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Full Length Research Paper

The frequency of slime, adhesin and methicillin resistance genes among staphylococci isolated from nasal samples of multiple sclerosis patients

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It was aimed to determine the carriage rate of *Staphylococcus aureus* and the occurrence of methicillin resistance, slime and adhesin genes in staphylococcal strains isolated from the nasal cavities of multiple sclerosis (MS) patients. The presence of *mecA* and *femA*, and the genes implicated in adhesion were determined by multiplex PCR in all strains. The *femA* gene was detected in 46.6% of 105 MS patients. While 18.1% of isolates carried the *mecA* gene, 81.9% of isolates were negative for the *mecA* gene. The presence of *icaA/icaD* genes was determined in a total of 84.8% of all strains. While 85.7% of *Staphylococcus epidermidis* isolates were positive in terms of slime genes, this ratio was determined as 81.6% among the *Staphylococcus aureus* strains. The occurrence of *clfA* gene was determined in 29 of 49 (59.2%) *S. aureus* isolates. Also, 45 out of 49 (91.8%) *S. aureus* was found to carry the *fnbA* gene. The carriage rate of the *cna* gene was determined in 40 (81.6%) isolates among the 49 *S. aureus* strains. The rate of methicillin resistance gene, slime production and the frequency of adhesin genes in MS patients were also significantly higher than the healthy control population. Determination of the nasal *S. aureus* carriers and the virulence of these strains will be important for prediction of the MS prognosis in these patients. And treating these *S.aureus* carriers will be very useful in preventing MS relapses.

Key words: Multiple sclerosis, *Staphylococcus aureus*, nasal carriage, methicillin resistance, adhesins, slime.

INTRODUCTION

Multiple sclerosis is a chronic, autoimmune disease that affects the central nervous system. The etiology of multiple sclerosis is unknown. It is believed that CD4 + Th1 cells play an important role in the occurrence of the disease. It is characterized as lymphocyte and macrophage infiltration in patients with active lesions in the brain (Rio et al., 2011).

Genetic predisposition, T cell activation and environmental factors such as various infections have been implicated in the initiation of the autoimmune disease. Microbial diseases are known to be the important cofactors in autoimmune disease initiation and persistence (Wucherpfennig, 2001). It was reported that the presence of autoreactive T cells for the development of MS is one of the most important factors (Mikulkova et al., 2011). Infectious agents are reported to be responsible for the activation of autoreactive T cells. In previous studies, it has been shown that multiple sclerosis exacerbations are frequently associated with bacterial and viral infections (Oikonen et al., 2011; Tselis, 2011).

Infections caused by *S. aureus* are a growing public health problem in our country as well as in other

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countries of the world. *S. aureus* is one of the major pathogens which can cause various human infections such as skin and soft tissue infections, and serious infections (pneumonia, bloodstream infections, and bone and joint infections) (Gorwitz et al., 2008).

The resistance to antibiotics in staphylococci has emerged as a major public health problem. Especially, the methicillin resistance is reported to be extremely important in terms of human health. Most of staphylococcal infections in humans are caused by methicillin-resistant staphylococci (MRS). Nasal carriage of methicillin-resistant *S. aureus* (MRSA) is reported to be an important risk factor for staphylococcal infections (Sedighi et al., 2011).

It is shown in various studies about *S. aureus* that nasal colonization can cause an infection when the host-pathogen balance is disturbed (Seybold et al., 2011). Since MS patients have T lymphocyte function disorder they are considered in immunocompromised or non-immunocompetent group. For this reason, it is very important to identify the MS patients colonized with *S. aureus*. The knowledge of the bacteria and their virulence is very critical for the prevention of infections, since one of the main reasons which aggravate the MS attacks is infection.

Limited studies were conducted in MS patients on nasal *S. aureus* carriage in the literature. Previously, in a study, it was showed that the rate of nasal carriage of *S. aureus* in patient with MS was higher than in healthy persons (Mulvey et al., 2011). Therefore, the nasal carriage and subsequent dissemination of *S. aureus* in multiple sclerosis patients is extremely important for triggering multiple sclerosis relapses.

