UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA – BOTUCATU

INFLUÊNCIA DA MASTITE CLÍNICA CAUSADA POR DIFERENTES PATÓGENOS NOS ÍNDICES REPRODUTIVOS DE VACAS LEITEIRAS E NA RESPOSTA DE PROTEÍNAS DE FASE AGUDA

Felipe Morales Dalanezi

Botucatu - SP

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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor em Medicina Veterinária.

Orientador: Prof. Hélio Langoni
Coorientadores: Prof. Dr. Ronaldo Luis Aoki Cerri e Profa. Dra. Elizabeth Moreira dos Santos Schmidt

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Nome do autor: Felipe Morales Dalanezi

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BANCA EXAMINADORA

Prof. Dr. Hélio Langoni
Presidente e orientador
Departamento de Higiene Veterinária e Saúde Pública FMVZ – UNESP – Botucatu /SP

Prof. Dr. Paulo Francisco Domingues
Membro
Departamento de Higiene Veterinária e Saúde Pública FMVZ – UNESP – Botucatu /SP

Prof. Dr. Márcio Garcia Ribeiro
Membro
Departamento de Higiene Veterinária e Saúde Pública FMVZ – UNESP – Botucatu /SP

Prof. Dr. Roberto Sartori Filho
Membro
Departamento de Zootecnia – ESALQ - USP – Piracicaba/SP

Prof. Dr. José Jurandir Fagliari
Membro
Departamento de Clínica e Cirurgia Veterinária - FCAV – UNESP – Jaboticabal/SP

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LISTA DE ABREVIATURAS

AGP: α-1 glicoproteína ácida (Alpha-1 Acid Glycoprotein)

AI: Inseminação artificial (artificial insemination)

APP: Proteína de fase aguda/ Acute phase proteins

CL: Corpo Lúteo

CMT: California Mastitis Test

CNS: Coagulase-negative *Staphylococcus*

CRP: Proteína C reativa (C-Reactive Protein)

DIM: Days in milk

DNA: Acido desoxirribonucleico

E2: Estradiol

ELISA: Enzyme-linked immunosorbent assay

FSH: Hormônio folículo estimulante (Follicle Stimulating Hormone)

FSHr: Receptor de FSH

GnRH: Hormônio liberador de gonadotrofinas

Hp: Haptoglobina (Haptoglobin)

HR: Hazard ratio

HRP: Horse-radish peroxidase

IBGE: Instituto Brasileiro de Geografia e Estatística

LBP: Proteína Ligadora de Lipopolisacarídeos (lipopolysaccharide biding protein)

LH: Hormônio luteinizante (Luteinizant Hormone)

LHr: Receptor de LH

LPS: Lipopolisacarídeo (Lipopolysaccharide)

mRNA: RNA mensageiro

M-SAA3: Isoforma 3 do SAA (mammary SAA isoform 3)

NMC: National Mastitis Council

P4: Progesterona

PGF2α: Prostaglandina F2α

SAA: Amilóide A Sérico (Serum Amiloid A)

SCC: Contagem de células somáticas

TMR: Total mixed ration

TNFα: Fator de Necrose tumoral α

SPARCL: Spatial Proximity Analyte Reagent Capture Luminescence

Sumário

RESUMO

DALANEZI, F.M. Influência da mastite clínica causada por diferentes patógenos nos índices reprodutivos de vacas leiteiras e na resposta de proteínas de fase aguda. Botucatu – SP. 2019, p.81. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

A mastite bovina é responsável por grandes perdas econômicas na bovinocultura de leite, além de aspectos de saúde pública que devem também ser considerados. Animais com mastite clínica ou subclínica apresentam diversas alterações reprodutivas. No primeiro artigo da tese, os resultados obtidos demonstraram que a mastite causada por patógenos maiores (20,1%) ou por gram-negativos (15,4%) levaram a queda na taxa de prenhez na primeira inseminação comparado com o grupo controle (32,6%). Foi observado maior taxa de perda gestacional para os grupos de patógenos maiores (22,2%) e gram-negativos (30,1%) comparados com o controle (12,8%). Foi observado diferença entre todos os grupos para o número de dias em aberto. O grupo controle apresentou o menor intervalo (126,5 dias) seguido pelo grupo patógenos menores (162,0 dias) e pelo grupo de patógenos maiores (175,1 dias). Comportamento similar observado para patógenos gram-positivo (172,7 dias) e patógenos gram-negativos (191,1 dias). Portanto, é importante controlar a mastite em rebanhos leiteiros e, para tanto, o diagnóstico é uma etapa importante. Diferentes proteínas de fase aguda foram descritas como bons biomarcadores para o diagnóstico da mastite. No segundo artigo da tese, foi observado que diferentes patógenos podem alterar a liberação de proteínas de fase aguda no leite. Bactérias que causam inflamações mais graves (*E. coli* e *Klebsiella pneumoniae)* levaram maior concentração das APP. Patógenos que causam infecções crônica (*Streptococcus* ambientais *Staphylococcus* aureus, Mycoplasma spp.) levaram a concentrações intermediarias das APP Micro-organismos que causam mastite menos graves (*Staphylococcus* coagulase negativa e *Enterococcus* spp.) levaram a menores concentrações das APP. Hp, AGP e CRP apresentaram correlação forte, positiva e significativa.

Palavras-chaves: Bactérias, bovinos, reprodução, glândula mamária, proteínas de fase aguda

ABSTRACT

DALANEZI, F.M. Influence of clinical mastitis caused by different pathogens on reproductive indices of dairy cows and on acute phase proteins response. Botucatu – SP. 2019, p.81. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

Bovine mastitis is responsible for major economic losses in dairy cattle farming, as well as public health aspects that should also be considered. Animals with clinical or subclinical mastitis present several reproductive alterations. In the first article of this thesis, the results showed that mastitis caused by major pathogens (20.1%) or gram-negative (15.4%) led to a decrease in pregnancy rate in the first insemination compared to the control group (32.6%). Higher gestational loss rates were observed for the major (22.2%) and gram-negative (30.1%) pathogen groups compared with the control (12.8%). The difference was observed between all groups for the number of days open. The control group had the shortest interval (126.5 days) followed by the minor pathogens group (162.0 days) and the major pathogens group (175.1 days). Similar behavior observed for gram-positive pathogens (172.7 days) and gram-negative pathogens (191.1 days). Therefore, it is important to control mastitis in dairy herds and, so, diagnosis is a critical step. Different acute phase proteins have been described as good biomarkers for the diagnosis of mastitis. In the second article of this thesis, it was observed that different pathogens may alter the release of acute phase proteins in milk. Bacteria that cause more severe inflammation (*E. coli* and *K. pneumoniae*) led to a higher concentration of APP. Pathogens causing chronic infections (Environmental *Streptococcus*, *S. aureus*, *Mycoplasma* spp.) led to intermediate concentrations of APP. Less severe mastitis causing microorganisms (Coagulase-negative *Staphylococcus* and *Enterococcus* spp.) led to lower concentrations of APP. Haptoglobin, AGP, and CRP showed strong, positive and significant correlations.

Keywords: Bacteria, bovine, reproduction, mammary gland, acute phase proteins.

CAPÍTULO 1

INTRODUÇÃO E REVISÃO DE LITERATURA

1. INTRODUÇÃO

Em 2016 o Brasil produziu 33 bilhões de litros de leite, com 19,7 milhões de cabeças, constituindo uma média de produção anual de 1.709 litros/vaca (IBGE, 2016). Alguns fatores levam a queda da produção leiteira no Brasil tais como, estresse térmico (RHOADS et al., 2009) e alta prevalência de doenças infecciosas (PEREIRA et al., 2013), com destaque para a mastite, que é a principal afecção do gado destinado à produção leiteira (LANGONI, 2013).

A mastite apresenta impacto significativo na produção leiteira mundial, apresentando custo anual de aproximadamente 175 milhões de euros. Estima-se que 89 milhões de euros são gastos por ano com o tratamento dos animais, enquanto ações preventivas custam em torno de 5 milhões de euros por ano (HALASA et al., 2007). No Brasil, a mastite bovina causa prejuízo médio de R$ 170.225,94 por ano. A redução na produção e o descarte de leite são os principais fatores que levam a perda de capital. A mastite subclínica causa custo médio anual de R$ 318,65/vaca/lactação, e indica que o investimento na prevenção da mastite é a alternativa mais viável, visto que o custo com esse procedimento representa menos de 20% dos gastos médios com a doença (LOPES et al., 2013). Assim, ressalta-se que deve-se conhecer e solucionar os fatores que diminuem os índices reprodutivos, e um dos fatores responsáveis pela queda dos índices é a mastite clínica e subclínica (SANTOS et al., 2004). Há necessidade de adoção de rigoroso programa de controle desta doença na propriedade (LANGONI, 2013).

A mastite é caracterizada pela infecção da glândula mamária de múltipla etiologia, causada frequentemente por bactérias. Pode ser classificada como mastite clínica ou subclínica. A forma clínica da doença se caracteriza pela produção anormal de leite, e pode ou não ter alterações na glândula mamária ou sinais clínicos sistêmicos (PINZÓN-SÁNCHEZ; RUEGG, 2011). Já a forma subclínica apresenta a mobilização de células inflamatórias para a glândula mamária, acarretando aumento na contagem de células somáticas (SCC) (GUIMARÃES et al., 2013).

Para o diagnóstico da mastite, rotineiramente são realizados dois testes principais: o California Mastitis Test (CMT) e o teste de Tamis (RADOSTITS et al., 2006), dos quais o primeiro para mastite subclínica e o segundo para mastite clínica. Atualmente, novos estudos têm demonstrado que algumas proteínas

produzidas pelo animal nas inflamações podem servir como biomarcadores para o diagnóstico de mastite (JAIN; GAUTAM; NASEEM, 2011). Algumas dessas proteínas chamadas de proteínas de fase aguda (APP) apresentam alta concentração no leite de vacas com mastite, como a haptoglobina (Hp), o amilóide A Sérico (SAA) e a proteína C reativa (CRP) (SADEK; SALEH; AYOUB, 2017; THOMAS et al., 2018). Esses novos biomarcadores são promissores e podem, hipoteticamente, substituir a SCC no diagnóstico, visto que possuem menos fatores que interferem no aumento da concentração das APPs quando comparado com a SCC (HUSSEIN et al., 2018).

A ocorrência da mastite clínica e/ou subclínica em vacas pode afetar negativamente os índices reprodutivos (LAVON et al., 2011; HUDSON et al., 2012). A ocorrência de mastite clínica próximo a inseminação leva a queda nos índices de concepção (SANTOS et al., 2004). Igualmente, o estabelecimento da mastite subclínica após a inseminação também diminui a taxa de concepção (LAVON et al., 2011; HUDSON et al., 2012).

Vacas com mastite clínica apresentaram maior intervalo entre o parto e primeira inseminação, maior taxa de perda gestacional, além de diminuir a produção de leite e a porcentagem de gordura no leite (CHEBEL et al., 2004; BOUJENANE; EL AIMANI; BY, 2014). Vacas com SCC maior que 200.000 cs/mL apresentaram queda de 10 pontos percentuais na taxa de concepção (BIJKER et al., 2015). Recentemente, observou-se que animais com mastite clínica apresentaram aumento no intervalo entre o parto e a primeira inseminação, aumento dos dias em aberto e aumento do número de inseminações por concepção (KUMAR et al., 2017b).

O presente estudo teve como finalidade relacionar a ocorrência da mastite clínica e as características reprodutivas de bovinos leiteiros, com ênfase nos patógenos de maior ocorrência nas infecções intramamárias clínicas.

2. REVISÃO DE LITERATURA

2.1 Mastite

A mastite clínica pode ser classificada em leve, moderada e grave, ou escores 1, 2 e 3, respectivamente (WENZ; GARRY; BARRINGTON, 2006), nas infecções por coliformes. A mastite leve é caracterizada pela secreção de leite

anormal, ausência de alterações sistêmicas ou na glândula mamária. Acredita-se que 60 a 90% dos casos de mastite clínica sejam leves (WENZ et al., 2005). Casos moderados de mastite apresentam secreção de leite anormal juntamente com alteração de um dos seis parâmetros a serem avaliados: secreção serosa pelo quarto afetado, diminuição da motilidade ruminal, desidratação, aumento da temperatura retal, aumento na frequência cardíaca ou respiratória. A mastite grave leva a secreção de leite anormal, pode ou não causar alterações na glândula mamária e acarreta a alteração de dois dos parâmetros: febre, taquicardia, taquipnéia, desidratação e diminuição da motilidade ruminal. Animais com mastite grave requerem atenção imediata podendo a afecção levar a morte do animal (ROBERSON, 2012).

A principal forma de contaminação da glândula mamária ocorre via ascendente pelo esfíncter do teto, por onde o patógeno adentra a cisterna do quarto e infecta as células (RAINARD; RIOLLET, 2006). A diminuição da imunidade no período pós-parto e no início da lactação facilita a instalação de agentes causadores da mastite (BURVENICH et al., 2003). Outra evidência da relação entre queda da imunidade mamária e episódio de infecção provavelmente decorre da queda na SCC anteriormente ao início da mastite (SURIYASATHAPORN et al., 2000). Após a invasão do micro-organismo, há transferência de leucócitos, principalmente neutrófilos, do sangue para o interior da cisterna, com o objetivo de combater a invasão dos patógenos (RAINARD; RIOLLET, 2006).

