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RESEARCH PAPER

Distribution of Staphylococcal Enterotoxin Genes among Clinical Isolates

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Abstract

Background: *Staphylococcus aureus* is an important pathogen which produces numerous numbers of toxins including enterotoxins those cause many diseases in both human and animal. It is very important to know the extent of distribution of these toxins, as they are concern of public health problems including food poisoning and toxic shock syndrome.

Objective: This study was conducted to estimate the distribution of enterotoxin genes among the clinical isolates of the *Staphylococcus aureus* by multiplex PCR.

Methods: This cross-sectional study was carried out in the Department of Microbiology& Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka during the period from March 2014 to February 2015. A total 125 isolates of *S. aureus* from different clinical specimens were identified by standard microbiological methods. Multiplex PCR assay was performed by using standard protocol with specific primers to detect genes for staphylococcal enterotoxins A to E (*sea, seb, sec, sed* and *see*) from identified *S. aureus* isolates.

Results: Out of 125 *S. aureus* isolates, 63 (50.4%) were enterotoxin genes positive in which the predominant gene was *sec*, which was present in 36% of tested *S. aureus* isolates followed by *sea* (17.6%) and *see* (13.6%). Multiple enterotoxin genes combination was common in *S. aureus* isolates and the predominant combination was *sea+sec* genes. Out of 76 *Staphylococcus aureus* isolated from indoor patients, 45 (59.2%) were positive for enterotoxin genes which were higher than outdoor patients 18 (36.7%).

Conclusion: The enterotoxin genes are frequently present in *S. aureus* isolates. The most frequent gene is *sec* followed by *sea* and *see*. Moreover, multiple genes are more commonly present in *S. aureus* strains which support the strong virulent potential of these strains.

Keywords: Enterotoxin genes, Superantigens, Polymerase chain reaction, *Staphylococcus aureus*.

Introduction

Staphylococcus aureus is a poten-tially virulent human pathogen, which causes toxin-mediated diseases, such as food poi-soning, toxic shock syndrome and staphy-lococcal scalded skin syndrome.[1] The ability of *S. aureus* to cause human disease depends on the production of cell surface adhesins, antiphagocytic factors and secreted exotoxins.[2] These exotoxins exhibit superantigen activity, stimulating large number of nonspecific polyclonal T-cell proliferation, with no need of prior antigen presenting cell (APC) processing.[3] Intermediaries of the toxin action are cytokines, interleukin 1 (IL-1), interferon-ã and tumour necrosis factor (TNFα). These massive cytokines release account for the most severe manifestation of superantigen mediated illnesses.[4]

To date, more than 23 distinct super antigenic toxins are known to be produced by *S. aureus*, which include staphylococcal enterotoxins (SEs), exfoliative toxins (ETs) and toxic shock syndrome toxin-1 (TSST-1).[5]

Staphylococcal enterotoxins (SEs) are the main source of food poisoning in most of the countries. Especially in South East Asia, rate of food poisoning is still higher because of warm and humid climate. Between 25-50% of the population are the carriers of *S. aureus* and 15-20% of the strains are

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enterotoxigenic.[6] Food poisoning caused by staphylococcal enterotoxins is characterized by prominent vomiting and watery non-bloody diarrhoea. The fatality rate of staphylococcal food poisoning is low (0.03%) for the general public but may reach 4.4% for extreme age groups. Quantities of less than 1ìg of toxin are sufficient to trigger vomiting in human.[7] Among the all enterotoxins, staphylococ-cal enterotoxins SEA, SEB and SED are the most common causes of outbreaks of food poisoning, but SEB can also cause respiratory symptoms and in severe cases can lead to pulmonary oedema and respiratory failure.[8]