It was reported that bacterial virulence factors have an important immunostimulatory capacity which can induce relapses of autoimmune diseases (Schiffenbauer et al., 1998). Adhesions [*clfA* (clumping factor A), *fnbA* (fibronectin-binding protein), *cna* (collagen binding protein)] and slime production in *S. aureus* were shown to be important bacterial virulence factors in a variety of studies (Duran et al., 2010a). The isolates carrying the adhesin and slime genes have been reported to cause serious infections. Because of the dysregulated immunity in MS patients, infections caused by virulent bacterial strains in MS patients may cause serious consequences. In these patients, such infections can trigger MS attacks. Therefore, the determination of nasal carriage and virulence determinants related with infection severity in MS patients may help in preventing MS relapses.

Because of the dysregulated immune balance in multiple sclerosis, we think that the occurrence of adhesins, slime production and methicilline resistance in *S. aureus* strains may mediate its pathogenicity. Taking these into consideration, the aim of this study was to determine the carriage rate of *S. aureus* and the occurrence of methicillin resistance, slime and adhesin genes in these strains isolated from the nasal cavities of MS patients.

MATERIALS AND METHODS

Patients and bacteriological specimens

This study was carried out between March 2009 and June April 2011 in Department of Microbiology and Clinical Microbiology, Medical Faculty of Mustafa Kemal University, Hatay. This study was conducted with 105 MS patients who had the diagnosis of „definite MS" according to Poser and McDonald"s diagnostic criteria and a control group including 40 healthy individuals (Polman et al., 2005).

Nasal swap samples were taken from the nasal cavities of each patient and healthy person for the isolation of staphylococci. A total of 56 *Staphylococcus epidermidis* and 49 *S. aureus* isolates were included the study. Informed consent was obtained from all participants and the study protocol was approved by the ethics committee. The identification of all staphylococci was made according to conventional microbiological criteria. The identification of all staphylococcal isolates was done on the basis of colony morphology, Gram staining, biochemical tests such as catalase and coagulase tests.

DNA isolation

For this purpose, all staphylococcal isolates were subcultured in Brain-heart infusion broth obtained from Merck (Merck, Germany) overnight with shaking at 37°C. The DNA isolation procedure was carried out as previously described by Johnson et al. (1991). In accordance with this procedure, staphylococcal strains were harvested from the cultures by centrifugation at 3.000 × g for 10 min.

Then the cell pellet was resuspended with PBS (phosphate-buffered saline) containing lysostaphin (100 µg/ml) (Sigma, USA), and incubated at 37°C for 30 min. Nucleic acid extraction was made using the classic phenol/chloroform extractions from all staphylococcal samples, and the DNA was precipitated with ethanol. The precipitate was dissolved in 50 µl of TE buffer (10 mM 10 mM Tris-Cl; 1 mM EDTA [pH 8.0], and stored at -20°C until processing.

PCR detection of *mecA* and *femA* genes

Specific primers sequences for the *mecA* and *femA* genes were selected from the study of Mehrotra et al. (2000) (Table 1). The PCR amplification of the genes was performed in a 25 µl reaction mixture. The PCR was performed under the following parameters: The PCR mixture containing 2.5 µl 10x standard reactive buffer [(without $MgCl_2$) (Promega Corp.)]; 200 µM of each deoxynucleoside triphosphate [AB Gene, UK], 3 mM $MgCl_2$; 20 pmol of primers for *femA* and *mecA*, and approximately 10 ng of template DNA, and brought up to a 25 µL final volume with distilled water. Reactions were started for 5 min at 94°C and placed on ice, and 1 U of *Taq* polymerase (Fermentas, USA) was added. Reaction mixtures were subjected to 35 PCR cycles (94°C for 2 min, 2 min at 55°C and 1 min at 72°C). A final elongation step at 72°C for 7 min was also applied in a thermal cycler (Bioder/Thermal Blocks xp cycler, Tokyo Japan).

PCR detection of *icaA* and *icaD* genes

Specific primers sequences for the *icaA* and *icaD* genes were selected from the study of Vasudevan et al. (2003) and Cramton et al. (1999), respectively (Table 1). The amplification of the *icaA* and *icaD* genes in all staphylococcal isolates was detected by multiplex PCR assay. PCR and amplification conditions was achieved as in

Table 1. The oligonucleotide sequence and predicted sizes used in the multiplex PCRs.

