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Assessment of Staphylococcal Toxins Acting as Superantigens in Different Nasal Specimens in The Etiology of Chronic Rhinosinusitis

Farklı Klinik Örneklerde Saptanan ve Kronik Rinosinüzit Etiyolojisinde Süperantijen olarak Görev Yapan Stafilokokal Toksinlerin Değerlendirilmesi

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ABSTRACT

The mechanism of development of chronic rhinosinusitis(CRS) is not fully known. However, bacteria are thought to play an important role in this clinic. It has been suggested that toxins with superantigen(SAgs) properties produced by one of these bacteria, Staphylococcus aureus(*S.aureus*), play a role in the development of inflammation associated with sinusitis. In this study, *S.aureus* was isolated by taking endoscopic sinus biopsy samples and nasal swab samples from patients with CRS and the control group. It was aimed to examine the frequency of *S.aureus* presence in the samples taken, the presence of toxin genes showing superantigen quality in these isolated bacteria, and to evaluate the roles of these parameters in the development of CRS. More *S.aureus* was isolated in the samples taken from patients with CRS than in the control group. The isolated *S.aureus* samples were analysed by real-time PCR method. The presence of enterotoxin A, B, C and D genes in the *S.aureus* samples isolated from the patient group were found at the rates of 54%, 32%, 16% and 16%, respectively, while these rates were 46%, 24%, 14% and 14% in the control group. The Toxic Shock Syndrome Toxin-1(TSST-1) gene was detected in 20% of the samples isolated from the patient and 46% in the control group bacteria. The fact that *S.aureus* was isolated in 20% of the patients shows that this bacterium is not necessary for CRS. The frequency of superantigen toxin genes in *S.aureus* isolates shows that these toxins are not necessary for the development of the disease.

Key Words

S. aureus, enterotoxin, toxic shock syndrome toxin-1, chronic rhinosinusitis.

ÖZ

Kronik rinosinüzitin gelişim mekanizması tam olarak bilinmemektedir. Bununla birlikte bakterilerin bu klinikte önemli rol oynadıkları düşünülmektedir. Bu bakterilerden biri olan Staphylococcus aureus (*S.aureus*) tarafından oluşturulan süperantijen özelliği gösteren toksinlerin sinüzit ile ilişkili inflamasyonun gelişiminde rol oynadığı ileri sürülmektedir. Bu çalışmada, kronik rinosinüzitli hastalardan ve kontrol grubundan, endoskopik sinüs biyopsi örnekleri ile burun sürüntüsü örnekleri alınarak *S.aureus* izole edilmiştir. Alınan örneklerde *S. aureus* bulunma sıklığı, izole edilen bu bakterilerde süperantijen niteliği gösteren toksin genlerinin varlığının incelenmesi ve bu parametrelerin kronik rinosinüzit gelişimindeki rollerinin değerlendirilmesi amaçlanmıştır. Kronik rinosinüziti olan hastalardan alınan örneklerde, kontrol grubuna göre daha fazla *S.aureus* üretilmiştir. Üretilen *S.aureus* örnekleri real-time PCR yöntemiyle incelenmiştir. Hasta grubundan izole edilen *S.aureus* örneklerinde, enterotoksin A, B, C ve D gen varlığı sırasıyla % 54, % 32, %16 ve %16 oranlarında bulunurken kontrol grubunda bu oranlar % 46, % 24, %14 ve %14 olarak bulunmuştur. TSST-1 geni, hasta grubundan izole edilen örneklerde % 20, kontrol grubu bakterilerinde ise % 46 oranında saptanmıştır. Hastaların % 20'sinde *S.aureus* izole edilmiş olması bu bakterinin kronik rinosinüzit kliniği oluşumunda mutlak gerekli olmadığını göstermektedir. Süperantijen niteliği gösteren toksin genlerinin *S.aureus* suşlarında bulunma sıklığı bu toksinlerin hastalık gelişiminde mutlak gerekli olmadığını göstermektedir.

