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Enterotoxigenic Genes of *Staphylococcus aureus* Isolated from Raw Milk and Milk Products in Mizoram, India

D. Paul, E. Motina, D. Deka, R. Das[1]

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ABSTRACT

Background: Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases worldwide resulting from the contamination of food by preformed *S. aureus* enterotoxins and milk is considered to be a major source of SFP. This study aimed at the PCR based detection of *S. aureus* and its enterotoxigenic genes in raw milk and milk products.

Methods: A total of 300 samples of raw milk and milk products were collected randomly from Aizawl district of Mizoram and were subjected to phenotypic and genotypic (*nuc* gene) detection of *S. aureus* and subsequent detection of enterotoxigenic genes namely, *sea, seb*, *sec* and *sed* in *S. aureus* isolates

Result: A proportion of 30.67% *S. aureus* isolates were found to be positive for species specific *nuc* gene and 28.26%, 2.17%, 4.34% and 5.43% *S. aureus* strains were positive for *sea, seb, sec* and *sed* genes, respectively. Most contamination of raw milk was observed with *S. aureus* harbouring highest proportion of *sea* gene. Combination of two enterotoxigenic genes, *sea* with another gene, was also possessed by the *S. aureus* isolates from milk and milk products.

Key words: Enterotoxin genes, Milk products, Raw milk, *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus is an opportunistic human and animal pathogen that colonizes the mucosal surfaces and the anterior nares are the primary reservoir sites of *S. aureus*. However, extra-nasal colonization of *S. aureus* also takes place in several locations such as skin, pharynx and gastrointestinal tract (Nair *et al*., 2014). Among the various food borne pathogens, *S.aureus* has grabbed the interest from food safety point of view because of their ubiquity and adaptability, inhabiting the skin and mucous membrane of humans, other mammals and birds. The factors contributing to the rise of this organism as a formidable pathogen involve multiple mechanisms of virulence including its acquired ability to resist antibiotics and evade host defences, production of an arsenal of virulence factors and secreted toxins such as enterotoxins (SEs) that are responsible for food poisoning (Joo *et al.,* 2011). Staphylococcal enterotox ins are heat stable which are potent gastrointestinal exotoxins causing food borne intoxication. Classical SEs have been divided into five serological types (SEA through SEE) having emetic properties on the basis of their antigenicities (Argudin *et al.,* 2010) and possess super-antigenic activity and are encoded by accessory genetic elements including plasmids, prophages, pathogenicity islands, vSa genomic islands or by genes located next to the staphylococcal cassette chromosome (SCC) implicated in methicillin resistance (Zhenzhen *et al*., 2018). The SEs are a major cause of food poisoning, which typically occurs after ingestion of different foods like processed meat and dairy products contaminated with *S. aureus* due to improper handling and subsequent storage at elevated temperatures. Food handlers carrying enterotoxin producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination through direct contact or through respiratory secretions (Argudin *et al*., 2010).

Milk is considered to be a good medium for *S. aureus* growth and enterotoxin production and contaminated raw milk has been often involved in SFP (Asao *et al.,* 2003). Mastitis *by S. aureus* is a serious problem in dairy production and milk from infected animals may contaminate bulk milk. Moreover, human handlers, milking equipment, environment, udder and teat skin of dairy animals may be other possible sources of bulk milk contamination and subsequently contaminate the milk products. Thus, enterotoxigenic *S. aureus* may be a potential risk of SFP after consumption of raw milk and milk products (Jorgensen *et al.,* 2005). The present study was taken up for molecular detection of

Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and Animal Husbandary, Central Agricultural University, Selesih, Aizawl-796 014, Mizoram, India.
[1]Immunology Section, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243 122, Uttar Pradesh, India.

Corresponding Author: D. Deka, Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and Animal Husbandary, Central Agricultural University, Selesih, Aizawl-796 014, Mizoram, India. Email: drdevajani@gmail.com

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S. aureus and its enterotoxigenic genes from raw milk and milk products in Aizawl district of Mizoram, India.

