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RESEARCH ARTICLE

CHARACTERIZATION OF TOXIN GENES IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MILK OF COWS WITH MASTITIS

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ABSTRACT

In this study *Staphylococcus aureus* isolated from milk of the cow with bovine mastitis were used for screening the production of exoproteins, presence of enterotoxin genes and comparison of pathogenic strains which can aid in better understanding of evolution of virulence factors among the species and also in developing the specific vaccine against the *S.aureus* infection. In this investigation exoproteins like alpha and beta toxins were screened by hemolysin test, PCR technique was used for the molecular identification of *S.aureus* using 23S rRNA gene, detection of enterotoxin gene such as Staphylococcal enterotoxin a (Sea), Staphylococcal enterotoxin b (Seb) and comparison of pathogenic isolates was carried out by PCR-RAPD technique which was found to be 87.50% polymorphism.

INTRODUCTION

Staphylococcus aureus is a gram positive bacteria that causes many serious diseases in humans as well as animals and it is found to be the most predominant bacteria causing bovine mastitis with relevant losses in the dairy industry (Kurjogi and Kaliwal, 2011) Staphylococcus produce many potential virulence factors such as toxins like alpha, beta and gamma toxin leukocidin, toxic shock syndrome toxin (TSST) and different types of enterotoxins. Alpha and beta toxins are exotoxins hemolysins in nature cause lysis of erythrocytes by pore formation. A wide range of cell types is affected by alpha toxins including erythrocytes, monocytes, lymphocytes, macrophages and epithelial cells (Bhakdi and Tranum, 1991; Bhakdi *et al.*, 1989 1988). Alpha toxin secreted as a water soluble single polypeptide chain of 34 kDa, zone is formed along the streaking on the blood agar and induces concentration dependent cell death via apoptotic pathway (Essmann *et al.*, 2003). On the other hand beta toxin also has the molecular weight of 34 kDa though it has shown that the purified protein induces inflammatory changes in the mouse mammary glands but still its role in pathogenesis remains uncertain (Ward *et al.*, 1979). Staphylococcus enterotoxins are the major cause of food poisoning, which typically occurs after ingestion of food particularly processed meat and dairy products, contaminated sub clinical bovine mastitis milk. Symptoms are of rapid onset include nausea and vomiting with diarrhea. Staphylococcus enterotoxins have been classified according to their serological differences they are designated as Sea to Sew all the toxins have low molecular weight ranging between 20-30 kDa. Sea is the most common cause of staphylococcal food poisoning worldwide. Enterotoxin are synthesized throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase (Betley, *et al.*, 1992; Bergdoll, 1979; Czop and Bergdoll, 1974; Derzelle *et al.*, 2009). These toxins are resistant to heat as well as proteolytic enzyme produced in the digestive tract after ingestion therefore, thermal treatment of food and digestion in the stomach cannot destroy the enterotoxins of *S.aureus* (Karima, 2006). In addition to their nature of potent gastrointestinal toxin enterotoxins of *S.aureus* act as pyrogenic toxins which cause immunosuppression and non-specific T-cell proliferation and their activities are referred to as super antigen activity (white *et al.*, 1989; Marrack and Kappler, 1990).

The objective of the present study was to isolate bacteria from the milk of cows with mastitis, identification of *S.aureus* by PCR method, screening for the production of exotoxins like alpha and beta toxins and detection of enterotoxin genes by using polymerase chain reaction. The present study was also conducted to compare the genotype of toxin producing *S.aureus* isolates by using PCR-RAPD technique.

MATERIALS AND METHODS

Isolation and identification of bacteria

A total of 185 bacterial isolates were recovered from 120 milk samples out of which 87 isolates were found to be staphylococcal species, 10 pure culture of *S.aureus* isolates were selected for the present investigation. All the *S.aureus* isolates were identified by colony morphology and conventional methods like gram staining, microscopic observation and biochemical test. The isolates were further confirmed by the amplification of species specific part of the gene encoding the 23S rRNA by using PCR

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Hemolysin test

All the confirmed cultures of *S.aureus* were streaked on the blood agar plates to study its hemolytic toxin types blood agar plates were prepared by using 2.5% of human blood in a nutrient agar

