterile microtube (which was used for extraction). Ten microliter lysozyme (10 mg/mL) was added and the microtube was left to stand at room temperature for 15 min, with vortexing every 5 min. After this period, 10 μL proteinase K (20 mg/mL) was added and the mixture was vortexed. The microtube was incubated for 15 min at 56 °C, with vortexing every 5 min. This mixture was then transferred to an extraction microcolumn and centrifuged at 11,000*g* for 1 min. The filtrate was discarded and 500 μL washing solution was added to the microcolumn. The column was again centrifuged at 11,000*g* for 3 min. The supernatant was discarded, the microcolumn was transferred to a new sterile microtube, and 200 μL Milli-Q water previously heated to 70 °C was added. The microcolumn was kept at room temperature for 1 min and centrifuged at 11,000*g* for 1 min. The columns were discarded and the filtered material was frozen until analysis by the polymerase chain reaction (PCR).

Extraction of yeast DNA Yeast DNA was extracted according to the protocol proposed by McCullough et al. [11]. The isolates were seeded onto inclined Sabouraud agar and incubated for 36 h at 37 °C. A loopful of this culture was resuspended in a 2-mL tube containing 1 mL 1 M sorbitol, 125 mM EDTA, and 500 mg glass beads. The tube was shaken twice in a Precellys® homogenizer for 45 s and centrifuged at 13,000*g* for 10 min. The supernatant was discarded and the sediment together with the glass beads was resuspended in 500 μL of a buffer solution containing 50 mM Tris–HCl, 50 mM EDTA and 2 % SDS and incubated for 1 h at 65 °C. After incubation, 500 μL 3 M sodium acetate was added. The mixture was homogenized by inverting the tube and kept on ice for 2 h, followed by centrifugation for 10 min at 25 °C. The supernatant was transferred to a 1.5-mL centrifugation microtube containing 1 mL ice-cold absolute ethanol, homogenized by inversion, and centrifuged for 10 min at 4 °C. The supernatant was discarded and the DNA retained on the tube wall was resuspended in 50 μL autoclaved Milli-Q water and frozen until the time of PCR.

Genotypic identification of the isolates

Polymerase chain reaction of bacteria Gram-positive bacteria of the genus *Staphylococcus* that belonged to the group of coagulase-negative staphylococci (CoNS) were identified by internal transcribed spacer PCR (ITS-PCR) using primers targeting conserved sequences adjacent to the 16S and 23S genes: G1 (5′-GAAGTCGT AACAAGG-3′) and L1 (5′-CAAGGCATCCACCGT-3′) [12, 13].

The remaining isolates of Gram-positive cocci and Gram-negative bacilli were submitted to PCR carried out in 0.2-mL microtubes containing 15.8 μL Milli-Q water, 10 pM of the forward primer and 10 pM of the reverse primer, 100 μM triphosphate desoxyribonucleotides (GE Healthcare), 10 U Taq DNA polymerase (Biotools), 20 mM $MgCl_2$-free buffer (Biotools), 0.75 mM $MgCl_2$ (Biotools), and 3 μL DNA. Primers targeting conserved sequences of each species were used for DNA amplification. The temperature and time parameters and number of amplification cycles reported in the literature and described in Table 1 were used.

The efficiency of the amplifications was monitored by electrophoresis on 2 % agarose gel prepared in 1× TBE buffer (89 mM Tris (pH 7.6), 89 mM boric acid, and 2 mM EDTA) and stained with SYBR® Safe at 90 V for 60 min (Figs. 1, 2). The following international reference strains were used as controls: *Acinetobacter baumannii* (ATCC 19606), *Enterobacter cloacae* (ATCC 23355), *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecium* (ATCC 6569), *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 4352), *Morganella morganii* (ATCC 8019), *Proteus mirabilis* (ATCC 15290), *Pseudomonas aeruginosa* (ATCC 15442), *Serratia marcescens* (ATCC 14756), *Staphylococcus aureus* (ATCC 25923), and *Stenotrophomonas maltophilia* (ATCC 13637).

Figures 1 and 2 illustrate the genotypic identification of *Enterobacter cloacae* and *Escherichia coli*, respectively.

DNA sequencing of bacteria The yeast and Gram-positive bacillus isolates were sequenced for identification to species level. Gram-negative bacilli identified as *Enterobacter aerogenes* by the conventional phenotypic methods and by the automated test were sequenced for species confirmation.

Amplification and purification of bacterial DNA for sequencing The bacterial isolates identified as Gram-positive bacilli by Gram staining and the Gram-negative bacilli identified as *Enterobacter aerogenes* by the conventional phenotypic methods and automated test were submitted to simple PCR. The efficiency of the reactions was monitored as described in item 2.4.1. For PCR, protocols described in the literature were followed using universal primers for Gram-positive bacilli [14] and primers of enterobacteria for *Enterobacter aerogenes* isolates [15]. The temperature and time parameters and number of amplification cycles are shown in Table 1. The amplified DNA fragments were purified using the HiYield Gel/PCR DNA Fragments Extraction Kit (RBC).

Amplification and purification of yeast DNA The yeast isolates were submitted to ITS-PCR as described by

Table 1 Primers used for the identification of some bacterial species by PCR

Microorganism	Gene	Annealing temperature (°C)	Amplicon size (bp)	Reference
Acinetobacter baumannii	blaOXA_51-like	52	353	[44]
Acinetobacter lwoffii	blaOXA_154-like	52	223	[45]
Enterobacter cloacae	*Hsp60* (housekeeping)	52	343	[46]
Enterococcus faecalis	16S chromosomal region	60	143	[47]
Enterococcus faecium	*sodA* (superoxide dismutase)	45	216	[21]
Escherichia coli	*gadA* (alpha-glutamate decarboxylase)	65	373	[48]
Klebsiella pneumoniae	*rpoB* (β subunit of RNA polymerase)	52	364	[49]
Morganella morganii	16S chromosomal region	62	809	[50]
Proteus mirabilis	*rsbA* (quorum sensing)	55	236	[51]
Pseudomonas aeruginosa	Genome fragment	55	724	[52]
Serratia marcescens	16S chromosomal region	52	1058	[53]
Staphylococcus aureus	16S chromosomal region	52	442	[54]
Stenotrophomonas maltophilia	*atpD* (housekeeping)	52	854	[55]
Bacillus spp.	*rpoB* (β subunit of RNA polymerase)	72	400	[14]
Enterobacter aerogenes	16SrDNA	55	280	[15]

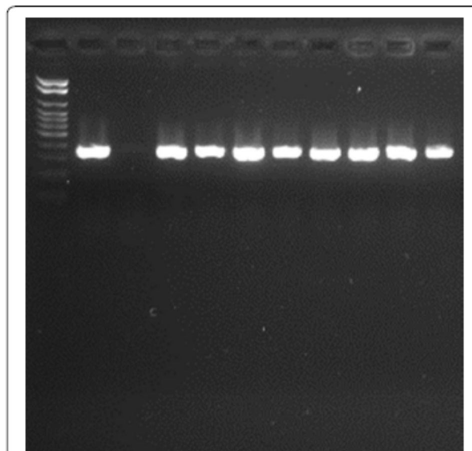

Fig. 1 Agarose gel electrophoresis for detection of *Hsp60* (343 bp) in *Enterobacter cloacae* (stained with SYBR® Safe) showing the amplified products positive control, negative control and some samples studied. A 100-bp ladder was used as molecular size marker

Kurtzman et al. [16]. These reactions were carried out in 0.2-mL microtubes containing a mixture of 6.7 μL Milli-Q water, 10 μM forward primer and 10 μM reverse primer, 12.5 μL GoTaq®Green Master Mix, and 3 μL DNA. The efficiency of the amplifications was monitored by electrophoresis (60 min, 80 V) on 1.5 % agarose gel prepared in 1× TBE buffer and stained with SYBR® Safe. Strains previously identified at the Laboratory of Fungal Biology, Department of Microbiology and Immunology, IBB, were used as controls. The amplified fragments were purified using the Illustra™ ExoProStar™ Kit (GE Healthcare Life Sciences).

DNA sequencing reaction of yeast and bacteria The sequencing reactions were carried out in a mixture containing 3.25 μL water, 1.75 μL 5× BigDye buffer (Applied Biosystems), 1 μL of each primer (5 pmol/μL), 2 μL of the PCR product, and 2 μL BigDye (Applied Biosystems). The cycle sequencing reaction was initiated at 96 °C for 1 min, followed by 40 cycles of denaturation at 96 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 4 min. The sequencing reaction product was precipitated with 1 μL 125 mM EDTA, 1 μL 3 M sodium acetate, and 25 μL 100 % ethanol. After homogenization, the solution was left to stand for 15 min and then centrifuged at 3000*g* for 15 min at 4 °C. The supernatant was removed by inverting the tube and 35 μL 70 % ethanol was added. The solution was centrifuged at 1650*g* for 15 min at 4 °C. After removal of the supernatant by inversion, 10 μL HiDi formamide (Applied Biosystems) was added and the mixture was left to stand for 5 min at 95 °C and for 2 min on ice. The product was run on an 8-capillary ABI 3500 sequencer (50 cm) using POP7 polymer (Applied Biosystems).

Analysis of the DNA sequences of yeast and bacteria The MEGA 5.0 (Molecular Evolutionary Genetics Analysis) and Lasergene programs were used for visualization and alignment of the DNA sequences obtained, which were compared to sequences published and stored in the GenBank database.

