RESEARCH ARTICLE

Virulence Genes Detection in *Streptococcus uberis* and *Streptococcus dysgalactiae* Isolated from Bovine Mastitis in Gujarat, India

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Abstract

One of the most expensive illnesses in the dairy sector is mastitis. Due to decreased milk production and increased treatment costs, it generates significant financial losses to the dairy industries. *Streptococcus uberis* and *Streptococcus dysgalactiae* are two common environmental bacteria which cause bovine mastitis and have several virulence factors that play major role in pathogenicity. The objective of this study was to detect some important virulence genes of *S. uberis* and *S. dysgalactiae*. Overall, sixteen *S. uberis* and ten *S. dysgalactiae* were isolated from 320 milk samples. The virulence genes *hasC*, *gapC* and *sua* were found in 50%, 100% and 81.25% isolates of the *S. uberis*, respectively, while in *S. dysgalactiae* isolates, *napr* and *eno* genes were found in 60% and 20% isolates, respectively. This work helps us to understand the virulence traits and mechanisms underlying how novel mastitis strains emerge in response to preventative and curative measures.

***Key words*:** Environmental pathogen, Mastitis, *Streptococcus,* Virulence gene.

Ind J Vet Sci and Biotech (2024): 10.48165/ijvsbt.20.1.07

Introduction

Due to the loss of farm profitability, lower milk production and treatment expenses, bovine mastitis has been deemed the most significant illness on dairy farms (Jabber and Bashima, 2021). Streptococci are amongst the major microorganisms thought to be responsible for bovine mastitis globally (Gomes and Henriques, 2016).

Pathogens that cause mastitis can be categorized as contagious and environmental (Cheng and Han, 2020). *Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli* and *Klebsiella* spp. are most common environmental pathogens (Pawanjit *et al*., 2018; Savita *et al*., 2020; Shabaz *et al*., 2020). Contagious pathogens are intended to live inside their hosts and mostly pass from cow to cow during milking. Within a herd, contagious microorganisms have the potential to spread more quickly and widely. Environmental infections, in contrast, may thrive without the host and are a natural component of the microflora in the area around the cow. Exposure to environmental streptococci happens at the time of milking and after milking, in the dry season, or just before parturition (Kabelitz *et al*., 2021). Pathogen exposure is correlated with the environmental abundance of the pathogens, which is influenced by factors like temperature and humidity. Environmental microorganisms enter into the udder when the teat canal is opened after milking or following injury (Hogan and Smith, 2012).

The capacity of the pathogen to enter the immunological host cells depends upon variety of virulence factors associated with adhesion, invasion and multiplication (Parasana *et al*., 2022). Producing the surface sticky molecule SUAM (*Streptococcus uberis* adhesion molecule) is one of the key virulence factors that contribute to persistence in the mammary gland. When this protein binds to the lactoferrin in milk and a receptor on the surface of the bovine mammary epithelial cells, a molecular bridge is formed that allows the bacteria to adhere and internalize into the epithelial cells, where humoral host defenses and antimicrobials in the milk are essentially ineffective (Almeida *et al*., 2011). The development of biofilms and the synthesis of hyaluronic acid capsules, which render the bacteria resistant to phagocytosis and decrease the impact of antibiotics, are additional

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How to cite this article: Parasana, D. K., Javia, B. B., Barad, D. B., Ghodasara, S. N., & Fefar, D. T. (2024). Virulence Genes Detection in *Streptococcus uberis* and *Streptococcus dysgalactiae* Isolated from Bovine Mastitis in Gujarat, India. Ind J Vet Sci and Biotech. 20(1), 31-34.

Source of support: Nil

Conflict of interest: None

Submitted 19/09/2023 **Accepted** 25/10/2023 **Published** 10/01/2024

significant contributors (Ward *et al*., 2001; Kromker *et al*., 2014). Plasminogen activator proteins like *PauA* (Rosey *et al*., 1999), lactoferrin binding proteins (Moshynskyy *et al*., 2003), a surface dehydrogenase protein called *GapC* and *Opp* proteins involved in the active transport of solutes necessary for growth in milk are recognized virulence factors that have been identified in *S. uberis* (Smith *et al*., 2002). Although the causes of virulence are not fully understood, it is hypothesized that different strains of bacteria would display them differently (Kromker *et al*., 2014). The virulence-associated *S. dysgalactiae* determinants, such as α-enolase, β-haemolysin, nephritis-associated plasminogen-binding receptor and Laminin-binding protein are essential for the pathogenesis of the organism (Shen *et al*., 2021). However, no any study has been carried out about these traits in relation to the *S. uberis* and *S. dysgalactiae* identified from bovine mastitis in Gujarat, India. So this study was designed for identification of virulence genes to understand the molecular pathogenesis of bovine mastitis caused by *S. uberis* and *S. dysgalactiae* in Saurashtra region of Gujarat.

