

Identification of methicillin-resistant staphylococci isolated from milk and milk products of Southern Assam, India and evaluation of their enterotoxigenicity

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Abstract

In this study, methicillin-resistant staphylococci isolated from milk and milk products were evaluated for their enterotoxigenic potential with emphasis on the three classical enterotoxin genes namely, SEA, SEB and SEC. 11(35.48%) out of 31 staphylococcal isolates were found to be methicillin-resistant, being positive for *mecA* gene. Of these 11 methicillin-resistant isolates, 8 (72.73%) were positive for either SEA or SEC. Sequencing of the 16S rDNA helped identify the methicillin-resistant isolates as *S. aureus*, *S. sciuri*, *S. epidermidis* and *S. saprophyticus*. Phylogenetic tree constructed with the 16S rDNA sequences revealed a close relationship among the isolates that accounts for dissemination of genes like those for antibiotic resistance and enterotoxins. The presence of methicillin-resistant enterotoxigenic strains of *Staphylococcus* in popular food products like milk and milk products analysed in this study indicates potential health risk for consumers and emphasizes the necessity of hygienic handling of these food products.

Keywords

Methicillin resistance

Enterotoxigenic

Staphylococci

Milk

Milk products

16S rDNA

Southern Assam

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Introduction

Staphylococcus is a genus of Gram-positive bacteria that houses both pathogenic and non-pathogenic strains. However, in recent years, there has been a remarkable change in the outlook regarding the pathogenicity of this clinically relevant group of microorganisms. For long, our knowledge regarding the pathogenicity of staphylococci was mostly limited to the coagulase-positive *Staphylococcus aureus*, but recently many coagulase-negative *Staphylococcus* (CNS) have been shown to be equally capable of producing clinically significant infections in humans and animals. In the food context, staphylococcal enterotoxins (SEs) produced by *S. aureus* and many CNS as recently reported by various authors, are perhaps one of the major threats associated with staphylococcal contamination in foods (Tsen *et al.*, 1998; Martin *et al.*, 2003; Sandel and McKillip, 2004; Kerouanton *et al.*, 2007; Vancraeynest *et al.*, 2007). These SEs secreted by staphylococci in contaminated foods are highly thermostable and the ingestion of these exotoxins leads to staphylococcal food poisoning. Milk and milk products serve as good substrates for staphylococcal contamination. It is thought that staphylococci can gain access to milk directly from infected udder or through

human manipulation. Dairy products can also get contaminated with staphylococci during storage and processing as a result of mishandling by workers given that staphylococci are commonly found on human skin and mucous membranes. Increasing resistance of staphylococci to commonly used antibiotics is another clinically important issue. Methicillin-resistant staphylococci was first reported in 1961 and by 1970, it had become endemic in many countries (Voss and Doebbeling, 1995). The resistance mechanism for methicillin is related to the presence of *mecA* gene. The *mecA* gene is located on a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (*SCCmec*). Horizontal, interspecies transfer of this element is speculated to be the cause of dissemination of methicillin-resistance (Bloemendaal *et al.*, 2010). Like methicillin resistance genes, enterotoxin genes are also carried by transmissible genetic elements and therefore, foods contaminated with such staphylococcal strains could play a key role in transmission of antibiotic-resistant enterotoxigenic staphylococci to humans imposing health risk (Chajecka-Weirzchowska *et al.*, 2012).

A survey conducted by Grace *et al.* (2009) in the North-eastern part of India on sweet making in various stalls revealed that 13% did not meet national standards for bacterial count. Additionally,

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Table 1. Primers used in this study along with the amplicon size

