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Development of Multiplex PCR for Rapid Detection of Toxigenic Strains of *Staphylococcus aureus* from Food Samples

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Abstract:

This study developed a multiplex PCR method for the screening and detection of five toxigenic strains of *Staphylococcus aureus*. The multiplex PCR procedure, which uses five pairs of primers produced specific amplicon of expected sized of SEA, SEB, SEC, SED and SEE genes which are responsible for producing Enterotoxins. These are the target genes for m-PCR detection. Overall results for the present study indicates that m-PCR is a potential technique for the rapid detection of all the five toxin producing strains of *Staphylococcus aureus*, which is one of the pathogenic food borne bacteria for routine monitoring and risk assessment of foods.

Keywords: *Staphylococcus aureus, Food Borne pathogen. Multiplex PCR*

Introduction:

Staphylococcus aureus is a bacterial pathogen considered a principle etiological agent of food poisoning. They are the second most common pathogen associated with outbreaks of food poisoning [1].*Staphylococcus aureus,* a Gram positive bacterium is one of the most common clinical and food borne pathogens worldwide. *Staphylococcus aureus* produces many important virulence factors including Staphylococcus enterotoxins SEs which are the main causes of diarrhea, vomiting and other symptoms associated with *Staphylococcus aureus* infections [2]. There are several distinct serological enterotoxins(SE) such as SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SES, SET. Out of all the *Staphylococcus aureus* enterotoxin SEA, SEB, SEC, SED, SEE showed emetic activity [3].The biological effects of all these enterotoxins include pyrogenicity, enhancement of lethal endotoxin shock and inflammatory cytotoxins. *Staphylococcus aureus* causes enterocolitis [4]

Microbial and immulogical techniques, presently in use are time consuming and laborious. In addition the toxin escape during sterile filtration are heat resistant and therefore this necessitates the development of more sensitive technique for identifying the toxigenic *Staphylococcus aureus*. Therefore early identification by this method will help in minimizing the infections there by preventing diarrheal diseases. This technique will also be useful in the food testing laboratories and medical diagnostic laboratories by health centers and at field levels.

Materials and Methods:

Isolation and Identification of *Staphylococcus aureus* from various food samples:

Food samples such as Ready to eat samples, Diary and dairy products, Fermented foods, bakery products were collected. They were selected according to their availability and popularity of consumption. The samples were taken in the sterile plastic bags in icebox. They were refrigerated rapidly and transferred to the test laboratory [5]

Preparation of food: 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.

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.

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 [6]

Extraction of DNA:

1.5 ml of overnight grown culture in LB broth were 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: Isoamyl alcohol (24:1) was added to the solution. Invert the eppendorf to and fro gently for proper mixing and centrifuged it for 6000rpm for 10 min at 4°C. Aqueous phase were transferred to a separate clean eppendorf. To this 0.6ml of ice cold Isopropanol were added and mixed gently. The precipitate was left undisturbed for 1 hr overnight at room temperature or at -20°C. 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.

Agarose gel electrophoresis:

The DNA extracts were loaded 2% W/V gel for electrophoreses and checked the bands under Transilluminator. Likewise the plasmid extracts are also run on Agarose gel electrolphoreses and confined its presence by visualizing the bands.

Primer designing:

The Primes are selected specifically from Primer 3 and synthesized by Chromous Biotech PVT LTD. All the 5 sets of primes for SEA, SEB, SEC, SED, SEE used in this study with their correct gene targets and size of expected amplification were studied. The DNA and Plasmid aliquots were used as the template for PCR amplification using the synthesized primes. (Table-2)

Muliplex PCR amplification:

Muliplex PCR amplification carried out in total of 50µl reaction mixture, which contained 5µl of 1 x buffer, 5 µl of 20mM $Mgcl_2$, 4 µl of 10 mM dNTPS, 0.5 µl of 10 µM of each specific primes and 0.1 µl of 2U Taq polymerese, 5µl of template with 29.9 µl of MQ water [7]. Samples are cycled 40 times in the denaturation cycle at 90° c for 30 sec with annealing at 55° for for 30 sec and extension at 73°C for 90 sec with final extension for 5 min at 72° C. The amplified products are analysed by Agarose gel electrophoreses.