These superantigen enterotoxins are considered to be major virulence factors of *S. aureus*. *S. aureus* carrying more toxin genes are responsible for more severe infections.[9] Most of the genes encoding these toxins are located on mobile genetic elements, such as the genes for SEB (*seb*) and SEC (*sec*) are located on the chromosomes. Genes for SEA (*sea*) and SEE (*see*) harboured by a bacteriophage vector and gene for SED (*sed*) carried by a plasmid (plB485).[10, 11] This association implies a horizontal transfer of these genes between staphylococcal strains and an important role in the evolution of *S. aureus* as a pathogen.[2]

Detection of toxigenic strains of *S. aureus* is also important for epidemiological reasons. For epidemiological surveillance, the methods most frequently used for the detection of staphylococcal toxins are immune diffusion, agglutination, radioimmunoassay and ELISA. Among the techniques used to identify toxin genotypes, DNA-DNA hybridization and PCR have been reported to be very successful and reliable. Low levels of excreted toxin(s) or cross-reactive antigens can be easily misidentified by immunologic methods.[10] Presence of enterotoxin genes should always be considered as an indicative of the ability of the organism to produce toxin in favourable environment.

The prevalence of enterotoxin genes among clinical isolates of *S. aureus* in different countries are – about 57% in India, 35.6% in Pakistan, 68.5% in Mexico and 75.7% in Japan.[12-15] The distribution of predominant classical SE genes also vary from country to country, such as: in Pakistan, *sec;* in Mexico *sea*; in China, *seb* and *see*.[13, 14, 16, 17] In Bangladesh, there are very limited data about the prevalence and distribution of staphylococcal enterotoxins. One study in Bangladesh showed that about 40% clinical isolates of *S. aureus* produce enterotoxins by using reverse passive latex agglutination test (RPLA) from culture supernatants.[18] To date, no valid data are available concerning the prevalence and genetic distribution of staphylococcal enterotoxin genes from clinical specimens in Bangladesh. But it is necessary to know the distribution pattern of these toxin genes for proper treatment and a better understanding of different toxin mediated diseases. This study was designed to determine the prevalence and distribution of enterotoxin genes in *S. aureus,* isolated from different clinical samples.

Materials and Methods

This cross-sectional study was carried out in the department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University, Dhaka during the period of March 2014 to February 2015. All the confirmed isolates of *S. aureus* from different clinical samples obtained during this period were included in the study.

Sample collection and processing: A total of 125 isolates of *S. aureus* from different clinical specimens were obtained from the laboratories of Microbiology of Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Medical College (DMC). The sites of infection were categorized in three categories depending on the type of specimens. Skin and soft tissue infection (SSTI) included pus, wound swab and aural swab, blood stream infection (BSI) included blood and central venous catheter tip and urinary tract infection (UTI) included urine specimens. Out of these 125 *S. aureus*, 93 were isolated from skin & soft tissue infection, 19 from blood stream infection & 13 from urinary tract infection. These 125 isolates of *S. aureus* were identified as by colony morphology, haemolytic property, pigment production, Gram staining, catalase test, coagulase test (slide and tube method) and mannitol fermentation test in mannitol salt agar media as per standard methods.[19]

Detection of enterotoxin genes: Multiplex PCR assays were used for the detection of genes for staphylococcal enterotoxins *sea, seb, sec, sed, see* (for enterotoxin A-E). *S. aureus* specific gene *fem*A was used as positive control to confirm the presence of *S. aureus* and to validate PCR condition. *fem*A is universally present in all *S. aureus* isolates. As negative control, PCR was tested with sterile water (table-I).[20]

Table-I: The primer sequences for amplification of the Staphylococcus aureus enterotoxin genes

Gene	Primer sequences (5' - 3')	Size of amplified product
sea	GGTTATCAATGTGCGGGTGGCGGCACTTTTTTCTCTTCGG	102bp
seb	GTATGGTGGTGTAACTGAGCCCAAATAGTGACGAGTTAGG	164bp
sec	AGATGAAGTAGTTGATGTGTATGGCACACTTTTAGAATCAACCG	451bp
sed	CCAATAATAGGAGAAAATAAAAGATTGGTATTTTTTTTCGTTC	278bp
see	AGGTTTTTTCACAGGTCATCCCTTTTTTTTCTTCGGTCAATC	209bp
*fem*A	AAAAAAGCACATAACAAGCGGATAAAGAAGAAACCAGCAG	132bp

Three major steps of PCR: include DNA extraction from bacterial pellets, DNA amplification in thermal cycler and visualization /documentation under UV light.