MATERIALS AND METHODS

The study was conducted in the department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and animal husbandary, Aizawl, Mizoram during 2020-21.

Collection of samples

A total of 300 samples, 90 raw cow milk, 30 pasteurised milk and 200 milk products including 50 curd, 50 rasmalai, 50 ice-cream and 30 paneer, were collected randomly from different unorganised farms, milk vendors, milk co operatives and sweet shops of Aizawl district, Mizoram.

Isolation and identification of *S. aureus*

The isolation and identification of *S. aureus* from raw milk and milk products was done following standard bacteriological method (Cowan and Steel, 1993). One ml or one gm of sample was mixed with 9 ml sterile 0.1% peptone water for enrichment and was centrifuged at 8000 rpm for 2-3 minutes for extrication of the sample in the broth and incubated for 24 hours at 37°C. A volume of 0.1 ml of broth culture showing cloudy discolouration was streaked aseptically on Baired Parker agar (BPA) plates and incubated at 37°C for 24-48 hours. The characteristic colonies on BPA plates were streaked on Mannitol Salt agar (MSA) plate and incubated at 37°C for 24 hours. The *S. aureus* isolates were phenotypically characterized based on morphological characteristics like colonies of jet black colour with halo zone on BPA and yellow colonies with yellow colouration of media on MSA and positive reactions in Gram stainingand coagulase test (Quinn *et al.*, 1999).

Molecular detection of *S. aureus* and its enterotoxigenic genes

All the presumptive *S. aureus* isolates were processed for bacterial lysate preparation using boiling and snap chilling method. A purified colony of isolate was inoculated into one ml of Luria Bertani (LB) broth and incubated at 37°C for 24 hours and the cells were pelleted by centrifugation at 8000 rpm for 10 minutes at 4°C. The pellet was washed three times with sterile normal saline solution (0.85%) and finally re-suspended in 200 µl of nuclease free sterile distilled water. The cell suspension was heated in a boiling water bath for 5 minutes followed by immediate chilling. The cellular debris was sediment by centrifugation at 5000 rpm for 5 minutes and the supernatant was used as DNA template for PCR assay.

The DNA templates of *S. aureus* isolates were subjected for detection of species specific *nuc* gene by using published primers and subsequently all the *nuc* gene positive *S. aureus* strains were screened for enterotoxin producing genes, namely*sea, seb, sec* and *sed* using published primers (Table 1) as described by Blaiotta *et al.* (2004). The details of various thermal cycling conditions for species specific gene (*nuc*) and different enterotoxigenic genes are given in Table 2. The amplified PCR products were further analysed by agarose gel electrophoresis.

The statistical analysis of data was carried out as per the method of Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Phenotypic and molecular detection of *S. aureus*

Out of 300 raw milk and milk product samples analysed, 51.33% samples revealed jet black colonies with halo zone

Table 1: Oligonucleotide primers used for PCR detection of enterotoxigenic genes of *S. aureus.*

Enterotoxigenic genes	Published gene sequence	Base pair	Reference
nuc	F- GCG ATT GAT GGT GAT ACG GTT	279	Brakstad *et al.* (1992)
	R- AGC CAA GCC TTG ACG AAC TAA AGC		
Sea	F- TAA GGA GGT GGT GCC TAT GG	180	Cremonesi *et al.* (2007)
	R- CAT CGA AAC CAG CCA AG TT		
Seb	F-TCG CAT CAA ACT GAC AAA CG	478	Johnson *et al.* (1991)
	R- GCA GGT ACT CTA TAA GTG CC		
Sec	F- ACC AGA CCC TAT GCC AGA TG	371	Cremonesi *et al.* (2007)
	R-TCC CAT TAT CAA AGT GGT TTC C		
Sed	F- TCA ATT CAA AAG AAA TGG CTC A	339	Cremonesi *et al.* (2007)
	R- TTT TTC CGC GCT GTA TTT TT		

Table 2: Thermal cycling conditions for *nuc* and enterotoxigenic genes of *S. aureus.*