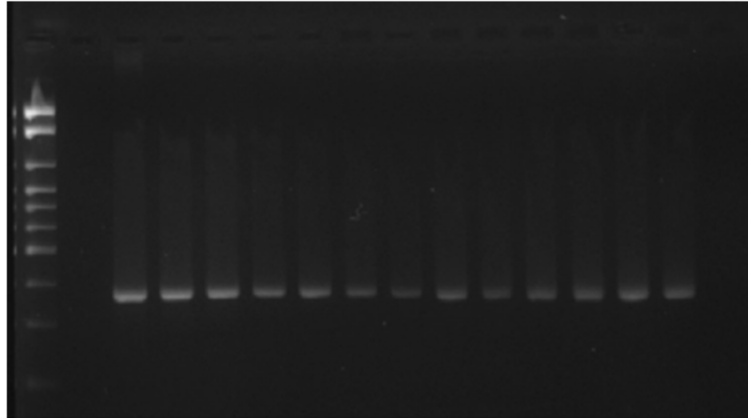

Fig. 2 Agarose gel electrophoresis for detection of *gadA* (373 bp) in *Escherichia coli* (stained with SYBR® Safe) showing the amplified products positive control, negative control and some samples studied. A 100-bp ladder was used as molecular size marker

Statistical analysis

Agreement between the results obtained with the different identification methods was analyzed statistically by the kappa test using the SPSS 20 program (IBM, Armonk, NY, USA). Genotypic identification was used as the gold standard. The accuracy of the conventional phenotypic identification methods and identification by the VITEK® 2 system was evaluated by calculating sensitivity and specificity according to Fletcher et al. [17].

Results

The results of identification with the automated VITEK® 2 system showed overall agreement of 94.7 % with the results of the genotypic methods (Tables 2, 3). Overall agreement of 98.7 % was observed between the results obtained by phenotypic identification using conventional methods and the results of the genotypic methods.

All yeast isolates (15/15) were correctly identified by the VITEK® 2 system using the YST card. The same result was obtained with the GN card for the identification of Gram-negative bacilli, with 100 % correct identifications of the 165 strains isolated.

The rate of correct identification obtained with the GP card used for the identification of Gram-positive cocci was 92.6 % for the microorganisms isolated (199/215). The agreement between the results of automated identification and those obtained with the other identification methods for *Enterococcus* spp. was 91.7 % due to the incorrect identification of one *Enterococcus faecalis* strain by the VITEK® 2 system. The agreement between species of the genus *Staphylococcus* was 97.5 % (198/203). The VITEK® 2 system correctly identified all *Staphylococcus aureus* isolates (17/17) and incorrectly identified 15 of the 186 (9.19 %) isolates belonging to the group of CoNS. The highest rate of incorrect identification was observed for *Enterococcus faecalis* with 12.5 % (7/8), followed by *Staphylococcus epidermidis* with 12.3 % (10/81), *Staphylococcus hominis* with 8.6 % (3/35), *Staphylococcus capitis* with 5.5 % (1/18), and *Staphylococcus haemolyticus* with 2 % (1/50).

The VITEK® 2 system incorrectly identified the isolates because some biochemical tests failed during the identification process performed by the device, exhibiting divergent characteristics of the species isolated. These errors are shown in Table 4.

Fewer errors occurred when the conventional phenotypic methods were used for identification compared to the automated VITEK® 2 system. Among Gram-positive cocci, the conventional phenotypic methods correctly identified 211/215 (98.1 %) isolates and the few errors observed mainly occurred in the identification of CoNS species, showing divergent results for a given species. The conventional phenotypic methods correctly identified all yeasts (15/15) and 164/165 (99.4 %) Gram-negative bacilli, with the errors described in Table 4.

One hundred percent discrepant results were obtained for the identification of five isolates of Gram-positive bacilli by the VITEK® 2 system (ANC card) and the genotypic identification methods.

Statistical analysis of the identification results revealed a kappa value of 0.945 ($p < 0.001$), indicating almost perfect agreement according to the criteria of Landis and Koch [18] (Table 5).

Table 2 Comparison of the results of identification of blood culture isolates obtained with the automated VITEK® 2 system, conventional phenotypic methods, and genotypic methods

Microorganism isolated (number)	Automated identification	Conventional methods	Genotypic identification
Bacillus licheniformis (N = 2)	0	NP	2
Corynebacterium amycolatum (N = 3)	0	NP	3
Enterococcus faecalis (N = 8)	7	8	8
Staphylococcus epidermidis (N = 81)	71	81	81
Staphylococcus hominis (N = 35)	32	33	35
Staphylococcus capitis (N = 18)	17	18	18
Staphylococcus haemolyticus (N = 50)	49	48	50
Staphylococcus aureus (N = 17)	17	17	17
Enterobacter cloacae (N = 13)	13	13	13
Proteus mirabilis (N = 5)	5	5	5
Escherichia coli (N = 13)	13	13	13
Serratia marcescens (N = 22)	22	22	22
Acinetobacter baumannii (N = 39)	39	39	39
Acinetobacter lwoffii (N = 3)	3	3	3
Candida albicans (N = 5)	5	5	5
Candida glabrata (N = 5)	5	5	5
Candida krusei (N = 2)	2	2	2
Candida tropicalis (N = 3)	3	3	3
Enterobacter aerogenes (N = 8)	8	8	8
Enterococcus faecium (N = 4)	4	4	4
Klebsiella pneumoniae (N = 43)	43	42	43
Morganella morganii (N = 3)	3	3	3
Pseudomonas aeruginosa (N = 15)	15	15	15
Staphylococcus cohnii (N = 1)	1	1	1
Staphylococcus warneri (N = 1)	1	1	1
Stenotrophomonas maltophilia (N = 1)	1	1	1

NP identification not performed

Table 3 Discrepant results between the automated VITEK® 2 system and the other identification methods of blood culture isolates

No.	Automated identification	Conventional methods	Genotypic identification
4	*Staphylococcus hominis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
3	*Corynebacterium urealyticum*	NP	*Corynebacterium amycolatum*
2	*Corynebacterium urealyticum*	NP	*Bacillus licheniformis*
2	*Staphylococcus lentus*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
2	*Staphylococcus haemolyticus*	*Staphylococcus hominis*	*Staphylococcus hominis*
1	*Enterococcus gallinarum*	*Enterococcus faecalis*	*Enterococcus faecalis*
1	*Staphylococcus cohnii*	*Staphylococcus capitis*	*Staphylococcus capitis*
1	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus*	*Staphylococcus epidermidis*
1	*Staphylococcus lugdunensis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
1	*Staphylococcus scuri*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
1	*Staphylococcus capitis*	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
1	*Staphylococcus warneri*	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus*
1	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus*	*Staphylococcus hominis*

NP identification not performed

Table 4 Biochemical tests that failed during the identification process by the VITEK® 2 system and by the conventional phenotypic methods

No.	Genotypic identification	Conventional phenotypic method		VITEK® 2 system	
	Identification	Identification	Incorrect test	Identification	Incorrect test
3	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	0	*Staphylococcus hominis*	dMNE−; TRE +
2	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	0	*Staphylococcus lentus*	PolyB−
1	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	0	*Staphylococcus cohnii*	SUC−; dTRE +
3	*Staphylococcus hominis*	*Staphylococcus hominis*	0	*Staphylococcus haemolyticus*	PyrA+; dMAN+
2	*Staphylococcus hominis*	*Staphylococcus epidermidis*	THIO+	*Staphylococcus hominis*	0
2	*Staphylococcus haemolyticus*	*Staphylococcus warneri*	URE+	*Staphylococcus haemolyticus*	0
1	*Enterococcus faecalis*	*Enterococcus faecalis*	0	*Enterococcus gallinarum*	dSOR−; dRAF+
1	*Staphylococcus capitis*	*Staphylococcus capitis*	0	*Staphylococcus cohnii*	βGAL+; βGUR+; SUC−; dTRE+
1	*Staphylococcus epidermidis*	*Staphylococcus haemolyticus*	URE−	*Staphylococcus haemolyticus*	PyrA+; URE−, PolyB−; TRE+; dMNE−
1	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	0	*Staphylococcus lugdunensis*	PyrA+; dTRE+
1	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	0	*Staphylococcus scuri*	PolyB−; βGUR+; NAG (+); dMAN+; dTRE+
1	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	0	*Staphylococcus capitis*	SUC−; dMAL−; PolyB−; URE−
1	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus*	0	*Staphylococcus warneri*	0
1	*Klebsiella pneumoniae*	*Klebsiella oxytoca*	Indol+	*Klebsiella pneumoniae*	0

dMNE D-mannose, *dTRE* D-trehalose, *PolyB* polymyxin B, *SUC* sucrose, *PyrA* L-pyrrolidonylarylamidase, *dMAN* D-mannitol, *dSOR* D-sorbitol, *dRAF* D-raffinose, *URE* urea, *βGAL* beta-galactosidase, *THIO* thioglycolatebroth, *βGUR* beta-glucuronidase, *NAG* *N*-acetylglucosamine, 0 no errors in the tests

Table 5 Kappa value according to Landis and Koch

Kappa value	Level of agreement
<0.00	No agreement
0.00–0.20	Slight
0.21–0.40	Fair
0.41–0.60	Moderate
0.61–0.80	Substantial
0.81–1.00	Almost perfect

Agreement between the conventional phenotypic methods and genotypic identification was higher than that between the genotypic method and automated identification by the VITEK® 2 system (Table 6).

Comparison of the sensitivity of the conventional phenotypic methods and the VITEK® 2 system showed a better performance of the former (Table 7).

