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Development of PCR assay for identification of *Staphylococcus aureus* and their enterotoxins from spoiled food samples

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Abstract
Food-borne diseases due to microbial pathogens, have become a major issue of concern as they represent serious threat to the health of millions of people world - wide. Serious outbreaks of food borne disease have been documented on every continent in the past decades, illustrating both the public health and social significance of these diseases. Food Contamination is of major health hazards in India leading to food poisoning. The food borne diseases due to food contamination or spoilage is one of the important problems to be addressed. *Staphylococcus aureus* has been found to be one of the major cause of food poisoning among all the other food pathogens.
S. aureus produces very important virulence factors including Staphylococcus enterotoxins (SEs) which are the main causes of diarrhoea, vomiting and other symptoms associated with food poisoning. Therefore to minimize infection in food and water, the etiological agents and harmful toxins produced by them must be identified. Early identification will be helpful in minimizing food borne infections which helps in the prevention of diarrheal diseases. The conventional methods used for identification of *S. aureus* are limited to their Biological characterization only that are not only time consuming rather have limited reliability. Currently the molecular techniques based on PCR amplification of 16S rRNA of *S. aureus* for rapid and specific detection is widely used approach. This study focuses on rapid detection of *S. aureus* strains obtained from various food samples by development of a specific PCR Assay including a novel primer set and standardized PCR Conditions. A multiplex PCR assay has also been developed for specific Staphylococcal enterotoxins genes (SEA, SEB, SEC, SED, and SEE) produced by different isolates. Out of the 59 isolates of *Staphylococcus aureus* obtained from various food samples, found positive for enterotoxin infection, 15, 21, 6, 12 and 5 were found to be producing SEA, SEB, SEC, SED and SEE respectively further employing that these strains were capable of producing only one type of enterotoxin. The developed multiplex polymerase chain reaction assays will be useful for rapid detection of *S. aureus* and respective enterotoxins being produced from foods, clinical samples and environmental surveys. This may lead to early diagnosis of infection and help in timely prophylaxis.

Keywords: *Staphylococcus aureus*, PCR assay, enterotoxin, food poisoning, SEA, SEB, SEC, SED, SEE

Introduction
With the rapid increase in the out breaks of food poisoning Surveillance of food borne diseases is of an increasingly high priority in the public health agenda worldwide. Among all, Staphylococcus aureus is one of the most common clinical and food borne pathogen. It is reported that more than 70% of *S. aureus* strains produced one or more enterotoxins [7]. Staphylococcus aureus is a major human pathogen and causes a variety of food borne infections, nosocomial and community acquired infections [11]. Because of their thermal stability they remain a great hazard even in heat processed foods. Multiplex PCR were developed to detect *S. aureus* toxin for SEA, SEB, SEC, SED and SEE. All these toxins show emetic activity on humans. The 16S rRNA gene of Staphylococcus contain DNA sequence that are highly conserved at genus level but variable among other bacterial genera. The primer pair used in a study has been shown to be conserved in 19 Staphylococcus species and subspecies [2, 3] therefore combined detection of 16S rRNA and all the five Staphylococcal enterotoxin genes may be a reliable set of markers for the detection of Staphylococcus aureus. Primers designed for 16S rRNA gene for Staphylococcus will be a useful tool for identification and confirmation of Staphylococcus species.

Materials and Method
Sample collection and processing: A total of 75 food samples classified into following three groups were collected from different food stores in and around Hyderabad
1. Milk and Milk products- e g cheese, butter, milk sweets, raw milk, curd.
2. Meat and Meat products – e g non-veg burgers and soups, chicken, mutton.

3. Ready to eat food- e g cakes, pastries, sandwiches.

The criterion of inclusion was the selection of perishable food items with defined expiry dates. While their selection it was made a mandate that the food items should not have crossed the expiry dates. Cereals, oils, pickles, ketchups, dry fruits and other such food stuffs that can be stored for long were excluded. All these food samples were deliberately allowed to get contaminated by random bacteria by incubating them for 1-2 weeks at room temperature and were detected for Staphylococcus aureus phenotypically followed by bacteriological examinations.

Preparation of food Samples and Isolation of *Staphylococcus aureus*: 10gm of each collected food samples was aseptically weighed and macerated and 10ml of sterile distilled water was added to them to perform serial dilution. Master plates were made in Nutrient Agar media for all of the 75 samples from 10^{-10} dilution by pour plate or spread plate method.

