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Amplification of 16sr RNA for *Staphylococcus Aureus* Isolates From Food Samples

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Abstract:

Staphylococcus aureus, a gram positive bacterium, is one of the most common clinical and food borne pathogens worldwide. *S .aureus* produces very important virulence factors including Staphylococcus enterotoxins (SEs) which are the main causes of diarrhea, vomiting and other symptoms associated with *S.aureus* infections. The conventional methods used for identification of *S.aureus* are time consuming and the reliability of these methods have been reported to be between 80-90%. Currently the molecular techniques based on PCR amplification of 16S rRNA of *S.aureus* for rapid and specific detection is widely used approach. In this study *S.aureus* strains are obtained from various food samples which are further confirmed by molecular characterization using 16S rRNA primer. The amplification of 1267 base pair fragments specific for 16SrRNA of *S.aureus* revealed positive amplification with all the isolates previously identified as *S.aureus* with bacteriological examination and with Staphylococcus enterotoxins genes (SEA, SEB, SEC, SED, and SEE). Two sets of primer pairs were used, the first was SauF 234 and the other was SauR 1501. The objective of the study was to examine the recovered strains phenotypically by conventional methods and genotypically by PCR for direct detection of *S.aureus* 16SrRNA gene which serves as internal control. This became the confirmation for the developed multiplex PCR for the identification of *S.aureus* isolated from various types of food samples. The developed multiplex polymerase chain reaction (PCR) method will be useful for the detection and identification of *S.aureus* from foods, clinical samples and environmental surveys.

Key words- *Staphylococcus aureus* , Enterotoxin, food poisoning, SEA,SEB,SEC,SED,SEE.

Introduction

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 [1]. 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 this method 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:

1. Bacterial Strains and Culture Medium

A total of 96 strains of *S.aureus* were used in this study. All the strains are derived from the food samples. All the Staphylococcus strains are previously identified phenotypically and by bacteriological examinations.

Preparation of food Samples: From each collected food samples, 25g were aseptically weighed and macerated and added with 225ml of sterile distilled water. Serial dilutions were performed till 10^{-10} and from each dilution 1ml were plated by pour plate or spread plate method [4].

Isolation of bacteria: Colonies obtained from the food samples are cultivated on specific medium for isolating *Staphylococcus aureus*. Specific medium for isolating *Staphylococcus aureus* were Mannitol Salt agar, Staphylococcus medium and Baird parker agar [5, 6]

Identification of *Staphylococcus aureus* by Biochemical Tests: Staining tests includes Grams and capsular Staining. Biochemical tests for confirming *Staphylococcus aureus* includes Coagulase Test, Catalase Test, Anaerobic Utilization

of Glucose, Mannitol and Production of Thermostable Nuclease [7, 8]

2. Extraction of DNA:

1.5 ml of overnight grown culture in LB broth was taken and centrifuged at 12000 rpm for 6 min. The pellet was collected and CTAB solution were added which was pre-warmed at 60°C. The solution was incubated for 1 hr at 60°C and after the incubation 0.8ml of Chloroform: Iso amyl alcohol (24:1) was added to the solution. Eppendorf is inverted to and fro gently for proper mixing and centrifuged it at 6000rpm for 10 min at 4°C. Aqueous phase were transferred to a separate clean eppendorf [9]. To this 0.6ml of ice cold Isopropanol was added and mixed gently. The precipitate was left undisturbed for 1 hr at room temperature or at -20°C overnight. The samples later were centrifuged at 6000 rpm for 15 min at 4°C. The supernatant were removed and 200µl of 10mM CH_3COONa and 76% ethanol were added to the pellet. The samples centrifuged at 12000 rpm for 15 min at 4°C.The pellets were collected and 500µl of 70% ice cold Ethanol added and centrifuged again at 12000rpm for 15min at 4°C. The DNA samples were stored at -20°C and then run on Agarose gel electrophoresis to confirm the DNA Bands [10, 11].

3. Primer Designing:

For amplification of 16SrRNA specific for *S.aureus*, two sets of primers were used (Table 1). These primers amplify 1267 bp fragments to specify 16SrRNA of Staphylococcus aureus strains [12, 13].

4. PCR Amplification:

The reaction mixtures consists of 5µl of exacted DNA template from the bacterial isolates, 5µl 10X PCR Buffer containing 75mM Tris HCl, with pH 9.0 ,2mM $MgCl_2$,50 mM KCl, 20mM $(NH4)_2SO_4$, 1µl dNTPs (40µM), 1µl (1U Ampli Taq DNA polymerase), 1µl (50pmol) from forward and reverse primers [14]. Each primer pair was used separately and the volume of the reaction mixture was completed to 50µl using DDW. Thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 70°C for SauF234 and SauR 1501 primers and extension at 72°C for 1 min. final extension carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected [15, 16].

5. Agarose Gel Electrophoresis:

The PCR products were tested for positive amplification by Agarose gel electrophoresis using 1.5 Kb molecular weight markers. The DNA extracts were loaded 2% W/V gel for electrophoreses and checked the bands using ladder under Transilluminator [17].