DNA extraction and quantification

DNA Extraction was carried out using SDS-Ammonium acetate method modified from Moore *et al.,* 2004(1). 2-5 µg (Loopfull) of culture was suspended in 0.2 ml extraction buffer (50mM Tris-HCl, 50mM EDTA, 250 mM NaCl, 1.5% Sucrose) and 50 µl of SDS (20%) was added to it. The solution was vortexed and incubated for 30 min at 65 ºC and 100 µl of 7.5 M Ammonium acetate was added to it. The tubes were incubated at 4 ºC for 15 minutes. DNA was extracted using 1 volume of chloroform: isoamyl alcohol mixture (24:1) and centrifuging at 10000 rpm for 5mins. Double volume of ethanol (96-100%) was added to the aqueous phase in a new tube, inverted twice and allowed to stand at 4 ºC for 30 minutes. Further, the mixture was centrifuged at 10,000 rpm for 15 minutes. After drying for few seconds pellet was dissolved in 50 µl elution buffer (10 mM Tris-HCl, 1 mM EDTA). Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at -20 ºC for further use.

Polymerase chain reaction

PCR amplification was performed using Applied Biosystem verti thermal cycler. All the primers for PCR amplification were obtained from MWG Biotech. Oligonucleotide sequences of primers for the 23S rRNA, Sea, Seb, Sed and TSST were published by Akineden *et al.,* (2001). The PCR was performed in 20 µl reaction mixture containing 2 µl of 10X assay buffer, 1.5 µl dNTP mix of 2.5 mM, 0.5 µl of $mgcl_2$, 1 µl each of forward and reverse primer (5 pmol), 0.3 µl of Taq polymerase, 1 µl of template DNA and 12.7 µl of HPLC grade water with the following amplification for 23 S r RNA gene initial denaturation at 94ºC for 3 min followed by 35 cycles of denaturation, annealing and extension (94ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min) and final extension at 72 ºC for 10 min followed by hold for infinity at 4 ºC. for Sea and Seb gene initial denaturetaion at 94 ºC for 3 min followed by 35 cycles of denaturetaion , annealing and extension (94 ºC for 1 min, 1 min at 57 ºC for Sea gene and 55 ºC for Seb gene and 72 ºC for 1 min) and final extension at 72 ºC for 10 min followed by hold for infinity at 4 ºC. PCR products were kept at -20 ºC until further use. The presence of PCR products was determined by 1.5% agarose gel electrophoresis and to analyse the size of amplified PCR product DNA markers of 100 bp and 1 kb was used which was provided by the Merck

PCR-RAPD technique was used to compare the genotype of toxin producing *S.aureus* isolates by using 5 primers (Table 1)

Table 1 Primers used for PCR amplification

Primer	Sequences 5'-3'
OPP01	GTAGCACTCC
OPQ01	GGGACGATGG
OPC13	AAGCCTCGTC
OPC20	ACTTCGCCAC
OPK08	GAACACTGGG

PCR amplification

PCR amplification was performed using Biometra thermal cycler. The PCR mixture contained 2.5 µl of 10 X buffer, 1µl of primer, 2.5 µl of 2.5 mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA. The PCR amplification cycle consisted of, a cycle of 5 min at 94 °C; 35 cycles of 45 sec at 94 °C, 45 sec at 37 °C, 1 min and 30 sec at 72 °C; and 1 cycle of 5 min at 72 °C.

Gel electrophoresis

Gel electrophoresis was performed using 1.4% agarose to analyse the size of amplified PCR product. DNA marker of Qarta bio (10,000 – 300 bp) Fermentas (10,000-250 bp) was used.

Rapid amplification of polymorphic DNA (RAPD)

RAPD data were scored for presence (1), absence (0) or as a missing observation. These bands were considered as polymorphic when they were absent in one among those two samples, in a frequency greater than 1% (Jorde, 1995) and changes in band intensity were not considered as polymorphism. The band size generated by five primers ranged from 150 to 2000 bp.

