PCR analysis of the isolates for the presence of staphylococcal enterotoxin gene from contaminated food samples

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1. Introduction

*Staphylococcus aureus* is facultative anaerobic gram-positive cocci, which occur singly, in pairs, and irregular clusters. *S. aureus* is non-motile, non-spore forming, catalase and coagulase positive. Typical colonies are yellow to golden yellow in color, smooth, entire, slightly raised, and haemolytic on 5% sheep blood agar. However, many strains may appear dirty white and non-haemolytic. It also gives a positive mannitol fermentation and deoxyribonuclease test.

*S. aureus* is widely distributed in nature and carried by 25-33% of normal individuals in the anterior snares and skin. It can colonize and infect both healthy, immunologically competent people in the community and hospitalized patients with decreased host defences. *S. aureus* is one of the commonest and most important Gram-positive hospital-acquired organisms.

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It has a high propensity to colonize abnormal skin surfaces and open wounds, where it may merely reside rather than cause active infection. It colonizes humans as well as domestic animals, and is a common opportunistic pathogen. Staphylococcal enterotoxins (SEs) belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), sharing common phylogenetic relationships, structure, function and sequence homology (Balaban and Rasooly, 2000). SEs when ingested by humans give rise to symptoms of acute gastroenteritis. These bacterial proteins are known to be pyrogenic and are connected to significant human diseases that include food poisoning and toxic shock syndrome.

The second most common food borne illness in the world is the Foodborne intoxication with the consumption of foods contaminated with SEs (Di Pinto et al., 2004). It expresses a wide array of cell-associated and secreted virulence factors. These properties make it a versatile pathogen capable of a wide range of infections. The secreted factors include various enzymes, cytotoxins, exotoxins, and exfoliate toxins.

The chief function of these enzymes is to turn host components into nutrients that the bacteria may use for growth. Most genes coding for SEs are located on mobile elements such as plasmids, bacteriophages or pathogenicity islands. Thus, horizontal transfer between strains is not rare. SEs are resistant to environmental conditions (freezing, drying, heat treatment, low pH) that easily destroy the enterotoxin-producing strain and the proteolytic enzymes retaining their activity in the digestive tract after ingestion (Bergdoll, 1999).

A recent study showed that most S. aureus isolates obtained from three separate hospitals had more than one enterotoxin gene. The median number of enterotoxin genes in the S. aureus isolates in that study was five and some contained up to 12 enterotoxin genes. Although there are more than 20 distinct staphylococcal enterotoxins, only a few of them have been studied in depth.

The most common Staphylococcal enterotoxins (SEs) are Staphylococcal enterotoxins A (SEA), Staphylococcal enterotoxins B (SEB) and Staphylococcal enterotoxins D (SED). SEA is the most common toxin in Staphylococcus-related food poisoning. SEB is associated with food poisoning, which has been studied for potential use as an inhaled bio-weapon. SED is suggested to be the second most common Staphylococcal toxin associated with food poisoning worldwide, and one study showed that only very small amounts of this toxin were needed to induce food poisoning.
PCR analysis for the presence of staphylococcal enterotoxin gene

Staphylococcal food-borne diseases acquired from eating enterotoxin contaminated food are the second most commonly reported types of food-borne diseases. The high incidence of Staphylococcal food poisoning is due to the insufficient pasteurization/decontamination of originally contaminated product source or its contamination during preparation and handling by individuals who are carriers of the organism. The amount of toxin needed to cause disease is less than 1 μg.

In an outbreak due to SEA contaminated chocolate milk, the amount of toxin was reported to be only 0.5 ng/mL. The disease has a short incubation period that ranges from just a few minutes to hours since the toxin is preformed. Symptoms include nausea, vomiting, abdominal pain, cramps and diarrhoea. SEA is responsible for approximately 80% of the cases of food poisoning outbreaks in the USA, while SEB is responsible for 10% of the cases. The disease is usually self-resolving, is rarely lethal and the elderly are more susceptible. It has been demonstrated that ingestion of SEs within food cause food poisoning, which is characterized by severe vomiting and diarrhoea.

Those symptoms occur within hours after eating of SE contaminated food. SE food poisoning leads to inflammatory changes throughout the gastrointestinal tract with severe lesions in the jejunum and ileum. The direct inhibitory effect of purified SEs on intestinal tone, contractility and colonic transit has been noted in the dog model.

