CLARISSE MAXIMO ARPINI

GENES DE VIRULÊNCIA DE *Streptococcus agalactiae* ASSOCIADOS À MASTITE BOVINA EM REBANHOS DE MINAS GERAIS

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Doenças Infecciosas, para a obtenção do título de Mestre.

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APROVADA em 17 de fevereiro de 2011.

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Ao meu pai, José Cleomar, que sempre foi minha inspiração diária de perseverança.

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"Hoje levantei cedo pensando no que tenho a fazer antes que o relógio marque meia noite.
É minha função escolher que tipo de dia vou ter hoje.

Posso reclamar porque está chovendo ou agradecer às águas por lavarem a poluição.
Posso ficar triste por não ter dinheiro ou me sentir encorajado para administrar minhas finanças, evitando o desperdício.
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Tudo depende só de mim."

(Charles Chaplin)

RESUMO

O Brasil possui o segundo maior rebanho leiteiro do mundo e, até 2008, ocupava o sexto lugar entre os países produtores de leite, com uma produção de cerca de 27,75 bilhões de quilos de leite. Minas Gerais é o maior produtor de leite do Brasil e, com um rebanho de 7 milhões de vacas, responde por cerca de 30% de toda a produção do país. O Estado produz mais de 7 bilhões de litros por ano. A mastite bovina é a doença que causa maiores perdas na indústria leiteira sob o ponto de vista econômico, pois, mesmo com todos os avanços tecnológicos no setor, mantém alta prevalência e resposta limitada à terapia, podendo ser causada por mais de cem agentes etiológicos diferentes, principalmente bactérias. Calcula-se que a perda na produção leiteira, por mastite, atinja entre l2% e l5%. Esta enfermidade se desenvolve devido a alterações metabólicas, fisiológicas, lesões traumáticas ou, mais frequentemente, a infecções por microrganismos. Qualquer que seja sua origem há alterações químicas e físicas no leite, acompanhadas por alterações patológicas no tecido glandular. O leite é considerado como um dos alimentos mais nobres por conter grandes quantidades de proteína, gordura, carboidratos, sais minerais e vitaminas em sua composição, podendo ser um excelente meio de cultura para o desenvolvimento de microrganismos e transmissão de algumas zoonoses ao homem, que, juntamente com a mastite podem deixar o leite e seus derivados impróprios para consumo. O *Streptococcus agalactiae* é altamente contagioso e ubíquo na glândula mamária, sendo um dos principais agentes etiológicos da mastite. A elucidação dos fatores de virulência deste agente é de grande importância para a prevenção e tratamento da mastite, já que esta doença é de difícil controle e apresenta resposta limitada às terapias existentes. Devido aos poucos estudos publicados com *S. agalactiae* isolados de bovinos este trabalho tem como objetivo comparar amostras de origem clínica e subclínica da mastite em relação à presença dos genes de virulência relacionados à cápsula polissacarídica rica em ácido siálico, hialuronato liase, proteína ligante de fibrinogênio e pili. *Primers* foram desenhados para amplificar os genes *fbs*A, *cps*C, *cps*D, *cps*E, *cps*K, *neu*B e para o cluster PI-1 de 16 isolados de *Streptococcus agalactiae* provenientes de mastite clínica e 51 isolados de mastite subclínica, oriundos de 21 rebanhos de Minas Gerais. As amostras também foram caracterizadas quanto à morfologia da colônia, hemólise em ágar, teste catalase, cultura em ágar esculina e ágar bile-esculina, CAMP teste e determinação do grupo de Lancefield. Análises moleculares mostraram a presença do gene *fbs*A em 85,07% dos isolados, *hyl*B em 38,80%, *cps*C, *cps*D e *cps*E em 4,48%, cpkJ, *cps*K e *neu*B em 79,10% e do cluster PI-1 em 1,49%. Observou-se diversidade dos isolados entre e dentro dos diferentes rebanhos, no

entanto, não foi observada relação dos fatores de virulência avaliados com o grau de severidade da infecção.

Palavras-chave: *Streptococcus agalactiae.* Fatores de virulência. Mastite bovina.

ABSTRACT

Brazil has the second largest dairy herd in the world and by 2008, occupied the sixth place among the producers of milk, with an output of around 27.75 billion kilos (61.18 billion pounds) of milk. Minas Gerais is the largest milk producer in Brazil and, with a flock of 7 million cattle and accounts for about 30% of all production in the country. The state produces more than 7 billion (1.85 billion gallons) liters per year. The mastitis is a disease that causes major losses in the dairy industry under the economic point of view, because even with all the technological advances in the industry, maintains a high prevalence and limited response to therapy and may be caused by more than one hundred different etiologic agents mainly bacteria. It is estimated that the loss in milk production by untreated, reach between l2 and l5%%. This disease develops due to metabolic and physiologic alterations, traumatic injury or more frequently, infections by microorganisms. Whatever its origin, there is chemical and physical changes in milk, accompanied by pathological changes in the glandular tissue. Milk is considered one of the finest foods because it contains large amounts of protein, fat, carbohydrates, minerals and vitamins in its composition, can be an excellent mean of culture for microorganisms development and transmission of some zoonotic diseases to humans, which along with mastitis can leave the milk and dairy products unfit for consumption. *Streptococcus agalactiae* is highly contagious and ubiquitous in the mammary gland, is a major etiological agents of mastitis. The elucidation of the virulence factors of this agent is of great importance for the prevention and treatment of mastitis. Because of the few published studies with *S. agalactiae* isolates from cattle this study aims to compare isolates from clinical and subclinical mastitis in relation to the presence of virulence genes related to polysaccharide capsule rich in sialic acid, hyaluronate lyase, fibrinogen binding protein and pili. Primers were designed to amplify the genes *fbs*A, *cps*C, *cps*D, *cps*E, *cps*K, *neu*B and the PI-1 cluster of 16 isolates of *Streptococcus agalactiae* from clinical mastitis and subclinical mastitis of 51 isolates, from 21 herds in Minas Gerais. The strains were also characterized for colony morphology, hemolysis on agar, catalase test, agar culture, esculin and bile-esculin agar, CAMP test and determination of Lancefield group. Molecular analysis showed the presence of gene fbsA in 85.07% of the isolates, 38.80% in *hyl*B, *cps*C, *cps*D and *cps*E at 4.48%, cpkJ, *cps*K and *neu*B 79.10% in the cluster and PI-1 at 1.49%. Observed diversity of strains within and between different flocks, however, no relationship was observed among virulence factors evaluated and the severity of infection.

Keywords: *Streptococcus agalactiae.* Virulence factors. Bovine mastitis.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Até 2008 o Brasil ocupava o sexto lugar entre os países produtores de leite (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2010) e, dentre os estados brasileiros, Minas Gerais é o principal produtor com, aproximadamente, 7 bilhões de litros de leite por ano, porém um dos maiores problemas enfrentados pelos produtores é a mastite.

A mastite, também conhecida como mamite, é a inflamação da glândula mamária causada por microrganismos e suas toxinas, miíases, traumas físicos ou agentes químicos irritantes. Aproximadamente 95% das infecções que resultam em mastite são causadas pelas bactérias *Streptococcus agalactiae*, *Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis* e *Escherichia coli*. Os 5% restantes são causados por outros microrganismos (GERMANO; GERMANO, 2001).

É uma das principais causas mundiais de prejuízos econômicos para os produtores de leite (FREITAS et al., 2005). Segundo Costa (1998) a perda na produção leiteira, por mastite, atinge entre l7% e 20%, o que significa um total de 5,5 bilhões de litros por ano em relação à produção anual.

Apesar dos avanços tecnológicos no setor, verifica-se que a mastite provocada por *S. agalactiae* mantém alta prevalência e resposta limitada às terapias disponíveis (CARNEIRO; DOMINGUES; VAZ, 2009). Para que se consiga controlar de forma mais eficiente as infecções ocasionadas por este agente, é imprescindível o conhecimento sobre os fatores de virulência do mesmo envolvidos na colonização e infecção.

No Brasil, existem poucos trabalhos sobre fatores de virulência para *S. agalactiae* isolados de bovinos. Desta forma, o presente estudo teve como

objetivos avaliar a presença dos genes de virulência *fbsA*, *hylB*, *cps* e do cluster PI-1 em isolados de *S. agalactiae* de casos de mastite bovina de rebanhos do Estado de Minas Gerais, Brasil, comparando a frequência dos fatores de virulência nos isolados associados a casos clínicos e subclínicos de mastite.

2 REFERENCIAL TEÓRICO

2.1 *Streptococcus agalactiae*

S. agalactiae, também conhecido por *Streptococcus* do grupo B (GBS) segundo a classificação de Lancefield (1933), é uma bactéria Gram-positiva, esférica ou ovóide com 0.6-1.2 μm de diâmetro, não formadora de esporos e não móvel. Tem crescimento ótimo a 37ºC e frequentemente cresce em cadeias no leite e meios de cultura líquidos. Possui metabolismo fermentativo, produzindo principalmente lactato, e é catalase negativo (HOLT et al., 1994; QUINN et al., 2005; RAJAGOPAL, 2009). Apesar da grande maioria das amostras provenientes de seres humanos ser beta-hemolítica (FACKLAM, 2002), no que se refere às amostras provenientes de mastite bovina são descritas cepas alfa-hemolíticas (hemólise parcial), beta-hemolíticas (hemólise total) e gama-hemolíticas (sem hemólise) (QUINN et al., 1995).

Segundo Hillerton et al. (2004), *S. agalactiae* é um microrganismo bem adaptado à glândula mamária e, geralmente, está envolvido em doenças clínicas agudas e infecções subclínicas persistentes. *S. agalactiae* é altamente contagioso e comumente encontrado na glândula mamária (FONSECA; SANTOS, 2000). Embora, frequentemente, não invada o tecido glandular, pode causar fibrose e abscessos (FONSECA; SANTOS, 2000).

S. agalactiae é capaz de causar doença em diversos hospedeiros. Foi isolado, inicialmente, na glândula mamária de bovinos, causando mastite e, posteriormente, no ser humano, causando meningite neonatal (MAIONE et al., 2005). A alta incidência da infecção neonatal por *S. agalactia a* partir da década de 70 chamou a atenção de médicos americanos, sendo que os motivos dessa alta incidência não foram totalmente elucidados. Uma possível explicação seria a infecção de humanos por *S. agalactiae* de origem bovina (JENSEN, 1985).

Manifestações clínicas em seres humanos adultos variam desde infecções na pele, tecidos moles e trato urinário inferior, bacteremia, pneumonia, artrite e endocardite (FONSECA; SANTOS, 2000).

Atualmente essa bactéria tem sido associada a casos de meningoencefalite e septicemia em peixes e, ocasionalmente, também pode causar infecções em outras espécies como ratos, gatos, cães, hamsters e sapos (ELLIOT; FACKLAM; RITCHTER, 1990; SPELLERBERG, 2000). Em peixes, surtos de estreptococose são responsáveis por grandes prejuízos econômicos para os produtores, pois podem causar mortalidade de até 90% do plantel (EVANS et al., 2002).

2.2 Mastite

As afecções intramamárias ocorrem quando um agente (infeccioso, químico, mecânico ou térmico) agride a glândula mamária, produzindo reação inflamatória e danos ao epitélio glandular, caracterizando o quadro de mastite (PRESTE et al., 2002). Nestas infecções, a extensão da resposta inflamatória varia de acordo com a natureza do estímulo e a capacidade de reação do animal. Reações brandas, sem alterações macroscópicas detectáveis, porém com alterações químicas e microbiológicas do leite, caracterizam a mastite subclínica. Respostas inflamatórias mais severas, denominadas de mastite clínica, resultam em mudanças no aspecto da secreção láctea, incluindo as alterações verificadas na forma subclínica, havendo, contudo, visíveis mudanças no tecido mamário e alguns efeitos sistêmicos, como hipertermia, prostração e tremores musculares (HILLERTON et al., 2004).

A epidemiologia da mastite varia consideravelmente dependendo da espécie, quantidade, patogenicidade e infectividade do agente envolvido e há evidências de que os fatores de risco diferem conforme essas características. Os

microrganismos que comumente causam mastite podem ser divididos em dois grupos, baseados na sua origem: patógenos contagiosos, representados pelos *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae e Corynebacteurim bovis,* e patógenos ambientais, representados principalmente pelos coliformes e *Streptococcus uberis* (PEELER et al., 2000).

2.3 Fatores de virulência

Os patógenos envolvidos na etiologia da mastite bovina possuem muitos fatores de virulência que facilitam a colonização e infecção da glândula mamária. Alguns patógenos podem escapar das defesas do hospedeiro ao se aderirem às células epiteliais, produzindo cápsulas que dificultam a captura e destruição pelos neutrófilos ou produzindo endotoxinas e exotoxinas que destroem ou inativam os leucócitos, podendo manter-se no interior das células para escapar à resposta imune do hospedeiro (BRADLEY, 2002; CARNEIRO; DOMINGUES; VAZ, 2009).

Os fatores de patogenicidade representam uma série de estratégias das quais o microrganismo se utiliza para invadir um hospedeiro. Para causar doença, o *S. agalactiae* deve entrar em contato com o hospedeiro, atravessando barreiras epiteliais (BARON; KASPER, 2005; PIETROCOLA et al., 2005). Para isto, o microrganismo utiliza estruturas presentes em suas superfícies ou secreta produtos no ambiente circundante. Em muitos, casos é vital para a sobrevivência do microrganismo a utilização de vários mecanismos com sobreposição de funções (BARON; KASPER, 2005). Em todos os organismos vivos, a regulação da expressão gênica em resposta aos estímulos externos é realizada pelo Sistema de Transdução de Sinal, conhecido como STS. O STS responde a sinais externos regulando a função dos fatores transcricionais do DNA mobilizado, sendo mais comum em bactérias o Sistema de Duplo-

Componente (TCS) (RAJAGOPAL, 2009). O sequenciamento do genoma de *S. agalactiae* tem revelado a presença de 17 a 20 TCS, que podem responder a mudanças no ambiente externo (GLAZER et al., 2002).