Materials and Methods

Bacterial Isolates and Extraction of DNA

In the present study, *S. uberis* (n=16) and *S. dysgalactiae* (n=10), were isolated from 320 bovine mastitis milk samples during 2020-2021 from Junagadh district, Gujarat, India and were processed for detection of virulence associated gene.

According to the manufacturer's instructions, the bacterial DNA from the cultures was extracted using the Nucleo-pore gDNA Fungal/Bacterial Mini Kit (Genetix brand, India) using a column-based DNA extraction method.

Virulence Gene Detection by PCR

The primers as mentioned in Table 1 were used for virulence genes detection in *S. uberis* and *S. dysgalactiae*. The standard programme for PCR reaction mixture (25 µL) included 2X PCR master mix 12.5 µL, forward and reverse primers (10 pmole/µL) 1.0 µL each, DNA template 3.0 µL and nuclease free water 7.5 µL. The PCR cycling conditions were: Initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C (*hasC* and *sua* gene), 55°C (*gapC* gene), 50°C (*eno* gene), 52°C (*napr* gene) for 45 s and extension at 72°C for 45 s with a final extension step at 72°C for 10 min.

Results and Discussion

Virulence Gene Detection in *S. uberis* Isolates

From 16 *S. uberis* isolates, 8 isolates amplified 300 bp product of *hasC* gene. All 16 *S. uberis* isolates amplified 200 bp product of *gapC* gene and 13 isolates amplified 776 bp product of *sua* gene (Fig. 1).

In the present study, of the 16 *S. uberis* isolates *hasC*, *gapC* & *sua* genes were detected in 8 (50%), 16 (100%) and 13 (81.25%) isolates, respectively. Out of 16 *S. uberis* isolates, 8 (50%) isolates were found positive for all 3 virulence genes, while in 8 (50%) isolates both *hasC* and *gapC* genes were detected, 8 (50%) isolates found positive for both *hasC* and *sua* genes and in 13 (81.25%) isolates both *gapC* and *sua* genes were detected. The findings presented are consistent with previous research conducted by other scientists. Reinoso *et al.* (2011) studied virulence-associated gene distribution in 78 *S. uberis* strains. They observed *hasC* gene in 70 (89.7%), *sua* gene in 65 (83.3%) and *gapC* in 62 (79.4%) isolates. Parin *et al.* (2017) found *hasC*, *gapC* and *sua* genes in 86%, 91% and 83% isolates, respectively. Zhang *et al.* (2020) found highest *gapC* (100%), followed by *hasC* (87.5%) and *sua* (81.3%) isolates. Kaczorek *et al.* (2017) found *hasC*, *gapC* and *sua* genes in 83%, 98% and 96% isolates, respectively. Zouharova *et al.* (2022) found *hasC* in 62%, while *gapC* and *sua* in 100% isolates.

The formation of capsules, which is reliant on the *has* operon, is one of the recognized defense strategies used by bacteria (Ward *et al*., 2001). Despite the fact that *hasABC* genes were more frequent in isolates linked to an illness, this suggests that the capsule is necessary for some elements of intra-mammary infection (IMI) and the pathogenesis. The *gapC* gene was another housekeeping gene that was detected in all of our *S. uberis* isolates. It produces the GAPDH protein, which is characterized as potentially being linked to virulence because of its capacity to bind a few host proteins or to provide protection from reactive oxygen species produced by the phagocytic cells of the host (Reinoso *et al*. 2011). Studies on vaccines have focused on GAPDH, which is also

Table 1: Virulence gene specific primer sequences

Primer (5' to 3')	Target gene	Product size	Reference
F: TGCTTGGTGACGATTTGATG R: GTCCAATGATAGCAAGGTACAC	*hasC*	300 bp	Field *et al.* (2003)
F: GCTCCTGGTGGAGATGATGT R: GTCACCAGTGTAAGCGTGGA	*gapC*	200 bp	Reinoso *et al.* (2011)
F: ACGCAAGGTGCTCAAGAGTT R: TGAACAAGCGATTCGTCAAG	*sua*	776 bp	Reinoso *et al.* (2011)
F: ATGTCAATTATTACTGATGT R: CTATTTTTTTAAGTTATAGA	*eno*	1308 bp	Abdelsalam *et al.* (2015)
F: GTTAAAGTTGGTATTAACGGT R: TTGAGCAGTGTAAGACATTTC	*napr*	963 bp	Abdelsalam *et al.* (2015)

regarded as a useful immunomodulatory protein (Fontaine *et al*., 2002). *sua* gene encodes the SUAM surface protein necessary for adhesion to and internalization into mammary epithelial cells after lactoferrin binding (Chen *et al*., 2011). The relevance of these pathogenic tactics in *S. uberis* mastitis is confirmed by the high prevalence of the *sua* gene in mastitis isolates. The SUAM is also regarded as a promising mastitis vaccine candidate (Almeida *et al*., 2015).