Oral and intra-duodenal administration of SEA to weanling pigs was associated with increased numbers of lymphocytes and polymorphonuclear cells in the jejunum and duodenum, quick emetic and neurobehavioral responses, suggesting that intestine is a site of SEA action.

Staphylococcal enterotoxins bind to class II MHC (major histocompatibility complex) molecules on APCs (antigen-presenting cells) outside of the antigenic peptide binding groove. SEA has two distinct binding sites on both sides of the peptide binding groove of class II MHC. SEA molecules must be bound to both sites for optimal activity, which allows for class II MHC cross linking, and stable interactions with T-cells.

The methods for the detection of enterotoxin in foods need to be much more sensitive to detect less than 1 ng of enterotoxin/g of food that may be present (Bergdoll, 1991). Immuno-assays are based on a quantitative reaction of an antigen (bacterial metabolite, e.g., toxin) with its antibody. Therefore, they are suited for detection of microorganisms based on their production of specific antigens and for quantitative detection of bacterial toxins (Notermans and Wernars, 1991). In a study conducted by Ewald (1988), two ELISA kits were employed to detect
staphylococcal enterotoxin A, B, C and D in foods to which enterotoxin had been added or which was artificially contaminated with enterotoxin-producing strains of Staphylococcus aureus. The sensitivity was found satisfactory and enterotoxins were detected by both ELISA kits in all positive samples. Enterotoxins A, B and C detected in small amounts (2-22 ng/ml) were not detectable after heating at 80°C, whereas enterotoxin D detected in considerably greater amounts (783 ng/ml) was reduced to 0.4% of this amount. Towbin et al., (1979) demonstrated that proteins immobilized on nitrocellulose sheets can be used to detect their respective antibodies.

**Clinical manifestations**

Infections caused by *S. aureus* range from minor skin disorders such as wound infections, furuncles and carbuncles, and bullous impetigo, through locally invasive diseases such as cellulitis, osteomyelitis, sinusitis, and pneumonia, to major life-threatening septicemia and meningitis. It is also a frequent cause of medical device-related infections such as intravascular line sepsis and prosthetic joint infections. Although minor skin infections may resolve naturally without antibiotic intervention, once *S. aureus* invades deeper structures, it often spreads haematogenously to other organ systems, leading to metastatic infection. Endocarditic and septicemia have significant morbidity and mortality despite aggressive antimicrobial therapy.

Toxin mediated disease include the toxic shock syndrome that presents with profound hypotension and a generalized erythematous rash. While TSS was commonly associated with menstruation and the use of hyperabsorbable tampons, the nonmenstrual form is commonly associated with wounds from different surgical procedures. Staphylococcal food poisoning occurs with a short incubation period of 2-6 hours and is characterized by nausea and vomiting, that is followed by abdominal cramps and diarrhoea, which can be hemorrhagic. It is mediated by enterotoxin B and occurs due to ingestion of food contaminated with preformed toxins.

*S. aureus* can produces a large diversity of exoproteins belonging to the family of superantigens, stimulating polyclonal T-cell proliferation through co-ligation between major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and the variable portion of the T-cell antigen receptor β chain or α chain with no need for prior antigen-presenting cells processing. T-cell/ antigen-presenting cells activation by these toxins leads to the release of large amounts of various cytokines/lymphokines which are deleterious for the host. Twenty
different enterotoxins have been described, among them, staphylococcal toxic shock syndrome toxin (TSST-1), staphylococcal enterotoxin A, B, C or enterotoxins coded by egc cluster. Beside their superantigenic properties, they are also pyrogenic and enteropathogenic for the majority, thus explaining their implication in both staphylococcal toxic shock syndrome (TSS) and food poisoning. The enterotoxins have also been implicated in a number of autoimmune disorders (rheumatic arthritis, etc.) and other abnormal immunologic states such as psoriasis, atopic dermatitis and Kawasaki syndrome.

In the present study, *Staphylococcus aureus* strains were isolated from different food samples after prior enrichment step by a variety of biochemical tests. These strains are checked for the presence of staphylococcal enterotoxin A gene and enterotoxin A by PCR and Western blot analysis respectively. This procedure can access whether food samples carry any pathogenic strains that can cause food poisoning. The procedure for Agarose gel Electrophoresis and Western blot analysis were refereed from Molecular Cloning: A laboratory manual by Maniatis *et al.*, (1982).

