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ISOLATION AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN A GENE IN STAPLE FOOD OF SOUTH INDIAN REGION

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ABSTRACT

Staphylococcus aureus (*S. aureus*) associated food borne disease is one of the major health concerned around the globe. A set of enterotoxins (encoded by enterotoxin genes) produced by *S. aureus* are actively involved in growth and survival of the bacterium in diverse conditions. These enterotoxins are the major cause of gastroenteric syndrome, in food poisoning. In the Indian subcontinent, different food items are consumed that might contaminate during the preparation and storage. Therefore, we aimed to investigate the prevalence of *S. aureus* in regularly consumed food items from the southern part of India. Total 224 food samples were collected and categorized as, Processed and canned food, Home sterilized food and Raw and unsterilized food. Out of 224, total 51 food samples were identified *S. aureus* positive using culture based morphological identification, biochemical assay, and molecular techniques. Results indicate that about 20 % of Processed and canned food, 10 % of Home sterilized food and 70 % of Raw and unsterilized food were *S. aureus* positive. Furthermore, quantitative PCR based profiling was performed to investigate the frequency of *S. aureus* enterotoxin (SEA) gene among the *S. aureus* positive samples.

Observation indicates that 16 food samples were positive to enterotoxin SEA gene, mainly in milk (12.5%), idali (6.2%), coconut paste (6.2%), fruit juice (25%), sweet (12.5%), panipoori (18.7%) and raw meat (18.7%). The current study investigated that prevalence of *S. aureus* in regularly consumed food items and their possible route of contamination from south India.

Keywords: food poisoning, enterotoxin, SEA gene, *Staphylococcus aureus, qPCR*

INTRODUCTION

Food-borne disease is a one of the major health concern around the globe, including food borne infections and intoxications **[1, 2]**. As per reference to World Health Organization (WHO) report published in 2015, it has been estimated about five billion people affected to food-borne disease, and nearly two million deaths annually assumed worldwide **[3, 4]**. A broad range of microorganisms have been identified that causes food-borne associated diseases, including bacterial species belong to the *Campylobacter*, *Clostrodium*, *Salmonella*, *Vibrio* and *Staphylococcus* species **[5]**. *Staphylococcus aureus* (*S. aureus*) is well identified pathogen which is a one of the major causative agent of food-borne disease. It is linked to sporadic food-borne diseases with symptoms of nausea, vomiting, abdominal cramps, and diarrhea, in different region of the world **[5, 6]**.

Genomic size of *S. aureus* is nearly 2.3 million base pair which consists of different housekeeping, virulence and regulatory genes required for growth and survival **[7, 8]**. Pathogenesis of *S. aureus* is controlled by group of thirty genes, including basic survival gene and virulent genes **[7-9]**. These gene are responsible for survival of bacterium in extreme temperature condition (low to high) and, in variable pH range **[10]**. Moreover, severe food poisoning by *S. aureus* has been linked to release of different type of enterotoxins, such as staphylococcal enterotoxins and toxic shock syndrome toxin 1 **[11-13]**. Staphylococcal enterotoxins are the heat-stable exotoxin and fifteen SEs have been identified classified as, SEA-SEE, SEG-SER and SEU, in which, few SEs cause gastro-enteric syndrome in human.

Identification of *S. aureus* can be performed using traditional biochemical, culture based techniques as well as molecular techniques. However, these laboratory practices are efficient but time consuming process for the evaluation and confirmation of *S. aureus* for diagnostic purpose. Misdiagnosis of *S. aureus* could lead the mortality risk of infected person. Furthermore, lack of standard diagnostic tool is a one of the major cause of misinterpretation the medical report. This further increases the risk of mishandling the specimens which upsurges the error in reported prevalence of *S. aureus* **[2, 14, 15]**. Therefore, for the detection of enterotoxin, various methods have been developed. Out of which, Reversed passive latex agglutination assay is one of the established method. Although, it has certain limitation, that it recognize only four enterotoxin, SEA to SED. Other methods to recognize enterotoxins at gene level, include PCR, sequencing and microarray **[16]**. However, various approaches are required to investigate the enterotoxins at gene and protein level.