Target gene	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	References
16S rDNA	27f	AGAGTTTGATCMTGGCTCAG	1450	Weisburg et al., 1991
	1492r	TACGGYTACCTTGTACGACTT		
sea	SEA-F	ATTAACCGAAGGTTCTGTAGA	552	Tang et al., 2006
	SEA-R	TTGCGTAAAAAGTCTGAATT		
seb	SEB-F	TGTATGTATGGAGGTGAAC	270	Shama et al., 2000
	SEB-R	ATAGTGACGAGTTAGTA		
sec	SEC-F	ACCAGACCCTATGCCAGATG	371	Cremonesi et al., 2005
	SEC-R	TCCCATTATCAAAGTGGTTTCC		
mecA	MECA-F	GTAGAAATGACTGAACGTCCGAT AA	310	Perez-Roth et al., 2001
	MECA-R	CCAATTCCACATTGTTTCGGTCT AA		
coa	COA-F	CCTCAAGCAACTGAAACAACA	151	Fan et al., 2008
	COA-R	TGAATCTTGGTCTCGCTTCAT		

sporadic cases of food poisoning from various regions of Assam had been reported time to time but the lack of proper investigation has resulted in poor knowledge of the cause and epidemiology of the food poisoning events. To our knowledge, there has been little information regarding potential staphylococcal contamination in milk and milk products of this region. With the backdrop of such a situation, the present study is an attempt to identify methicillin-resistant staphylococcal strains in milk and milk products of Southern Assam and to evaluate the presence of the classical enterotoxin genes SEA, SEB, SEC in the strains isolated.

Materials and Methods

Collection of samples and screening of staphylococcal isolates

Milk samples from cows, goats, buffaloes and locally made sweet samples from various sweet stalls were collected aseptically from regions in and around Southern Assam. The samples were immediately transported to the laboratory in cold conditions and were cultured in Mannitol Salt Agar (MSA) and Baird-Parker Agar (BPA) with an incubation period of 24-48 hours at 37°C (Rohinishree and Negi, 2011) following enrichment in *Staphylococcus* enrichment broth for 24-48 hours at 37°C. Yellow/creamy colonies growing on MSA and black colonies on BPA were subjected to Gram's staining, catalase test and coagulase test for confirmation and preliminary classification of staphylococci.

Determination of methicillin resistance

Phenotypic resistance to methicillin was determined by the Kirby-Bauer disk diffusion method using cefoxitin (30 mcg) antibiotic disc according to the guidelines of the Clinical and Laboratory Standards Institute. Results were recorded after 24 hours of incubation at 35°C on Mueller Hinton

Agar and interpreted as per Clinical and Laboratory Standards Institute (2010) standards. Among the isolates, one identified as *S. sciuri* (A14) and the other as *S. saprophyticus* (A19) were included in this study from a previous work (Mohanta et al., 2015) for evaluation of methicillin resistance.

Methicillin resistance of the staphylococcal isolates was confirmed by PCR amplification of the *mecA* gene (Table 1). To 12.5 µl of 2X Master mix containing 3mM MgCl₂, 150 mM Tris HCl, 0.4 mM dNTPs and 0.05 units/µl Taq DNA polymerase, 1 µl each of the forward and reverse primers in the concentration of 10pM/ µl, 1 µl of extracted DNA and 5.5 µl of nuclease-free water were added. 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 45 seconds, extension at 72°C for 45 seconds followed by final extension for 5 minutes at 72°C.

Identification of the methicillin-resistant staphylococcal isolates

All the staphylococcal isolates positive for *mecA* were identified according to the presence of the target genes 16S rDNA (*Staphylococcus* genus specific) and *coa* (specific for coagulase-positive *Staphylococcus*). For amplification of the 16S rDNA (Table 1), 15 µl of 2X Master mix was mixed with 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 *coa* gene was amplified simultaneously in a multiplex PCR with *mecA* gene. The amplified 16S rDNA PCR products were sequenced and the sequences were compared to already reported sequences in GenBank database using the Basic Local Alignment Search Tool (BLAST).

The isolates were identified based on their highest percentage homology with the already reported sequences. Multiple sequence alignments were performed using ClustalW and phylogenetic tree was constructed using Mega5.0. The evolutionary history was inferred using the Maximum-likelihood method and the evolutionary distances were computed using the Kimura 2- parameter method.

