Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 23 No. 3 Ser. No. 80 2022

IRANIAN JOURNAL OF VETERINARY RESEARCH

Short Paper

Detection of virulence associated genes in *Streptococcus agalactiae* isolated from bovine mastitis

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10.22099/IJVR.2022.43305.6311

(Received 10 Mar 2022; revised version 5 Jul 2022; accepted 30 Jul 2022)

Abstract

Background: Mastitis is one of the most expensive diseases in the dairy industry. It causes heavy monetary losses by decreasing milk production and treatment cost. *Streptococcus agalactiae*, the cause of contagious bovine mastitis, possesses various virulence factors that contribute to pathogenicity. **Aims:** The main aim of the study was to evaluate the distribution of virulence genes of *S. agalactiae*. **Methods:** In the current work, 98 *Streptococcus* species were isolated from 320 milk samples, collected from Veterinary Clinical Complex, Junagadh. Out of the isolates, 42 *S. agalactiae* isolates were used for virulence genes detection. **Results:** All *Streptococcus* spp. were confirmed at genus level by targeting *tuf* gene, and *S. agalactiae* was identified at species level by targeting *16S rRNA* gene. For virulence gene detection, *scpB*, *cfb*, and *cylE* genes were targeted. Of 42 *S. agalactiae* isolates, 15, 16, and 10 isolates possessed *scpB*, *cfb*, and *cylE* genes, respectively. **Conclusion:** This study aids us to know virulence characteristics and mechanisms responsible for the development of new strains in mastitis epidemiology in response to prevention and control strategies.

Key words: Bovine mastitis, Molecular detection, *S. agalactiae*, Virulence genes

Introduction

Bovine mastitis is a multi-etiological condition and occurs based on factors related to the animals and environment, which leads to harmful effects on animal health and decrease profit for dairy farmers. Mastitis-causing bacteria are divided into contagious and environmental bacteria. *Streptococcus agalactiae* comes under the contagious bacteria which is responsible for chronic mastitis. *S. agalactiae* infection increases somatic cell counts in milk and decreases milk production. Bovine mastitis caused by *S. agalactiae* is a serious problem after *S. aureus* that disturbs animal health and farm productivity (Richards *et al*., 2011; Radtke *et al*., 2012; Javia *et al*., 2018). The ability of the bacteria to invade the immune host cells needs various virulence factors to begin growth and multiplication. *S. agalactiae* possesses several virulence factors, including structural components, toxins, and enzymes that play an important role in intra mammary infections. In *S. agalactiae*, C5a peptidase enzyme is encoded by *scpB* gene, which increases bacterial invasion to epithelial cells by reducing neutrophil enrolment (Beckmann *et al*., 2002). The *cylE* gene, encoding β-haemolysin, causes tissue injury and systemic spread of the bacteria (Doran *et al*., 2003). The CAMP factor of *S. agalactiae* increases the activity of the beta toxin of *Staphylococcus* spp. The CAMP factor may have a cytotoxic action on mammary tissue. The present study was conducted in a very important geographic area that represents the rearing of elite milch breeds of bovine *viz*; Jaffarabadi buffaloes and Gir cows. Thus, the identification of *S. agalactiae* causing bovine mastitis with its molecular characterization of virulence genes will help to recognize the epidemiology and pathogenesis of intramammary infection caused by *S. agalalctiae*.

Materials and Methods

Sample collection

The present work was carried out on 320 milk samples from mastitis cases presented at Veterinary Clinical Complex, Veterinary College, Junagadh, as well as various milk samples from livestock owners, farms, and Gaushalas in and around Junagadh district of Gujarat state, India.

Isolation and biochemical characterization

All mastitic milk samples were enriched into *Streptococcus* selection broth (Himedia, USA), for 6 h at 37°C with 5-10% CO_2. Then, it was cultured on brain heart infusion (BHI) agar and incubated at 37°C for 48 h for pure culture. Biochemical tests, including catalase, oxidase, and CAMP were carried out as per the standard procedures (FDA, 2001; Harley and Prescott, 2002; Javia *et al*., 2020).

Determination of CAMP test

The CAMP test was carried out as per the method described by Sandholm *et al*. (1995) with some modifications to identify *S. agalactiae*. The standard strain of *S. aureus* (ATCC-43300) was grown on 5% sheep blood agar (SBA) plates overnight at 37°C. *S. aureus* producing β-haemolysin was inoculated by a narrow streak at the center of the SBA plate onto a plate. The *Streptococcus* isolates were streaked at 90 degree angle without touching the *S. aureus* streak. Then, the plate was incubated at 37°C for 24 h. An "arrowhead"-shaped enhanced zone of β-hemolysis suggest the positive CAMP test.