RESULTS

Identification of bacteria

All the 10 isolates used in the present study could be identified as *S. aureus* based on the colony morphology, gram staining and biochemical test. The identification of isolates as *S.aureus* were confirmed by PCR amplification of the gene encoding the 23s rRNA . the amplicon of the 23S r RNA gene showed a uniform size of about 1250bp (Fig.1)

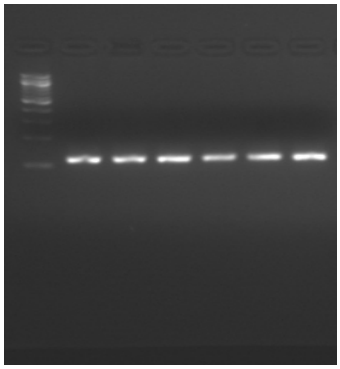


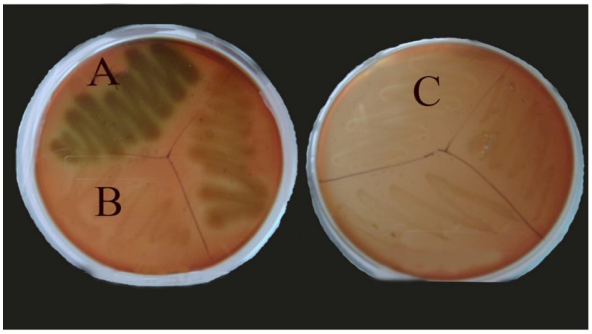
Fig. 1 PCR amplicon of 23S rRNA gene, lane 1 was DNA marker of 1 kb

Hemolysin toxin test

Among 10 *S.aureus* isolates the highest hemolysin toxin produced was found to be alpha toxin that is 6 isolates showed a green zone along the streaking on the blood agar. Followed by beta toxin that is 3 isolates showed a clear zone around the streaking on the blood agar and 1 isolate was found to be non hemolytic (Fig 2)

Enterotoxin gene detection

All the 10 *S.aureus* isolates were screened for the presence of enterotoxin gene encoding Sea, Seb, Sed, and tsst among 10 isolates 1 isolate contained the gene encoding Sea and 1 isolate showed amplification for the gene encoding Seb. None of the isolates harbored the genes encoding Sed and tsst (Fig 3).



A – α- haemolytic B – non-haemolytic C – β-haemolytic

Fig. 2 Plates showing hemolysin test

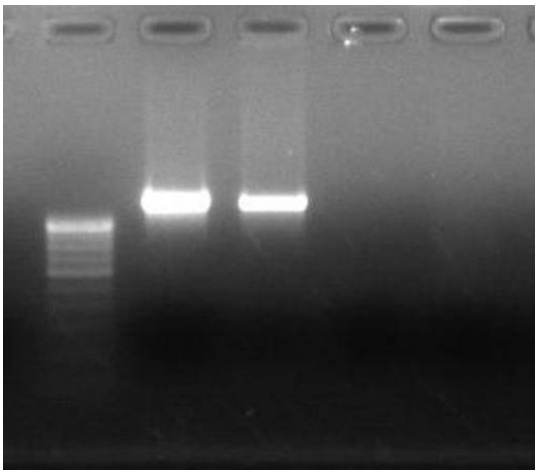


Fig. 3 PCR amplicon of Sea and Seb gene,
lane 1 was DNA marker of 100 bp lane 2: Sea lane 3: Seb, lane 4: Sed and lane 5: Tsst

Interspecies Comparative RAPD analysis

The bands of RAPD analysis between the two species *Staphylococcus aureus* 1 and *Staphylococcus aureus* 2 (SA1 and SA2) were considered as polymorphic when they were absent in one among those two samples (Table 2-6 Fig 4-6). Based on 0-1 data generated the genetic polymorphism was studied. Out of 24 loci observed 21 loci are polymorphic i.e. 87.5% (Table 7).

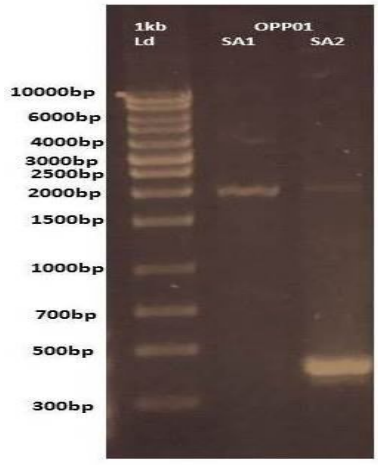


Fig. 4 RAPD pattern using OPP01
Lane 1: Qartabio DNA marker (10000-300bp) Lane 2: PCR product of SA1 Lane 3: PCR product of SA2