**Objectives:**

1. Isolation of *Staphylococcus aureus* from contaminated food samples using biochemical methods.
2. Extraction of DNA from the isolated *Staphylococcal aureus* organisms.
3. PCR analysis of the isolates for the presence of staphylococcal enterotoxin gene.
4. Western blot analysis of the PCR positive samples for checking the presence of staphylococcal enterotoxin A in the culture supernatant utilizing commercial polyclonal sera that detects specifically staphylococcal enterotoxin A and not any other enterotoxin.

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

**Materials:**

1. Contaminated food samples
2. Conical flasks with BHI broth
3. Incubator
4. Baird-Parker agar plates
5. Mannitol-salt agar plates
6. Plates containing DNase agar with toluidine blue.
Method:

- Contaminated food samples were taken and grinded with sterile BHI broth in a pestle and mortar in a sterile chamber.
- The grinded samples were suspended in to conical flasks containing BHI broth and incubated overnight at 37°C for enrichment.
- The enriched sample was plated on to the Baird Parker agar plates for the selective isolation of colonies with shiny black colonies with a white halo precipitate around them which indicates their lecithinase activity.
- The colonies with lecithinase activity were considered presumptive *S.aureus* isolates and they are streaked on to BHI agar plates for further studies.
- The presumptive isolates were plated on to the mannitol-salt agar plates and DNAsE agar plates for testing their mannitol fermentation activity, capacity to grow at high salt concentration and DNAse activity.
- Mannitol-salt agar plates selects for organisms positive for mannitol fermentation when the plates turn from red to yellow, and it also selects for organisms that grows at high salt concentrations (> 10%).
- The isolates which were positive for mannitol fermentation, withstands high salt concentration and positive for DNAse activity were considered as *S. aureus* isolates.

The above isolates were processed for DNA extraction for checking the presence of staphylococcal enterotoxin A gene by PCR. The PCR positive isolates were further processed for checking the presence of staphylococcal enterotoxin A in the culture supernatants which is a exotoxin.

**PCR analysis:**

PCR was performed for checking the presence of staphylococcal enterotoxin A gene. The following primers were used for this study.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer sequence</th>
<th>Annealing temp.</th>
<th>Product size.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA</td>
<td>F - gttataaatgcgggtg</td>
<td>58.0</td>
<td>120 bp</td>
</tr>
<tr>
<td></td>
<td>R - ggcactttttctctcgg</td>
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</table>
PCR for checking the presence of staphylococcal enterotoxin A gene of *Staphylococcus aureus* was performed in the following way:

- Amplification of each gene was performed in a 20μl reaction mixture containing 1x PCR buffer, 1.8mM of MgCl₂, 100 μM (each) dNTP, 10 pmol of each primer, 1 U of DNA polymerase (Sigma Aldrich, USA), and 50-100 ng of template DNA.
- PCR was performed on Eppendorf Pro Gradient thermal cycler.
- The thermal profile involved initial denaturation for 4 min at 95°C and 30 cycles with the following steps: 0.5 min of denaturation at 94°C, 0.5 min of annealing at 58°C, and 1 min of extension at 72°C, with a final extension for 10 min at 72°C.
- The PCR products were run on 1.5 % Agarose gel and stained with ethidium bromide to visualize the products under a UV transilluminator.

**Preparation of Agarose gel:**

**Materials required:**

1. Agarose gel running apparatus.
2. Gel trays with appropriate combs.
3. Gel casters
4. 5x TBE buffer
5. 0.5 x TBE buffer
6. Agarose
7. Ethidium bromide
8. Standard DNA ladders
9. Heating mantle

**Procedure:**

- 1.5 gm of agarose was dissolved in to 100 ml of 0.5x TBE buffer and boiled on a heating mantle until the agarose dissolved completely.
- It was allowed to cool for 10 minutes and later 10 μl of ethidium bromide (10 mg/ml conc.) was added and mixed thoroughly and poured on to the gel tray with combs set on a gel caster.
- The gel was allowed to solidify for half an hour and then the gel was removed from the caster, combs were removed and placed in to the tank filled with 0.5x TBE buffer.
• The PCR samples were mixed with 6x gel loading dye and loaded in to the wells along with standard DNA ladders and run at 100 volts.
• Once the dye front reached the edge of the gel, the gel was placed on the UV transilluminator and observed under UV light for visualization of the PCR amplified products.
• Lanes with bright orange bands in the required regions were taken as positives for that particular gene.
• The gels were photographed in the gel documentation system for further reference.

