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Detection of Enterotoxin Genes (*seg, seh* and *sei*) in *Staphylococcus aureus* Milk Isolates from Cow with Subclinical Mastitis

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ABSTRACT

Enterotoxigenic *Staphylococcus aureus* can be isolated from various sources and are responsible for food poisoning in addition to many suppurative infections. In the present study, from 82 physically normal milk samples from cattle subclinical mastitis 33(40.24%) were isolated to *S. aureus*. Out of these, 22 (66.66%) *S. aureus* isolates had either one or more enterotoxin genes. Both *seg* and *sei* enterotoxin genes were carried by 19 (57.5%) *S.aureus* isolates while only three (9.09%) isolates were positive for *seh* gene. This study showed the evidence of presence of enterotoxin genes *seg*, *seh*, and *sei* in *S. aureus* isolates from subclinical mastitis. The presence of these enterotoxigenic *S. aureus* strains in milk indicates potential public health concern.

Keywords: *seg* gene, *seh* gene, *sei* gene, *Staphylococcus aureus*, Subclinical mastitis

Staphylococcus aureus is one of the main contagious pathogen that causes subclinical mastitis (Guimaraes *et al*., 2010). It remains in the infected quarter which act as reservoir (Matos *et al*., 1991; Roberson *et al*., 1994; Petersson-Wolfe *et al*., 2010) and rapidly spreads in dairy herds during milking (Petersson-Wolfe *et al*., 2010). It stays adhered to and finally invade mammary epithelial cells (Dego *et al*., 2002). *Staphylococcus aureus* affected 50% subclinical mastitis cases have been reported to later convert into clinical form (Pearson and Mackie, 1979). The pathogenic properties of *S. aureus* is mainly due to various virulence factors such as Protein A, clumping factor, coagulase, fibronectin, heamolysin, nucleases, exfoliative toxins and enterotoxins (Peacock *et al.*, 2002; Haveri *et al.*, 2007; Fournier *et al.,* 2008).

Staphylococcal enterotoxins are extracellular toxins secreted by various strains of *S. aureus* and are responsible for food poisoning (Bergdoll, 1979). They are classified into five classical serological types (*SEA, SEB, SEC, SED and SEE*) but other enterotoxins have also been described which includes *SEG, SEH and SEI* (Omoe *et al*., 2002; Bania *et al*., 2006). They show the property of pyrogenicity and superantigenicity and are heat stable (Argudin *etet al.,* 2010; Hennekinne *et al.*, 2012).

The gene responsible for production of enterotoxins *SEG*, *SEH* and *SEI* are located on chromosome (*seg* and *sei*), enterotoxin gene cluster (*seg* and *sei*) and transposon (*seh*) of *S. aureus* bacteria (Argudin *et al*., 2010; Medved'ová *et al.*, 2017). These genes are located non uniformly among different strains of *S. aureus* (El-Huneidi *et al*., 2006; Omoe *et al*., 2002) and also varies greatly interms of their presence on accessory and core genome (Moore and Lindsay, 2001). The present study was designed to detect the occurrence of *seg*, *seh* and *sei* genes in *S.aureus* isolates from cow milk with subclinical mastitis.

MATERIALS AND METHODS

Sample collection

A total of 82 physically normal milk samples in 5-10 ml amount each, were collected from cattle (pooled from all quarters) belonging to different farms in and around Bikaner (Rajasthan). The samples collected in sterilized

test tubes were immediately transferred to the laboratory on ice for further processing.

Somatic cell count and determination of subclinical mastitis

The modified technique of leukocyte count described by Prescott and Breed (1910) was followed for total somatic cell count. A 10 µl amount from each properly shaken milk samples was withdrawn with the help of micropipette. The milk was spread evenly on a glass slide in an area of one square centimetre, dried in air and then few drops of xylene were poured on it and kept for 1 minute to dissolve out fat globules of milk. The smear was then fixed with methanol for 2 minutes, washed with distilled water and stained with methylene blue (aquous) for 3-5 minutes. The smear was washed with phosphate buffer saline solution (pH 7.0), kept in a couplin jar for 10 minutes, blot dried and cell counting was done. A total of 20 fields selected randomly, were examined under oil immersion objective. The total numbers of cells counted in 20 fields were multiplied by a common factor 15923.55 to determine the total somatic cell count per ml of milk sample.

In the present investigation presence of 2.5 lakh somatic cells per mL of milk with positive bacteriological culture was taken as threshold value for determination of subclinical mastitis (Anonymous, 1987).

Isolation and identification of *S. aureus*

The organisms were isolated and identified as described by Cowan and Steel (1975) and Quinn *et al*. (1994).

Genotypic confirmation of *S. aureus*

Bacterial DNA isolation for PCR was done according to method described by Nachimuttu *et al.* (2001) with some modifications. The genotypic confirmation of *S. aureus* based on 23S rRNA was carried out as per method described by Straub *et al*. (1999). The primer pairs used in PCR are depicted in Table 1. The reaction was carried out for 25µL of final volume of PCR. The volume of isolated DNA used was 3µL. The primers were used at concentration of 2pmol each. The PromegaGoTaq® PCR Core System kit was used for PCR with 5µL buffer (5X GoTaq® Flexi), 3µL of 25mM magnesium chloride, 1 µL of 10 mMdNTP mix and 0.2 µL of 5U/µL GoTaq®pol. The volume was made upto 25µL with nuclease free water.

