Resistant Strains of Enterotoxigenic *Staphylococcus aureus; Unknown Risk for Multiple Sclerosis Exacerbation*

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**Background:** Despite all advances in neurological sciences, there are unknown aspects in the epidemiology of multiple sclerosis (MS). Based on this hypothesis, the enterotoxigenic strains of *Staphylococcus aureus* (*S. aureus*) are possible risk factors for exacerbations of MS.

**Materials and Methods:** Two-hundred nasal swab samples were collected from non-MS (*n* = 80), MS stable (*n* = 60) and MS exacerbation (*n* = 60) groups. Samples were cultured and those that were *S. aureus*-positive were analyzed for the presence of enterotoxins, using polymerase chain reaction (PCR). Antimicrobial susceptibility was performed using disk diffusion method.

**Results:** Ninety out of 200 nasal samples (45%) were positive for *S. aureus*. The highest levels of nasal colonization were seen in MS exacerbation group (68.33%). The most commonly detected enterotoxins were sea (30.55%) and sec (11.11%). There were significant differences between *S. aureus* colonization and type of samples (P = 0.026) and, also, between type of samples and prevalence of enterotoxins (P = 0.022). The highest levels of enterotoxigenic genes were seen in MS exacerbation group. The *S. aureus* strains had the highest levels of resistance against tetracycline (80%), ampicillin (72.22%), methicillin (66.66%), erythromycin (66.66%), oxacillin (63.33%), trimethoprim-sulfamethoxazole (61.11%) and cotrimoxazole (55.55%).

**Conclusions:** Our findings should raise awareness about the role of *S. aureus* enterotoxins, in resistant strains of *S. aureus*, as a risk factor for MS exacerbation. It is better to keep MS patients away from polluted environments of hospitals and health centers.

**Keywords:** Multiple Sclerosis; Antibiotic Resistance; Iran; Enterotoxins; *Staphylococcus aureus*

1. **Background**

*Staphylococcus aureus* (*S. aureus*) is a significant human pathogen, which colonizes the anterior nares of 20% - 60% of humans (1, 2). The *S. aureus* strains can cause a number of diseases, ranging from skin and soft tissue infections to urinary and respiratory infections, myocardial endocarditis, pneumonia, endocarditis and osteomyelitis (1, 2).

Several of the *S. aureus* strains secrete a group of extra-cellular enzymes, which stimulate tissue extinction and dispersal and mitigate damaging toxins that cause catalytic effects on host cells and tissue damage (3, 4). The staphylococcal enterotoxins (SEs) are a group of low-molecular-weight single chain proteins that are similar in composition and biological activity, which differ in antigenicity (sea to sec) (5, 6).

Enterotoxigenic genes of *S. aureus* may be involved in the fundamental etiology of multiple sclerosis (MS). The MS is a chronic disease of the central nervous system, with incompletely known etiology (4, 7-9). It is widely accepted as a complex autoimmune disease, generally targeting young adults (10). Although discovery of the activation of CD4+ T immune cells represents a major step in disease pathology, the specific causes, responsible for activating this autoimmune disease, are unknown (10). Therefore, it is important to know the exact or potential mechanisms and also the risk factors of MS.

Although the possible role of *S. aureus*, in the occurrence of MS, is not entirely known, however, the presence of high levels of antibiotic resistance in the *S. aureus* strains increases the importance of this matter (11-17). According to the available data, almost 15% of *S. aureus* hospital infections were methicillin resistant (MRSA) (11-13) and between 20% - 70% of them were multi-drug resistant (14-17).

2. **Objectives**

As far as we know, there were scarce available data on the prevalence of *S. aureus* and its enterotoxins, in the cases of MS. Therefore, the present study was carried out to...
investigate the prevalence of \textit{S. aureus} in the nasal swabs of MS patients and, also, investigate the role of enterotoxigenic genes of this bacterium in the exacerbation of MS.

3. Materials and Methods

3.1. Samples and \textit{Staphylococcus aureus} Identification

From September 2013 to August 2014, a total of 200 nasal swab samples were collected from non-MS persons (n = 80), patients who had not experienced a relapse of MS in the past 6 months (MS stable group) (n = 60) and those who had suffered a relapse of MS within 30 days of study recruitment (MS exacerbation group) (n = 60). All samples were collected from the educational hospitals and private health centers of the Tehran province, Iran. The swab samples were rapidly transferred to the laboratory in cooler, with ice-packs.

