B-4940
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RESEARCH ARTICLE Indian Journal of Animal Research

Analysis of Virulence Genes of *Staphylococcus aureus*, *Streptococcus*, *E. coli* Isolated from Bovine Subclinical Mastitis

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10.18805/IJAR.B-4940

ABSTRACT

Background: The major mastitis-producing organisms are *Staphylococcus aureus, Streptococcus agalactiae*, *Streptococcus dysgalactiae, Streptococcus uberis and Escherichia coli.* Molecular characterization of the major bacterial pathogens for some genes responsible for their virulence should be considered the reduction of risk factors responsible for the presence and spread of contagious pathogens through milk.

Methods: The two hundred milk samples were collected from cows and buffaloes of four different tehsils of the Sirohi district of Southern Rajasthan. Out of 200 milk samples, 74 milk samples that were found positive for SCM were cultured for primary isolation of predominant bacterial pathogens. Out of these 74 positive samples, total of 97 isolates were recovered from these milk samples either as a single or mixed infection. To genotypically characterize *S. aureus* isolates, genes encoding virulence determinants (*spa*-IgG-binding and *Coa*), Staphylococcal enterotoxins *(bac* and *bca*) and *E. coli* Shiga toxins (*stx1* and *stx2*) were investigated.

Result: The etiological prevalence of SCM caused by different bacteria was, *S. aureus,* (27%), *Streptococcus* spp. (15%) and *E. coli* (6.5%) respectively either as single and or as mixed infections. All *S. aureus* isolates were tested by PCR for the presence of the *spa* gene and *coa* gene results revealed that 40 isolates (74.0%) carried both s*pa* (IgG-binding) and *coa* gene. 11 isolates of *S. agalactiae* (36.6%) carried the *bca* gene. The *bca* gene codes for Alpha-C protein, a surface protein that helps the bacteria to enter the host cells. *E. coli* isolates were also screened for the presence of virulence gene *stx1*and *stx2* gene. Out of 13 isolates tested, 6 isolates harboured both *stx1* and *stx2* genes.

Key words: Bacterial, Gene, Mastitis, Prevalence, Virulence.

INTRODUCTION

Subclinical mastitis (SCM) is a herd problem, which acts as a repository of microorganisms that leads to the spread of infection to the other animals undetectable to naked eyes. The sub clinically affected animals remain a continuous source of infection to other herd mates. If the infection persists for longer periods, then it may form a fibrous tissue barrier between the organisms and the antibiotic preparations, thus limiting their efficacy.

A variety of virulence factors are responsible for subclinical and persistent intramammary infection, Fursova *et al.,* (2020). Molecular characterization of these virulence factors should be considered for the reduction of risk factors responsible for the presence and spread of contagious pathogens, minimizing the usage of antibiotics and to prevent the mutation of micro-organisms, Pérez *et al.,* (2020). Indiscriminate usage of antibiotics has led to the development of antibiotic resistance conferred by specific genes (Liu and Pop, 2009).

Pathogenicity of staphylococcus associated with mastitis is an extremely important feature in the disease process that requires a better understanding. The ability of *Staphylococcus aureus* to cause various infections and intoxication results from the production of different virulence factors Aung *et al.,* (2011). Slime production is considered a virulence factor that inhibits the immune response of the host and facilitates the adhesion of the pathogen according to Atkin *et al.,* (2014). Similarly, Pang *et al.,* (2017) identified unique signal transduction genes from the complete genome of that isolated pathogen and amplified these genes using gene-specific primers for a selected pathogen-like virulence gene of *S. agalactiae i.e., hylB*, *gapC*, *cspA*, *dltA*, *fbsA*, fbsB and bibA. Ismail *et al.,* (2020) also studied the virulence genes of *Escherichia coli i.e.* eaeA, aer, traT, stx1, stx2, fimH and Cnf genes from bovine mastitis.

Virulence associated genes of *Staphylococcus aureus* has been evaluated by various researcher *i.e.,* coagulase gene (Coa) and spa genes by Khan *et al.,* (2013); Parth *et al.,* (2016) of enterotoxin A to E (sea, seb, sec, sed, see), (coa),

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How to cite this article: Solanki, S. and Devi, D. (2022). Analysis of Virulence Genes of *Staphylococcus aureus*, *Streptococcus*, *E. coli* Isolated from Bovine Subclinical Mastitis. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4940.

