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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 epidemiol[illegible] of multiple sclerosis (MS). Based on this hypothesis, the enterotoxigenic strains of *Staphylococcus aureus* (*S. aureus*) are possible risk factors for exacerbations of MS.
Objectives: The present study was carried out to investigate the role of resistant strains of enterotoxigenic *S. aureus* in MS exacerbation.
Materials and Methods: Two-hundred nasal swab samples were collected from non-MS (n = 80), 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 [illegible].
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 [illegible] 55%) and *seb* (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.[illegible]%), erythromycin (66.66%), oxacillin (63.33%), trimethoprim-sulfamethoxazole (61.11%) and cotrimoxazole (55.55%).
Conclusions: Our findings should raise awareness about the role of *sea* and *seb* enterotoxins, in resistant strains of *S. aureus*, as a risk factor for MS exacerbation. It is better to keep MS patients away from pollut[illegible] environments of hospitals and health centers.

Keywords: Multiple Sclerosis; Antibiotic Resistance; Iran; Enterotoxin; *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, life-threatening endocarditis, pneumonia, endocarditis and osteomyelitis (1, 2).

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

Enterotoxigenic genes of the *S. aureus* can activate a high proportion of T cells, due to their ability to bind to both major histocompatibility complex (MHC) molecules in antigen-presenting cells and specific V-β regions, in the T-cell receptor (4, 7). This activation results in the polyclonal stimulation of T cells and an increased production of proinflammatory cytokines (4, 7). Based on the controversial theories and the results of previous studies, the 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 $CD4^+$ Th1 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 *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 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 *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, staphylococci were identified on the basis of colony characteristics, Gram staining, pigment production, hemolysis and the following biochemical reactions: catalytic activity, coagulated test (rabbit plasma), oxidase test, glucose O/F test, resistance to bacitracin (0.04 U), mannitol fermentation on mannitol salt agar (MSA) (Merck, Darmstadt, Germany), urease activity, nitrate reduction, novobiocin resistance, phosphatase, deoxyribonuclease (DNase) test and carbohydrate (x) sucrose, trehalose and maltose, fructose, 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 *S. aureus* strains against 16 commonly used antibiotics in the cases of urinary tract infections was determined using the instruction of clinical and laboratory standards institute (CLSI) guidelines (19). Susceptibilities of *S. aureus* isolates were tested against ampicillin (10 u/disk), gentamycin (10 μg/disk), amikacin (30 u/disk), imipenem (30 u/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 u/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 standard (19). *S. aureus* ATCC 25923 and *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 cultured culture were harvested with centrifugation at 5 minutes, at 14000 rpm. The cell pellet was resuspended and lysed in 200 μL of lysis buffer (40 mM Tris-acetate, pH 7.8, 20 mM sodium-acetate, 1 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulphate) by vigorous 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 transferring the clear supernatant into a new eppendorf tube, an equal 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 H_2O. 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 *S. aureus* in each DNA sample was confirmed using the Banada et al. method (21). The polymerase chain reaction (PCR) reaction mix consisted of 1 X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.001% (w/v) gelatin) with 4 mM $MgCl_2$, 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 1. The Oligonucleotide Primers and the Polymerase Chain Reaction Programs Used for Amplification of Enterotoxins of *Staphylococcus aureus* Isolates of Nasal Swabs [a,b]

Target Gene	Primer Sequence (5' – 3')	PCR Product, b	Annealing Temperature, °C
sea		120	50
F	TTGGAAACGGTTAAAACGAA		
R	GAACCTTCCCATCAAAAACA		
seb		478	50
F	TCGCATCAAACTGACAAACG		
R	GCAGGTACTCTATAAGTGCC		
sec		257	50
F	GACATAAAAGCTAGC TT		
R	AAATCGGATTAA TTA		
sed		317	50
F	CTAGTTTGGTAATA CCT		
R	TA CTATATCTTATAGGG		
see		209	50
F	AGGT AGGTCATCC		
R	TTTTTCTTCGGTCAATC		
seg		287	55
F	GTAGACATTTTTGGCGTTCC		
R	AGAACCATCAAACTCGTATAGC		
seh		213	46.4
F	GTCTATATGGAGGTACAACACT		
	GACCTTTACTTATTTCGCTGTC		
sei		454	50
F	GGTGATATTGGTGTAGGTAAC		
R	ATCCATATTCTTTGCCTTTACCAG		
sej		142	50
F	CATCAGAACTGTTGTTCCGCTAG		
R	CTGAATTTTACCATCAAAGGTAC		

[a] 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.
[b] PCR volume, 50 μL: 5 μL PCR buffer, 10X 1.5 mM $Mgcl_2$, 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 = 0.02). 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