DNA extraction: DNA was extracted by using commercial kits (Qiagen, Hilden, Germany). At first preserved colonies from the nutrient agar slants were inoculated into 0.5 ml of brain heart infusion broth in a sterile 1.5ml microcentrifuge tube and incubated overnight at 37^0C temperature. Then total DNA was extracted from these broth culture by using the Qiagen DNA extraction kit (QIAamp DNA mini kit), in accordance with the manufacturer's guideline for Gram positive bacteria.

DNA amplification:

Primer used for multiplex PCR: Multiplex PCR assay were used for the detection of genes for staphylococcal superantigen enterotoxins *sea, seb, sec, sed,* and *see*. Six pairs of primers were used to target the structural genes for enterotoxins A to E (*sea, seb, sec, sed, see*), along with *fem*A. Detection of *fem*A gene was used as an internal positive control to confirm the presence of *S. aureus* and to validate PCR condition. As negative control, were tested with sterile water. The primer sequences that were used in the multiplex PCR are listed in Table- I.[20]

Mixing of master mix and primer mix with template DNA: Sterile 0.2 ml microcentrifuge tube was taken and the tube was labeled with date and identification number.10 µl master buffer composed of mixture of PCR buffer, $MgCl_2$, and deoxy nucleoside triphosphate/dNTP (Texas Bio Gene Inc, USA) and 0.2 µl of taq polymerase (Geneaid Biotech Ltd, Taiwan) were taken in PCR tube. Then 0.5µl of each gene specific primers were added. Then mixture of master mix, primers and taq polymerase was vortexed and then spinned for a brief time. Afterwards 2.5 µl of extracted DNA from each separate sample was added to the tube. Then PCR tube was centrifuged for 5 seconds.

DNA amplification in thermal cycler (Applied bio-system 2720): Amplification was carried out in an automated DNA thermal cycler and comprised initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification.[35] Each cycle consists of –

1. Denaturation at 94°C for 2 min,
2. Annealing at 57°C for 2 min, and
3. Extension at 72°C for 1 min), and

After completion of 35 cycles a final extension was done at 72°C for 7 min.

Then the product was held at 4^0C. After amplification the product was processed for gel documentation or kept at -20^0C till tested.

Amplicon detection by agarose gel electrophoresis: The amplified product was detected by electrophoresis in 2 % agarose gel containing 0.002% ethidium bromide. During electrophoresis, the gel with the stand was placed in horizontal electrophoresis apparatus containing 1x TAE buffer.

The amplified product (10µl) was slowly loaded into the wells using disposable micropipette tips. 10 µl of amplified product of negative control were also loaded into different well marker of DNA of known bp (100 bp ladder) was loaded in one well to determine the size of amplified PCR products. Electrophoresis was carried out at 100 volts for 90 minutes in 2% agarose gel, pre-stained with ethidium bromide in a submerge gel apparatus.

Visualization of the gel: The gel was observed under UV trans-illuminator for DNA bands. The DNA bands

were identified according to their molecular size by comparing with the molecular weight marker (100bp DNA ladder) loaded in a separate lane. The amplified products were visualized as 102bp (*sea*), 164bp (*seb*), 451bp (*sec*), 278bp (*sed*), 209bp (*see*), 132bp (*fem*A), by UV trans-illuminator (figure 1). The presence of gene *fem*A confirms the presence of *S. aureus.*