**Western blot analysis for detection of staphylococcal enterotoxin A positive strains from recovered S. aureus food isolates:**

**Protein sample preparation:**

• For the analysis of any exotoxins secreted by *Staphylococcus aureus* by immunoassays, the toxins secreted may not be in sufficient quantities to be detected directly.
• Therefore a prior concentration step is necessary for analysis by immunoassays commonly by Western blotting.
• All the isolates to be tested were inoculated in to BHI broth and incubated overnight with shaking.
• 1.5 ml of broth was collected and centrifuged at 12,000 rpm for 5 minutes to separate bacterial cells. One ml of culture supernatant was taken in a different tubes, 250μl of 100% trichloroacetic acid was added and dissolved.
• The tubes were incubated at 4°C for 10 minutes, centrifuged at 12,000 rpm for 10 minutes and the supernatant was discarded.
• The pellet was washed with ice cold acetone and vortexed thoroughly to collect the protein precipitated on to the walls of the eppendorf tubes.
• The tubes were centrifuged at 12,000 rpm for 10 minutes. The pellet was resuspended in PBS and lysis buffer (2 x) in 1:1 ratio and loaded on to the SDS-PAGE gels for the separation of proteins.

**PAGE analysis:**

The concentrated protein samples were run on 12% SDS- Polyacrylamide gel and later transferred on to a nitro-cellulose membrane for analysing the strains of *Staphylococcus aureus* producing staphylococcal enterotoxin A (SEA) using commercial polyvalent antisera raised against SEA in rabbits.
• The resolving gel was made by mixing the following reagents for making one gel (5ml)
  30% acrylamide/bis-acrylamide solution-2.0 ml
  Distilled H₂O- 1.65 ml
  Tris-Cl (1.5 Mm,pH -8.8)- 1.25 ml
  10 % SDS- 50 μl
  10 % APS- 50 μl
  TEMED – 3.0 μl
• The above reagents were mixed thoroughly without producing bubbles and poured the mixture in between the glass plates fixed on the casting apparatus. 200 μl of isobutanol was poured over the surface of the gel mixture to remove any air bubbles.
• The gel mixture was allowed to solidify for 20 minutes. Later the isobutanol was poured off and the surface of the gel was washed with distilled water.
• The stacking gel mixture (5 ml) was prepared by mixing the following components:
  30 % acrylamide/bisacrylamide solution- 0.8 ml
  Distilled water- 2.5 ml
  Tris-Cl (0.5 mM, pH-6.8)- 1.25 ml
  10 % SDS (sodium do-decyl sulphate)- 50 μl
  10% APS (ammonium persulphate) – 50 μl
  TEMED – 8 μl
• The above reagents were mixed thoroughly and poured above the resolving gel combs were placed and allowed to solidify for 20 minutes. Combs were removed and wells were washed with distilled water to remove any debris and unsolidified components.
• The concentrated samples were loaded on to the wells along with prestained protein ladder and run at 70 volts. Once the samples reached resolving gel they were run at 100 volts until bromophenol blue dye reaches the edge of the gel.

**Blotting procedure:**

• Later the resolving gel was cut and removed carefully without tearing and the separated proteins were transferred on to the charged activated PVDF membrane.
• Pre-wet materials in transfer buffer. Stack in the following order:
1. Case (clear side)
2. Sponge
3. Whatman paper
4. PVDF membrane
5. Gel
6. Case (black side)

- Transfer apparatus was placed with black side facing black.
- All the cassettes with gel and membrane sandwiches were placed in proper orientation and run at 70 volts for 70-80 minutes.

**ELISA:**

- The blotting assembly was removed and the membrane was transferred in to a box with 5% non-fat milk solution for blocking overnight at 4°C.
- After blocking the membrane was washed with PBST for two minutes four times with shaking.
- Later the membrane was incubated with polyclonal antisera raised against staphylococcal enterotoxin A in rabbit for 30 minutes.
- The membrane was washed with PBST for four times each wash for two minutes.
- Later the membrane was incubated with an anti-rabbit secondary antibody conjugated to horse-radish peroxidise enzyme for 30 minutes.
- Unbound secondary conjugate was washed with PBST for 6 times each wash for 2 minutes.
- The membrane was developed with Diamino Benzidine (DAB) and 
  \( \text{H}_2\text{O}_2 \) in phosphate buffered saline. Lanes with bright brown bands at 28 kDa were taken as positive for the production of enterotoxin A. In addition these SEA positive strains should be correlating to PCR results for staphylococcal enterotoxin A gene.

