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Molecular epidemiology of *Streptococcus agalactiae* isolated from mastitis in Brazilian dairy herds

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ABSTRACT

Streptococcus agalactiae is one of the most common pathogens leading to mastitis in dairy herds worldwide; consequently, the pathogen causes major economic losses for affected farmers. In this study, multilocus sequence typing (MLST), genotypic capsular typing by multiplex polymerase chain reaction (PCR), and virulence gene detection were performed to address the molecular epidemiology of 59 bovine (mastitis) *S. agalactiae* isolates from 36 dairy farms located in the largest milk-producing mesoregions in Brazil (Minas Gerais, São Paulo, Paraná, and Pernambuco). We screened for the virulence genes *bac*, *bca*, *bibA*, *cfb*, *hylB*, *fbsA*, *fbsB*, PI-1, PI-2a, and PI-2b, which are associated with adhesion, invasion, tissue damage, and/or immune evasion. Furthermore, five capsular types were identified (Ia, Ib, II, III, and IV), and a few isolates were classified as non-typeable (NT). MLST revealed the following eight sequence types (STs): ST-61, ST-67, ST-103, ST-146, ST-226, ST-314, and ST-570, which were clustered in five clonal complexes (CC64, CC67, CC103, CC17, and CC314), and one singleton, ST-91. Among the virulence genes screened in this study, PI-2b, *fbsB*, *cfb*, and *hylB* appear to be the most important during mastitis development in cattle. Collectively, these results establish the molecular epidemiology of *S. agalactiae* isolated from cows in Brazilian herds. We believe that the data presented here provide a foundation for future research aimed at developing and implementing new preventative and treatment options for mastitis caused by *S. agalactiae*.

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Introduction

According to the World Food Organization,[1] the global production of milk reached 782,000,000 tonnes in 2013. Brazil is among the top producers of milk, ranking 4th globally and producing 34.4 million tonnes. Milk production worldwide is affected by numerous factors, but one of the most important factors is mastitis: the disease can lead to reduced milk production by the affected animals and/or to the production of low-quality milk, which is invariably discarded, resulting in large economic losses.[2] Furthermore, mastitis is currently treated with antibiotics, which, when used indiscriminately, can lead to the emergence of antibiotic-resistant bacteria that can be further propagated.[3] Thus, the type and the pathogenic nature of various mastitis-related bacterial species must be investigated in order to develop improved treatment and preventative options that will not only limit the health complications for the affected animals but also lessen the impact on farmers, particularly in countries such as Brazil.

The major bovine mastitis-causing pathogenic bacteria are *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Staphylococcus aureus*.[2,4,5] *S. agalactiae*, also known as Group B *Streptococcus* (GBS), has been shown to cause both clinical and subclinical mastitis in cattle, and has been detected in 60% of Brazilian dairy herds.[2,6] Moreover, GBS is recognized to be the causative agent of several diseases in various other animal species, including humans, where it is the most common life-threatening disease in newborn humans, causing pneumonia, septic shock syndrome and meningitis with high mortality rates.[7–9] In these various species, GBS also appears to be capable of colonizing different tissues in the body, a characteristic that has been linked to several specific bacterial virulence genes that enable the microorganism to colonize, invade, and spread in the host. In GBS, these genes include the following: bacterial immunogenic adhesin (*bibA*), fibrinogen-binding protein A (*fbsA*), and fibrinogen-binding protein B (*fbsB*), which are related to adhesion; Pilus Island 1 (PI-1), PI-2a, and PI-2b, which are related to adhesion and invasion; *cfb* and *hylFB*, which are related to tissue damage; and *bac* and *bca*, which are related to immune evasion.[9,10] Furthermore, these virulence genes might also be associated with adaptation and clinical manifestations in various host species.

Because GBSs exert a major effect on both animal and human health, several tools have been developed for epidemiological typing of this pathogen.[11–13] The capsule of this bacterium has been shown to be the first bacterial virulence factor that enables the bacterium to evade the immune system and invade the host. Thus, capsular serotyping is a classic method used in epidemiological studies of GBS. To date, 10 GBS capsular polysaccharide serotypes (CPSs) have been identified (Ia, Ib, and II–IX), and their distribution in humans is directly related to ethnic and geographic regions.[8] Furthermore, capsular genotyping is considered to be highly suitable for epidemiological investigations because the serotypes can be identified in the presence or absence of CPS expression.[6] Multilocus sequence typing (MLST) is a method based on the amplification and sequencing of bacterial housekeeping genes, and it has been used to investigate, characterize, and distinguish specific clones among GBSs isolated from humans and animals from diverse geographical regions.[12,14,15] Although MLST and molecular capsular typing have been widely employed in the epidemiological characterization of GBSs, no published study has reported the use of these tools for identifying GBS isolates in mastitis-affected Brazilian dairy herds.

Therefore, in this study, the first aim was to genotypically characterize GBS isolates from bovine mastitis from multiple Brazilian farms located in various regions of the country. The data obtained were then used to address the molecular epidemiology of the strains. Lastly, the presence of 10 GBS virulence genes was analysed in order to identify the genes that have the greatest impact in this population.

Materials and methods

Bacterial strains

We evaluated 65 bacterial strains from the culture collection of the Bacteriology Laboratory/DMVP UFLA, previously identified as *S. agalactiae* phenotypically by the catalase test, CAMP test, hippurate hydrolysis test and the PYR test, as described by MacFaddin.[16] These strains were isolated from the milk of 65 animals from 36 dairy farms located in the largest Brazilian cow milk producing states (Minas Gerais, São Paulo, Paraná, and Pernambuco) in the mesoregions of the country, between 2010 and 2011 (Material supplementary 1). The reference strains NEM 316 and ATCC BAA-611 (also designated 2603V/R) were used as positive controls and a *Streptococcus dysgalactiae* strain (ATCC 27957) was used as the negative control in the multiplex-polymerase chain reaction (PCR) and PCR assays.

Specific PCR, molecular capsular typing and sequencing

Genotypic analyses were then performed to confirm this *S. agalactiae* classification by using *S. agalactiae*-specific PCR as previously described.[17] All isolates that were positive in this PCR analysis were subjected to CPS typing by using a multiplex-PCR assay, as described by Poyart et al.[13] Strains that were not amplified in the multiplex PCR were subjected to serotype IX-specific PCR analysis.[18] All reactions were performed twice to ensure data reproducibility. The amplification products were analysed using 1.5% (w/v) agarose gel electrophoresis with 1× Tris–acetate buffer (0.04 M Tris–acetate, pH 8.4, 1 mM EDTA) and were visualized with a UV transilluminator after staining with 0.5× GelRed™ (Biotium, USA). A 100-bp DNA ladder (New England Biolabs, USA) was used as a molecular marker in each electrophoresis assay. PCR products were purified using a Wizard PCR Prep kit (Promega, USA). Sequencing reactions were performed using an Applied Biosystems BigDye Terminator Cycle Sequencing Kit and run on an ABI 3730xl DNA analyser (Applied Biosystems, USA).

Multilocus sequence typing and clonal group assignment

MLST was performed by sequencing the internal fragments of seven housekeeping genes (*adhP*, alcohol dehydrogenase;

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