DNA extraction from colony of bacteria

The bacterial DNA of the cultures was extracted by the method of column-based DNA extraction using Nucleo-pore gDNA Fungal/Bacterial Mini Kit (Genetix brand, India), according to the manufacturer's instruction.

PCR-based detection of *Streptococcus* genus and species

The primers as stated in Table 1 were used for amplification of *Streptococcus* genus and species specific sequence. The reaction mixture was prepared as per Table 2. The PCR cycling condition was kept as follows: Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C (*tuf* gene), 60°C (*16s rRNA* gene) for 45 s and an extension at 72°C for 45 s with a final extension step at 72°C for 10 min. The amplification reactions were carried out using a programmable thermal cycler (M/s Applied Biosystems, USA).

Table 2: Constituents of the reaction mixture for PCR

Constituents	Amount (µL)
Master mix	12.5
Forward primer (10 pM/µL)	1.0
Reverse primer (10 pM/µL)	1.0
Nuclease free water	7.5
DNA	3.0

PCR-based detection of virulence genes

The primers as detailed in Table 3 were used for virulence genes detection in *S. agalactiae*. The PCR cycling condition was kept as follows: Initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 47°C (*scpB* gene), 52°C (*cfb* gene), 55°C (*cylE* gene) for 45 s and an extension at 72°C for 45 s with a final extension step at 72°C for 10 min.

Results

Isolation and biochemical characterization

Very small pinpoint colonies with a chain of Gram-positive cocci under a microscope were presumptively considered as *Streptococcus* spp. Of 320 milk samples, 98 samples were found positive for major *Streptococcus* species. All 98 isolates were found catalase and oxidase negative. Out of 98 *Streptococcus* isolates, 42 were found positive for CAMP test (Fig. 1).

PCR-based detection of *Streptococcus* genus and species

All of the 98 *Streptococcus* spp. yielded 197 bp product of *tuf* gene (Fig. 2). From 98 isolates of *Streptococcus* spp., 42 isolates were positive for *16S rRNA* gene of *S. agalactiae* with specific 405 bp product (Fig. 3).

Table 1: Specific primer sequences of *Streptococcus* genus (*tuf*) and species (*16S rRNA*)

Primer (5´ to 3´)	Target gene	Product size	Reference
F: GTACAGTTGCTTCAGGACGTATC R: AGCTTCGATTTCATCACGTTG	*tuf*	197 bp	Picard *et al*. (2004)
F: CGCTGAAGGTTTGGTGTTTACA R: CACTCCTACCAACGTTCTTC	*16S rRNA*	405 bp	Riffon *et al*. (2001)

Table 3: Specific primer sequences of virulence genes

Primer (5´ to 3´)	Target gene	Product size	Reference
F: ACAACGGAAGGCGCTACTGTTC R: ACCTGGTGTTTGACCTGAACTA	*scpB*	255 bp	Dmitriev *et al*. (2004)
F: ATGGGATTTGGGATAACTAAGCTAG R: AGCGTGTATTCCAGATTTCCTTAT	*cfb*	193 bp	Dmitriev *et al*. (2002)
F: TGACATTTACAAGTGACGAAG R: TTGCCAGGAGGAGAATAGGA	*cylE*	248 bp	Bergseng *et al*. (2007)

