Distribution of Virulence Genes Among Methicillin-resistant Staphylococcus Aureus of Clinical and Environmental Origin

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Research note

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Abstract

Objective: This study was designed to discover the dissemination of virulence genes in Methicillin-resistant Staphylococcus aureus from clinical and environmental settings.

Results: The virulence gene such as sea (n=54), seb (n=21), eta (n=27), etb (n=2), cna (n=24), ica (n=2) and tst (n=30) was revealed from this study. Different SCCmec types such as type I, type II, type III, type IV, type V, type VI, type VII, type VIII and type XII were detected among sixty three MRSA isolates where SCCmec type II having ST1551 and type V with ST2416 were found to be associated with multidrug resistance and were highly prevalent in the study area.

Introduction

Incidence of community associated as well as hospital acquired staphylococcal infection has created an awful scenario over last few decades. Staphylococcus aureus has the ability to cause a wide variety of infections ranging from septicaemia to toxic shock syndrome [1]. Rich diversification in mobile genetic element Staphylococcus cassette chromosome mec (SCCmec) leads to the expansion of antibiotic resistance determinants as well as virulence factors, which is naturally designed for the stable maintenance of the core genome environment. These virulence determinants are the genes that facilitate the successful colonisation and endurance of the organism or interrupt with the defence system of the host [2]. The dissemination of SCCmec types and along with virulence determinants in Methicillin Resistant Staphylococcus aureus isolates among the hospital as well as environmental settings constitute a vast reservoir for potential spread of infection. Accumulation of toxins, virulence factors, surface proteins and enzymes have became an attributing factor of S. aureus along with the rapid development of multidrug resistance. The pathogenicity of an organism depends on surface proteins, which help in attachment and colonisation of the bacteria to the host and these are cellular protein, proteases and toxins which help by inhibiting phagocytosis leaving primary immune response ineffective [1]. Staphylococcus aureus enterotoxins are the most commonly implicated toxin in Staphylococcal food poisoning. Both sea and seb gene are associated with food poisoning [3]. The exfoliative toxins which are serine proteases also known as epidermolytic toxin, mainly associated with the loss of keratinocytes, cell-cell adhesion, inducing blister formation [4]. CNA (collagen binding adhesion) is a virulence factor belongs to the family of adhesions. It contains a signal peptide on A domain located on N terminus and β repeats, cell anchoring region [5]. Biofilm which acts as a pathogenic pool associated with antimicrobial resistance. Intercellular adhesion cluster (ica) is a gene which is synthesised for the development of biofilm [6]. Toxic Shock Syndrome toxin (TSST), an exoprotein of 22kD molecular weight, which belongs to pyrogenic toxin super antigen (PTAgS) encoded by a gene tst. The presence of this protein affects the cells of immune system eventually leading to deaths [7]. Fibronectic proteins (fnbA and fnbB) are very important for their involvement in adhesion to cells as well as also favour internalization by cells which leads to intercellular persistence and chronic staphylococcal infections [8]. So far no study had been carried out to explore the detail in involvement of virulence gene in Staphylococcal infection in this study area. Therefore, a comparative analysis on prevalence of virulence genes in the Methicillin Resistant Staphylococcus aureus isolates among hospital, community as well as environmental setting is undertaken in the present study.

Main Text

Methodology:

Study location:

Isolates were collected from hospital, community and environment from three districts of the state of Assam in India. Clinical isolates were collected from the patient with wound infections, UTI, Post surgical infections from various wards
and those visited the clinic of Silchar Medical College and Hospital, Assam, India while the environmental samples were collected from the soil, water and sewage water for a period of one year from June 2017 to May 2018.

**Identification And Characterization Of Isolates:**

Isolates were cultured on Mannitol Salt broth for specifying the growth of Staphylococcal isolates and incubated for 24 hrs for observing the visible growth of the organism. Organisms are then spread onto MRSA Chrom agar, Baird Parker agar (Himedia) and the plates were incubated overnight. The presumptive *S.aureus* isolates were confirmed through 16 s rDNA sequence analysis. Additionally phenotypic characterization was performed through gram staining, rapid coagulase (Rapid *H.aureus* Coagulase confirmation kit, Himedia) and latex agglutination testing (HiStaph™ Latex Test Kit, Himedia). Screening of the methicillin resistance was performed by cefoxitin agar disk diffusion and further genotypically confirmed by *coa, nuc, femB, mecA* and *mecC* PCR [9, 10, 11, 12, 13].