Gene	Primer	Oligonucleotide sequence (5'-3')	Size of amplified product (bp)
femA	fem A-1	AAAAAAGCACATAACAAGCG	132
	fem A-2	GATAAAGAAGAAACCAGCAG	
mecA	mec A-1	ACTGCTATCCACCCTCAAAC	163
	mec A-2	CTGGTGAAGTTGTAATCTGG	
clf A	clf A-1	CCGGATCCGTAGCTGCAGATGCACC	1000
	clf A-2	GCTCTAGATCACTCATCAGGTTGTTCAGG	
fnb A	fnb A-1	GATACAAACCCAGGTGGTGG	191
	fnb A-2	TGTGCTTGACCATGCTCTTC′	
cna	cna-1	AAAGCGTTGCCTAGTGGAGA	192
	cna-1	AGTGCCTTCCCAAACCTTTT	
ica A	ica A-1	CCTAACTAACGAAAG GTAG	1315
	ica A-2	AAGATATAGCGATAA GTGC	
ica D	ica D-1	AAACGTAAGAGAGGT GG	381
	ica D-2	GGCAATATGATCAAG ATAC	

our previous study (Duran et al., 2010a). The existence of the *icaA* and *icaD* genes was investigated in staphylococcal strains isolated from the MS patients and healthy control groups and the reference strains. To determine the expected bp lengths (381, for the *icaD*, 1315, for the *icaA* bp), DNA marker with defined molecular weights in the range 100 to 2000 and reference strain were used. The PCR products were analyzed in a 2% (w/v) agarose gel in 1xTAE buffer (40 mmol/L, Tris-acetate, 1 mmol/L EDTA). Ethidium bromide (0.5 µg/mL TAE)-stained DNA amplicons were visualized using a gel imaging system (Wealtec, Dolphin-View, USA).

PCR detection of *fnbA*, *cna* and *clfA* genes

The oligonucleotide primers for the *fnbA*, *cna* and *clfA* genes were selected from the study of Arciola et al. (2005) and McDevitt et al. (1995) (Table 1). The PCR amplification was carried out as in our previously study (Duran et al., 2010a).

Slime production

For the determination of slime production, negative and positive controls were studied at the same time. For this purpose, the slime-producing reference strain of *S. epidermidis* (ATCC 35984) and non-slime-producing reference strain of *S. epidermidis* (ATCC 12228) were selected as control strains. The slime production of the strains was evaluated by the CRA (Congo Red Agar) plate test as previously described by Freeman et al. (1989).

Statistical analysis

All data were analyzed with the Chi-square test, comparing the frequency of virulence genes (slime production, mecA gene, and adhesins) in staphylococci both the MS patients and healthy control group. The p value <0.05 was considered significant. The statistical analyses in the present study were done by using Statistical Package for Social Sciences (SPSS1 for Windows V. 11.5, Chicago, USA) software.

RESULTS

A total of 105 MS patients and 60 healthy persons were included to this study. It was found that the isolation rate of *S. aureus* was 46.7% (49/105), and the isolation rate of *S. epidermidis* was 53.3% (56/105). As for the healthy control group, the nasal carriage rate of *S. aureus* was found to be 20% (12/60) in this group.

In this study, we investigated the rates of the adhesin genes (*fnbA*, *cna* and *clfA*), methicilline resistance gene and the slime genes (*icaA* and *icaD*) and phenotpically slime production on CRA in all staphylococcal isolates.

The presence of *icaA*/*icaD* genes was determined in 84.8% (89/105) of all strains. The ratio of slime genes was 85.7% (48/56) among the *S. epidermidis* and 81.6% (40/49) among the *S. aureus* isolates (Figure 1). There was no statistically significant difference between *S. epidermidis* and *S. aureus* isolates in terms of the presence of slime genes (p>0.05).

As for healthy control group, the presence of *icaA*/*icaD* genes was determined in 35 isolates of 60 (58.3%) strains. The ratio of slime genes was 56.3% (27/48) among the *S. epidermidis* and 58.3% (7/12) among the *S. aureus* isolates. Similarly to MS patients groups, it was not observed a statistically significant difference between