Determination of enterotoxigenicity

All the methicillin resistant staphylococcal isolates were examined for the presence of the classical enterotoxin genes SEA, SEB, and SEC. A multiplex PCR was carried out for simultaneous amplification of SEA, SEB, SEC (Table 1). The reaction mixture consisted of 12.5 µl of 2X Master mix, 1 µl each of the forward and reverse primers in the concentration of 10pM/ µl, 1 µl of extracted DNA and 5.5 µl of nuclease-free water. 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 45 seconds, extension at 72°C for 45 seconds followed by final extension for 5 minutes at 72°C.

Reference strains

S. aureus ATCC 25923 was used as reference strain for PCR-based analysis of *coa* and enterotoxin genes SEA, SEB, SEC. For evaluation of phenotypic methicillin resistance and PCR-based analysis of *mecA*, a tested and verified strain of methicillin-resistant *S. aureus* (MRSA) was procured from Department of Microbiology, Assam University, Silchar.

Nucleotide sequence accession numbers

The 16S rDNA sequences of the isolates determined in this study were submitted to GenBank database, the accession numbers of which are mentioned in Table 2.

Results

Methicillin-resistant staphylococcal isolates

From a pool of 31 staphylococcal isolates, methicillin-resistant staphylococci were recovered from 11 samples (35.48%), seven from cow milk, two from milk products and one each from goat milk and buffalo milk (Table 2). All of these 11 isolates tested positive for *mecA* gene and hence considered methicillin-resistant. However, phenotypic antibiotic resistance with the disk diffusion method showed only two isolates exhibiting resistance to cefoxitin.

Table 2. Methicillin-resistant isolates identified in this study along with their source of collection, presence of enterotoxin genes and GenBank accession numbers.

Isolate	Genbank accession number	Source	Enterotoxin gene	Best BLAST match (% similarity)
A14	KJ854368	Milk product	sea	<i>S. sciuri</i> (97)
KC12	KP337595	Cow milk	sec	<i>S. epidermidis</i> (98)
KC14	KP337596	Cow milk	sec	<i>S. aureus subsp. aureus</i> (93)
HCM22	KJ854373	Cow milk	none	<i>S. sciuri</i> (99)
A19	KJ854370	Milk product	sea	<i>S. saprophyticus</i> (93)
KRC33	KP337599	Cow milk	none	<i>S. sciuri</i> (96)
DCG43	KP337597	Cow milk	sec	<i>S. epidermidis</i> (95)
SMCM25	KP701478	Cow milk	sec	<i>S. epidermidis</i> (99)
SBM15	KP701477	Buffalo milk	none	<i>S. aureus subsp. aureus</i> (99)
HBM6	KJ854371	Cow milk	sec	<i>S. aureus subsp. aureus</i> (87)
KG22	KP337598	Goat milk	sec	<i>S. aureus subsp. aureus</i> (91)

Identification of isolates

Based on 16S rDNA sequencing results, four *Staphylococcus* species were identified in 11 isolates that included four *S. aureus*, three *S. sciuri*, three *S. epidermidis* and one *S. saprophyticus* (Table 2). The four isolates identified as *S. aureus* by 16S rDNA sequencing also tested positive for *coa* gene in a multiplex PCR conducted for simultaneous detection of *coa* and *mecA* genes (Figure 1). Rest of the seven isolates were negative for *coa* gene indicating prevalence of coagulase-negative staphylococci.

Enterotoxigenicity of the isolates

Multiplex PCR for simultaneous detection of the enterotoxin genes SEA, SEB and SEC revealed that 8 (73.73%) among the 11 methicillin resistant isolates were positive for either SEA or SEC. Six isolates were positive for SEC while only two isolates were positive for SEA (Figure 2). None of the isolates were found to harbour SEB.

Phylogenetic tree

From the phylogenetic tree (Figure 3) it is evident that the isolates obtained from this study share a close relationship. This suggests the possibility of horizontal gene transfer which accounts for dissemination of genes like those for antibiotic resistance or enterotoxins among the staphylococcal strains.