Samples were directly cultured onto 7% sheep blood agar (Merck, Darmstadt, Germany) and incubated aerobically at 37°C, for 48 hours. After incubation, suspicious colonies were examined by the use of morphologies compatible with \textit{Staphylococcus} spp. (microscopical morphology, catalase and coagulase production). Studied colonies were cultured on tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and tryptic soy agar (TSA) (Merck, Darmstadt, Germany). After growth, \textit{Staphylococci} were identified on the basis of colony characteristics, Gram staining, pigment production, fermentation and the following biochemical reactions: catalytic activity, coagulated test (rabbit plasma), oxacilin broth, glucose O/F test, resistance to bacitracin (0.04 U), mannitol fermentation on mannitol salt agar (MSA) (Merck, Darmstadt, Germany), urease active, nitrate reduction, novobiocin resistance, phosphatase (de phosphoribonuclease (DNase) test) and carbohydrate (1% sucrose, trehalose and maltose, fructose, lactose, mannose) fermentation test (18).

3.2. Antimicrobial Susceptibility Test

The pattern of antimicrobial resistance was studied using the simple disk diffusion technique. The Mueller-Hinton agar (Merck, Darmstadt, Germany) medium was used for this purpose. Antibiotic resistance of \textit{S. aureus} strains against 16 commonly used antibiotics in the case of urinary tract infections was determined using the instruction of clinical and laboratory standards institute (CLSI) guidelines (19). Susceptibilities of \textit{S. aureus} isolates were tested against ampicillin (10 μg/disk), gentamicin (10 μg/disk), amikacin (30 μg/disk), imipenem (30 μg/disk), methicillin (30 μg/disk), tetracycline (30 μg/disk), vancomycin (5 μg/disk), ciprofloxacin (5 μg/disk), norfloxacin (30 μg/disk), cotrimoxazole (30 μg/disk), clindamycin (2 μg/disk), trimethoprim-sulfamethoxazole (25 μg/disk), penicillin G (10 μg/disk), oxacillin (1 μg/disk), erythromycin (15 μg/disk) and azithromycin (15 μg/disk) antibiotic agents (Oxoid, Basingstoke, UK). The plates containing the discs were allowed to stand for at least 30 minutes before incubation at 35°C for 24 hours. The diameter of the zone of inhibition produced by each antibiotic disc was measured and interpreted using the CLSI zone diameter interpretative standards (19). \textit{S. aureus} ATCC 25923 and \textit{Escherichia coli} ATCC 25922 were used as quality control organisms in antimicrobial susceptibility determination.

3.3. DNA Extraction and Polymerase Chain Reaction Confirmation

Total genomic DNA was extracted from the bacterial colonies. A single colony was inoculated in 5 mL of brain heart infusion broth and incubated overnight at 37°C. Then, 1.5 mL of a 24 hours culture were harvested with centrifugation, after 5 minutes, at 14000 rpm. The cell pellet was resuspended in 200 μL of lysis buffer (40 mM Tris-acetate, 1 M, 20 mM sodium-acetate, 1 mM ethylene-diaminetetraacetic acid, 1% sodium dodecyl sulphate) by vortexing and pipetting. To remove most proteins and cell debris, 66 μL of 5M NaCl solution were added and mixed well, and then, the viscous mixture was centrifuged at 12000 rpm for 10 minutes, at 4°C. After centrifugation, the clear supernatant into a new eppendorf tube, and the final volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely formed. Following centrifugation at 14000 rpm for 5 minutes, the supernatant was then removed to another eppendorf tube and double volume of 100% ethanol was added. The tubes were inverted five to six times, gently, and then centrifuged at 10000 rpm, for 5 minutes. The supernatant was discarded and 1 mL of ethanol (70%) was added to the pellet, and tubes were centrifuged at 10000 rpm, for 5 minutes. Finally, the supernatant was discarded and the pellet was dried for 10 minutes at room temperature, after which it was resuspended by 100 μL H2O. The stock was kept at -20°C until use. The DNA concentration has been determined by measuring absorbance of the sample at 260 nm using spectrophotometer (20).

