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EVALUATION OF DEVELOPED MULTIPLEX PCR TECHNIQUE OF STAPHYLOCOCCUS AUREUS FOR SPECIFICITY AND ITS EFFICIENCY ON FOOD SAMPLES

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ABSTRACT

Staphylococcus aureus is one of the most significant pathogens causing community acquired infections. Among the secreted Staphylococcus virulence factors, there is growing list of enterotoxins which can induce gastroenteric syndrome and toxic shock syndrome. There are up to 15 staphylococcal enterotoxins known but not all play a role in food poisoning. This study focuses on only five enterotoxins that are SEA, SEB, SEC, SED and SEE. Staphylococcal food borne diseases resulting from consumption of food contaminated with Staphylococcal enterotoxins (SEs) produced by certain strains of *Staphylococcus aureus.* Analytical methods are essential for routine monitoring purposes and safeguard public health. This new technique will reduce the time involved in identification and subsequently evaluation of their toxicity. Therefore early identification by this method will help in minimizing the infections, thereby preventing diarrheal diseases. The evaluation for specificity of mPCR method for *Staphylococcus aureus* was done by SDS PAGE and confirmed by western blotting with specific antibodies against SEA, SEB, SEC, SED and SEE Staphylococcal enterotoxins. The efficiency of mPCR method was tested on live food samples and identified based on the band length and markers. This technique will be useful in quality control of food products produced by food and dairy industries, food testing laboratories and medical diagnostic laboratories especially at health centers and at field level.

Key words- *Staphylococcus aureus*, Food poisoning, Gastroenteric syndrome, Staphylococcal enterotoxins, Toxic shock syndrome

INTRODUCTION

Food contamination is a major health problem especially in developing countries like India. Microorganisms are associated in a variety of ways, with all of the food that we eat [1].Among all the food poisoning organisms the *Staphylococcus aureus* remains one of the most common type of food poisoning in both developed and developing countries [2]. *Staphylococcus aureus* is ubiquitous bacterium being both a human and zoonotic commensal. It is found in dairy products, vegetables, raw and fermented foods. *Staphylococcus aureus* is found to be a potentially hazardous in foods because of its production of heat stable Enterotoxins [3]. These toxins may be present in processed foods. This bacterium is also highly salt tolerant, resistant to nitrites and capable of growing with a low water activity.

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Therefore to minimize infection in food and water it is essential to identify the responsible etiological agents and also the presence of harmful toxins produced by them. Early identification will help in minimizing these infections thereby help in preventing diarrheal diseases.

Toxin producing strains of *Staphylococcus aureus* are the most commonly encountered food pathogens causing various episodes of food poisoning. Clinical isolates of *Staphylococcus aureus* can produce a spectrum of extracellular protein, toxins and virulence factors which contribute to the pathogenecity of the organism. The staphylococcal enterotoxins are recognized agents of intoxication, staphylococcal food poisoning syndrome and are also involved in other type of infections with sequelae of shock in humans and animals [4, 5]. Serologically five toxin groups have been recognized and designed as Staphylococcal enterotoxin (SEs) SEA, SEB, SEC, SED and SEE. SEA is the most often responsible for food poisoning [6, 7 and 8]. As little as 1 mcg of toxin can cause illness [9]. Staphylococcal enterotoxins (SEs) are antigenic and various immunological methods have been described for their detection [10, 11 and 12]. However there are difficulties in preparing high yields of specific antibodies by conventional immunological procedures and the supply of good antisera is limited. These polyclonal antibodies also give rise to cross reactions. Production of monoclonal antibodies to all the five toxins is not only expensive but also time consuming and requires the use of large number of animals. Moreover commercially available imported kits are very costly. Microbiological methods presently employed are laborious, tedious, time consuming. The requirement for time consuming enrichment steps and biochemical characterization has lengthened most analysis time to several days. Conventional methods for the detection of Staphylococcal enterotoxins in culture media are immunodiffusion, agglutination and ELISA. Often these methods do not allow the detection of very low concentrations of toxins and potential toxin producing strains may not be detected. Furthermore, the production of Staphylococcal enterotoxins (SEs) is influenced by the culture conditions (medium, pH, temperature) that might not represent conditions present in vivo. Hence direct detection of genes for SEs is a clear indication of the toxin producing capacity of a strain. As a result conventional methods are inadequate for making fast and timely assessments on the microbiological safety of foods [13]. Therefore the development of newer techniques is essential for rapid and accurate identification of the etiological agents either in food or stool sample.

Recently much emphasis is focused on molecular biology techniques like PCR and DNA methods for rapid and accurate identification of the etiological agents either in food or a stool sample.

MATERIALS AND METHOD

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 [17].

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 [17].

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 [17, 22].

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 [20].