A mastite é a infecção da glândula mamária causada por diversos agentes, principalmente bacterianos. Os principais agentes bacterianos causadores da mastite são: *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli* e *Corynebacterium bovis.* Menos frequentemente são relatados *Pseudomonas aeruginosa, Enterobacter* spp., *Mycoplasma* spp., *Mycobacterium bovis, Brucella abortus* e *Listeria monocytogenes* (MARTINS et al., 2010). No Brasil, os principais agentes causadores da mastite bovina são *Corynebacterium bovis, Streptococcus dysgalactiae* e *Staphylococcus aureus* (LANGONI et al., 2011). Já em países de clima temperado como Estados Unidos e Reino Unido, os principais agentes causadores da mastite bovina são *Streptococcus uberis* e *Escherichia coli* (BRADLEY et al., 2007; OLIVEIRA; HULLAND; RUEGG, 2013).

A mastite pode ser classificada como contagiosa, quando causada por agentes transmitidos de um animal para outro ou por fômites durante a ordenha, ou ambiental quando causada por bactérias presentes na cama, água ou no solo onde os animais permanecem nos períodos entre ordenhas (ROBERSON, 2012). Os principais micro-organismos causadores de mastite contagiosa são: *Staphylococcus aureus* e *Streptococcus agalactiae.* Durante os anos 80, estes dois patógenos eram responsáveis por 87% dos isolamentos bacterianos (OLIVER; MITCHELL, 1984). Entretanto, estudos realizados nos Estados Unidos e Canadá apontaram para uma nova realidade de agentes causadores de mastite, com aumento da incidência de coliformes, *Streptococcus* não-*agalactiae* e *Staphylococcus* coagulase-negativos, enquanto que apenas 7% dos isolados são *S. aureus* e nenhum isolamento de *S. agalactiae* (LAGO et al., 2011). Corroborando esses achados, outro estudo revelou o isolamento principalmente de *Streptococcus dysgalactiae, Escherichia coli, Streptococcus uberis* e *Klebsiella* sp. (SCHUKKEN et al., 2013).

Estes estudos demonstraram que os esforços para combater a mastite contagiosa apresentaram resultados expressivos, aumentando a porcentagem de isolamento de agentes ambientais (ROBERSON, 2012). Por outro lado, o aumento da ocorrência de mastite ambiental levou a um novo questionamento sobre o tratamento da mastite, pois alguns autores defendem a hipótese que as infecções causadas por bactérias Gram negativas não devem ser tratadas (LAGO et al., 2011). Ao contrário, estudo demonstrou que vacas não tratadas apresentaram menor taxa de cura e maior contaminação do rebanho quando comparado com vacas tratadas (SCHUKKEN et al., 2011). Para determinar então se a vaca deve ou não ser tratada é preciso considerar alguns fatores ligados ao animal como: idade, estágio de lactação, SCC e o histórico clínico de mastite. O responsável pelo tratamento precisa ser treinado para definir quais animais deve tratar ou quais animais devem ser manejados de outra forma como a secagem precoce do quarto afetado (ou de todos os quartos), ablação química da glândula afetada ou o descarte do animal (ROYSTER; WAGNER, 2015).

Para evitar a contaminação mamária com agentes ambientais é preciso que seja realizada a correta redução da contaminação do ambiente onde o animal permanece 12 a 14 horas por dia fazendo com que o teto esteja sempre em contato com os contaminantes presentes no material (HOGAN; SMITH,

2012). A sujidade da pele e a concentração de bactérias que recobrem os tetos indicam a dimensão da contaminação da cama onde a vaca permanece (MANZI et al., 2012). Visto que as bactérias ambientais não sobrevivem por longos períodos na pele do animal, alta população bacteriana na pele do teto indica contaminação recente deste animal (HOGAN; SMITH, 2012).

Já para os agentes causadores da mastite contagiosa, a ordenha representa o maior risco de infecção (KEEFE, 2012). Mastite contagiosa é transmitida diretamente de uma vaca infectada para outra saudável ou de utensílios de ordenha. Esse fato se deve a baixa sobrevivência dos patógenos fora da glândula mamária (KEEFE, 1997). Procedimentos realizados durante a ordenha reduzem o risco de mastite contagiosa (DUFOUR et al., 2012), tais como: uso de toalhas individuais para limpeza do úbere, uso de luvas pelo ordenhador, manutenção adequada da ordenha mecânica e antissepsia dos tetos depois da ordenha (KEEFE, 2012).

Apesar de alguns estudos mostrarem a queda na porcentagem de mastites causadas por *S. aureus* (LAGO et al., 2011; SCHUKKEN et al., 2013), no Brasil esse patógeno ainda é importante agente causador das mastites (MARTINS et al., 2010). *S. aureus* possui ampla variedade de fatores de virulência, a saber: produção de enterotoxinas, hemolisinas, nucleases, proteases e biofilme (TODAR, 2002), além de ser considerado importante agente de intoxicação alimentar (SÁ et al., 2004).

O controle da mastite no rebanho depende principalmente do diagnóstico. Para a determinação da instalação do quadro de mastite alguns testes podem ser utilizados como o *California Mastitis Testis* (CMT). O CMT utiliza a reação entre o DNA das células presentes no leite e detergente específico (SCHALM; NOORLANDER, 1957) levando a formação de gel, possibilitando análise da presença de células inflamatórias presentes no leite (WHYTE et al., 2005). A análise quantitativa do número de células no leite pode ser realizada pela SCC. Alguns fatores como: nível de infecção, estágio de lactação, idade, raça e transporte do leite podem alterar a SCC (DEB et al., 2013).

É considerado indicativo de mastite quando a SCC ultrapassa 200.000 células/mL (DOHOO; MEEK, 1982). Outro procedimento importante do diagnóstico é a realização do cultivo microbiológico para isolamento do agente

causador da mastite. Para tanto, amostras de leite devem ser semeadas em meios de cultivo para o isolamento microbiano (DEB et al., 2013).

Proteínas de fase aguda relacionadas à mastite

Nos bovinos, as principais APP são a Hp, o SAA, a proteína ligadora de lipopolisacarídeo (LBP) e a α1 glicoproteína ácida (AGP) (CECILIANI et al., 2012). As APP podem também ser classificadas como maiores, moderadas ou menores de acordo com a sua resposta frente a inflamação, sendo Hp, SAA, LBP e AGP classificadas como maiores. Hp, SAA e CRP foram mensuradas no leite e no sangue indicando propriedades como biomarcadores da mastite (ECKERSALL et al., 2001; THOMAS et al., 2015, 2018; SIMÕES et al., 2018), tornando assim essas proteínas importantes tanto em casos agudos septicêmicos como em casos crônicos de mastite subclínica.

A Hp é uma das principais APP encontrada em vacas, apresentando concentração menor que 0,01mg/mL em animais saudáveis e aumentada em até mil vezes em animais com resposta imunológica ativada (CECILIANI et al., 2012). A Hp é utilizada como marcador da inflamação sistêmica causada por várias doenças que acometem vacas de leite, como mastite e esteatose hepática (ECKERSALL; BELL, 2010). A produção da Hp é realizada principalmente no fígado, porém a expressão extra-hepática foi demonstrada na glândula mamária, em leucócitos e no abomaso (CECILIANI et al., 2012). As principais funções da Hp na corrente sanguínea são: capturar e retirar do sangue a hemoglobina livre resultante de hemólise (KRISTIANSEN et al., 2001), diminuir a concentração de radicais livres provenientes do estresse oxidativo (TSENG et al., 2004), regular a resposta do sistema imune por meio dos leucócitos, efeito bacteriostático e auxiliar na produção de proteínas (CECILIANI et al., 2012). Durante o processo inflamatório, a Hp apresenta funções específicas como o recrutamento de neutrófilos para o foco inflamatório (EL GHMATI et al., 1996).

Dados sobre a produção de Hp na glândula mamária são escassos em condições patológicas. O aumento da sua expressão foi notado após a infecção por *E. coli* (BUITENHUIS et al., 2011). Foi observada a expressão de RNA mensageiro (mRNA) de Hp no epitélio alveolar da glândula mamária (THIELEN et al., 2007) e aumento focal da expressão desse mRNA no local de infecção por *E. coli* (RINALDI et al., 2010). A quantificação da concentração de Hp no leite foi

mensurada pela expressão de mRNA, após a inoculação de lipopolisacarídeo (LPS) presente na membrana de bactérias gram-negativas, na cisterna da glândula mamária. Foi observado aumento da concentração de mRNA no leite 3 horas e no soro 9 horas após a inoculação (HISS et al., 2004).

O SAA pertence a uma grande família de proteínas conhecida por ser uma das mais reativas APP (BING et al., 1999). A maior parte das isoformas do SAA é produzida no fígado e liberada na corrente sanguínea (UHLAR; WHITEHEAD, 1999). A isoforma 3 do SAA (M-SAA3), identificada no leite bovino, é produzida por células da glândula mamária e atua combatendo a inflamação local do úbere (WEBER et al., 2006). Vacas saudáveis quando estimuladas com prolactina apresentam aumento na produção do M-SAA3 pelas células epiteliais da mama. Entretanto, esse aumento não foi acompanhado pela produção do SAA, mostrando assim que o M-SAA3 é a isoforma mais importante para a glândula mamária (LARSON et al., 2005). Observou-se também que o M-SAA3 é um bioindicador de mastite mais confiável que a Hp, pois possui maior relação com a inflamação da glândula mamária que a Hp (ECKERSALL et al., 2006).

O M-SAA3 possui a função de estimular a resposta inata do sistema imunológico na glândula mamária, além de apresentar ação anti-bacteriana contra *E. coli, S. uberis e Pseudomonas aeruginosa* (MOLENAAR et al., 2009). Vacas com mastite experimental por *S. aureus* apresentaram aumento na concentração de M-SAA3 primeiro no leite que no sangue (ECKERSALL et al., 2006). Infecções extra mamárias podem elevar a concentração sérica do SAA. Porém, não alteram a concentração de M-SAA3 no leite, evidenciando que essa isoforma produzida na mama não é influenciada por inflamações concorrentes, indicando sua importância no diagnóstico da mastite (NIELSEN et al., 2004). Estudo recente demonstrou que a concentração de M-SAA3 no leite é mais confiável que a SCC para o diagnóstico de mastite subclínica (HUSSEIN et al., 2018).

A CRP também é uma APP produzida no fígado e liberada na corrente sanguínea, além de ser responsável pela resposta mais rápida no processo inflamatório (CLYNE; OLSHAKER, 1999). A CRP possui a função de diminuir o dano tecidual, destruir patógenos e auxiliar a regeneração dos tecidos a estimular a fagocitose de patógenos pelos macrófagos (BAUMANN; GAULDIE,

1994; HORADAGODA et al., 1999). Apesar de considerada uma APP menor, por possuir pouca variação na sua concentração sanguínea nos episódios inflamatórios, na mastite em vacas leiteiras a concentração sérica de CRP observada foi de 1.083 ng/mL, enquanto que vacas sadias apresentam concentração de 82 ng/mL (SCHRODL et al., 1995). Todavia, no leite de vacas com mastite clínica a concentração média foi de 32,64 ng/mL, podendo variar entre 1,8 ng/mL e 172,47 ng/mL (THOMAS et al., 2015). Adicionalmente foi demonstrado que a CRP não acompanha a variação na contagem de células somáticas no leite de vacas, demonstrando assim que sua liberação é mais sensível a infecções da glândula mamária que a SCC. A CRP, juntamente com Hp e SAA, apresentam elevação da sua concentração no colostro e no leite de vacas nos primeiros dias de lactação (THOMAS et al., 2016). Esse fato é importante, pois as APP só podem ser utilizadas no diagnóstico de mastite após o sexto dia de lactação, para que não ocorra resultados falso positivos.

AGP é uma APP classificada como moderada, devido ao fato de aumentar 2 a 3 vezes durante o processo inflamatório (ECKERSALL et al., 2001). É uma lipocalina que pertence à família das proteínas capazes de se ligar e transportar moléculas hidrofóbicas. Essa função, quando exercida durante a resposta inflamatória, pode auxiliar a transportar moléculas que combatem a fonte da inflamação (FLOWER; NORTH; SANSOM, 2000). Essa proteína foi adicionada a uma subfamília das lipocalina chamada imunolipocalinas, capazes de imunomodular a reação inflamatória por meio da agregação neutrofílica no local da inflamação, diminuir a quantidade de espécies reativas de oxigênio e inibir a apoptose de células brancas (LOGDBERG; WESTER, 2000). Além das funções previamente descritas, a AGP pode proteger o hospedeiro contra infecções bacterianas, inibindo a ligação dos patógenos com as células alvo (LIBERT et al., 2002). Assim, como as APPs citadas anteriormente, a AGP é produzida no fígado e liberada na corrente sanguínea. Porém estudos também revelaram a produção e liberação pelas células da glândula mamária de bovinos saudáveis, bem como aumento da produção após infecção com *Staphylococcus aureus* (CECILIANI et al., 2005, 2007; WHELEHAN et al., 2011). Essa proteína foi descrita também no leite e colostro de vacas leiteiras. Indicando que, durante o início da lactação, a determinação da concentração dessa APP no leite pode ser alterada (CECILIANI et al., 2005). Foi demonstrado que vacas apresentando

mastite subclínica apresentaram aumento da concentração de AGP no leite (BIAN; LV; LI, 2014).