Submitted: 23-05-2022 **Accepted:** 28-09-2022 **Online:** 11-10-2022

(spa) (femA), (mecA) El-Tawab *et al.,* (2016), nuc (thermo nuclease) and Coa, beta-lactamases (blaZ, mecA) and (pvl and tst) genes Awad *et al.,* (2017), Similarly, the cfb gene of *S. agalactiae*, mig gene of *S. dysgalactiae*, Krishnaveni *et al.,* (2014) and eae, stx, let est and hlyA gene of *E. coli.* by Ahmed *et al.,* (2018) has been studied.

Molecular genetics permits the isolation of specific genes from bacterial pathogens, the elucidation of their structure and function and the modification of their expression. A powerful approach based on Koch's postulates has been proposed as. The supposed virulence factor should be associated with pathogenic strains of the bacterial species under investigation. The genes encoding this supposed virulence factor should be isolated and the inactivation of the genes should lead to a significant loss of virulence. The re-introduction of the genes of origin in the modified strains should restore the virulence. During the past decade, this modern molecular approach has been developed to study virulence factors of microorganism from bovine mastitis.

MATERIALS AND METHODS

The entire research was performed in the laboratory of the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Navania, Vallabhnagar, Udaipur. This study was conducted with all animal welfare and ethical considerations in mind and was approved by the Establishment's Animal Ethics Board. Screening of the SCM was conducted by modifying the California mastitis test.

Isolation and biochemical characterization

A total of 74 milk samples based on CMT were subjected to bacteriological examination for the isolation and identification of bacterial species in the milk samples, the techniques as per standard procedures by Markey *et al.,* (2013) were implemented.

Identification and biochemical analysis of *Staphylococcus aureus,* Streptococcus spp. and *E. coli.*

Pure cultures of isolates were submitted for gram staining and further by catalase test. The catalase-positive cultures were streaked on nutrient agar obliques and preserved at 4°C. From these slants, the pure cultures were subjected to various biochemical tests as per standard procedures Markey *et al.,* (2013). The isolated bacteria were identified up to specie level based on colony characteristics of individual primary isolate.

Extraction of bacterial DNA

Isolation of bacterial genomic DNA directly from milk samples was done based on the protocol described by Phuektes *et al.,* (2001) with some modifications in the initial steps. Before milk is used for DNA extraction it was subjected to centrifugation and fat was removed from the top. The remaining milk was discarded and the pellet was used for the DNA isolation.

Molecular detection of virulence genes

The oligonucleotide primer sequences and the corresponding amplicon sizes for the detection of virulence genes in different PCR tests are mentioned in Table 1. All the PCR tests for the detection of virulence genes were carried out in a final volume of 25 µl. The optimum concentrations of different reagents for PCR are mentioned in Table 2. The cyclic conditions for each reaction are given in Table 3.

RESULTS AND DISCUSSION

The virulence genes examined in this study would be helpful to suggest possible association in the pathogenesis of mammary infections. Several virulence factors are involved in the adhesion to and invasion of host cells, as well as in the immune system evasion. Both virulence factors and the ability to resist antimicrobial drugs in bacterial infection contribute to successful host-microbe colonization and dissemination into a population. Thus, it is not difficult to envisage a synergistic action between these features during infection. In our study, the *spa* (IgG) and *coa* gene of *Staphylococcus aureus*, *bca* and *bac* gene of Streptococcus and genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*) for *E. coli.* were discovered from subclinical mastitis.

Table 1: Oligonucleotide primer sequences and amplicon sizes for virulence gene.

Bacterial species and their gene	Oligonucleotide primer sequence (5′-3′)	Amplicon size (bp)	Reference
S. aureus Spa (IgG-binding)	CACCTGCTGCAAATGCTGCG GGCTTGTTGTTGTCTTCCTC	970	Kalorey *et al.,* (2007)
S. aureus (coa)	ATAGAGATGCTGGTACAGG GCTTCCGATTGTTCGATGC	627	Kalorey *et al.,* (2007)
Streptococcus genus (*bca)*	TAACAGTTATGATACTTCACAGAC ACGACTTTCTTCCGTCCACTTAGG	535	Manning *et al.,* (2006)
Streptococcus genus (*bac)*	CTATTTTTGATATTGACAATGCAA GTCGTTACTTCCTTGAGATGTAAC	592	Manning *et al.,* (2006)
E. coli (stx1)	AAATCGCCATTCGTTGACTACTTCT TGCCATTCTGGCAACTCGCGATGCA	366	Momtaz *et al.,* (2012)
E. coli (stx2)	CGATCGTCACTCACTGGTTTCATCA GGATATTCTCCCCACTCTGACACC	282	Momtaz *et al.,* (2012)