Discussion

The need for the rapid and efficient identification of microorganisms isolated from blood cultures has encouraged studies that investigated automated identification systems to reduce the time of identification. Several of these studies have used direct inoculation from blood culture bottles, but the results were not as efficient as those obtained in studies using standard inocula from subcultures of microorganisms grown for 24 h on solid media. The poor performance of the VITEK® 2 system for the identification of microorganisms using direct inoculation from blood culture bottles is probably due to the small number of cells or to contamination with other microorganisms that impair the correct identification of the causative agent of infection [19–22]. The VITEK system has been investigated for more than two decades and improvement of the ID-GPC (Gram-positive cocci) and ID-GNB (Gram-negative bacilli) identification cards to the GP (Gram-positive cocci) and GN (Gram-negative bacilli) cards has made the system more efficient. Wallet et al. [23] compared the old and new identification cards and found that the GP and GN cards correctly identified 235/249 (94.4 %) Gram-positive cocci and 321/331 (97 %) Gram-negative bacilli, while the ID-GPC and ID-GNB correctly identified 218/249 (87.5 %) and 295/331 (89.1 %) isolates, respectively.

The present study was conducted over a period of 18 months and evaluated the accuracy of the VITEK® 2 system in identifying 400 microorganisms (Gram-positive cocci, Gram-positive bacilli, Gram-negative bacilli, and yeasts) isolated from blood cultures and inoculated by the standard method onto GP, GN, YST (yeast) and ANC (Gram-positive bacilli) cards. The results were compared to genotypic identification (gold standard) and 94.7 % agreement was observed. Similar rates have been reported by De Cueto et al. [24] (95.0 %, 95/100)

Table 6 Kappa values of agreement between automated identification by the VITEK®2 system, the conventional phenotypic methods, and genotypic identification

Group	Number of isolates	Kappa	
		Conventional method x genotypic method	Automated method x genotypic method
Gram-positive cocci	255	0.969	0.904
CoNS	186	0.969	0.886
Gram-negative bacilli	165	0.993	1.000
Gram-positive bacilli	5	NP	0
Yeasts	15	1.000	1.000
Total	400	0.958	0.945

CoNS coagulase-negative staphylococci, *NP* not performed

and by Nakasone et al. [25] (95.8 %, 454/474) who also used standard inocula. Studies using direct inoculation from blood cultures bottles obtained lower agreement of 91.4 % [5] and 81.0 % [6].

All 15 yeasts isolated during the study period were correctly identified by the VITEK® 2 system. Correct identification of all yeast isolates (56/56) has also been observed by Nakasone et al. [25]. Studies involving a larger number of strains and species found lower agreement, 92.1 % (222/241) reported by Graf et al. [26] and 78.9 % (277/351) by Won et al. [27].

The VITEK® 2 system has shown satisfactory identification rates of Gram-negative bacilli for decades, as also observed in this study in which 100 % correct identifications of the isolates were obtained. Funke et al. [28] and Ling et al. [29] analyzed 845 and 281 isolates, with correct identification rates of 84.7 % (716/845) and 95 % (267/281), respectively. Nakasone et al. [25], Gherardi et al. [5] and Prod'hom et al. [6] analyzed 181, 91 and 95 Gram-negative bacilli and obtained correct identifications of 96.7 % (175/181), 100 % (91/91) and 98.8 % (92/95), respectively, with the VITEK® 2 system. Studies using direct inoculation from blood culture bottles reported lower rates of correct identification of 82 [30] and 93 % [31]. De Cueto et al. [24] compared direct inoculation from blood cultures with inoculation from subcultures in 50 isolates. The result was 100 % correct identifications for the standard method and 62 % (31/5) correct identifications for direct inoculation. Similar results have been reported by Kerremans et al. [32] who analyzed 161 isolates; 90 % (145/161) of the isolates were correctly identified by subculture and 80 % (129/161) by direct inoculation from blood cultures.

The identification of Gram-positive cocci by the automated VITEK® 2 system showed 92.6 % agreement with genotypic identification, which is compatible with the rate reported by Ligozzi et al. [33] who obtained 91.5 % (351/381) correct identifications. De Cueto et al. [24] obtained 100 % (50/50) correct identifications of the isolates. Funke and Funke-Kissling [34], Nakasone et al. [25] and Chatzigeorgiou et al. [35] reported higher rates of correct identification than those obtained in this study of 94.5 % (344/364), 96.1 % (226/235) and 97.9 % (144/147), respectively. Gherardi et al. [5] and Prod'hom et al. [6] obtained correct identifications of 75 % (36/48) and 74 % (133/180), while Lupetti et al. [36] found a rate of 89 % (49/55). These rates are lower than those observed in this study and are outside the acceptable parameter of 90 % correct identifications; however, these studies used direct inoculation from blood culture bottles.

With respect to genera of Gram-positive cocci, a difference in the efficiency of the VITEK® 2 system was observed. Agreement was 91.7 % (11/12) for the genus *Enterococcus*, similar to the rates reported by Nakasone et al. [25] and Jin et al. [37]. Lower efficiencies of 77.8, 83.1, 87.5, 72 and 77.8 % correct identifications of strains of this genus have been observed by Ligozzi et al. [33], d'Azevedo et al. [38], Moore et al. [39] and Paim et al. [42], respectively. In the case of *Staphylococcus*, a higher rate of incorrect identifications was observed for CoNS isolates (91.9 %), in agreement with Ligozzi et al. [33]

Table 7 Sensitivity and specificity of the conventional phenotypic methods and of the automated VITEK® 2 system in the identification of microorganisms

Microorganism	VITEK® 2 system		Phenotypic methods	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Enterococcus faecalis	87.5	100	100	100
Staphylococcus capitis	94.4	99.5	100	100
Staphylococcus cohnii	100	99.0	100	100
Staphylococcus epidermidis	87.6	100	100	98.5
Staphylococcus haemolyticus	98.0	96.5	96.0	100
Staphylococcus hominis	91.4	98.3	94.2	100
Staphylococcus warneri	100	99.5	100	99.0

(86 %), Funke and Funke-Kissling [34] (93.7 %), Kim et al. [40] (87.5 %), Delmas et al. [41] (78.8 %), and Paim et al. [42] (72.9 %). These unsatisfactory results are due to the fact that automated identification systems are unable to perform a fully reliable differentiation between different CoNS species because of the variable expression of phenotypic characteristics in these microorganisms [43]. The slow metabolism of certain species leads to ambiguous results in their identification, a fact observed by Ligozzi et al. [33]. All *Staphylococcus aureus* isolates were correctly identified (17/17), as also reported in the studies of Delmas et al. [41] (6/6), Chatzigeorgiou et al. [35] (52/52), Paim et al. [42] (11/11), and Funke and Funke-Kissling [34] (45/45). Ligozzi et al. [33], who evaluated a larger number of isolates, found 99 % agreement (99/100). These rates of correct identification demonstrate a satisfactory performance of the VITEK® 2 system for the identification of *Staphylococcus aureus*.

The failure of the ANC card to identify Gram-positive bacilli can be explained by the variability in the genera and species of these microorganisms and the consequent difficulty in developing cards that contain variable biochemical tests necessary for correct identification. These microorganisms were not identified by conventional phenotypic methods since this identification is infeasible in routine clinical microbiology because it requires numerous expensive and time-consuming biochemical tests.

The better performance of the conventional methods for CoNS identification was responsible for the higher sensitivity of these methods compared to the VITEK® 2 system. This finding can be explained by the fact that the conventional methods used consisted of specific tests for each CoNS species and by the incubation period of 72 h, which is necessary for this correct identification since some species have a slower metabolism on some substrates.

Conclusions

The kappa values indicate reliability of the results obtained with the VITEK® 2 system. Analysis of specificity showed a performance higher than 90 % as required for commercial systems in clinical microbiology, demonstrating that this system is suitable for the identification of microorganisms isolated in routine clinical microbiology laboratories.

Authors' contributions

ACMM conceived and designed the study, performed the tests, analyzed and interpreted the data, and wrote the manuscript. CMCBF substantially contributed to the conception of the study and performed the statistical analyses. AMF collected the blood cultures and performed the automated identification. RSC participated in the data collection and analysis. EB identified the yeast isolates. MLRSC participated in the conception of the study, data analysis, and writing and revision of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

The data set supporting the results of this article is included within the article.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Faculdade de Medicina de Botucatu, Botucatu, São Paulo State, Brazil (Protocol 347/2012-CEP).

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Evaluation of the outcome of patients with blood culture positive for coagulase-negative staphylococci: death and hospital discharge

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ABSTRACT

Coagulase-negative staphylococci (CoNS) are responsible for more than 30% of bloodstream infections. However, assessment of the clinical significance of these microorganisms when isolated from blood culture continues to be difficult. The objective of this study was to follow up patients with CoNS-positive blood cultures addressing two outcomes: first, the isolation of CoNS from more than one blood culture compared to the detection of these agents in a single blood culture, and to evaluate the different species of these microorganisms and the clinical data of patients associated with outcome variable death. A cohort study was conducted over 18 months including patients at the time of their first CoNS-positive blood culture, and an observational cross-sectional study conducted at the time of inclusion of the patient in the cohort to verify the isolation of CoNS from more than one blood culture compared to a single blood culture. Blood cultures were obtained upon medical request and positive samples were submitted to the isolation and identification of microorganisms by molecular biology techniques. Multivariate analysis of the outcome variable death in patients with a CoNS-positive blood culture revealed positive associations with AIDS and with a *Staphylococcus hominis*-positive blood culture, while a negative association was observed with meropenem use. Analysis of multiple CoNS-positive blood cultures showed a positive association with patients with septic shock (requiring vasoactive drugs), suggesting that the severity of sepsis (shock) is a predictor of the clinical significance of CoNS.

INTRODUCTION

Bloodstream infections (BSI) are a public health problem since they increase the rate of patient morbidity and mortality, length of hospital stay, and treatment costs[1]. The increase in the occurrence of BSI can be explained by advances in healthcare and by the use of invasive devices, especially intravenous catheters, for the treatment and monitoring of critically ill patients in the intensive care unit (ICU)[2-5].