Evidences indicates that *Staphylococcus* species are ubiquitously present in wide range of food materials, from raw to cooked food items (including ready-to-eat foods stuff which increase the risk of social health burden **[17, 18]**. Therefore, the current study is aimed to detect the presence of *S. aureus* in regularly consumed food specimens collected from southern part of India. Furthermore, we aimed to identify the expression profile of SEA gene in collected food items.

MATERIALS AND METHODS

Collection food specimen

Food specimens were collected from road-side hawkers, local store and home using sterile sample collection method from the region of south India. Based on the availability food materials, specimens were classified in three independent categories (**Table 1**), such as: (I) Processed and canned food (II) Home sterilized food and (III) Raw and unsterilized food.

Table 1: Detail of collected food samples and quantity utilized for the current study.

S. No.	Processed and canned food		Home sterilized food		Raw and unsterilized food	
	Food material	*(n)*	*Food material*	*(n)*	*Food material*	*(n)*
1	Sweet	14	Idli	12	Fresh juice	19
2	Canned sweet	12	Steamed Rice	16	Milk	28
3	Instant curry (Veg)	10	Homemade Curry (Veg)	12	Noodles	6
4	Instant curry (Non-Veg)	12	Homemade Curry (Non-Veg)	10	Coconut Paste (Chutney)	14
5	Canned juice	10	Bhajji	8	Pani Poori (Juice)	13
6	Instant Idly batter	10	Dosa paste	8	Raw meat	10
	∑ =	68		66		90

n = number of samples

Preparation of the bacterial culture

All collected specimens (1mg) were separately macerated/mashed and homogenized by adding 10ml of sterile distilled water. 0.1ml homogenously mixtures were used for spreading onto the nutrient agar plates, under laminar airflow conditions and incubated at 37°C for overnight.

Morphological and biochemical screening of bacterial strains

All 224 specimens containing master plates were observed for morphological identification of *S. aureus*. We have adopted standardized morphological method to identify the Staphylococcus colonies with golden yellow color and watery consistency **[19]**. *S. aureus*-10652 was used for SEA positive bacterial strain. All identified colonies were characterized using grams staining and biochemical assay namely, Catalase, Coagulase, Methyl Red Voges Proskauer, Mannitol and DNase Tests **[20-22]**.

Genomic DNA extraction and purification

On the basis of preliminary observation, all positive samples of *S. aureus* were subjected for total genome isolation using QIAamp DNA mini kit (Qiagen, Hilden, Germany), as described by the manufacturer. Further, isolated DNA was purified using Qiagen DNA-purification kit, Qiagen, Hilden, Germany), as described by the manufacturer. Freshly purified DNA were quantified using NanoDrop-2000 (Thermo Scientific, Massachusetts, USA), and quality of DNA was accessed by absorbance ratio at both 260/280 and 230/260 nm.

Oligonucleotide primers and TaqMan probes

Quantitative expression profile of *S. aureus* bacterial specimens were performed by TaqMan quantitative reverse PCR method. The oligonucleotide primers and fluorescence-labeled hybridization probes of the *S. aureus* enterotoxin A genes (SEA) were adopted from previous published work from Klotz and his group **[23]**. 50µl PCR reaction mixture was prepared by mixing 10X PCR buffer, 10mM dNTPs, 6 mM MgCl2, 50 pmol forward primer, 50 pmol reverse primer, 150 nM TaqMan probe, 0.5 of AmpErase, and 2U of AmpliTaq. 10 µl (about 100ng) of purified DNA template was added in the PCR reaction mixture. The sequences of specific primers and FAM labeled probe are shown in **Table 2**. *S. aureus*-specific genomic marker femB was used as a housekeeping gene. Real-time quantitative PCR was performed using standard protocols on an ABI 7900HT Sequence Detection System (Applied Biosystems, Bethesda, USA) equipped with a 96-well reaction plate. The PCR condition program was initiated 50°C for 2 min, to hold at 95°C for 10 min, and to complete 40 cycles of 94°C for 15 second and 60°C for 1 min. The result of quantitative amplification was observed using intensity of fluorescence signal of FAM detected by the ABI 7900 HT sequence detector.