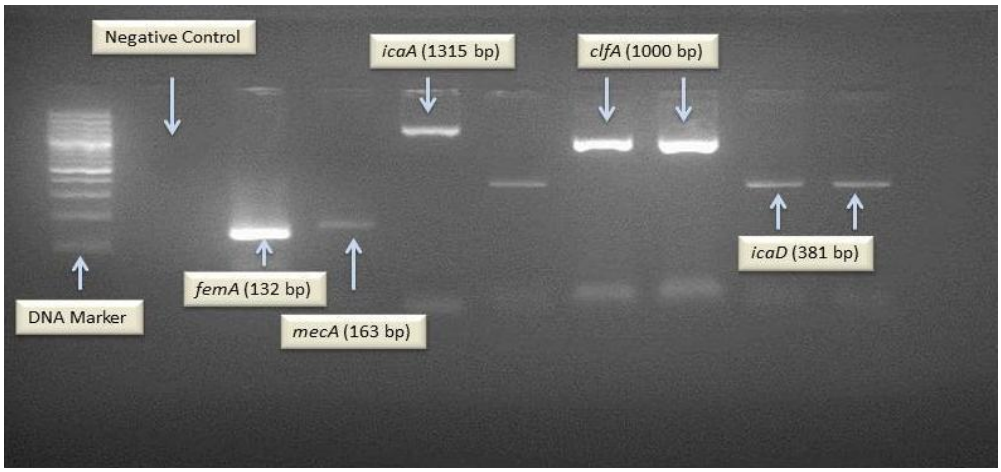

Figure 1. Ethidium bromide-stained multiplex PCR products after gel electrophoresis for the *mecA* and *femA*, *icaA*, *icaD* and *clfA* genes. Lane 1: DNA molecular size marker (100 bp ladder), Lanes 2: Negative control, Lane 3; *femA* (132 bp), Lane 4: *mecA* (163 bp), Lane 5: *icaA* (1315 bp), Lane 6, 9 and 10 (381 bp): *icaD*, Lane 7 and 8: *clfA* (1000 bp).

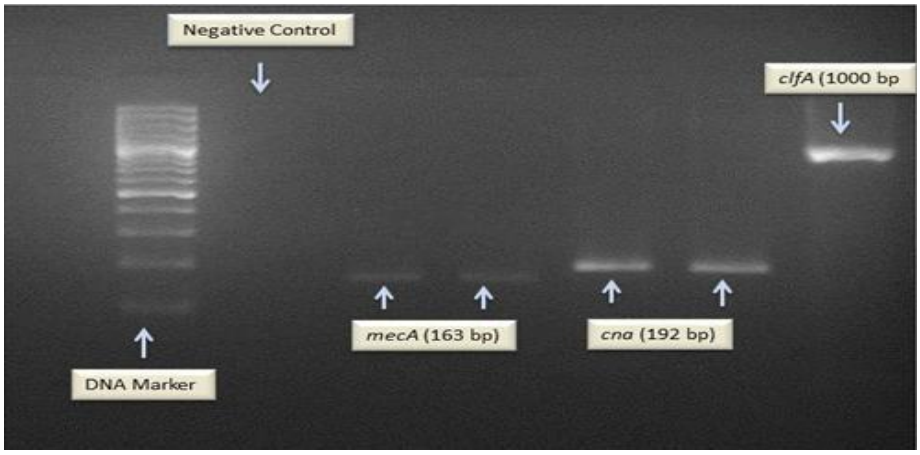

Figure 2. Ethidium bromide-stained multiplex PCR products after gel electrophoresis for the mecA, cna and clfA genes. Lane 1: DNA molecular size marker (100 bp ladder), Lanes 2: Negative control, Lane 3 and 4: mecA (163 bp), Lane 5 and 6: cna (192 bp), Lane 7: clfA (1000 bp).

S. epidermidis and *S. aureus* isolates in terms of the presence of slime genes (p>0.05).

The phenotypic expression of slime production was evaluated by Congo red agar plate test. Slime production in the isolated strains which have both *icaA* and *icaD* genes was as follows: of the 105 strains**,** slime production was found in 87 (82.9%, 87/105) isolates (Figure 2). This ratio was determined as 80.4% (45/56) and 77.6% (38/49) in *S. epidermidis* and *S. aureus* isolates, respectively.

Frequency of the *femA* and the *mecA* genes

In this study, it was found that a total of 49 (46.6%; 49/105) isolates (*femA*-positive) were *S. aureus* and that the other 56 (53.3%; 56/105) (*femA*-negative) were *S. epidermidis* (Figure 1). While a total of 19 (18.1%; 19/105) isolates carried the *mecA* gene (methicillin resistance gene), 86 of 105 (81.9%) isolates were negative for *mecA* gene. Whilst 8 of 49 (16.3%) *S. aureus* strains carried the *mecA* gene and 12 of 56 (21.4%) were found in *S. epidermidis* isolates.