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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 were collected and CTAB solution were added which was pre-warmed at 60^0C. The solution were incubated for 1hr. at 60^0C and after the incubation 0.8ml of Chloroform: Isoamyl alcohol (24:1) were added to the solution. Invert the eppendorf to and fro gently for proper mixing and centrifuged it for 6000rpm for 10 min at 4^0C. Aqueous phase were transferred to a separate clean eppendorf. To this 0.6ml of ice cold Isopropanol were added and mixed gently. The precipitate were left undisturbed for 1 hr – overnight at room temperature or at -20^0C. The samples later were centrifuged at 6000 rpm for 15 min at 4^0C. 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 4^0C.The pellets were collected and 500μl of 70% ice cold Ethanol added and centrifuged again at 12000rpm for 15min at 4^0C. The DNA samples were stored at -20^0C and then run on Agarose gel electrophoresis to confirm the DNA Bands [14].

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 electrolphoresis and confined its presence by visualizing the bands [14].

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.

Multiplex PCR Amplification
Muliplex PCR amplification carried out in total of 50μl reaction mixture, which contained 5μl of 1X buffer, 5μl of 20mM $Mgcl_2$, 4μl of 10mM dNTPS, 0.5μl of 10μM of each specific primes and 0.1μl of 2U Taq polymerese, 5μl of template with 29.9 μl of MQ water [3]. Samples are cycled 40 times in the denaturation cycle at 90^0 c for 30 sec with annealing at 55° for for 30 sec and extension at 73^0C for 90 sec with final extension for 5 min at 72^0 C. The amplified products are analyzed by Agarose gel electrophoreses [2].

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 [19].

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

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 1gm of food samples each and incubated at 37^0 C for 1 week [16]. 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. All the samples were detected by specific molecular weight for each toxin [19, 22].

SDS-PAGE and Immunoblotting
Aliquots of protein extracts was added with an equal volume of sample buffer heated at 95^0C for 3min and loaded on to 12.5% SDS-PAGE homogenous gel. Protein molecular weight marker

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was also loaded on the gel. The gels were run at 150V for 3hr. in electrophoresis system then electroblotting with semidry transfer unit to a nitrocellulose membrane at 90mA for 3 hrs. The membrane was blocked with blocking solution overnight and incubated with polyclonal rabbit antisera of anti-sea, anti-SEB, anti-SEC, anti-SED and anti-SEE raised against SEA, SEB, SEC, SED and SEE.

RESULTS AND DISCUSSIONS

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 biochemical reactions specific to *Staphylococcus aureus*., such as Coagulase, nuclease test Catalase test, Anaerobic utilization of Glucose and Mannitol and identified to be *Staphylococcus aureus*.

DNA and Plasmid Extraction

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

Multiplex PCR Amplification

The DNA and Plasmid aliquots were used as template for multiplex PCR amplification. All the bands for all toxins from all the strains of *Staphylococcus aureus* were observed with specific molecular weight were confirmed. Each toxins are confirmed based on it specific molecular weight which are compared using 1.5 kb ladder. 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.

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 4x100 and 4x100 CFU respectively. All the samples were detected by multiplex PCR method (Table-2). 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 4^0C.The pellets were collected and 500μl of 70% ice cold Ethanol added and centrifuged again at 12000rpm for 15min at 4^0C. The DNA samples were stored at -20^0C and then run on Agarose gel electrophoresis to confirm the DNA Bands [1].

Table-2: 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	SEE
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

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 [14].

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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-3).

Table-3: 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-1 SEA-2	TTGCAGGGAACAGCTTTAGG TACCACCCGCACATTGATAA	247
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
16SrRNA	16SrRNA 16SrRNA	CGATTCCCTTAGTAGCGGCG CCAATCGCACGCTTCGCC	1267

Multiplex PCR method for identification of toxigenic strains of *Staphylococcus aureus* for SEA,SEB,SEC,SED,SEE and 16SrRNA.

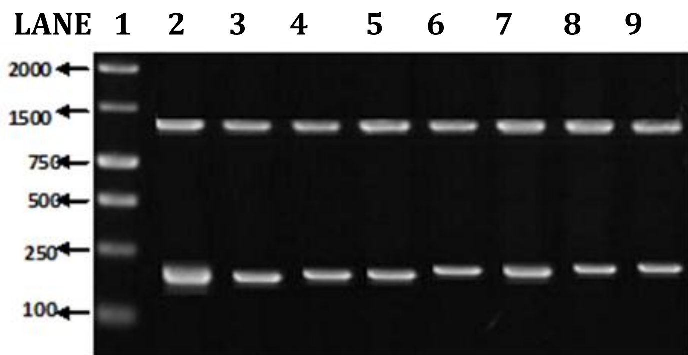

Fig:1 SEB and 16SrRNA (LANE 1-MARKER, LANE-2 –POSITIVE CONTROL LANE 3-9: TEST SAMPLE)

