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Identification of Enterotoxigenic Coagulase Negative *Staphylococcus spp.* Isolated from Bovine Milk and Milk Products of Southern Assam, India

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Coagulase negative staphylococci (CNS) have been frequently isolated from milk and milk products but the pathogenicity of CNS has long been underrated.The objective of this study was to detect the presence of the classical enterotoxin genes *sea,seb, sec* in CNS and identify the strains that harboured these genes. 50 CNS isolates were selected for the present study. 27 out of these 50 isolates (54%) tested positive for the enterotoxin genes with *sec* being the most predominant followed by *sea* and *seb*. On the basis of their phenotypic characters, 5 representative isolates were selected for 16S r RNA gene sequencing for identification and their phylogenetic relationship was studied. The enterotoxin gene harbouring CNS strains were identified as *Staphylococcus succinus*, *Staphylococcus sciuri*, *Staphylococcus kloosii* and *Staphylococcus saprophyticus*. The present study confirms the enterotoxin producing capability of the coagulase negative group of *Staphylococcus* and emphasizes the need for their precise identification in order to devise appropriate measures in controlling the pathogens.

Key words: Coagulase negative staphylococci, bovine milk, milk products, enterotoxin genes, 16S r RNA.

Foods of animal origin especially milk and dairy products have been involved in food borne diseases worldwide[1 2].Staphylococcal food poisoning (SFP) is one among the most predominant types of food poisoning. Results from statistical studies in 15 countries showed milk and dairy products to be the most common foods involved in staphylococcal food borne illness [3]. Given the health benefits of milk and dairy products and the high popularity of these food products among consumers, the risk of SFP through these products is a matter of major concern.

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SFP is caused as a result of ingestion of staphylococcal enterotoxins (SEs) that are preformed in the food contaminated through human manipulation or raw materials obtained from animals[4]. These SEs are highly thermostable and retain their activity even after pasteurization[1]. Apart from the coagulase positive *Staphylococcus aureus* which has been the major pathogen associated with SEs, many coagulase negative *Staphylococcus* (CNS) species have been, of late, shown to be potential producers of SEs. Five of these SEs (SEA, SEB, SEC, SED, SEE) are reported to be the major causes of SFP with SEA, SEB, SEC being the most common enterotoxins found associated with food-poisoning cases[5].The recent identification of new SEs has increased the perceived frequency of the enterotoxigenic strains

suggesting higher pathogenic potential of *Staphylococcus* than what was estimated before[6]. There has been discrepancies among reports on the enterotoxigenic potential of CNS with some investigators failing to detect toxin genes or toxin production while many reporting CNS to harbour SEs with potential toxin production capability[7 8 9 4 10]. In the light of these findings, the coagulase negative group of staphylococci demands greater attention and their proper identification becomes imperative.

A study conducted by Grace*et al.,* (2009) in the North Eastern part of India on sweet making in various stalls showed that 13% did not meet national standards for bacterial count thus suggesting poor storage conditions and consequent health risk for consumers[11].To our knowledge, there have been few reports on the enterotoxigenic strains of *Staphylococcus spp*. obtained from milk and dairy products from this region. In the present study an attempt has been made to screen coagulase negative staphylococcal isolates harbouring enterotoxin genes (*sea, seb, sec*) and to identify the strains using 16S r RNA gene sequencing.

MATERIALS AND METHODS

Collection of samples and screening of isolates

Staphylococcal isolates used in this study were obtained from bovine milk and milk products (locally made sweets from various local sweet stalls) in and around Southern Assam. The collected samples were immediately transported to the laboratory in ice-cold conditions and were cultured in Mannitol Salt Agar and Baird-Parker agar (HiMedia, India) with an incubation period of 24-48 hours at 37°C [12]following incubation in *Staphylococcus* enrichment broth for 24-48 hours at 37°C. Primary screening of the isolates was done on the basis of morphology, Gram's stain, catalase, coagulase, mannitol fermentation and oxidase tests according to standard protocols. 50 coagulase-negative isolates were selected for this study of which 33 were obtained from milk and the remaining 17 from milk products. Of these 50 isolates, 27 isolates tested positive for enterotoxin genes *sea*, *seb*and *sec*. Based on variations in biochemical characters and colony morphology of these enterotoxigenic isolates, 5 representative isolates among these 27 were finally selected for sequencing of the 16S r RNA gene.