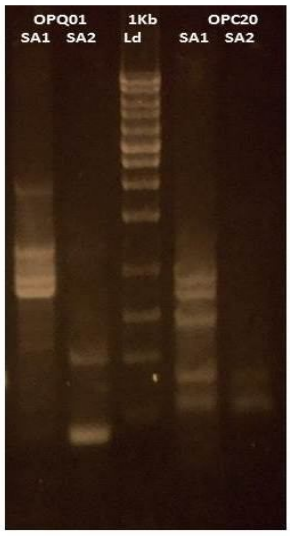


Fig 5 RAPD pattern using OPQ01 and OPC20

Lane 1: PCR product of SA1 using OPQ01 Lane 2: PCR product of SA2 using OPQ01 Lane 3: DNA marker (10000-300bp) Lane 4: PCR product of SA1 using OPC20 Lane 5: PCR product of SA2 using OPC20 Qartabio DNA marker: 10000bp, 8000bp, 6000bp, 5000bp, 4000bp, 3000bp, 2500bp, 2000bp, 1500bp, 1000bp, 700bp, 500bp and 300bp.

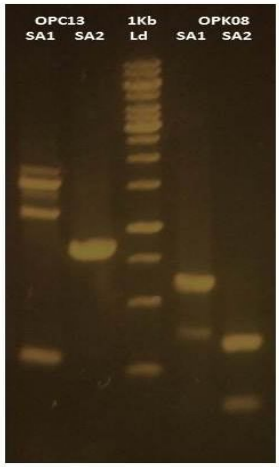


Fig 6 RAPD pattern using OPC01 and OPK08

Lane 1: PCR product of SA1 using OPC13 Lane 2: PCR product of SA2 using OPC13 Lane 3: Fermentas DNA marker (10000-250bp) Lane 4: PCR product of SA1 using OPK08 Lane 5: PCR product of SA2 using OPK08 Fermentas DNA marker: 10000bp, 8000bp, 6000bp, 5000bp, 4000bp, 3500bp, 3000bp, 2500bp, 2000bp, 1500bp, 1000bp, 750bp, 500bp and 250bp.

Table 2 Comparative RAPD analysis of SA1 and SA2 using primer OPP01

Primer	Culture name	
OPP01	**SA1**	**SA2**
2000	1	1
400	0	1

Table 3 Comparative RAPD analysis of SA1 and SA2 using primer OPQ01

Primer	Culture name	
OPQ01	SA1	SA2
2000	1	0
1200	1	0
900	1	0
850	1	0
550	1	0
500	0	1
250	0	1

Table 4 Comparative RAPD analysis of SA1 and SA2 using primer OPC13

Primer	Culture name	
OPC13	SA1	SA2
1700	1	0
1500	1	0
1400	1	0
1200	1	0
800	0	1
300	1	0

Table 5 Comparative RAPD analysis of SA1 and SA2 using primer OPC20

Primer	Culture name	
OPC20	SA1	SA2
900	1	0
800	1	0
680	1	0
400	1	1
350	1	1

Table 6 Comparative RAPD analysis of SA1 and SA2 using primer OPK08

Primer	Culture name	
OPK08	SA1	SA2
600	1	0
400	1	0
380	0	1
150	0	1

causing bovine mastitis in the area that work has been carried out (Kurjogi and Kaliwal, 2011). In the present study all the *S.aureus* isolates were identified by conventional and PCR methods, the use of primers for the amplification of gene encoding 23S rRNA was similar to that of the author Akineden *et al*., (2001), this allowed the rapid identification of the species. Which revealed that the amplification of 23S rRNA with a size of approximately 1250 bp, the results of the present study were in corresponding with the results of Dimitar *et al.,* (2004) and Akineden (2001).

The production of bacterial virulence factors is frequently influenced by various extracellular and intracellur signals exoproteins like alpha toxin and beta toxin are the major virulence factor involved in *S.aureus* pathogenesis. In the present study alpha toxin producing strains were found to be highest followed by beta toxin whereas it has been reported that beta toxin producing strains were highest among the coagulase negative staphylococci isolated from food and starter culture (Christiane *et al.,* 2008). Bovine mastitis with *S.aureus* are usually treated with antibiotics and reports revealed that low doses of antibiotic treatment enhance the hemolytic activity of the bacteria (lorian, 1971; Lorian and Gemmel 1991, Vymola and lochman, 1974). Kernodle (1995) has reported that beta lactums induces alpha toxin production *in vitro* and also increases the lethal activity of broth filtrates in a mouse model.