Anahtar Kelimeler

S. aureus, enterotoksin, toksik şok sendromu toksini-1, kronik sinüzit.

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INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease that affects the nasal cavity and paranasal sinuses. This disease is among the most common chronic respiratory tract diseases affecting humans [1,2]. CRS is a multifactorial inflammatory disorder whose precise pathogenesis remains unknown. Various immunopathological processes with persistent inflammation in the nasal mucosal surface lead to CRS [1]. CRS reduces patients' quality of life and is highly prevalent worldwide. This disease lasts at least four weeks, results in the usage of antibiotics for an extended period and requires high treatment costs. Antibiotic treatment does not always yield a complete cure, and sometimes surgery may be required too. The pathophysiology of chronic rhinosinusitis should be well-defined to develop more effective treatments. However, up to date, despite its impact on a societal and individual level, the etiology of CRS remains still unclear. In recent years, research has focused on the multifactorial interactions of host and environmental factors that determine susceptibility to CRS [3]. Many factors, such as allergy, upper respiratory tract infections, immunodeficiency, and some environmental effects (smoking, air pollution), play a role in transforming the repetitive acute sinusitis picture into the chronic phase [4,5]. Within these factors, bacteria were claimed to play an essential role in the etiology of CRS. A previous study reported that *S. aureus* nasal colonization was detected in 67% of patients with CRS with nasal polyps [6, 7]. *S. aureus* is a bacterium with many enzymes and toxins being virulence factors. It has been claimed that Staphylococcal enterotoxins, a subgroup of Staphylococcal superantigen (SAgs) [8,9] and Toxic Shock Syndrome Toxin-1 (TSST-1) may be the cause of the development of chronic sinusitis resulting inflammation on nasal mucosa [5,10-15]. *S.aureus* is the most virulence staphylococcus species. The pathogenesis of staphylococcal infections depends on the production of surface proteins (such as collagen-binding protein, fibronectin-binding protein, clumping factor) that allow the bacteria to adhere to host tissues and the release of extracellular proteins such as specific toxins and hydrolytic enzymes [16]. The term 'superantigen' is justified when contrasting the few T cells that react to conventional antigens (one of 104–105) with up to 20% of T cells that can be activated by a SAgs [16,17]. SAgs are characterized by their ability to activate human T cells, regardless of the T cell's antigen specificity. SAgs achieve this property by linking directly to the major histocompatibility complex class II (MHC-II) on antigen-presenting cells with TCR, provided that an appropriate T cell receptor β-chain variable (TCRVβ) element is present. Thus, SAgs can bypass the strict requirements for T cell antigen recognition, namely antigen uptake and processing by an antigen-presenting cell followed by presenting the resulting peptides on MHC molecules [8,18].

In this study, the prevalence of *S. aureus* was determined in the biopsy samples of the patients with chronic rhinosinusitis compared to the control group, which has no CRS but carries *S. aureus*. It also aims to investigate the enterotoxin A, B, C, D, and TSST-1 gene expressions of these isolated *S. aureus* isolates.

MATERIALS and METHODS

Patients' specification

Adult patients who applied to Gazi University Hospital Otorhinolaryngology (ORL) polyclinic with the symptoms like nasal congestion, nasal flow, postnasal flow, headache, and sneeze were included in the study where 712 patients were examined. Among them, 240 patients were clinically and radiological diagnosed with CRS. Nasal swabs were taken from all these patients with rhinosinusitis and investigated microbiologically regarding the existence of *S. aureus*. Besides, patients with chronic rhinosinusitis were investigated using endoscopic surgery biopsy sampling for the existence of *S. aureus* as well. Functional endoscopic sinus surgery operation was done by the Messerklinger technique [19]. This technique has been applied to only a limited number of patients; 50 *S. aureus* isolates obtained from these patients by both surgical technique and traditional methods were included in the study. The remaining 472 patients were used as the control group. Biopsy samples were put in the tubes, including 0,9% NaCl and brought to the microbiology laboratory. Nasal swab samples were transported to the laboratory with species containing carrier medium. The existence of enterotoxin A, B, C, and D and toxin gene TSST-1 were investigated by real-time PCR technique in the total amount of 100 *S. aureus* isolates isolated both from patients with chronic rhinosinusitis (n=50) and the control group (n=50).

Ethics Committee Approval

This study was approved by Gazi University ethics committee with the decision number 095. Consent forms were obtained from all patients participating in the study.