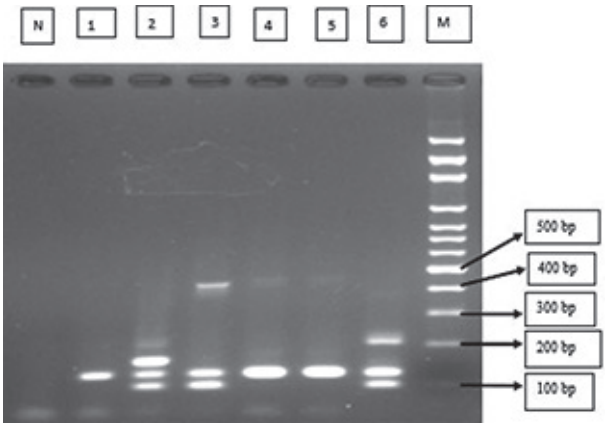

Figure 1: Gel electrophoresis of amplified DNA. Lane M: DNA molecular size marker (100bp ladder), lane N: negative control, lane 1: femA, lane 2: femA+sea+seb, lane 3: femA+sea+sec, lane 4: femA, lane 5: femA, lane 6: femA+sea+see.

Data analysis: All the data were analysed using SPSS (version-20). *p* value was calculated from chi-square test using 2x2 contingency table.

Results

The present study was conducted to investigate the enterotoxin genes among clinical isolates of *S. aureus*. For this purpose 125 *S. aureus* isolates were collected from laboratories of Microbiology department of Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Medical College (DMC). Out of 125 study isolates, 63 (50.4%) were positive for one or more enterotoxin genes. Frequency of enterotoxin genes in isolates from SSTI, UTI and BSI were 50 (53.8%), 7 (53.8%) and 6 (31.6%) respectively (table II).

Table-II: Enterotoxin genes positivity according to sites of infection

Sites of infection	No. of toxin genes positive *S. aureus* (%)
Urinary tract infection (n=13)	07 (53.8)
Skin & soft tissue infection (n=93)	50 (53.8)
Blood stream infection (n=19)	06 (31.6)
Total (n=125)	63 (50.4)

Out of 125 study isolates, predominant gene was *sec* gene 45 (36%) followed by *sea* gene 22 (17.6%) and *see* gene 17 (13.6%) (table III).

In both UTI and SSTI, *sec* was predominant gene which was 5 (38.5%) & 38 (40.9%) respectively and in BSI it was 2 (10.5%). The predominant gene in BSI was found *sea* (4, 21.1%). The detection rate of *sea* gene in SSTI and UTI was (16, 17.2%) and (2, 15.4%). No isolates from UTI and BSI was found to be positive for *seb*, *sed* genes and *see* gene was also absent in isolates from BSI (table III).

Out of 76 *S. aureus* isolates from indoor patients, 45 (59.2%) were found to be positive for enterotoxin genes. In contrary out of 49 isolates from outdoor patients, 18 (36.7%) were positive for enterotoxin genes (figure 2).

Table-III: Distribution of different staphylococcal enterotoxin genes in different sites of infection

Enterotoxin genes	Urinary tract infection (n=13)	Skin & soft tissue infection (n=93)	Blood stream infection (n=19)	Total no. of positive *S. aureus* (n=125)
sea	02 (15.4)	16 (17.2)	04 (21.1)	22 (17.6)
seb	00	08 (8.6)	00	08 (6.4)
sec	05 (38.5)	38 (40.9)	02 (10.5)	45 (36)
sed	00	05 (5.4)	00	05 (4.0)
see	01 (7.7)	16 (17.2)	00	17 (13.6)

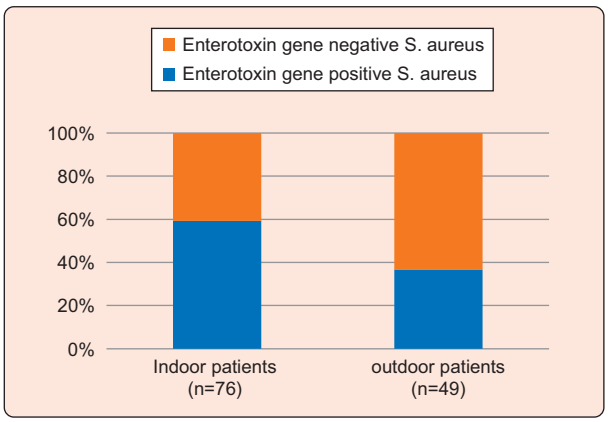

Figure 2: Enterotoxin gene positive Staphylococcus aureus among indoor and outdoor patients