Statistical analysis

All set of data were analyzed using GraphPad Prism 6.0 (GraftPad Software, San Diego, California). All experiments were carried out in triplicate and results were expressed as mean ± SEM (standard error of mean). Statistical significance would be defined at P-values less than 0.05 ($P<0.05$). Additional the following tests were decided to be performed for the analysis; Pearson's correlation test, One-Way ANOVA test and Fisher's Exact Test and others as per requirements.

Table 2: Details of specific primers and FAM labeled probe for *S. aureus* enterotoxin A genes (SEA)

Target Gene	Primers/Probes	Sequence length
SEA	Forward primer	AAAATACAGTACCTTTGGAAACGGTT
	Reverse primer	TTTCCTGTAAATAACGTCTTGCTTGA
	Probe (FAM labeled)	AACGAATAAGAAATGTAACTGTTCAGGAGTTGGATC

RESULTS AND DISCUSSION

Species Confirmation:

Fifty one of the 224 food samples tested positive for the presence of *S. aureus*. These positive isolates were obtained from different food sources which was broadly categorized as (I) Processed and canned food, (II) Home sterilized food and (III) Raw and unsterilized food. **Table 3** summarizes the result of biochemical assays used for testing *S. aureus*.

Thus, the identification based on morphological and biochemical assay confirmed the presence of *S. aureus* in 51 food specimens belong to processed and canned food (19.6%, 10/51), Home sterilized food (9.8%, 5/51), and Raw and unsterilized food (70.6%, 36/51), demonstrated in **Figure 1A**. **Figure 1B-1D** elaborate the frequency of *S. aureus* positive samples among the total number of categorized food. For instance, 14.7% (n=10) *S. aureus* positive specimens out of total 68 processed and canned food), likewise, sweet (05 out of 14 specimens), canned sweet (02 out of 12 specimens), canned juice (03 out of 10 specimens); home sterilized food (9.8%, 5 *S. aureus* positive specimens out of total 66 home sterilized food), such as idli (04 out of 12 specimens), and dosa paste (01 out of 08 specimens); and raw and unsterilized food (40%, 36 *S. aureus* positive specimens out of total 90 raw and unsterilized food), such as fresh juice (07 out of 19 specimens), milk (12 out of 28 specimens), , coconut paste (Chutney; 06 out of 14 specimens), pani poori juice (05 out of 13 specimens), and raw meat (06 out of 10 specimens), shown in **Figure 1**.

Few of the bacterial specimens were characterized on the basis of molecular genetic profiling using specific 16S rRNA gene of *S. aureus* amplification, sequencing and phylogenetic analysis, published previously **[24]**. Observation based on molecular genetic profiling indicates that all bacterial specimens were identified 99-100% homology to *S. aureus*.

It was observed out of 51 sample tested positive for *S. aureus*, 70% belong to Raw and unsterilized food specimens, while 20% were Processed and canned food specimens. Home sterilized food specimens constituted only 10% of the positive samples. It has been known that *S. aureus* usually does not compete with native microflora of raw food. So, the contamination of food product can be easily linked to in proper handling that had led to transmission of bacterium either through respiratory secretion or direct contact by the handlers. Other major sources of *S. aureus* infection in these food sources can be improper storage or through air dust and surface where the food was kept.