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Base pair	Number of cycles
nuc	95°C 5 for min	95°C for 45 sec	58°C for 45 sec	72°C for 45 sec	72°C for 5 min	279	30
sea	94°C for 5 min	94°C for 1 min	58°C for 1 min	72°C for 1 mint	72°C for 5 min	180	30
seb	94°C for 5 min	94°C for 1 min	51°C for 2 min	72°C for 1 min	72°C for 7 min	478	30
sec	94°C for 5 min	94°C for 1 min	52°C for 1 min	72°C for 1 min	72°C for 7 min	371	30
sed	95°C for 5 min	94°C for 1 min	56°C for 1min	72°C for 1 min	72°C for 7 min	339	30

in BPA and subsequently 46.00% samples revealed yellow colonies on MSA. The bacterial colonies showing positive Gram reaction and catalase reaction are presumptively identified as *S. aureus*. A total of 30.67% *S. aureus* isolates were confirmed by amplification of species specific *nuc* gene (279 bp) (Fig 1) from 138 (46.00%) presumptively identified *S. aureus* isolates distributed in raw milk (43.33%), rashmalai (34.00%), ice cream (46%), paneer (23.33%) and pasteurized milk (20.00%) (Table 3). Wide variations in the prevalence of *S. aureus* in milk and milk products from different places were reported by Siriken *et al.* (2016) (56%), Sutejo *et al.* (2017) (97%) and Haque *et al.* (2018) (19.44%) from Turkey, Indonesia and Bangladesh, respectively. Kandil *et al.* (2018) reported the prevalence of *S. aureus* as 80% and 40% in raw milk and ice-cream, respectively from Egypt. In India, Sudhanthiramani *et al.* (2015), Hamid *et al.*(2017), Begum *et al.* (2018) and Bhati *et al.*(2018) reported 39.09%, 21.21%, 92.31% and 63.80% *S. aureus* in raw milk and milk products from Tirupati, Jammu and Kashmir, Chennai and Rajasthan, respectively. Although pasteurization of milk is an important food safety phenomenon, the occurrence of *S. aureus* in pasteurized milk is not uncommon and reported in variable rates which might be due to cross contamination during storage and selling along with raw milk, local conditions, sample sizes, sample sources and geographic location (Dai *et al*., 2019). The abundance of *S. aureus* in the air, dust, waste, water, milk, food or food equipment, environmental surfaces, humans or animals including cow's udders may increase the occurrence of the organism in milk and milk products during their unhygienic production, processing, storage and selling. Ojokoh (2006) and Jadhav and Raut (2014) reported that hands, skin and clothing of handlers and droplets produced by coughing, talking and sneezing can settle on food and may act as possible sources of this organism in milk products.

Detection of enterotoxigenic genes in *S. aureus* strains

From 92 (30.67%) *nuc* positive *S. aureus* strains from milk and milk products, 40.22% strains were found to be positive for different enterotoxigenic genes contributing to 71.79% in raw milk, 17.65% in rasmalai, 13.04% in ice-cream, 14.28% in paneer, 33.33% in pasteurized milk and absent in curd. The prevalence of enterotoxigenic genes was significantly ($P<0.05$) higher in raw milk than pasteurized milk and other milk products (Table 4) (Fig 2). Begum *et al.* (2018) and Haque *et al.* (2018) confirmed *S. aureus* by