Culture and Identification of S.aureus

Biopsy samples were homogenized by squeezing in a sterilized tube (with glass beads) inside 1 ml of physiological saline using a sterilized glass stick. Later the samples were collected from the top-level liquid using a 0.5 µl calibrated bacteriological loop and inoculated into the 5% sheep blood agar plate. Inoculated plates were incubated at 37° C for 24 to 48 hours. Growing bacteria were investigated for colony morphology, gram stain property, hemolysis property, catalase activity, and other biochemical properties. The pure culture was made if the bacterium were thought to be staphylococcus. The coagulase activity of the growing staphylococcus was investigated using a tube coagulase test. The identification reliability of the bacteria, which the classical methods can identify, was confirmed using a gram-positive bacteria identification kit (API Staph. Bio-Merioux, France). *S. aureus* isolates were stored at -20° C in tryptic soy broth.

Investigating the Existence of Toxin Gene

The existence of TSST-1 and enterotoxin A, B, C, and D genes in *S. aureus* isolates were investigated using the real-time polymerase chain reaction (Real-time PCR) technique [14,20].

DNA Isolation

S.aureus isolates in tryptic soy broth culture solution were centrifuged at 4000 rpm at 4°C for 5 minutes and washed two times with the washing solution having diethyl pyrocarbonate (DEPC)-treated water molecular biology grade (Merck&Co). 100 µl from each *S. aureus* sediment were transferred to other Eppendorf tubes. 0.8 mg silica beads were added to the tube and vortexed for 5 minutes. 100 µl lysis buffer (TRIS 10nM, EDTA 10 nM, Nacl 50 nM, SDS 2%) and 20 µl Proteinase-K (100 mg/ml) were added to the samples in the Eppendorf tube, and tubes were incubated for 2 hours at 65°C. Denaturation of Proteinase-K was achieved by applying a heat block at 95°C for 5 minutes. Then the tubes were centrifuged at 10000 rpm for 5 minutes. 150 µl was taken from the top-level liquids, including DNA, and put into the clean Eppendorf tubes, and 150 µl phenol-chlorophorm-isoamilalcohol (25:24:1) mixture was added to the tubes. After stirring well and centrifuging at 14000 rpm for 5 minutes, the top clean liquids were transferred to other Eppendorf tubes without touching the muddy sediment. 200 µl chloroform-isoamyl alcohol (24:1) mixtures were added to the tubes and vortexed gently. Again, the tubes were centrifuged at 14000 rpm for 5 minutes, and the top clean liquids were transferred to clean Eppendorf tubes. 100% ethanol was added at a rate of 1:2.5 to the liquids and washed. After gentle vortexing, the tubes were centrifuged for 20 minutes at 14000 rpm. A straw removed the alcohol at the top of the tubes. The tubes were washed twice by adding 200 µl 70% ethanol. The samples were dried to remove the remaining alcohol, and 50 µl water plus DEPC- treated water were added to the final product DNA. Purified DNA was calculated by spectrometer from the OD260 channel and Qubit fluorometric system (Invitrogen).

Identification of toxin genes by Real-Time PCR.

Real-Time PCR was made by LightCycler® 480 Multiwell Plate 96 equipment. In this study, forward and reverse primers were used for each gene to investigate the gene existence of enterotoxins A, B, C, and D and TSST-1 toxin genes. 16S rRNA gene array was used as a calibration sample to normalize the internal control gene and input quantities. Primers used by the guidance of the literature knowledge are shown in Table 1 [21,22].

LightCycler® 480 SYBR Green I Master kit was used for real-time PCR. 10 µl master mix, 1 µl (5 ng) forward primer, 1 µl (5 ng) reverse primer, 3 µl water and 5 µl from each DNA sample were added to each hole of the microplate according to the kit instruction. PCR was conducted by these steps: Denaturation phase: 5 minutes, 95°C. Amplification: 1 minutes, 95°C. Binding: 1 minutes, 55°C. Extension: 5

RESULTS and DISCUSSION

S. aureus was isolated from six biopsy samples from 30 patients (20%) while not isolated from any of the biopsy samples from control group. *S. aureus* was isolated from 44 (21%) of the 210 nasal swabs from patients with CRS. *S. aureus* was isolated from 50 (11%) nasal swabs of the 442 people in the control group without CRS. Much more *S. aureus* was isolated from the biopsy samples and nasal swabs of the patients with CRS compared to the control group, and the difference was statistically significant ($p<0,05$). Identification rates of *S. aureus* isolated from the patient and the control groups are given in Table 2.

Table 1. The primers used.