Enterotoxin gene positive *S. aureus* isolates from indoor patients were significantly higher than outdoor patients ($p<0.01$) [Not shown in figure 2]. *p* value for Indoor patients was calculated by chi-square test using 2x2 contingency table.

Out of 125 *S. aureus* isolates, single toxin gene were present in 36 (28.8%) isolates, two and three toxin genes combination were present in 20 (16%) isolates and 07 (5.6%) isolates respectively (figure 3).

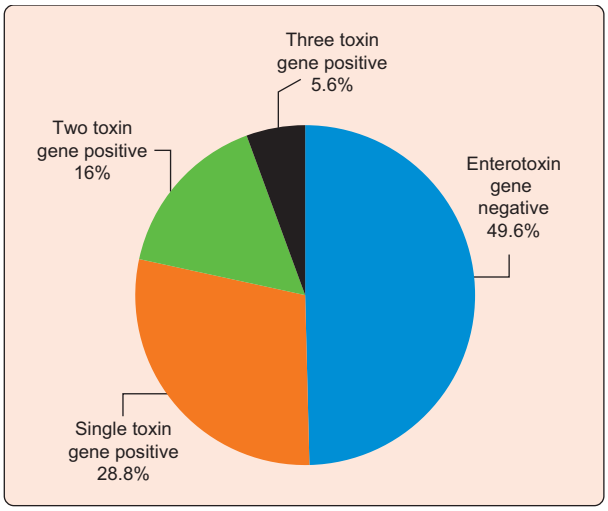

Figure 3: single and multiple enterotoxin gene positive Staphylococcus aureus.

The most frequent toxin gene was found *sec* either alone or in combination with other toxin genes. No isolate was found positive for *seb* and *sed* alone. The highest toxin genes combination detected was *sea+sec* 7 (5.6%) followed by *sec+see* 5 (4%).

Table-IV: Distribution of single and combinations of enterotoxin genes among Staphylococcus aureus strains (Total isolates, n=125)

Toxin genotypes	No of toxin gene positive isolates (%)
sea	08 (6.4)
seb	00
sec	25 (20)
sed	00
see	03 (2.4)
sea+sec	07 (5.6)
sec+see	05 (4.0)
sec+sed	02 (1.6)
sea+see	02 (1.6)
seb+see	02 (1.6)
sea+seb	01 (0.8)
seb+sec	01 (0.8)
seb+sec+sed	01 (0.8)
sec+sed+see	02 (1.6)
sea+seb+see	02 (1.6)
sea+seb+sec	01 (0.8)
sea+sec+see	01 (0.8)
Total	63 (50.4)

Discussion

In the current study, out of 125 *S. aureus* isolates, 63 (50.4%) were positive for enterotoxin genes, which was in agreement with study by Reina et al in Spain (53.4%), Taj et al in Pakistan (48.5%), Nada et al in Egypt (54%), Jassim et al in Iraq (55%), Leke et al in France (56.2%).[13,21-24] But higher detection rate of toxin genes in *S. aureus* isolates were reported by Elazhari et al, 2011 in Morocco (76.4%) Adame-G´omez et al, 2020 in Mexico (68.5%) and Hu et al, 2008 in Japan (75.7%).[14,15,25] Because, they included newly published staphylococcal enterotoxin genes (*seg, seh, sei, sej, sek* and many more) along with classical toxin genes.