The DNA bands were irradiating the pre stained gel under UV illuminator are photographed. This study was monitored by the use of Controls.(Table-1) The quality control positive (QCP) were used sufficient amount standard specific strains of S.aureus for each toxins are selected to give positive result across all primes sets. PCR reaction mix were loaded and separated on 2 % W/v agarose gel electrophoreses , along with 1.5 kb ladder as a molecular weight standard [8]

Confirmation of PCR products by artifical contamination of Food Sample:

Food samples were collected and artificially contaminated with *Staphylococcus aureus* strains specific for each type of toxins.The food samples were previously confirmed to be derived of *Staphylococcus aureus* 2-3 colonies were picked up, inoculated in 1 gm of food samples artificially contaminated with *Staphylococcus aureus* at levels of $4x10^1$ and $4x10^0$ CFU respectively. All the samples were detected by multiplex PCR method. TABLE-3 showed specific bands for SEA, SEB, SEC, SED, SED and SEE respectively from various food sample such as ready to eat foods, fermented foods, milk and milk products and concerned foods. The efficacy of this method is further evaluated with 25 food sampleseach and incubated at 37° C for 1 week.

Following procedures were done to confirm the presence of toxin specific *Staphylococcus aureus* by cultural characteristics, by biochemical reaction and also multiplex PCR amplification using synthesized primers and analyzed for the DNA bands for respectively. All the samples were detected by specific molecular weight for each toxin.

Results and discussions:

1. **Isolation of pathogenic strains of *Staphylococcus aureus* from various food samples.**

From all different varieties of food samples S.auress were isolated and identified. S.aureus were isolated in Nutrient Agar, Mannitol salt agar, Staphylococcus medium and and Baired parkes medium. The calories should positive reaction for all biochemica reactions specific to *Staphylococcus aureus*., such a Coagulase, nuclease test Catalase test, Anaerobi

lization of Glucose and Mannitol and identified to *Staphylococcus aureus*.

DNA and Plasmid Extraction:

NA and Plasmid extracts were isolated from the *aphylococcus aureus* and all the aliquots isolated m all the strains of *Staphylococcus aureus*, lated from different food samples were confirmed its presence by loading in 1 % - 0.8% w/v arose gel and confirmed by the DNA bands cked under UV illuminator.

Multiple PCR amplification:

e DNA and Plasmid aliquots were used as plate for multiplex PCR amplification. All the nds for all toxins from all the strains of *phylococcus aureus* were observed with specific lecular weight were confirmed. Each toxins are nfirmed based on it specific molecular weight ich are compared using 1.5 kb ladder. The DNA nds were irradiating the pre stained gel under UV minator are photographed. This study was nitored by the use of Controls (Table-1). The quality control positive (QCP) were used sufficient amount standard specific strains of *Staphylococcus aureus* for each toxins are selected to give positive result across all primes sets. PCR reaction mix were loaded and separated on 2 % w/v agarose gel electrophoresis, along with 1.5 kb ladder as a molecular weight standard.

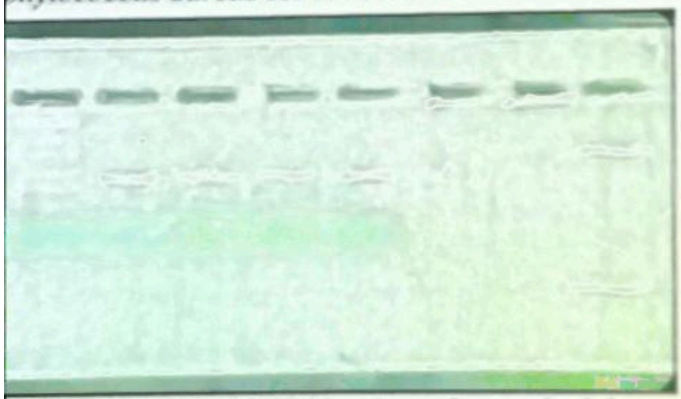
4. Evaluation of developed mulitplex PCR Technique in artificially contaminated food samples:

The analysis indicated that all the primer pairs were confirmed for their corresponding toxin producing genes of *Staphylococcus aureus* and showed specific bands for SEA, SEB, SEC, SED, and SEE respectively from various food sample such as ready to eat foods, fermented foods, milk and milk products and concerned foods. The efficacy of this method is further evaluated with 25 food samples artificially contaminated with *Staphylococcus aureus* at levels of $4x10^1$ and $4x10^0$ CFU respectively. All the samples were detected by multiplex PCR method. TABLE-3.