Pyörälä et al. (2011) afirmaram que mastites clínicas causadas por diferentes patógenos podem causar maior ou menor aumento da concentração de APPs no leite. Foi observado que a mastite clínica grave gera maior liberação de proteínas quando comparado com mastites de graus leve e moderado. Acredita-se, dessa forma, que agentes causadores de mastites mais graves, como gram negativos, estimulem maior liberação dessas proteínas, comparado com agentes que causam mastites leve e moderada, como os cocos gram positivos. Estudo recente demonstrou que a concentração de Hp, SAA e CRP no leite de vacas com mastite pode variar de acordo com o patógenos causadores da infecção (THOMAS et al., 2018).

Já foi descrito que SAA, Hp e a CRP (KALMUS et al., 2013; THOMAS et al., 2015, 2018; SIMÕES et al., 2018) podem ser usadas como indicadores de inflamação da glândula mamária. Visto que para determinação dessas proteínas são utilizadas técnicas de imunoensaio, acredita-se que essa mensuração possa ser avaliada juntamente com outras análises realizadas durante a ordenha.

Reprodução Bovina

A vaca, assim como todas as fêmeas mamíferas, nasce com milhares de oócitos inclusos em folículos pré-antrais. Porém, apenas pequena porcentagem desses folículos chega a ser ovulada. A maioria é descartada durante a fase de desvio folicular por meio do processo de atresia (YANG; FORTUNE, 2008).

Nos bovinos, a foliculogênese ocorre em duas fases: a fase pré-antral e antral (ERICKSON, 1966). A fase pré-antral é constituída de folículos primordiais, folículos primários e folículos secundários. Em contraste, a fase antral é dividida em crescimento inicial e final dos folículos terciários (FAIR et al., 1997).

Mecanismos endócrinos são preponderantes na regulação do desenvolvimento folicular antral, destacando-se as gonadotrofinas, como hormônio folículo estimulante (FSH), hormônio luteinizante (LH) e os hormônios esteroides ovarianos, estradiol (E2) e progesterona (P4). Entretanto, estudos

sustentam a participação de fatores ovarianos parácrinos nos mecanismos de controle, inclusive na mediação e modulação da ação das gonadotrofinas (MONNIAUX et al., 1997; WEBB et al., 2003).

Os bovinos são animais poliéstricos anuais e apresentam ciclo estral de 21 dias de duração, com 2 a 3 ondas de crescimento folicular (SENGER, 2003). Em cada onda folicular, o período inicial é caracterizado por uma fase de desenvolvimento comum, onde os folículos crescem em diâmetro, em resposta ao aumento dos níveis séricos do FSH. O FSH possui papel chave na regulação da emergência das ondas foliculares e manutenção do crescimento dos folículos durante o período inicial de desenvolvimento, antes da divergência folicular (ADAMS et al., 1992). Em espécies monovulatórias, como a bovina, apenas um folículo é selecionado e passa a exercer dominância sobre os demais que compõem a onda folicular. Cada onda envolve o recrutamento de um “pool” de folículos e a seleção de um folículo dominante, que continua a crescer até o estágio pré-ovulatório, enquanto os outros da mesma onda sofrem atresia (WEBB et al., 1999).

As concentrações plasmáticas de FSH começam a diminuir nos dias 2 e 3 após a emergência e, então, alguns folículos da onda cessam o desenvolvimento e entram em atresia, com exceção do folículo dominante que adquire “independência” ao FSH; que continua o crescimento mesmo em ambiente de baixa concentração plasmática dessa gonadotrofina. Essa mudança no ritmo de desenvolvimento folicular é denominada divergência ou desvio folicular e inicia com redução nas taxas de desenvolvimento dos folículos subordinados, em contraste com a manutenção ou aumento (FORTUNE et al., 2001) da taxa de desenvolvimento do futuro folículo dominante. A divergência folicular ocorre quando o maior folículo se encontra com diâmetro em torno de 8 mm (TORTORELLA et al., 2017). O conjunto de eventos que transformam o folículo primordial em folículo pré-ovulatório é chamado de foliculogênese, e compreende a formação, o crescimento e a maturação dos folículos (VAN DEN HURK; ZHAO, 2005).

Por meio da secreção de E2 e inibina o folículo dominante causa redução dos níveis circulantes de FSH, que se tornam insuficientes para a manutenção do crescimento dos folículos subordinados. Acredita-se que a refratariedade do

folículo dominante aos níveis decrescentes de FSH se deve, em parte, à aquisição de receptores para o LH, gonadotrofina responsável pelo desenvolvimento e maturação folicular terminal (GINTHER et al., 1996). Finalmente, se a regressão do corpo lúteo ocorre, ainda durante a fase de crescimento do folículo dominante, a queda dos níveis de P4 possibilita o aumento da frequência dos pulsos de LH, culminando no pico de LH necessário à ovulação e à maturação oocitária com retomada da meiose. Caso contrário, o folículo dominante regride e deixa de inibir a secreção de FSH, possibilitando a emergência de nova onda folicular (MONNIAUX et al., 1997).

A produção de estrógeno e o crescimento dos folículos dependem da ação coordenada do FSH e LH em seus receptores. O modelo de duas células/dois hormônios (FORTUNE; QUIRK, 1988) sugere que células da granulosa e células da teca estão envolvidas na produção de E2 pela interação do FSH com seus receptores (FSHr) presentes na granulosa e do LH com seus receptores (LHr) presentes nas células da teca. No momento da seleção morfológica, o folículo dominante tem maiores concentrações intra-foliculares de E2, o que condiz com a menor produção estrogênica de folículos subordinados em relação aos dominantes, como verificado em estudos “in vitro” (FORTUNE; QUIRK, 1988).

Após o pico de LH inicia-se a formação do corpo lúteo (CL), no folículo dominante, células da teca e da granulosa que supriam a produção de E2 e andrógenos, respectivamente, passam a sintetizar P4 estimuladas pelo LH. As células da granulosa se transformam em células luteais grandes, enquanto as da teca tornam-se células luteais pequenas (REYNOLDS; GRAZUL-BILSKA; REDMER, 2002). Nessa fase, a ocitocina parece desempenhar papel importante na produção de P4 pelas células da granulosa (CHANDRASEKHERT; FORTUNE, 1990).

O CL é um órgão endócrino transitório formado pela ruptura do folículo ovulatório e sua função primária é a produção de P4, responsável pela preparação do endométrio, implantação e manutenção da gestação (MILVAE, 2000). Após a ovulação, as células da teca e da granulosa sofrem hipertrofia e luteinização para produzirem P4. As concentrações de P4 apresentam aumento

constante até o dia 16 do ciclo estral e, se não houver fecundação, o CL sofre regressão (SARTORI et al., 2004).

O CL apresenta períodos regulares de formação, funcionamento e luteólise marcados por intensas remodelações teciduais, caracterizadas pela proliferação, migração, diferenciação e morte celular. O CL possui populações heterogêneas de células com características morfofuncionais e bioquímicas distintas, classificadas como células esteroidogênicas e não-esteroidogênicas (MARTIN; FERREIRA, 2009).

Na ausência do embrião no ambiente intrauterino, são produzidos sinais no endométrio para a síntese de prostaglandina F2α (PGF2α) que desencadeará a luteólise. A luteólise pode ser classificada como funcional e estrutural. Na luteólise funcional é evidenciada queda significativa na produção de P4 (STOUFFER et al., 2001). Na luteólise estrutural, as células esteroidogênicas e do endotélio vascular sofrem processo de degradação por apoptose e nota-se diminuição da irrigação do CL (MIYAMOTO et al., 2005).

A síntese de PGF2α é mediada principalmente pelo (E2) e a ocitocina. Durante o ciclo estral da vaca, a P4 inibe a formação de receptores para E2 no endométrio, causando estímulo para a produção de receptores de ocitocina, sem a produção da PGF2α. Entre 10 a 12 dias após a ovulação, os receptores de P4 começam a diminuir, aumentando a síntese de receptores de ocitocina, com a subsequente produção de PGF2α (MARTIN et al., 2013).

Influência da mastite na reprodução

Vários estudos demonstraram o efeito deletério da mastite nos índices reprodutivos (HUDSON et al., 2012; KUMAR et al., 2017a), porém não se sabe ao certo como a infecção mamária influencia nas falhas reprodutivas. A influência negativa da mastite na reprodução foi observada tanto em animais da raça holandesa como em animais cruzados (KUMAR et al., 2017b, 2017a). Algumas hipóteses foram aventadas na tentativa de explicar esse fenômeno. Vacas com mastite podem apresentar sinais sistêmicos como febre, aumento da permeabilidade vascular, leucocitose e aumento das frequências respiratória e cardíaca (SOTO; NATZKE; HANSEN, 2003; HANSEN; SOTO; NATZKE, 2004), além de maior concentração de cortisol circulante (HOCKETT et al., 2000).

A reação inflamatória induzida pela mastite origina a liberação de várias moléculas bioativas, que podem causar agravos aos órgãos do aparelho reprodutivo (HANSEN; SOTO; NATZKE, 2004). Vacas com mastite apresentaram aumento de interleucinas, fator de necrose tumoral – α (TNFα) (RIOLLET; RAINARD; POUTREL, 2001), óxido nítrico (BLUM et al., 2000) e PGF2α (GIRI et al., 1984). O óxido nítrico, o TNFα e a PGF2α podem diminuir a fertilidade atuando no oócito e no embrião (HANSEN; SOTO; NATZKE, 2004). O TNF-α pode ainda inibir o desenvolvimento dos oócitos em blastocistos, estimular apoptose de células ovarianas e causar luteólise prematura (SOTO; NATZKE; HANSEN, 2003; SILVA et al., 2017; CAMPOS et al., 2018). A mastite diminui a liberação de hormônios reprodutivos como o hormônio liberador de gonadotrofinas (GnRH) (BATTAGLIA et al., 1997), o LH e o E2 (HOCKETT et al., 2005). Em búfalos apresentando mastite clínica ou subclínica foi observado diminuição no diâmetro do folículo pré-ovulatório e no CL levando a diminuição na concentração sérica de E2 e PGF2α (MANSOUR; HENDAWY; ZEITOUN, 2016; MANSOUR; ZEITOUN; HUSSEIN, 2017).

Outro fator importante para a diminuição dos índices reprodutivos devido a mastite são os grupos bacterianos envolvidos. Vacas diagnosticadas com mastite por agentes gram-negativos apresentam queda de trinta e dois pontos percentuais na taxa de concepção (HERTL et al., 2010). O principal fator responsável por causar inflamação em infecções por gram-negativos é o LPS, um lipopolissacarídeo pertencente a membrana celular. Foi demonstrado que o lipídeo A é o responsável por ativar o sistema imune do hospedeiro, estimulando a liberação de citocinas e fatores pró-inflamatórios (SCHUKKEN et al., 2012). Acredita-se que as substâncias produzidas em decorrência da resposta inflamatória induzida pelo LPS, acarrete problemas reprodutivos (CAMPOS et al., 2018). Em outro estudo observou-se que a administração de LPS em vacas leiteiras, reduziu a produção de E2, levou à falha na ativação de oócitos primordiais, reduziu a competência oocitária, diminuiu a vascularização do CL, reduziu a concentração plasmática de P4 e antecipou a luteólise (BROMFIELD; SHELDON, 2013). A infecção por bactérias gram-negativas (*E. coli*) aparenta ser mais prejudicial ao desenvolvimento oocitário quando comparada com bactérias gram-positivas (*S. aureus*). Entretanto, a infecção por bactérias gram-positivas

apresentou menor porcentagem de blastocistos, indicando assim que cada bactéria apresenta uma forma de ação distinta, embora ambas infecções sejam deletérias para a reprodução (ASAF et al., 2014). A mastite causada por agentes gram-negativos diminuiu a probabilidade de prenhez, independente do período de ocorrência. Tanto antes como depois da inseminação houve queda na taxa de prenhez (HERTL et al., 2014).

Poucos estudos acerca da influência da mastite na reprodução em vacas leiteiras têm sido realizados no Brasil, havendo a necessidade de melhor entendimento dos efeitos da mastite clínica no desempenho reprodutivo em vacas leiteiras, fato que motivou a proposta do presente estudo.

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OBJETIVOS E HIPÓTESES

GERAL

Investigar a influência da mastite clínica causada por diferentes patógenos nos parâmetros reprodutivos, em vacas da raça holandês.

ESPECÍFICOS

Comparar os parâmetros reprodutivos de vacas holandesas, com ou sem diagnóstico de mastite clínica, no período entre o parto e a confirmação de nova prenhez;

Investigar a influência da mastite clínica causada por diferentes grupos de patógenos nos índices reprodutivos de vacas holandesas;

Quantificar a concentração de proteínas de fase aguda no leite de vacas com mastite clínica, causada por diferentes patógenos.

HIPÓTESES

Os patógenos maiores podem alterar mais intensamente as características reprodutivas quando comparados aos patógenos menores;

Alterações nas características reprodutivas nos grupos com mastite clínica serão mais intensas que as observadas no grupo controle;

Patógenos causadores de mastite com maior acometimento da glândula mamária estimularão maior produção de proteínas de fase aguda, com maior concentração dessas no leite.

CAPÍTULO 2

ARTIGO 1 – INFLUENCE OF PATHOGENS CAUSING CLINICAL MASTITIS ON REPRODUCTIVE VARIABLES OF DAIRY COWS

Artigo 1 – Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows.