Isolation results

Out of these 74 positive samples for SCC, 72 samples had bacterial growth and while in 02 samples there was absence of bacterial growth. Out of the 72 samples that showed bacterial colonies, only 40 had single bacterial growth whereas rest of the 32 samples had mixed growth. A total of 97 isolates were recovered from these milk samples. The prevalence of mastitis caused by *Staphylococcus aureus,* (54/200, 27%), Streptococcus spp. (30/200, 15%) and *E. coli* (13/200, 6.5%) respectively either as single and or as mixed infections. The similar results were also reported by Lakshmi and Jayavardhanan (2016) which found 36% *Staphylococcus aureus* and 27% *E. coli*. Omar, and MatKamir, (2018) find Staphylococcus spp. (73.2%). Coagulase negative staphylococci encompassing 68.3% of the isolates, whereas 4.9% was coagulase positive staphylococci. Similarly, Sztachanska *et al.,* (2016) reporting 31.6% Coagulase negative staphylococci, 15.6% Streptococcus *(Str.) agalactiae*, 12.1% *Staphylococcus aureus* from subclinical mastitis.

Table 2: Optimum concentration of different reagents for PCR targeting virulence genes.

Component	Volume/reaction (µl)
10x Taq DNA polymerase buffer	2.5
25 mM M_gcl_2	1.5
10 mM dNTPs	0.2
10 pM forward primer	1
10 pM reverse primer	1
5 U/µl Taq DNA polymerase	0.3
Template DNA	3
Nuclease free water	15.5
Total volume	25 µl

Virulence associated genes of *S. aureus*

All 40 isolates (74.0%) of *S. aureus* carried both s*pa* (IgG-binding) and *coa* gene. Whereas, 14 isolates (25.9%) found spa negative. All positive isolates showed amplification products of 970 bp size and 627 bp size respectively, (Fig 1 and 2). Such *spa* negative *S. aureus* isolates have earlier been reported by some workers, Santos *et al.,* (2014) Momtaz *et al.,* (2010) Khichar *et al.,* (2014). Choudhary *et al.,* (2018). The IgG -binding of the *spa* gene can be used in the study of genetic diversity in the *S. aureus* strains as a molecular marker for epidemiological research of origin and origins of infection.

Virulence associated genes of streptococcus

The results of the present study revealed that 11 isolates of Streptococcus (36.6%) carried the *bca* gene as they showed amplification products of 535 bp size (Fig 3). The *bca* gene

Table 3: Cyclic conditions for PCR amplification of virulent genes.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
Spa (IgG)	94°C/5min	94°C/1min	55°C/30 sec	72°C/1 min	72°C/5 min	36
coa	93°C/5min	94°C/1 min	55°C/30 sec	72°C/1 min	72°C/5 min	36
bca	93°C/3min	93°C/1 min	52°C/1 min	72°C/1 min	72°C/5 min	35
bac	93°C/3 min	93°C/1 min	52°C/1 min	72°C/1 min	72°C/5 min	35
(stx1)	93°C/3 min	93°C/1 min	52°C/1 min	72°C/1 min	72°C/5 min	35
(stx2)	93°C/3 min	93°C/1 min	52°C/1 min	72°C/1 min	72°C/5 min	35

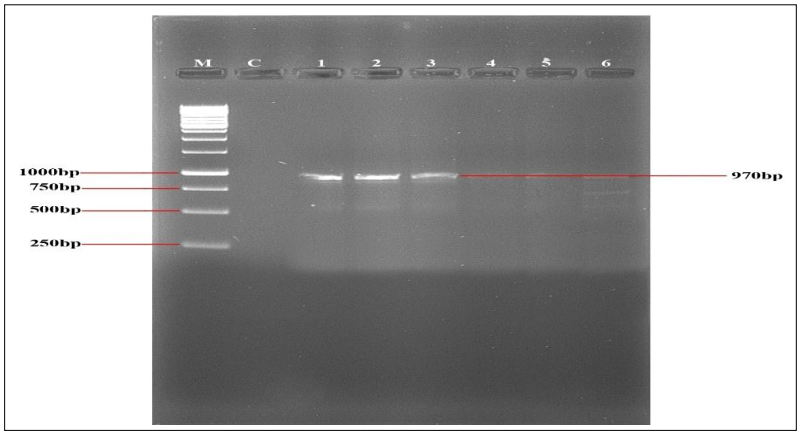

Fig 1: Detection of virulence gene by PCR amplification of 970 bp *Spa* (IgG-binding) gene of *S. aureus* isolated from subclinical bovine mastitis milk.