Presence of \textit{S. aureus} in each DNA sample was confirmed using the Banada et al. method (21). The polymerase chain reaction (PCR) reaction mix consisted of 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.001% (w/v) gelatin) with 4 mM MgCl2, 250 mM of each nucleotide (deoxynucleoside triphosphate), 0.5 mM of each primer (forward and reverse), 4 ng of the molecular beacon and 4 U of Jumpstart Taq DNA polymerase (Fermentas, Vilnius, Latvia).

3.4. Polymerase Chain Reaction Amplification for Enterotoxigenic Genes

The PCR method was used to study the distribution of
sea, seb, sec, sed, see, seg, seh, sei, and sej enterotoxins of the S. aureus (3, 22-24). Oligonucleotide primers, annealing temperature, PCR programs and size of products are shown in Table 1. A programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device was used in all PCR reactions. All runs included a negative DNA control, consisting of PCR grade water and strains of S. aureus ATCC 13565 (sea), ATCC 14458 (seb), ATCC 19095 (sec), FRI 361 (sed, seg, sei, and sej), ATCC 27664 (see) and FRI 137 (seh) that were used as positive controls.

3.5. Statistical Analysis
The results were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) for significant relationship between incidences of enterotoxigenic genes of S. aureus isolated from the nasal swab samples of non-MS, MS stable and MS exacerbation groups. The chi-square test and Fisher’s exact two-tailed test analysis were performed in this study. Statistical significance was considered at a P < 0.05.

3.6. Ethical Considerations
The present study was accepted by the ethical committees of the educational Hospitals. Written informed consent was obtained from all of the study patients or their parents.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence (5’ – 3’)</th>
<th>PCR Product, bp</th>
<th>Annealing Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>sea</td>
<td>F TTGGAAACGGTTAAAACGAA</td>
<td>120</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R GAACCTTCCCATCAAAAACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seb</td>
<td>F TCGCATCAAACGTCAAAACG</td>
<td>478</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R GCAGGTACTCTATAAGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sec</td>
<td>F GACATAAAAGCTAGGCTTT</td>
<td>257</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R AAATCGGATTATTTATTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sed</td>
<td>F CTTTTGCCGAAATCTCCTT</td>
<td>371</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R TACATCATCTATTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>see</td>
<td>F AGGTACACCCTAGGTACATCC</td>
<td>209</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R TTTTTCGCCGGCAGATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seg</td>
<td>F GTGACATTTTGCCGGTTCC</td>
<td>287</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R AGAACCATCAAACCTGTATAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seh</td>
<td>F GTCTATATGGAGTTAACAAC</td>
<td>213</td>
<td>46.4</td>
</tr>
<tr>
<td></td>
<td>R GACCTTACTTATTTGCGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>454</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R GGTGATATTGGTAGTACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>142</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R CATCGAAGGCTGCTTCCAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR programs: one cycle at 94°C, for 5 minutes; 30 cycles at 94°C for 2 minutes, 72°C for 1 minute and one cycle at 72°C, for 5 minutes.

PCR volume, 50 μL: 5 μL PCR buffer, 10X 1.5 mM MgCl₂, 200 μM dNTP (Fermentas), 0.5 μM of each primers F (forward) and R (reverse), 1.25 U Taq DNA polymerase (Fermentas), 2.5 μL DNA template.
4. Results

The study enrolled 200 patients, 80 in the non-MS, 60 in the MS stable groups and 60 patients in the MS exacerbation group. Of 200 nasal swabs collected for this study, 90 (45%) were positive for *S. aureus* (Table 2), with significant differences identified between MS exacerbation group and non-MS group (*P* = 0.019) and also, between MS stable groups and non-MS group (*P* = 0.032). On the other hand, the most commonly infected group was MS exacerbation group (68.33%).

