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RESEARCH ARTICLE Agricultural Science Digest, Volume Issue : ()

Distribution of Staphylococcal Enterotoxin (SE) Genes among Different *Staphylococcus* Species Isolated from Bovine Mastitis

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10.18805/ag.D-6122

ABSTRACT

Background: Mastitis is an economically important disease of bovine species. Among the causative agents of mastitis, *Staphylococcus* species are the leading cause of contagious mastitis and are also responsible for milk-borne intoxication in consumers. The study aims to assess the distribution of enterotoxins in various species of coagulase-positive and coagulase-negative Staphylococci isolated from bovine mastitis milk samples.

Methods: A total of 135 mastitis bovine milk samples collected were subjected to cultural isolation followed by biochemical and molecular identification. The distribution of nine enterotoxins among coagulase-positive and coagulase-negative Staphylococcus species was done by PCR.

Result: About 74.07% (100 isolates) of mastitis samples were found positive for Staphylococci of which 44% were coagulase-positive and 56% coagulase-negative. Totally sixteen different species were isolated which included two species of coagulase-positive and fourteen species of coagulase-negative staphylococci. Out of nine Staphylococcal Enterotoxin (SE) genes screened, seven SE encoding genes namely *sea, seb, sec, sed, seg, sei* and *sej* were detected among the isolates with a range of one to six SE genes per isolate; however, *see* and *seh* could not be detected in any of these isolates. About 84.09% and 96.42% of CPS and CNS isolates were shown to have SE genes respectively and a maximum of six SE genes (*sea, seb, sed, seg, sei* and *sej*) were detected in a CNS isolate belonging to *S. caprae*. The high prevalence of SE encoding genes in both CPS and CNS isolated from bovine mastitis cases highlights the possible risk of staphylococcal food poisoning.

Key words: Bovine, Enterotoxin, Genes, Mastitis, Staphylococci.

INTRODUCTION

Staphylococci are one of the leading causes of mastitis in animals and food poisoning in humans. In dairy cows, mastitis is considered an economically important infectious disease and *Staphylococcus aureus* has long been identified as the predominant pathogen causing mastitis in bovine and small ruminants (Nesaraj *et al.,* 2023; Doley Sharmita *et al*., 2017). These pathogens produce several toxins such as α, β, γ and δ toxins, toxic shock syndrome toxin (TSST-1), enterotoxins, nuclease, coagulase, catalase, hyaluronidase, phosphatase, lipase, staphylokinase and proteases which can have direct effects on mammary tissue and or lead to an uncontrolled host inflammatory response by acting as superantigens (Fayisa *et al.,* 2023). These toxins are highly stable and are resistant to heat and most of the proteolytic enzymes such as pepsin and trypsin and are often considered as the most common bacterial toxins involved in food poisoning in humans (Abdallah *et al*., 2024; Narayan *et al.,* 2023). *Staphylococcus aureus* is the world's third most important cause of food-borne illness (Rahimi and Alian, 2013) and milk and milk products are considered to be the common vehicles for this species (De Buyser *et al.,* 2001; Jorgensen *et al.,* 2005). Among different species of *Staphylococcus*, the coagulase-producing (CPS) staphylococci were considered more pathogenic than the coagulase-negative species; however, several studies have proved that coagulase-negative species (CNS) as important cause of mastitis in ruminants (Seker *et al*., 2023; Deng

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How to cite this article: Bhavani, P., Antony, P. X., Mukhopadhyay, H.K., Pillai, R. M., Thanislass, J., Vijayalakshmi, P., Mangadevi, N., Vadhana A.P. and Pesingi, P.K. (2024). Distribution of Staphylococcal Enterotoxin (SE) Genes among Different *Staphylococcus* Species Isolated from Bovine Mastitis. Agricultural Science Digest. 1-6. doi: 10.18805/ag.D-6122.

Submitted: 11-07-2024 **Accepted:** 11-10-2024 **Online:** 06-01-2025

et al., 2023) and many CNS have been isolated from human blood cultures and nosocomial infections (Akgul and Bora, 2023).