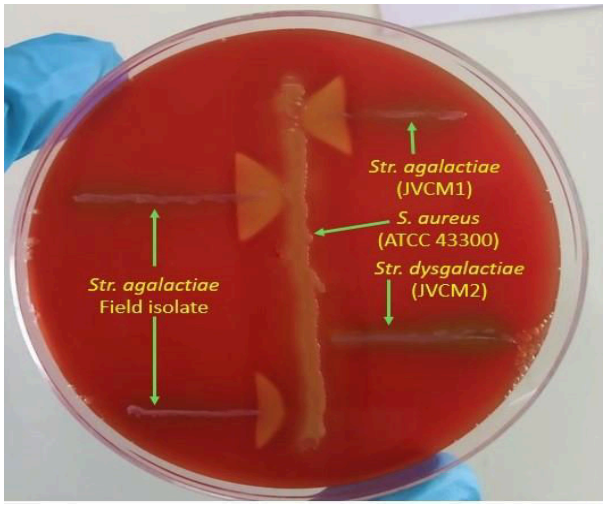

Fig. 1: CAMP test on 5% sheep blood agar

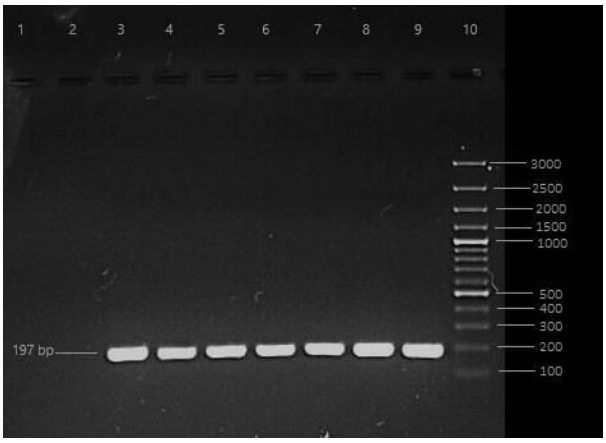

Fig. 2: Genus specific PCR of *Streptococcus* spp. for *tuf* gene (197 bp). Lane 1: Negative template control, Lane 2: *E. coli* (MTCC 722) as negative control, Lane 3: *S. uberis* (ATCC 700407) as positive control, Lane 4 to 9: *Streptococcus* spp. recovered from milk samples, and Lane 10: 100 bp ladder

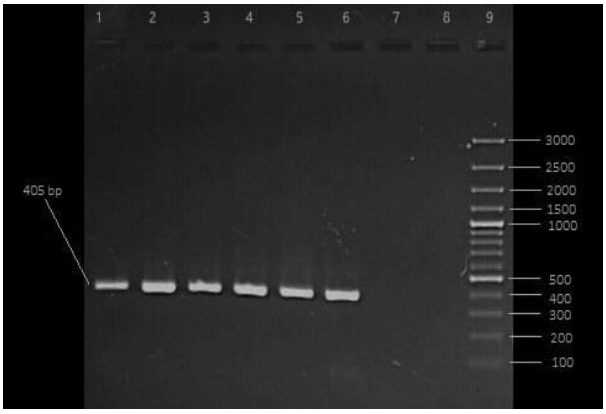

Fig. 3: Species specific PCR of *S. agalactiae* for *16s rRNA* gene (405 bp). Lane 1: *S. agalactiae* (JVCM1) as positive control, Lane 2-6: *S. agalactiae* isolates from samples, Lane 7: *S. aureus* (ATCC 43300) as negative control, Lane 8: Negative template control, and Lane 9: 100 bp ladder

PCR-based detection of virulence genes

From 42 *S. agalactiae* isolates, 15 isolates were yielded 255 bp amplicon of *scpB* gene (Fig. 4). Out of 42 *S. agalactiae* isolates, 16 isolates were yielded 193 bp amplicon of *cfb* gene (Fig. 5). Out of the 42 *S. agalactiae* isolates only 10 isolates were yielded 248 bp amplicon of *cylE* gene (Fig. 6).

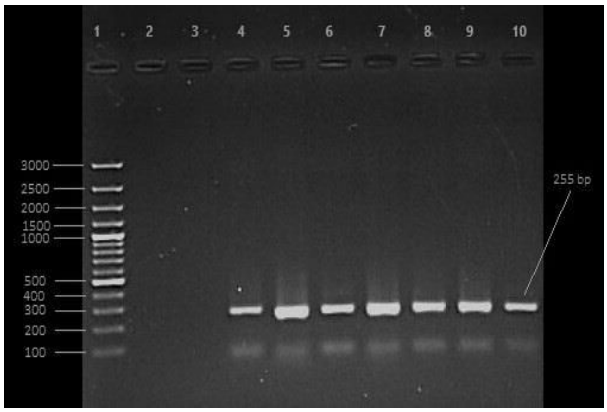

Fig. 4: *scpB* gene specific PCR for *S. agalactiae* (255 bp). Lane 1: 100 bp ladder, Lane 2: Negative template control, Lane 3: *S. aureus* (ATCC 43300) as a negative control, and Lane 4 to 10: *S. agalactiae* isolates from bovine milk samples

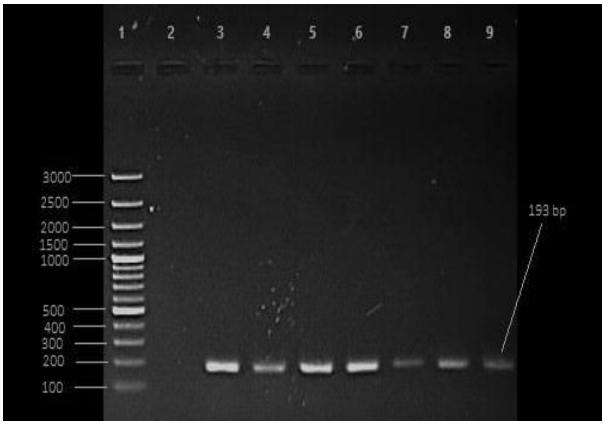

Fig. 5: *cfb* gene specific PCR for *S. agalactiae* (193 bp). Lane 1: 100 bp ladder, Lane 2: *S. aureus* (ATCC 43300) as negative control, and Lane 3 to 9: *S. agalactiae* isolates from bovine milk samples