**Antibiotic Susceptibility Testing And Minimum Inhibitory Concentration:**

Kirby-Bauer disk diffusion method was used for susceptibility testing against different groups of antibiotic viz. ciprofloxacin (5 µg/ml), gentamicin (10 µg/ml), co-trimoxazole (25 µg/ml), erythromycin (10 µg/ml), clindamycin (10 µg/ml), doxycycline (10 µg/ml), and tetracycline (30 µg/ml) (Hi-media, Mumbai) and the results were interpreted according to the CLSI recommendation. *Staphylococcus aureus* ATCC 25923 was used as control. While minimum inhibitory concentration testing was performed for oxacillin (Himedia), vancomycin, linezolid and teicoplanin as per CLSI recommendations [14].

**Determination Of Virulence Factor By Multiplex Pcr Assay:**

Genes encoding virulence factors such as *sea* (enterotoxinA), *seb* (enterotoxinB), *eta* (exfoliative toxin A), *etb* (exfoliative toxin B), *cna* (collagen binding adhesion), *fnb* (fibronectin binding protein), *ica* (intercellular adhesion protein), *tst* (toxic shock syndrome) were studied by multiplex PCR amplification for all the MRSA isolates. Virulence specific primers were used for the study (Table 1). Three sets of multiplex PCR were employed for the study which was initiated with the 25 µl of the reaction mixture which includes (12.5 µl of the Go green Taq mixture (Promega, India), 10 pmol of each primer, nuclease free water and 50 pmol of the DNA product) with varied reaction conditions (Additional file 1: Table S1).

**Distribution of toxin encoded virulence genes among SCC mec types:**

SCC mec types were checked in the MRSA strains carrying different virulence factors which were described in (Additional file 2: Table S2).The protocol describing SCC mec type amplification was mentioned earlier.

**Multilocus sequence typing of SCC mec types carrying Virulence genes:**

To establish the relatedness of SCC mec types with the virulence factors, amplification of MLST gene was performed according to Enright *et al.*, 2000 for all the MRSA isolates carrying SCC mec types in combination with virulence gene. Seven housekeeping genes were targeted for the MLST study and the primers were used for amplification by targeting the genes viz; *arcC, aroE, glp, gmk, pta, tpi* and *yqiL*. The sequence were obtained and analysed in Centre for Genomic Epidemiology MLST 2.0 website (https://cge.dtu.dk/services/MLST-2.0) [15].
**Result**

Among five hundred collected samples of different location, 161 staphylococcal isolates were isolated, of which 85 isolates were confirmed as *Staphylococcus aureus*. Further, presence of femB and mecA genes was also observed and sixty three isolates exhibited methicillin resistance. Antibiotic susceptibility testing showed linezolid (66.6%) was the most effective one followed by minocycline (55.5%) and doxycycline (40%). Presence of different virulence genes were noted in the current study where 44 isolates carried enterotoxin A (sea) gene, 7 isolates were harbouring enterotoxin B (seb) gene while 15 isolates found carrying both the enterotoxin A and B gene. Exfoliative toxin gene eta was found in 26 isolates and etb gene in one while one isolate was harbouring both the exfoliative genes eta and etb. Collagen binding adhesion (cna) gene was found in 24 isolates, and presence of toxic shock syndrome toxin (tsst) gene was observed in 30 isolates. Intercellular adhesion protein (ica) were found to be present in 2 isolates. It was observed that enterotoxin A (sea) gene (92.06%) was the most predominant staphylococcal toxin followed by toxic shock syndrome toxin (tsst) (50.79%), exfoliative toxin A (eta) gene (47.61%), cna (38.09%), seb (33.33%) and ica and etb gene (3.1%). It was also observed that, environmental sources acted as reservoir as majority of the sea gene (47.6%) was prevailing, followed by tsst (28.57%) and cna (26.98%) (Table no. 1).

The MRSA isolates having combination of the virulence genes were also focused in this study and it was found that twenty nine different combination of virulence genes coexisted (Table no. 3). Moreover it was also observed from the current study that three isolates were having a combination of six virulence genes, seven isolates with a combination of five virulence genes, and thirteen isolates showed different combinations with four virulence genes whereas combination three virulence genes were observed in 19 isolates. (Table no. 2)

SCCmec typing showed that majority of the isolates with virulence genes were of SCCmec type V (47.61%), followed by SCCmec type II (39.68%) and SCCmec type III (23.80%), while less number of isolates were associated with SCCmec type IVa (0.15%), typeVI (0.63%), type VII (0.95%), type VIII (0.3%) and type XII (0.15%). Nine different STs of isolates with SCCmec types were observed where isolates of SCCmec type I, type II, and type VI belonged to ST 2472, ST 2039 and ST 2459 and isolates carrying SCCmec type IV, SCCmec type III were found associated with ST1551 and ST2302 while ST672, ST 5152 and ST2884 were observed with SCCmec type V, SCCmec type VII, type VIII and type XII respectively.