Staphylococcal enterotoxins (SEs) are one of the toxins produced by enterotoxigenic *Staphylococcus* species. These SEs act as potent superantigens and help the organism to persist in the mammary gland resulting in recurring mastitis in the infected herds. There are at least five major classical types of SEs that include *sea, seb, sec, sed* and *see*. Among these classical types, *sea* and *seb* have frequently been detected in milk and milk products but recently, several other toxins like *seg, seh, sei, sek, sel, sem, sen, seo, sep, seq, ser* and *seu*, have also been detected in staphylococci isolated from cases of bovine mastitis (Wiśniewski *et al.,* 2023). The pathogenicity of *Staphylococcus* is determined by its ability to produce different toxins and the potency of toxin to produce clinical forms of disease in animals and humans. Therefore, in the present study, we aimed to analyze the distribution of the different enterotoxins among CPS and CNS species isolated from bovine mastitis cases.

MATERIALS AND METHODS

A total of 135 milk samples from bovine clinical mastitis cases were aseptically collected from Pondicherry and processed in the Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education and Research, Pondicherry, India. About one milliliter of milk sample was centrifuged at 6000 rpm for 5 min and the sediment was streaked onto Sheep Blood Agar plates and incubated at 37°C for 48 h. The characteristic colonies were stained with Gram's staining. Different species of staphylococci were identified and differentiated from other cocci by a series of biochemical reactions that included catalase production, oxidase production, nitrate reduction, oxidation-fermentation test, sugar fermentation (lactose, maltose, mannitol, mannose, sucrose and trehalose), ability to produce coagulase and urease and Voges-Proskauer test. CPS and CNS were differentiated based on, colony pigments, the ability to produce coagulase and acetoin production.

DNA was extracted from isolates as per the protocol (Chomczynski and Rymaszewski, 2006). Briefly, 2 to 3 fresh staphylococcal colonies were dissolved in 500µl of alkaline polyethylene glycol (AL-PEG) reagent and the suspension was held in a water bath set at 60°C and kept for 10 min. The lysed suspension was centrifuged at 10,000 rpm for 5 min and the obtained supernatant was diluted with nuclease-free water at a 1:10 ratio and was used as a DNA template. PCR for *Staphylococcus* genus-specific, *S. aureus* species-specific and enterotoxins were carried out using the primers listed in Table 1.

RESULTS AND DISCUSSION

Isolation and identification of different *Staphylococcus* species from milk samples

About 74.07% (100 isolates) of samples were found positive for *Staphylococcus* species based on cultural

Table 1: Details of the primers used in the study.

Name	Target gene	Primer sequences (5'-3')	Amplicon Size (bp)	Reference
Genus specific	16S rRNA	Forward: AACTCT GTTATTAGCGAAGAACA Reverse: CCACCTTCCTCCGGTTTGTCACC	756	Zhang *et al.,* 2004
S. aureus specific	*nuc*	Forward: GCGATTGATGGTGATACGGT Reverse: AGCCAAGCCTTGACGAACTAAAGC	270	Brakstad *et al.,* 1992
Enterotoxins	*sea*	Forward: GCAGGGAACAGCTTTAGGC Reverse: GTTCTGTAGAAGTATGAAACACG	520	Monday and Bohach,1999
	seb	Forward: ACATGTAATTTTGATATTCGCACTG Reverse: TGCAGGCATCATGTCATACCA	667	Lovseth *et al.,* 2004
	sec	Forward: CTTGTATGTATGGAGGAATAACAA Reverse: TGCAGGCATCATATCATACCA	284	Monday and Bohach,1999
	sed	Forward: GTGGTGAAATAGATAGGACTGC Reverse: ATATGAAGGTGCTCTGTGG	385	Monday and Bohach,1999
	see	Forward: TACCAATTAACTTGTGGATAGAC Reverse: CTCTTTGCACCTTACCGC	171	Monday and Bohach,1999
	seg	Forward: AAGTAGACATTTTTGGCGTTCC Reverse: AGAACCATCAAACTCGTATAGC	287	Omoe *et al.,* 2002
	seh	Forward: CAACTGCTGATTTAGCTCAG Reverse: GTCGAATGAGTAATCTCTAGG	359	Monday and Bohach,1999
	sei	Forward: CAACTCGAATTTTCAACAGGTACC Reverse: CAGGCAGTCCATCTCCTG	466	Monday and Bohach,1999
	sej	Forward: CATCAGAACTGTTGTTCCGCTAG Reverse: CTGAATTTTACCATCAAAGGTAC	142	Monday and Bohach,1999