Sepsis, the clinical condition resulting from the aggravation of BSI[6], is the second leading cause of death in non-coronary ICUs in the United States[7] and the main cause of death in Latin American countries[8]. In Brazil, sepsis was identified as the leading public health problem in the Brazilian Sepsis Epidemiological Study (BASES study)[9]. The Centers for Disease Control and Prevention (CDC) found that cases of sepsis increased in the United States from 621,00 in 2000 to 1,141,000 in 2008[10]. Globally, an estimated 17 million cases of sepsis occur each year, 600,000 in Brazil alone[7].

Bloodstream infections are caused by different microorganisms. In the last four decades, the prevalence of agents causing these infections has changed and coagulase-negative staphylococci (CoNS) have become responsible for more than 30% of these cases[11]. A survey conducted by the National Healthcare Safety Network (NHSN) between 2009 and 2010 indicated CoNS as the main causative agents of infections in North American hospital[12]. The SCOPE (Surveillance and Control of Pathogens of Epidemiological Importance) study, a North American project created to monitor nosocomial infections, analyzed data from March 1995 to September 2002 and identified CoNS as the main causative agents of BSI[4]. Several smaller-scale studies conducted between 2003 and 2013 reported *Staphylococcus* spp. to be the main microorganisms isolated from blood cultures[13-18].

CoNS are involved in different infections (urinary infection, endocarditis, peritoneal dialysis-associated peritonitis) in both immunocompromised hosts and healthy individuals[19].

This group of bacteria is part of the human microbiota, i.e., they are natural inhabitants of the skin and mucosa of humans and live in symbiosis with their host. In cases of skin barrier disruption, inoculation through needles or implantation of medical devices (e.g., catheters, prostheses), these microorganisms can cause serious infections[20,21]. CoNS have emerged as important pathogens during the last decades, especially in infections affecting immunosuppressed patients, preterm infants and patients with prosthetic implants. They are recognized as essentially opportunistic microorganisms that prevail in numerous organic situations to produce serious infections[22,23]. In the hospital environment, CoNS are the leading cause of BSI, with most cases involving ICU patients[20].

Blood cultures that are positive for CoNS raise doubts on the part of healthcare workers since, despite their pathogenic potential, these microorganisms are frequently contaminants as inhabitants of human skin[24]. Although CoNS are frequently isolated from blood cultures (38%), only 10% of BSI are caused by these species.

Regardless of whether or not they are contaminants, *Staphylococcus* spp. are the most frequently isolated bacteria in clinical microbiology laboratories and the study of their epidemiology is therefore important to clarify the clinical significance of these microorganisms in order to permit prophylaxis and adequate treatment of infections caused by these species.

An observational study was conducted to address two outcomes of interest: first, the isolation of CoNS from more than one blood culture compared to the detection of these agents in a single blood culture and, second, to analyze factors associated with the death of patients with blood cultures positive for this group of microorganisms. As the study design, the two outcomes were evaluated in a cohort using an initial cross-sectional approach.

MATERIALS AND METHODS

1. Place

The study was conducted at two ICUs of the University Hospital of the Botucatu Medical School (HC-FMB) that receive adult patients. The two units of the Specialized Intensive Care Service (Serviço Especializado de Terapia Intensiva - SETI) with 16 and 9 beds admit clinical and surgical patients.

2. Study design

Prospective cohort of patients admitted to the ICUs of HC-FMB who had at least one CoNS-positive blood culture over a period of 18 months (August 2012 to February 2014).

A cross-sectional study was conducted at the time of inclusion of the patients in the cohort whose outcome were multiple positive blood cultures (versus a single positive blood culture). For this analysis, all factors studied were collected during the period prior to inclusion. Next, the cohort design was used to study factors associated with death within 30 days as outcome.

Criteria for inclusion were an age of 18 years or older, hospitalization in the ICU during the study period, and at least one positive blood culture. Exclusion criteria were the presence of a blood culture positive for any other microorganism during the 30 days prior to collection of the blood culture positive for a CoNS species. Criteria for exit from the cohort (censorship) were hospital discharge, a blood culture positive for other agents, or transfer to another hospital during the study period.

3. Sample collection

The blood samples were collected upon medical request using the standard protocol of the hospital. The samples were inoculated into blood culture bottles and incubated in a BACTEC™ 9050 system.

4. Isolation and identification of microorganisms present in the collected samples

4.1 Extraction of bacterial DNA

Bacterial DNA was extracted directly from the blood sample using the Illustra Kit (GE Healthcare) according to manufacturer instructions, with modifications[26,27].

4.2 Polymerase chain reaction (PCR)

The internal transcribed spacer (ITS) region, which is located between the 16S and 23S rDNA genes, was amplified by PCR using conserved primer sequences adjacent to the G1 (5' GAAGTCGTAACAAGG3') and L1 genes (5' CAAGGCATCCACCGT3'), according to Barry *et al.* (1991) and Couto *et al.* (2001).

The efficiency of the amplifications was monitored by electrophoresis on 2% agarose gel prepared in 1X TBE buffer (89 mM Tris (pH 7.6), 89 mM boric acid, and 2 mM EDTA) and stained with SYBR® Safe at 90 V for 60 min. The following international reference strains were used as controls: *Staphylococcus capitis* subsp. *capitis* (ATCC 27843), *Staphylococcus capitis* subsp. *urealyticus* (ATCC 49325), *Staphylococcus cohnii* (ATCC 49330), *Staphylococcus cohnii* subsp. *cohnii* (ATCC 29974), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus haemolyticus* (ATCC 29970), *Staphylococcus hominis* (ATCC 27844), and *Staphylococcus warneri* (ATCC 10209).

5. Statistical analysis

The clinical data collected from the patient records were entered into Epi Info™ (a public domain software package created by the CDC) and the information extracted from the database were used in the statistical analyses performed using the SPSS 20 program (IBM, Armonk, NY, USA).

According to the study design, the outcomes were analyzed in a cohort, with an initial cross-sectional approach (Figure 1). In line with our objectives, the cross-sectional data were analyzed using common nonparametric statistical procedures (chi-squared test, Fisher's exact

test and Mann-Whitney U test, when indicated) and logistic regression. Analysis of the cohort was performed using time-to-event analysis (Kaplan-Meier and Cox regression). These methods permit censored patients of the cohort to contribute data of the period studied.

For selection of the variables to be included in the multivariate models, a hierarchical strategy (Victora *et al.*, 1997) with sequential inclusion of groups of data was used (Figure 2). In this case, the models were constructed for each group and variables with a p-value < 0.05 were included in the model of the following group. Demographic data and comorbidities were included as distal variables and the presence of healthcare-associated infections (HAIs; only for the death outcome variable) and CoNS species (for both outcomes) as proximal variables.

RESULTS

1. Sample

During the 18 months of the study, 2,367 blood cultures were collected from patients admitted to the ICUs of the Botucatu University Hospital. Microbial growth was detected in 417 (17.6%) of these cultures, with growth of CoNS in 186 (44.6%). These 186 CoNS isolates were obtained from 129 patients. The difference between the number of patients and strains isolated is due to the fact that more than one sample was collected from the same patient because of the duration of hospitalization and clinical evolution of the patient. The following CoNS were identified: 81 (43.5%) *Staphylococcus epidermidis*, 50 (26.9%) *Staphylococcus haemolyticus*, 35 (18.8%) *Staphylococcus hominis*, 18 (9.7%) *Staphylococcus capitis*, 1 (0.54%) *Staphylococcus warneri*, and 1 (0.54%) *Staphylococcus cohnii* (Table 1).

1.1 Patients included in the cohort

According to the inclusion criteria, of the 129 patients with CoNS-positive blood cultures, 110 patients with 159 blood culture isolates were included in the cohort: 69 (43.4%) *Staphylococcus epidermidis*, 44 (27.7%) *Staphylococcus haemolyticus*, 31 (19.5%)

Staphylococcus hominis, 14 (8.8%) *Staphylococcus capitis*, and 1 (0.6%) *Staphylococcus cohnii* (Table 1).

1.2 Patients excluded from the study

Nineteen (14.7%) patients were excluded because they had a blood culture positive for another microorganism during the 30 days preceding the CoNS-positive blood culture in order to avoid possible bias in the statistical analysis of the data.

2. Time of permanence in the study of patients with blood cultures positive for different CoNS species

Comparison of the time of permanence in the study between the different CoNS species showed that patients with blood cultures positive for *S. epidermidis* and *S. haemolyticus* remained longer in the study, on average 21 days. Patients with blood cultures positive for *S. capitis* and *S. hominis* remained on average 17 and 14 days, respectively, while the only patient with a blood culture positive for *S. cohnii* remained one day in the study (Table 2).

3. Factors associated with the isolation of coagulase-negative staphylococci from more than one blood culture

A cross-sectional study was conducted at the time of inclusion in the cohort whose outcome was multiple positive blood cultures (versus a single positive blood culture). All factors analyzed were collected during the period preceding inclusion. The results of univariate and multivariate logistic regression revealed a significant positive association between patients with septic shock (requiring vasoactive drugs) and multiple CoNS-positive blood cultures. No statistically significant results were observed for the other factors analyzed, including the different CoNS species (Table 3).

4. Factors associated with death in patients with CoNS-positive blood cultures

A cohort study was used to analyze the factors associated with death within 30 days using time-to-event analysis. The criteria for exit from the cohort were: hospital discharge,

transfer from the hospital, or a blood culture positive for another microorganism. The results of univariate analysis of factors associated with the outcome variable death revealed significant negative associations with diabetes mellitus, surgery and meropenem and vancomycin use, and positive associations with AIDS and a *S. hominis*-positive. In multivariate analysis of the cohort, only AIDS and a *S. hominis*-positive blood culture remained positively associated with death (Table 4).