The PCR cycle included initial denaturation at 95°C for 1 min, 30 cycle of three steps (denaturation at 94°C for 90s, annealing at 55°C for 90s and extension at 72°C for 75s) and final extension at 72°C for 10 min.

Multiplex PCR for detection of *seg*, *seh* and *sei* genes

Multiplex PCR for detection of *seg*, *seh* and *sei* genes was done according to method as described by Omoe *et al*. (2002) with little modification. The primer pairs used in PCR are depicted in Table 1.The reaction was carried out for 25µL of final volume of PCR. The volume of isolated DNA used was 3µL. The primers were used at concentration of 2pmol each. The PromegaGoTaq® PCR Core System kit was used for PCR with 5µL buffer (5X GoTaq® Flexi), 3 µL of 25 mM magnesium chloride, 1.2µL of 10mM dNTP mix and 0.2µL of 5U/µL GoTaq®pol. The

Table 1: Primers used for detection of different genes in present study

Sl. No.	Gene	Primer sequence	Size (bp)	Reference
1	23S rRNA	F-5'-ACG GAG TTA CAA AGG ACG AC-3'	1250	(Straub *et al*., 1999)
		R-5'-AGC TCA GCC TTA ACG AGT AC-3'		
2	*Seg*	F-5'-AAG TAG ACA TTT TTG GCG TTC C-3'	287	(Omoe *et al*., 2002)
		R-5'-AGA ACC ATC AAA CTC GTA TAG C-3'		
3	*Seh*	F-5'-GTG TAT ATG GAG GTA CAA CAC T-3'	213	(Omoe *et al*., 2002)
		R-5'-GAC CTT TAC TTA TTT CGC TGT C-3'		
4	*Sei*	F-5'-GGT GAT ATT GGT GTA GGT AAC-3'	454	(Omoe *et al*., 2002)
		R-5'-ATC CAT ATT CTT TGC CTT TAC CAG-3'		

volume was made upto 25µL with nuclease free water. The PCR cycle included initial denaturation at 95°C for 3min, 30 cycle of three steps (denaturation at 94°C for 60s, annealing at 50°C for 60s and extension at 72°C for 60s) and final extension at 72°C for 5min.

Agarose gel electrophoresis

The PCR product of 23S rRNA and enterotoxin genes were analysed by electrophoresis on 0.8% and 1% agarose gel, respectively.

RESULTS AND DISCUSSION

Detection of subclinical mastitis due to *S. aureus*

In the present study, out of 82 milk samples, 65 milk samples had somatic cell count more than 2.5 lakhs and were culture positive but *S. aureus* was isolated from only 33 samples. The colonies of 33 *S. aureus* isolates showedgolden yellow pigmentation on nutrient agar and fermented mannitol on mannitol salt agar.

Though *S. aureus* could be identified by conventional methods in the present investigation but the genotypic confirmation was also carried out with a PCR based method involving specific primer targeted against 23S rRNA gene. An amplicon of 1250 bp (Fig. 1) was obtained with all the 33 isolates from cattle subclinical mastitis which was specific to *S. aureus.* This method was developed by Straub *et al.* (1999) and has been used by various workers (Akineden *et al.*, 2001; Salasia *et al.*, 2004; Sanjiv *et al.*, 2008; Upadhyay *et al.*, 2012; Nathawat *et al.,* 2015; Nathiya *et al*., 2018; Bhati *et al.,* 2018) for identification of *S. aureus* from various sources. Similarly, Bhati *et al*. (2016) also used this technique targeting 23S rRNA for confirmation of *S. aureus* isolates from subclinical mastitis of cattle from the same study area.

The overall prevalence of subclinical mastitis due to *S. aureus* in cattle was 40.24% which is similar to the report submitted by Bhati *et al.* (2016) from the same area. In other reports from different areas, the incidence of *S. aureus* subclinical mastitis was 6.7% and 22.58% by Fagundes *et al.* (2010) and Mpatswenumugabo *et al.* (2017), respectively. These findings are much lower (6.7% and 22.58% *S. aureus* subclinical mastitis) than ours in present study which indicates that the incidence of subclinical mastitis due to *S. aureus* is dominant in this area.