Lane M: 1 kb DNA ladder.

Lane C: Negative control.

Lane 1-3: PCR amplified 970 bp product of *S. aureus* isolates.

Lane 3-6: No template.

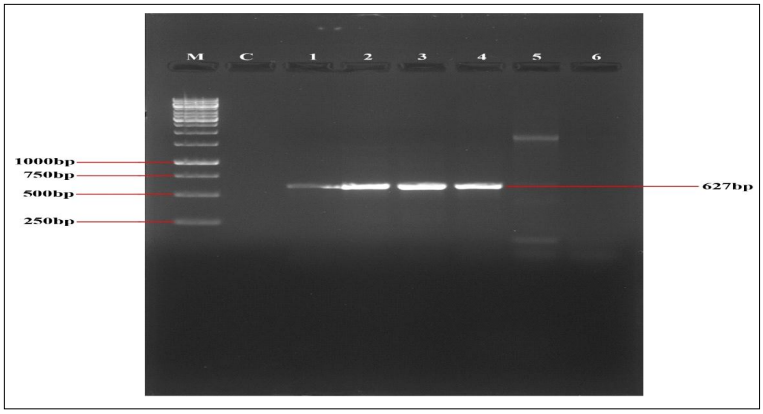

Fig 2: Detection of virulence gene by PCR amplification of 627 bp *coa* gene of *S. aureus* isolated from subclinical bovine mastitis milk.
Lane M: 1 kb DNA ladder.
Lane C: Negative control.
Lane 2-4: PCR amplified 627 bp product of *S. aureus* isolates.
Lane 5-6: No template.

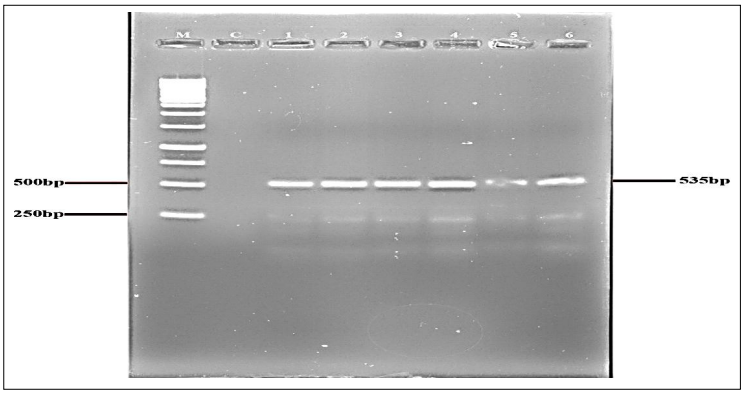

Fig 3: Detection of virulence gene by PCR amplification of 535 bp *bca* gene of Streptococcus isolated from subclinical bovine mastitis milk.
Lane M: 1 kb DNA ladder.
Lane C: Negative control.
Lane 1-6: PCR amplified 535 bp product of Streptococcus isolates.

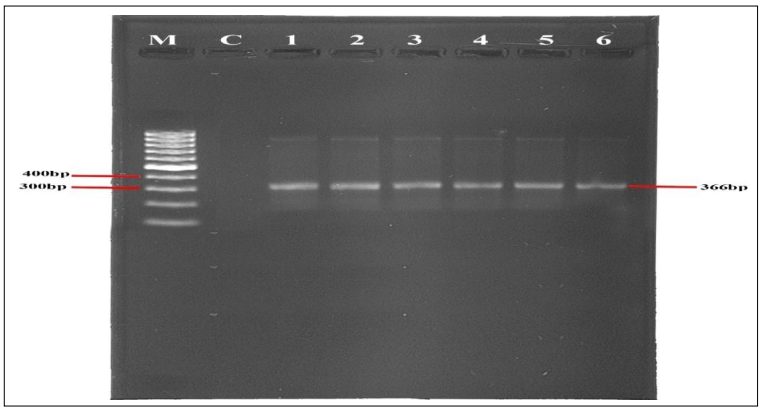

Fig 4: Detection of virulence gene by PCR amplification of *stx1* gene (366 bp) of *E. coli* isolated from subclinical bovine mastitis milk.
Lane M: 100 bp ladder.
Lane C: Negative control.
Lane 1-6: PCR amplified 366 bp product of *E. coli* isolates.

