Molecular detection of virulence genes and multi-drug resistance patterns in *Streptococcus agalactiae* in clinical bovine mastitis: Tehran and Alborz provinces, Iran

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ABSTRACT

Streptococcus agalactiae is one of the important causes of mastitis in cows. The ability of *Streptococcus agalactiae* to cause disease depends on the production of a large number of virulence factors encoded by different genes. The overuse of antibiotics to treat mastitis can lead to antibiotic resistance. This research was conducted to detect some virulence genes and the antibiotic resistance of *Streptococcus agalactiae*. For this purpose, a total of 30 samples of *Streptococcus agalactiae* isolated from the milk of different cows presenting clinical mastitis in Tehran and Alborz, out of these, 24 samples were confirmed as *Streptococcus agalactiae* through the detection of the two *16S-23S rRNA* genes. Disk diffusion method for a panel of 10 antimicrobial agents showed a large number of strains resistant simultaneously to six antibiotics. Five virulence genes *bac*, *bca*, *cylE*, *hylB*, and *cfb* were screened by polymerase chain reaction (PCR). The *cfb* and *hylB* genes were found in 95.83 % of the isolates. *cylE* gene was detected in 29.16 % of the isolates. *bca* and *bac* genes were not detected in any of the isolates. The *bac* and *bca* genes likely have minimal impact on the pathogenesis of *Streptococcus agalactiae* mastitis in dairy cows, while the *hylB* and *cfb* genes play a crucial role in this condition. The results presented here are one of the first molecular data concerning these five virulence genes in *Streptococcus agalactiae* isolates causing bovine mastitis in the Tehran and Alborz provinces that provide a foundation for the development of diagnostic, preventive, and therapeutic methods.

Key words: Antibiotic resistance, Dairy cow, Mastitis, *Streptococcus agalactiae*, Virulence genes.

1. Introduction

Streptococcus agalactiae, the only known member of group B streptococci, was initially differentiated from other streptococci by Rebecca Lancefield in the 1930s after being isolated from milk and cows with bovine mastitis (1). This bacterium causes mastitis in cows, pneumonia and meningitis in human infants (2,3).

In cases of mastitis, the genus Streptococcus accounts for 25 to 50% of the isolated pathogens in the world (4). Meanwhile, *Streptococcus agalactiae* is a significant cause of mastitis in cows. *Streptococcus agalactiae* can persist in the mammary gland for extended periods without causing symptoms. The disease progresses slowly (5,6). *Streptococcus agalactiae* is transmitted through infected mammary glands and contaminated environmental sources, such as milking machines and bedding (2). *Streptococcus agalactiae* infection in dairy cows is a major factor in reducing milk production and the quality of milk products. Milk from cows with mastitis reduces the quality of dairy products. Changes in milk composition not only decrease its nutritional value and cause processing issues but also shorten the shelf life of liquid milk products (5,7,8).

The ability of *Streptococcus agalactiae* to cause disease depends on the production of a large number of virulence factors, each encoded by different genes. For instance, the virulence factors alpha protein C, beta protein C, hyaluronidase, CAMP factor, and B-hemolysin are encoded by *bca*, *bac*, *hylB*, *cfb*, and *cylE* genes, respectively are some virulence genes that were reported in some *Streptococcus agalactiae* that were isolated from mastitis milk samples (9,10). Previously, Ahmadiet al. (2009) in Urmia, Iran and Momtaz et al (2012) in Isfahan, Iran detected Streptococcus agalactiae among the bacteria extracted from milk samples by PCR method (11,12).

The most common treatment for mastitis is the administration of intramammary antibiotics in the infected parts of the udder and injection (13). The overuse of antibiotics to treat mastitis over a long period can lead to antibiotic resistance. This can result in the need to increase the dosage of antibiotics, leading to the accumulation of high levels of antibiotics in milk and dairy products, which can then be transferred to humans (14). Antibiotic resistance has been described as one of the most significant global threats of the 21st century for this reason (15). Therefore, it is crucial to determine antibiotic resistance in bacteria isolated from mastitis cases for effective treatment of this disease (16).

Therefore, this study aimed to determine drug resistance and describe the distribution of virulence genes in isolates to aid in the prevention and control of bovine mastitis.