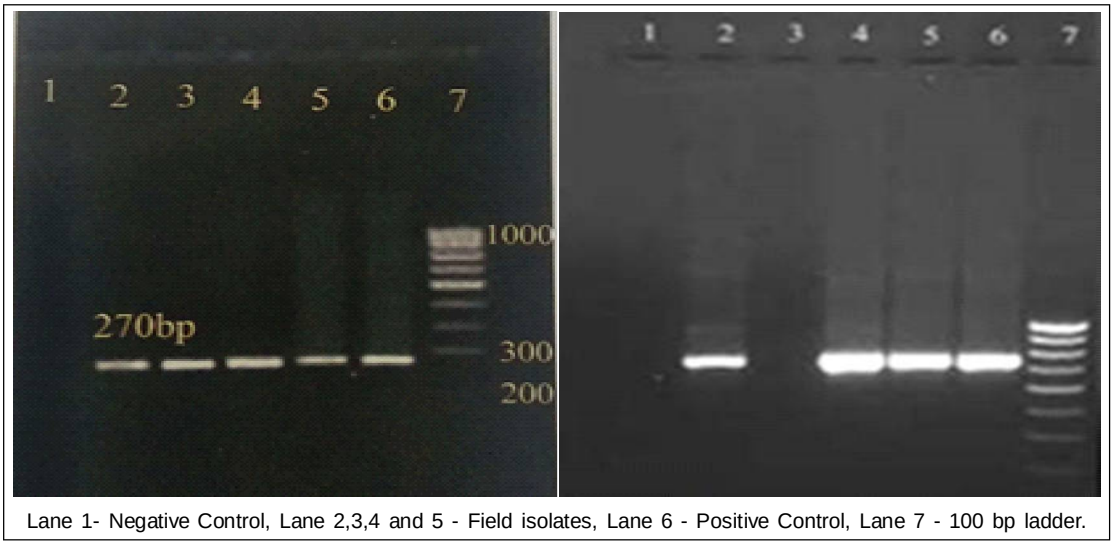
identification, Gram staining and biochemical tests. The isolates were further confirmed at the genus level by polymerase chain reaction amplifying 756 bp fragment of 16s rRNA (Fig 1a). *Staphylococcus aureus* species were identified by amplifying the *nuc* gene fragment (Fig 1b). The coagulase test revealed that 44 isolates belonged to coagulase test-positive *Staphylococcus* (CPS) and 56 were coagulase-negative *Staphylococcus* (CNS). Out of 44 CPS, 42 isolates were identified as *Staphylococcus aureus* and two isolates were *Staphylococcus intermedius.*

CNS further characterized up to the species level by series of biochemical tests and they were identified as *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus hominis*, *Staphylococcus xylosus*, *Staphylococcus caprae*, *Staphylococcus capitis*, *Staphylococcus sciuri*, *Staphylococcus lugdenensis*, *Staphylococcus saccharolyticus*, *Staphylococcus lentus*, *Staphylococcus auricularis*, *Staphylococcus hemolyticus* and *Staphylococcus arlettae*.

Molecular screening for staphylococcal enterotoxin genes

All *Staphylococcal* isolates were subjected to molecular screening by PCR for detection of 9 different Staphylococcal enterotoxins (SE) encoding genes namely *sea, seb, sec, sed, see, seg, seh, sei* and *sej*. Overall, PCR detected 7 SE genes (*sei, sea, seg, sec, sej, sed* and *seb*) in CPS and CNS isolates (Fig 2) however, none of these isolates revealed *see* and *seh*. Among CPS isolates, *sei* gene was found the highest number of isolates, followed by *sea, seg, sec, sej, sed* and *seb* while, in CNS isolates, *sei* was found in the highest number of isolates followed by *sea, seg, sed, seb, sej* and *sec*. The genes encoding SE production were detected in 84.09% and 96.42% of CPS and CNS isolates respectively. The distribution of SE among different species is detailed in Table 2. The overall distribution of SE genes was higher in CNS than in CPS isolates and a maximum of six SE genes (*sea, seb, sed, seg, sei* and *sej*) were detected in a CNS isolate belonging to *S. caprae.*