Polymerase Chain Reaction

For the amplification of *coa* gene,3 µl of 2X Master Mix (HiMedia, India) was added to 0.5 µl of each of the forward and reverse primers (10pM/ µl), 2 µl of extracted DNA and 8µl of nuclease-free water. The reaction tube was heated to 94°C for 3 minutes prior to 30 cycles of denaturation for 30 seconds at 94°C, annealing for 45 seconds at 50°C and extension for 45 seconds at 72°C followed by final extension for 5 minutes at 72°C.

A multiplex PCR was carried out for the amplification of enterotoxin genes *sea, seb, sec*. The reaction mixture consisted of 12.5 µl of 2X Master mix (HiMedia, India),1 µl each of the forward and reverse primers in the concentration of 10pM/ µl , 1 µl of extracted DNA and 5.5 µl ofnuclease-free water resulting in a reaction volume of 25 µl. The reaction tube was heated to 94 °C for 1 minute before 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 52°C for 45seconds, extension at 72°C for 45 seconds followed by final extension for 5 minutes at 72°C.

For amplification of 16S r DNA,15 µl of 2X Master mix (HiMedia, India) was added to 0.8 µl each of forward and reverse primers (10pM/ µl), 2.5 µl of extracted DNA and 26 µl of nuclease-free water. The reaction tube was then heated to 95 °C for 5 minutes prior to 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds, extension at 72 °C for 1 minute 30 seconds followed by a final extension at 72 °C for 7 minutes.

The 2X Master mix used in the amplification reactions consisted of 3mM $MgCl_2$, 150mMTrisHCl, 0.4 mMdNTPs and 0.05 units/µl Taq DNA polymerase.

Sequence analysis of 16S r RNA and identification of the isolates

The purified PCR product was sequenced in an automated DNA sequencing facility (Department of Biotechnology, Assam University, Silchar) using Applied Biosystems 3500 Genetic Analyzer according to manufacturer's instructions.

Both forward and reverse sequences determined in this study were aligned to generate a single sequence using BioEdit software (version 7.2.3; www.mbio.ncsu.edu/bioedit/bioedit.html) and blasted in NCBI database for identification of the isolates. The sequences were then compared

with the available 16S r RNA sequences of *Staphylococcus* in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) and the isolates were identified on the basis of highest percentage homology with the available sequences. Multiple sequence alignments were performed using ClustalW (www.genome.jp/tools/clustalw/). Phylogenetic tree was constructed using Mega

Table 1. Oligonucleotide primers for detection of coa (coagulase gene), 16S rRNA gene and enterotoxin genes sea, seb, sec

Target gene	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
16S r RNA	27f	AGAGTTTGATCMTGGCTCAG	1450	19
	1492r	TACGGYTACCTTGTTACGACTT		
coa	COA-F	CCTCAAGCAACTGAAACAACA	151	20
	COA-R	TGAATCTTGGTCTCGCTTCAT		
sea	SEA-F	ATTAACCGAAGGTTCTGTAGA	552	21
	SEA-R	TTGCGTAAAAAGTCTGAATT		
seb	SEB-F	TGTATGTATGGAGGTGTAAC	270	22
	SEB-R	ATAGTGACGAGTTAGGTA		
sec	SEC-F	ACCAGACCCTATGCCAGATG	371	23
	SEC-R	TCCCATTATCAAAGTGGTTTCC		

Table 2. Variations in morphological and biochemical characters of the isolates that were positive for enterotoxin genes

Isolate	Colony morphology	Gram's test	Catalase test	Coagulase test	Oxidase test	Mannitol fermentation
A1	Small, yellowish	+	+	_	_	+
A2	Small,creamy	+	+	_	_	+
A4	Small, yellowish	+	+	_	_	+
A5	Medium, Creamy	+	+	_	+	_
A6	Medium, creamy	+	+	_	+	_
A7	Small, yellowish	+	+	_	_	+
A8	Small, creamy	+	+	_	_	+
A9	Medium, creamy	+	+	_	+	_
A10	Small, yellowish	+	+	_	_	+
A11	Medium, yellowish	+	+	_	+	+
A12	Small, yellowish	+	+	_	_	+
A13	Medium, yellowish	+	+	_	+	+
A14	Medium,yellowish	+	+	_	+	+
A15	Medium, Creamy	+	+	_	+	_
A16	Small, creamy	+	+	_	_	_
A17	Small, Creamy	+	+	_	_	_
A18	Medium, creamy	+	+	_	+	_
A19	Small ,yellowish	+	+	_	_	+
A20	Small, creamy	+	+	_	_	_
A21	Small, creamy	+	+	_	_	_
A22	Medium, yellowish	+	+	_	+	+
A23	Small ,yellowish	+	+	_	_	+
A24	Small, creamy	+	+	_	_	_
A25	Small ,yellowish	+	+	_	_	+
A26	Small, yellowish	+	+	_	_	+
A27	Medium, creamy	+	+	_	+	_
A28	Small, yellowish	+	+	_	_	+

