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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 *hylB*, *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 hlyB protein called hyaluronate lyase [*HlyB*], 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 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 [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 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 [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 StrC4 [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 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. [34]: 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 *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), 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 *cpsC* to *neuB* [27].

Genes		Sequences	GenBank Access	Amplicon
Cluster *PI-1*	*Pil*F	5'CTCATCAGTTGACGATTGTTC3'	EU929554.1	751
	*Pil*R	5'CCATTGCCTGTTGCTCAC3'		
*hyl*B	*Hyl*F	5'GCAACAGCCACTCATAGCA3'	CP000114.1	1180
	Hyl R	5'GAGCGAGGGACACCGAT3'		
*fbs*A	*Fbs* F	5'GCTTTGGCTTTATATGGGAG3'	AJ437620.1	1662
	Fbs R	5'GCTACATTAGTAACCTGAGA3'		
cps C, D, E	*Cps* F	5'GCTAATGCTTGCGATGGTT3'	AB017355.1	1852
	Cps R	5'CTGGTCTTTCTTTTCTAAGGA3'		
cps J, K, e neu B	*Neu* F	5'GGATTAGCCTTTATCACACTT3'	AB017355.1	668
	Neu R	5'GCAACTTCTTTAGTATTGTATA3'		

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

The PCR for all virulence genes were made in a total volume of 30 µl, containing 1 µL of each primer (10 pmol), 0.5 U Taq Flexi DNA polymerase (Promega®, Wisconsin, USA) 3 µL enzyme buffer full (10 x), 1 µL mix of dNTPs (100 µmoles of each base) and 5µL of DNA template (50 ng/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. 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 genes are described in Table 2. Some isolates showed no amplification products for any of the genes evaluated.

Strains	Herd	Mastitis form	*fbsA*	*PI-1*	*hylB*	*cps C, D e E*	*cps J, K, neuB*
S. agalactiae 167	B	Clinical	P	N	P	N	N
S. agalactiae 199	B	Clinical	P	P	N	N	P
S. agalactiae 461	D	Clinical	P	N	P	P	P
S. agalactiae 477	E	Clinical	P	N	P	N	P
S. agalactiae 518	E	Clinical	P	N	P	N	P
S. agalactiae 568A	F	Clinical	P	N	P	N	P
S. agalactiae 589	F	Clinical	P	N	N	N	P
S. agalactiae 609A	G	Clinical	P	N	N	N	P
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 1026	N	Clinical	P	N	N	N	P
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 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 506A	E	Subclinical	P	N	P	P	P
S. agalactiae 516A	E	Subclinical	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 580A	F	Subclinical	P	N	P	N	N
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 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 1027	N	Subclinical	P	N	N	N	P
S. agalactiae 1051A	O	Subclinical	P	N	P	N	P
P=Presence, N=Not presence							

Table 2: 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.

PCR for the detection of *fbsA* showed the presence of the gene *fbsA* in 82% of the isolates studied. Among the isolates from clinical cases, amplification of this gene was detected in 100% of the strains. It is believed that this gene has key role in the virulence of *S. agalactiae*, and it is involved even in cases of hemorrhage [22,37,38].

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 2, Appendix). 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 [10,31,39]. 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 [40,41].

Only *S. agalactiae* strains 477, 506A and 1460 were positive for the region throughout the region *cps* operon (Table 2, Appendix), 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 *cpsJ*, *cpsK* and *neuB* 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 *cpsJ*, *cpsK* and *neuB*, but showed amplification of genes for other virulence factors. Poyart et al. [27] 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 *hylB* (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 *S. agalactiae* of human origin and two of bovine origin were compared for virulence and presence of gene *hylB*. This virulence gene was founded in all strains. In work published by Sukhnanand et al. [8], involving strains of *S. agalactiae* from humans and cattle, in 52 strains of bovine origin tested, only nine had the gene *hylB*. In another study published by Yildirim, Lammle and Fink [25], *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 *hylB* 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 *hylB*.

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 *PI-1* and the presence of the *cps* gene.

	*fbs*A	*PI-1*	*hyl*B	*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

Table 3: 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.

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].

	Set 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
0=No amplification; 1=only one gene amplified; 2=two types amplified; 3=three types amplified; 4=four types amplified; 5=all genes amplifieds.						

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.

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
S. agalactiae 1385	R	Subclinical	648
S. agalactiae 1388		Subclinical	617
S. agalactiae 1438	S	Subclinical	686
S. agalactiae 1453		Clinical	627
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 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 gene in which there annealing of primers. 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 frequence 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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