Artigo enviado e em tramitação para publicação na revista *Journal of Dairy Science* segundo as normas estabelecidas: https://www.journalofdairyscience.org/content/inst-auth

INTERPRETIVE SUMMARY

Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows. *By Dalanezi et al*. Some authors demonstrated that clinical and subclinical mastitis could negatively affect reproductive efficiency in dairy cows. This study aimed at evaluating the effect of clinical mastitis, caused by different pathogens, in reproduction variables of dairy cows. Animals from control group (without clinical mastitis) had less days open, improved conception at first artificial insemination (AI) and decreased pregnancy loss compared with groups with clinical mastitis. Major pathogens of mastitis lead to more days open compared to minor pathogens, but no significant association was observed between those groups in pregnancy at first AI and pregnancy loss. When comparing pathogens by Gram classes (positive and negative), cows affected by Gram-negative bacteria had worse results in days open, pregnancy at first AI and decreased pregnancy loss.

Running head: CLINICAL MASTITIS, PATHOGENS AND FERTILITY

Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows

F.M. Dalanezi*, S.F. Joaquim*, F.F. Guimarães*, S.T. Guerra*,

B.C. Lopes*, E.M.S. Schmidt†, R.L.A. Cerri‡, H. Langoni*[1]

*****Department of Veterinary Hygiene and Public Health, São Paulo State University (UNESP), campus Botucatu, Faculty of Medicine Veterinary and Animal Science, Brazil 18168-681

† Department of Veterinary Clinical Science, São Paulo State University (UNESP), campus Botucatu, Faculty of Medicine Veterinary and Animal Science, Brazil 18168-681

‡ Applied Animal Biology, Faculty of Land and Food Systems, University of British Columbia, Vancouver, Canada V6T 1Z4

[1]Corresponding author: Helio Langoni, St. Prof. Dr. Walter Mauricio Correa, s/n – UNESP Botucatu, SP, Brazil, 18618-681; Tel: +55 14 3880 2094; email: helio.langoni@unesp.br

ABSTRACT

Mastitis is responsible for pronounced economic losses in dairy farms, as well as for causing public health concerns. Reproductive efficiency of commercial dairy herds is vital for the economic success of dairy operations and tightly associated with the health status of cows. Mastitis has previously been linked with decreased fertility of dairy cows, but the effect of specific pathogens on the severity of fertility reduction is still unclear. The aim of the study was to investigate the effect of clinical mastitis, in dairy cows, caused by different pathogen in the reproductive variables. Dairy cows with mastitis by major pathogens (*Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella pneumoniae, Mycoplasma* spp., and environmental *Streptococcus*) had lower Percentage of pregnancies at first artificial inseminations (**AI**) (20.1±0.03%) than healthy animals (32.6±0.02%), no difference was observed between cows infected by minor pathogens (Coagulase-negative *Staphylococcus* and *Corynebacterium* spp.) (26.2±0.03%) and healthy animals nor major pathogens group. Pregnancy loss was lower in the control group (12.8±0.02%) compared with the major pathogens group (22.2±0.02%); however, there was no difference when compared with the minor pathogen group (16.5±0.02%). Cows diagnosed with mastitis caused by major pathogens had more days open (175.1±3.7 days) than by minor pathogens (162.0±4.1 days) and control group (129.5±1.9 days). When only the Gram-staining was considered to categorize the pathogens, the mastitis cases caused by gram-negative bacteria decreased

pregnancy at first AI (15.4±0.03%), and increased days open (191.1±7.4 days) and pregnancy losses (30.1±0.03%) compared with the control group. Cows diagnosed with gram-positive bacteria during mastitis cases also increased days open (172.7±4.1 days) compared with control cows; but no difference was observed to percentage of pregnancy to first AI (23.8±0.02%) nor pregnancy loss (17.2±0.02%). This study shows that bacteria causing mastitis can affect fertility of cows differently. Major pathogens and gram-negative bacterial lead to the most dramatic decrease in reproduction efficiency.

Keywords: Bacteria, dairy cow, artificial insemination, mammary infection

INTRODUCTION

Mastitis presents a significant challenge to global milk production, being responsible for an annual cost of approximately two billion dollars to dairy farmers in the United States alone (Smith and Hogan, 2001). Major costs related to mastitis were: 1) reduction of milk production (US$ 102/cow/year), 2) discard of milk (US$ 24/cow/year) and animal replacement (US$ 33/cow/year), and 3) bringing whole cost of mastitis to around US$ 172/cow/year (Smith and Hogan, 2001).

Mammary infections are characterized by multiple etiologies, most frequently caused by bacteria (Deb et al., 2013), that trigger an inflammatory response (Fuenzalida et al., 2015). Mastitis can be classified as clinical or subclinical. Clinical mastitis is branded by abnormal milk, and could or not present udder alteration or clinical systemic signs (Pinzón-Sánchez and Ruegg, 2011). Subclinical mastitis causes mobilization of inflammatory cells to the udder without changes in milk or udder characteristics, resulting in increased somatic cells count (**SCC**; Guimarães et al., 2013).

Occurrence of clinical or subclinical mastitis can negatively affect reproduction (Lavon et al., 2011; Hudson et al., 2012). Cows with clinical mastitis require more artificial insemination (AI) events as they have a decreased conception rate (Barker et al., 1998; Santos et al., 2004). Furthermore, cows diagnosed with clinical mastitis showed longer intervals between calving and first AI, greater pregnancy losses, extended days open, decreased milk production, and a decreased percentage of milk fat when compared with healthy cows (Chebel et al., 2004; Boujenane et al., 2014; Kumar et al., 2017). Similarly, subclinical mastitis after AI also has been associated with a decreased conception rate (Hudson et al., 2012). Cows with SCC greater than 200 $x10^3$ cells/mL had a 10% point decrease in pregnancy per AI compared with non-infected cows (Bijker et al., 2015). The aim of the study was to investigate the effect of clinical mastitis, in dairy cows, caused by different pathogen in the reproductive variables.

MATERIAL AND METHODS

The study was approved by the Ethics Committee on Animal Use (CEUA) guidelines of the School of Veterinary Medicine and Animal Science (FMVZ) - São Paulo State University (UNESP), Brazil (Protocol number: 196/2016 - CEUA).

Animals and trial period

This study was conducted in five commercial dairy herds with average milk production of 30 Kg/day/cow, SCC lower than $400x10^3$ sc/mL, program of mastitis control (with herd management software) and at least 200 cows in milk. Eight hundred and thirty-three lactating Holstein cows were enrolled in this trial. Herd of one farm were housed cows in cross-ventilation free-stall barns using a carousel type milking parlor; animals were milked three times a day. Other herds were milked in Herringbone parlor, cows were kept in free-stall

barns equipped with sprinklers and fans for cooling; cows were milked twice a day and fed a TMR with water access *ad libitum*. Farms were monitored for a period of 24 months, and samples were collected by trained staff at the moment of clinical mastitis diagnosis during milking using tamis or strip cup test (Constable et al., 2016). All other animal related data were recorded and stored on monthly basis by the authors. Animals were classified in accordance with occurrence or not of clinical mastitis. Cows diagnosed with mastitis were classified by pathogenicity of the agent isolated as suggested by Schepers et al.,(1997) and were: **Major pathogens** (*Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella pneumoniae*, *Mycoplasma* spp., *Streptococcus uberis, and Streptococcus dysgalactiae*), and **Minor pathogens** (Coagulase-negative *Staphylococcus* and *Corynebacterium* spp.). Cows were also divided according to the Gram-stain classification: **gram-positive** (*Staphylococcus aureus,* Coagulase-negative *Staphylococcus, Streptococcus agalactiae, Streptococcus uberis,* and *Streptococcus dysgalactiae*), and **gram-negative** (*Escherichia coli* and *Klebsiella pneumoniae*). Cows classified as control without mastitis diagnosed between calving and pregnancy diagnosis. Cow without mastitis had data and milk samples collected after 60 DIM until confirmation of pregnancy at around 60 days of gestation.

Microbiologic culture

Milk samples from cows presenting clinical mastitis were collected for microbial isolation using the tamis or strip cup test (i.e. presence of blood, color change or lumps). Milk samples from each mammary gland quarter diagnosed with mastitis were collected aseptically, and transported back to the laboratory under refrigeration (4-8 ºC). Milk samples (100 µL) were plated on bovine blood agar at 5% and MacConkey agar, kept at 37ºC in aerobic conditions for 72 h with observation of microbial growth every 24 h. Isolates of microorganisms were identified according to culture, morphology (Gram-stain) and biochemistry characteristics

(Quinn et al., 2011). Samples that had at least three colonies after 72 h of incubation were considered positive. Samples with more than three different colonies were considered contaminated, following NMC recommendations (NMC, 2004).

The isolation of *Mycoplasma* spp. was performed by culture of milk in each Petri plate filled with Hayflick medium supplement, in accordance with Whitford et al. (1994). Incubation was conducted in the microaerophylic atmosphere of a CO_2 heating chamber, with subsequent observation every two days until the 15th day. Isolation of *Mycoplasma* spp. was carried out by visualization in stereomicroscope of culture plates; the culture was positive when observed colonies appeared with aspect of "fried eggs" and formation of films and smear like described by Pretto et al. (2001).

Reproductive indices

Farms involved in the study used herd management software (DairyPlan C21, GEA, Germany) to store data, such as calving, health disorders, vaccinations, and time of AI. Reproduction data was collected by farm personnel and the veterinarian responsible for reproductive exams, and recorded on a daily/weekly basis depending on standard operation procedures of each farm. Reproductive indices of each animal enrolled in the study was comprised of pregnancy per AI at first service; pregnancy loss (from 30 to 60 days of gestation) and days open.

Reproductive management used in the study was as follow: at 60 DIM animals were monitored using an automated activity monitor (Boumatic Heat-seeker-TX, Boumatic Dairy Equipment, Madison, WI), for evaluation of cow's movement and detection of estrus. Cows that had a relative increase in physical activity that surpassed a set threshold determined by the system were examined to confirm estrus and later artificially inseminated. Pregnancy diagnosis was performed by *per rectum* ultrasonography (Mindray - 2200VET DP; Shenzhen

Mindray Bio-Medical Electronics CO., Shenzhen, China) at 30 days after AI, then again at 60 days after AI for confirmation. Cows considered pregnant at 30 days post AI, then non-pregnant at 60 of gestation were classified as having a pregnancy loss. Metritis, retained placenta and lameness were diagnosed by the farm veterinarian using the clinical criteria described by LeBlanc (2008) and Shearer et al. (2012). Milk production data was recorded during the 30 days prior to the diagnosis of mastitis, whereas in the control group the milk production record was done during the month of AI.

Statistical analysis

Sample size was calculated using a single proportion (pregnancy per AI at 30 days of gestation) power analysis (alpha = 0.05/beta = 0.20) assuming a null hypothesis proportion of 35% (control) and an expected difference of 10% units (25%; Major). The variables pregnancy at first AI and pregnancy loss were analyzed by logistic regression using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC). The model included the effects of the initial group of bacteria (Major vs Minor), Gram-stain (positive or negative), farm, parity, metritis, lameness, retained placenta, milk production, and interactions. Effects of group of pathogens (major or minor and Gram positive or negative) on hazard risk of pregnancy by 30 days after AI were analyzed with PHREG procedure of SAS. The Cox-proportional hazard regression models included days open as the outcome variable and group, Gram-stain, farm, parity, metritis, lameness, retained placenta, and milk production as explanatory variables. Observations were right-censored at culling or at 300 DIM if pregnancy had not been previously confirmed. Results are presented by means ± SEM and considered significantly different if $P < 0.05$ and a tendency if $0.05 < P < 0.10$. Significant effects were reported by least squares means. The non-significant variables were excluded from the model according to the Wald's criterion ($P > 0.20$).

RESULTS

The study analyzed 2,519 milk samples from participating herds. No growth culture, contaminated culture, or pathogens not part of this investigation were 1,958 samples. The final analysis comprised of 273 animals in control group, 191 in the minor pathogens group, 369 in the major pathogens group, 123 in the gram-negative group, and 395 in the gram-positive group. The minor pathogens group was composed of 84.3% (161/191) coagulase-negative *Staphylococcus* and 15.7% (30/191) *Corynebacterium* spp. Major pathogens group was composed of 55.2% (204/369) environmental *Streptococcus*, 25.5% (94/369) *Escherichia coli*, 7.9% (29/369) *Klebsiella pneumoniae*, 4.1% (15/369) *Streptococcus agalactiae*, 4% (15/369) *Staphylococcus aureus,* and 3.3% (12/369) *Mycoplasma* spp. Gram-negative group was composed of 76.4% (94/123) *E. coli* and 23.6% (29/123) *Klebsiella pneumoniae*. Gram-positive group was composed of 55.4% (219/395) environmental *Streptococcus*, 40.7% (161/395) coagulase-negative *Staphylococcus*, and 3.9% (15/395) *Staphylococcus aureus*.

For pregnancy to first AI a difference ($P = 0.01$) between control and major group was found, but no differences were observed between minor and the two other groups. A difference was observed between control and gram-negative group ($P = 0.004$), but no difference was observed between gram-positive group and control or gram-negative groups (Table 1).

Only the control and the major pathogens groups were considered different ($P = 0.01$) for pregnancy loss percentage. No differences were observed between minor and the two other groups. The pregnancy loss was found to be greatest ($P = 0.003$) in the gram-negative group compared with the gram-positive and control groups (Table 1).