5.0 (www.megasoftware.net).The evolutionary history was inferred using the Neighbour-Joining method and the evolutionary distances were computed using the Kimura 2- parameter method.

Nucleotide sequence accession numbers

The 16S rRNA sequences of the isolates determined in this study were submitted to GenBank database, the Accession Numbers of which are mentioned in Table 3.

RESULTS AND DISCUSSION

50 CNS isolates were screened on the basis of biochemical coagulase test and PCR of the *coa*gene. Of the 50 CNS isolates, 66% was isolated from milk and the rest 34% from milk products. These 50 isolates tested negative for coagulase both phenotypically and genotypically. All the 50 isolates showed positive Gram's reaction, positive catalase reaction and negative coagulase reaction but there was considerable variation in morphological characters (colony size and colour) results of oxidase and mannitol fermentation.

27 among the total CNS isolates tested positive for enterotoxin genes . 9 out of 27 isolates carried *sea* gene alone (33%), 1 carried *seb* gene alone (4%), 1 carriedboth *sea* and *seb*, (4%), 16 carried *sec* gene alone (59%) (Table 3). The percentage of enterotoxin positive isolates from milk and milk products were 67% and 33% respectively. The 27 isolates showed considerable variations in morphology and biochemical characters (Table 2) from which 5 representative isolates (A2, A14, A15, A17, A19) were selected for 16S r RNA gene sequencing. On comparing the 16S r RNA gene sequences generated from these five selected isolates with those available in the GenBank database, the highest similarity of the reported sequences with sequences generated in this study was observed for *Staphylococcus sciuri*

Table 3. Detection of enterotoxin genes by PCR in the CNS isolates, source of isolation and corresponding Genbank accession numbers

Isolates	Source	Accession Numbers	sea	seb	sec
A1	Milk product		+	-	-
Staphylococcus succinus(A2)	Milk	KJ854367	-	-	+
A4	Milk		-	-	+
A5	Milk		-	-	+
A6	Milk		+	-	-
A7	Milk product		-	-	+
A8	Milk		+	-	-
A9	Milk		-	-	+
A10	Milk product		-	-	+
A11	Milk product		-	-	+
A12	Milk		-	-	+
A13	Milk product		-	-	+
Staphylococcus sciuri(A14)	Milk product	KJ854368	+	-	-
Staphylococcus kloosii(A15)	Milk		-	+	-
A16	Milk		-	-	+
Staphylococcus kloosii(A17)	Milk	KJ854369	+	+	-
A18	Milk		+	-	-
Staphylococcus saprophyticus(A19)	Milk product	KJ854370	+	-	-
A20	Milk		-	-	+
A21	Milk		+	-	-
A22	Milk product		-	-	+
A23	Milk		+	-	-
A24	Milk product		-	-	+
A25	Milk		-	-	+
A26	Milk		-	-	+
A27	Milk		+	-	-
A28	Milk		-	-	+

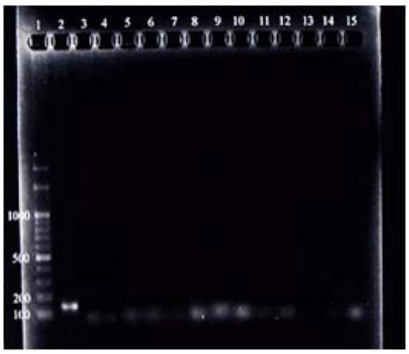

Fig. 1. Showing PCR analysis of *coa* gene

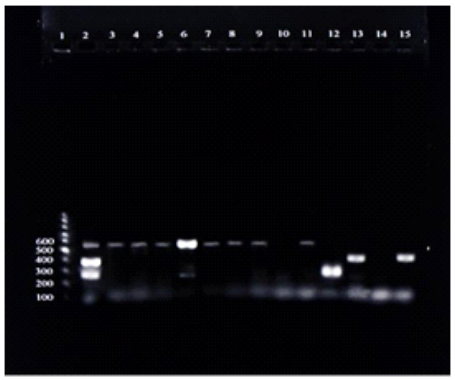

Fig. 2.