**Discussion**

Virulence factors play a key step for pathogenic invasion leading to staphylococcal infection in the hospital, community and in environmental settings. Rich diversity of virulence associated genes within *S. aureus* is reported worldwide [16, 17]. It was observed in the present study that the virulence determinants such as sea, tsst and eta gene were found to be more predominant in the study isolates. However, in an earlier study conducted by Mojtabi et al., 2018, reported high prevalence of eta, etb and etd genes in *S aureus* among clinical isolates [18]. In another study by Wu et al., 2011 and van Trijp et al., 2010, low prevalence of eta gene was found as compared to our study [19, 20]. Studies from different countries revealed that sea is the most common enterotoxin recovered followed by seb and sed and also distribution of different Staphylococcal enterotoxins (SE's) were found from the food poisoning outbreaks [21]. Studies from Korea by Lim et al., 2010, stated that Staphylococcal enterotoxin (seg, sei, sec) genes and toxic shock syndrome toxin (tsst) genes were most common in clinical MRSA isolates [22]. Thus from the above study it can be explained that the high frequency of exfoliative toxin (eta) which facilitated colonization and invasion may be due to wound infection and presence of enterotoxin pose a high risk of food borne intoxication.

Our study involved MRSA targeting virulence genes and showed 31% of the occurrence rate which was comparatively lower to the studies of Liang et al., 2019 (55%) and Koosha et al., 2013 (87.6%) [23, 24]. Current study observed sixty
three MRSA isolates of which 85.71% of the isolates were found to harbour enterotoxin A (sea) gene. This rate is found to be higher than the previous studies [25, 26].

Mobile genetic element (SCCmec) carries both resistance as well as virulence genes. Different SCCmec types carry different virulence genes. It has been found from the study of Lim et al., 2010 that 97.6% of SCCmec type II carried sec, seg, sei and tst gene; 73% of SCCmec type III strains carried sea gene and 89.7% of SCCmec type IV strain carried sec; seg; sei genes [22]. While our study reveals that 28.2% of SCCmec type II isolates carried sea, seb, cna, tst and eta genes, 10% of SCCmec type III carried sea, seb, cna and tst gene, 34.1% of SCCmec type V was found to carry sea, seb, ica, cna, tst eta and etb genes and 20% of SCCmec type VII carried sea, seb, cna, tst, eta and etb genes. However the rest isolates with SCCmec types I, IV, VI, VIII and XII showed less prevalence rate as compared to the above.

The study conducted by Liang et al., 2016 showed that ST239-SCCmecAll-t37 clone was more prevalent one as reported from china whereas our current study showed that ST2884-SCCmec type V was more predominant than the other sequence type [23].

This study involved a comparative analysis of virulence genes found to be prevailing in hospital, community as well as in environmental settings. It was recorded that majority of the isolates containing virulence gene were found in the environmental sources which is in contrast to the other studies where virulence genes were observed in clinical settings [16, 24] and underscores the risk of acting environmental sources as reservoir.

Limitations

This study warrants a proteomic approach to analyse the mode of transfer of virulence gene which may be from environment setting to the hospital and community origin and vice versa.

Abbreviation

SCCmec: Staphylococcal cassette chromosome mec; MLST: Multi-locus Sequence Typing; MRSA: Methicillin resistant Staphylococcus aureus

Declarations

Author’s contribution:

DB performed the experimental work, data collection and analysis and prepared the manuscript. SC and BJD analyzed the data. DDC have designed work plan and corrected manuscript. AB has conceived the plan and supervised the whole study. All authors ensured that this is the case. All authors read and approved the final manuscript.

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Competing interests:
The authors declare that they have no competing interests.

**Availability of data and materials:**

All the data generated in this research work are presented in this research article. In case of any additional information requirement corresponding author will be providing the necessary information as per ethical guidelines.

**Consent for publication:**

Not applicable.

**Ethics approval and consent to participate:**

The work was approved by Institutional Ethical committee of Assam University, Silchar. The authors confirm that participants provided their written informed consent to participate in this study.