Screening and expression profiling of SEA gene

To determine the prevalence of SEA gene in *S. aureus* positive samples quantitative PCR was performed using Taqman PCR based assay. Observations based on TaqMan PCR assay revealed that 16 *S. aureus* positive food samples were expressed the SEA gene. The following showed the positive expression profile - milk (Specimen Number [(S. No.) MKSA-1 and MKSA-3)], idli (S. No. IDSA-4), Coconut paste (S. No. CPSA-6), Fruit juice (S. No. FJSA-2 and FJSA-3), Sweet (S. No. SWSA-5 and SWSA-8), Pani poori juice (S. No. PPSA-5, PPSA-9 and PPSA-12), and Raw meat (S. No. RMSA-4, RMSA-6 and RMSA-7). Interestingly, canned sweet (CSSA), canned juice (CJSA) and dosa paste specimens were negative to *S. aureus* enterotoxin A gene.

Quantitative PCR is a well a stabilized methodologies to detect the minimal amount of gene and also allow to analyze large number of sample at a time. Therefore, development of quantitative PCR based assay is more appropriate in the detection of toxin profile produced by different pathogens. In order to determine the presence of SEA in different *S. aureus* culture prepared from selected food specimens, we investigate the presence of SEA in 51 *S. aureus* positive food specimens, including Milk, Idli, Coconut paste, Fruit juice, Sweet, Pani poori juice, and Raw meat. Observation indicates that out of 51, 16 specimens of *S. aureus* culture were positive with SEA gene. In which, *S. aureus* culture prepared from Milk (2 out of 12; 16% positive), Idli (1 out of 4; 25% positive), Coconut paste (1 out of 6;

16% positive), Fruit juice (4 out of 7; 57% positive), Sweet (2 out of 5; 40% positive), Pani poori juice (3 out of 5; 60% positive), and Raw meat (3 out of 6; 50% positive) samples were positive with SEA gene **(Figure 2)**.

QRT analysis revealed that SEA gene expression was higher in raw and unprocessed food items which can be attributed to longer storage time with handling errors. Further, in food items like fresh fruit juices, Pani poori syrup and local sweet likelihood of contamination due to traditional storage practices, ice, and human contact.

Table 3: Preliminary observations of *S. aureus* using morphological and biochemical assay.

S. No.	Type of detection assay	Test	Observations	Results
1	Culture based assay	Nutrient Agar medium culture	Yellow pigmented circular colonies with smooth surface	Positive
2		Gram staining test	Purple-red stained coccus	Positive
3	Biochemical based assay	Catalase slide test	Showing brisk effervescence, bubbles of oxygen observed	Positive
4		Coagulase tube test	Clot formation observed	Positive
5		Coagulase slide test	Clot formation observed	Positive
6		DNase test	Clear zone around the colony	Positive
7		Methyl-Red test	Development of stable red color	Positive
8		Voges-Proskauer test	Development of reddish brown color	Positive