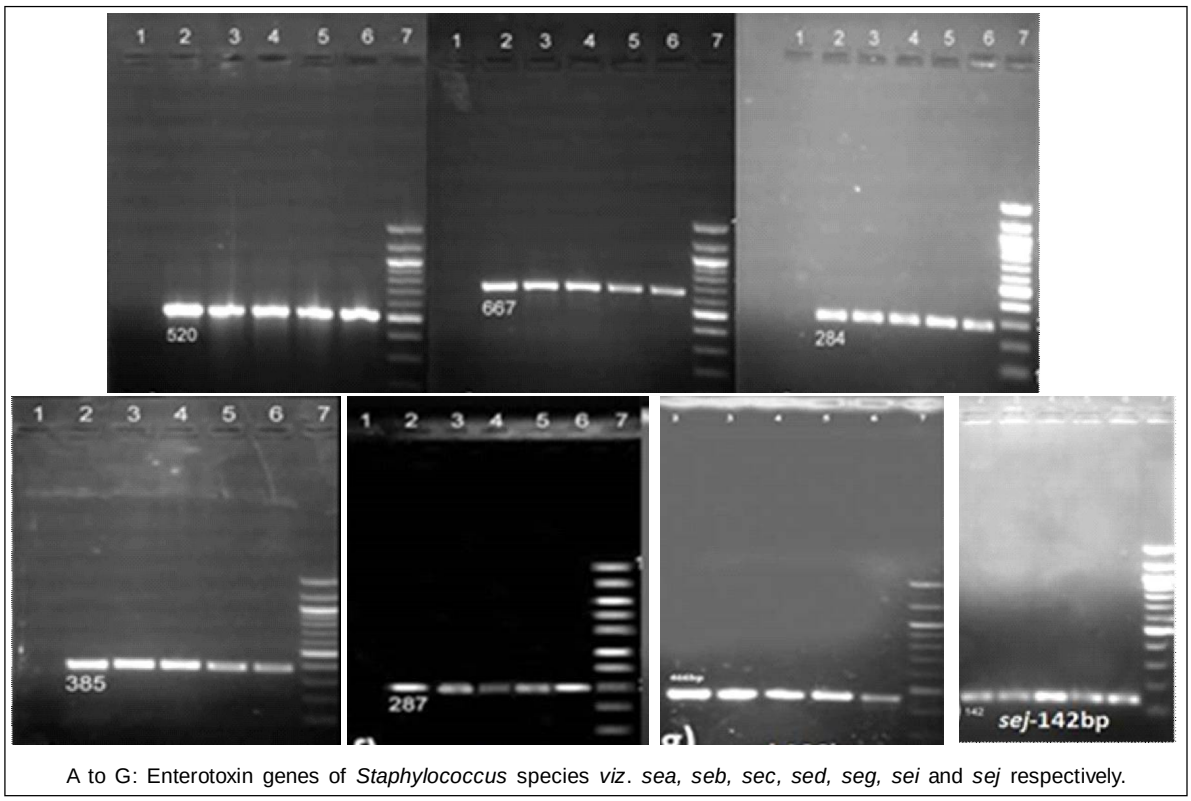
Staphylococci species are reported to be a major pathogen causing mastitis in dairy cattle and are also associated with a widespread spectrum of infections and food poisoning in humans (Waseem *et al*., 2020). Coagulase-positive *S. aureus is* considered the most pathogenic strain of its genus and is an etiological factor for various clinical manifestations including food poisoning (Yiğin-Akın *et al*., 2018). Staphylococcal food poisoning is a kind of foodborne intoxication that occurs due to ingestion of preformed SEs and is characterized by vomition, gastroenteritis, diarrhea painful contraction of gastrointestinal smooth muscles with a short incubation period usually 30 minutes to 8 h (Le *et al.,* 2003, Podkowik *et al.,* 2013). *S. aureus* was considered the only species of its genus capable of producing enterotoxins however, recent reports have shown the involvement of CNS species in hospital-acquired infections (Podkowik *et al.,* 2013). CNS are capable of producing various virulence factors similar to *S. aureus* including enterotoxins (Podkowik *et al.,* 2012). Moreover, CNS such as *S. simulans*, *S. chromogenes* are now considered as emerging pathogens of bovine mastitis (Pyorala and Taponen, 2009). Therefore, it is important to understand the distribution of the SEs in both CPS and CNS isolated from the bovine mastitis milk. In the present study, sixteen species of staphylococci from bovine mastitic milk were isolated and identified by a series of biochemical tests. Biochemical tests most commonly used tool for the differentiation of different Staphylococci (Thorberg *et al*., 2009). *S. aureus* was most prevalent followed by *S. chromogens, S. epidermidis, S. simulans* and *S. caprae*. Both CPS and CNS are capable of producing enterotoxins; however, the pattern of toxin-producing genes is highly variable. Out of nine SEs screened, seven different enterotoxin genes namely *sei, sea, seg, sec, sej, sed* and *seb* were detected by PCR and their prevalence was higher



Lane 1- Negative Control, Lane 2,3,4 and 5 - Field isolates, Lane 6 - Positive Control, Lane 7 - 100 bp ladder.