Investigating the *S.aureus* isolates for the presence of entrotoxin genes revealed that out of 10 tested isolates SA1 showed the presence of Sea gene and SA2 showed the presence of Seb gene, none of the isolates showed amplification for the gene Sed and TSST. Whereas Zschock *et al.,* (2000) have reported 3 strains harboured the Sea gene, 2 isolates had Seb genes, 22 were positive for Sec gene and sed was found in 4 isolates. Authers also report that 46.4% of tested clinical isolates were found to carrying sea gene (Pourmand *et al.*, 2009). Another study in Jordan by Naffa *et al.,* (2006) have showed that in clinical isolates Sea, Sec and Sea along with Sec were detected in 15.4%, 4% and 4% of the strains respectively. However, none of the isolates had Sed and Seb. The study of Koltz *et al.,* (2003) have revealed that the number of isolates carrying Seb, Sec and Sed were 9.7%, 21.5% and 14.0% respectively.

Table 7 Comparative RAPD analysis of SA1 and SA2

Primer	sample	Total Loci	Polymorphic loci	% of polymorphism
OPP01	SA1	2	1	50.00
	SA2			
OPQ01	SA1	7	7	100.00
	SA2			
OPC13	SA1	6	6	100.00
	SA2			
OPC20	SA1	5	3	60.00
	SA2			
OPK08	SA1	4	4	100.00
	SA2			
Total		24	21	87.50

DISCUSSION

Many studies have been conducted for the detection of enterotoxin and exotoxin in clinical isolates but a few researchers have been carried out the studies on toxins and their genes in bovine mastitis milk isolates. *S.aureus* was choosen for the present study as it was predominant organism

A lower prevalence of enterotoxin positive *S.aureus* that is only 5 and 4% was reported by *Lopes et al.,* (1990) and Harvey and Gilmour (1985) respectively by using mastitis strains. In another study of *S.aureus* isolated from clinical samples at Bulgaria reported that among the gene encoding classical enterotoxins that most prevalent toxin gene was Seb,

followed by Sea, Sed and the least was TSST (Dimitar *et al*., 2004). Considering the findings of the present study PCR technique is proven to be more sensitive and specific method for the identification and detection of enterotoxin genes

In recent years, molecular DNA markers have been used extensively for biodiversity analysis and identification purposes. The RAPD technique is simple yet efficient technique for the biodiversity analysis. It has been widely used for studying genetic polymorphism, DNA fingerprinting, varietal identification, classification and population genetics (Williams *et al.,* 1990; Rollinson and Stothard, 1994; Loxdale *et al.,* 1996). A RAPD marker has the greatest advantage in its capability to scan across all regions of the genome hence highly suited for phylogeny studies at species level (Wilikie *et al*., 1993; Demeke 1992).

The present study has been carried to find out the genetic variation among two bacterial species (SA1 and SA2) using five-RAPD primers. Based on band pattern observed using five RAPD primers, the overall polymorphisms detected across two species were 87.50%, whereas another study in Turkey by Guler *et al.,* (2005) have used Coa gene primers for finding out the epidemiological relatedness among *S.aureus* isolated from bovine mastitis cases, similarly several authors used Coa gene for typing of *S.aureus* isolates of human and bovine origin (Raimunda *et al.,*1999; Lange *et al.,*1999;)

CONCLUSION

The results of the present study revealed the presence of toxin producing genes in the *S.aureus* isolated from milk of the cow with bovine mastitis, where as PCR RAPD analysis results concluded that genetic similarity among the enterotoxin producing strains were 87.50%. However, the relationship between enterotoxin and the exotoxins like alpha toxin and beta toxin remains elusive. This finding indicates the need for control of *S.aureus* bovine mastitis as the toxins produced by *S.aureus* can neither be destroyed by heat treatment nor hydrolysed by digestive enzymes and also suggest the public authorities that grading of milk has to be done after conducting the milk test for the presence of bovine mastitis. Finally veterinarian and researchers could gain a knowledge of toxins of *S.aureus* isolated from milk of cow with bovine mastitis, assuming that the present investigation contribute significantly to the field of research which will certainly provide new exciting findings in the forth coming years.

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