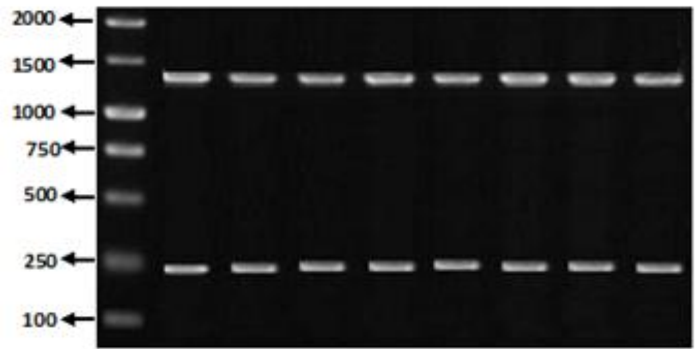

Fig.: 2 SEE and 16SrRNA (LANE 1-MARKER, LANE-2 –POSITIVE CONTROL LANE 3-9: TEST SAMPLE)

Fig.:3 SEA and 16SrRNA (LANE 1-MARKER, LANE-2 –POSITIVE CONTROL LANE 3-9: TEST SAMPLE)

Fig.: 4 SEC and 16SrRNA (LANE 1-MARKER, LANE-2 –POSITIVE CONTROL LANE 3-9: TEST SAMPLE)

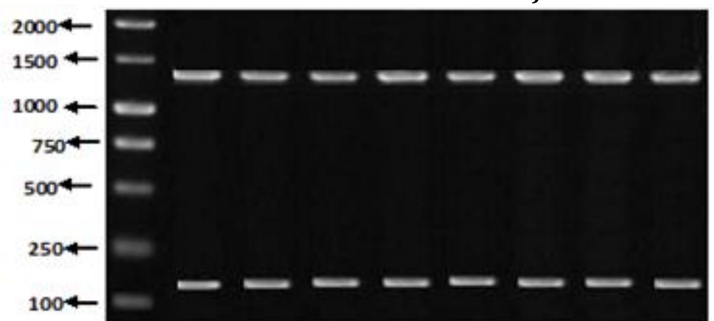

Fig.: 5 SED and 16SrRNA (LANE 1-MARKER, LANE-2 –POSITIVE CONTROL LANE 3-9: TEST SAMPLE)

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SDS-PAGE and Immunoblotting

The culture filtrates from enterotoxin S.aureus strains isolated from food samples and detected for SEA, SEB, SEC, SED, SEE by SDS-PAGE (fig. 6) and were confirmed by immunoblotting analysis (fig. 7).

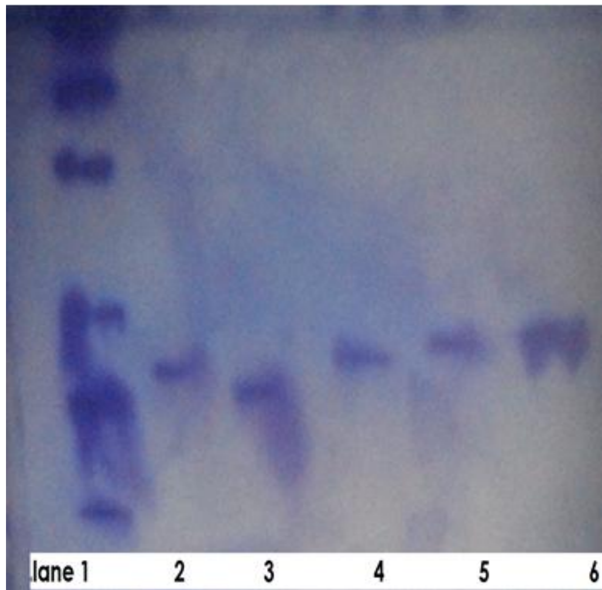

Fig.:6 Electrophoretic separation of Five Toxins SEA, SEB, SEC, SED and SEE Along With Protein Markers. (Lane 1- Protein Markers, Lane2-SEA, Lane3-SEB, Lane 4-SEC, Lane5-SED, Lane 6-SEE)

The immunoblotting analysis of culture filtrates showed specific bands of SEA, SEB, SEC, SED and SEE as expected. The bands of positive control and test gave same results.

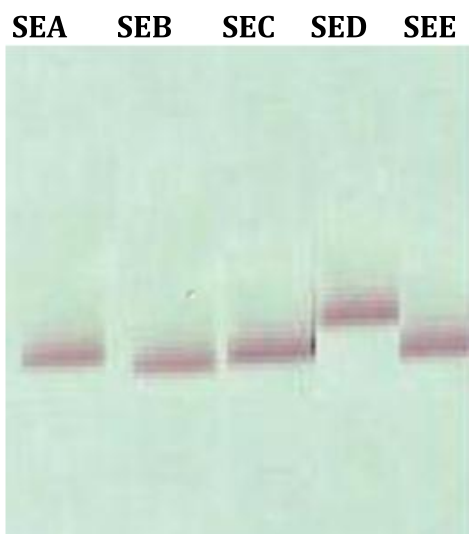

Fig. 7 Immunoblotting result for SEA, SEB, SEC, SED and SEE

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. The strains of S. aureus developing five toxins are further identified by SDS-PAGE and confirmed by immunoblotting technique. Hence the developed mPCR method will help in faster diagnosis of *Staphylococcus aureus* food poisoning. 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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