Distribution of enterotoxigenic genes of the *S. aureus* strains of various studied groups is shown in Table 3. Results of the gel electrophoresis for enterotoxigenic genes of the *S. aureus* are shown in Figures 1 - 4. The most commonly detected enterotoxins were *sea* (30%), *sec* (15.55%), and *seb* (11.11%). There were no positive results for *see* and *seh* enterotoxins. Significant differences were seen between the prevalence of *sea* and *seg* (*P* = 0.015), *sea* and *sei* (*P* = 0.018), *sea* and *sed* (*P* = 0.033), *sec* and *seg* (*P* = 0.035) and *seb* and *sei* (*P* = 0.041) genes. Significant differences were also seen for the prevalence of *sea* gene, between MS exacerbation and MS stable groups (*P* = 0.020) and also, between MS exacerbation and non-MS groups (*P* = 0.023). In addition to *sea*, there were significant differences for the prevalence of *sec* gene between MS exacerbation and MS stable groups (*P* = 0.039).

Antibiotic resistance pattern of *S. aureus* isolated from various studied groups is shown in Table 4. We found that the *S. aureus* strains of MS exacerbation group had the highest levels of resistance to various types of antibiotics (*P* = 0.028). The *S. aureus* isolates of our investigation had the highest levels of resistance against tetracycline (80%), ampicillin (72.22%), methicillin (66.66%), erythromycin

**Table 2. Distribution of Staphylococcus aureus in Various Studied Groups**

<table>
<thead>
<tr>
<th>Studied Groups of Patients</th>
<th>No. Samples Collected</th>
<th>No. of Positive Samples a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-MS group</td>
<td>80</td>
<td>19 (23.75)</td>
</tr>
<tr>
<td>MS stable group</td>
<td>60</td>
<td>30 (50)</td>
</tr>
<tr>
<td>MS exacerbation group</td>
<td>60</td>
<td>41 (68.33)</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>90 (45)</td>
</tr>
</tbody>
</table>

a Values are presented as No. (%).

**Table 3. Distribution of Enterotoxigenic Genes of Staphylococcus aureus in Various Studied Groups a**

<table>
<thead>
<tr>
<th>Studied Groups of Patients</th>
<th>No. of Positive Samples</th>
<th>sea</th>
<th>sec</th>
<th>sed</th>
<th>see</th>
<th>seg</th>
<th>seh</th>
<th>sei</th>
<th>sej</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-MS group</td>
<td>19</td>
<td>1 (5.26)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MS stable group</td>
<td>30</td>
<td>3 (10)</td>
<td>1 (3.33)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MS exacerbation group</td>
<td>41</td>
<td>22 (53.65)</td>
<td>10 (24.39)</td>
<td>3 (7.31)</td>
<td>1 (2.43)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>27 (30)</td>
<td>10 (11.11)</td>
<td>14 (15.55)</td>
<td>4 (4.44)</td>
<td>1 (1.11)</td>
<td>1 (1.11)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

a Values are presented as No.

**Figure 1. Results of the Gel Electrophoresis for Identification of Entero-toxigenic Genes in Staphylococcus aureus Strains**

![Image](M: 100 bp DNA ladder (Fermentas, Vilnius, Latvia), lines 1 - 4: positive samples for sed (317 bp), seb (478 bp), sea (120 bp) and sec (257 bp), lines 5 - 8: positive controls and line 9: negative control.)
Figure 3. Results of the Gel Electrophoresis for Identification of Enterotoxigenic Genes in Staphylococcus aureus Strains

M: 100 bp DNA ladder (Fermentas, Vilnius, Latvia), lines 1: positive samples for seh (213 bp), line 2: positive controls and line 3: negative control.

Figure 4. Results of the Gel Electrophoresis for Identification of Enterotoxigenic Genes in Staphylococcus aureus Strains

M: 100 bp DNA ladder (Fermentas, Vilnius, Latvia), lines 1: positive samples for seg (287 bp), line 2: positive controls and Line 3: negative control.

Table 4. Antibiotic Resistance Pattern of Staphylococcus aureus Isolated From Various Studied Groups a,b

<table>
<thead>
<tr>
<th>Type of Samples</th>
<th>No. of Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-MS group</td>
<td>19 (52.63)</td>
</tr>
<tr>
<td>MS stable group</td>
<td>30 (66.66)</td>
</tr>
<tr>
<td>MS exacerbation</td>
<td>41 (85.36)</td>
</tr>
<tr>
<td>Total</td>
<td>90 (72.22)</td>
</tr>
</tbody>
</table>

Values are presented as No. (%).