5. Patient survival according to CoNS species isolated from blood culture

Survival analysis according to the CoNS species isolated from blood culture by the Kaplan-Meier time-to-event method (Figure 3) showed that patients with a *S. hominis*-positive blood culture progressed to death faster than patients with blood cultures positive for other CoNS species. Higher survival rates were observed for patients with blood cultures positive for *S. epidermidis* and *S. haemolyticus*. The only patient from which *S. cohnii* was isolated died on the first day.

DISCUSSION

The frequencies of CoNS species found here agree with previous studies, which are unanimous in reporting *S. epidermidis* as the most common CoNS species isolated from HAIs[31], including a survey of the NHSN published in 2013[12]. In the present study, *S. epidermidis* accounted for 81 of the 186 isolated and identified CoNS, a frequency of 43.5%. This number is lower than the 60% found by Viana *et al.* (2011) and higher than the 34% reported by Keim *et al.* (2011). *Staphylococcus haemolyticus* is the second most common CoNS species isolated from infections[31]. In the study of Fernandes *et al.* (2011), *S. haemolyticus* was the second most common CoNS species isolated from blood cultures, with a frequency of 13.8%, a percentage lower than that found in the present study which was 26.9% (50/186). *Staphylococcus hominis* was the third most common species, with a frequency of

isolation of 18.8% (35/186). This frequency is higher than that found by Fernandes *et al.* (2011) (1.3%) and Keim *et al.* (2011) (2%), but much lower than the frequency of 40.2% reported by Kilic *et al.* (2014). *Staphylococcus capitis* was the fourth most common species isolated (9.1%), a value higher than the 2% reported by Fernandes *et al.* (2011). The isolated species *S. warneri* and *S. cohnii* were not related to cases of BSI and the patient with a *S. warneri*-positive blood culture was excluded from the study because of a blood culture positive for another microorganism. The patient with a *S. cohnii*-positive blood culture died within one day due to his severe clinical condition which, however, was not associated with BSI.

The longest time of permanence in the study was observed for patients with blood cultures positive for *S. epidermidis* and *S. haemolyticus*. As mentioned earlier, these species are the most common CoNS isolated from blood cultures of patients hospitalized over a long period of time. This explanation is supported by the demonstration of hospital clones of *S. epidermidis* and *S. haemolyticus* in a study conducted in 2015 that isolated the same *S. epidermidis* and *S. haemolyticus* clones over a period of 12 years at the same hospital where the present study was conducted[35]. The survival of patients with *S. epidermidis* or *S. haemolyticus* isolated from blood cultures was also greater when compared to the survival of patients with blood cultures positive for other CoNS species, suggesting that these species are more common and that many isolates are contaminants. Another finding indicating that in this study these species were associated with contamination is the fact that 61.7% of the patients with isolation of *S. epidermidis* and *S. haemolyticus* had only one positive blood culture. An American multicenter study published in 2010 reported that 38% of the collected blood cultures were positive for CoNS, but only 10% were true BSI[25].

The definition to characterize patients with severe infections is a problem that healthcare workers have been facing for years. The most commonly used definitions are those created in 1992 during a meeting of the Society of Critical Care Medicine (SCCM) and the

American College of Chest Physicians. According to this recommendation, septic shock is a state of acute circulatory failure characterized by persistent arterial hypotension and the need for vasopressors[8]. Patients with septic shock exhibit a completely compromised immune response to primary infection, which is the trigger of sepsis[36]. This suggests that at this time of shock any invasion of the bloodstream by an opportunistic microorganism will have a negative impact on the clinical presentation of the patient. Thus, multiple CoNS-positive blood cultures positively associated with patients with septic shock (requiring vasoactive drugs) suggest that the severity of sepsis (that progressed to septic shock) is a predictor of the clinical significance of CoNS, with isolation from more than one positive blood culture, one of the most common criteria used by the CDC to define BSI caused by these microorganisms[37].

Patients infected with human immunodeficiency virus (HIV) or with advanced AIDS are at an increased risk of acquiring BSI[38] and of colonization and infection with opportunistic pathogens[39]. It is known that CoNS of the human microbiota are opportunistic pathogens in immunocompromised patients[19] and infections caused by these microorganisms are easily found in patients with HIV. Cohort studies involving patients with HIV and BSI published by Adeyeme *et al.* (2010) and Declercq *et al.* (2015) have identified CoNS as the main causative agents of BSI. This scenario explains why in this study AIDS was positively associated with death in the cohort of patients with CoNS-positive blood cultures.

Despite the higher frequency of blood cultures positive for *S. epidermidis* and *S. haemolyticus* (43.4% and 27.7%, respectively), the isolation of *S. hominis* from blood cultures was positively associated with death. Analysis of the cohort showed the inclusion of 10 patients with blood cultures positive for this CoNS species who progressed to death. Two of these patients had more than one *S. hominis*-positive blood culture. To our knowledge, this is the first study demonstrating the importance of *S. hominis* as an etiological agent of BSI and the greater severity when compared to patients infected with other CoNS species such as *S. epidermidis*

and *S. haemolyticus*, which have been more frequently isolated and more commonly associated with infections in previous studies. The survival of patients with *S. hominis* isolated from blood cultures was also lower when compared to patients with blood cultures positive for other CoNS species. This fact highlights the clinical significance of this species and the need for more attention when these microorganisms are isolated from blood cultures.

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TABLES

Table 1

Coagulase-negative staphylococcal species isolated from blood cultures of patients admitted to the ICUs of the Botucatu University Hospital.

CoNS species	No. of isolates	Selected for the cohort
Staphylococcus epidermidis	81	69 (81.2%)
Staphylococcus haemolyticus	50	44 (88%)
Staphylococcus hominis	35	31 (88%)
Staphylococcus capitis	18	18 (78%)
Staphylococcus warneri	1	0
Staphylococcus cohnii	1	1 (100%)
Total	186	159 (85%)

CoNS: coagulase-negative staphylococci.

Table 2

Mean and median time of permanence of patients in the study according to coagulase-negative staphylococcal species.

CoNS species	Mean (95% confidence)			
	Time (days)	Lower limit	Upper limit	Standard deviation
Staphylococcus epidermidis	21.82	18.45	25.19	1.72
Staphylococcus haemolyticus	21.80	17.67	25.93	2.10
Staphylococcus capitis	16.81	7.68	25.94	4.66
Staphylococcus hominis	13.59	7.41	19.76	3.15
Staphylococcus cohnii	1.0	1.0	1.0	0.0

CoNS: coagulase-negative staphylococci.

Table 3

Univariate and multivariate analysis of single and multiple positive blood cultures.

Predictor	Univariate analysis				Multivariate analysis	
	Multiple cultures (n=40)	Single culture (n=70)	OR (95%CI)	p	OR (95%CI)	p
Demographic data						
Female sex	15 (37.5%)	37 (52.9%)	0.54 (0.24-1.18)	0.12	...	...
Age (years), median (quartiles)	61.5 (54.3-72.8)	60.5 (50-74.3)	...	0.50	...	...
Comorbidities						
Heart disease	11 (27.5%)	16 (22.9%)	1.28 (0.53-3.12)	0.59	...	...
Lung disease	3 (7.5%)	12 (17.1%)	0.39 (0.10-1.48)	0.16	...	...
Kidney disease	8 (20.0%)	10 (14.3%)	1.50 (0.54-4.18)	0.44	...	...
Diabetes mellitus	11 (27.5%)	16 (22.9%)	1.28 (0.53-3.1)	0.59	...	...
Neoplasm	4 (10.0%)	7 (10.0%)	1.00 (0.27-3.65)	1.00	...	...
AIDS	2 (5.0%)	2 (2.9%)	1.79 (0.24-13.22)	0.62	...	...
Central nervous system disease	11 (27.5%)	19 (27.1%)	1.02 (0.43-2.43)	0.97	...	...
Trauma	6 (15.0%)	8 (11.4%)	1.37 (0.44-4.27)	0.57	...	...
Charlson score, median (quartiles)	2 (0-4)	2 (0-3)	...	0.67	...	...
Clinical data and/or hospitalization						
Previous days, median (quartiles)	3.5 (0-9.5)	5 (1-12.5)	...	0.43	...	...
Surgery	20 (50.0%)	39 (56.5%)	0.77 (0.35-1.68)	0.51	...	...
Drains	12 (30.0%)	24 (34.3%)	0.82 (0.36-1.90)	0.65	...	...
Nasogastric tube	37 (92.5%)	59 (84.3%)	2.30 (0.60-8.29)	0.21	...	...
Parenteral nutrition	3 (7.5%)	2 (2.9%)	2.76 (0.44-17.25)	0.35	...	...
Fever (in the last 24 hours)	24 (23.2%)	40 (57.1%)	1.29 (0.57-2.90)	0.54	...	...
Leukocytes x 1000, median (quartiles)	12.3 (9.6-19.1)	15.1 (10.3-18.9)	...	0.32	...	...
Neutrophilia	35 (65.8%)	51 (72.9%)	0.72 (0.31-1.68)	0.51	...	...
PCR (mg/dL), median (quartiles)	27.3 (10.7-34.0)	24.2 (6.7-33.1)	...	0.56	...	...

Table 3 continued.