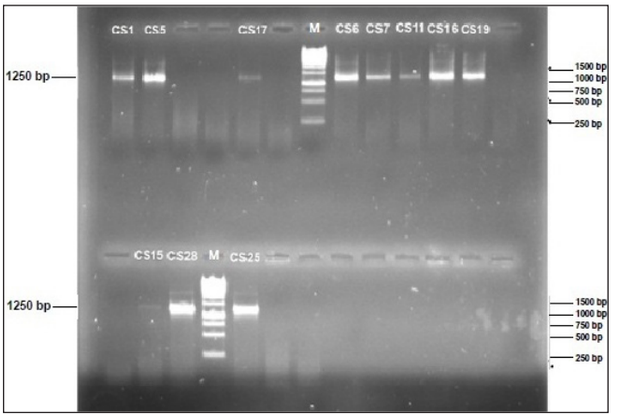

Fig. 1: Agarose gel electrophoresis showing 1250 bp PCR products of amplicon of 23S rRNA of *S. aureus* isolates from cattle subclinical mastitis; M=Ladder (1kbp)

Prevalence of enterotoxin genes *seg*, *seh* and *sei*

In this study, 22(66.66%) out of 33 *S. aureus* isolates were positive for either one or more *seg*, *seh* and *sei* genes (Fig. 2). Enterotoxin gene *seg* and *sei* was detected in 19 (57.50%) and *seh* gene was detected in 9.09% of the *S. aureus* isolates.

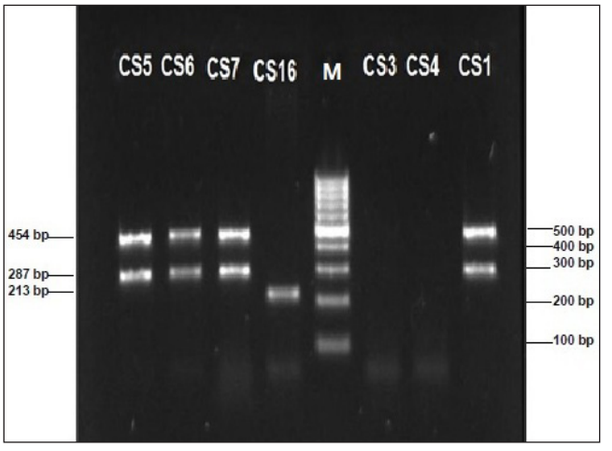

Fig. 2: Agarose gel electrophoresis showing amplicons of *seg* (287 bp), *seh* (213 bp) and *sei* (454 bp) genes of S. aureus isolated from cattle subclinical mastitis; M=Ladder (100bp)

The *S. aureus* isolates obtained from different geographical locations are known to harbour various enterotoxin genes (Omoe *et al.,* 2002; Lim *et al.* 2004; Salasia *et al*., 2004; Kastuda *et al*., 2005 and Silva *et al*., 2005). The recently described enterotoxin genes *seg*, *seh* and *sei* are widely distributed in *S. aureus* isolates obtained from clinical and subclinical cases of mastitis (Zschock *et al.*, 2005 and Freitas *et al.*, 2008). A high percentage (57.5%) of *S. aureus* carrying *seg* and *sei* gene was obtained in present study. Such a higher percent of *S. aureus* strain carrying *seg* and *sei* gene was also reported from bovine clinical mastitis and raw milk isolates (Omoe *et al*., 2002) which suggest their probable role in pathogenesis. Although lower (36% *seg* genes and 22% *sei* genes) percent of *S. aureus* isolates having *seg* and *sei* genes from subclinical mastitis in cattle was also reported by Zschock *et al*. (2005).

Enterotoxin gene *seh* was detected in lesser *S. aureus* isolates which was also reported by Omoe *et al.* (2002) in raw milk. Comparatively higher (15.09%) percent of *S. aureus* strains carrying *seh* gene from subclinical mastitis was reported by Freitas *et al.* (2008). This gene is carried on transposons (Malachowa and DeLeo, 2010) and are reported less frequently.

In this study both *seg* and *sei* gene existed together in *S. aureus* isolates which was also reported by El-hunedi *et al.* (2006) in food poisoning isolates of *S. aureus* and by Omoe *et al.* (2002) in *S. aureus* isolates from raw milk and clinical mastitis cases of cattle. The probable reason behind this coexistence is occurrence of both of these genes as part of enterotoxin gene cluster which contain five genes *seg*, *sei*, *sek*, *sel* and *sem* together (Jarraud *et al*., 2001).

The consumption of raw milk containing enterotoxigenic *S. aureus* strain from subclinical mastitis as reported in this study can cause food poisoning (Fagundes *et al*., 2010). Even though the *S. aureus* organism can die at high temperature, the property of enterotoxin remains stable at high temperature (Argudin *et al.,* 2010; Hennekinne *et al.*, 2012) and worsens the scenario. The enterotoxin gene detection merely doesn't prove the production of enterotoxin (Omoe *et al.,* 2002) and their role in food poisoning. Hence, further study is required to ensure their role.

In conclusion, the present study revealed the presence of *S. aureus* strains carrying recently described enterotoxin genes from cow with subclinical mastitis. The studies of enterotoxin gene pattern and enterotoxin production in these *S. aureus* strains isolated from subclinical mastitis will help in better understanding of pathogenicity of this organism and consequently to control subclinical mastitis. Furthermore, the presence of enterotoxigenic *S. aureus* strains in milk affected with subclinical mastitis is a potential public health concern.

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