2.3.1 Toxinas formadoras de poros

As toxinas formadoras de poros promovem a entrada de patógenos nas células hospedeiras e facilitam a sobrevivência intracelular e disseminação sistêmica. O *S. agalactiae* apresenta no seu genoma pelo menos dois genes que codificam toxinas formadoras de poros, conhecidas como Beta-hemolisina/citolisina (Beta-H/C) e Christie Atkins Munch Peterson, mais conhecida como fator CAMP (RAJAGOPAL, 2009).

A Beta-H/C é uma proteína de superfície que promove a invasão do *S. agalactiae* nas células hospedeiras, permitindo que este atravesse barreiras como as células epiteliais e endoteliais e, inclusive, a barreira hematoencefálica (DORAN; LIU; NIZET, 2003; GIBSON; NIZET; RUBENS, 1999; NIZET, 2002). Além disso, podem induzir à apoptose e liberar citocinas, permitindo a invasão celular e a resistência à fagocitose (DORAN; NIZET, 2004; NIZET, 2002). O fator CAMP é uma proteína secretada com propriedades formadoras de poros e que tem sido relatada como importante para a patogênese de *S. agalactiae* (JURGENS; STERZIK; FEHRENBACH, 1987; LANG; PALMER, 2003). Esta proteína promoveu a formação de poros discretos nas membranas além de se ligar à porção Fc de IgG e IgM, impedindo a ação destes anticorpos (DORAN; NIZET, 2004; LANG; PALMER, 2003). Além da ação das toxinas formadoras de poros, que facilitam a sobrevivência de GBS no hospedeiro, é essencial para o GBS destruir as defesas imunológicas inatas (RAJAGOPAL, 2009).

2.3.2 Hialuronato liase

Uma proteína também importante para a patogênese do *S. agalactiae* é a hialuronato liase (HylB) codificada pelo gene *hyl*B (GASE; OZEGOWSKI; MALKE, 1998). Ela pertence a um grupo especial de enzimas, as Hialuronidases, responsáveis pela degradação de polissacarídeos, principalmente a N-acetilglicosamina, que compõe o ácido hialurônico (AKHTAR; KRISHNAN; BHAKUNI, 2006). Ela também está presente no genoma de vários microrganismos Gram-positivos como várias espécies de *Streptococcus, Staphylococcus, Peptostreptococcus, Propionibacterium, Streptomyces* e *Clostridium*. No entanto, em microrganismos Gram-negativos, as hialuronidases são encontradas nas regiões periplasmáticas ao invés de serem excretadas para o meio extracelular e, por este motivo, apresentam pouca importância na patogênese (HYNES; WALTON, 2000).

O ácido hialurônico é um dos principais componentes do organismo e a enzima hialuronato liase pode clivá-lo, assim como a condroitina e sulfatos de condroitina (AKHTAR; KRISHNAN; BHAKUNI, 2006; LI; JEDRZEJAS, 2001). A hialuronato liase facilita a difusão do *S. agalactiae* durante a infecção (AKHTAR; KRISHNAN; BHAKUNI, 2006; LIU; NIZET, 2004; NIZET; RUBENS, 2001; RAJAGOPAL, 2009).

2.3.3 Cápsula polissacarídica rica em ácido siálico

Outro fator de virulência que pode estar presente em *S. agalactiae* é a cápsula polissacarídica rica em ácido siálico (CPS) localizada ao redor da membrana celular que exemplifica o mimetismo molecular, o que permite que o microrganismo invada o organismo do hospedeiro sem que seja percebido pelo

sistema imunológico (BARON; KASPER, 2005; CAMPBELL; BAKER; EDWARDS, 1991; MARQUES et al., 1992).

O ácido siálico, também conhecido como N-acetilneuramínico, é encontrado de forma abundante no organismo de vertebrados, principalmente nas regiões terminais de unidades de carboidratos em glicoproteínas e glicolipídeos, estando diretamente envolvido em vários processos fisiológicos e patológicos, incluindo processos infecciosos (ANGATA; VARKI, 2002; YU et al., 2006).

A cápsula, presente em *S. agalactiae*, tem a capacidade de promover a aderência do microrganismo às superfícies epiteliais, além de inibir a fagocitose pelos macrófagos e neutrófilos (HULSE et al., 1993; NIZET et al., 1997; NIZET; RUBENS, 2001; TAMURA et al., 1994). O ácido siálico é um fator essencial para a patogenicidade, pois impede a deposição do componente C3b do sistema complemento, bloqueando a fagocitose (JARVA et al., 2003). O gene *neu*, localizado na extremidade a jusante do operon *cps* (POYART et al., 2007; YAMAMOTO et al., 1999) é responsável pela produção do ácido siálico e sialização da cápsula (LEWIS et al., 2006).

A maioria das proteínas de superfície anexas à CPS contribui para a capacidade de aderência, invasão e escape do sistema imunológico do hospedeiro (BARON; KASPER, 2005).

2.3.4 Superóxido dismutase

A resistência aos patógenos pelas espécies reativas de oxigênio, codificadas pelo hospedeiro, é de grande importância na evasão imunitária (HAMILTON et al., 2006; RAJAGOPAL, 2009). Os *S. agalactiae* possuem, em seu genoma, o gene *sod*A, que é capaz de codificar uma superóxido dismutase com cofator de Mn^{2+} que induz a resistência às espécies reativas de oxigênio e

evasão imune (RAJAGOPAL, 2009). A superóxido dismutase converte oxigênio simples ou ânions de superóxidos (O^{-2}) em molécula de oxigênio (O_2) e de peróxido de hidrogênio (H_2O_2), que é subsequentemente metabolizado por catalases e peroxidases (RAJAGOPAL, 2009), embora as bactérias do gênero *Streptococcus* sejam catalase negativas (QUINN et al., 1995).

2.3.5 Proteína ligante de fibrinogênio

Várias bactérias patogênicas aderem às células hospedeiras por meio de proteínas de superfície que se ligam à matriz extracelular (SCHUBERT et al., 2004). A matriz extracelular de tecidos de mamíferos é composta por glicoproteínas, como colágeno, laminina, fibronectina e fibrinogênio, formando uma estrutura macromolecular subjacente às células epiteliais e endoteliais (ARAUJO et al., 2008; SCHUBERT et al., 2004; SUHR et al., 2010). O fibrinogênio é uma proteína de fase aguda sintetizada pelo fígado e tem sua liberação aumentada no processo inflamatório (VECINI; PATRÍCIO; CIARLINI, 2006). Várias pesquisas têm demonstrado interações de GBS com as proteínas da matriz extracelular e, para cada uma dessas proteínas, existem receptores específicos (SCHUBERT et al., 2004; SPELLERBERG et al., 1999).

A aderência de *S. agalactiae* aos tecidos do hospedeiro é importante no início do processo infeccioso (FROST; WANASINGHE; WOOLCOCK, 1977; RAJAGOPAL, 2009). Enquanto nos *Streptococcus* pertencentes aos outros grupos de Lancefield, a ligação ao fibrinogênio é feita por uma proteína de membrana denominada M (FISCHETTI, 1989; VASI et al., 2000), a ligação de *S. agalactiae* ao fibrinogênio da matriz extracelular é mediada por duas proteínas conhecidas como FbsA e FbsB, que podem ligar tanto ao fibrinogênio solúvel quanto ao imobilizado de humanos e de bovinos (JACOBSSON, 2003; JONSSON et al., 2005; SCHUBERT et al., 2002). Estudos têm demonstrado que

a proteína FbsA também tem função de agregação plaquetária, podendo causar outros agravos durante a infecção (PIETROCOLA et al., 2005) como também pode estar envolvida no mecanismo de escape ao sistema imunológico, evitando a opsonização por macrófagos e neutrófilos (PIETROCOLA et al., 2005; SCHUBERT et al., 2002).

Segundo Lindahl, Stälhammar-Carlemalm e Areschoug (2005), uma mutação no gene *fbs*A pode causar uma redução na habilidade do *S. agalactiae* em se desenvolver em sangue de seres humanos, o que sugere que a proteína FbsA contribui para a resistência à fagocitose.

2.3.6 Pili

Estudos recentes demonstram que os *S. agalactiae* codificam pequenos apêndices na superfície celular, conhecidos como pili (DRAMSI; TRIEU-CUOT; BIERNE, 2005; LAUER et al., 2005). Estas estruturas representam alguns dos mais importantes fatores de virulência para a infecção em um organismo de um mamífero, consistindo de subunidades protéicas repetidas, que se estendem desde a superfície bacteriana até o meio circundante (DRAMSI; TRIEU-CUOT; BIERNE, 2005; MARESSO; SCHNEEWIND, 2008), permitindo o desenvolvimento de infecções invasivas em seres humanos, principalmente infecções urinárias, genitais e gastrointestinais, podendo contribuir para a ocorrência de meningite e septicemia neonatal (DORAN; NIZET, 2004; MAISEY et al., 2007). Estudos sobre estes fatores de virulência em *S. agalactiae* associados à mastite em bovinos são escassos.

A extremidade dos pili geralmente apresenta propriedades adesivas, que promovem a ligação bacteriana à matriz extracelular e / ou a ligação a receptores celulares do hospedeiro (MARESSO; SCHNEEWIND, 2008). Em *S. agalactiae*, os pili mediam resistência aos Peptídeos Catiônicos Antimicrobianos (AMPs) e

também facilitam a aderência e ataque do patógeno às células hospedeiras (DRAMSI; TRIEU-CUOT; BIERNE, 2005; MAISEY et al., 2007, 2008; PEZZICOLI et al., 2008). Além destas funções, um estudo realizado por Konto-Ghiorgh et al. (2009) revelou que os pili de *S. agalactiae* também estão envolvidos na formação de biofilmes.

Os pili são codificados pelos genes *pil*A, *pil*B e *pil*C (MAISEY et al., 2007) que estão localizados em dois clusters, ilha-1 de pilus (PI-1) e ilha-2 de pilus (PI-2), sendo que este último apresenta duas variantes, a PI-2a e PI-2b (LAUER et al., 2005; RAJAGOPAL, 2009; ROSINI et al., 2006). Estas estruturas são formadas por três subunidades protéicas: PilA, PilB e PilC e a sua montagem envolve duas classes de proteínas sortases tipo C, StrC3 e StrC4 (KONTO-GHIORGH et al., 2009). As proteínas sortases pertencem a uma família de proteínas de organismos procariotos que estão envolvidas na formação de pili (LEMIEUX; WOOD; CAMILLI, 2008).

Acredita-se que as sortases tipo C possam polimerizar os pili pela formação de ligações covalentes entre diferentes subunidades. Essas proteínas já foram descritas em outros microrganismos além dos GBS, como em *Actinomyces viscosis, Actinomyces naeslundii, Bacillus cereus, Clostridium perfringens, Enterococcus faecalis, Streptococcus pneumoniae* e *Streptococcus pyogenes* (DRAMSI; TRIEU-CUOT; BIERNE, 2005; MARESSO; SCHNEEWIND, 2008).

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SEGUNDA PARTE - ARTIGO

VIRULENCE GENES OF THE *Streptococcus agalactiae* ASSOCIATED WITH BOVINE MASTITIS IN MINAS GERAIS LIVESTOCK HERDS, BRAZIL

Artigo redigido conforme norma da revista Infection and Immunity

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Abstract

Brazil has the second largest dairy herd in the world and until 2008, occupied the sixth place among the milk producers, with a production of about 27.75 billion kilos of milk. Minas Gerais is the largest milk producer in Brazil and, with a flock of 7 million cattle and accounts for about 30% of all production in the country. The state produces more than 7 billion liters per year. The mastitis is a disease that causes major losses in the dairy industry under the economic point of view, because even with all the technological advances in the industry, maintains a high prevalence and limited response to therapy and may be caused by more than one hundred different etiologic agents mainly bacteria. Estimated that the loss in milk production by untreated, reach between 12 and 15%, which means a total of 2.8 billion gallons per year of the annual production of 21 billion liters. Approximately 17% to 20% of the population of dairy cattle in at least one point in their lives is affected by mastitis. This disease develops due to metabolic, physiologic, traumatic injury or more frequently, infections by microorganisms. Whatever its origin, there is chemical and physical changes in milk, accompanied by pathological changes in the glandular tissue. Milk is considered one of the finest food because it contains large amounts of protein, fat, carbohydrates, minerals and vitamins in its composition and can be an excellent culture medium for the development of microorganisms and transmission of some zoonotic diseases to humans, who, along with mastitis can leave the milk and dairy products unfit for consumption. *Streptococcus agalactiae* is highly contagious and ubiquitous in the mammary gland, is a major etiological agents of mastitis. The elucidation of the virulence factors of this agent is of great importance for the prevention and treatment of mastitis, since this disease is difficult to control and has limited response to existing therapies. Because of the few published studies with *S. agalactiae* isolates from cattle this study aims to compare isolates from clinical and subclinical mastitis in relation to the presence of virulence genes related to polysaccharide capsule rich in sialic acid, hyaluronate lyase, fibrinogen binding protein and pili. Primers were designed to amplify the genes *fbs*A, *cps*C, *cps*D, *cps*E, *cps*K, *neu*B and the PI-1 cluster of 16 isolates of *S. agalactiae* from clinical mastitis and subclinical mastitis of 51 isolates, from 21 herds in Minas Gerais. The strains were also characterized for colony morphology, hemolysis on agar, catalase test, agar culture, esculin and bile-esculin agar, CAMP test and determination of Lancefield group. Molecular analysis showed the presence of gene *fbs*A in 85.07% of the isolates, 38.80% in *hyl*B, *cps*C, *cps*D and *cps*E at 4.48%, *cps*J, *cps*K *neu*B and 79.10% in the cluster and PI-1 at 1.49%. Observed diversity of strains within and between different flocks, however, no relationship was observed among virulence factors evaluated and the severity of infection.

Introduction

Mastitis is an inflammation of the mammary gland caused by microorganisms and their toxins, myiasis, physical trauma or chemical irritants. Approximately 95% of infections that result in mastitis are caused by the bacteria *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli*. The remaining 5% are caused by other microorganisms (11).

It is one of the main causes of economic losses to dairy producers. Estimated the loss in milk production by untreated, affects between 17% and 20%, which means a total of 5.5 billion liters per year of the annual production in Brazilian dairy herds (11).

S. agalactiae, also known as Group B *Streptococcus* (GBS) following the classification of Lancefield (23). This is a highly contagious agent and commonly found in the mammary gland of cattle (11), usually associated with acute clinical mastitis and persistent subclinical infections (17).

Despite technological advances in the industry, it appears that mastitis caused by *S. agalactiae* has high prevalence and limited response to available therapies (7). In order to be able to control more efficiently the infections caused by this agent, it is essential knowledge about the virulence factors of this agent involved in colonization and infection because the pathogenicity factors represent a range of strategies from which the organism uses to invade a host. In many cases it is vital to the survival of the microorganism using various mechanisms with overlapping functions (3).

The *fbs*A gene is responsible for encoding the protein FbsA, which allows the binding of *S. agalactiae* to fibrinogen, soluble or mobilized from extracellular matrix of the host organism (19, 21, 44). The adherence of *S. agalactiae* to host tissues is important early in the infection process (12, 42), and recent studies have shown that the protein FbsA also has platelet function and

may cause other problems during infection (38) but may also be involved escape mechanism in the immune system, preventing opsonization by macrophages and neutrophils (38, 44).

The gene is responsible for hlyB protein called hyaluronate lyase (HlyB), which is very important for the pathogenesis of *S. agalactiae* (14). This protein belongs to a special group of enzymes, hyaluronidase, responsible for the degradation of polysaccharides such as chondroitin, chondroitin sulfate, and especially the N-acetylglucosamine, which is part of the composition of hyaluronic acid (1, 27), facilitating the spread of *S. agalactiae* during infection (1, 29, 35, 42).

The *cps* cluster is responsible for the formation of the polysaccharide capsule and its sialidation. The polysaccharide capsule rich in sialic acid (PSC), located around the cell membrane, allows the organism to invade the host's body without being perceived by the immune system, which exemplifies the molecular mimicry (3, 6, 32). The sialic acid, also known as N-acetylneuraminic acid, is found abundantly in the body of vertebrates, being directly involved in various physiological and pathological processes, including infectious processes (2, 49).

The capsule is present in *S. agalactiae*, has the ability to promote the adherence of microorganisms to epithelial surfaces in addition to inhibiting phagocytosis by macrophages and neutrophils (18, 34, 35, 46). The sialic acid is an essential factor in pathogenicity because it prevents the deposition of the C3b component of complement system, blocking phagocytosis (20). The *neu* gene, located on the downstream end of the cps operon is responsible for production of sialic acid and sialidation capsule (26, 39, 47).

Recent studies show that the *S. agalactiae* encode small appendages on the cell surface, known as pili (9, 25). The pili are encoded by genes dick, Pilbara and Pilc (30) which are located in two clusters of a pilus island (PI-1)

and the pilus island-2 (PI-2), but the latter has two variations PI-PI-2a and 2b (25, 42, 43). These structures are formed from three protein subunits: PilA, PilB and PilC and their assembly involves two classes of proteins sortases type C, and StrC3 StrC4 (22). These structures represent some of the most important virulence factors for infection in different microorganisms, allowing the development of invasive infections in humans (8, 30).

There are few studies on the virulence factors in *S. agalactiae* associated with mastitis in cattle. Thus, this study aimed to evaluate the presence of virulence genes *fbs*A, *hyl*B, *cps* cluster and the PI-1 in *S. agalactiae* strains isolated from cases of bovine mastitis in dairy herds from state of Minas Gerais, Brazil, comparing the frequency of virulence factors in isolates associated with clinical and subclinical cases of mastitis.

Materials and methods

Bacterial strains

Were isolated from 67 strains of *S. agalactiae* in 21 cattle herds in the dairy region of Minas Gerais in the period between 2004 and 2010, with 16 isolates from clinical mastitis and 51 isolates from subclinical mastitis. The isolates are part of the bank of bacterial strains from the Department of Veterinary Medicine, Federal University of Lavras, Minas Gerais (DMV / UFLA) and kept in BHI (Brain Heart Infusion) containing 10% glycerol at -70°C.

Phenotypic characterization

Strains of *S. agalactiae* were characterized by routine tests, according to Quinn et al. (41): colony morphology, Gram stain, hemolysis on agar, catalase test, agar culture, esculin and bile-esculin agar and CAMP test and determination of Lancefield group SLIDEX Strepto-Kit (BioMerieux, France).

Molecular characterization

For extraction of total DNA, the bacterial isolates were cultured on blood agar supplemented with 5% horse blood for 24 to 48 hours at 37°C and then transferred to BHI for 24 hours at 37°C. Total DNA was extracted by Genomic DNA Miniprep kit Bacterial (Axygen, Biosystems ®), according to the manufacturer's instructions.

Primers (Table 1) for the *fbs*A genes (encoding fibrinogen-binding protein), *hyl*B (encodes the enzyme hyaluronan liase), *cps* (encodes the protein responsible for formation of the polysaccharide capsule), *neu*B (encodes the protein responsible for the production of sialic acid) and cluster IP-1 (encoding the proteins of pili) were designed with the aid of software ITD

(http://www.idtdna.com/Home/Home.aspx), DNAME (version 4.0 Lynnon Corporation, Canada) and BLAST (http / /: www.ncbi.nlm.nih.gov / BLAST).

The *cps* and *neu* genes were evaluated together to assess the presence in the region of the *cps* operon from the gene that corresponds to the gene *cps*C to *neu*B (39).

Table 1 Sequences of oligonucleotides designed for amplification of virulence genes, f*bs*A, *hyl*B, *cps*, and *neu*B cluster PI-1 of *Streptococcus agalactiae*.

Genes	**Sequences**		**GenBank Access**
Cluster PI-1	PilF	5'CTCATCAGTTGACGATTGTTC3'	EU929554.1
	PilR	5'CCATTGCCTGTTGCTCAC3'	
*hyl*B	HylF	5'GCAACAGCCACTCATAGCA3'	CP000114.1
	HylR	5'GAGCGAGGGACACCGAT3'	
*fbs*A	FbsF	5'GCTTTGGCTTTATATGGGAG3'	AJ437620.1
	FbsR	5'GCTACATTAGTAACCTGAGA3'	
cps C, D, E	CpsF	5'GCTAATGCTTGCGATGGTT3'	AB017355.1
	CpsR	5'CTGGTCTTTCTTTTCTAAGGA3'	
cps J, K, e *neu* B	NeuF	5'GGATTAGCCTTTATCACACTT3'	AB017355.1
	NeuR	5'GCAACTTCTTTAGTATTGTATA3'	

The PCR for all virulence genes were made in a total volume of 30ul, containing 1µL of each primer (10 pmol), 0.5 U Taq Flexi DNA polymerase (Promega ®, Wisconsin, USA) 3µL enzyme buffer full (10x), 1µL mix of dNTPs (100 nmoles of each base) and 5µL of DNA template (50ng/µL). The amplification was performed in 0.2 mL tubes in a device model Peltier Thermal Cycler Multi-Purpose (Biocycle ®, China). For all genes we used the same annealing temperature. The initial cycle was 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 57 ° C for 1 minute, 72°C for 2 minutes. The

final extension was 72°C for 10 minutes. Amplification products were subjected to electrophoresis on agarose gel 1.0%, which was stained with Sybr Green (Invitrogen ®, California, USA).

Amplification products were sequenced at the Central Laboratory of Molecular Biology UFLA using the same primers for PCR's. The alignments were performed using the software Mega 4.1 (http://www. Megasoftware.net/mega4/mega41.html). The identity values for nucleotide sequences were determined using the BLAST software and was compared to the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical Analysis

We compared the frequencies of virulence genes in isolates associated with clinical and subclinical mastitis using the F test, using the software SPSS 17.0 (SPSS Inc., Chicago, USA).

Results and Discussion

Phenotypic characterization

All strains were considered pure after assessment of purity by Gram staining. All samples were appeared as Gram-positive cocci arranged in a chain, with negative results in tests for catalase and esculin fermentation, lack of growth in medium containing bile-esculin and belonging to the Lancefield group B by testing SLIDEX Strepto-Kit (BioMerieux, France). These results confirm the specie *S. agalactiae* for all isolates.

CAMP test in only two strains from different herds (*S. agalactiae* 654 and *S. agalactiae* 615) obtained from subclinical mastitis were negative (Table 2, Appendix A). This is unusual result for *S. agalactiae*, because this test is used to characterize the species. However, Hensler et al. (16) also reported the

existence of nonhemolytic *S. agalactiae* strains which showed no genes encoding CAMP factor. These same strains were not attenuated for systemic virulence which may be due to the presence of another virulence factor, called β-Hemolisina/Citosina, who is also a toxin, capable of a compensatory function when the gene for factor CAMP is absent or repressed (16).

As for the phenotypic assessment of hemolysis, 5.97% of the isolates showed beta-hemolysis, 14.92% were alpha-hemolytic and 79.11% were gamma-hemolytic (Table 2, Appendix A). The predominance of hemolysis range found in the isolates tested is aligned with the result presented by Duarte et al. (10) that in cattle from Minas Gerais, Sao Paulo and Rio de Janeiro, about 50% of the isolates showed betha-hemolysis. It is known that the pattern of beta hemolysis is common in *S. agalactiae* isolated from humans (10), but in isolated bovine only a few studies.

Molecular characterization

The PCR's were optimized for oligonucleotide designed (Table 1) and the results of amplification of different virulence genes are described in Table 3 (Appendix A). Some isolates showed no amplification products for any of the genes evaluated.

PCR for the detection of *fbs*A showed the presence of the gene *fbs*A in 82% of the isolates studied (Figure 1, APPENDIX B). Among the isolates from clinical cases, amplification of this gene was detected in 100% of the strains (Table 3, APPENDIX A). It is believed that this gene has key role in the virulence of *S. agalactiae*, and it is involved even in cases of hemorrhage (13, 15, 34).

The gene encoding IP-1 that is part of the formation of pili was only amplified in the strain 199 of *S. agalactiae* was isolated from clinical case (Table 3, APPENDIX A). Although, the negative results for major strains tested

does not indicate that these isolates did not provide other genes that encode proteins forming pili, because there are many genes related to formation of this structure and polymorphisms occur within these genes (31, 42, 43). Studies have shown that strains that have undergone deletion of genes for pili, keep presenting capacity of adhesion and invasion, has been proposed action of other mechanisms (4, 37).

Only *S. agalactiae* strains 477, 506A and 1460 were positive for the region throughout the region cps operon (Table 3, APPENDIX A), and two strains, one from a clinical case and other from subclinical mastitis case, were obtained from the same herd.

PCR for operon of genes ***cps*J, *cps*K and *neu*B (Figure 2, Appendix B)** resulted positive for 53 of isolates tested, indicating that these isolates have the gene for the production of acid sialic to be integrated into the polysaccharide capsule. Among the strains isolated from clinical mastitis, only two showed no gene amplification *cps*J, *cps*K and *neu*B, but showed amplification of genes for other virulence factors. Poyart et al. (39) found in a study of strains of *S. agalactiae* from infections in humans, that when there is a large deletion of an internal region of the operon, the genes located downstream to the region of deletion may not be active, but the region located upstream of the deletion are still expressed.

A total of 24 strains (35.8%) showed gene amplification *hyl*B (Table 3, APPENDIX A). These isolates belong to ten of the 21 herds examined. Although, there was no significant difference regarding the presence of this virulence factor among strains obtained from clinical and subclinical cases ($p > 0.05$). In a study conducted by Cooke et al. (7) strains of *S. agalactiae* of human origin and two of bovine origin were compared for virulence and presence of gene *hyl*B. This virulence gene was founded in all strains. In work published by Sukhnanand et al. (45), involving strains of *S. agalactiae* from humans and

cattle, in 52 strains of bovine origin tested, only nine had the gene *hyl*B. In another study published by Yildirim, Lammle and Fink (48), *S. agalactiae* strains isolated from humans, cattle, pigs, monkeys, otters, dogs, cats and rabbits were analyzed for production of hyaluronate lyase. In this study, approximately 81% of the isolates showed positive activity of hyaluronate lyase, but there was *hyl*B gene amplification in 78% of the phenotypically negative strains. In these strains no activity for hyaluronate lyase was attributed to one insertion sequence responsible for gene inactivation *hyl*B.

Comparing the PCR results of isolates from subclinical origin with those of clinical origin, it appears that there is a higher frequency of virulence factors studied in isolates of clinical mastitis (Table 4 and Figure 3, APPENDIX B), but statistical analysis failed to confirm this observation.

Table 4 Results from PCR for virulence genes of *Streptococcus agalactiae* isolates from clinical and subclinical cases of bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010

	fbsA	**PI-1**	***hylB***	***cps* C, D e E**	***cps* J, K, *neu*B**
% of positive results obtained for strains associated to subclinical cases	80,39	0	39,21	5,88	76,47
% of positive results obtained for strains associated to clinical cases	100	6,25	37,50	6,25	87,50

By analyzing individual strains according to their origin, clinical or subclinical case, and the presence of virulence genes (Table 3, APPENDIX A, and Table 5), one realizes that in clinical isolates is higher frequency of genes of virulence (Figure 4, Appendix B). However, when analyzing by means of Fisher's test, the frequencies of occurrence of genes according to the type of mastitis, it was found that there is no relationship ($p > 0.05$) between the presence of virulence factors and the presentation of mastitis, confirming studies on the pathogenesis of the disease, explaining that this condition depends not

only on the specie, quantity, pathogenicity and infectivity of the agent involved, but also the host immune response and the environment in which host and agent are (17, 36).

Table 5 Frequencies of virulence genes in *Streptococcus agalactiae* isolates from clinical and subclinical mastitis cases in dairy herds of Minas Gerais state in the period 2004-2010

	Number of amplified genes					
	0	**1**	**2**	**3**	**4**	**5**
% of positive results for strains obtained subclinical cases	5,88	19,61	49,01	23,53	1,96	0
% of positive results for strains obtained clinical cases	0	6,25	62,50	25,00	6,25	0

These data confirm the work carried out with isolates of human origin, describing the association of two or more virulence factors, with also the possibility of a compensatory effect when the factors cannot be expressed (16, 24, 28, 42).

Sequencing

The variations in patterns of bands verified in electrophoresis of PCR products from gene *fbs*A suggested the occurrence of the polymorphism in these gene, within e among herds (Table 6, Appendix A). The polymorphism in this gene was confirmed by sequencing of PCR products. Schubert et al. (44) reported that *fbs*A gene in different strains of *S. agalactiae* of human origin, showed great variation in numbers of nucleotides in addition to variation in the composition of the repeating units in the protein, indicating genetic instability, allowing intragenic recombinations.

No differences were founded in fragments length among strains associated to clinical and suclinical mastitis cases, however, according to Schubert et al. (44) changes in repeat regions can directly interfere with the

binding of FbsA to fibrinogen, with the increase in the number of repetitions in FbsA providing a larger number of binding sites for fibrinogen and increased virulence.

Analyses of nucleotide identity performed by BLAST for genes sequenced in some sequences revealed *fbs*A (*S. agalactiae* 12 and *S. agalactiae* 252) identity values quite high, reaching 100%. Some strains showed low identity with sequences already deposited in GenBank (Table 10, Appendix A). This can be justified by the fact that there are no deposits of sequences of these genes to isolates of *S. agalactiae* of bovine origin, and comparative analyses were realized with sequences obtained from isolates of human origin. However, this result demonstrates the existence of genetic variations among isolates from human and bovine isolates, which may have effects on virulence of the isolates and the encoded protein.

The two high gene identities *fbs*A occurred in strains of *S. agalactiae* 12 and *S. agalactiae* 252, reaching a value of 100%. Each isolate obtained this value of identity with two different genetic human sequences deposited in GenBanK , which could be expected since the isolates are from different herds. When performing alignment between the sequences that showed above 85% identity with GenBank AJ437619.1 strain was verified that there is a region of conservation in the genes of approximately 500 nucleotides between them (Figure 5, APPENDIX B). This is explained because there is a conserved region of the active site of the gene in which there annealing of primers. The alignment between all isolates showed that there is little conservation of gene *fbs*A (Figure 6, APPENDIX B).

After analysis of gene *fbs*A alignment was possible to confirm the amplification of the region of the gene mat peptide (Figure 7, APPENDIX B).

Genes *hyl*B, *cps*C, *cps*D, *cps*E, *cps*J, *cps*K *neu*B and were even less conserved after alignment (Figures 8, 9 and 10, Appendix B). The amplicons for

genes *cps*C, *cps*D, *cps*E, *cps*J, *cps*K, *neu*B showed greater nucleotide variation not only among the herds, but also within each herd (Table 7, Appendix A and Table 8), which demonstrates the polymorphism of these genes. In both cases, there are no previous reports about the presence/absence of these virulence factors for *S. agalactiae* isolates from cattle.

The sequencing of amplicon products for the gene *hyl*B showed nucleotide variations within and between herds (Table 9), suggesting polymorphism of this gene. The presence of this virulence factor for *S. agalactiae* isolates from cattle has not been reported.

The highest identity for genes *cps*C, *cps*D and *cps*E was obtained for one isolate (*S. agalactiae* 506A), with 98% identity (Table 10, Appendix A) with strains of human and tilapia origins (Oreochromis niloticus), while for genes *cps*J, *cps*K and *neu*B, the highest identity was 93% (Table 10, Appendix A) in isolated *S. agalactiae* 516A and *S. agalactiae* 1026, also with a strain of human origin. Only one strain had a result of significant identity to the gene *hyl*B (Table 10, Appendix A), *S. agalactiae* 1516, with 83% identity. These results may reflect the lack of information from *S. agalactiae* of bovine origin for comparison, resulting in low homology to most isolates, some showing no significant homology.

S. agalactiae is considered a contagious pathogen that is transmitted from animal to animal normally during milking. Thus, it was expected that there were low diversity among strains obtained from the same herd. PCR amplification and sequencing of genes indicated the existence of genetic heterogeneity in isolates of *S. agalactiae* involved in the clinical and subclinical bovine mastitis, as well as among isolates within and between herds, indicating the existence of population diversity in the population of *S. agalactiae* in herds. These results contradict previous studies (5, 10, 33) that showed high identity to isolates of this agent among herds.

Research with *S. agalactiae* from bovine mastitis are still very scarce and, in Brazil, practically nonexistent, which makes this study important because it contributes to the elucidation of virulence mechanisms of the population of this agent and the generation of knowledge applicable in the control and prevention bovine mastitis.

Table 8 Approximated numbers of nucleotides determined after the sequencing of amplification products of genes *cps*C, *cps*D and *cps*E in isolates of *S. agalactiae* from bovine mastitis in cattle in Minas Gerais in the period 2004-2010

Strain	**Herd**	**Mastitis form**	**Amplicon**
S. agalactiae 461	D	Clinical	1032
S. agalactiae 506A	E	Subclinical	1170
S. agalactiae 516A		Subclinical	764
S. agalactiae 1460	S	Subclinical	956

Table 9 Approximated numbers of nucleotides determined after the sequencing of the gene *hyl*B in isolates of *Streptococcus agalactiae* from bovine mastitis in cattle in Minas Gerais in the period 2004-2010

Strains	Herd	Mastitis form	Amplicon
S. agalactiae 12	A	Subclinical	1655
S. agalactiae 40		Subclinical	1993
S. agalactiae 167	B	Clinical	1672
S. agalactiae 461	D	Clinical	1948
S. agalactiae 477	E	Clínica	1737
S. agalactiae 506A		Subclinical	656*
S. agalactiae 516A		Subclinical	1885
S. agalactiae 518		Clinical	1776
S. agalactiae 522		Subclinical	1750
S. agalactiae 529		Subclinical	405*
S. agalactiae 552A	F	Subclinical	1406
S. agalactiae 568A		Clinical	1605
S. agalactiae 580A		Subclinical	683*
S. agalactiae 654	H	Subclinical	194*
S. agalactiae 728	I	Subclinical	1543
S. agalactiae 730		Subclinical	1614
S. agalactiae 767	J	Subclinical	1473
S. agalactiae 794		Subclinical	354*
S. agalactiae 813	K	Subclinical	1507
S. agalactiae 910	L	Subclinical	458*
S. agalactiae 1051A	O	Subclinical	1234
S. agalactiae 1220	Q	Subclinical	2130
S. agalactiae 1230		Subclinical	1611
S. agalactiae 1385	R	Subclinical	1487
S. agalactiae 1516	T	Clinical	1185
S. agalactiae 1565	U	Subclinical	2108

*Parcial sequencing

Conclusões

Os testes moleculares apontaram a presença dos genes de virulência *fbs*A, *hyl*B, *cps*C, *cps*D, *cps*E, *cps*J, *cps*K, *neu*B e PI-1 na população de *S. agalactiae* estudada.

A maior frequência dos genes de virulência avaliados para os isolados provenientes de mastite clínica não é sugestiva de maior virulência dos mesmos.

Existe diversidade genética entre os isolados de *S. agalactiae* envolvidos na forma clínica e subclínica da mastite bovina, bem como entre os isolados dentro e entre rebanhos.

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Appendix A - Tables

Table 2 Results of phenotypic tests of strains of *S. agalactiae* isolates from bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010

Strains	Herd	Mastitis form	CAMP	Hemolysis
S. agalactiae 12	A	Subclinical	+	Gamma
S. agalactiae 34A	A	Subclinical	+	Gamma
S. agalactiae 40	A	Subclinical	+	Gamma
S. agalactiae 160	B	Subclinical	+	Gamma
S. agalactiae 162	B	Subclinical	+	Gamma
S. agalactiae 164	B	Subclinical	+	Gamma
S. agalactiae 167	B	Clinical	+	Alpha
S. agalactiae 199	B	Clinical	+	Gamma
S. agalactiae 252	C	Subclinical	+	Gamma
S. agalactiae 436	D	Subclinical	+	Gamma
S. agalactiae 440	D	Subclinical	+	Gamma
S. agalactiae 458	D	Subclinical	+	Gamma
S. agalactiae 461	D	Clínica	+	Gamma
S. agalactiae 477	E	Clínica	+	Alpha
S. agalactiae 506A	E	Subclinical	+	Gamma
S. agalactiae 516A	E	Subclinical	+	Gamma
S. agalactiae 518	E	Clinical	+	Alpha
S. agalactiae 522	E	Subclinical	+	Beta
S. agalactiae 529	E	Subclinical	+	Gamma
S. agalactiae 552A	F	Subclinical	+	Gamma
S. agalactiae 568A	F	Clinical	+	Gamma
S. agalactiae 580A	F	Subclinical	+	Gamma
S. agalactiae 589	F	Clinical	+	Gamma
S. agalactiae 609A	G	Clinical	+	Gamma
S. agalactiae 615	G	Subclinical	-	Gamma
S. agalactiae 617A	G	Subclinical	+	Gamma
S. agalactiae 618A	G	Subclinical	+	Gamma
S. agalactiae 654	H	Subclinical	-	Gamma
S. agalactiae 728	I	Subclinical	+	Gamma
S. agalactiae 730	I	Subclinical	+	Gamma
S. agalactiae 767	J	Subclinical	+	Alpha
S. agalactiae 794	J	Subclinical	+	Beta
S. agalactiae 813	K	Subclinical	+	Alpha
S. agalactiae 910	L	Subclinical	+	Alpha
S. agalactiae 926	L	Subclinical	+	Gamma
S. agalactiae 941	L	Clinical	+	Alpha
S. agalactiae 960	M	Clinical	+	Gamma
S. agalactiae 999A	N	Clinical	+	Gamma
S. agalactiae 1001	N	Subclinical	+	Alpha
S. agalactiae 1007	N	Subclinical	+	Alpha
S. agalactiae 1013	N	Subclinical	+	Gamma
S. agalactiae 1026	N	Clinical	+	Gamma

Table 2, conclusion

S. agalactiae 1027	N	Subclinical	+	Gamma
S. agalactiae 1051A	O	Subclinical	+	Gamma
S. agalactiae 1092	O	Subclinical	+	Gamma
S. agalactiae 1093	O	Clinical	+	Gamma
S. agalactiae 1097	O	Subclinical	+	Beta
S. agalactiae 1102	P	Subclinical	+	Gamma
S. agalactiae 1137	P	Subclinical	+	Gamma
S. agalactiae 1205	Q	Subclinical	+	Gamma
S. agalactiae 1220	Q	Subclinical	+	Gamma
S. agalactiae 1230	Q	Subclinical	+	Beta
S. agalactiae 1385	R	Subclinical	+	Gamma
S. agalactiae 1388	R	Subclinical	+	Gamma
S. agalactiae 1427	R	Subclinical	+	Gamma
S. agalactiae 1438	S	Subclinical	+	Gamma
S. agalactiae 1453	S	Clinical	+	Gamma
S. agalactiae 1457	S	Subclinical	+	Gamma
S. agalactiae 1460	S	Subclinical	+	Gamma
S. agalactiae 1495	T	Subclinical	+	Gamma
S. agalactiae 1496	T	Subclinical	+	Gamma
S. agalactiae 1497	T	Subclinical	+	Gamma
S. agalactiae 1514	T	Clinical	+	Gamma
S. agalactiae 1516	T	Clinical	+	Gamma
S. agalactiae 1528	U	Subclinical	+	Alpha
S. agalactiae 1540	U	Subclinical	+	Gamma
S. agalactiae 1565	U	Subclinical	+	Gamma

Table 3 Results of the individual's PCR for amplification of virulence genes of *Streptococcus agalactiae* isolates from bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010