In Bangladesh Islam et al found lower rate of toxins production by *S. aureus* which was 40%. In their study they only tested for toxin production by RPLA test.[18] But in this study, we used PCR method which is more sensitive than RPLA detection of toxin production by *S. aureus*. Because, RPLA method can be affected by the growth conditions of *S. aureus* including temperature, pH, and water activity and the produced toxin levels might be lower than the detection limits.[26]

Alternatively, the toxin gene may not be expressed due to mutation either in the coding region or in a regulatory region, for example, *agr* (accessory gene regulator).[27] On the other hand, PCR technique permits the detection of toxin genes independent of their expression.[28]

In this study, out of total 125 study isolates, *sec* gene (36%) alone or in combination with other toxin genes was the most frequently found gene followed by *sea* (17.6%) and *see* (13.6%). These results are in consistent with the results of Taj et al, from Pakistan and of Asiimwe et al, from south West Uganda.[13, 29] They also found higher frequency of *sec* in their studies. However, our results are in contrast to other studies where they showed that most human clinical *S. aureus* isolates were positive for *seb* gene by Yang et al, from China, *sea* gene by Adame-G´omez et al, from Mexico and *see* gene by Rong et al, from China.[14,16, 17]

In the present study out of 125 study isolates, *seb* gene was present in 6.4% and *sed* gene in 4.0% of isolates. Similar results of *seb* and *sed* genes were also reported in other studies.[14]

These different frequencies of enterotoxin genes in different studies may be due to geographical differences, source of origin of the sample (food, human, animal) and genes which have been detected. The variable distribution of *S. aureus* enterotoxin genes in different areas may be explained by the fact that the enterotoxin genes are mostly carried by mobile genetic elements, which can be exchanged among bacteria of the same or different species, accounting for differences in the geographical distribution of staphylococcal enterotoxin genes.[30]

In this study, enterotoxin genes were positive in highest number of strains isolated from skin and soft tissue infections and urinary tract infection (53.8%) than other sites of infection. Similar results were found in study by Reina et al, they found 61.7% of strains from SSTI were positive for enterotoxins.[21] In this study, *sec* was predominant gene in the isolates from SSTI and UTI, and in BSI, *sea* gene (21.1%) was predominant. All these results were similar as reported by Taj et al from Pakistan. They showed that, *sec* gene was predominant in SSTI and UTI whereas *sea* gene was highest in BSI.[13]

In the current study, enterotoxin genes containing *S. aureus* isolates were significantly higher ($p<0.01$) in hospitalized patients (59.2%) in contrast to outdoor patients (36.7%). Because, infection by methicillin resistance *Staphylococcus aureus* (MRSA) strains are higher in hospitalized patients and MRSA strains carry more toxin genes so, toxin genes containing isolates were also higher among them.[13]

The highest number of enterotoxin genes combination was *sea+sec* (5.6%) followed by *sec+see* (4%). Reina et al from Spain also found *sea+sec* combination in 5.4% of *S. aureus* isolates.[21] However, these results were in contrast to other studies which showed that most common enterotoxin genes combinations were *seg+sei* genes, *selm+selo* genes, *sec+seg+sei+ sell+selm+seln+selo+tst* genes.[15,25,31] They included newly described enterotoxin genes along with classical toxin genes for these reason toxin genes combinations they found were different from the present study.

Conclusion

Analysing the findings of present study, about half of the *S. aureus* isolated from clinical specimens harboured single or multiple enterotoxin genes, which is not negligible. Among enterotoxin genes *sec* gene alone or in combination with other toxin genes are most frequently found gene in *S. aureus* isolates. Enterotoxin genes positive *S. aureus* strains are more common in indoor patients than outdoor patients. The existence of these toxin genes does not indicate the ability of bacteria for toxin production and pathogenesis; but generally, isolates with more virulence genes show higher pathogenesis abilities, resulting in more severe and invasive infections.

Further studies are needed in Bangladesh, on the occurrence of these enterotoxin mediated diseases in the community and the role of these toxins producing *S. aureus* in these diseases.

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