Identification of *S aureus* by Gram's Staining and Biochemical characterization: From all of the master plates prepared a total of 100 isolated Colonies showing the characteristic morphology of smooth and circular yellow colonies were further sub cultured and Gram stained. 72 isolates were showing gram positive cocci in groups that were further biochemically characterized [9]. A total of 64 such isolates gave positive biochemical characterization as that of S aureus. The results are summarised as follows:

Table 1: Showing morphological and Biochemical characters of *S. aureus*

Culture medium	**Results observed**
Nutrient Agar medium	Yellow pigmented circular colonies with smooth surface
Gram Staining	Positive coccus
Biochemical Tests	**Results observed**
Catalase	Positive; showing brisk effervescence, bubbles of oxygen observed
Coagulase	Clot formation observed
DNase	Clear zone around the colony
MR	Development of stable red color
Mannitol fermentation (phenol red indicator)	Turns yellow due to acid production and bubble appeared in durhams tube due to gas production

Extraction of genomic DNA: 500ml of LB broth was prepared and all of the 64 isolates were inoculated separately in it and incubated at 37ºC for 18 – 24 hrs.
1.5ml of each culture was taken and centrifuged at 10000rpm for 10mts. The supernatant was discarded and 500ul of lysis buffer (50mM Tris HCl, 20mM EDTA, 1.5% SDS) was added to each of the 64 pellets. All of them were gently vortexed and incubated at 60 – 65ºC for 20mts with gentle vortex at every 10mts. They were cooled to room temperature and 50ul of 3M sodium acetate was added to each. They were kept in ice cold condition for 10mts to prevent exothermic reaction and centrifuged at 10000rpm for 10mts. To the supernatant equal volume of Isopropyl alcohol was added and kept in ice cold condition for ½ hr. They were again centrifuged at 12000rpm for 10mts. The collected pellets were air dried and 50ul of TE buffer was added to each of them.

Primer designing and assay development: Novel primers were designed for amplification of 16S rRNA gene of *S. aureus* as well as five different SEs by using Primer BLAST and Primer3 Tools. The two sets of primers for each of the gene were standardized and the optimum PCR conditions were designed for them. In multiplex PCR more than one DNA sequence are simultaneously amplified as if many separate PCR reactions are performing all together in one reaction. This process amplifies DNA in samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. All of the primer pairs have to be optimized so that all of them can work at the same annealing temperature during PCR.
The PCR assay is developed by performing different gradients for temperature, time and number of cycles for denaturation, annealing and extension steps of PCR.

Table 2: Showing output of Primer designing tools for 16S rRNA and Enterotoxins of *S. aureus*

Sr. no	**Gene**	**Tool**	**Sequence**	
1	16S rRNA	Primer BLAST	FWD	CACCTTCCGATACGGCTACC
			REV	CGTCAAATCCCATCATGCCC
		Primer 3	FWD	GAAAGCCACGGCTAACTACG
			REV	CATTTCACCGCTACACATGG
2	SEA	Primer BLAST	FWD	ACCAACGGTTCTTTTGAGGT
			REV	GCATCAGAACTGTTGTTCCGC
		Primer 3	FWD	TCAGAACTGTTGTTCCGCTAGA
			REV	AACCAACGGTTCTTTTGAGG
3	SEB	Primer BLAST	FWD	ACTCGCCTTATGAAACGGGA
			REV	CTGGTGCAGGCATCATGTCA
		Primer 3	FWD	TGTTCGGGTATTTGAAGATGG
			REV	GGTGCAGGCATCATGTCATA
4	SEC	Primer BLAST	FWD	AGACCCTACGCCAGATGAGT
			REV	GTCAAACTTATCGCCTGGTGC
		Primer 3	FWD	GAAACACAATTTCTTTTGAAGTGC
			REV	AAACTTATCGCCTGGTGCAG
5	SED	Primer BLAST	FWD	GTGTCACTCCACACGAAGGT
			REV	CGCGCTGTATTTTTCCTCCG
		Primer 3	FWD	GAGGTGTCACTCCACACGAA
			REV	TTTTCCGCGCTGTATTTTTC

6	SEE	Primer BLAST	FWD	GAGGAAAAATACAGCGCGGA
			REV	TGAAGGTGCTCTGTGGATAATGT
		Primer 3	FWD	GAAAAATACAGCGCGGAAAA
			REV	TCAATATGAAGGTGCTCTGTGG

PCR Amplification: Five groups of 64 gDNA samples each were made and each sample within a group was amplified with primer of one of the enterotoxin along with that of the 16S rRNA gene. The reaction mixtures consists of 2µl of extracted DNA template from the bacterial isolates, 5µl of 10X PCR Buffer [containing 75mM Tris HCl, with pH 9.0, 2mM $MgCl_2$, 50 mM KCl, 20mM $(NH4)_2SO_4$, 1µl dNTPs (40 M), 1 µl of 1U Ampli Taq DNA polymerase], 1µl (50pmol) each of forward and reverse primers [10]. The volume of the reaction mixture was made up to 50µl using double distilled water. For all the novel primer designed the temperature, time and number of cycles for PCR were standardized to develop the assays. The PCR products were tested for positive amplification on 1.2% Agarose gel electrophoresis using 1Kb molecular weight marker and checked under Transilluminator of the Gel Documentation System.