A difference ($P < 0.001$) was found between control and infected cows (major and minor), as well as between the minor and major groups ($P = 0.02$) (Table 1 and Figure 1) for days

open. The hazard ratio risk (HR) of pregnancy were HR = 1.92; $P < 0.001$, HR = 1.22; $P = 0.02$, and HR = 0.81; $P = 0{,}02$ in the control, minor and major pathogens groups, respectively. On the other hand, gram-positive affected cows had more days open, compared to gram-negative affected cows (Table 1 and Figure 2). Pregnancy HR for cows infected by gram-positive pathogens was 1.36; $P = 0.004$, while for gram-negative-infected cows was 0.73; $P = 0.004$. Similarly, to the pathogens' analyses, days open in the control group was significantly lower than infected cows, particularly gram-negative cows, which were more negatively affected than cows in the gram-positive group ($P < 0.001$).

DISCUSSION

This study aimed to analyze the effect of clinical mastitis etiology on dairy cow's reproduction performance. It was observed that cows affected by mastitis caused by more pathogenic bacteria (major pathogens group) had worse reproductive results compared with those in the control and less pathogenic bacteria (minor pathogens group) groups. Similarly, cows affected by mastitis caused by gram-negative pathogens had worse reproductive results compared to the control and gram-positive groups. Another interesting result was that the minor pathogens group and gram-positive group had similar results, suggesting that pathogens causing mild clinical mastitis were able to disturb reproductive performance, but not as markedly as pathogens in the major pathogens and gram-negative groups.

Cows diagnosed with mastitis had a lower percentage of pregnancy at first AI (Barker et al., 1998; Chebel et al., 2004), which was also observed in this study. The control group had a higher percentage of pregnancy at first AI compared to infected cows (major and minor groups). No changes were detected between major and minor groups, and also between minor and control group. These results support the hypothesis that minor pathogens could cause impairments in the reproductive performance, but it was not as significant as for the major

group. Gram-negative bacteria had a stronger influence in reproductive results compared to gram-positive bacteria. Other authors (Soto et al., 2003; Hansen et al., 2004) reported that cows affected by mastitis caused by gram-negative bacteria also had a more negative effect on reproductive performance than cows affected by gram-positive bacteria. In a recent study, the pregnancy rates after embryo transfer to cows diagnosed with mastitis were also worst in infected cows (by any categorization) (Barbosa et al., 2018). The authors also found major pathogens and gram-negative groups to cause the greatest reduction in reproduction performance. The current study (using AI) found more pronounced differences between minor pathogens and gram-positive bacteria when compared with the non-infected control group. Generally, the study using embryo transfer had the minor pathogens group and the gram-positive (particularly the contagious sub-division) to be similar to the control group (Barbosa et al., 2018). Although both studies can only speculate on the underlying causes for the differences, it is possible that cows diagnosed with mastitis from minor pathogens and gram-positive bacteria may benefit from embryo transfer technologies. Perhaps, by skipping key steps during follicle development, oocyte quality, fertilization, and early embryo development up to the blastocyst stage. There were no changes between control and minor group which was formed by CNS and *Corynebacterium* spp., supporting the findings of the previous authors.

Cows from the major pathogens group had a greater percentage of pregnancy loss compared with the control group. No significant difference was observed between minor pathogens and the other two groups. A difference between control and major pathogens was expected; as the udder infection has been shown to influence conception and pregnancy maintenance (Hansen et al., 2004; Hockett et al., 2005). In contrast, pathogens that caused less severe mastitis seem to have no influence on pregnancy loss in cattle. The udder inflammatory reaction releases substances, such as interleukins, TNF-α, LPS, nitric oxide, and

prostaglandins, into the bloodstream that could lead to luteolysis or embryo death (Hansen et al., 2004). The gram-negative group had a relevant increase in pregnancy loss when compared with cows from the control and gram-positive groups. Apparently, the LPS in the gram-negative bacteria causes a significant disturbance in embryo development leading to an increase in pregnancy loss, which has also been observed by elsewhere (Bromfield et al., 2015; Ibrahim et al., 2015; Magata and Shimizu, 2017; Campos et al., 2018). No difference in pregnancy loss was observed for cows with subclinical mastitis after embryo transfer (Barbosa et al., 2018). This could be explained by the fact that the authors used seven-day-old embryos, which could possibly have avoided the effect of the inflammatory reaction on the oocytes and on the ovulation and fertilization event. Another important point is the fact that the authors worked with subclinical mastitis, which may cause a milder effect on the reproductive tract.

In this study the days open period were longer in the groups of cows with clinical mastitis compared with the control group. These findings are in accordance with literature that reported that infected cows had more days between calving and conception (Kumar et al., 2017). A significant difference was observed between major and minor pathogens groups in pregnancy at first AI, indicating that the severity of mastitis could influence the number of days open. Supporting these findings, a few reports confirmed that mastitis causes reproductive impairments because of disturbances in oocyte and embryo development, decreased production of reproductive hormones, follicular growth disruption, deficiency of ovulation and lower estrous manifestation (Hockett et al., 2000, 2005; Soto et al., 2003; Herath et al., 2007; Williams et al., 2008). When different Gram class groups were evaluated, gram-negative bacteria caused an increase of days open compared to gram-positive bacteria. It was observed that gram-positive bacteria raised the number of days open compared to the control group. These findings support the theory that the LPS present in the gram-negative

membrane bacteria causes relevant disruption to reproductive tissues leading to fertility problems (Hansen et al., 2004) and consequently more days open.

It is well known that the LPS from gram-negative bacteria infecting the mammary gland causes deleterious consequences for bovine fertility (Hansen et al., 2004). However, the manner in which LPS impacts reproduction is unclear. Some authors have been studying this mechanism, trying to understand the underlying pathway: LPS could delay oocyte development in cows (Soto et al., 2003), decrease the release of estradiol from granulosa cells (Williams et al., 2008), inhibit ovulation (Williams et al., 2008), lead to early apoptosis of oocytes (Zhao et al., 2019), affect oviducts promoting early embryo death (Ibrahim et al., 2015), delay cytoplasm maturation in oocytes causing embryonic developmental changes (Magata and Shimizu, 2017), cause problems in pregnancy maintenance (Bromfield et al., 2015; Campos et al., 2018), and reduce the primordial ovarian follicle pool, to a long term effect on fertility (Bromfield and Sheldon, 2013). These findings support the results discussed in this investigation. The effects mentioned above could be responsible for causing failure in the pregnancy at first AI, as well as pregnancy loss and long-term effects on fertility leading to more days open. As gram-negative-mastitic cows had more markedly decrease in reproductive parameters, efforts would be done to control these pathogens. Some control actions as pre dipping, quality of bed materials, cleaning of beds and water quality, could help to decrease mastitis caused by gram-negative (Hogan and Smith, 2012).

CONCLUSION

In conclusion, cows that were not diagnosed with clinical mastitis had higher pregnancy at first AI and reduced pregnancy loss and days open compared to cows with mastitis. These results provide circumstantial evidence that pathogens causing clinical

mastitis could lead to deleterious effects in reproductive performance of dairy cows, reinforcing the relevant of mastitis control programs in dairy herds. There are differences between pathogens causing clinical mastitis and the effects on reproduction, which further supports the concept that the bacteria had distinct ways to affect reproductive variables. Cows diagnosed with mastitis caused by major pathogens and gram-negative bacteria lead to more substantial losses in reproductive performance.

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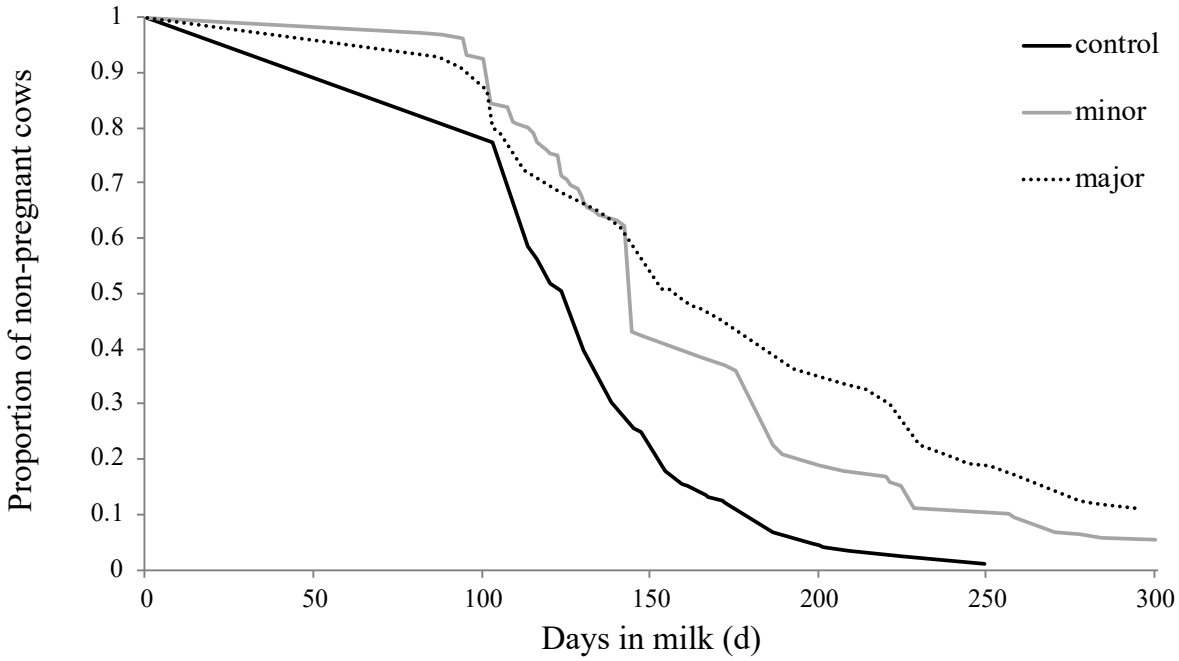

Figure 1. Survival curve of DIM to conception until 300 DIM for each group of cows (*P* =0.02). Control (n=273) – non-infected cows among calving and new pregnancy. Minor (n=191) – cows with mastitis caused by coagulase-negative *Staphylococcus* or *Corynebacterium* spp. Major (n=369) – cows with mastitis caused by *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella pneumoniae*, *Mycoplasma* spp., *Streptococcus uberis,* and *Streptococcus dysgalactiae*.

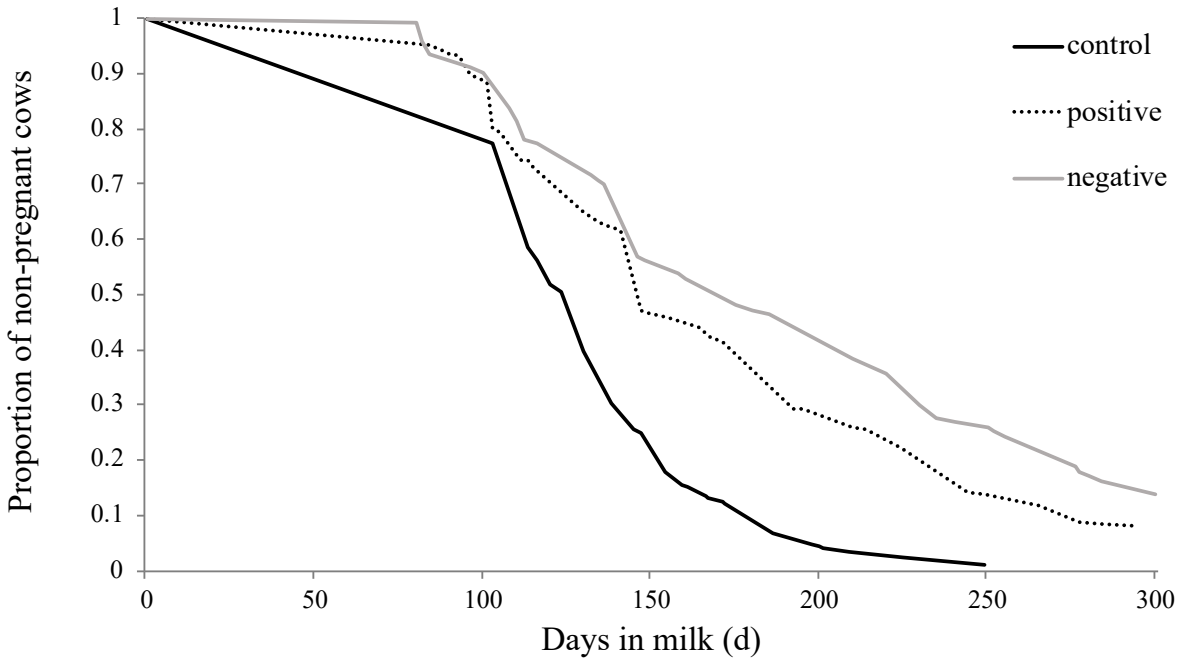

Figure 2. Survival curve of DIM to conception until 300 DIM for gram bacteria class ($P < 0.001$). Control (n=273) – non-infected cows among calving and new pregnancy. Positive (n=395) – cows with mastitis caused by *Staphylococcus aureus,* Coagulase-negative *Staphylococcus, Streptococcus agalactiae, Streptococcus uberis,* and *Streptococcus dysgalactiae*. Negative (n=123) – cows with mastitis caused by *Escherichia coli* and *Klebsiella pneumoniae*.