The frequency of adhesins genes (*fnbA, cna and clfA*)

In the present study, the occurrence of *clfA* gene was determined in 29 of 49 (59.2%) *S. aureus* isolates (Figure 4). Also, 45 out of 49 (91.8%) *S. aureus* isolates analyzed

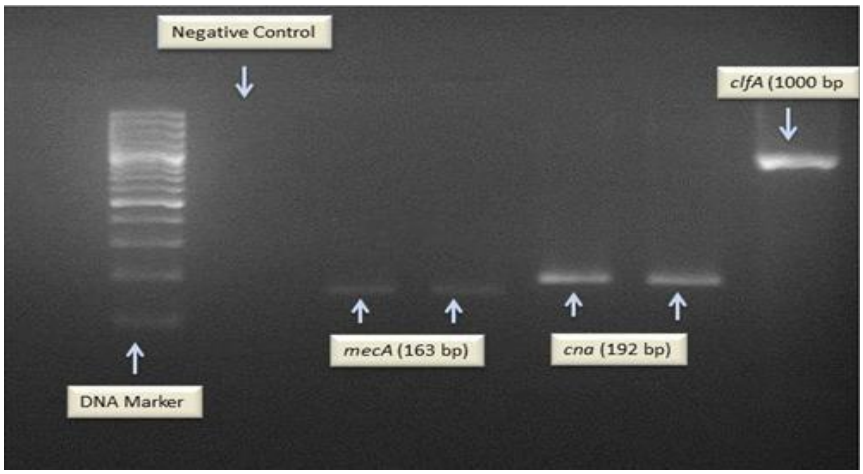

Figure 3. Multiplex PCR amplification products by agarose gel electrophoresis for the fnbA (191 bp) gene. Lane 1: DNA molecular size marker (100 bp ladder), Lanes 2: Negative control, Lane 3 and 4: fnbA (191 bp).

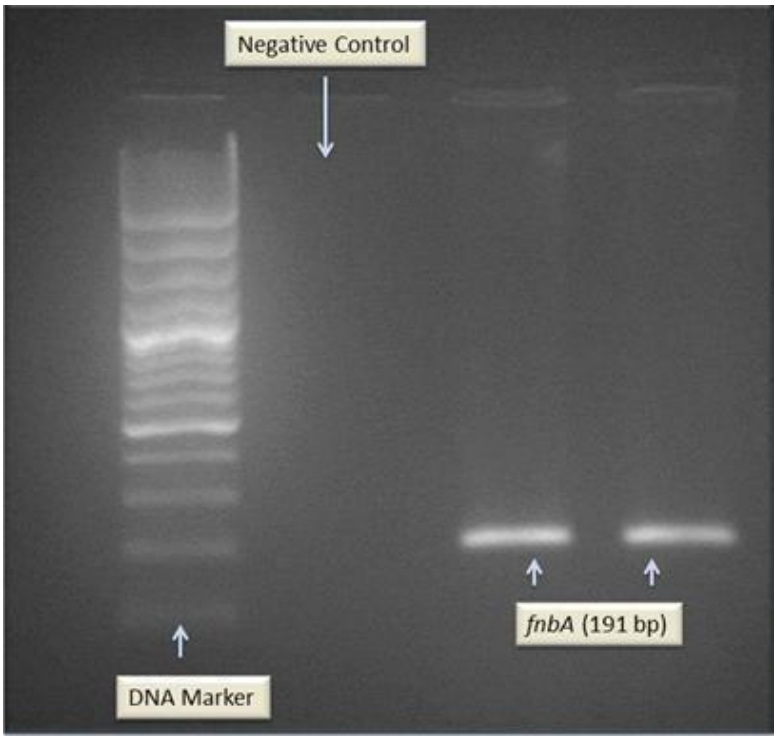

Figure 4. Multiplex PCR amplification products by agarose gel electrophoresis for the *fnbA* (191 bp) gene. Lane 1: DNA molecular size marker (100 bp ladder), Lanes 2: Negative control, Lane 3 and 4: *fnbA* (191 bp).

were found to carry the *fnbA* gene. The carriage rate of the *cna* gene was determined in 40 (81.6%) isolates among the 49 *S. aureus* strains. The frequency of the *clfA, fnbA* and *cna* genes was determined as 33.3 (4/12), 58.3 (7/12) and 50% (6/12), respectively in the healthy control group (Figures 3 and 4).