2. Materials and Methods

2.1. Collection of isolates of *Streptococcus agalactiae* and 16S rRNA sequence analysis

30 isolates of *Streptococcus agalactiae* were isolated from 400 milk samples of mastitis-affected cows in 10 herds in industrial cattle farms in Alborz and Tehran provinces by the Mabna laboratory, located in Mehrshahr, Karaj, Alborz, Iran. The samples were frozen in 30 microtubes with a size of 2 ml containing 1% glycerol and paraffin at -20˚C transferred to Karaj branch of Islamic Azad University research laboratory. All *Streptococcus agalactiae* isolates were confirmed with 16S rRNA polymerase chain reaction (PCR).

2.2. Analysis of antimicrobial susceptibility

All confirmed isolates underwent susceptibility testing for 10 commonly used antimicrobial agents in Tehran and Alborz provinces dairy farms, including erythromycin (15 µg), ceftiofur (30 µg), penicillin (10 µg), ciprofloxacin (5 µg), streptomycin (10 µg), kanamycin (30 µg), tetracycline (30 µg), neomycin (30 µg), florfenicol (30 µg), and clindamycin (2 µg) using the disc diffusion method on Mueller-Hinton agar plates, supplemented with 5% sheep blood. The cultures were incubated overnight (16–18 h) at 37˚C in atmosphere with 5% CO_2, and the results were interpreted by the recommendations of the Clinical and Laboratory Standards Institute (CLSI).

2.3. Genomic DNA extraction

The template DNA was obtained by boiling bacterial colonies. Therefore, each bacterial isolate was cultured in 2 mL of Muller Hinton broth, then transferred to 2 mL microtubes, and centrifuged (Hermle Z233MK-2) at 5000 rpm (2374 x g) for 10 minutes. Then the supernatant was discarded and 200 microliters of distilled water was added to the remaining sediment. Then the microtubes were placed in the hot block (Techne-DB.2D) for 10 minutes at 100°C to disrupt the bacterial walls and release the bacterial genome. The microtubes were once again placed in a centrifuge at 5000 rpm (2374 x g) for 10 minutes. Ultimately, the liquid supernatant was utilized as the genomic DNA.

2.4. Detection of virulence genes

All confirmed isolates were screened for the presence of the following virulence genes: *bac* (C-β protein), *bca* (C- α protein), *cfb* (CAMP factor), *cylE* (β-hemolysins/cytolysin) and *hylB* (hyaluronidase) (9,17).

The concentrations of components in the reaction mixtures used for amplifying gene fragments were selected based on experimental results and references shown in Table 1. For each gene, 12.5 µl of 2xTaq DNA Polymerase Master Mix RED 1.5mM MgCl2 (Ampliqon Co. Denmark), 0.5 µl of each primer (0.4 µM for *bca*), and 1 µl of template DNA were placed in each microtube. Then the total volume of each microtube reached 25 µl with distilled water. Each reaction included a positive control (DNA isolate containing the tested gene) and a negative control (nuclease-free water) in thermocycler (Applied Biosystems- en61327). The primer sequences and conditions used for amplification of DNA fragments are presented in Table 1. Also, PCR temperatures and conditions are shown in footnotes of the Table 1.

(Please insert Table 1 here)

3. Results

3.1. Antimicrobial susceptibility

Antimicrobial susceptibility testing of the isolates showed that 100% of the 24 confirmed isolates (Unconfirmed isolates are number 2, 8, 10, 25, 26, and 30) of Streptococcus agalactia were susceptible to penicillin, ciprofloxacin, and ceftiofur and that 75% were susceptible to florfenicol. All 24 isolates were resistant to the streptomycin, kanamycin, tetracycline and neomycin. The resistance rate for clindamycin and erythromycin were 95.8% and 91.6%, respectively (Table 2).

3.2. Prevalence of virulence genes

Presence of five virulence genes of *Streptococcus agalactiae* (*bca*, *cylE*, *cfb*, *hylB*, and *bac*) were tested for all of the 24 confirmed isolates are shown in figures 1 to 6. The results showed that *cfb* and *hylB* genes were detected in 95.8% of *Streptococcus agalactiae* isolates. Also, *cylE* gene was detected in 29.1% of these isolates. The *bac* and *bca* genes were not detected in these isolates. Three distinct virulence gene profiles were identified and the virulence gene profile *cfb-hylB* was common among isolates as shown in Table 2.