**Results:**

**Isolation of S. aureus from food and milk samples:**

Totally 58 food and milk samples were processed for isolation of *staphylococcus aureus* after a prior enrichment step overnight in BHI broth.

20 strains of *S. aureus* were recovered from the above samples, DNA was extracted from all the recovered isolates and checked by agarose gel electrophoresis and staining with ethidium bromide.
PCR analysis for the presence of staphylococcal enterotoxin gene

<table>
<thead>
<tr>
<th>Food samples</th>
<th>Presumptive isolates</th>
<th>Positive S. aureus isolates</th>
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<tbody>
<tr>
<td>Pastries</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>Chicken samples</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Mutton samples</td>
<td>10</td>
<td>5</td>
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<tr>
<td>Milk samples</td>
<td>11</td>
<td>4</td>
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</table>

PCR results for staphylococcal enterotoxin A:

DNA was extracted from all the isolates and PCR was carried out in 20 μl reaction in Eppendorf Pro thermocycler.

Overall 8 strains were positive for the presence of staphylococcal enterotoxin A gene by PCR after running the PCR products in Agarose gel stained with ethidium bromide.

<table>
<thead>
<tr>
<th>No. of isolates tested</th>
<th>Strains positive for SEA gene by PCR</th>
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<tr>
<td>20</td>
<td>7</td>
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Western blot analysis results:

All the strains which were positive for the presence of SEA gene by PCR were processed for checking the presence of toxin by Western blot analysis along with positive and negative controls.

Over all 6 strains were found to produce staphylococcal enterotoxin A (28 kDa) by Western blot analysis in the culture supernatants after the proteins were concentrated by precipitation with trichloroacetic acid.

The bands present in the 45-55 kDa region of the membrane were due to the non specific binding of IgGs to the staphylococcal protein A (SPA) which is present in majority of *Staphylococcus aureus* strains.

<table>
<thead>
<tr>
<th>No. of isolates tested</th>
<th>SEA PCR positive samples</th>
<th>Samples positive for toxin by Western blot analysis</th>
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<td>20</td>
<td>7</td>
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Pictures:

Mannitol-salt agar selects *Staphylococcus aureus* organism because of their characteristic biochemical properties like mannitol fermentation and the capacity to grow at high salt concentration.
1. **Mannitol-salt agar plate:**

![Image of Mannitol-salt agar plate]

- Control plate
- S. aureus on Mannitol-salt agar plates
- Negative control

2. **Staphylococcus aureus on Baird-Parker agar plate:**

![Image of Staphylococcus aureus on Baird-Parker agar plate]

- S. aureus colony having characteristic clearing because of lecithinase activity

3. **DNase agar with toluidine blue plate:**

![Image of DNase agar with toluidine blue plate]

- Negative control
- S. aureus produces a zone of clearing on DNase plate because of DNase activity.
4. *S. aureus* on BHI agar plate:

![Image of BHI agar plate showing characteristic colonies of *S. aureus*](image)

5. Agarose gel showing the PCR amplified products:

![Image of agarose gel showing PCR amplified products](image)

Picture showing the PCR amplified products run on agarose gel stained with ethidium bromide.

6. Western blot results for checking the enterotoxin A of *S. aureus*:

![Image of Western blot results](image)

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- Non specific reaction by protein A.
- SEA (28 kDa)

1. Isolate-1. 2. Isolate-2. 3. Isolate-5. 4. Isolate-6. 5. Isolate-8.
Conclusions:

1. A large number of food samples were found harboring strains of *Staphylococcus aureus* which are carrying virulent gene like SEA.
2. 20 strains of *Staphylococcus aureus* were recovered from 58 food samples of various types.
3. 7 strains of the recovered 20 isolates were carrying SEA when tested by PCR.
4. Six strains of staphylococcal enterotoxin A was observed in the culture supernatants of the PCR positive *S. aureus* strains.

References: