Virulence Genes of the *Streptococcus agalactiae* Associated with Bovine Mastitis in Minas Gerais Livestock Herds, Brazil

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**Abstract**

Brazil has the second largest dairy herd in the world. Minas Gerais is the largest milk producer in Brazil and accounts for about 30% of all production in the country. The mastitis is a disease that causes major losses in the dairy industry under the economic point of view, because 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 12 and 15%. Whatever its origin, there are chemical and physical changes in the milk, accompanied by pathological changes in the glandular tissue. *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 *fbsA*, *cpsC*, *cpsD*, *cpsE*, *cpsK*, *neuB* and the PI-1 cluster of 16 isolates of *Streptococcus agalactiae* from clinical mastitis and subclinical mastitis. Molecular analysis showed the presence of gene *fbsA* in 85.07% of the isolates, 38.80% in *hyb*, *cpsC*, *cpsD* and *cpsE* at 4.48%, *cpkJ*, *cpsK* and *neuB* 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

**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 [1].

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 [1].

*S. agalactiae*, also known as Group B *Streptococcus* (GBS) following the classification of Lancefield [2]. This is a highly contagious agent and commonly found in the mammary gland of cattle [1], usually associated with acute clinical mastitis and persistent subclinical infections [3].

Despite technological advances in the industry, it appears that mastitis caused by *S. agalactiae* has high prevalence and limited response to available therapies [4]. 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 [5].

The *fbsA* 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 [6-8]. The adherence of *S. agalactiae* to host tissues is important early in the infection process [9,10], and recent studies have shown that the protein *FbsA* also has platelet function and may cause other problems during infection [11] but may also be involved escape mechanism in the immune system, preventing opsonization by macrophages and neutrophils [8,11].

The gene is responsible for *hyb* protein called hyaluronate lyase (*HyalB*), which is very important for the pathogenesis of *S. agalactiae* [12]. 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 [13,14], facilitating the spread of *S. agalactiae* during infection [10,14-16].

The *cps* cluster is responsible for the formation of the polysaccharide capsule and its sialylation. 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 [5,17,18]. 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 [19,20].

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 [15,21-23]. The sialic acid is an essential factor in pathogenicity because it prevents the deposition of the C3b component of complement system, blocking phagocytosis [24]. The neu gene, located on the downstream end of the cps operon is responsible for production of sialic acid and sialidation capsule [25-27].

Recent studies show that the *S. agalactiae* encode small appendages on the cell surface, known as pili [28,29]. The pili are encoded by genes *pilA*, *pilB* and *pilC* 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 [10,30,31]. 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 StrtC4 [32]. These structures represent some of the most important virulence factors for infection in different microorganisms, allowing the development of invasive infections in humans [30,33].

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 *fbsA*, *hylB*, *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 (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. [34]: colony morphology, Gram stain, hemolysis on agar, catalase test, agar culture, esculin and bile-esculin agar 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 *fbsA* genes (encoding fibrinogen-binding protein), *hylB* (encodes the enzyme hyaluronate lyase), *cps* (encodes the protein responsible for formation of the polysaccharide capsule), *neuB* (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), DNA NAME (version 4.0 Lynam Corporation, Canada) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

The *cps* and *neuB* genes were evaluated together to assess the presence in the region of the *cps* operon from the gene that corresponds to the gene *cpsC* to *neuB* [27].

### Table 1: Sequences of oligonucleotides designed for amplification of virulence genes, *fbsA*, *hylB*, *cps*, and *neuB* cluster PI-1 of *Streptococcus agalactiae*.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences</th>
<th>GenBank Access</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cluster PI-1</em></td>
<td>F 5’CTCATCAGTGGACGTGTTC3’&lt;br&gt;R 5’GCACTATGCTGAGCAGG3’</td>
<td>EU929554.1</td>
<td>751</td>
</tr>
<tr>
<td><em>HyB</em></td>
<td>F 5’CCATTGCGTGGTCTACCG3’&lt;br&gt;R 5’GGATTAGCCTTTATCACAC3’</td>
<td>CP000114.1</td>
<td>1180</td>
</tr>
<tr>
<td><em>fbsA</em></td>
<td>F 5’GCCTTGGCTTTTATAGGGA3’&lt;br&gt;R 5’GGATTAGCCTTTATCACAC3’</td>
<td>AJ437620.1</td>
<td>1682</td>
</tr>
<tr>
<td><em>cps C, D, E</em></td>
<td>F 5’GCTATGCTGAGGAG3’&lt;br&gt;R 5’GGATTAGCCTTTATCACAC3’</td>
<td>AB017355.1</td>
<td>1852</td>
</tr>
<tr>
<td><em>neu B</em></td>
<td>F 5’GCAACTTCTTTAGTATTGT3’&lt;br&gt;R 5’GGATTAGCCTTTATCACAC3’</td>
<td>AB017355.1</td>
<td>668</td>
</tr>
</tbody>
</table>
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. This is unusual result for S. agalactiae, because this test is used to characterize the species. However, Hensler et al. [35] 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 [35].

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. The predominance of hemolysis range found in the isolates tested is aligned with the result presented by Duarte et al. [36] that in cattle from Minas Gerais, Sao Paulo and Rio de Janeiro, about 50% of the isolates showed beta-hemolysis. It is known that the pattern of beta hemolysis is common in S. agalactiae isolated from humans [36], 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 products are described in Table 2. Some isolates showed no amplification products for any of the genes evaluated.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Herd</th>
<th>Mastitis form</th>
<th>fbsA</th>
<th>PI-1</th>
<th>hyfB</th>
<th>cps C, D e E</th>
<th>cps J, K, neuB</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. agalactiae 167</td>
<td>B</td>
<td>Clinical</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>S. agalactiae 199</td>
<td>B</td>
<td>Clinical</td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>S. agalactiae 461</td>
<td>D</td>
<td>Clinical</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>S. agalactiae 477</td>
<td>E</td>
<td>Clinical</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
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<td>S. agalactiae 518</td>
<td>E</td>
<td>Clinical</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>S. agalactiae 568A</td>
<td>F</td>
<td>Clinical</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
</tbody>
</table>

capsule. Among the strains isolated from clinical mastitis, only two showed no gene amplification of \textit{cpsf}, \textit{cpsK} and \textit{neuB}, but showed amplification of genes for other virulence factors. Poyart et al. [27] found in a study of strains of \textit{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 \textit{hybB} (Table 2, Appendix). 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 Correa et al. [42] strains of \textit{S. agalactiae} of human origin and two of bovine origin were compared for virulence and presence of gene \textit{hybB}. This virulence gene was founded in all strains. In work published by Sukhnanand et al. [8], involving strains of \textit{S. agalactiae} from humans and cattle, in 52 strains of bovine origin tested, only nine had the gene \textit{hybB}. In another study published by Yildirim, Lammle and Fink [25], \textit{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 \textit{hybB} 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 \textit{hybB}.

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 3), but statistical analysis failed to confirm this observation. According to Usein et al., [43], may exist between the presence of \textit{PI-1} and the presence of the \textit{cps} gene.

Table 3: Results from PCR for virulence genes of \textit{Streptococcus agalactiae} isolates from clinical and subclinical cases of bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010.

By analyzing individual strains according to their origin, clinical or subclinical case, and the presence of virulence genes (Table 4), one realizes that in clinical isolates is higher frequency of genes of virulence. 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 [3,44].

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 [10,35,45,46].

Table 4: 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.

Sequencing

The variations in patterns of bands verified in electrophoresis of PCR products from gene *fbsA* suggested the occurrence of the polymorphism in these genes, within e among herds (Table 5). The polymorphism in this gene was confirmed by sequencing of PCR products. Schubert et al. [31] reported that *fbsA* 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.

### Table 5: Approximated numbers of nucleotides determined after the sequencing of the gene *fbsA* in *Streptococcus agalactiae* isolated from...
bovine mastitis in dairy herds from Minas Gerais in the period 2004-2010.

No differences were founded in fragments length among strains associated to clinical and subclinical mastitis cases, however, according to Schubert et al. [31] 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 fbsA (S. agalactiae 12 and S. agalactiae 252) identity values quite high, reaching 100%. Some strains showed low identity with sequences already deposited in GenBank. 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 fbsA 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. This is explained because there is a conserved region of the active site of the fibrinogen-binding protein. The alignment between all isolates showed that there is little conservation of gene fbsA.

After analysis of gene fbsA alignment was possible to confirm the amplification of the region of the gene mat peptide.

Genes hylB, cpsC, cpsD, cpsE, cpsJ, cpsK, neuB and were even less conserved after alignment. The amplicons for genes cpsC, cpsD, cpsE, cpsJ, cpsK, neuB showed greater nucleotide variation not only among the herds, but also within each herd, 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 hylB showed nucleotide variations within and between herds, 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 cpsC, cpsD and cpsE was obtained for one isolate (S. agalactiae 506A), with 98% identity (Table 5) with strains of human and tilapia origins (Oreochromis niloticus), while for genes cpsJ, cpsK and neuB, the highest identity was 93% 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 hylB, 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 [36,47,48] 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.

Discussion

The molecular tests showed the presence of the virulence genes fbsA, hylB, cpsC, cpsD, cpsE, cpsJ, cpsK, neuB and PI-1 in population of S. agalactiae analysed in this work.

The major frequency of the virulence genes analysed from the clinical mastitis doesn't means that exist a major virulence of de bacterial isolates.

Exist a genetic diversity between the isolates of S. agalactiae involved in clinical and subclinical bovine mastitis, and between isolates within and between herds.

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