TABLE-1: Standard Strains

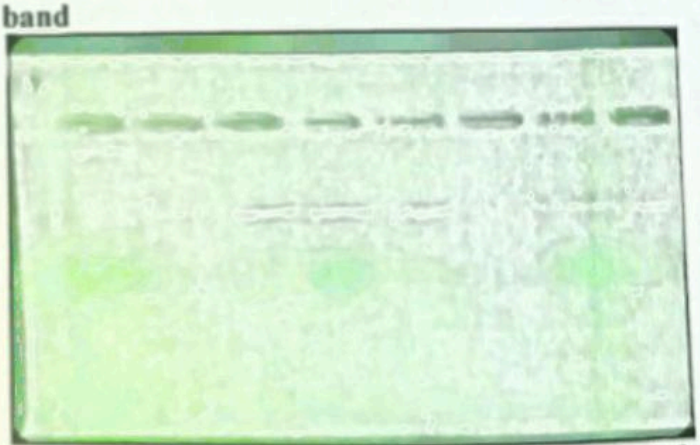
GENES	STANDARDS
SEA	*Staphylococcus aureus* ATCC 13565
SEB	*Staphylococcus aureus* ATCC 14458
SEC	*Staphylococcus aureus* ATCC 19095
SED	*Staphylococcus aureus* FRI 361
SEE	*Staphylococcus aureus* ATCC 27664

1: Multiplex Pcr Developed For Detecting *phylococcus aureus* for All Five Toxins



e 1: showing 1.5 kb ladder. Lane 2: standard for gene. Lane 3-5 showing positive for sea from samples. Lane 6-8 showing negative result.

Fig 2: Multiplex PCR for SEB showing 243 bp band



Lane1: showing 1.5 kb ladder. Lane2: standard for seb gene. Lane 3,4,5,7,8 showing positive for seb from food samples. Lane 6 showing negative result.

		TACCACCCGCACATTGATAA	
Seb	SEB-1 SEB-2	CGCATCAAACTGACAAACGA CCGTTTCATAAGGCGAGTTG	243
Sec	SEC-1 SEC-2	TCCGTTGGCTTTTCACTTTT GTTAAATCGGGTGGTGCAAT	163
Sed	SED-1 SED-2	CTAGTTTGGTAATATCTCCT TAATGCTATATCTTATAGGG	317
See	SEE-1 SEE-2	TAGATAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	270

Table-3: multiplex pcr results from food samples for five strains of Staphylococcus aureus

Products	Total samples collected	Food samples Positive for *S.aureus*	DNA Isolation	Gene Amplified				
				SEA	SEB	SEC	SED	SED
DIARY AND DIARY PRODUCTS	20	17	17	6	6	-	3	2
MEAT AND MEAT PRODUCTS	30	19	19	3	8	4	3	1
READY TO EAT FOODS	50	44	44	30	5	2	3	4
TOTAL	100	80	80	39	19	6	9	7

Fig 3: Multiplex PCR for SEC and SED showing 163 bp Band and 317 bp Band

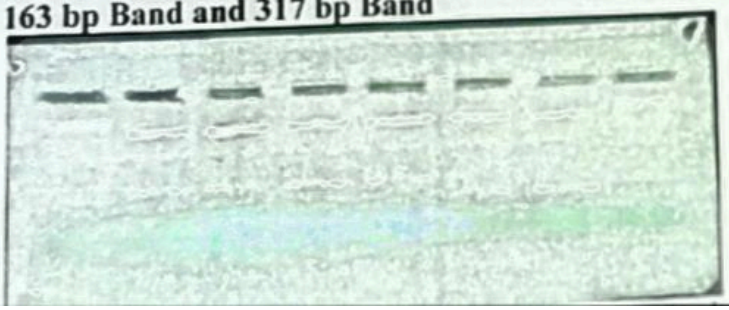
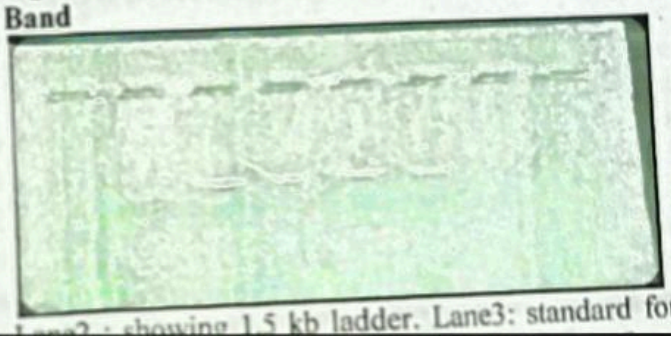


Fig 4:Multiplex PCR for SEE showing 270 bp Band



Lane2 : showing 1.5 kb ladder. Lane3: standard for

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 nosocomial and community acquired infections. 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.

Aknowledgement:

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