Strains	**Herd**	**Mastitis form**	***fbsA***	**PI-1**	***hylB***	***cps* C, D e E**	***cps* J, K, *neu*B**
S. agalactiae 12	A	Subclinical	P	N	P	N	P
S. agalactiae 34A	A	Subclinical	N	N	N	N	N
S. agalactiae 40	A	Subclinical	P	N	P	N	P
S. agalactiae 160	B	Subclinical	P	N	N	N	P
S. agalactiae 162	B	Subclinical	P	N	N	N	P
S. agalactiae 164	B	Subclinical	P	N	N	N	P
S. agalactiae 167	B	Clinical	P	N	P	N	N
S. agalactiae 199	B	Clinical	P	P	N	N	P
S. agalactiae 252	C	Subclinical	P	N	N	N	N
S. agalactiae 436	D	Subclinical	P	N	N	N	P
S. agalactiae 440	D	Subclinical	P	N	N	N	P
S. agalactiae 458	D	Subclinical	N	N	N	N	P
S. agalactiae 461	D	Clinical	P	N	P	N	P
S. agalactiae 477	E	Clinical	P	N	P	P	P
S. agalactiae 506A	E	Subclinical	P	N	P	P	P
S. agalactiae 516A	E	Subclinical	P	N	P	N	P
S. agalactiae 518	E	Clinical	P	N	P	N	P
S. agalactiae 522	E	Subclinical	P	N	P	N	N
S. agalactiae 529	E	Subclinical	P	N	P	N	P
S. agalactiae 552A	F	Subclinical	P	N	P	N	N
S. agalactiae 568A	F	Clinical	P	N	P	N	P
S. agalactiae 580A	F	Subclinical	P	N	P	N	N
S. agalactiae 589	F	Clinical	P	N	N	N	P
S. agalactiae 609A	G	Clinical	P	N	N	N	P
S. agalactiae 615	G	Subclinical	N	N	N	N	N
S. agalactiae 617A	G	Subclinical	P	N	N	N	P
S. agalactiae 618A	G	Subclinical	P	N	N	N	P
S. agalactiae 654	H	Subclinical	N	N	P	N	N
S. agalactiae 728	I	Subclinical	N	N	P	N	P
S. agalactiae 730	I	Subclinical	N	N	N	N	P
S. agalactiae 767	J	Subclinical	P	N	P	N	N
S. agalactiae 794	J	Subclinical	P	N	P	N	P
S. agalactiae 813	K	Subclinical	P	N	P	N	P
S. agalactiae 910	L	Subclinical	N	N	P	N	P
S. agalactiae 926	L	Subclinical	P	N	N	N	N
S. agalactiae 941	L	Clinical	P	N	N	N	N
S. agalactiae 960	M	Clinical	P	N	N	N	P
S. agalactiae 999A	N	Clinical	P	N	N	N	P
S. agalactiae 1001	N	Subclinical	N	N	N	N	N
S. agalactiae 1007	N	Subclinical	P	N	N	N	N
S. agalactiae 1013	N	Subclinical	N	N	N	N	P
S. agalactiae 1026	N	Clinical	P	N	N	N	P
S. agalactiae 1027	N	Subclinical	P	N	N	N	P
S. agalactiae 1051A	O	Subclinical	P	N	P	N	P

Table 3, conclusion

S. agalactiae 1092	O	Subclinical	N	N	N	N	P
S. agalactiae 1093	O	Clinical	P	N	N	N	P
S. agalactiae 1097	O	Subclinical	P	N	N	N	N
S. agalactiae 1102	P	Subclinical	P	N	N	N	P
S. agalactiae 1137	P	Subclinical	P	N	N	N	P
S. agalactiae 1205	Q	Subclinical	P	N	N	N	P
S. agalactiae 1220	Q	Subclinical	P	N	P	N	P
S. agalactiae 1230	Q	Subclinical	P	N	P	N	P
S. agalactiae 1385	R	Subclinical	P	N	P	N	P
S. agalactiae 1388	R	Subclinical	P	N	N	N	P
S. agalactiae 1427	R	Subclinical	N	N	N	N	P
S. agalactiae 1438	S	Subclinical	P	N	N	N	P
S. agalactiae 1453	S	Clinical	P	N	N	N	P
S. agalactiae 1457	S	Subclinical	P	N	N	N	P
S. agalactiae 1460	S	Subclinical	P	N	N	P	P
S. agalactiae 1495	T	Subclinical	P	N	N	N	P
S. agalactiae 1496	T	Subclinical	P	N	N	N	P
S. agalactiae 1497	T	Subclinical	P	N	N	N	P
S. agalactiae 1514	T	Clinical	P	N	N	N	P
S. agalactiae 1516	T	Clinical	P	N	P	N	P
S. agalactiae 1528	U	Subclinical	P	N	P	N	P
S. agalactiae 1540	U	Subclinical	P	N	N	N	P
S. agalactiae 1565	U	Subclinical	P	N	P	N	P

N (Not amplified) e P (presence)

Table 6 Approximated numbers of nucleotides determined after the sequencing of the gene *fbs*A in *Streptococcus agalactiae* isolated from bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010.

Strains	Herd	Mastitis form	Amplicon
S. agalactiae 12	A	Subclinical	562
S. agalactiae 40		Subclinical	669
S. agalactiae 160	B	Subclinical	547
S. agalactiae 162		Subclinical	570
S. agalactiae 164		Subclinical	557
S. agalactiae 199		Clinical	538
S. agalactiae 252	C	Subclinical	218
S. agalactiae 436	D	Subclinical	270
S. agalactiae 440		Subclinical	320
S. agalactiae 461		Clinical	328
S. agalactiae 477	E	Clinical	724
S. agalactiae 506A		Subclinical	278
S. agalactiae 516A		Subclinical	276
S. agalactiae 518		Clinical	274
S. agalactiae 522		Subclinical	282
S. agalactiae 529		Subclinical	265
S. agalactiae 552A	F	Subclinical	327
S. agalactiae 568A		Clinical	319
S. agalactiae 580A		Subclinical	339
S. agalactiae 589		Clinical	361
S. agalactiae 609A	G	Clinical	697
S. agalactiae 617A		Subclinical	652
S. agalactiae 618A		Subclinical	764
S. agalactiae 767	J	Subclinical	524
S. agalactiae 794		Subclinical	589
S. agalactiae 813	K	Subclinical	328
S. agalactiae 926	L	Subclinical	337
S. agalactiae 960	M	Clinical	538
S. agalactiae 999A	N	Clinical	331
S. agalactiae 1007		Subclinical	355
S. agalactiae 1026		Clinical	325
S. agalactiae 1027		Subclinical	337
S. agalactiae 1051A	O	Subclinical	522
S. agalactiae 1093		Clinical	586
S. agalactiae 1097		Subclinical	539
S. agalactiae 1102	P	Subclinical	603
S. agalactiae 1137		Subclinical	567
S. agalactiae 1205	Q	Subclinical	581
S. agalactiae 1220		Subclinical	708
S. agalactiae 1230		Subclinical	247

Table 6, conclusion

S. agalactiae 1385	R	Subclinical	648
S. agalactiae 1388		Subclinical	617
S. agalactiae 1438	S	Subclinical	686
S. agalactiae 1453		Clinical	626
S. agalactiae 1457		Subclinical	717
S. agalactiae 1460		Subclinical	604
S. agalactiae 1495	T	Subclinical	537
S. agalactiae 1496		Subclinical	547
S. agalactiae 1497		Subclinical	717
S. agalactiae 1514		Clinical	622
S. agalactiae 1516		Clinical	592
S. agalactiae 1528	U	Subclinical	331
S. agalactiae 1540		Subclinical	583
S. agalactiae 1565		Subclinical	562

Table 7 Approximated numbers of nucleotides determined after the sequencing of amplification products of genes cpsJ, cpsK NeuB and in *S. agalactiae* isolated from bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010

Strains	Herd	Mastitis form	Amplicon
S. agalactiae 12	A	Subclinical	785
S. agalactiae 40		Subclinical	881
S. agalactiae 160	B	Subclinical	1302
S. agalactiae 162		Subclinical	840
S. agalactiae 164		Subclinical	840
S. agalactiae 199		Clinical	1004
S. agalactiae 436	D	Subclinical	1113
S. agalactiae 440		Subclinical	1052
S. agalactiae 458		Subclinical	703
S. agalactiae 461		Clinical	1032
S. agalactiae 477	E	Clinical	927
S. agalactiae 506A		Subclinical	1170
S. agalactiae 516A		Subclinical	764
S. agalactiae 518		Clinical	953
S. agalactiae 529		Subclinical	950
S. agalactiae 568A	F	Clinical	1248
S. agalactiae 589		Clinical	1009
S. agalactiae 609A	G	Clinical	1037
S. agalactiae 617A		Subclinical	1034
S. agalactiae 618A		Subclinical	942
S. agalactiae 728	I	Subclinical	977
S. agalactiae 730		Subclinical	862
S. agalactiae 794	J	Subclinical	594
S. agalactiae 813	K	Subclinical	1097
S. agalactiae 910	L	Subclinical	1060
S. agalactiae 960	M	Clinical	988
S. agalactiae 999A	N	Clinical	959
S. agalactiae 1013		Subclinical	904
S. agalactiae 1026		Clinical	799
S. agalactiae 1027		Subclinical	862
S. agalactiae 1051A	O	Subclinical	957
S. agalactiae 1092		Clinical	985
S. agalactiae 1093		Subclinical	1033
S. agalactiae 1102	P	Subclinical	892
S. agalactiae 1137		Subclinical	882
S. agalactiae 1205	Q	Subclinical	883
S. agalactiae 1220		Subclinical	941
S. agalactiae 1230		Subclinical	1072
S. agalactiae 1385	R	Subclinical	1042
S. agalactiae 1388		Subclinical	958
S. agalactiae 1427		Subclinical	1136

Table 7, conclusion

S. agalactiae 1438	S	Subclinical	872
S. agalactiae 1453		Clinical	797
S. agalactiae 1457		Subclinical	960
S. agalactiae 1460		Subclinical	956
S. agalactiae 1495	T	Subclinical	899
S. agalactiae 1496		Subclinical	894
S. agalactiae 1497		Subclinical	810
S. agalactiae 1514		Clinical	769
S. agalactiae 1516		Clinical	824
S. agalactiae 1528	U	Subclinical	970
S. agalactiae 1565		Subclinical	788

Table 10 Nucleotide identity of *S. agalactiae* virulence genes isolated from bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010 with *S. agalactiae* sequences deposited in genBank

Strains	Genes	Identity values	Identified sequences GenBank	Origin species
***S. agalactiae* 12**	*fbsA*	100%	*S. agalactiae* - AJ437619.1	Human
	hylB	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 40**	*fbsA*	90%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437621.1	
	hylB	nss		
	*cps*J e K, *neu*B	86%	*S. agalactiae* - AY375362.1	Human
			S. agalactiae - EF990365.1	
			S. agalactiae - EF990364.1	
			S. agalactiae - CP000114.1	
			S. agalactiae - AL766849.1	
			S. agalactiae - AF163833.1	
			S. agalactiae - AB028896.2	
			S. agalactiae - AB017355.1	
***S. agalactiae* 160**	*fbsA*	90%	*S. agalactiae* - AL766848.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 162**	*fbsA*	94%	*S. agalactiae* - AL766848.1	Human
	*cps*J e K, *neu*B	86%	*S. agalactiae* - AY375362.1	Human
			S. agalactiae - EF990365.1	
			S. agalactiae - EF990364.1	
			S. agalactiae - CP000114.1	
			S. agalactiae -AL766849.1	
			S. agalactiae - AB028896.2	
			S. agalactiae GenBank: AB017355.1	
***S. agalactiae* 164**	*fbsA*	92%	*S. agalactiae* GenBank: AJ421083.1	Human
			S. agalactiae GenBank: AJ437622.1	
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 167**	*fbsA*	nss		
	hylB	nss		

Table 10, continuation

***S. agalactiae* 199**	*fbsA*	94%	*S. agalactiae* - AJ437622.1	Human
			S. agalactiae - AJ421083.1	
			S. agalactiae - AJ437621.1	
	PI-1	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 252**	*fbsA*	100%	*S. agalactiae* - AJ437620.1	Human
***S. agalactiae* 436**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 440**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 458**	*cps*J e K, *neu*B	nss		
***S. agalactiae* 461**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437619.1	
	hylB	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 477**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AE009948.1	
	hylB	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 506A**	*fbsA*	97%	*S. agalactiae* - AE009948.1	Human
	hylB	nss		
	cps C, D e E	98%	*S. agalactiae* - GU217534.1	Tilapia
			S. agalactiae - GU217533.1	
			S. agalactiae - EF524088.1	Human
			S. agalactiae - EF524086.1	
			S. agalactiae - DQ652541.1	
			S. agalactiae - DQ652540.1	
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 516A**	*fbsA*	98%	*S. agalactiae* - AL766848.1	Human
	hylB	nss		
	*cps*J e K, *neu*B	93%	*S. agalactiae* - AM498296.1	Human
***S. agalactiae* 518**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ421083.1	
	hylB	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 522**	*fbsA*	98%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ421083.1	

Table 10, continuation

			S. agalactiae - AJ437619.1	
	hylB	nss		
***S. agalactiae* 529**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437619.1	
	hylB	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 552A**	*fbsA*	96%	*S. agalactiae* - AL766848.1	Human
	hylB	nss		
***S. agalactiae* 568A**	*fbsA*	96%	*S. agalactiae* - AJ437619.1	Human
			S. agalactiae - AE009948.1	
	hylB	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 580A**	*fbsA*	93%	*S. agalactiae* - AJ437619.1	Human
	hylB	nss		
***S. agalactiae* 589**	*fbsA*	95%	*S. agalactiae* - AJ437619.1	Human
	*cps*J e K, *neu*B	86%	*S. agalactiae* - EF990365.1	
			S. agalactiae - EF990364.1	
			S. agalactiae - CP000114.1	
			S. agalactiae - AY375362.1	
			S. agalactiae - AL766849.1	
			S. agalactiae - AF163833.1	
			S. agalactiae - AB028896.2	
			S. agalactiae - AB017355.1	
***S. agalactiae* 609A**	*fbsA*	92%	*S. agalactiae* - AJ437621.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 617A**	*fbsA*	92%	*S. agalactiae* - AL766848.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 618A**	*fbsA*	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 654**	*hylB*	nss		
***S. agalactiae* 728**	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 730**	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 767**	*fbsA*	96%	*S. agalactiae* - CP000114.1	Human
	*hyl*B	nss		
***S. agalactiae* 794**	*fbsA*	91%	*S. agalactiae* - AL766848.1	Human
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		