DISCUSSION

The front of the nasal mucosa in humans is the most important area for the colonization of *S. aureus*. The detection and treatment of nasal *S. aureus* carriers in immunocompromised persons is vital. Any infection with

the microorganisms in their nasal mucosa via autoinoculation may have serious consequences. It is very important to know the nasal *S. aureus* carriage in MS patients with T-cell dysfunction. To identify methicilline resistance, adhesins and slime genes that are associated with microorganism virulence in immunocompromised patients such as MS is exremely important (Mikulkova et al., 2011; Sedighi et al., 2011).

The rate of *S. aureus* nasal carriage in immunocompromised patients was found to be statistically higher than in the healthy population. In a study conducted by Ghasemian et al. (2010), the infection rates in immunocomprimised hemodialysis patients was found to be higher than in non-carriers (Ghasemian et al., 2010). Since MS patients have impaired T lymphocyte function, they are also considered in immunocompromised group.

In literature, there are a very limited number of studies conducted on *S. aureus* nasal carriage in multiple sclerosis patients. Furthermore, we could not find any study where the methicillin resistance, slime production, slime and adhesin genes were investigated in staphylococcal strains isolated from MS patients. Conducting such a study in MS patients is important to emphasize staphylococcal infections (Mikulkova et al., 2011).

In various studies, the correlation has been reported to be a relationship between the frequency of MS attacks and infections. The colonization of highly pathogenic *S. aureus* strains (methicillin resistant, slime producing and containing various adhesion genes) in these patients is a potential risk for infection and, it may also cause an MS attack (Tselis 2011; Duran et al., 2010a; Bach, 2005).

In a study conducted by Mulvey" et al. (2011) the rate of nasal *S. aureus* carriage was found to be higher in MS patients than in the healthy population. In our previous study, the rate of *S. aureus* carriage was also higher in the hemodialysis patients correlated with Mulvey" et al. (2011) and Duran et al., (2006). In the present study, nasal carriage rate was significantly higher in the MS patients compared with the healthy (control) population. *S. aureus* carriage rate was 46.6% in MS group while healthy group had only 20% carriers. The nasal carriage rate in MS patients was found statistically significantly higher than in the control group ($p< 0.01$).

Nasal *S. aureus* carriage is the most important cause of occurrence and spreading of staphylococcal infections. The numbers of studies conducted on *S. aureus* nasal carriage in patients with MS are very limited. There is also a very rare study which focuses on methicillin resistance, adhesine and slime genes in this patient group. To know the rate of *S.aureus* carriage and to determine the virulence genes with genotypic methods instead of phenotypic ones will be an important progress revealing the disease progression in MS patients. It is a well known fact that MS attacks are aggravated with infections (Gorwitz et al., 2008; Sedighi et al., 2011).

Determination of nasal *S. aureus* carriage and methicillin resistance rates in isolated strains by nasal swaps cultures has been reported to be important (Ammerlaan et al., 2011). Methicillin resistance in *S.aureus* strains also causes resistance to some antibiotics which makes the treatment and control of the infections of this microorganism. The *mecA* gene is responsible for methicillin resistance in staphylococci. PB2a is encoded by *mecA* gene and almost all MRSA isolates produces an additional penicillin binding protein called PB2a. PB2a has a lower affinity to beta-lactams than PB2. Since MRSA isolates have lower affinity to PB2a, they are also resistant to beta-lactam antibiotics. It was reported that the methicillin resistance may vary from region to region in various studies. It can even differ in various clinics of the same hospital. For example, MRSA nasal colonization in a study from the USA was observed in 3.0% of outpatients (Jernigan et al., 2003). In our country, a very low prevalence (1%) of MRSA nasal carriage was reported, although the MSSA nasal carriage rate was 8.8% among outpatients (Arabaci and Oldacay, 2008; Erdenizmenli et al., 2004). In this study, a total of 18.1% of the staphylococcal strains isolated from nasal swab culture of MS patients was carrying the *mecA* gene which was responsible for methicillin resistance.

In a study conducted in 2011, the correlation between superantigen-producing *S. aureus* strains and multiple sclerosis relapses were investigated in MS patients with *S.aureus* nasal carriers. It was determined that *S. aureus* strains isolated from MS patients were found to be more virulent than the strains isolated from the healthy control group. In various studies, a significant correlation between the virulent *S. aureus* strains and MS relapses was reported (Mulvey et al., 2011).