Studied Groups of Patients	No. Samples Collected	No. of Positive Samples [a]
Non-MS group	80	19 (23.75)
MS stable group	60	30 (50)
MS exacerbation group	60	41 (68.33)
Total	[illegible]	90 (45)

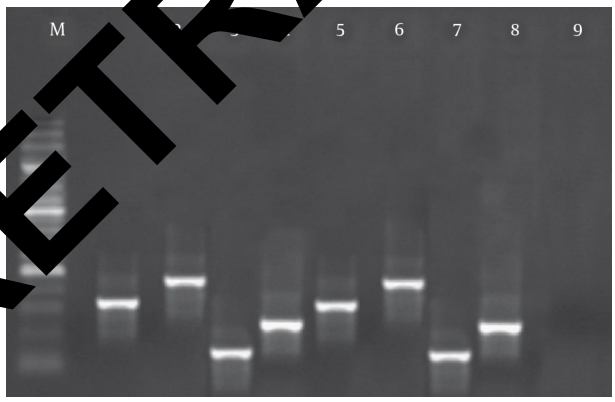
[a] Values are presented as No. (%).

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

Studied Groups of Patients	No. of Positive Samples	*sea*	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*sej*
Non-MS group	19	1 (5.26)	1 (5.26)	1 (5.26)	-	-	-	-	-	-
MS stable group	[illegible]	[illegible]	3 (10)	3 (10)	1 (3.33)	-	-	-	-	-
MS exacerbation group	41	22 (53.65)	6 (14.63)	10 (24.39)	3 (7.31)	-	1 (2.43)	-	1 (2.43)	-
Total	90	27 (30)	10 (11.11)	14 (15.55)	4 (4.44)	-	1 (1.11)	-	1 (1.11)	-

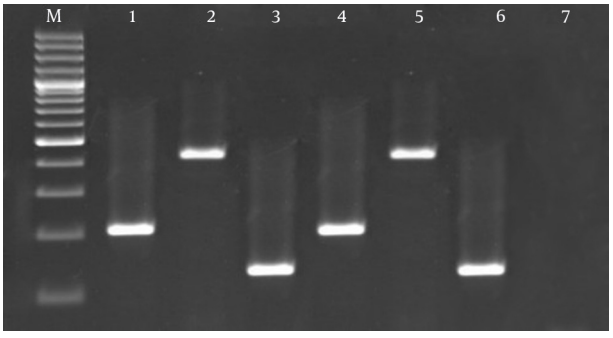
[a] Values are presented as No.

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



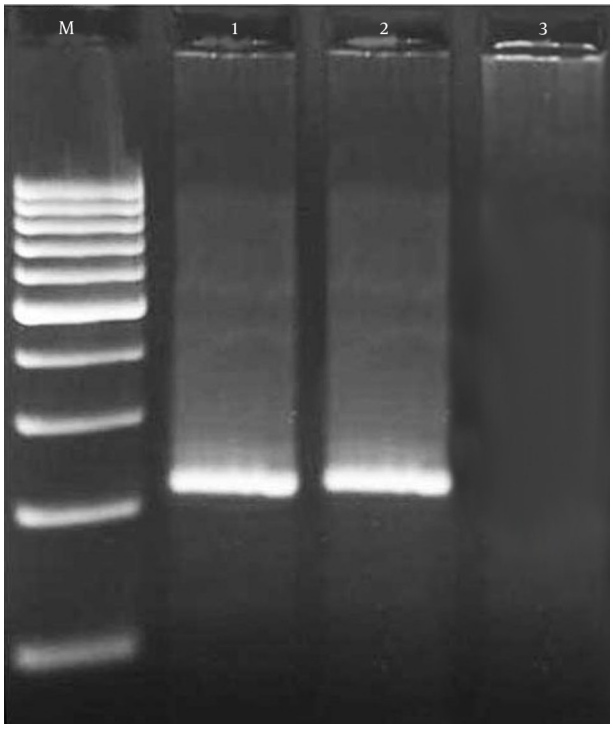
M: 100 bp DNA ladder (Fermentas, Vilnius, Latvia), lines 1 - 4: positive samples for *sed* (317 bp), *seb* (478 bp), *sea* (120 bp) and *sec* (257 bp), lines 5 - 8: positive controls and line 9: negative control.