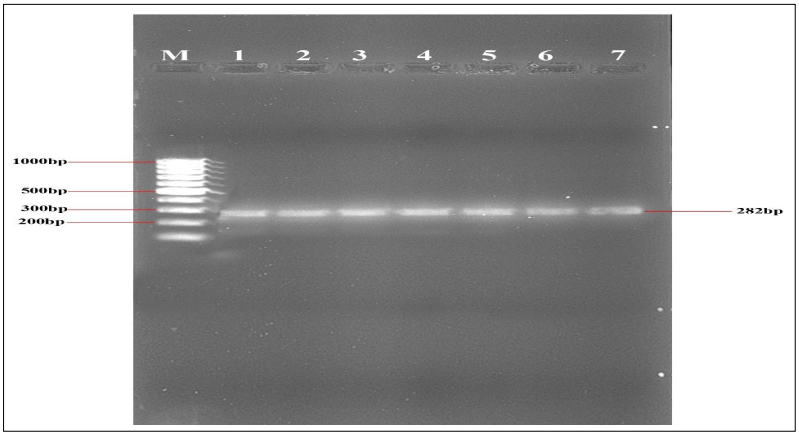

Fig 5: Detection of virulence gene by PCR amplification of *stx2* gene (282 bp) of *E. coli* isolated from subclinical bovine mastitis milk.
Lane M: 100 bp ladder.
Lane 1-7: PCR amplified 282 bp product of *E. coli* isolates.

Table 4: Prevalence of virulence-associated genes.

Bacterial isolates	Target genes	No of positive sample	Percentage	No of negative sample	Percentage
Staphylococcus aureus (n=54)	*Spa (IgG)*	40	74.0	14	25.9
	coa	40	74.0	14	25.9
Streptococcus spp. (n=30)	*bca*	11	36.6	19	63.33
	bac	NA	-	NA	-
E. coli (n=13)	*(stx1)*	6	46.15	7	53.8
	(stx2)	6	46.15	7	53.8

codes for Alpha-C protein, a surface protein that helps the bacteria to enter the host cells according to Bolduc *et al.,* (2002). All the 30 isolates of Streptococci were subjected to PCR targeting virulence-associated bac gene revealed that out of 30 isolates of Streptococcus none was carrying the bac gene. A similar result was found by Eldesouky *et al.,* (2016) Duarte *et al.,* (2004) and Duarte *et al.,* (2005) Behiry *et al.,* (2015) Ding *et al.,* (2016).

Virulence associated genes of *E. coli*

In the present study, the *E. coli* isolates from SCM were also screened for the presence of virulence gene *stx1* and *stx2* gene. Out of 13 isolates tested, 6 isolates harbored both *stx1* and *stx2* genes (46.15%) as they showed amplification products of 366 bp size and 282 bp size respectively, (Fig 4 and 5). These findings are by the earlier studies that indicated that genes encoding Shiga toxins 1 and 2 (stx1 and stx2) were the most prevalent virulence factors isolated from subclinical mastitis and clinical bovine mastitis. Moussa *et al.,* (2010) confirmed in their study that the stx2 gene was the most prevalent virulence factor in an animal environment contaminated by feces. It is also a frequent cause of bovine or subclinical mastitis. *E. coli* isolates usually possess one or more virulence factors that may help in the establishment at the infection site and subsequently cause subclinical mastitis and clinical bovine mastitis. The prevalence of virulence- associated genes is presented in the Table 4.

CONCLUSION

In conclusion, the present study's findings revealed the presence of virulence genes of different bacteria isolated from bovine subclinical mastitis. This is an alarming situation so attention must be paid to the implementation of new ways for effective prophylaxis, control and treatment of such infections on dairy farms. Further, it is concluded that PCR assays alone can be used as rapid and sensitive diagnostic tools to detect virulence factors that help in the detection of the severity of infection, distribution and stating preventive and control strategies related to affiliations caused by milk consumption in Humans.

Authors' contributions

Sudeep Solanki participated in the Conceptualization, Formal analysis, Investigation and Writing - of the original draft of the manuscript. Durga Devi participated in the design of the study, performed the statistical analysis and reviewed and editing the manuscript. All authors read and approved the final manuscript.

Funding

The research was self-funded.

Ethics approval and consent to participate

This study was conducted with all animal welfare and ethical considerations in mind and was approved by the Establishment's Animal Ethics Board". The study was done

on milk samples collected from cows and buffaloes with the consent of the owner. Additionally, all ethical measures were taken to reduce animal pain during sampling and all criteria regarding inclusion and exclusion of their animals were explained for owners.

Competing interests

The authors declare that they have no competing interests.

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