For enteretoxin A
SEA1: TTGGAAACGGTTAAAACGAA,
SEA2: GAACCTTCCCATCAAAAACA,
For enteretoxin B
SEB1: TCGCATCAAACTGACAAACG,
SEB2: GCAGGTACTCTATAAGTGCC,
For enteretoxin C
SEC1: GACATAAAAGCTAGGAATTT,
SEC2: AAATCGGATTAACATTATCC
For enteretoxin D
SED1 : CTAGTTTGGTAATATCTCCT,
SED2: TAATGCTATATCTTATAGGG,
For Toxic Shock Syndrome Toxin-1
TSST1: TGTAGATCTACAAACGATAATATAAAGGAT,
TSST2: ATTAAGCTTAATTAATTTCTGCTTCTATAGTT
For 16s rRNA
16sF: CCGCCTGGGGAGTACG,
16sR3: AAGGGTTGCGCTCGTTGC

Table 2. *S. aureus* identification rates from patient and control group samples

S.aureus	Positive	Negative
Biopsy sample		
Patient group n:30	6 (20%)	24(80%)
Control group n:30	0 (0%)	30(100%)
Nasal swab		
Patient group n:210	44 (21%)	166(79%)
Control group n:442	50 (11%)	392(89%)
Total samples		
Patient group n:210	50 (21%)	190(79%)
Control group n:442	50 (11%)	422(89%)

The presence of the genes of enterotoxin A, B, C, and D found by Real-Time PCR analyses from the *S. aureus* isolates isolated from the patients with CRS and the control group is given in Table 3. The enterotoxin A gene was detected in 54% of the *S.aureus* (27/50) isolated from the patients, and 46% (23/50) of *S.aureus* from the control group also exhibited its presence. Within the patient group, genes of toxin A were found more frequent, but between the patient and control groups, it was found to be not statistically significant. In the *Staphylococci* isolated from the patient group, enterotoxin B existed at 32 % (16/50), and in the control group bacteria, it was found at 24 % (12/50). At the same time, enterotoxin C and D were found in 16 % isolates of *S. aureus* (8/50). On the other hand, in 14% isolates of the *S. aureus* (7/50) isolated from the control group, enterotoxin C and D were found.

The results were statistically evaluated with the Chi-Square test. According to the Chi-Square test, these four types of enterotoxins were not statistically significant between the patient and control groups (p>0,2).

While TSST-1 gene was identified in 10 (20%) of the 50 *S. aureus* isolates obtained from the patients' group, TSST-1 gene was identified in 23 (46%) of the 50 *S. aureus* isolates obtained from the control group. More TSST-1 gene existence was identified from the bacteria isolated from the control group, and the difference was statistically significant (p<0,2). The prevalence of the TSST-1 gene in the *S. aureus* isolates obtained from the patients with CRS and the control group is given in Table 4.

Table 3. Presence of Enterotoxin genes in *S. aureus* isolates.

Enterotoxins			
Enterotoxin A	Positive	Negative	
Patient group n:50	27 (54%)	23 (46%)	(p>0,2)
Control group n:50	23 (46%)	27 (54%)	
Enterotoxin B			
Patient group n:50	16 (32%)	34 (68%)	(p>0,2)
Control group n:50	12 (24%)	38 (76%)	
Enterotoxin C			
Patient group n:50	8 (16%)	42 (84%)	(p>0,2)
Control group n:50	7 (14%)	43 (86%)	
Enterotoxin D			
Patient group n:50	8 (16%)	42 (84%)	(p>0,2)
Control group n:50	7 (14%)	43 (86%)	

Table 4. Presence of TSST-1 gene in S. aureus isolates (p<0,2).

TSST-1	Positive	Negative
Biopsy sample		
Patient group n:30	10 (20%)	40 (80%)
Control group n:30	23 (46%)	27 (54%)

CRS is one of the common respiratory tract infections in our country and throughout the world. It is known that in the development of CRS, microorganisms, and various factors play a significant role. *S. aureus* is one of the most commonly blamed bacteria in CRS. During the development of the CRS, it was claimed that some exotoxins of *S. aureus* qualifying as superantigens have an impact. The findings of various studies supported the possible effects of TSST-1 and enterotoxins in the pathogenesis of CRS. Determining how superantigens affect the development and aggravation of chronic rhinosinusitis might help understand this disease's pathogenesis. While some studies support this toxin hypothesis, some indicate that it was impossible to reach a particular conclusion on this issue [1,23,24].

The number of *S. aureus* isolated from the group of patients was significantly higher than the control group. However, in various studies, the number of *S. aureus* isolated from both groups was lower than those isolated from patient and control groups in this study [12,28,29].