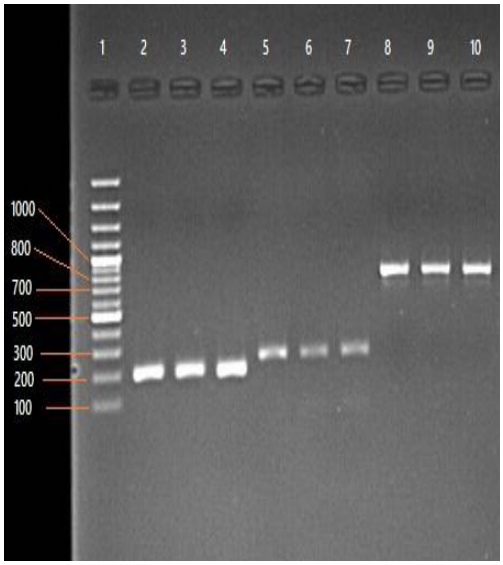

Fig. 1: Virulence gene specific PCR for *S. uberis:* Lane 1: 100 bp plus ladder, Lane 2,3,4: *gapC* gene products (200 bp), Lane 5,6,7: *hasC* gene products (300 bp), Lane 8,9,10: *sua* gene products (776 bp)

Detection of Virulence Genes in *S. dysgalactiae* Isolates

From 10 *S. dysgalactiae* isolates, 6 isolates amplified 963 bp amplicon of *napr*, and only 2 isolates amplified 1308 bp amplicon of *eno* gene (Fig. 2).

In the present study, of the 10 *S. dysgalactiae* isolates, *napr* and *eno* genes were detected in 6 (60%) and 2 (20%) isolates, respectively. Both these (*napr* and *eno*) virulence genes were detected in 2 (20%) isolates. Kaczorek *et al*. (2017) found *napr* and *eno* genes in 83% and 76% isolates, respectively. Shen *et al*. (2021) studied virulence gene detection in 60 *S. dysgalactiae* isolates. They found *napr* gene in 100%, while *eno* gene in only 16.67% isolates. The host is infected and colonized by the host-binding plasminogen protein encoded by *napr* and *eno* genes. Plasminogen recruitment to the bacterial surface has been recognized as a crucial pathogenic step that facilitates bacterial adhesion to cell surfaces (Fulde *et al*., 2013). Therefore, the primary cause of *S. dysgalactiae* infection in dairy cows may be the *napr* gene, which encodes nephritis-associated plasminogen-binding receptor.

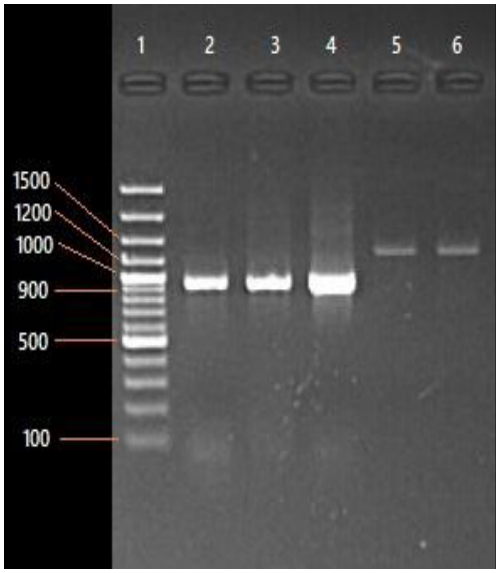

Fig. 2: Virulence gene specific PCR for *S. dysgalactiae:* Lane 1: 100 bp plus ladder, Lane 2,3,4: *napr* gene products (963 bp), Lane 5,6: *eno* gene products (1308 bp)

Conclusion

This study concludes that the *napr* gene may act as the main invasive factor in *S. dysgalactiae* that causes clinical mastitis, which significantly influences the progression and management of bovine mastitis. Due to their high frequency and highly conserved character, a number of virulence-associated genes are thought to make excellent candidates for vaccine development. These results provide guidance for the protection of public health and advancement to our knowledge focused on mastitis prevention initiatives. Further investigation is required to identify additional crucial virulence-related variables and to clarify each crucial stage in the pathogenesis of *S. uberis* and *S. dysgalactiae* intramammary infection.

Acknowledgement

The Kamdhenu University, Gandhinagar, administration and the Dean of the College of Veterinary Science & AH, KU, Junagadh are thanked for their assistance and facilities.

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