(Please insert Table 2 here)

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4. Discussion

Streptococcus agalactiae is considered one of the major mastitis pathogens. To the best of our knowledge, this is one of the first molecular study that characterizes *Streptococcus agalactiae* isolates circulating among cattle with mastitis in Tehran and Alborz provinces, Iran. Of the 30 original strains identified as *Streptococcus agalactiae* by biochemical tests, only 24 were confirmed genetically, for an isolation rate of 80.0%. The presence of virulence factors in a pathogen significantly influences disease progression (9). Concerning the virulence genes screened in this study, five virulence genes were detected, which included *bac*, *bca*, *cfb*, *hylB*, and *cylE*.

The *hylB* gene encodes the hyaluronidase protein (18). Hyaluronidase increases the spread of infection by hydrolyzing the hyaluronic acid in the connective tissue (20). In previous studies the frequency of *hylB* virulence gene has been reported in more than 95% of the investigated isolates (7,18-24). In this study, the virulence gene *hylB* was seen in 23 of the 24 confirmed isolates of *Streptococcus agalactiae* (95.83%). This indicates the importance of *hylB* in improving of mastitis by *streptococcus agalactiae*.

The *cfb* virulence gene encodes the CAMP factor, which induces the formation of pores in the host cell membrane (9,18). The frequency of *cfb* virulence gene was more than 90% in most researches (7,9,18,19,21,24-26). The next reported frequency was 68.96% and the lowest frequency of this gene was 38.09% (27). With these interpretations, we can conclude that this

virulence gene is also one of the most abundant virulence genes of *Streptococcus agalactiae*. In this research, *cfb* virulence gene was founded in all isolates but one (95.83%).

The virulence gene *cylE*, by encoding the B-hemolysin protein, increases the invasion of this bacterium into host cells (9). Different frequencies have been reported in different countries for this virulence gene. The highest frequency reported for the *cylE* virulence gene was 100% (19-21). Also, in some researches, the frequency of this gene has been reported as 93% (18,26). Frequencies of 78% and 68.2% were reported (9,22). The lowest mentioned frequency for this gene was 23.80% (27). In this research, *cylE* virulence gene was found in 7 isolates out of 24 confirmed isolates of *Streptococcus agalactiae* (29.16%) and according to the clinical reports, the cows that affected with these isolates showed sever clinical mastitis.

Among the reviewed articles from various countries, the *bca* and *bac* virulence genes have the lowest frequency of occurrence. The virulence gene, *bca*, encodes surface protein C alpha antigen. This protein mediates the adhesion of bacteria to the epithelial cells of the host. The *bac* virulence gene encodes surface protein C beta antigen, responsible for binding to immunoglobulin A (9). In most studies, the frequency of the *bca* and *bac* virulence genes was less than 10% and, in some cases, even 0% (7,9,18-21,25). In this study, *bac* and *bac* virulence genes were not found in any of the 24 confirmed isolates of *Streptococcus agalactiae* (0%), This can be related to the relatively small size of the samples collected in this study.

These results indicated that the *bac* and *bca* virulence genes probably do not significantly contribute to the pathogenesis of mastitis caused by *Streptococcus agalactiae* in dairy cows and these two genes are less important in the virulence of *Streptococcus agalactiae* than the virulence genes *hylB*, *cylE*, and *cfb*. It can be concluded that the *hylB* and *cfb* genes play a significant role in the pathogenesis of mastitis caused by *Streptococcus agalactiae* in dairy cows.

The most common treatment for mastitis involves administering antibiotics directly into the infected teats of udder and giving intramuscular injections (13). In this study, we conducted susceptibility testing for 10 commonly used antibiotic agents to treat clinical mastitis in dairy cows in Tehran and Alborz Provinces. We found that all 24 isolates showed 100% resistance rate to streptomycin, neomycin, tetracycline, and kanamycin, while they exhibited high sensitivity to penicillin, ciprofloxacin, and ceftiofur. Also, the resistance rate in 24 isolates was over than 90% for clindamycin and erythromycin and it was 12.5% for florfenicol.

The results of this study indicate that three antibiotics, namely penicillin, ciprofloxacin, and ceftiofur, may be suitable drug choices for treating *streptococcus agalactiae* mastitis in the provinces of Tehran and Alborz. However, *Streptococcus agalactiae* can eventually develop resistance to these antimicrobial agents. Therefore, these three antibiotics should not be considered a long-term solution. Also, the virulence genes investigated in this study can provide helpful data for the preparation of vaccines for use in livestock in the Tehran and Alborz provinces.