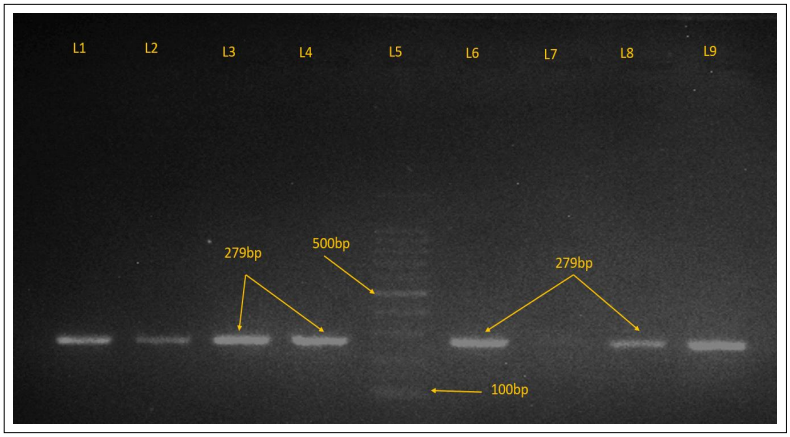

Fig 1: Agarose gel electrophoresis plate showing PCR amplicons of *nuc* gene (279bp); L5: 100 bp ladder; L2 and L8: Positive control; L1, L3, L4, L6 and L9: Positive sample; L7: Negative control.

Table 3: Phenotypic and molecular detection of *S. aureus* isolates from milk and milk products in Aizawl district, Mizoram.

Type of sample	Number of samples analysed	Number of *S. aureus* isolates		
		Phenotypic method		Molecular method (*nuc* gene)
		No. of isolates in BPA (%)	No. of isolates in MSA (%)	No. of isolates positive for *nuc* gene (%)
Raw cow milk	90	72	70 (77.78)	39 (43.33)
Curd	50	0	0 (0.00)	0 (0.00)
Rasmalai	50	26	20 (40.00)	17 (34.00)
Ice-cream	50	32	29 (58.00)	23 (46.00)
Paneer	30	15	13 (43.33)	7(23.33)
Pasteurized milk	30	9	6 (20.00)	6 (20.00)
Total	300	154 (51.33)	138 (46.00)	92 (30.67)

detecting the *nuc* gene of *S. aureus* in milk and milk products as 92.31% and 24. 66% from Chennai (India) and Mymensingh district of Bangladesh, respectively.

Among the enterotoxigenic genes, the prevalence of *sea* gene (28.26%) was significantly (P<0.05) higher than *seb* (2.08%), *sec* (4.34%) and *sed* (5.43%) genes. The *sea* gene was recorded in *S. aureus* isolates from raw milk and all milk products except curd with highest occurrence in strains from raw milk (53.85%). The *seb, sec* and *sed* genes occurred in lower variable rates or absent (Table 4). Enterotoxin A is the most frequently encountered staphylococcal enterotoxin among staphylococcal food poisoning cases. Similar to the present findings, Normanno *et al.* (2007) reported that the *sea* gene is the most commonly reported enterotoxigenic gene in *S. aureus* isolates obtained from different types of foods. Afifi *et al.* (2011) also isolated *S. aureus* in 50% of raw milk and 35% of ice-cream samples from Egypt with major classical enterotoxin *sea* (29.30%) followed by *sec* (16.10%) and *sed* (10.10%). Saka and Tergi (2018) revealed 5(41.60%) *sea*, 2(16.60%) *sec* and 1 (8.30%) *sed* in raw milk and milk product from Turkey. Gandhale *et al.* (2017) isolated 17 *S. aureus* strains with

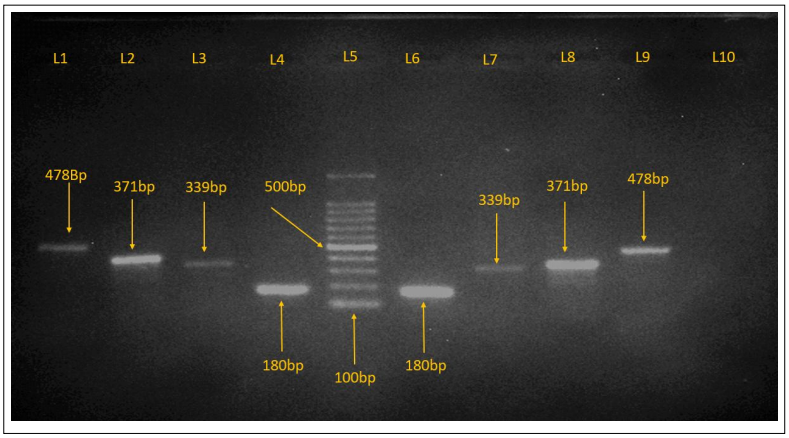

Fig 2: Agarose gel electrophoresis plate showing PCR amplicons of *enterotoxigenic genes*(*sea, seb, sec* and*sed*); L5: 100bp ladder; L1to L4: Positive control of (*seb, sec, sed*and*sea*), respectively; L6, L7, L8and L9: Positive sample of (*sea, sed, sec*and*seb*), respectively; L10: Negative control.