Table1. Effect of mastitis caused by different groups of pathogens (non-infected - control, minor or major) or by Gram class (positive or negative) on pregnancy at first AI (% ± SEM), pregnancy loss (% ± SEM), and days open (days ± SEM) in dairy cows.

	Pregnancy/first AI	Pregnancy loss	Days open
Group			
Control	32.6±0.02^{aA}	12.8±0.02^{aA}	129.5±1.9^{aA}
Minor	26.2±0.03^{ab}	16.5±0.02^{ab}	162.0±4.1^{b}
Major	20.1±0.02^{b}	22.2±0.02^{b}	175.1±3.7^{c}
Gram-positive	23.8±0.02^{AB}	17.2±0.02^{A}	172.7±4.1^{B}
Gram-negative	15.4±0.03^{B}	30.1±0.03^{B}	191.1±7.4^{C}

Pregnancy/first AI – cows diagnosed pregnant after first AI, after calving. Pregnancy loss – cows diagnoses pregnant 30 days after AI, and diagnosed non-pregnant at 60 days, after AI, check. Days open – number of days between calving and new conception. Control – non-infected cows among calving and new pregnancy. Minor cows with mastitis caused by coagulase-negative *Staphylococcus* or *Corynebacterium* spp. Major – cows with mastitis caused by *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella pneumoniae*, *Mycoplasma* spp., *Streptococcus uberis,* and *Streptococcus dysgalactiae*. Gram-positive – cows with mastitis caused by *Staphylococcus aureus,* Coagulase-negative *Staphylococcus, Streptococcus agalactiae, Streptococcus uberis,* and *Streptococcus dysgalactiae*. Gram-negative – cows with mastitis caused by *Escherichia coli* and *Klebsiella*

pneumoniae. [a, b, c] Numbers in the column followed by distinct letters are different ($P < 0.05$).

[A, B, C] Numbers in the same column followed by distinct letters are different ($P < 0.05$).

CAPÍTULO 3

ARTIGO 2 – CONCENTRATION OF ACUTE PHASE PROTEINS IN MILK FROM COWS WITH CLINICAL MASTITIS CAUSED BY DIFFERENT PATHOGENS.

Artigo 2 – Concentration of acute phase proteins in milk from cows with clinical mastitis caused by different pathogens.

Artigo a ser enviado a revista Journal of Proteomics seguindo as normas estabelecidas: https://www.elsevier.com/journals/journal-of-proteomics/1874-3919/guide-for-authors

INTERPRETIVE SUMMARY

Concentrations of acute phase proteins in milk from cows with clinical mastitis caused by different pathogens. *By Dalanezi et al*. Mastitis is an important disease in dairy cattle. To control the infection quickly and accurately diagnosis is vital. This study aimed at evaluating the effect of clinical mastitis, caused by different pathogens, in concentration of acute phase proteins in milk. We observed different concentrations of APPs between the different pathogens, with Hp, CRP, and AGP found in similar concentrations, across of pathogens. Serum Amyloid A had a different pattern than expected for this protein. As significantly correlations were observed between Hp, CRP, and AGP concentrations, CRP and AGP could be as reliable as Hp in diagnosing clinical mastitis caused by different pathogens. This study demonstrated that SPARCL could be used as a replacement for enzyme-linked immunosorbent assay (ELISA) to determine the concentration of APPs in milk. Haptoglobin, CRP, and AGP can be used as biomarkers in diagnosis of mastitis caused by different pathogens.

Running head: CLINICAL MASTITIS, PATHOGENS AND BIOMARKERS

Concentrations of acute phase proteins in milk from cows with clinical mastitis caused by different pathogens.

F.M. Dalanezi*, S.F. Joaquim*, F.F. Guimarães*, S.T. Guerra*,

B.C. Lopes*, Chris Chadwick[§], E.M.S. Schmidt[†], R.L.A. Cerri[‡], H. Langoni*[1]

*Department of Veterinary Hygiene and Public Health, São Paulo State University (UNESP), campus Botucatu, Faculty of Medicine Veterinary and Animal Science, Brazil 18168-681

§Life Diagnosis, Inc. West Chester, Pennsylvania, United States of America 19380

† Department of Veterinary Clinical Science, São Paulo State University (UNESP), campus Botucatu, Faculty of Medicine Veterinary and Animal Science, Brazil 18168-681

‡ Applied Animal Biology, Faculty of Land and Food Systems, University of British Columbia, Vancouver, Canada V6T 1Z4

[1]Corresponding author: Helio Langoni, St. Prof. Dr. Walter Mauricio Correa, s/n – UNESP Botucatu, SP, Brazil, 18618-681; Tel: +55 14 3880 2094; email: helio.langoni@unesp.br

ABSTRACT

New diagnostic methods for mastitis detection are being developed, among which the acute phase proteins (APPs) are receiving special attention. These proteins can be produced by mammary gland cells, leading to localized increases in their concentration in milk. Some APPs, including haptoglobin (Hp), serum amyloid A (SAA), and C-reactive protein (CRP), have been described as biomarkers for mastitis diagnosis. The objective of this study was to detect APPs from milk samples from cows with clinical mastitis caused by different pathogens. Here, in 133 milk samples from cows with clinical mastitis caused by *Escherichia coli* (n=24), coagulase-negative *Staphylococcus* (CNS) (n=24), *Mycoplasma* spp. (n=18), *Enterococcus* spp. (n=18), *Klebsiella pneumoniae.* (N=18), environmental *Streptococcus* (n=16), and, *Staphylococcus aureus* (n=15) were determined the concentrations of Hp, SAA, α1-acid glycoprotein (AGP), and CRP; concentrations were determined using a new method knows as Spatial Proximity Analyte Reagent

Capture Luminescence (SPARCL). We observed different concentrations of APPs between the different pathogens, with Hp, CRP, and AGP found in similar concentrations, across of pathogens – *E. coli* (Hp: 164.1 µg/mL, AGP: 47.3 µg/mL and, CRP: 2.0 µg/mL); *K. pneumoniae* (Hp: 206.1 µg/mL, AGP: 34.9 µg/mL and, CRP: 1.6 µg/mL); *S. aureus* (Hp: 158.7 µg/mL, AGP: 51.3 µg/mL and, CRP: 0.8 µg/mL); environmental *Streptococcus* (Hp: 179.0 µg/mL, AGP: 29.7 µg/mL and, CRP: 0.6 µg/mL); *Mycoplasma* spp. (Hp:102.0 µg/mL, AGP: 24.7 µg/mL and, CRP: 0.6 µg/mL); *Enterococcus* spp. (Hp: 43.0 µg/mL, AGP: 17.9 µg/mL and CRP: 0.2 µg/mL) and, CNS (Hp: 0.0 µg/mL, AGP: 9.7 µg/mL and, CRP: 0.1 µg/mL). Serum Amyloid A had a different pattern than expected for this protein (*E. coli* – 20.5 µg/mL; *K. pneumoniae* – 52.4 µg/mL; *S. aureus* – 38.2 µg/mL; Environmental *Streptococcus* – 63.8 µg/mL; *Mycoplasma* spp. – 35.2 µg/mL; *Enterococcus* spp. 55.4 µg/mL and, CNS – 14.9 µg/mL). As significantly correlations were observed between Hp, CRP, and AGP concentrations, CRP and AGP could be as reliable as Hp in diagnosing clinical mastitis caused by different pathogens. A significant, but moderate, correlation was observed between Hp and SAA. This the first study to demonstrated that SPARCL could be used as a replacement for enzyme-linked immunosorbent assay (ELISA) to determine the concentration of APPs in milk. This is also the first study to determine concentrations of APP in milk from mastitis caused by *Mycoplasma* spp., *K. pneumoniae* and *Mycoplasma* spp. Haptoglobin, CRP, and AGP can be used to diagnose mastitis caused by different pathogens.

Keywords: Bacteria, bovine, mammary gland, SPARCL

INTRODUCTION

Mastitis is characterized by infection of the mammary gland. It has multiple etiologies, and the most frequent etiological agents are bacteria [1], which lead to an inflammatory reaction [2]. Cases are classified as clinical and subclinical. The clinical form is characterized by abnormal milk production, possible mammary gland tissue alteration or systemic symptoms [3,4], while the subclinical form involves the mobilization of inflammatory cells to the udder, increasing the somatic cell count (SCC) [5]. Mastitis control is dependent on diagnosis, with tests such as the California Mastitis Test (CMT); which exploits the reaction between the DNA from inflammatory cells in the milk and specific reagents, leading to the formation of a gel, the consistency of the gel allows subjective analysis of the quantity of cells in the milk sample [6,7]. Alternatively, the quantitative analysis of the number of cells in milk could be done using the SCC test. Some factors, such as the level of infection, lactation state, age, breed, and milk transport, could change the SCC [1]. Another important procedure to diagnosis is the microbiological culture used to isolate the pathogen causing the infection; a milk sample should be incubed in culture medium and checked for colony growth [1].

Recent studies have demonstrated that acute phase proteins (APPs) produced by cows with mastitis could be used as diagnostic biomarkers [8–10]. In bovine, the most relevant APPs are haptoglobin (Hp), serum amyloid A (SAA), lipopolysaccharide binding protein (LBP), and alpha-1-acid glycoprotein (AGP) [11]. Haptoglobin, SAA, and CRP have been identified in milk and serum, indicating their potential as biomarkers of mastitis [12,13]. Recently, AGP has been identified as a potential biomarker of mammary inflammation, however, research is currently limited [11].

Increases in Hp, SAA, CRP, and AGP production in mammary gland cells have been observed in cases of mastitis, indicating the local production of these APPs in response to bacterial infection [9,14]. In the near future, these biomarkers could replace the SCC test as a standard diagnostic method. In addition, this approach is considered better, as APPs have less interfering factors than the SCC test [15].

The first objective of this study was to detect APPs from milk samples from cows with clinical mastitis caused by different pathogens (*Staphylococcus aureus*, *Escherichia coli*, environmental *Streptococcus*, coagulase-negative *Staphylococcus* (CNS), *Mycoplasma* spp., *Enterococcus* spp., and *Klebsiella pneumoniae*). The second objective was to test a new method of detection of APPs in milk using the Spatial Proximity Analyte Reagent Capture Luminescence (SPARCL) technique, aiming to have a faster and easier option to replace enzyme-linked immunosorbent assay (ELISA) for APPs detection. We determined correlations between the concentrations of APPs, to understand the role of these proteins in diagnosing clinical mastitis caused by different pathogens.

MATERIAL AND METHODS

The study was approved by the Ethics Committee on Animal Use (CEUA) guidelines of the School of Veterinary Medicine and Animal Science (FMVZ) - São Paulo State University (UNESP), Brazil (Protocol number: 196/2016 - CEUA).

Microbiological culture

This study was conducted in five commercial dairy herds with average milk production of 30 Kg/day/cow, SCC lower than $400x10^3$ sc/mL, program of mastitis control (with herd management software) and at least 200 cows in milk. Herd of one farm were housed cows in cross-ventilation free-stall barns using a carousel

type milking parlor; animals were milked three times a day. Other herds were milked in Herringbone parlor, cows were kept in free-stall barns equipped with sprinklers and fans for cooling; cows were milked twice a day and fed a TMR with water access *ad libitum*. Milk samples from cows with natural occurrence of acute clinical mastitis were used for microbiological isolation. Samples were collected, by convenience, by trained staff at the moment of clinical mastitis diagnosis using tamis or strip cup test [16]. Individual samples were collected aseptically, following the antisepsis of the teats with a 1% iodine-alcohol solution, and transported under refrigeration (4-8ºC) to laboratory. Samples were cultured in bovine blood agar (5%), and MacConkey agar, incubated under aerobic conditions at 37ºC for 72 hours, and examined for microbial growth every 24 hours. Strains of microorganisms were identified according to culture, morphologic, and biochemical characteristics [17]. After pathogens were identified, milk samples were stored at -20ºC until APP determination.

Detection of *Mycoplasma* spp. was previously carried out as part of the standardized culture protocol followed for bovine milk samples, with cell numbers established as: 0.05 mL of each sample was cultured in a Petri dish filled with solid Hayflick medium, as described by Whitford et al. (1994)[18]. Incubation was carried out in a CO_2 incubator under a microaerobic atmosphere, with observations of microbial growth carried out for at least 15 days after incubation. Evaluation of the isolated *Mycoplasma* spp. was carried out on a stereo microscope, through visualization of colonies displaying characteristic “fried-egg” shapes and the formation of smears, as described by PRETTO et al. (2001)[19].

Pathogen identification

Microorganisms of the *Streptococcus* genus isolated from milk samples were differentiated by three tests: 1) The CAMP test: CAMP factor produced by the organism acts synergistically with beta-hemolysin produced by *S. aureus* in the blood agar; 2) Esculin hydrolysis: The main purpose is to verify if bacteria have the capacity to hydrolyze esculin as a source of carbon; 3) Bile-esculin hydrolysis: This test is based on the ability of *Streptococcus* to hydrolyze esculin in the presence of bile (4%) [17].

Samples containing *Staphylococcus* were categorized by the coagulase test [20]. Coagulase-positive strains were subjected to various biochemical tests to identify *S. aureus*, such as the fermentation of sugars (trehalose, maltose and mannitol), resistance to polymyxin B (300 UI), and sensitivity to novobiocin (5 mg) [17].