Figure 2. Results of the Gel Electrophoresis for Identification of Enterotoxigenic Genes in *Staphylococcus aureus* Strains



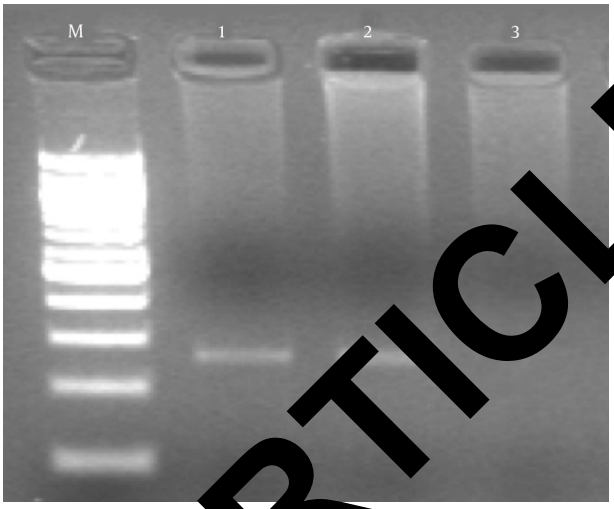
M: 100 bp DNA ladder (Fermentas, Vilnius, Latvia), lines 1 - 3: positive samples for *see* (209 bp), *sei* (454 bp) and *sej* (142 bp), lines 4 - 6: positive controls and Line 7: negative control.

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 sam s for *seh* (213 bp), line 2: positive controls and line 3: negative contro

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



M: 100 bp DNA ladder (Ferm , Vilnius, Latvia), lines 1: positive samples for *seg* (287 bp), li 2: positive rols and Line 3: negative control.

(66.66%), oxa lin %), trimethoprim-sulfamethoxazole (61.11%) a trimoxazole (55.55%). The most effective antibiotics re imipenem and vancomycin. Signifi- ferences were seen between bacterial resistance against tracycline and vancomycin (P = 0.019), ampicillin and i penem (P = 0.017), tetracycline and clindamycin .026), tetracycline and azithromycin (P = 0.032) and xacillin and vancomycin (P = 0.035).

Table 4. Antibiotic Resistance Pattern of *Staphyl s aureus* ed From Various Studied Groups [a,b]

Type of Samples	No. of Positive Samples	AM10	Gen10	Amk30	Imp30	Met30	Tet30	VAN	Cip5	Nor	Cotr	Clin	TM/SuL	Pen10	Ox	Em15	Az15
Non-MS group	19	10 (52.63)	6 (31.57)	5 (26.31)	5.26)	8 (4)	12 (63.15)	2 (10.52)	5 (26.31)	3 (15.78)	8 (42.10)	4 (21.05)	9 (47.36)	4 (21.05)	8 (42.10)	8 (42.10)	3 (15.78)
MS stable group	30	20 (66.66)	12 (40)	10 33.	3 ( )	17 (56.66)	22 (73.33)	5 (16.66)	8 (26.66)	7 (23.33)	13 (43.33)	8 (26.66)	15 (50)	9 (30)	15 (50)	16 (53.33)	9 (30)
MS exacerbation group	41	35 (6)	29 (73)	(5)	9 (21.95)	35 (85.36)	38 (92.68)	8 (19.51)	12 (29.26)	12 (29.26)	29 (70.73)	15 (36.58)	31 (75.60)	25 (60.97)	30 (73.17)	36 (87.80)	18 (43.90)
Total	9	(22)	47 (72.22)	39 (43.33)	13 (14.44)	60 (66.66)	72 (80)	15 (16.66)	25 (27.77)	22 (24.44)	50 (55.55)	27 (30)	55 (61.11)	38 (42.22)	57 (63.33)	60 (66.66)	30 (33.33)

[a] In th able 10 = ampicillin (10 u/disk), Gen10 = gentamycin (10 μg/disk), Amk30 = amikacin (30 u/disk), Imp30 = imipenem (30 u/disk), Met30 = meth illin (30 μg/disk), Tet30 = tetracycline (30 μg/disk), VAN = vancomycin (5 μg/disk), Cip5 = ciprofloxacin (5 μg/disk), Nor = norfloxacin (30 μg/disk), C = cotrimoxazol 0 μg/disk), Clin = clindamycin (2 μg/disk), TM/Sul = trimethoprim-sulfamethoxazole (25 μg/disk), Pen10 = penicillin G (10 u/disk), x = ox in (1 μg/disk), Em15 = erythromycin (15 μg/disk) and Az15 = azithromycin (15 μg/disk).
[b] Va s are pr sented as No. (%).

4. 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 in MS exacerbations, increases the possibility of new treatment choices for managing this disease. Potential antimicrobial decolonization regimes, which have been used successfully to decolonize individuals with MRSA in the hospital and community scenery, could be used in MS patients, colonized with *S. aureus* (28). 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%. It 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, gentamycin, penicillin, tetracycline, cotrimoxazole, trimethoprim-sulfamethoxazole, oxacillin and erythromycin, were 85.36%, 70.73%, 85.36%, 92.68%, 70.73%, 75.60%, 73.17% and 87.80%, 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* against the studied antimicrobial agents of our investigation (14, 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 imipenem 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.

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