We detected several virulence profiles associated with *Streptococcus agalactiae* intramammary infections. It can be concluded that the *bac* and *bca* virulence genes probably do not significantly contribute to the pathogenesis of mastitis caused by *Streptococcus agalactiae* in dairy cows. However, this can be influenced by the relatively small size of the samples collected in this study. Also, the *hylB* and *cfb* genes play a significant role in the pathogenesis of mastitis caused by *Streptococcus agalactiae* in dairy cows. On the other hand, according to the results of the disk diffusion test, we have determined that penicillin, ciprofloxacin, and ceftiofur are the most effective antibiotics for treating mastitis caused by *Streptococcus agalactiae*. These

data will assist us in closely monitoring *Streptococcus agalactiae* strains, improving diagnostic methods, and developing prevention, treatment, and perspective of producing a vaccine.

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A collection of *Streptococcus agalactia* isolates investigated in this study was gathered by the Mabna laboratory in March and April 2024.

Authors' Contribution

1 -Study concept and design: H.P.

2 -Acquisition of data: F.H.H., F.M.

3 -Analysis and interpretation of data: H.P., F.H.H., N.H., F.M.

4 -Drafting of the manuscript: F.H.H.

5 -Critical revision of the manuscript for important intellectual content: H.P., N.H.

6 -Statistical analysis: -

7 -Administrative, technical, and material support: H.P., N.H., F.M.

8- Study supervision: H.P., N.H., F.M.

Ethics

All experimental procedures were carried out with the utmost respect for the principles of ethical research, ensuring the welfare and safety of the participants.

Conflict of interest

The authors declare that there are no conflicts of interest in disclosing this work.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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Table 1. PCR primers of genes and cycling conditions used to identify and characterize *Streptococcus agalactiae*

Gene	Primer sequence (5'---> 3')	Amplicon size (bp)	Reference
16S-23S rRNA1[1]	Fw: TGTTTAGTTTTGAGAGGTCTTG Rv: CGTGGAATTTGATATAGATATTC	150	16
16S-23S rRNA2[1]	Fw: GGAAACCTGCCATTTGCG Rv: TAACTTAACCTTATTAACCTAG	281	16
bac[2]	Fw: AAGCAACTAGAAGAGGAAGC Rv: TTCTGCTCTGGTGTTTTAGG	479	16
bca[3]	Fw: TGATACTTCACAGACGAAACAACG Rv: TACATGTGGTAGTCCATCTTCACC	398	16
cfb[4]	Fw: TTTCACCAGCTGTATTAGAAGTA Rv: GTTCCCTGAACATTATCTTTGAT	153	16
cylE[5]	Fw: CATTGCGTAGTCACCTCCC Rv: GGGTTTCCACAGTTGCTTGA	380	17
hylB[2]	Fw: CACCAATCCCCACTCTACTA Rv: TGTGTCAAACCATCTATCAG	444	16

1. 94℃ (600 s); 30 cycles of 94°C (60 s), 55°C (60 s), 72°C (60 s); final extension 72°C (420 s)
2. 94°C (300 s); 30 cycles of 94°C (30 s), 53°C (30 s), 72°C (60 s); final extension 72°C (240 s)

3. 96°C (180 s); 30 cycles of 95°C (60 s), 58°C (45 s), 72°C (45 s); final extension 72°C (600 s)
4. 94°C (180 s); 40 cycles of 95°C (20 s), 55°C (30 s), 72°C (120 s); final extension 72°C (300 s)
5. 94°C (180 s); 34 cycles of 94°C (20 s), 56°C (20 s), 72°C (45 s); final extension 72°C (300 s)

Table 2. Multi-drug resistance patterns and virulence gene profiles in 24 *streptococcus agalactiae* isolates

	Patterns	Number of isolates	Frequency %
Antibiotics resistance patterns	N, CC, FF, TE, E, K, ST	3	12.5
	N, CC, TE, E, K, ST	19	79.16
	N, CC, TE, K, ST	1	4.16
	N, TE, K, ST	1	4.16
Virulence gene profile	*cylE*	1	4.16
	cfb, hylB	23	95.83
	cfb, hylB, cylE	6	25.00

N: Neomycin, CC: Clindamycin, FF: Florfenicol, TE: Tetracycline, E: Erythromycin, ST: Streptomycin