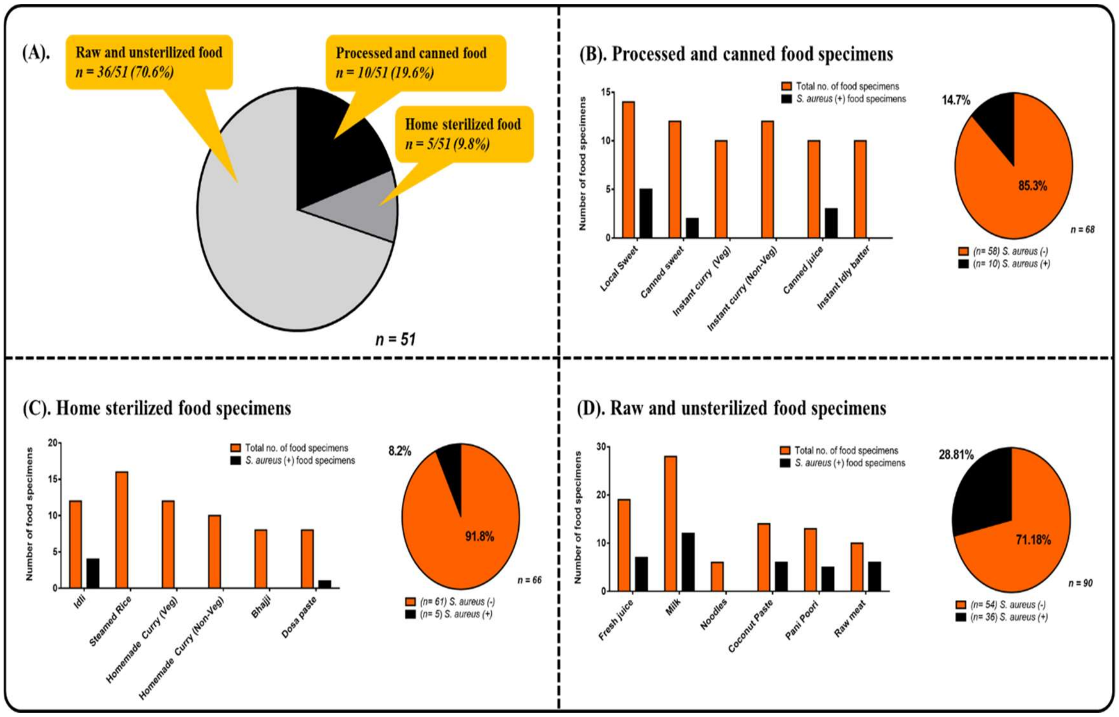
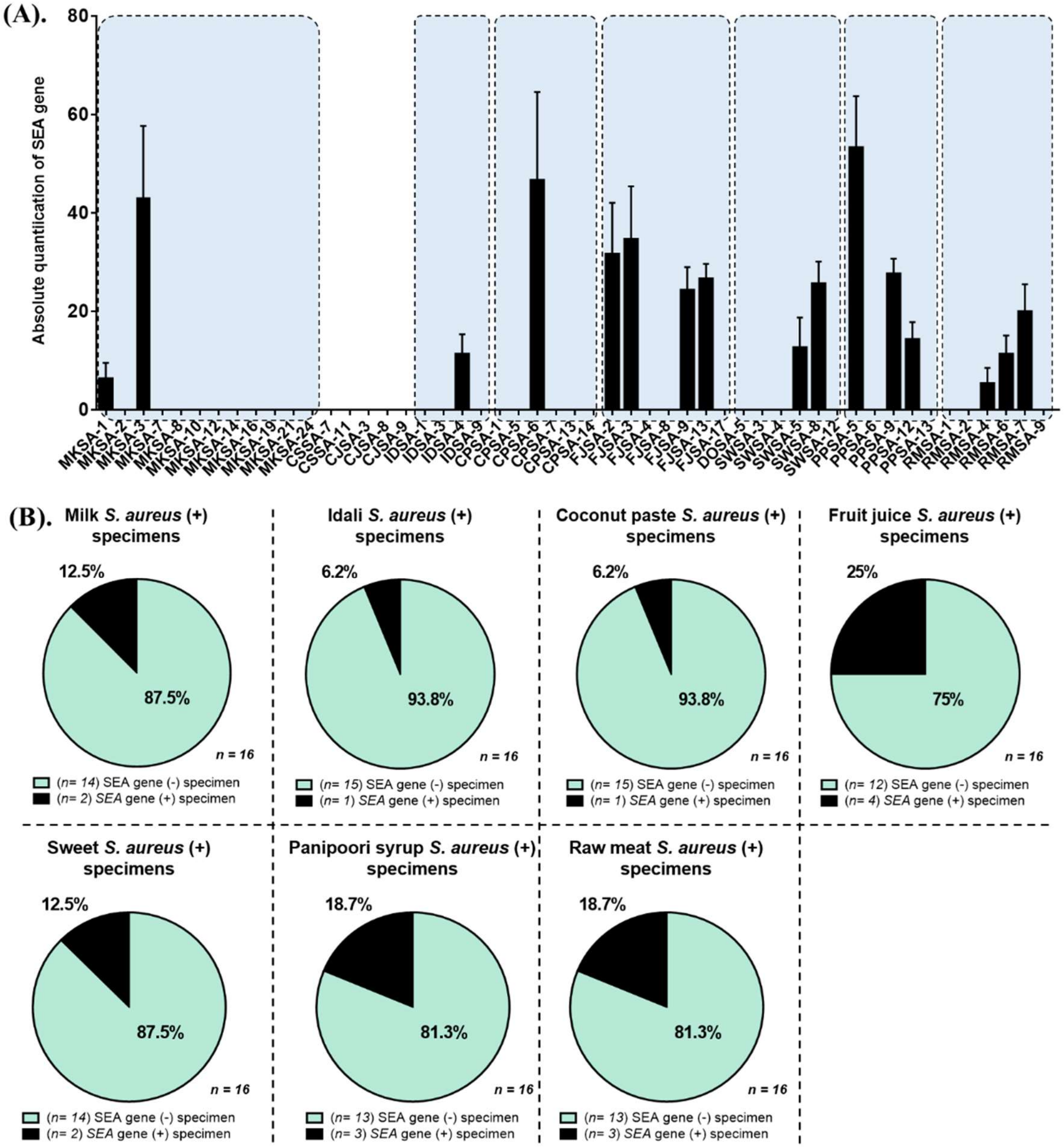