Predictor	Univariate analysis				Multivariate analysis	
	Multiple cultures (40)	Single culture (70)	OR (95%CI)	p	OR (95%CI)	p
Septic shock (requiring vasoactive drugs)	**21 (55.3%)**	**22 (31.4%)**	**2.70 (1.19-6.09)**	**0.02**	**4.26 (1.57-11.54)**	**0.004**
Antibiotic use						
Ampicillin	0 (0.0%)	2 (2.9%)	Undefined	1.00	...	...
Oxacillin	0 (0.0%)	2 (2.9%)	Undefined	1.00	...	...
Amoxicillin-clavulanate	9 (22.5%)	9 (12.9%)	1.97 (0.71-5.46)	0.19	...	...
Piperacillin-tazobactam	11 (27.5%)	19 (27.1%)	1.02 (0.43-2.43)	0.97	...	...
Cefazolin	5 (12.5%)	13 (18.8%)	0.63 (0.21-1.91)	0.41	...	...
Ceftriaxone	5 (12.5%)	2 (2.9%)	4.86 (0.89-26.32)	0.10	...	...
Cefepime	5 (12.5%)	11 (15.7%)	0.78 (0.25-2.39)	0.65	...	...
Imipenem	8 (20.0%)	11 (15.7%)	1.34 (0.49-3.67)	0.57	...	...
Meropenem	3 (7.5%)	12 (17.1%)	0.39 (0.10-1.48)	0.16	0.25 (0.03-1.94)	0.19
Levofloxacin	2 (5.0%)	9 (12.9%)	0.36 (0.07-1.74)	0.32	...	...
Ciprofloxacin	4 (10.0%)	8 (11.4%)	0.86 (0.24-3.06)	1.00	...	...
Clarithromycin	7 (17.5%)	8 (11.4%)	1.64 (0.55-4.93)	0.37	...	...
Clindamycin	3 (7.5%)	4 (5.7%)	1.34 (0.28-6.30)	0.70	...	...
Metronidazole	4 (10.0%)	8 (11.4%)	0.86 (0.24-3.06)	1.00	...	...
Vancomycin	9 (22.5%)	19 (27.1%)	0.78 (0.31-1.94)	0.59	...	...
Linezolid	1 (2.5%)	3 (4.3%)	0.53 (0.06-5.69)	1.00	...	...
Trimethoprim-sulfamethoxazole	3 (7.5%)	1 (1.4%)	5.60 (0.56-55.70)	0.14	...	...
Polymyxin B	0 (0.0%)	2 (2.9%)	Undefined	1.00	...	...
Colistin	9 (22.5%)	9 (10.9%)	1.97 (0.71-5.46)	0.19	1.92 (0.11-32.57)	0.65
CoNS species						
S. epidermidis (reference)	17 (42.5%)	34 (48.6%)	...	...	...	...
S. haemolyticus	14 (35.0%)	16 (22.9%)	1.75 (0.70-4.41)	0.24	2.50 (0.87-7.16)	0.09
S. hominis	3 (7.5%)	13 (18.6%)	0.46 (0.12-1.80)	0.27	0.33 (0.08-10.41)	0.14
S. capitis	2 (5.0%)	6 (8.6%)	0.67 (0.12-3.66)	0.64	1.64 (0.26-10.41)	0.59
S. cohnii	1 (1.4%)	0 (0.0%)	Undefined	1.0	Undefined	1.0
Multiple species	4 (10.0%)	0 (0.0%)	Undefined	1.0	Undefined	1.0

OR: odds ratio; 95%CI: 95% confidence interval; CoNS: coagulase-negative staphylococci.

Table 4

Risk factors associated with death and coagulase-negative staphylococcal species isolated from blood cultures.

Risk factor	Prevalence of death in the sample	Univariate analysis		Multivariate analysis	
		HR (95%CI)	p	HR (95%CI)	p
Demographic data					
Female sex	52 (47.3%)	1.17 (0.63-2.16)	0.62	...	...
Age (years), median (quartiles)	61 (51-73)	1.01 (0.99-1.03)	0.19	...	...
Comorbidities					
Heart disease	27 (24.5%)	1.17 (0.59-2.29)	0.66	...	...
Kidney disease	18(16.4%)	1.69 (0.83-3.45)	0.15	...	...
Lung disease	15 (13.6%)	2.28 (1.09-4.79)	0.03	...	...
Diabetes mellitus	27 (24.5%)	**0.31 (0.12-0.80)**	**0.02**	...	...
Central nervous system disease	30 (27.3%)	1.17 (0.61-2.26)	0.64	...	...
Neoplasm	11 (10%)	2.01 (0.92-4.36)	0.08	...	...
AIDS	4 (3.6%)	**4.43 (1.57-12.54)**	**0.005**	**4.76 (1.41-16.05)**	**0.01**
Trauma	14 (12.7%)	0.52 (0.16-1.69)	0.28	...	...
Charlson score, median (quartiles)	2 (0-3)	1.02 (0.99-1.05)	0.07	...	...
Clinical data and/or hospitalization					
Time prior to culture (days)	4 (1-12)	0.97 (0.93-1.01)	0.09	...	...
Nasogastric tube	96 (87.3%)	0.92 (0.35-2.20)	0.85	...	...
Parenteral nutrition	5 (4.5%)	0.56 (0.10-3.57)	0.84	...	...
Drains	36 (32.7%)	0.93 (0.31-1.26)	0.19	...	...
Surgery	59 (53.6%)	**0.46 (0.25-0.87)**	**0.02**	...	...
Pressure ulcer	48 (43.6%)	0.98 (0.53-1.83)	0.96	...	...
Antibiotic use					
Ampicillin	3 (2.7%)	1.44 (0.35-5.98)	0.62	...	...
Amoxicillin-clavulanate	5 (4.5%)	1.57 (0.38-6.53)	0.53	...	...
Piperacillin-tazobactam	27 (24.5%)	0.51 (0.23-1.16)	0.11	...	...
Cefazolin	3 (2.7%)	0.05 (0.00-93.10)	0.43	...	...
Ceftazidime	1 (0.9%)	1.95 (0.27-14.28)	0.51	...	...
Cefepime	14 (12.7%)	0.77 (0.30-1.95)	0.58	...	...

Table 4 continued.

Risk factor	Prevalence in the sample	Univariate analysis		Multivariate analysis	
		HR (95%CI)	p	HR (95%CI)	p
Imipenem	31 (28.2%)	1.21 (0.63-2.31)	0.56	...	...
Meropenem	21 (19.1%)	**0.13 (0.03-0.55)**	**0.005**	**0.17 (0.04-0.70)**	**0.02**
Ertapenem	1 (0.9%)	0.05 (0.00-∞)	0.77	...	...
Ciprofloxacin	7 (6.4%)	0.69 (0.17-2.87)	0.61	...	...
Levofloxacin	2 (1.8%)	0.05 (0.00-724.17)	0.54	...	...
Clarithromycin	7 (6.4%)	2.76 (0.96-7.95)	0.06	...	...
Vancomycin	51 (46.4%)	**0.44 (0.23-0.85)**	**0.01**	...	...
Teicoplanin	3 (2.7%)	2.29 (0.55-9.51)	0.26	...	...
Linezolid	11 (10.0%)	1.33 (0.56-3.17)	0.52	...	...
Polymyxin B	16 (14.5%)	0.51 (0.18-1.14)	0.20	...	...
Colistin	16 (14.5%)	0.66 (0.26-1.68)	0.39	...	...
Clindamycin	2 (1.8%)	1.14 (0.19-10.29)	0.74	...	...
Metronidazole	9 (8.2%)	0.48 (0.12-1.97)	0.31	...	...
Trimethoprim-sulfamethoxazole	3 (2.7%)	2.30 (0.55-9.56)	0.25	...	...
HAI					
VM-associated pneumonia	19 (17.3%)	1.27 (0.58-2.76)	0.35	...	...
Urinary infection	13 (11.8%)	0.63 (0.22-1.77)	0.38	...	...
Surgical site infection	2 (1.8%)	0.05 (0.00-150.22)	0.46	...	...
Bloodstream infection*	40 (36.4%)	0.52 (0.25-1.06)	0.07	...	...
CoNS species					
S. epidermidis (reference)	51 (46.4%)	1		...	...
S. haemolyticus	30 (27.3%)	1.03 (0.46-2.31)	0.94	0.84 (0.34-2.09)	0.71
S. hominis	**16 (14.5%)**	**2.61 (1.17-5.83)**	**0.02**	**2.33 (1.04-5.20)**	**0.04**
S. capitis	8 (7.3%)	1.87 (90.62-5.64)	0.27	1.29 (0.42-3.95)	0.65

* Data obtained from the report of the Healthcare-Associated Infection Control Committee.

HR: hazard ratio; 95%CI: 95% confidence interval; HAI: healthcare-associated infection; VM: mechanical ventilation; CoNS: coagulase-negative staphylococci.