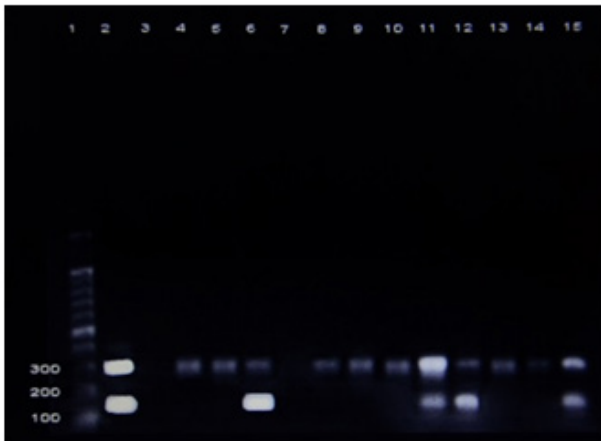


Figure 1. Multiplex PCR for *coa* and *mecA*. Lane 1: 100 bp ladder; lane 2: positive control; lanes 4,5,8,9,10,13,14: amplification of *mecA* alone at 310 bp; lanes 6, 11,12,15: co-amplification of *coa* at 151 bp and *mecA* at 310 bp .

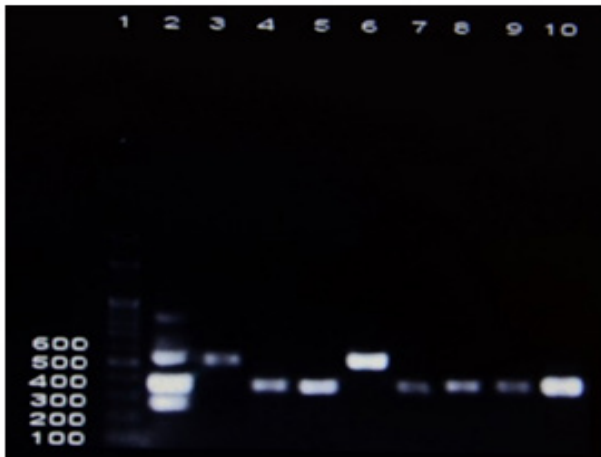


Figure 2. Multiplex PCR for SEA, SEB, SEC. Lane 1: 100 bp ladder; lane 2: positive control, lanes 3,6: amplification of SEA at 552 bp; lanes 4,5,7,8,9,10: amplification of SEC at 371 bp.

Discussion

The rationale behind this work was to perform molecular characterization of staphylococcal isolates from milk and milk products in order to have a better understanding of the risk associated with the consumption of these food products. PCR of *coa* gene and sequence analysis of 16S rDNA confirmed the prevalence of methicillin-resistant coagulase negative *Staphylococcus* (MR-CNS) in the analyzed food samples over methicillin-resistant *S. aureus* (MRSA). *S. epidermidis*, *S. sciuri* and *S. saprophyticus* were the CNS species identified in this study. This finding is in accordance with previous study wherein these species were reported to have been isolated from milk and milk products (Sawant *et al.*, 2009, Perillo *et al.*, 2012, Ruaro *et al.*, 2013, Chajęcka-Weirzchowska *et al.*, 2015). CNS are abundantly found on human and animal skin and mucous membranes. This could

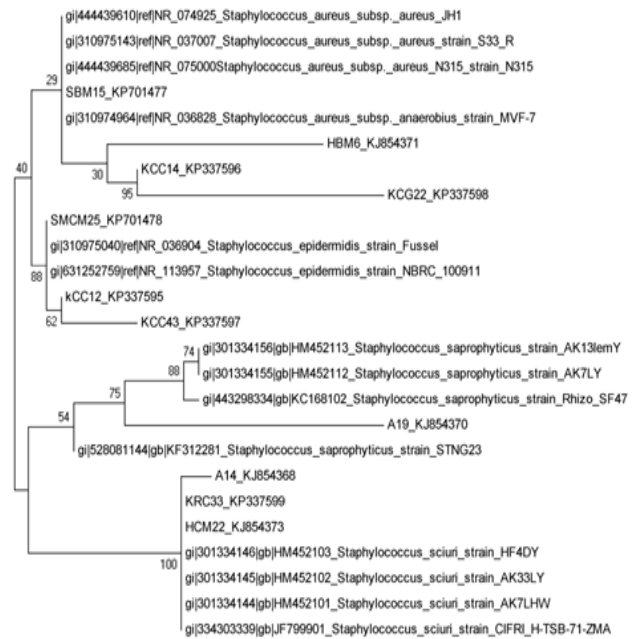


Figure 3. Phylogenetic relationship among the isolates.

explain the prevalence of CNS in the analyzed food products that might have been introduced into the food through improper handling by food workers.