5. Discussion

The results of our investigation showed that resistant strains of enterotoxigenic S. aureus may be the risk factors for MS exacerbation. We found that the total prevalence of S. aureus in the nasal swab samples of non-MS, MS stable and MS exacerbation groups were 23.75%, 50% and 68.33%, respectively. The high prevalence of S. aureus in the MS exacerbation group may be due to the fact that these patients are more frequently under treatment with immunosuppressing drugs. Therefore, the levels of immunity in this group of patients are reduced and several infections, like S. aureus, will occur. Higher prevalence of enterotoxigenic genes and antibiotic resistance were
also seen in the S. aureus strains of the MS exacerbation group. Mulvey et al. (4), in a similar study, which was conducted in Canada, showed that the total prevalence of S. aureus in non-MS, MS stable and MS exacerbation groups were 30%, 21.2% and 27.3%, respectively, which was entirely different from our results. Probably the S. aureus isolates of their investigation were related to the host. In fact, they showed that there is no difference in the host-pathogen interaction, making MS patients more susceptible to colonization with S. aureus, and this finding is related to those of Hu et al. (25).

The S. aureus may carry toxigenic genes, which can act as superantigens that trigger large numbers of CD4+ cells and have been involved in various autoimmune diseases, including MS, rheumatoid arthritis and Wegener’s granulomatosis (26). As far as we know, the present study is the first prevalence report of enterotoxigenic S. aureus in the anterior nares of MS patients, in Iran. We found that the total prevalence of enterotoxigenic genes in the S. aureus strains of MS exacerbation patients were higher than those of MS stable and non-MS patients. The total prevalence of sea, seb, sec, sed, seg and sei genes in the S. aureus isolates of MS exacerbation patients were 30%, 11.11%, 15.55%, 4.44%, 1.11% and 1.11%, respectively. Higher prevalence of superantigens and enterotoxigenic genes, in the patients who suffered from MS, were reported previously by Mulvey et al. (4), Franca et al. (7) and Kumar et al. (27).

The identification of S. aureus harbored enterotoxins and especially sea and sec, as a possible risk factor for MS exacerbations, increases the possibility of new treatment choices for managing this disease. Potential antimicrobial decolonization regimes, which have been successfully to decolonize individuals with MRSA in the hospital and community scenarios, could be used in MS patients, colonized with S. aureus (4). We found that all the S. aureus strains of our study were resistant to more than three antibiotics and the prevalence of resistance against methicillin was 66.66%, which was high, especially for patients who suffered from MS. Our results also indicate that the total prevalence of antibiotic resistance in the S. aureus strains of MS exacerbation group, against ampicillin, gentamicin, tetracycline, cotrimoxazole, trimethoprim-sulfamethoxazole, oxacillin and erythromycin were 85.36%, 70.73%, 85.36%, 92.68%, 70.73%, 75.60%, 73.39% and 72.80%, respectively, which was compared with those of MS stable and non-MS patients. Of the studies that have been conducted in this field, in Iran, all have shown high levels of antibiotic resistance of S. aureus in the studied antimicrobial agents of our investigation (24-29,33).

Although S. aureus enterotoxins and superantigens have been recognized as risk factors in other immunological diseases, like rheumatoid arthritis and Wegener’s granulomatosis, this is one of the first studies examining the potential association of enterotoxigenic genes in the S. aureus isolates of nasal swabs and the potential association with MS exacerbations. The data presented in this study seem to highlight the need for a more extensive trial, to better define the role of the colonization of S. aureus containing sea, seb, sec, sed, seg and sei enterotoxigenic genes, and their potential role in the etiology of MS. In the current setting of Iran, prescription of antibiotics, especially in the cases of MS, should be done based on the results of disk diffusion method. The results of our investigation showed that prescription of imipemcin and vancomycin, due to their low levels of antibiotic resistance, is effective.

Authors’ Contributions
Farzad Mehrabi: writing and editing, the main concept. Ali Asgari: submission and revision.

References