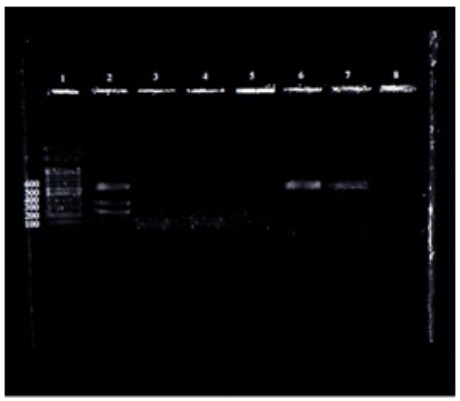

Fig. 3.

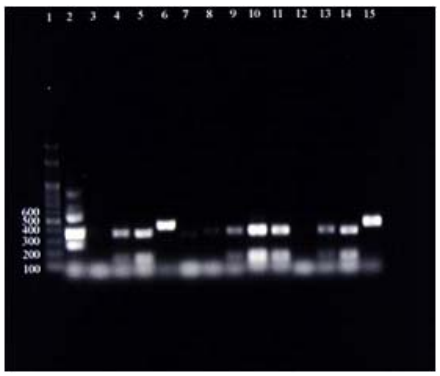

Fig. 4.

Fig. 5.

Fig. 6.

Lane 1 in Figure 1, 2, 3, 4,5,: 100 bp ladder; lane 4 in Figure 6 :100 bp ladder
Lane 2 in Figure 1-4: positive control *Staphylococcus aureus* ATCC 25923; lane 5 in Figure 5: positive control *Staphylococcus aureus* ATCC 25923; lane 1 in Figure 6: positive control *Staphylococcus aureus*ATCC 25923
Amplification of *sea*: lanes 3-9,11 in Figure 2 and lanes 6,7 in Figure 3; amplification of *seb*: lanes 6 and 12 in Figure 2; amplification of *sec*: lanes 13,15 in Figure 2 ,lanes 4,5,7-11,13,14 in Figure 4, lanes 6,9,10 in Figure 5 and lanes 2,3 in Figure 6.