FIGURES

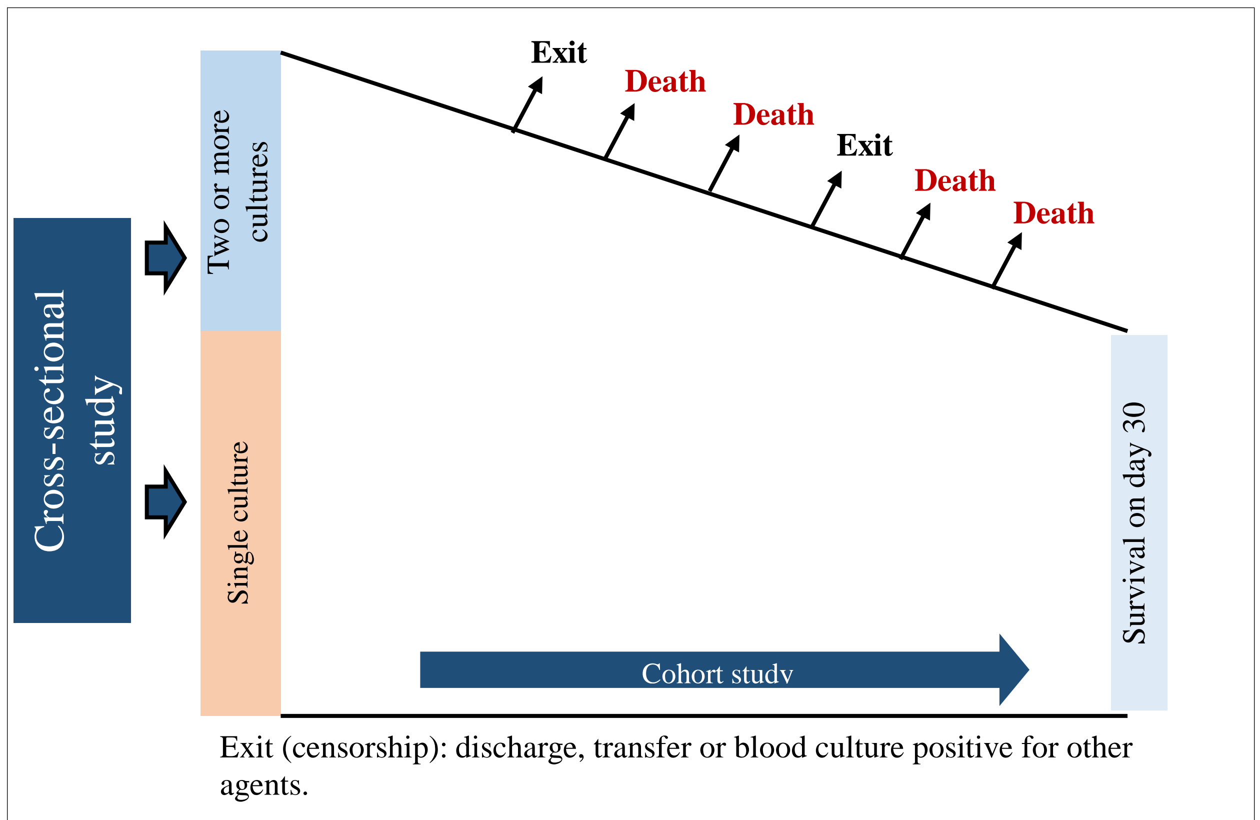

Figure 1: Schematic representation of the design of the cohort study.

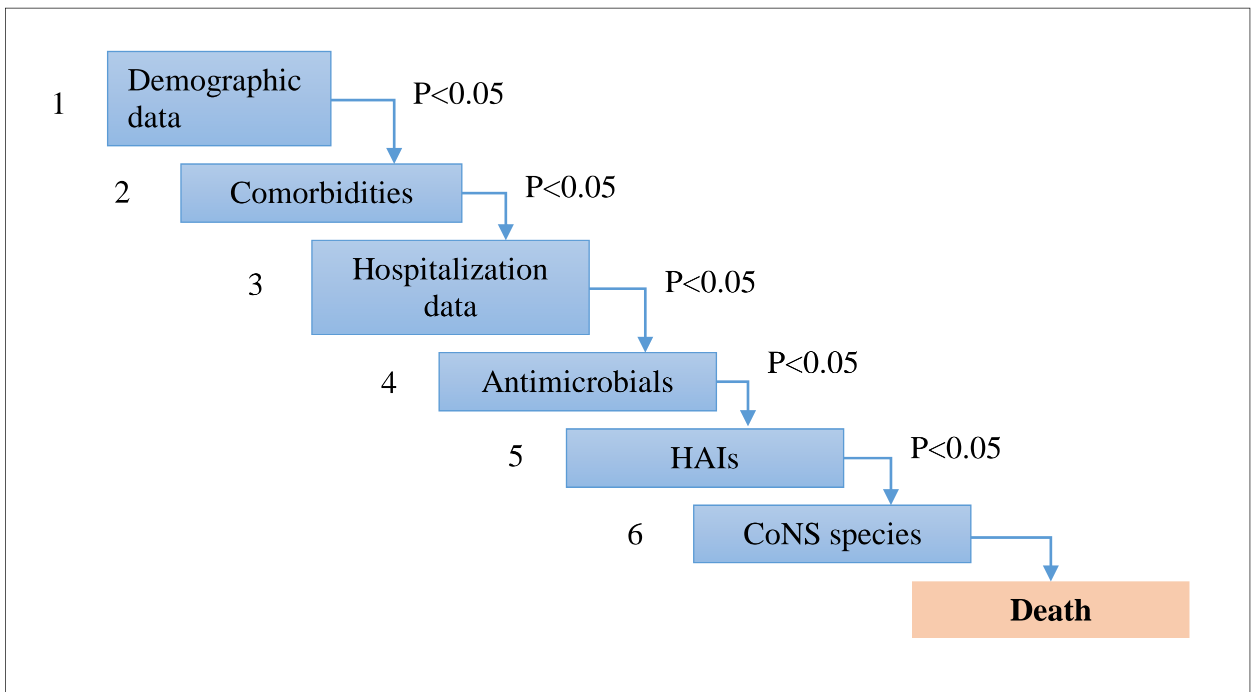

Figure 2: Schematic representation of the hierarchical strategy used for multivariate analysis of the outcomes (death and multiple positive blood cultures), excluding the presence of healthcare-associated infections (HAIs) in the second analysis. CoNS: coagulase-negative *staphylococci*.

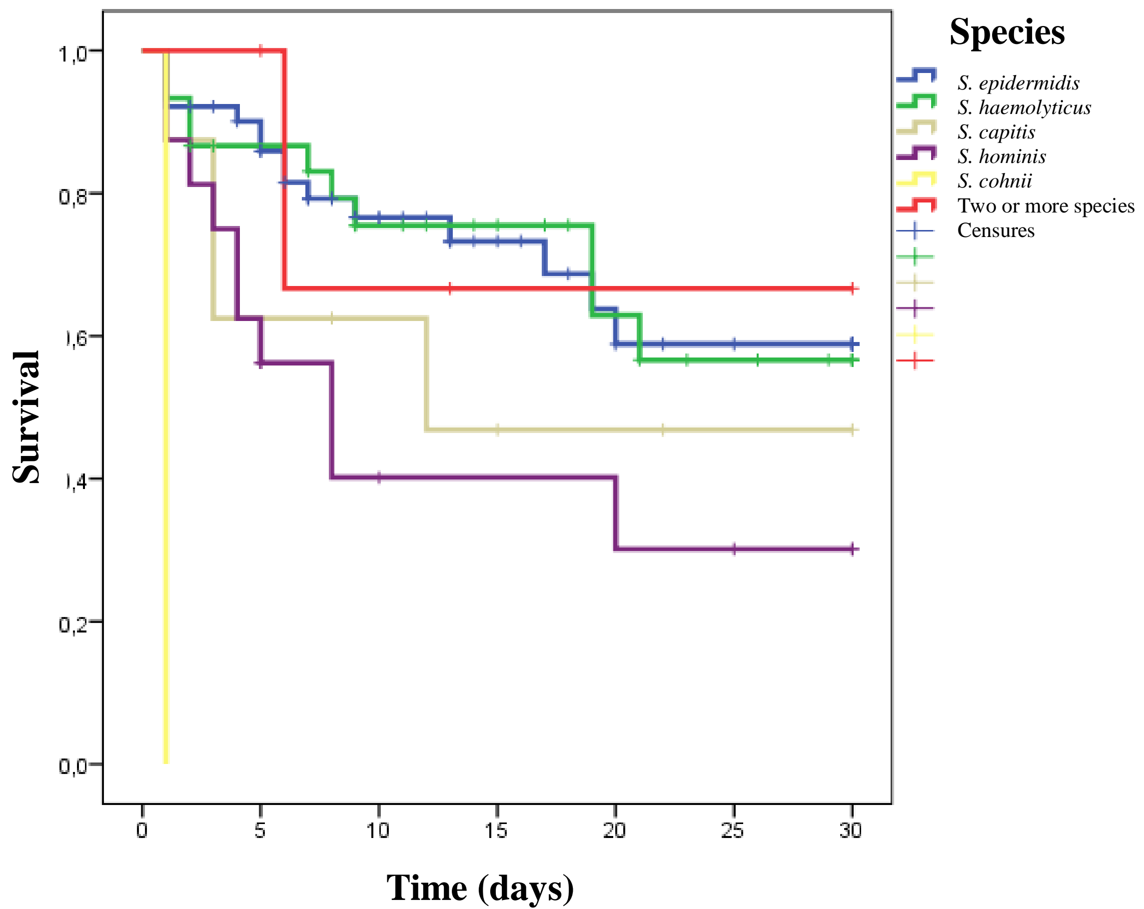

Figure 3: Kaplan-Meier survival curve of the cohort of patients infected with different coagulase-negative staphylococcal species and the outcome variable death.

6. Conclusões

- Embora os métodos convencionais tenham apresentado melhor desempenho comparado ao sistema VITEK® 2, os valores do teste de Kappa conferem confiabilidade aos resultados obtidos com o sistema VITEK® 2, e os testes de especificidade revelaram um desempenho superior aos 90% exigido dos equipamentos comerciais no campo da microbiologia clínica, demonstrando que esse equipamento é viável para a identificação dos micro-organismos isolados na rotina dos laboratórios de microbiologia clínica.

- Múltiplas hemoculturas positivas para ECN associadas de forma positiva com pacientes em choque (necessitando de droga vasoativa) sugerem que a gravidade da sepse (que se desenvolveu para choque) é um preditor da significância clínica de ECN.

- O estudo de coorte demonstrou que os pacientes infectados com o vírus da imunodeficiência humana (HIV) ou com AIDS em estado avançado e aqueles com hemocultura positiva para *S. hominis* apresentaram uma associação positiva ao óbito.