Results and Discussion

A total of 12 pairs of Primers were obtained from the two tools used for Primer designing. Considering the maximum product length and minimum GC content following set of primers were short listed for the target six genes:

Table 3: Showing details of short listed primers designed for 16S rRNA and Enterotoxins of *S. aureus*

Sr no	Gene	Tool	Sequence	Melting point	GC%	Product Length
1	16S rRNA	Primer BLAST	CACCTTCCGATACGGCTACC	59.97	60	1248
			CGTCAAATCCCATCATGCCC	59.33	55	
2	SEA	Primer 3	TCAGAACTGTTGTTCCGCTAGA	60.05	45.45	245
			AACCAACGGTTCTTTTGAGG	59.07	45	
3	SEB	Primer 3	TGTTCGGGTATTTGAAGATGG	59.81	42.86	247
			GGTGCAGGCATCATGTCATA	60.51	50	
4	SEC	Primer BLAST	AGACCCTACGCCAGATGAGT	60.03	55	156
			GTCAAACTTATCGCCTGGTGC	59.87	52.38	
5	SED	Primer 3	GAGGTGTCACTCCACACGAA	59.71	55	324
			TTTTCCGCGCTGTATTTTTC	60.21	40	
6	SEE	Primer 3	GAAAAATACAGCGCGGAAAA	60.21	40	276
			TCAATATGAAGGTGCTCTGTGG	60.13	45.45	

Assay was developed for all the six sets of amplifications as follows:

Table 4: PCR Cycle conditions for 16S rRNA and enterotoxins Amplification

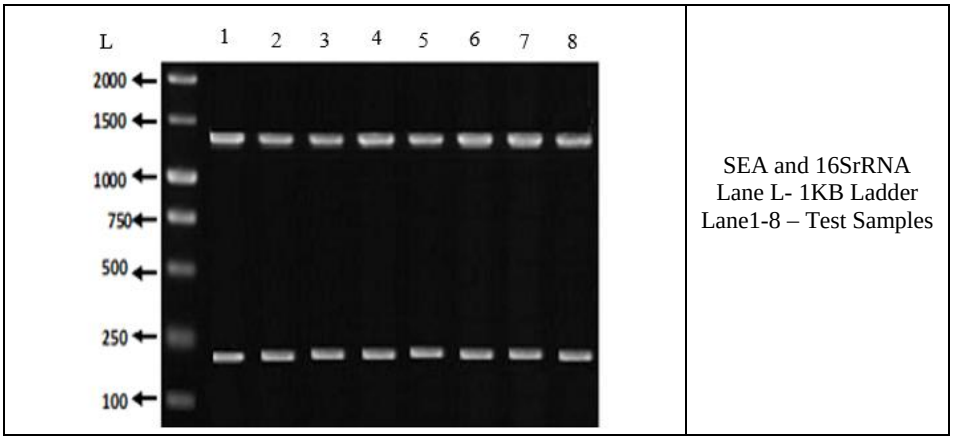
Step	Temperature	Time	No. of Cycle
Initial denaturation	94 ºC	3 min	1
Denaturation	94 ºC	30 sec	27
Annealing	56 ºC	45 sec	
Extension	72 ºC	90 sec	
Final Extension	72 ºC	5 min	1
Hold	4 ºC	Indefinitely	1

Out of all the 64 isolates in each of the five groups 61 samples gave positive amplification results with 16S rRNA primer in each group revealing that only 61 isolates were *S. aureus*, remaining three might be any other species of *Staphylococcus.* Out of these 61, 59 got successfully amplified with any one of the enterotoxins also summarized as follows:

Table 5: Number of isolates producing specific enterotoxin

Group No.	Primers Used	No. of positive isolates
1	16S rRNA + SEA	15
2	16S rRNA + SEB	21
3	16S rRNA + SEC	6
4	16S rRNA + SED	12
5	16S rRNA + SEE	5
Total		59

An important point to be noted here was none of the isolate were found to be positive for more than one enterotoxins employing that all of the S aureus strains obtained in the work were highly specific for Type of enterotoxins being produced while two of them did not show any amplicons for any of the enterotoxin.