Results and Discussion:

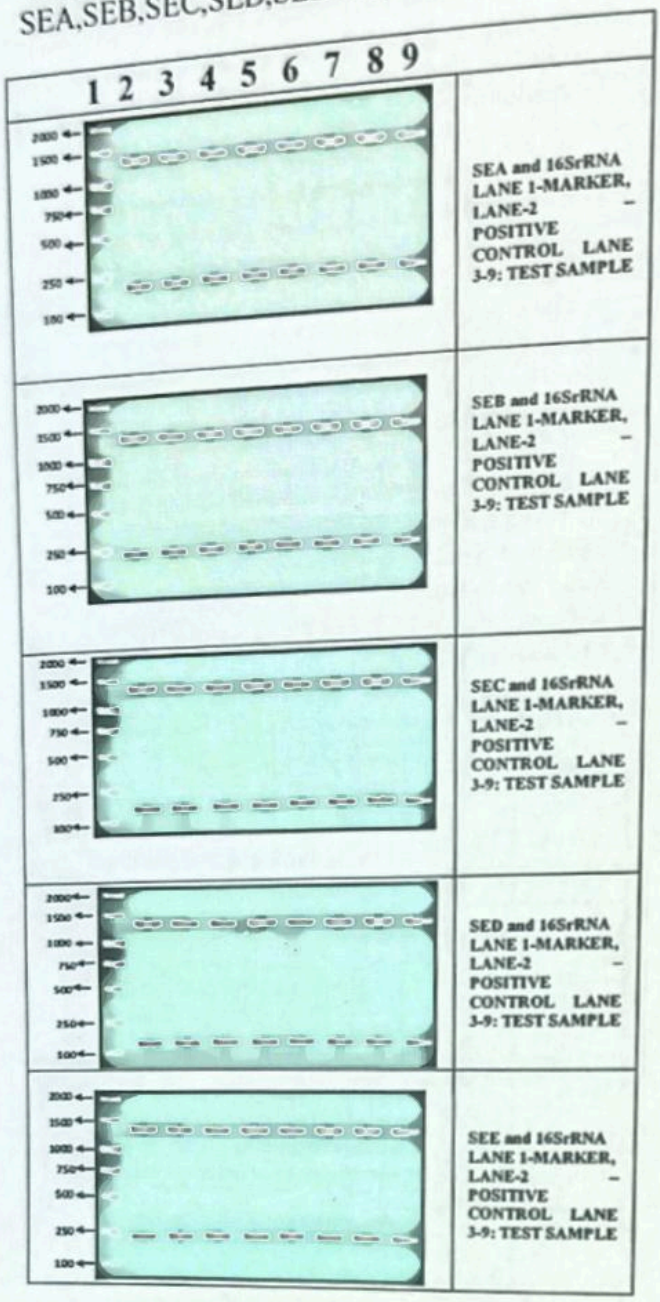
The amplification of 1267 base pair fragments specific for 16SrRNA of *Staphylococcus aureus* using SauF 234 and SauR 1501 primers revealed positive amplification of 1267 base fragments with all 85 isolates out of 96 isolates (88%) previously identified phenotypically as S.aureus with bacteriological examination.

Hence multiplex PCR combined with 16SrRNA and staphylococcus enterotoxins for SEA, SEB, SEC, SED and SEE were developed for rapid detection of *S.aureus* from food samples. The results of this study indicates that mPCR can be used for rapid detection of *S.aureus* from food samples.

TABLE-1: Primer sequences and expected size of PCR amplified gene targets of *Staphylococcus aureus*

Gene	Primer	Oligonucletide sequence (5' – 3')	Size of amplified product (bp)
SEA	SEA-F	TTGCAGGGAACAGCTTTAGGT	247
	SEA-R	CCACCCGCACATTGATAA	
SEB	SEB-F	CGCATCAAACTGACAAACGAC	243
	SEB-R	CGTTTCATAAGGCGAGTTG	
SEC	SEC-F	TCCGTTGGCTTTTCACTTT	163
	SEC-R	TTTAAATCGGGTGGTGCAAT	
SED	SED-F	CTAGTTTGGTAATATCTCCT	317
	SED-R	TAATGCTATATCTTATAGGG	
SEE	SEE-F	TAGATAAAGTTAAAACAAGC	270
	SEE-R	TAACTTACCGTGGACCCTTC	
16SrRNA	SauF 234	CGATTCCCTTAGTAGCGGCG	1267
	SauR 1501	CCAATCGCACGCTTCGCC	

Figure-1 Multiplex PCR for identification of Toxigenic strains of Staphylococcus aureus for SEA,SEB,SEC,SED,SEE and 16SrRNA:



Conclusion:

Staphylococcal infection is currently widespread throughout the world and has prompt interest and concern for the rapid detection of toxigenic strains of *Staphylococcus aureus*. *Staphylococcus aureus* is a major human pathogen and causes a variety of food borne infections, nosocomial and community acquired infections [18, 19, 20]. Molecular based methods have well grounded potential to overcome insufficiencies of identification procedures associated with the results based on biochemical characteristics. 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 a public safety concern in foods. The strains of *S.aureus* developing five toxins are further identified by SDS-PAGE and confirmed by immune blotting technique [21, 22]. Hence the developed mPCR method will help in faster diagnosis of *Staphylococcus aureus* food poisoning. The purpose ofthis study was firstly, characterization of recovered strains phenotypically by conventional methods and genotypically by PCR for direct detection of S.aureus 16SrRNA gene and staphylococcus enterotoxin genes foe SEA, SEB, SEC, SED and SEE. The most impressive advantages of PCR based detection method in comparison to the standard microbiological detection method are admittedly considered speed, sensitivity and exactness of the obtained results.

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