Table 10, continuation

***S. agalactiae* 813**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437619.1	
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 910**	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 926**	*fbsA*	97%	*S. agalactiae* - AJ437619.1	Human
***S. agalactiae* 960**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ421083.1	
			S. agalactiae - AJ437619.1	
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 999A**	*fbsA*	98%	*S. agalactiae* - AJ437619.1	Human
	*cps*J e K, *neu*B	87%	*S. agalactiae* - EF990365.1	
			S. agalactiae - EF990364.1	
			S. agalactiae - AY375362.1	
			S. agalactiae - CP000114.1	
			S. agalactiae - AL766849.1	
			S. agalactiae - AB028896.2	
			S. agalactiae - AB017355.1	
***S. agalactiae* 1007**	*fbsA*	98%	*S. agalactiae* - AJ437619.1	Human
***S. agalactiae* 1013**	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1026**	*fbsA*	96%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437619.1	
			S. agalactiae - AE009948.1	
	*cps*J e K, *neu*B	93%	*S. agalactiae* - AM498296.1	
***S. agalactiae* 1027**	*fbsA*	97%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ421083.1	
			S. agalactiae - AJ421083.1	
			S. agalactiae - AJ437620.1	
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1051A**	*fbsA*	94%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437621.1	
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		

Table 10, continuation

***S. agalactiae* 1092**	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1093**	*fbsA*	95%	*S. agalactiae* - AJ421083.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1097**	*fbsA*	92%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437621.1	
***S. agalactiae* 1102**	*fbsA*	91%	*S. agalactiae* - AJ421083.1	Human
			S. agalactiae - AL766848.1	
	*cps*J e K, *neu*B	91%	*S. agalactiae* - AM498296.1	Human
***S. agalactiae* 1137**	*fbsA*	96%	*S. agalactiae* - CP000114.1	Human
	*cps*J e K, *neu*B	84%	*S. agalactiae* - EF990365.1	
			S. agalactiae - EF990364.1	
			S. agalactiae - AY375362.1	
			S. agalactiae - CP000114.1	
			S. agalactiae - AL766849.1	
			S. agalactiae - AF163833.1	
			S. agalactiae - AB028896.2	
			S. agalactiae - AB017355.1	
***S. agalactiae* 1205**	*fbsA*	92%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437621.1	
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1220**	*fbsA*	89%	*S. agalactiae* - AJ421083.1	Human
			S. agalactiae - AL766848.1	
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1230**	*fbsA*	93%	*S. agalactiae* - AE009948.1	Human
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1385**	*fbsA*	94%	*S. agalactiae* - AJ421083.1	Human
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1388**	*fbsA*	93%	*S. agalactiae* - AJ421083.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1427**	*cps*J e K, *neu*B	nss		

Table 10, continuation

***S. agalactiae* 1438**	*fbsA*	90%	*S. agalactiae* - AJ421083.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1453**	*fbsA*	82%	*S. agalactiae* - AJ421083.1	Human
			S. agalactiae - AJ437622.1	
			S. agalactiae - AJ437619.1	
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1457**	*fbsA*	88%	*S. agalactiae* - CP000114.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1460**	*fbsA*	93%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437621.1	
	cps C, D e E	nss		
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1495**	*fbsA*	94%	*S. agalactiae* - AE009948.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1496**	*fbsA*	95%	*S. agalactiae* - AE009948.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1497**	*fbsA*	87%	*S. agalactiae* - CP000114.1	Human
	*cps*J e K, *neu*B	86%	*S. agalactiae* - CP000114.1	Human
			S. agalactiae - AL766849.1	
			S. agalactiae - AF163833.1	
			S. agalactiae - AB028896.2	
			S. agalactiae - AB017355.1	
***S. agalactiae* 1514**	*fbsA*	91%	*S. agalactiae* - AJ437621.1	Human
	*cps*J e K, *neu*B	nss		
***S. agalactiae* 1516**	*fbsA*	96%	*S. agalactiae* - AL766848.1	Human
			S. agalactiae - AJ437621.1	
	*hyl*B	83%	*S. agalactiae* - AE009948.1	Human
			S. agalactiae - AL766849.1	
			S. agalactiae - Y15903.1	
			S. agalactiae - U15050.1	
	*cps*J e K, *neu*B	92%	*S. agalactiae* - AM498296.1	Human
***S. agalactiae* 1528**	*fbsA*	92%	*S. agalactiae* - AJ421083.1	Human
			S. agalactiae - AJ437621.1	
	*hyl*B	nss		
	*cps*J e K, *neu*B	92%	*S. agalactiae* - AM498296.1	Human

Table 10, conclusion

***S. agalactiae* 1540**	*fbsA*	94%	*S. agalactiae* - AL766848.1 *S. agalactiae* - AJ421083.1	Human
***S. agalactiae* 1565**	*fbsA*	94%	*S. agalactiae* - AJ421083.1	Human
	*hyl*B	nss		
	*cps*J e K, *neu*B	nss		

nss (none significant similarity)

Appendix B – Figures

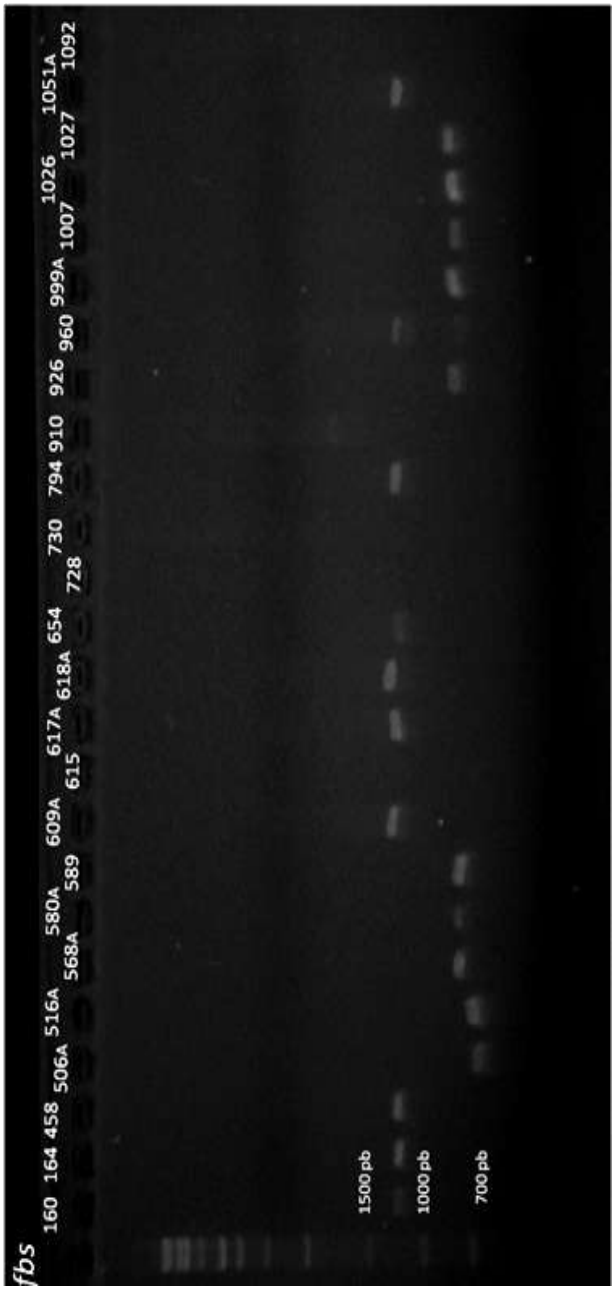

Figure 1 Electrophoresis of the *fbs*A gene. In the two gels is the presence of gene fragments that have varying sizes of amplicons of approximately 300pb to 1500pb.

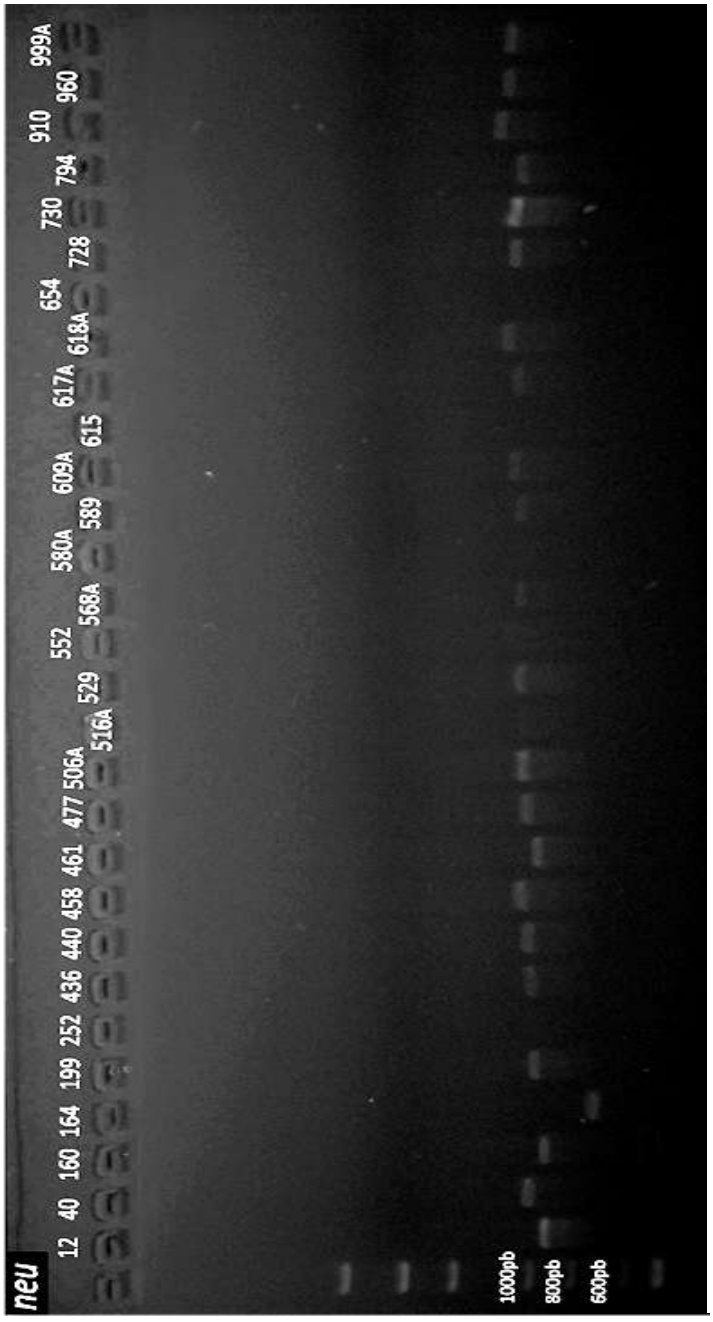

Figure 2 Electrophoresis of *cpsJ*, K / *neu*B gene. In the gel is present gene fragments that showed a size of amplicons ranging from approximately 600pb to 1000pb.

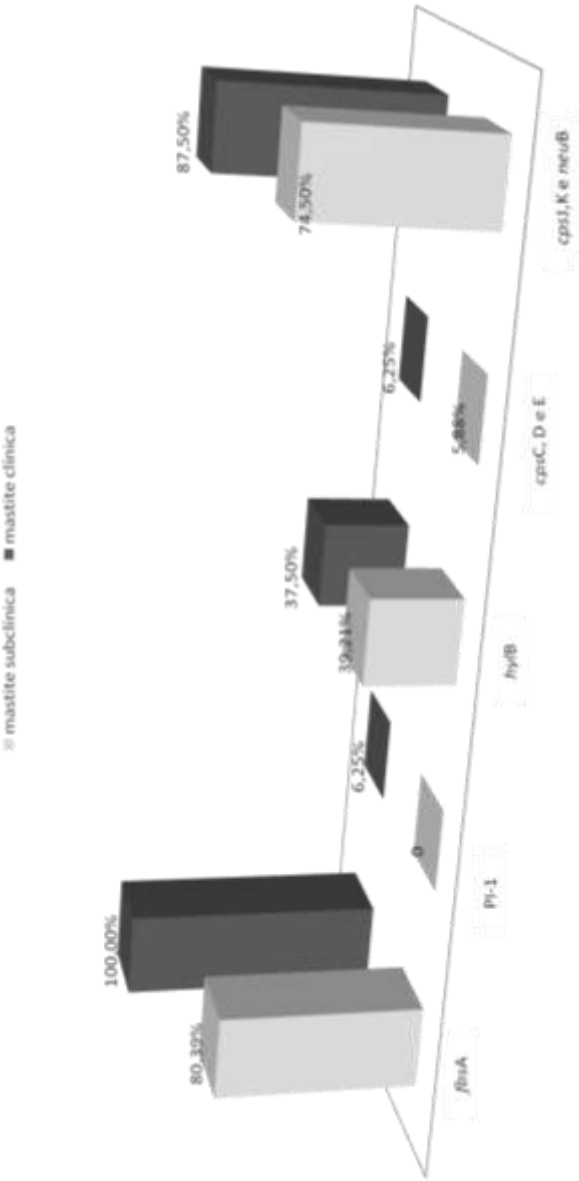

Figure 3 Percentage of gene amplification *fbs*A, PI-1, *hyl*B, *cps*C, D, E and K *neu*B *cps*J in isolates from clinical and subclinical mastitis.