Figure 1: Prevalence of *S. aureus* in different food specimens analyzed by morphological and biochemical assay (A) Details of categorized food samples identified positive to *S. aureus* in different food specimens. (B-D) Frequency of *S. aureus* positive processed and canned food specimens, home sterilized food specimens, and Raw and unsterilized food specimens



 # *abbreviations:* Milk *S. aureus* positive (SA+) (MKSA); Canned sweet SA+ (CSSA); Canned juice SA+ (CJSA); Idli SA+ (IDSA); Coconut paste SA+ (CPSA); Fruit juice SA+ (FJSA); Dosa paste SA+ (DPSA); Sweet SA+ (SWSA); Pani poori juice (PPSA); Raw meat SA+ (RMSA).

Figure 2: Expression profile of enterotoxin SEA gene among *S. aureus* positive food specimen samples (A) Bar graph shows the results of absolute quantitative expression of SEA gene. *S. aureus*-specific genomic marker femB was used for normalization of target gene expression. (B) Pie diagram represent the frequency of SEA gene expressed in *S. aureus* positive food specimen in milk (12.5%), idali (6.2%), coconut paste (6.2%), fruit juice (25%), sweet (12.5%), panipoori (18.7%) and raw meat (18.7%)

CONCLUSION

There is a high consumption rate of coconut paste, milk and raw meats, etc., in the south Indian region. The prevalence of high contamination of *S. aureus* in these foods in the current study has urged in a serious need to hygienic handling and storage of these foods especially in the local area to limit food contamination and possible food poisoning associated with spoiled food. Further, presence of enterotoxin gene expression the isolates may lead to severity of infection in patients affected by *S. aureus* food poisoning. Also, identification of *S. aureus* enterotoxin expression by quantitative PCR method, gives a better understanding of infectious particles in the food items. In summary this study provides a better view of *S. aureus* infection in local and street, south Indian foods thus, identifying areas at which the local government bodies can evaluate the handling and storage of these items. Moreover, identification of enterotoxins using qRT PCR by random sampling of street vendors, midday meals will provide early detection of contaminants and prevent suffering of general population because of food poisoning.

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Ethics declaration: Ethical clearance (Ref: NIU/NERB/SOS/BT-MB/18/106) was obtained from Institutional Ethical Committee (Ethics Review Board), Noida International University, Gautam Budh Nagar, UP, India.

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