7. Anexos da tese
Anexo 1: Autorização do Comitê de Ética em Pesquisa

Universidade Estadual Paulista
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Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 02 de julho de 2012 347/2012-CEP

Ilustríssima Senhora
Profª Drª. Maria de Lourdes Ribeiro de Souza Cunha
Departamento de Micro/Imuno do
Instituto de Biociências de Botucatu

Prezada Drª Maria de Lourdes,

De ordem do Senhor Coordenador deste CEP, informo que o Projeto de Pesquisa (Protocolo CEP 4285-2012) "Identificação fenotípica e genotípica de microorganismos presentes em hemoculturas de pacientes em Unidade Intensiva: Incidência e perfil patogênico de Stapylococcuss Coagulase-negativa", a ser conduzido por Aydir Cecília Marinho Monteiro, orientado por Vossa Senhoria, Co-orientado pelo Prof. Dr. Carlos Magno Castelo Branco Fortaleza, recebeu do relator parecer favorável, aprovado em reunião de 02/07/2012.

Situação do Projeto: APROVADO. Os pesquisadores deverão apresentar ao CEP ao final da execução do Projeto o "Relatório Final de Atividades".

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP

Anexo 2: Termo de Consentimento Livre e Esclarecido para participação em Pesquisa Científica

Termo de Consentimento Livre e Esclarecido para participação em Pesquisa Científica

Projeto de Pesquisa: "Identificação fenotípica e genotípica de micro-organismos presentes em hemoculturas de pacientes em unidade intensiva: incidência e perfil patogênico de *Staphylococcus* coagulase-negativa".

O objetivo dessa pesquisa é comparar os resultados de exames realizados manualmente com os resultados dos exames realizados por uma máquina. Para que a máquina venha fazer esses exames e o resultado, no futuro, fique pronto mais rápido precisamos de um sangue examinado manualmente para ser colocado na máquina. Além disso, estudaremos detalhadamente um dos tipos de bactérias que infeccionam o sangue, para sabermos melhor sobre essa infecção na nossa região.

Por essa razão, convido você a participar dessa pesquisa, na qual não precisará coletar material algum além do já pedido pelo médico e coletado pela enfermagem. Nós usaremos o sangue que sobrou do exame já realizado. Essa amostra já foi identificada e descartada pelo Laboratório de Análises Clínicas do Hospital. Analisaremos os dados anotados em seu prontuário médico, sem causar nenhum transtorno durante os procedimentos, faremos a consulta após o médico responsável liberar os prontuários. A pesquisadora se disponibiliza a responder quaisquer perguntas e você poderá retirar seu consentimento a qualquer momento. Uma via desse termo ficará com você, e o sigilo e privacidade dos seus dados são garantidos.

Qualquer dúvida adicional, você poderá entrar em contato com o Comitê de Ética em Pesquisa, através do fone: (14) 38116143.

Tendo sido informado sobre a pesquisa que será realizada sob a responsabilidade da biomédica Aydir Cecília Marinho Monteiro, orientada pela professora Dr.ª Maria de Lourdes Ribeiro de Souza da Cunha, do Departamento de Microbiologia e Imunologia do Instituto de Biociências da UNESP, declaro que concordo em participar da mesma, permitindo o uso da amostra de hemocultura já identificada e descartada pelo Laboratório de Análises Clínicas do Hospital.

Botucatu, ___ de ______________de 2012.

_________________________________ _________________________________

Assinatura da Pesquisadora

Assinatura do Paciente ou Responsável

Amostra Nº:

Nome do Paciente:

____

Assinatura do Paciente RG do Paciente:

(Entre 11 e 18 anos)

Endereço e telefone do pesquisador: Departamento de Microbiologia e Imunologia, Instituto de Biociências, UNESP, Botucatu, tel.14-3882-6240 / 14-97814932; aydir@terra.com.br.

Endereço e telefone da orientadora: Departamento de Microbiologia e Imunologia, Instituto de Biociências, UNESP, Botucatu, tel. 14-3811-6240; cunhamlr@ibb.unesp.br.

Anexo 3: Ficha usada para coleta dos dados clínicos dos pacientes que apresentaram hemoculturas positiva para Estafilococos coagulase-negativa.

FICHA DE AVALIAÇÃO CLÍNICA

1. INFORMAÇÕES GERAIS

Hospital: ___________________________

Código de identificação: _____________________

Nome: ____ Sexo:_______

Idade: _______ Número do Prontuário: _____________ Enfermaria: _______________

Data da Admissão Hospitalar: ___/___/____ Data de Admissão na UTI: __/__/____

Data de Saída Hospitalar: ___/___/____ Data de Saída da UTI: ___/___/____

Tipo de Saída: (   ) alta   (   ) transferência   (   ) óbito (   ) positividade para outro agente (   ) falha na terapia

Movimentação no hospital

Unidade: __________ Admissão: ___/___/___ Saída: ___/___/___

Unidade: __________ Admissão: ___/___/___ Saída: ___/___/___

Unidade: __________ Admissão: ___/___/___ Saída: ___/___/___

Unidade: __________ Admissão: ___/___/___ Saída: ___/___/___

Internações anteriores (no último ano)

- Neste hospital

Unidade: ___________________ Admissão: ___/___/___ Saída: ___/___/___

Unidade: ___________________ Admissão: ___/___/___ Saída: ___/___/___

- Em outro hospital

Hospital: ___________________ Admissão: ___/___/___ Saída: ___/___/___

Hospital: ___________________ Admissão: ___/___/___ Saída: ___/___/___

Diagnóstico principal: ______________________________________CID: _______

Co-morbidades

( ) Doença cardíaca ( ) Doença pulmonar( ) Doença renal ( ) Doença hepática

( ) Diabetes mellitus ( ) Doença do SNC ( ) Neoplasia sólida

( ) Linfoma/leucemia ( ) Aids ( ) Trauma

( ) Outra(s):_ ____

Charlson score

1 - ( ) IAM ( ) ICC ( ) Doença vascular periférica

( ) Demência ( ) DPOC ( ) Doença do tecido conjuntivo

( ) úlcera péptica ( ) Hepatopatia leve

( ) Doença cerebrovascular ( ) Diabetes

2 - ( ) Hemiplegia ( ) Doença renal moderada/severa

( ) Neoplasia maligna ( ) Leucemia ( ) Linfoma

( ) Diabetes com dano de órgão

3 - ( ) Doença hepática moderada/severa

6 - ( ) AIDS ( ) Tumor sólido metastático

Score: _______

2. DADOS ANTERIORES À COLETA DE HEMOCULTURA

Imunidade

( ) Neutropenia ( ) Esteróides ( ) Radioterapia

( ) Outro imunossupressor: _____________________

Procedimentos

Cirurgias: ______________ Data: ___/___/___

Duração _______ Anestesia ( ) geral ( ) peridural ( ) local

______________ Data: ___/___/___

Duração _______ Anestesia ( ) geral ( ) peridural ( ) local

Outros procedimentos invasivos: ____________ Data: ___/___/___

____________ Data: ___/___/___

____________ Data: ___/___/___

____________ Data: ___/___/___

Uso de Dispositivos

( ) Sonda nasogástrica/nasoenteral ( ) NPP

( ) SVD ( ) CVC

( ) Drenos ( ) Úlcera de Pressão

Uso de Antimicrobianos

1.___________________ de ___/___/___ a ___/___/___ total: ____ dias

2.___________________ de ___/___/___ a ___/___/___ total: ____ dias

3.___________________ de ___/___/___ a ___/___/___ total: ____ dias

4.___________________ de ___/___/___ a ___/___/___ total: ____ dias

5.___________________ de ___/___/___ a ___/___/___ total: ____ dias

6.___________________ de ___/___/___ a ___/___/___ total: ____ dias

7.___________________ de ___/___/___ a ___/___/___ total: ____ dias

8.___________________ de ___/___/___ a ___/___/___ total: ____ dias

3. DADOS POSTERIORES À COLETA DE HEMOCULTURA – 24h

1. Leucócitos: ________________ Leucocitose ( ) Leucopenia ( )
   Neutropenia ( ) Neutrofilia ( )
2. PCR: ____
3. Febre ( ) ___________
4. Hipotermia ( ) _______
5. Hipotensão ( ) _______

4. MICRO-ORGANISMOS ISOLADOS EM OUTRAS CULTURAS

__________________ material _________________ data: ___/___/___

___________________ material _________________ data: ___/___/___

___________________ material _________________ data: ___/___/___

___________________ material _________________ data: ___/___/___

5. DADOS EM ATÉ 28 DIAS APÓS COLETA DAS HEMOCULTURAS
Imunidade

( ) Neutropenia ( ) Esteróides ( ) Radioterapia

( ) Outros imunossupressores: ____________________________________

Procedimentos

Cirurgias: ______________ Data: ___/___/___

Duração _______ Anestesia ( ) geral ( ) peridural ( ) local

______________ Data: ___/___/___

Duração _______ Anestesia ( ) geral ( ) peridural ( ) local

Outros procedimentos invasivos: ____________ Data: ___/___/___

____________ Data: ___/___/___

____________ Data: ___/___/___

____________ Data: ___/___/___

Uso de Dispositivos

( ) Sonda nasogástrica/nasoenteral ( ) NPP

( ) SVD ( ) CVC

( ) Drenos ( ) Úlcera de Pressão

Uso de Antimicrobianos

1.___________________ de ___/___/___ a ___/___/___ total: ____ dias

2.___________________ de ___/___/___ a ___/___/___ total: ____ dias

3.___________________ de ___/___/___ a ___/___/___ total: ____ dias

4.____________________ de ___/___/___ a ___/___/___ total: ____ dias

5.____________________ de ___/___/___ a ___/___/___ total: ____ dias

6.____________________ de ___/___/___ a ___/___/___ total: ____ dias

7.____________________ de ___/___/___ a ___/___/___ total: ____ dias

8.____________________ de ___/___/___ a ___/___/___ total: ____ dias

Observações