Out of 31 staphylococcal isolates, 11 were positive for the *mecA* gene that is responsible for methicillin resistance in staphylococci. Interestingly, only two of these *mecA* gene positive isolates showed resistance to methicillin phenotypically. This discrepancy might be explained by the presence of a so-called 'silent gene' that expresses only *in vivo* or this could be the result of mutations in the coding or promoter regions of the gene detected by PCR. Presence of methicillin-resistant staphylococci from popular foods like milk and milk products is an alarming situation since these organisms pose health hazards for consumers serving as reservoir of resistance genes (Chajęcka-Weirzchowska *et al.*, 2015). Given the medical relevance of *S. aureus*, *S. epidermidis*, *S. sciuri* and *S. saprophyticus*, methicillin resistance shown by these organisms is a matter of major concern from a clinical perspective.

Among the twenty three serologically distinct enterotoxins identified so far, enterotoxins SEA, SEB and SEC are mostly implicated in food poisoning cases (Balaban and Rasooly, 2000). SEC is the most thermostable of the three followed by SEB and SEA (Notermans *et al.*, 1988). In our study we found SEC to be the most prevalent enterotoxin gene followed by SEA. Many authors have reported the prevalence of SEC in milk and dairy products with potential toxin production (Valle *et al.*, 1990, Veras *et al.*, 2008, Piechota *et al.*, 2014). Also, the isolates in our study tested positive for either SEA or SEC. No co-existence of enterotoxin genes was observed. This could be explained by the different locations in which

they are localized (SEA is carried on a prophage and SEC is localized on pathogenicity islands) and hence need not necessarily express together. In contrast to *S. aureus*, enterotoxigenicity of CNS has been a debatable issue with some authors suggesting their potential in enterotoxin production (Vernozy-Rozand *et al.*, 1996, Veras *et al.*, 2008) while others failing to find any association of CNS with enterotoxigenicity (Even *et al.*, 2010, Ruaro *et al.*, 2013). In our study, we found a good number of CNS isolates harbouring at least one of the classical enterotoxin genes suggesting the capability of CNS to pose health risk to the consumers. Although for confirmation of potential toxin production, specific tests need to be performed in order to quantify the toxins, it has been suggested that staphylococci carrying toxin genes should not be ignored because the possibility of these strains producing toxins *in vivo* can not be excluded (Schmitz *et al.*, 1998). Moreover, the perceived frequency of enterotoxigenic isolates might have further increased if, in this study, we had taken into consideration, the other enterotoxin genes that has been identified so far (Rall *et al.*, 2008). Like gene for methicillin resistance, enterotoxin genes are also located on transmissible genetic elements like pathogenicity islands (SEC), transposons, plasmids (SEB) and phage (SEA) (Cunha and Calsolari, 2007) which facilitates the dissemination of these genes through horizontal gene transfer.

From the phylogenetic tree it is further evident that the staphylococcal isolates are closely related to one another which increases the probability of gene transfer paving way for strains to accumulate methicillin resistance genes and enterotoxin genes. Although a small number of isolates were analyzed in this study, the high percentage of clinically relevant methicillin-resistant enterotoxigenic strains of staphylococci isolated from the analyzed foods is a matter of serious concern for public health.

Conclusion

The findings of this study highlight potential health risk for consumers unless strict hygiene and preventive measures are adopted to prevent staphylococcal infection in farm animals and food products during storage, processing and distribution. Further, contamination of food products with CNS should not be ignored since they also have the capacity to induce health hazards through enterotoxin production.

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