Colonies grown in MacConkey media were identified through conventional biochemical tests [17].

In the case of growth of non-specified pathogens causing mastitis, characterization was carried out according to the genus found, as described by Quinn et al. (2011)[17].

Determination of the concentrations of acute phase proteins

To determine the presence of APPs in milk, 133 samples from cows diagnosed with clinical mastitis caused by different pathogens were used. The pathogens included *Escherichia coli* (n=24), coagulase-negative *Staphylococcus* (CNS) (n=24), *Mycoplasma* spp. (n=18), *Enterococcus* spp. (n=18), *Klebsiella pneumoniae* (N=18), environmental *Streptococcus* (n=16), and *Staphylococcus aureus* (n=15). The concentrations of APPs (Hp, SAA, AGP, and CRP) were determined by the SPARCL assay. SPARCL assays uses two particular antibodies: one conjugated to

a chemiluminescent substrate (acridan) and another one to horse-radish peroxidase (HRP). When HRP and acridan antibodies bind to their target, they come closer. So, the adding of hydrogen peroxide, HRP catalyzes the oxidation of acridan, initiating a flash of chemiluminescence related to the biomarker concentration. Acridan molecules that are not close to HRP are not oxidized and do not generate luminescence. A consistent immunoassay, fast and sensitive, could be elaborated from this principle. We used commercial kits to carry out the technique. For each APP assayed for, specific kits were used: for Hp we used the Cow Haptoglobin Microplate SPARCL Assay (HAPT-SP-11), for SAA we used the Cow SAA Microplate SPARCL Assay (SAA-SP-11), for AGP we used the Cow AGP Microplate SPARCL Assay (AGP-SP-11), and for CRP we used the Cow CRP Microplate SPARCL Assay (CRP-SP-11). All kits were provided by Life Diagnostics Inc., West Chester, USA. Procedures were carried out according to the specification of the manufacturers.

Statistical analysis

Data were first analyzed for normality of distribution using Kolmogorov-Smirnov and Shapiro-Wilk tests. As the data was not normally distributed, a non-parametric (Wilcoxon test) comparisons among different pathogens and APPs, and Dunn's post hoc analysis (using Bonferroni error correction to adjust for multiple comparisons) were performed. A non-parametric (Spearman's correlation) analysis was used to check the correlation between the four APPs concentration. *P*-values were considered significant at $P<0.05$. All tests were performed using the SAS University® software (SAS Institute Inc., Cary, NC).

RESULTS

Cows enrolled in the study were in average at fourth lactation, and the percentage of quarters infected was: front right - 24.8% (33/133), front left - 32.3% (43/133), rear right - 24.8% (33/133), and rear left - 18.1% (24/133). Control group (n=8), no-infected cows and with SCC lower than $200x10^3$, had a range of concentration (μg/mL) for Hp: 0.0; SAA: 0.0 – 8.7; AGP: 6.1 – 8.8; and CRP: 0.07 – 0.13.

Concentrations of milk Hp (Table 1) were the significantly higher ($P<0.05$) in samples from mastitis caused by *E. coli*, *Klebsiella pneumoniae, S. aureus*, and environmental *Streptococcus,* and significantly lower ($P<0.05$) in samples from mastitis caused by *Enterococcus* spp. and CNS. Samples from mastitis caused by *Mycoplasma* spp. had moderate concentrations of Hp when compared to all other pathogens.

For milk SAA concentrations (Table 1), samples from cows infected with environmental *Streptococcus* were significantly higher ($P<0.05$), followed by cows infected with *Klebsiella pneumoniae* and *Enterococcus* spp. Milk samples from animals infected with *S. aureus* and *Mycoplasma* spp. had concentrations similar to those with *Klebsiella pneumoniae* and *Enterococcus* spp. infections, but different from those for with environmental *Streptococcus*. The significantly lower ($P<0.05$) concentrations were observed in samples from animals with *E. coli* and CNS infections.

Concentrations of milk AGP (Table 1) were significantly higher ($p<0.05$) in samples from mastitis caused by *E. coli* and *S. aureus*, followed by *Klebsiella pneumoniae* Samples from mastitis caused by environmental *Streptococcus* and *Mycoplasma* spp. had concentrations similar to those with *Klebsiella pneumoniae*

infection, but different from those with *E. coli* and *S. aureus.* Milk samples from mastitis caused by *Enterococcus* spp. and CNS had significantly lower ($p<0.05$) concentrations when compared to the other pathogens.

Milk CRP (Table 1) concentrations had significantly higher ($p<0.05$) in samples from cows with *E. coli* and *Klebsiella pneumoniae* infections, moderate concentrations for mastitis caused by *S. aureus,* environmental *Streptococcus,* and *Mycoplasma* spp., and significantly lower ($p<0.05$) concentrations for mastitis caused by *Enterococcus* spp. and CNS.

Significant positive correlations (Table 2) were observed between Hp and SAA ($r_s=0.39$, $P<0.001$), Hp and AGP ($r_s=0.60$, $P<0.001$), Hp and CRP ($r_s=0.55$, $P<0.001$), and AGP and CRP ($r_s=0.72$, $P<0.001$). No significantly correlation was observed between SAA and AGP ($r_s=0.11$, $P=0.18$), and SAA and CRP ($r_s=0.05$, $P=0.55$).

Table 1: Concentration of APPs (µg/mL) in milk of cows with clinical mastitis caused by different pathogens.

	***Klebsiella pneumoniae* (N=18)**	***E. coli* (N=24)**	***S. aureus* (N=15)**	**Environmental *Streptococcus* (N=16)**	***Mycoplasma* spp. (N=18)**	***Enterococcus* spp. (N=18)**	**CNS (N=24)**
Haptoglobin	206.1^{A}	164.1^{A}	158.7^{AB}	179.0^{A}	102.0^{B}	43.0^{C}	0.0^{C}
	(0.0 – 1113.3)	(0.0 – 2009.4)	(0.0 – 596.1)	(0.0 - 812.2)	(0.0 - 582.9)	(0.0 – 213.0)	(0.0 – 319.1)
	[126.3 – 468.1]	[71.7 - 305.1]	[0.0 – 300.0]	[130.2 – 363.7]	[0.0 – 332.8]	[0.0 – 127.3]	[0.0 - 66.3]
Serum amiloid A	52.4^{AB}	20.5^{C}	38.2^{B}	63.8^{A}	35.2^{B}	55.4^{AB}	14.9^{C}
	(2.0 – 178.8)	(0.0 – 264.0)	(13.3 – 129.5)	(21.0 – 151.4)	(0.0 – 102.2)	(0.0 – 250.0)	(0.0 – 141.7)
	[31.9 – 97.1]	[8.6 – 47.2]	[21.8 – 94.9]	[48.4 – 70.6]	[17.4 – 51.5]	[26.8 – 86.1]	[8.7 – 37.7]
α1-glicoprotein acid	34.9^{AB}	47.3^{A}	51.3^{A}	29.7^{B}	24.7^{B}	17.9^{C}	9.7^{C}
	(14.4 – 305.0)	(6.8 – 892.9)	(11.9 – 289.9)	(11.3 – 100.7)	(10.6 – 427.5)	(10.1 – 297.5)	(5.9 – 42.6)
	[22.4 – 145.3]	[24.8 – 155.7]	[17.3 – 207.4]	[19.0 – 49.3]	[15.1 – 79.2]	[15.8 – 22.2]	[7.8 – 9.7]
C reactive protein	1.6^{A}	2.0^{A}	0.8^{B}	0.6^{B}	0.6^{B}	0.2^{C}	0.1^{C}
	(0.1 – 8.0)	(0.1 – 7.8)	(0.0 – 9.2)	(0.1 – 5.4)	(0.1 – 6.8)	(0.1 – 2.6)	(0.1 – 3.5)
	[0.5 – 4.4]	[0.8 – 4.8]	[0.1 – 2.0]	[0.3 – 1.4]	[0.3 – 2.1]	[0.1 – 0.7]	[0.1 – 0.4]

*Data with distinct letters (A,B,C) in the lines are significantly different ($P<0.05$). Hp – Haptoglobin; SAA – Serum amyloid A; AGP – Alpha-1-acid glycoprotein; CRP – C-reactive protein; CNS – coagulase-negative *Staphylococcus*. The values indicate the median, (minimum – maximum), and [Q1 – Q3].

Table 2: Correlations between different milk APPs of cows with clinical mastitis (r_s values).

APPs	Hp	SAA	AGP	CRP
Hp	1.00			
SAA	0.39*	1.00		
AGP	0.60*	0.11	1.00	
CRP	0.55*	0.05	0.72*	1.00

*significantly different (P<0.05). Hp – Haptoglobin; SAA – Serum amyloid A; AGP – Alpha-1-acid glycoprotein; CRP – C-reactive protein.

DISCUSSION

This is the first study reporting on the use of the SPARCL technique to detect APPs in cows with clinical mastitis. Additionally, this is the first time that AGP was detected in milk samples from cows with clinical mastitis caused by different pathogens. Results of the study showed differences between the concentrations of Hp, SAA, CRP, and AGP in milk derived from clinical infection by different pathogens. Correlations found between the APPs were in accordance with previous studies, and APPs could be used as marker proteins in the diagnoses of clinical mastitis in dairy cows.

The main functions of Hp in local of inflammation are: bind iron (prevent use of iron by bacteria), bind hemoglobin (avoid oxidative damage), anti-inflammatory and, bacteriostatic effect [11]. Cows infected with *E. coli*, *Klebsiella pneumoniae*, and environmental *Streptococcus* had higher concentrations of Hp compared to other pathogens. This result has previously been reported for *E. coli* infection, and reflects

the strong response of the cells to bacterial damage [9,21,22]. This is the first study reporting the detection of Hp in milk from mastitis caused by *Klebsiella pneumoniae*, but the high level of this APP was expected, because, similar to *E. coli*., these bacteria can cause acute clinical mastitis. The LPS present in membrane of *E. coli* and *Klebsiella pneumoniae* can modulate production of APPs by stimulating production of Tumor Necrosis Factor α (TNF-α). The TNF-α lead to an increase in production of APPs in liver [23]. Was demonstrated that *E. coli* and *Klebsiella pneumoniae* has the same pattern to cause clinical mastitis, because both had similar molecules of LPS in bacterial membrane. However, *Klebsiella pneumoniae* usually causes more severe mastitis them *E. coli*, it is due to a little difference in one of the constitutive molecules of LPS [24], as was observed in the present study by concentration of Hp in milk. Together with the increase in TNF-α is observed an increase in migration of polymorphonuclear (PMN) to mammary gland, leading to increase of SCC and consequently to number of phagocytic cells entails to increase in the killed bacteria which can release more LPS stimulating the inflammatory response and APPs production [25]. Increment in migration of PMN to the udder could be stimulated by Hp, since one role of this proteins is recruitment of neutrophils [26]. The high concentration of Hp in milk infected by environmental *Streptococcus* was also expected, as previous articles have demonstrated a high level of this APP in mastitis caused by these pathogens [9,22].

Gram-positive bacteria is well known by causing more chronical mastitis them acute mastitis [27] because of the lack of LPS [28]. Although, was demonstrated that *Streptococcus uberis* (one of environmental *Streptococcus*), can lead to a striking

inflammatory response in mammary gland infections that could result in a sharp increase in APPs as Hp [29]. Gram-positive pathogens causes more slow elevation of APPs and cytokines, but for longer time when compared to gram-negative bacteria [27,29–32]. Thus, the lack of time between onset of infection and collection of milk sample can explain why the concentration of Hp in *Streptococcus*-infected cows was similar to coliform-infected cows. *E. coli*, *Klebsiella pneumoniae* and environmental *Streptococcus* can cause a large tissue damage releasing reactive oxygen species (ROS) [33], fought ROS is one of the functions of Hp [11], so the rise of ROS can cause a trigger to Hp production.

The presence of *Mycoplasma* spp. and *S. aureus* in the udder led to a moderate increase in Hp in the milk. The concentration of this APP was similar among *S. aureus*, *E. coli*, *Klebsiella pneumoniae* and, environmental *Streptococcus*, but was similar to *Mycoplasma* spp. also. While a low level of Hp in milk samples from cows with *S. aureus* infection has been observed in other studies [9], here, the moderate concentration of Hp for *S. aureus* infection was similar to that reported by Kalmus et al. (2013). This is the first study to report on the concentration of Hp in mastitic milk from *Mycoplasma* spp. infection. This pathogen has an analogous behavior to *S. aureus*-infection, resulting in a chronic and persistent disease [34], so was expected that they have a correlated production of Hp. Besides, Mycoplasma sp. has similar pattern of immune system stimulation as *S. aureus*, was demonstrated that this bacteria can promote the production of cytokines as coliform pathogens [35], indicating an inflammatory response between these two pathogens. *Mycoplasma bovis* can inhibits the innate immune response of the host by a series of mechanisms [36], it can explain

why *Mycoplasma* causes production of some inflammatory molecules, as TNF-α that is not produced in *S. aureus* infection [35], and had related pattern with this bacteria. *Mycoplasma* spp. and *S. aureus* had a characteristic of low bacterial grow [35], since Hp has bacteriostatic function [11]. The low speed of pathogens grow could lead to a slow increase in Hp concentration as observed in this study. Anti-inflammatory role was attributed to Hp [11], as these bacteria has a not so pronounced inflammatory response it is expected a moderate stimuli to Hp production.