SEA and 16SrRNA
Lane L- 1KB Ladder
Lane1-8 – Test Samples

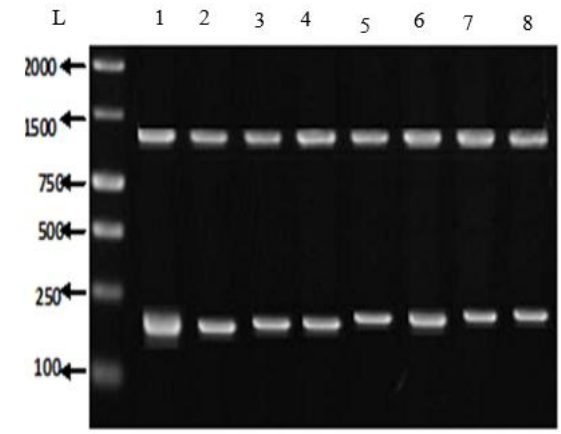
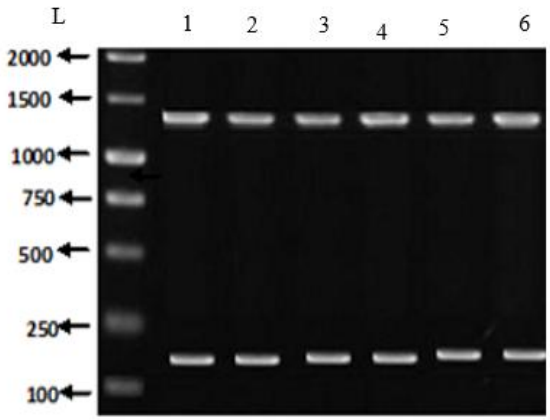
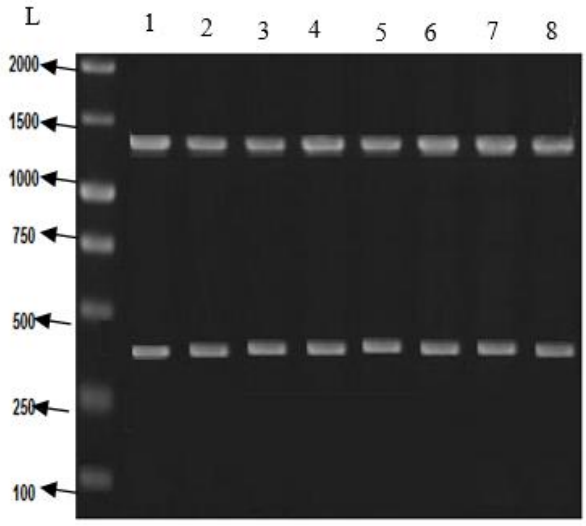
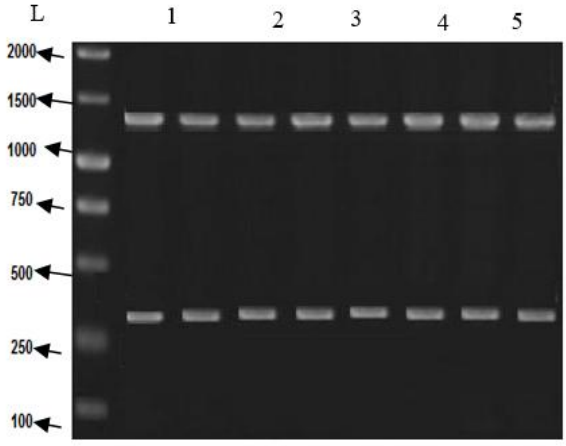
	SEB and 16SrRNA Lane L- 1KB Ladder Lane1-8 – Test Samples
	SEC and 16SrRNA Lane L- 1KB Ladder Lane1-6 – Test Samples
	SED and 16SrRNA Lane L- 1KB Ladder Lane1-8 – Test Samples
	SEE and 16SrRNA Lane L- 1KB Ladder Lane1-5 – Test Samples

Fig 1: Multiplex PCR amplicons showing Toxigenic strains of Staphylococcus aureus for different Enterotoxins and 16SrRNA

Conclusion

Food borne infection is currently widespread throughout the world and has evoked a prompt interest and concern for the rapid detection of toxigenic strains of *Staphylococcus aureus*. The most impressive advantages of PCR based detection method in comparison to the standard microbiological detection method are its speed, sensitivity and specificity and accuracy of the obtained results. Molecular based methods have well-grounded potential to overcome insufficiencies of identification procedures associated with the results based on biochemical characteristics. The purpose of our study was characterization of recovered strains phenotypically by conventional methods and genotypically by PCR for direct detection of *S. aureus* 16SrRNA gene and staphylococcus enterotoxin genes for SEA, SEB, SEC, SED and SEE. Out of the 59 isolates of *Staphylococcus aureus* obtained from various food samples, found positive for enterotoxin infection, 15, 21, 6, 12 and 5 were found to be producing SEA, SEB, SEC, SED and SEE respectively further employing that these strains were capable of producing only one type of enterotoxin. Developed PCR techniques on further investigations focus on the application to large scale of clinical samples so that this technique can be used in detecting toxigenic strain of *Staphylococcus aureus* for public safety concern in foods. Hence the developed mPCR method will help in faster diagnosis of *Staphylococcus aureus* food poisoning.

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