Fig 1: Screening of field isolates for species-specific (A) and genus-specific (B) detection of the *S. aureus*.

among CNS than in CPS. The number of SE genes per species was also highest in *S. caprae*, a CNS species with six SE encoding genes (*sea, seb, sed, seg, sei* and *sej*). The predominant SE encoding genes detected were *sei, sea* and *seg*. Park *et al.*, (2011) reported that the distribution of the super antigens such as *seb, seln* and *selq* were highest among the *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, *S. sciuri* subsp. *carnaticus*, *S.*



A to G: Enterotoxin genes of *Staphylococcus* species *viz*. *sea, seb, sec, sed, seg, sei* and *sej* respectively.

Fig 2: Representative electrophoretic images showing the PCR amplified products of different enterotoxin genes of Staphylococcus species isolated from the mastitis milk of dairy cow.

Table 2: Distribution of SE among different *Staphylococcus* species.

Specie of Staphylococci	Total isolates	Isolates positive for SE genes	Distribution of SE toxinotypes	Predominant SE toxinotype
S. aureus	42	35	*sei, sea, seg, sec, sej, sed, seb*	*sei*
S. intermedius	2	02	*sei, sea, seg*	*sei*
S. chromogens	10	09	*sei, seg, sea, sed, sej, seb, sec*	*sei*
S. epidermidis	8	08	*sei, seg, sea, sed, seb, sec, sej*	*sei*
S. simulans	6	06	*sei, sea, sed, sej, seg, seb*	*sei*
S. caprae	5	05	*sea, sei, seb, sed, seg, sec, sej*	*sea*
S. hominis	5	05	*sei, sea, seb, sec, sed, seg, sej*	*sei*
S. xylosus	5	05	*sea, sed, sei, seb, seg, sec*	*sea*
S. capitis	4	04	*sea, seg, sei, seb, sed*	*sea*
S. sciuri	3	03	*sea, sed, seg, sei*	*sea*
S. auricularis	2	02	*sei, sea, sed, seg*	*sei*
S. lentus	2	02	*sei, sea, sed, seg*	*sei*
S. lugdensis	2	02	*sei, sea, sed, sec, seg*	*sei*
S. sacchrolyticus	2	02	*sei, sea, seg,*	*sei*
S. hemolyticus	1	01	*sea, sec, sed, seg, sei,*	*sea*
S. arlettae	1	-	*	-
Total	100	91		

simulans and *S. succinus* which were CNS isolated from the bovine mammary infections. Similarly, de Freitas Guimaraes *et al.,* (2013) reported that 66% of CNS and 35% of CPS isolated from subclinical mastitis cases possessed the SE genes with a high frequency of *sea*, *seb* and *sec.* Among different SE encoding genes, *sea* genes lesser extent *sei* genes are mainly linked to staphylococcal food poisoning worldwide (Kwon *et al.,* 2004; Argudin *et al.,* 2011). Studies have also reported that all the *seg* producing staphylococci consistently carried *sei* gene but not vice-versa (Omoe *et al.,* 2002) which was further confirmed in the present study as well. The high prevalence of SE-encoding genes in CNS isolates of mastitis origin may pose a potential risk of food poisoning as these toxins are highly heat resistant even at pasteurization temperature.

CONCLUSION

The CNS that was once considered less pathogenic is becoming more pathogenic with increased enterotoxin-producing abilities. Although the mere presence of SE encoding genes does not confirm the toxin-producing ability, their presence suggests their potential to produce toxins under suitable conditions. Therefore, it is imperative to screen raw milk obtained from healthy udder, processed and stored milk and ready to eat milk products for different SE encoding genes to safeguard human health. Personal hygiene and hygienic practices during milking, transportation and storage might reduce the chances of infection and contamination; but, routine screening of milk and milk products for different pathogenic microbes and their toxins is essential to formulate suitable control strategies. Nevertheless, the present findings indicate a potential public health hazard and underscore the need to establish both effective bovine mastitis control programs and diagnostic methods to limit staphylococcal food poisoning.

ACKNOWLEDGEMENT

We thank the Dean, Rajiv Gandhi Institute of Veterinary Education and Research, (RIVER) Puducherry for providing the necessary facilities and financial support to complete this work. We sincerely thank the clinicians at Veterinary Teaching Hospital, RIVER and Veterinary Dispensaries, Puducherry for their kind cooperation in sample collection.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

We declare that none of the authors has a financial or personal relationship that could inappropriately influence or bias the manuscript's contents.

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