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### Tables

**Table No. 1: Prevalence of virulence genes among different settings**
| Virulence Genes | No. of Isolates (n=63) |
|----------------|------------------------|
|                | Hospital | Community | Environment |
| sea            | 15 (23.8%) | 13 (20.6%) | 30 (47.6%) |
| seb            | 4 (0.63%) | 5 (0.79%) | 12 (19.04%) |
| eta            | 4 (0.63%) | 11 (17.46%) | 13 (20.63%) |
| etb            | 1 (0.16%) | - | 1 (0.16%) |
| ica            | 1 (0.16%) | 1 (0.16%) | - |
| cna            | 4 (0.63%) | 3 (0.47%) | 17 (26.98%) |
| tst            | 5 (0.79%) | 9 (1.42%) | 18 (28.57%) |

Table No. 2: Virulence gene profile of Methicillin resistant *Staphylococcus aureus* w.r.t SCCmec type
| Sl. No. | Virulence gene                  | SCC\text{mec} types |
|--------|---------------------------------|---------------------|
|        |                                 | Type I | Type II | Type III | Type IV(a) | Type V | Type VI | Type VII | Type VIII | Type XII |
| 1.     | Nuc+sea+seb+cna+tst+eta         | 1      | 1       | 1        | 1          |        |        |          |          |         |
| 2.     | Nuc+sea+seb+cna+tst             |        |         | 1        |            |        |        |          |          |         |
| 3.     | Nuc+sea+seb+tst+eta             |        |         |          | 1          |        |        |          |          |         |
| 4.     | Nuc+sea+cna+tst+eta             |        |         |          | 1          |        |        |          |          |         |
| 5.     | Nuc+sea+seb+cna                 |        |         | 1        | 1          |        |        |          |          |         |
| 6.     | Nuc+sea+cna+tst                 |        |         |          | 1          |        |        |          |          |         |
| 7.     | Nuc+eta+etb+tst                 |        |         | 1        |            |        |        |          |          |         |
| 8.     | Etb+ica+sea+nuc                 |        |         | 1        |            |        |        |          |          |         |
| 9.     | Sea+seb+cna+nuc                 | 1      | 1       | 1        | 1          | 1      | 1      |          |          |         |
| 10.    | Nuc+sea+seb+tst                 | 1      | 2       | 1        | 1          |        |        |          |          |         |
| 11.    | Nuc+eta+sea+seb                 | 1      | 1       | 1        | 1          | 1      | 1      |          |          |         |
| 12.    | Seb+ica+nuc                     |        |         |          | 1          |        |        |          |          |         |
| 13.    | Sea+seb+nuc                     | 1      | 3       | 1        | 1          | 1      | 1      | 1        | 1        |         |
| 14.    | Nuc+sea+cna                     | 1      | 2       | 1        | 3          | 1      | 2      | 1        | 1        | 1       |
| 15.    | Nuc+seb+cna                     | 1      | 1       | 1        | 1          | 1      | 1      |          |          |         |
| 16.    | Sea+tst+nuc                     | 1      | 5       | 2        | 1          | 1      | 1      | 1        | 1        | 1       |
| 17.    | Seb+tst+nuc                     | 1      | 2       | 1        | 1          | 1      | 1      |          |          | 1       |
| 18.    | Nuc+tst+cna                     | 1      | 1       | 1        | 1          | 1      | 1      | 1        | 1        | 1       |
| 19.    | Nuc+eta+sea                     | 1      | 4       | 1        | 3          | 1      | 1      | 1        |          |         |

**No. of Isolates**
|   |   |   |   |   |
|---|---|---|---|---|
| 20. | Nuc+eta+tst | 1 | 1 | 1 |
| 21. | Nuc+ica+seb |   |   | 1 |
| 22. | Nuc | 1 | 25 | 15 | 1 | 30 | 4 | 6 | 2 | 1 |
| 23. | Sea | 1 | 7 | 4 | 1 | 10 | 5 | 2 | 1 |
| 24. | seb | 1 | 3 | 2 | 1 | 5 | 1 | 1 |
| 25. | ica |   |   |   |   |   |   |   |   | 1 |
| 26. | cna | 1 | 2 | 1 |   | 3 | 1 | 2 | 2 | 1 |
| 27. | tst | 1 | 6 | 2 |   | 3 | 1 | 4 | 1 | 1 |
| 28. | eta | 1 | 5 |   | 1 | 5 | 2 | 4 | 1 |
| 29. | etb |   |   |   |   | 1 | 1 |   |   |   |