There are a number of factors that can play a role for a triggering infection. Among these factors, microbial factors, host factors and environmental factors can be listed. It is considered that there are various virulence factors belonging to the bacteria which play a role in the development of the infection caused by bacteria. Bacterial adhesins were reported to be one of the most important factors initiating the infection in the host. One of the most important virulence factors is slime production in microorganisms. Slime factors play the important role among the microbial factors in staphylococci. Bacterial slime is a glycocalyx substance that consists of 40% carbohydrate and 27% protein. It has an extremely high antigenic structure. It is known that slime producing strains are more virulent and resistant to antibiotics. Besides, slime factor also supresses the cellular immune response, and inhibits neutrophil chemotaxis and phagocytosis (Duran et al., 2010b; Montanaro et al., 1999).

In our study, phenotypically slime production was detected in only 87 of the 105 (82.9%) staphylococcal isolates *in vitro*. A total of 89 (84.8%; 89/105) isolates were found to possess the *icaA/icaD* genes. In the healthy group, a total of 23 (57.5%; 23/40) isolates were

positive for the *icaA/icaD* genes, all of these isolates produced slime on CRA agar, phenotypically. There was a statistically significant difference between these two groups (MS and healty control group) in terms of slime production ($p<0.01$).

Structure of the cell wall, capsule, surface proteins and enzymes are the major factors which determine virulence of the staphylococci. Collagen, fibronectin binding proteins and clumping factor are surface proteins with similar chemical structure and location. These proteins are the most important factors for the colonization of the staphylococci to the host tissues (Marraffini et al., 2006).

Cna is an important adhesin protein that helps the attachment of microorganism to host cell surface. It was shown to be in various studies that *cna* gene was an important virulence factor in pathogenesis of septic arthritis and bone tissue infections (Kouidhi et al., 2010; Elasri et al., 2002). In our study, it was found that 81.6% of MS patients carried this gene. It was determined that the frequency of the *cna* gene was significantly higher in strains isolated from MS patients compared to the healthy population (50%) ($p<0.01$).

Colonization of *S. aureus* to host is the most important factor affecting the pathogenesis of the diseases. Staphylococci have some cell surface components on the cell surface. These cell surface components have an important role in the virulence of the microorganism. Cell surface components have roles in binding to host cell, occurrence of tissue damage in host tissue and in the protection against the phagocytic activity of neutrophils (Vergara-Irigaray et al., 2009; Sutter et al., 2011; Higgins et al., 2006). *ClfA* is a surface adhesin protein of *S. aureus* and increases the bacterial pathogenicity by binding of bacterial cells to fibrinogen (Hair et al., 2008).

It was found that *clfA* gene inhibited phagocytosis in the absence of fibrinogen, and showed enhanced inhibition in the presence of fibrinogen (Higgins et al., 2006). In our previous study, the *clfA* was shown to be an important virulence protein in *S.aureus* strains. In that study, we identified the *clfA* gene in more than half of *S.aureus* strains isolated from the wounds samples. In the present study, the occurrence rate of the *clfA* gene was detected as 59.2% in MS patients. The frequency of the *clfA* gene in MS patients was statistically significantly higher than in the control group (33.3%) ($p<0.01$). The findings of this study about the *clfA* gene were consistent with the earlier reported studies (Duran et al., 2010b; Campoccia et al., 2009).

As for *fnbA* proteins, they are one of the main virulence factors of *S. aureus* cell wall (Schwarz-Linek et al., 2006). It is also reported that the expression of the *fnbA* gene also increases the biofilm production (O'Neill et al., 2009).

As reported in the literature, the *fnbA* gene was found to be very frequent in *S. aureus* strains. In a similar study carried out in 2011, we almost determined the *fnbA* gene in *S.aureus* strains isolated from the patients treated in the orthopedic unit. Similarly in this study, the *fnbA* ratio was found as 91.8% in all patients included the study.

There was a statistically significant difference between MS patients and the control group ($p<0.001$).

In this study, *S. aureus* carriage rate in MS patients was found to be statistically significantly higher than in the healthy control population. The rate of methicillin resistance gene, slime production and frequency of adhesin genes were also significantly higher in strains isolated from the MS patients. Determination of the nasal *S. aureus* carriers and the virulence of these strains will be important for prediction of the MS prognosis in these patients and treating these *S. aureus* carriers will be very useful in preventing MS relapses.

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