Mastitis caused by both *Enterococcus* spp. and CNS resulted in low Hp concentrations, indicating that these bacteria lead to a mild inflammatory response in the udder cells. The Hp concentration found in mastitic milk from CNS infection was consistent with the literature, when compared to other pathogens [21,22]. Coagulase-negative *Staphylococcus* usually cause mild clinical or subclinical mastitis being considered a minor pathogen causing the disease [37]. Even elevating SCC this pathogen can survive for a long time in the udder [37]. However a low concentration of cytokines was detected in milk [38], this low level can indicate a mild stimulation of innate response by the udder cells leading to a low level of Hp. This is the first time that Hp was detected in milk from mastitis caused by *Enterococcus* spp., and this study demonstrated that these bacteria elicit a similar response to CNS. *Enterococcus* has been recently studied by its characteristic of develop multidrug resistance [39–41]. Studies showed that the production of biofilm could be related to pathogenicity of *Enterococcus* species, and this fact help the bacteria to invade host cells and avoid immune response, decreasing the local inflammation resulting in a less pronounced production of APPs like Hp [40,42].

Bind cholesterol (preventing its accumulation after cells die), modulation of innate immune reaction (mediate the attraction, migration and adherence of monocytes and neutrophils), opsonization of Gram-positive and negative pathogens [11] are functions of SAA. Concentrations of SAA were the highest in samples from mastitis caused by environmental *Streptococcus,* and lowest in *E. coli* and CNS samples. Chemoattractant of defense cells, is one of the recognized functions of SAA, so bacteria that cause markedly increase in SCC as *Streptococcus*, *Enterococcus* and, *Klebsiella pneumoniae* it is assumed that show an increase in SAA concentration in milk. Although there was no difference between environmental *Streptococcus*, *Klebsiella pneumoniae*, and *Enterococcus* spp., the latter two pathogens showed no difference when compared to *Mycoplasma* spp. and *S. aureus*, demonstrating a moderate concentration of SAA in samples of milk infected by these bacteria compared to those infected by environmental *Streptococcus*. *Mycoplasma* and *S.* aureus cause chronical mastitis in cows, subsequently it do not cause quickly and pronounce response by immune system [34], as SAA is the APP that had the fastest response after infection [43], a moderate concentration of this APP is probable. Both SAA and Hp is elevate more in acute cases than chronic cases [44], that help to understand the results found in the study. In *S. aureus*-infected samples SAA concentration was similar to those reported in previous studies [21,22], indicating that this detection is plausible and could help explain the concentrations found for other bacteria.

The low concentration of SAA in samples from CNS infection was expected, as these bacteria do not cause accentuated inflammation in the udder cells, so not stimulating the functions of SAA. *E. coli*-infected milk samples was previously demonstrated high

concentration of SAA [9,21,22], so the findings in our study is not in accordance with similar studies elsewhere. These data could be explained by the fact that a new technique was used to detect the APPs, and this technique might be better suited to detect one particular isoform of SAA that is present in a minor concentration in infections caused by *E. coli*. Was expected an increase in SAA concentration in milk by *E. coli*-infected animals, as was observed to *Klebsiella pneumoniae*, but these two pathogens has a little difference in one of the LPS factor [24], that could stimulate immune response in a different way producing an isoform of SAA not fully detected by the assay. Samples from CNS-infected cows showed low concentration of SAA that are in accordance with other studies [21,22,38], like cited before CNS usually cause mild clinical or subclinical mastitis leading to minor stimulation o immune system, production of cytokines and APPs.

All APPs are produced in the liver, and AGP is not different. But was demonstrated that AGP can be produced mainly by alveoli cells, but the major part of AGP found in milk is released by white blood cells [45]. The same authors demonstrated that AGP increase drastically during mammary infection, probably outcome of migration of somatic cells to the udder and, acting as a regulator of epithelial damage caused by this cells [45]. Beside that AGP can reduce apoptosis [46] and increase expression of anti-inflammatory cytokines by macrophages [47]. Comparison of AGP concentrations in milk from cows with or without mastitis and with minor or moderate clinical mastitis, has previously been carried out, with significant differences observed between animals with subclinical mastitis and healthy animals [48], but no difference was observed between animals with minor or moderate clinical mastitis [49]. The same authors

compared AGP concentrations in milk from cows with subclinical mastitis caused by *E. coli*, *Streptococcus* spp., and *Staphylococcus* spp., but no difference was found between the three bacteria [48]. This is the first study that reported significant differences between AGP concentrations in milk from cows with mastitis caused by different pathogens. Here, it was observed that mastitis caused by *E. coli* or *S. aureus* showed a high concentration of AGP. This elevation in concentration of the APP was showed in blood of cows challenged with LPS [23], so this response of udder, by AGP, to *E. coli* is in accordance with literature. Similar pattern was expected from AGP concentration by *Klebsiella pneumoniae* infections, since was demonstrated an increase in concentration of this protein after mastitis by this pathogen [50]. *Klebsiella pneumoniae* had a similar result to *E. coli* and *S. aureus*, as well as to environmental *Streptococcus* and *Mycoplasma* spp., and showed a moderate elevation of AGP concentration in milk. Recently was reinforced the difference of immune system reaction against gram-negative and positive bacteria [51]. The study showed that gram-negative bacteria can cause a markedly response by APPs, when gram-positive bacteria entail to a mild response, corroborating with the well-known behavior of causing acute or chronical mastitis respectively. Milk samples from cows infected with *Enterococcus* spp. or CNS, showed low concentration of AGP, indicating the mild effect of these pathogens in production and release of this APP. Like cited before gram-positive bacteria usually trigger bland cases of mastitis that induce slight damage to udder tissue and small mobilization of defensive cells, so it is expected that this cells result a not pronounced improve in AGP concentration in milk, since the major part of this protein is released in milk by the blood white cells.

Main functions of CRP are: regulate immune system during infections, destroy infectious agents, minimizing tissue damage and, help tissue repair and regeneration [44,52]. Results from cows sampled are in accordance with previous observations showing that different CRP concentrations are present in milk from cows with mastitis caused by different pathogens [9,53]. This research found that milk samples infected by *E. coli* and *Klebsiella pneumoniae* had a high CRP concentration, as previously observed in *E. coli* mammary infection [9], indicating that *Klebsiella pneumoniae* produce the same pattern of CRP release. Mastitis caused by gram-negative bacteria is known for cause severe clinical cases producing large tissue damage [54], and probably this damage stimulate action of CRP due the function of this APP. Mammary infections by *S. aureus*, environmental *Streptococcus,* and *Mycoplasma* spp. had moderate levels of CRP in the milk sampled. Thomas et al. (2018) found high CRP concentration for milk samples infected with *S. uberis* and *S. dysgalactiae* (environmental *Streptococcus*), different that found in this study. In the same study, the CRP concentrations of samples infected with *S. aureus* was in accordance with the data observed in this study, indicating a moderate damage by this pathogen to the epithelial cells of the udder. Although environmental *Streptococcus* are described as causing severe clinical mastitis, the concentration of AGP and CRP was moderate for these pathogens in our study. This behavior is similar to found for *S. aureus* indicating that these bacteria has the same stimulation pattern of inflammatory response. There are some difference among the APPs chain reaction [52], so maybe the pathogens studied produce a different response among the different APPs, like already was demonstrated for *S. uberis*, different strains can generate different responses from the host [55].

Mammary infection with CNS and *Enterococcus* spp. had a lower CRP concentration in comparison to the aforementioned bacteria. CRP detection in milk from CNS infection was in accordance with literature [9], indicating that *Enterococcus* spp. could induce a mild inflammatory response in the mammary gland, similar to that of CNS. Like CNS, *Enterococcus* usually cause chronic mastitis, so produce little tissue damage and reaction from the host. Probably these bacteria could not stimulate a large production and release of CRP, since the function of the protein is not strongly requested in the site of inflammation.

Spearman's rank test showed strong correlation between Hp, AGP, and CRP concentrations in milk from cows with mastitis. Based on these results, we could confirm that pathogens with similar patterns of concentrations of different APPs caused similar inflammatory responses in the epithelial cells of the udder. The correlation between Hp and SAA was moderate, and the concentrations determined for these proteins for different pathogens did not indicate a similar response to bacterial aggression. No correlation was found between SAA, CRP, and AGP, indicating that the detection of these APPs from different pathogens is not related and does not follow the same behavior across the bacteria. These data support previous reports in literature, where a moderate correlation between Hp and SAA was found (r_s=0.39) [21]. Other studies have found a strong correlation between Hp and M-SAA3, the mammary isoform of SAA [9]; the fact that a different SAA isoform was analyzed in this study might explain the difference between the data. In accordance with Thomas et al. (2018), the results of this study revealed a strong correlation between Hp and CRP (r_s=0.55). We propose that due to the strong correlation between AGP and Hp (r_s=0.60), and AGP

and CRP (r_s=0.72), AGP could be a good biomarker, presenting similarly to Hp and CRP, for clinical mastitis in cows caused by different pathogens.

Overall, this is the first study on the detection of APPs in milk following the SPARCL technique. Results are in accordance with the literature, demonstrating the feasibility of this new method for detection of APPs as Hp, CRP and, AGP in milk. This new technology is important for research and clinical use, as it is easier and faster, because it needs only one incubation and has no wash steps, than the standard ELISA technique commonly used for this purpose. However, the use of kits with antibodies against SAA could lead to the detection of one or more isoforms, different to M-SAA3; thus, more studies are necessary to improve the use of this kit in the detection of SAA in milk from cows with mastitis.

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5. DISCUSSÃO GERAL

A discussão dos resultados obtidos está descrita no corpo dos dois artigos que compõem essa tese. De maneira geral os resultados observados no estudo estão de acordo com a literatura consultada. Os resultados que diferem dos dados já apontados na literatura por outros autores, estão devidamente justificados e discutidos no corpo dos respectivos artigos.

6. CONCLUSÃO GERAL

Com base nos dois artigos que integram essa tese é possível concluir que, diferentes micro-organismos distribuídos nos grupos dos patógenos maiores, menores, bem como gram-positivos e gram-negativos, alteram os parâmetros reprodutivos com graus de intensidade variáveis. Acredita-se que essa diferença se deva ao potencial que os patógenos possuem em causar doença mais grave, muito provavelmente em função dos fatores de virulência do patógeno causador da mastite.

O segundo artigo dessa tese demonstra justamente a capacidade que as diferentes bactérias possuem em agredir a glândula mamária, também provavelmente relacionado aos fatores de patogenicidade e virulência dos patógenos envolvidos na infecção. Foi demonstrado que os diferentes patógenos levaram a liberações distintas de Hp, SAA, CRP e AGP. Esses padrões de liberação das proteínas permitem que essas sejam usadas no diagnóstico de mastite clínica causada por diversos agentes infecciosos.

7. CONSIDERAÇÕES FINAIS

Acredito que essa tese tenha contribuído para o melhor entendimento e conhecimento tanto da reprodução, como da resposta de proteínas de fase aguda em casos de mastite clínica em vacas leiteiras. Espero que todo o trabalho do autor, orientador e colaboradores, estimule novas pesquisas na área, que é muito importante para o agronegócio brasileiro e também para a saúde pública.

Pessoalmente essa tese levou a enormes ganhos científicos para o autor. Foi um grande desafio trabalhar com áreas distintas que envolvem a mastite bovina, entretanto foi um grande prazer também aprender diversas técnicas e metodologias para microbiologias e mensuração de proteínas. O fato de ter passado seis meses em outro país aprendendo sobre a bovinocultura canadense e também sobre análises estatísticas, são experiências que moldaram não só a forma como interajo com outras pessoas, mas também como avaliar e desenvolver atividades acadêmicas.

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fmvz - unesp
Faculdade de Medicina Veterinária e Zootecnia
Campus de Botucatu

ATESTADO

Atesto que o Projeto "INFLUÊNCIA DOS PATÓGENOS CAUSADORES DA MASTITE CLÍNICA NOS ÍNDICES REPRODUTIVOS DE VACAS LEITEIRAS" **Protocolo CEUA 0196/2016**, a ser conduzido por Felipe Morales Dalanezi, responsável/orientador Prof. Hélio Langoni, para fins de pesquisa científica/ensino - encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal - CONCEA.

Finalidade	PESQUISA CIENTÍFICA
Vigência do projeto	10/01/2017 a 10/01/2018
Nome Comum / Espécie / Linhagem	BOVINA / BOS TAURUS / Não se aplica
Raça	Holandês Preto e Branco
Nº de animais machos	0
Nº de animais fêmeas	700
Nº de animais sexo indefinido	0
Peso médio de animais machos	0
Peso médio de animais fêmeas	500 Kg
Peso médio de animais sexo indefinido	0
Idade	5 ano(s) e 0 mes(es) e 0 dia(s).
Procedência	Animais de rebanho comercial

Projeto de Pesquisa aprovado em renunião da CEUA em 08/12/2016

PROFª. ASS. DRª. IBIARA CORREIA DE LIMA ALMEIDA PAZ
Presidente da CEUA da FMVZ, UNESP - Campus de Botucatu

Faculdade de Medicina Veterinária e Zootecnia
Seção Técnica Acadêmica
Rua Prof. Dr. Walter Mauricio Corrêa, s/n
UNESP - Campus de Botucatu/SP - Cep 18618-681
(14) 3880-2176 - patrizia@fmvz.unesp.br - www.fmvz.unesp.br