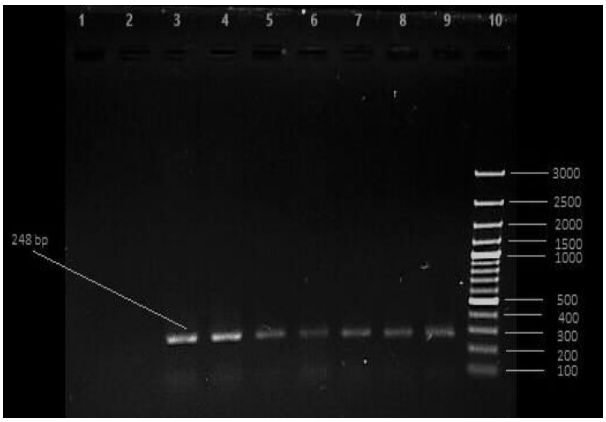

Fig. 6: *cylE* gene specific PCR for *S. agalactiae* (248 bp). Lane 1: Negative template control, Lane 2: *S. aureus* (ATCC 43300) as negative control, Lane 3 to 9: *S. agalactiae* isolates from bovine milk samples, and Lane 10: 100 bp ladder

Discussion

In the present study, out of 320 screened milk samples, 98 (30.62%) *Streptococcus* spp. were isolated. Similar findings found by many scientists. Atyabi *et al*. (2006) found 974 (33.54%) *Streptococcus* from screening of 2904 milk samples. Nithinprabhu (2010) screened 163 milk samples and recovered 40 (24.54%) *Streptococcus* isolates. Shrestha and Bindari (2012) found 27% prevalence of *Streptococcus*. While, Amosun *et al*. (2010) recovered 130 (65%) *Streptococcus* isolates from 200 milk samples. Kia *et al*. (2014) studied 700 milk samples collected from dairy farms and they found 525 (75%) positive samples for streptococci. Elkenany (2020) found 190 (65.72%) *Streptococcus* spp. from 288 milk samples collected from 72 mastitic animals.

In the present study, we recovered 42 (42.85%) *S. agalactaie* out of 98 *Streptococcus* isolates. Similar kinds of findings were reported by many scientists. Ghose and Sharda (2004) found 58.11% prevalence of *S. agalactiae*. El-jakee *et al*. (2013) found 19.3% prevalence of *S. agalactiae*. Yang *et al*. (2013) found 61% prevalence of *S. agalactiae*. Kia *et al*. (2014) found 52.95% prevalence of *S. agalactiae*. Ding *et al*. (2016) found 70.4% prevalence of *S. agalactiae*. Elkenany (2020) found 60% prevalence of *S. agalactiae*. In the present study, all 42 *S. agalactiae* isolates were CAMP positive. Similar findings were found by Dad *et al*. (2007), Amosun *et al*. (2010), Kia *et al*. (2014), Carvalho-Castro *et al*. (2017), and Abd Alkader and Hyyawi (2021).

In the present study, out of 42 *S. agalactiae* isolates, *scpB*, *cfb*, and *cylE* genes were found in 15 (35.71%), 16 (38.09%), and 10 (23.8%) isolates, respectively. Out of 42 *S. agalactiae* isolates, 2 (4.76%) isolates were found positive for all 3 virulence genes, while 4 (9.52%) isolates found positive for both *scpB*+*cylE* genes, and 4 (9.52%) isolates were found positive for both *cylE*+*cfb* genes. The results obtained here are in agreement with previous studies carried out by many scientists. Jain *et al*. (2012) carried out virulence genes detection on 27 isolates of *S. agalactiae*. He observed *scpB* and *cylE* genes in 6 (22.22%) isolates. Ding *et al*. (2016) found *scpB*, *cfb*, and *cylE* genes in 48.1%, 50.6%, and 4.9% isolates, respectively.

The study of virulence genes of *S. agalactiae* is helpful to understand the molecular basis of pathogenesis of *S. agalactiae* causing mastitis, which is observed as the predominant etiological agents amongst the major *Streptococcus* spp. involved in causing bovine mastitis. The results acquired from the current work are considered the beginning for more wide-ranging study of the virulence genes in evolving bovine mastitis caused by *S. agalactiae*.

Acknowledgements

The authors thanks to laboratory technician and laboratory attendant of Department of Veterinary Microbiology, Kamdhenu University, Junagadh who helped us in collecting the samples and performing the molecular experiments.

Conflict of interest

There are no conflicts of interest.

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