Fig. 2-6. Showing multiplex PCR of enterotoxin genes *sea, seb, sec*

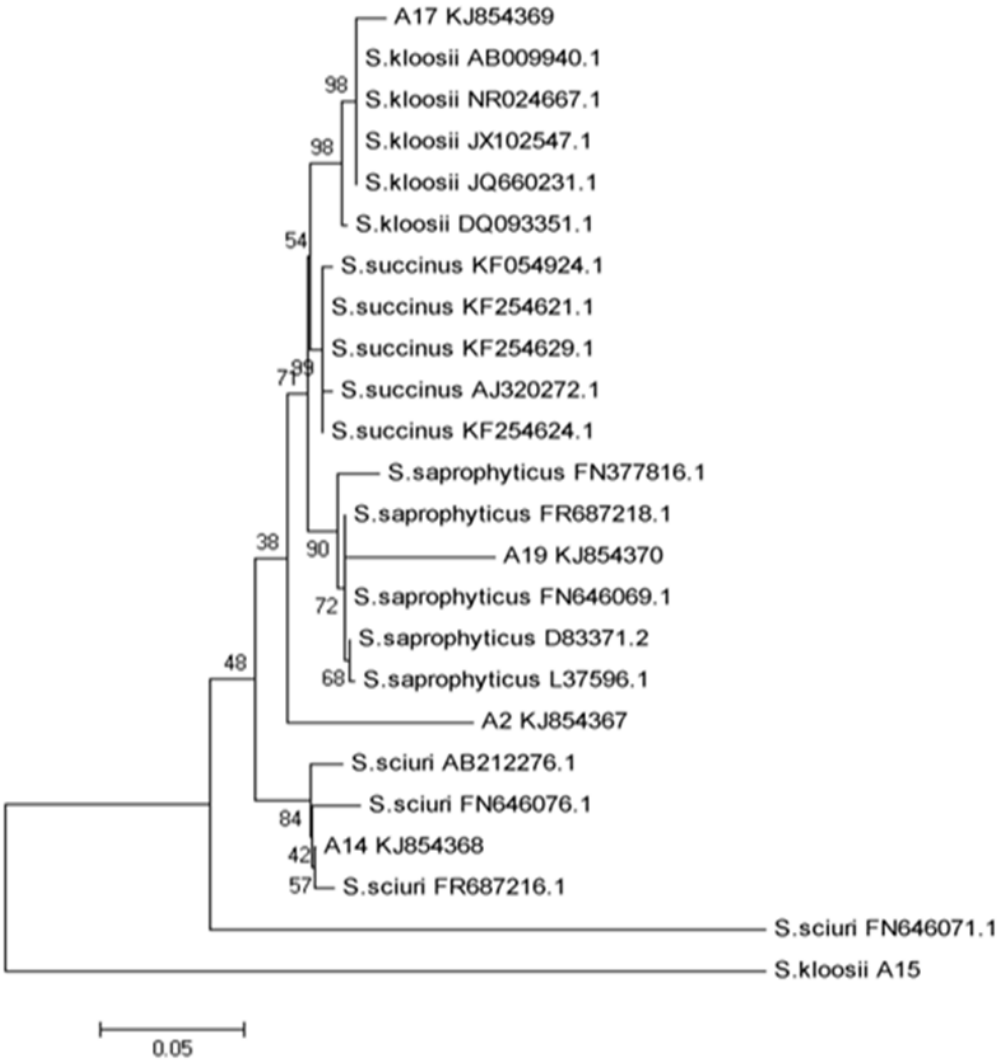

Fig. 7. Showing phylogenetic relationship among the isolates

K854368 (97%) followed by *Staphylococcus kloosii* KJ854369 (96%) *Staphylococcus saprophyticus* KJ854370 (93%) and *Staphylococcus succinus* KJ854367 (88%).

The isolates showed significant distance from one another as was evident from their variations in biochemical tests and morphology. One of the isolates (A15 in the phylogenetic tree) identified as *Staphylococcus kloosii* showed different individual branching in the phylogenetic tree (Figure 7) and hence its position in the phylogenetic tree could not be properly explained.

54% of the coagulase negative isolates in the study tested positive for at least one of the classical enterotoxins *sea, seb* and *sec* with *sec* being the most predominant followed by *sea*, and*seb*. This finding is in accordance with a few recent and past observations that throw light on the enterotoxigenicity of CNS isolated from milk and dairy products. In a recent study, Piechota*et al.,* showed CNS to be positive for enterotoxin genes. Further, they reported *sec* to be the most prevalent gene in staphylococcal isolates from bovine milk [13]. Veras*et al*.,(2008) also reported 63% CNS isolates in dairy products from food poisoning outbreaks carrying enterotoxin genes (*sea* and *sec* predominantly) with detectable corresponding toxins [10]. Valle*et al*.,(1990) in his study also reported

enterotoxigenic CNS strains and predominance of enterotoxin gene *sec* [14] .The remaining 46% isolates that did not amplify any of the enterotoxin genes tested for cannot be ignored since they could be carriers of other enterotoxin genes given that there are around 18 enterotoxin genes identified till date[15].

Sequencing of the 16S r RNA gene and phylogenetic analysis of the isolates helped identify five representative isolates that were positive for at least one of the enterotoxin genes. The results suggest enterotoxin producing potential of *Staphylococcus saprophyticus* and *Staphylococcus sciuri* as reported earlier by Valle*et al.*, Al- Tarazi*et al.*, Kamau*et al.*, [14 16 17]. The other 3 isolates identified as *Staphylococcus kloosii* (A15, A17) and *Staphylococcus succinus* (A2) were also positive for enterotoxin genes but to the best of our knowledge there is little literature available on the enterotoxigenicity of *Staphylococcus kloosii* and *Staphylococcus succinus* till date. However since the position of A15 in the phylogenetic tree could not be properly explained and because it showed comparatively less similarity with the already reported staphylococcal sequences, the identity of this isolate could not be properly assessed.

The results of the present study suggest the toxin producing capacity of coagulase negative staphylococci but for establishing the pathogenicity of the isolates the toxin concentration has to be determined. However strains containing genes for toxins cannot be simply ignored because they retain the potential of producing significant toxin to cause food poisoning [18].This report from Southern Assam establishes the threat associatedwith consumption of raw milk and necessitates evaluation of the processing and storage conditions of milk products in the sweet stalls of this region.

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