For instance, El Fiky et al. isolated 45 % of *S. aureus* from patients with nasal polyp CRS and 13 % from the control group [12]. In our study, the percentage determined in the control group and the researchers' findings are similar. Laudien et al. detected S. aureus in 28% of patients with chronic rhinosinusitis, while Niederfuhr and colleagues detected S. aureus in 39%. However, they did not find a significant difference compared to the control group [28,29].

In general, *S. aureus* has been found to be more frequent among patients with chronic sinusitis. However, parallel to other studies, no evidence was found in this study suggesting that *S.aureus* bacteria is the only etiological agent in patients. The rates of *S. aureus* isolated from all patients proves that this bacterium is only one of the effective microorganisms.

The existence of enterotoxin A, B, C, and D genes was examined using a real-time PCR method in *S. aureus* isolated from patient and control groups. The enterotoxin genes A, B, C and D were found in more inpatient group bacteria compared to control group bacteria. However, the difference was statistically significant for none of the enterotoxin types.

Considering these findings, it is not possible to conclude that enterotoxin formation has a role in CRS etiology. When the frequency of enterotoxin production from patient and control group bacteria is examined, it can be said that enterotoxins were not responsible for the prognosis of CRS alone. However, it can be a factor that has a role in the development of the illness. In a study conducted, mucus and tissue samples of patients with chronic sinusitis were examined. It was found that 33 % of the patients with nasal polyps had staphylococcal toxin, but no toxin was found in the control group samples [26]. In another study, while at least one *Staphylococcus* exotoxin was detected in nasal and mucosa tissues of 54% of the chronic sinusitis patients with nasal polyps, in control group tissues and chronic nasal sinusitis patients without nasal polyps, the existence of toxins was not found [24]. These findings prove that toxins might have a role in chronic sinusitis development, but they are not necessarily vital for the development of the illness. Some studies with similar findings as in our study were reported [13,28].

When TSST-1 toxin genes were evaluated in *S. aureus* isolates isolated from patient and control groups, contrary to the expectations, a higher existence of the TSST-1 gene was found in *S. aureus* isolates obtained from the control group. While 23% of gene existence was found in control group bacteria, in inpatient group bacteria, this percentage was 10%. In their studies, El Fiky et al. found the frequency of existence of TSST-1 in the bacteria of patients with chronic sinusitis which is higher compared to the control group [12]. Heymans et al., who conducted the most detailed study, also found TSST-1 in 13% of the isolates derived from patients with CRS, 19% of the bacteria derived from CRS patients with nasal polyp, and 32% of the bacteria derived from the control group [25]. The findings obtained from this study are compatible with those of the researchers. Our results and the results of the other research studies indicate that TSST-1 was inefficient for the development of CRS. The fact that *S. aureus* is carried to the nasopharynx among some people has a significant role in the formation of infections related to the bacteria and bacteria's spreading among people. A balance must be formed between the host and the microorganism for the improvement and the continuum of carriage. In the carriers, the bacterium decreases the oscillation of surface proteins and the oscillation of exotoxin [30]. Therefore, while researching this type of protein, it is possible to reach different results between bacteria from the culture medium and those from the lesion. As a result, the nasal carriage rate of *S. aureus* for people

with CRS proved to be higher than for those without CRS. According to the examination of enterotoxin A, B, C, and D genes in *S. aureus* isolates isolated from patient and control groups, no significant difference was found between the rate of the bacteria forming enterotoxin in both groups. Based on the results of the presence of the TSST-1 gene in the S. aureus isolates isolated from the patient and control groups; it may be speculated that this toxin is not vital for development of CRS. However, its presence in the microenvironment of the nasal tissues might have a role in the pathogenesis of the disease. Since enterotoxin and TSST-1 investigated in this study were considered as superantigens, a consensus could not be reached regarding their roles in the etiology of chronic rhinosinusitis. However, it is thought that knowing the effects of these toxins will be beneficial in the follow-up and treatment of the prognosis of the disease.

In conclusion, chronic rhinosinusitis, which cannot be based on a single cause, is a state which comes up under the conditions of the combined effect of several microorganisms, and because of the repetitive acute infections, the host's anatomic, allergic, genetic, and immune status becomes convenient. It is considered that knowing all the influential factors and eliminating the effects of these factors will increase the success of the treatment.

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