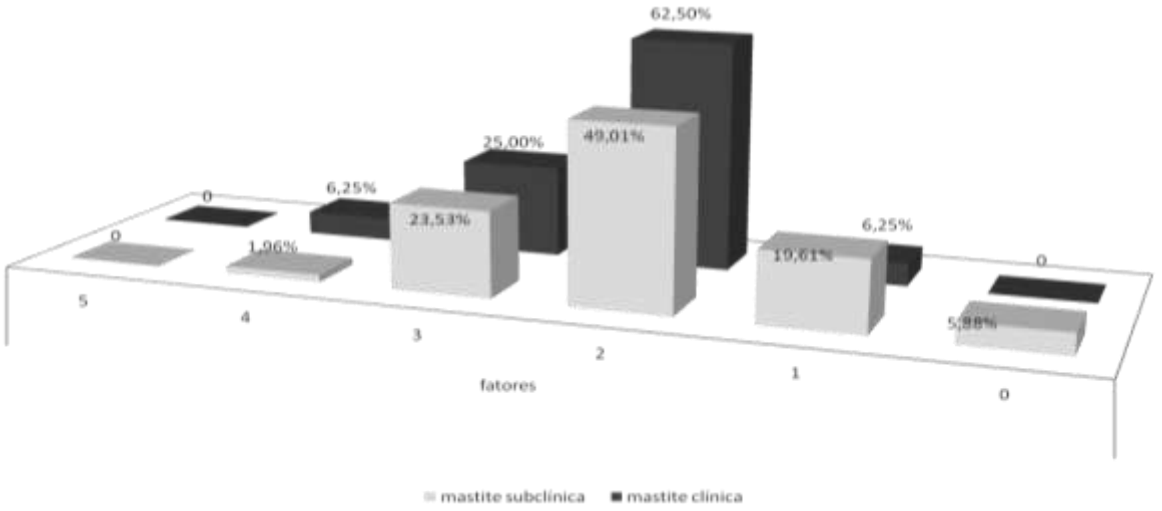

Figure 4 Graph comparing the combination of virulence factors. The columns show the occurrence of virulence factors, based on the results of amplificant ion of genes surveyed, each alone: 0 (no gene amplification), 1 (a gene amplified), 2 (two amplified genes), 3 (three genes amplified) 4 (four genes amplified) and 5 (five genes amplified).

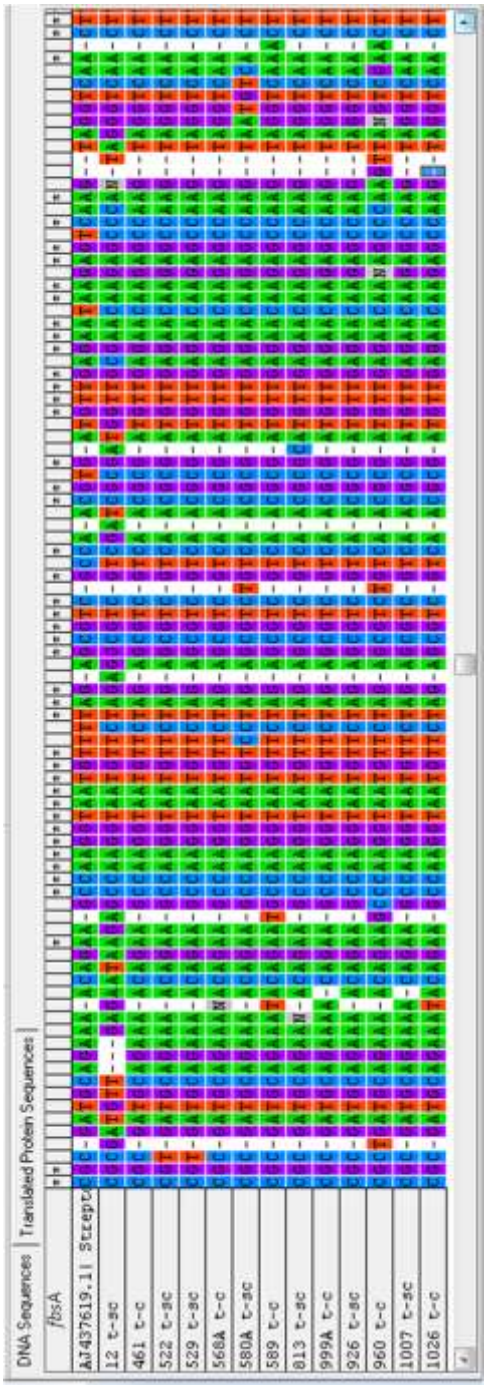

Figure 5 Alignment of the *fbs*A gene in isolates of *S. agalactiae* showed that nucleotide identity over 86% with the strain AJ437619 (GenBank) highlighting a region with greater conservation.

Figure 6 Alignment of the *fbs*A gene in isolates of *S. agalactiae*, highlighting the gene polymorphism in region among nucleotides 1940 and 2066. (Continued)

Figure 6 Alignment of the *fbs*A gene in isolates of *S. agalactiae*, highlighting the gene polymorphism in region among nucleotides 2067 and 2191. (Continued)

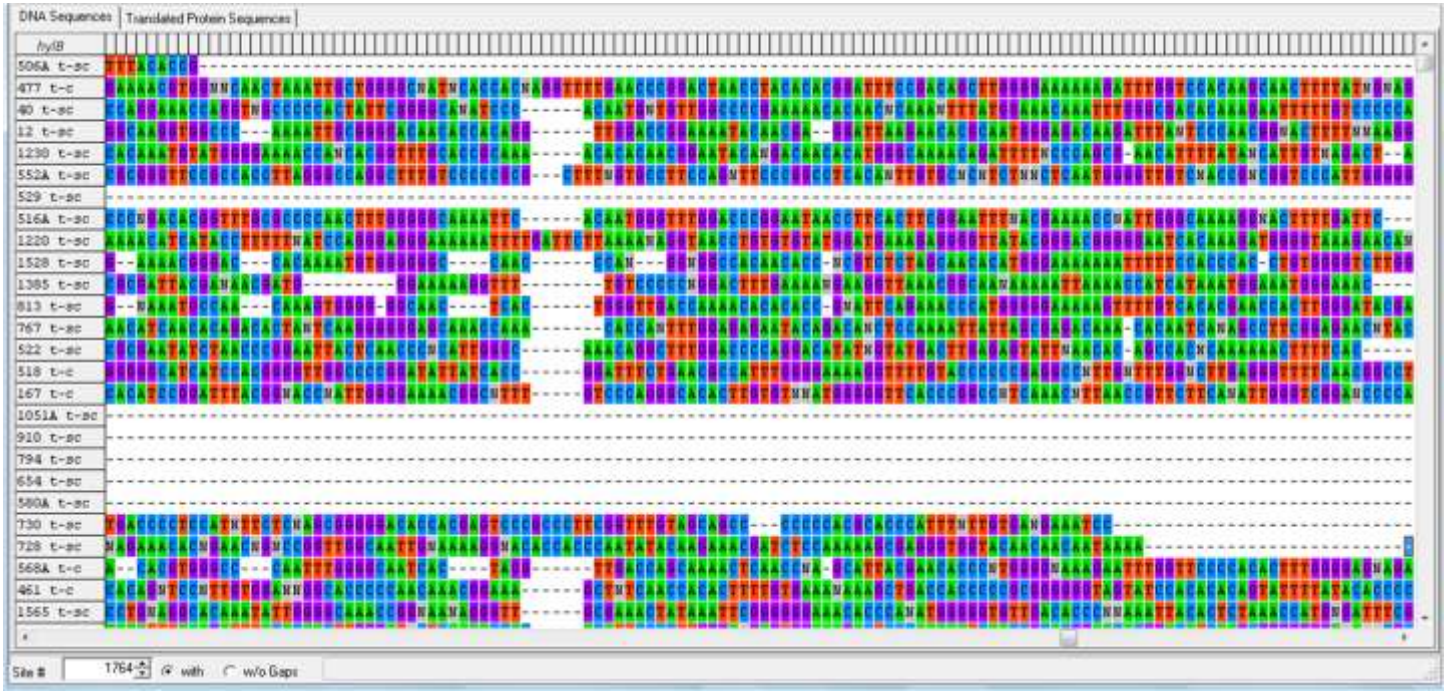

Figure 7 Alignment of the *fbs*A gene in isolates of *S. agalactiae*, highlighting the region between nucleotides 217 and 2349 that corresponds to the mat peptide.

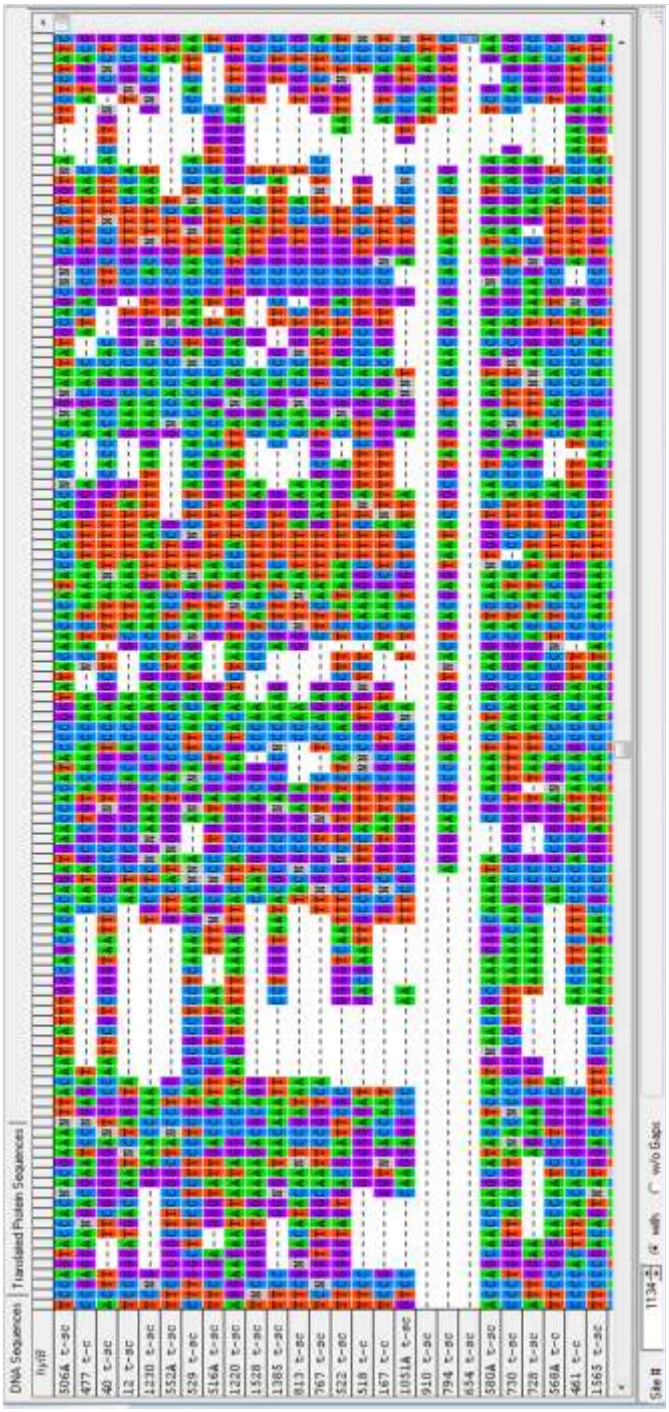

Figure 8 Alignment of the *hylB* gene in isolates of *S. agalactiae*, highlighting the gene polymorphism. Number region among nucleotides 1009 and 1134. (Continued)

Figure 8 Alignment of the *hylB* gene in isolates of *S. agalactiae*, highlighting the gene polymorphism. Number region among nucleotides 1135 and 1260. (Continued)

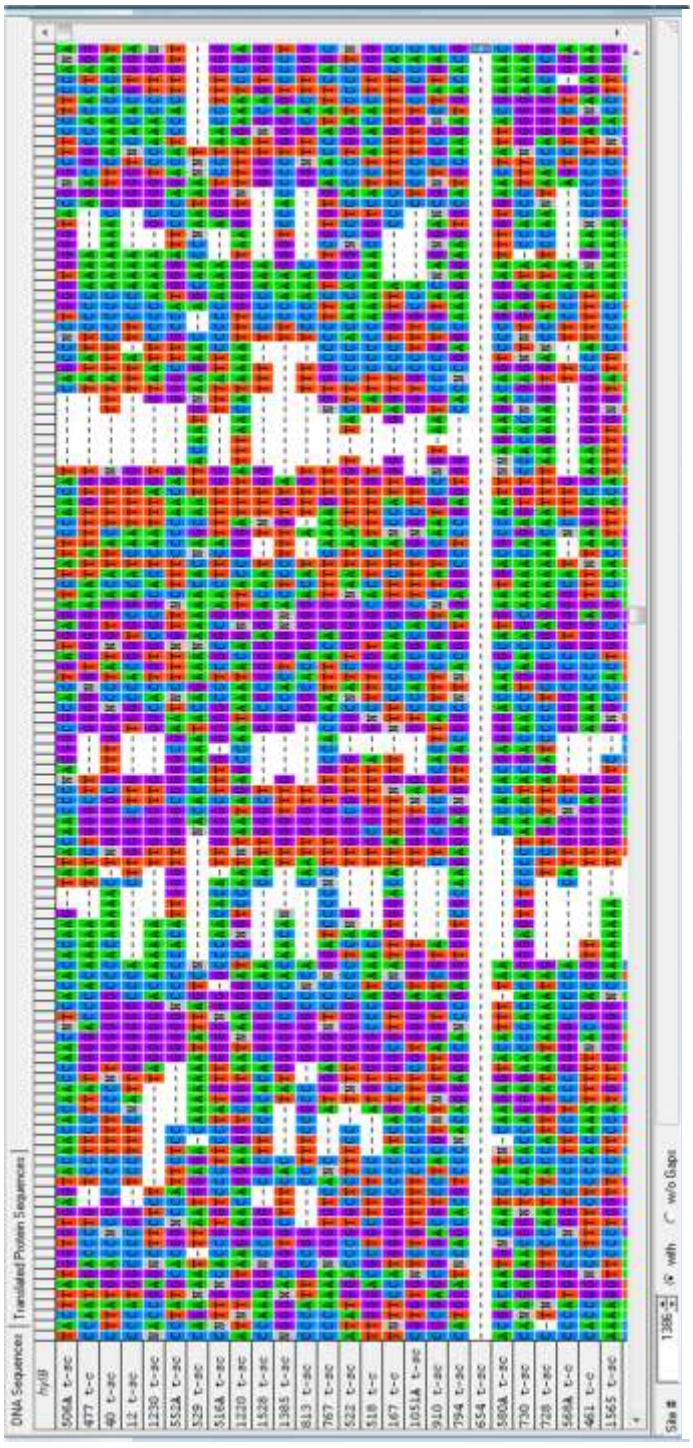

Figure 8 Alignment of the *hyl*B gene in isolates of *S. agalactiae*, highlighting the gene polymorphism. Number region among nucleotides 1261 and 1386. (Continued)