Table 4: Detection of enterotoxigenic genes of *S. aureus* strains isolated from milk and milk products.

Type of sample	No of sample analysed	Type of virulence genes (%)				Detection of enterotoxigenic genes (%)
		Sea	*Seb*	*Sec*	*sed*	
Raw milk	39	21 (53.85)	1 (2.56)	3 (7.69)	3 (7.69)	28 (71.79)[b]
Curd	0	0	0	0	0	0 (0.00)
Rashmalai	17	1 (5.88%)	0	0	2	3 (17.65)[a]
Ice cream	23	2 (8.70)	0	1 (4.35)	0	3 (13.04) [a]
Paneer	7	1 (14.29)	0	0	0	1 (14.28) [a]
Pasteurized milk	6	1 (16.67)	1 (16.67)	0	0	2 (33.33)[a]
Total	92	26 (28.26)	2 (2.08)	4 (4.34)	5 (5.43)	37 (40.22)

Table 5: Detection of enterotoxin genes of *S. aureus* in combination from milk and milk products.

Type of sample	*nuc* positive *S. aureus* samples analysed	No. of samples with combination of genes (%)	Isolate no	*sea*	*seb*	*sec*	*sed*	Total
Raw milk	28	6 (21.42)	RFL2 b	+	+	-	-	2
			1CF2c	+	-	-	+	2
			1CF5c	+	-	-	+	2
			ICF7a	+	-	-	+	2
			MM9b	+	-	+	-	2
			MM10b	+	-	+	-	2
Rashmalai	13	2 (15.38)	MRIa	+	-	-	+	2
			MRMIa	+	-	-	+	2
Pasteurized milk	3	1 (33.33)	PMu3a	+	+	-	-	2

enterotoxigenic genes *viz.seb* (1.92%), *sec* (21.15%), *sed* (7.69%) and *see* (1.92%) in bovine milk from Satara district of Maharashtra, India. Sharma *et al.* (2017) revealed that out of 30 isolates of *S. aureus* from bovine raw milk, 30% were positive for *sec*, 10% for *sea* and 3.30% for *seb* in Rajasthan, India.

Out of 92 *nuc* gene positive *S. aureus* strains, 10 (18.87%) numbers of strains were found to be positive for combination of two numbers of virulence genes, 21.42% strains in raw milk, 15.38% strains in rasmalai and 33.33% in pasteurized milk (Table 5). Normanno *et al.* (2007), Rall *et al.* (2008) and Afifi *et al.* (2011) revealed that *S. aureus* strains from bovine milk and milk products synthesized enterotoxins in different combination from Italy, Brazil and Egypt, respectively. Mansour *et al.* (2017) also detected *S. aureus* genotypically (*nuc* gene) in 16.30% of raw milk samples in Egypt and three strains expressed single and combination of two and three enterogenic genes and absence of enterotoxin A and D.

CONCLUSION

To conclude, enterotoxigenic *S. aureus* in raw milk and dairy products is an important public health problem especially in traditional dairy products. Staphylococcal enterotoxins may cause food poisoning due to consumption of raw or unpasteurized milk products. Present study provide the information regarding the genetic background of *S. aureus* strains isolated from raw and pasteurized milk and milk products samples in Mizoram, a hilly North Eastern state of India and it is the first molecular approach to demonstrate virulence properties of *S. aureus* isolates from milk and milk products which is very important from staphylococcal food poisoning point of view in consumers. These isolated *S. aureus* strains had virulence potential as which half of them carried enterotoxin genes either singly or in combination of two.

Conflict of interest: None.

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