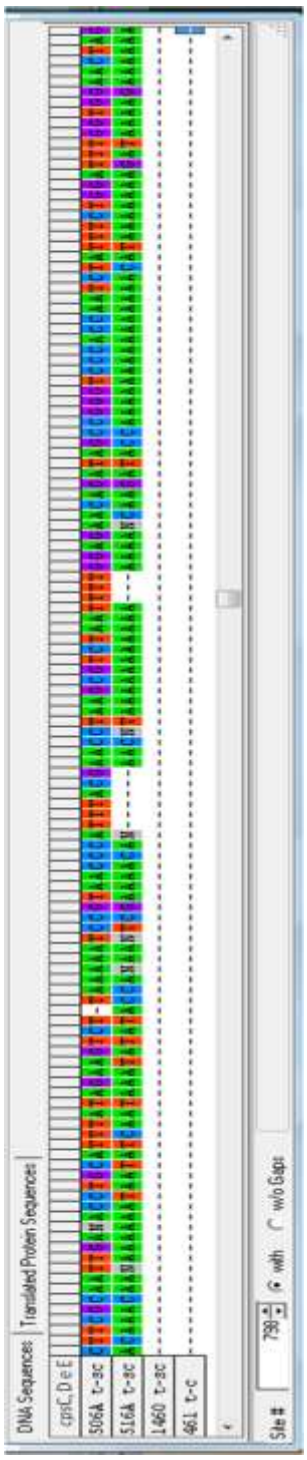

Figure 9 Alignment of the *cps*C, *cps*D and *cps*E genes in isolates of *S. agalactiae*, highlighting the gene polymorphism in region among nucleotides 670 and 798. (Continued)

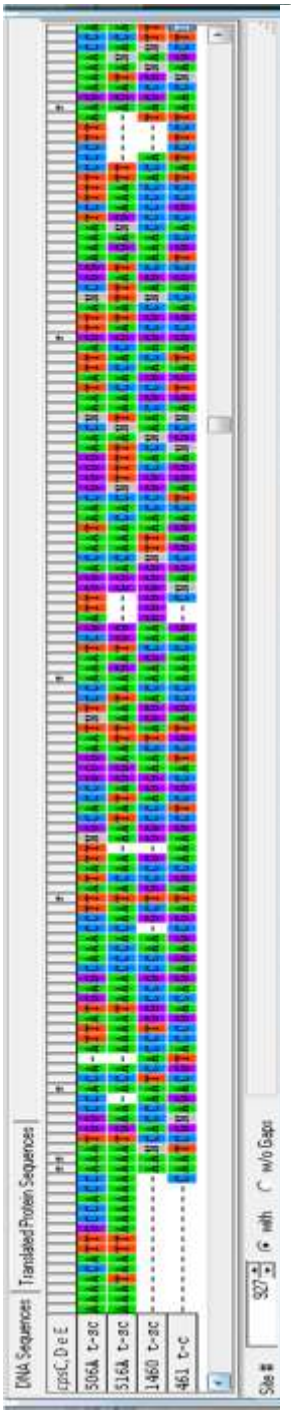

Figure 9 Alignment of the *cps*C, *cps*D and *cps*E genes in isolates of *S. agalactiae*, highlighting the gene Polymorphism in region among nucleotides 799 and 927. (Continued)

Figure 9 Alignment of the *cps*C, *cps*D and *cps*E genes in isolates of *S. agalactiae*, highlighting the gene Polymorphism in region among nucleotides 928 and 1055. (Continued)

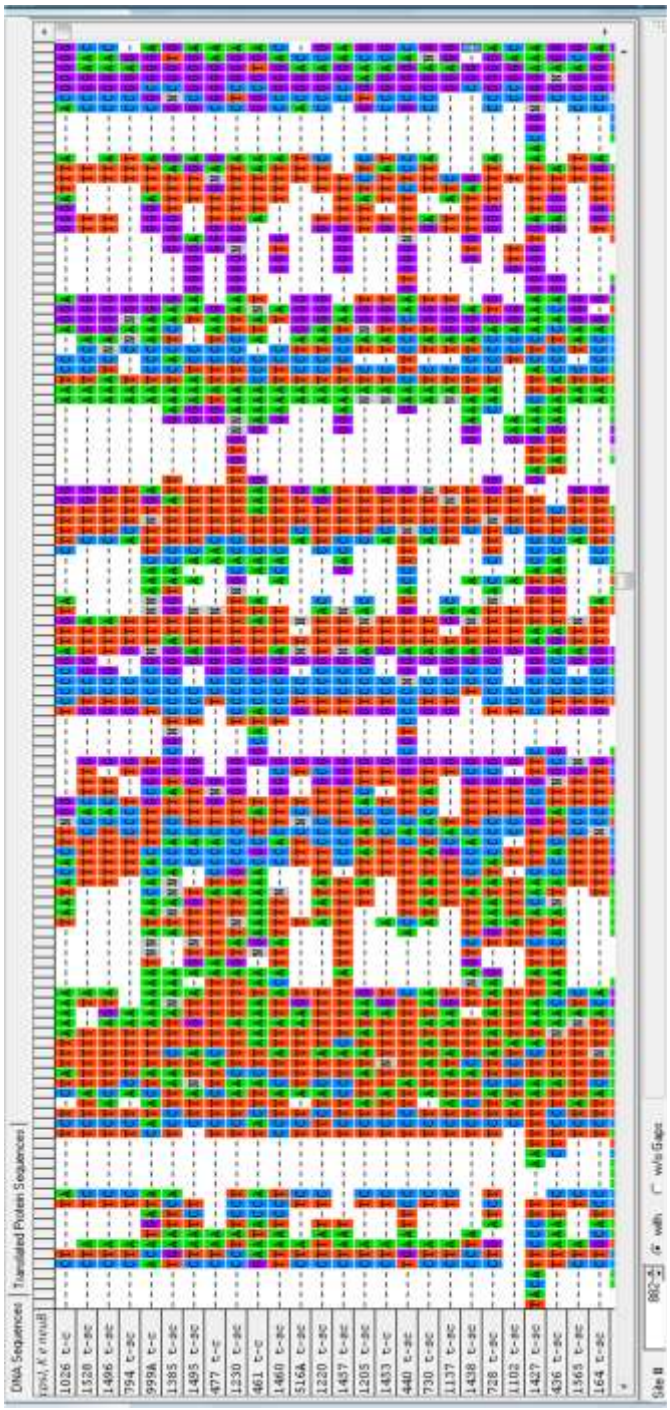

Figure 10 Alignment of the *cpsJ*, *cpsK* and *neuB* genes in isolates of *S. agalactiae*, highlighting the gene Polymorphism in region among nucleotides 757 and 882. (Continued)

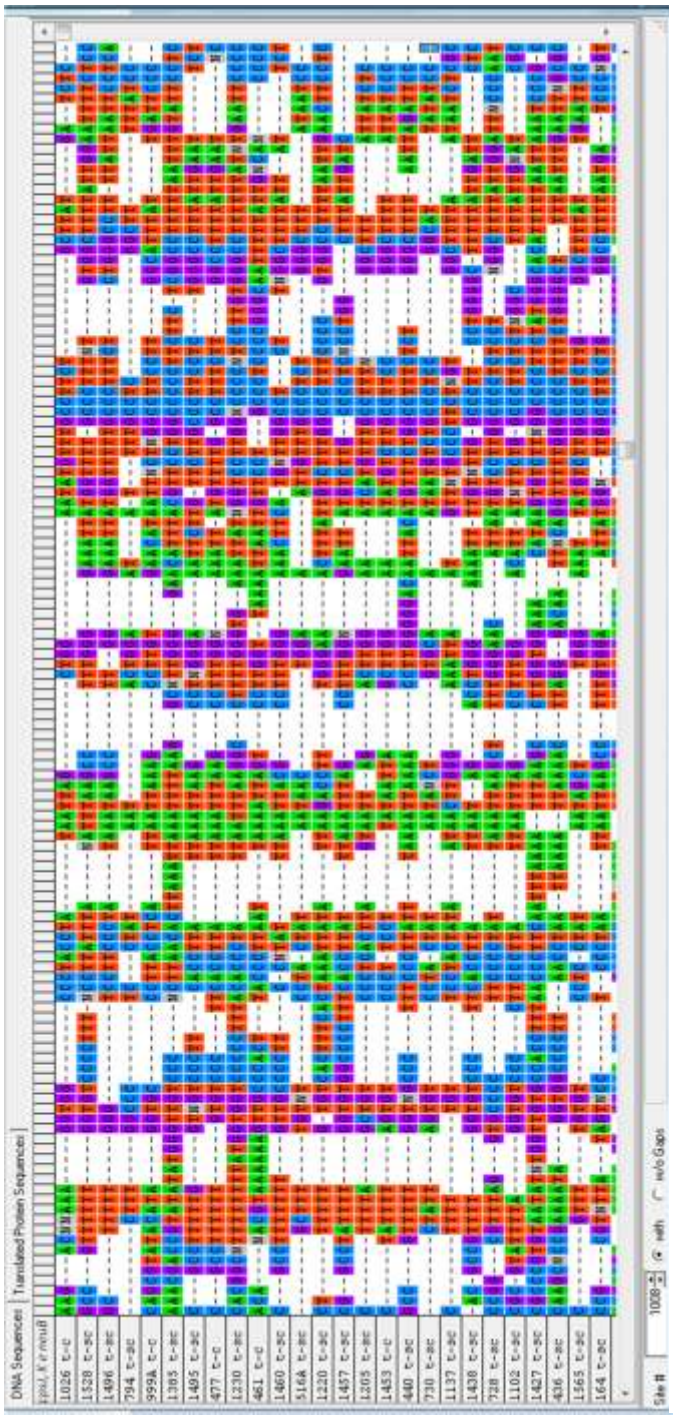

Figure 10 Alignment of the *cps*J, *cps*K and *neu*B genes in isolates of *S. agalactiae*, highlighting the gene Polymorphism in region among nucleotides 883 and 1008. (Continued)

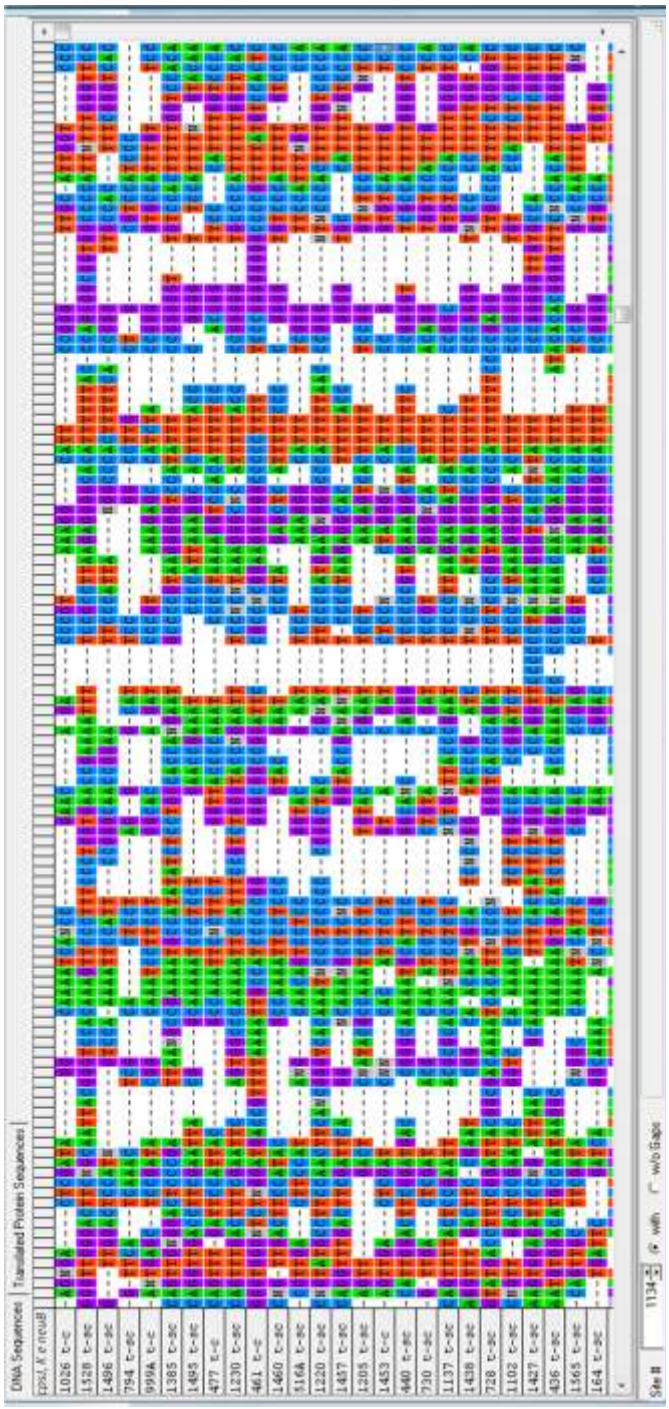

Figure 10 Alignment of the *cps*J, *cps*K and *neu*B genes in isolates of *S. agalactiae,* highlighting the gene Polymorphism in region among nucleotides 1009 and 1134. (Continued)