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Detection of Group A Beta Hemolytic Streptococci Species, *emm*, and Exotoxin Genes Isolated from Patients with Tonsillopharyngitis

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Abstract
Group A Beta Hemolytic Streptococci (GAS) is the most critical human pathogen that leads to tonsillopharyngitis. The aims of this study were to identify GAS isolates and to determine *emm* typing, the coverage rate of available vaccines, and the distribution of superantigen gene profiles. 15 GAS isolates were isolated from throat cultures of 200 patients with tonsillopharyngitis, who were admitted to Canakkale Health Application and Research Hospital between October 2017 and May 2018. Identification of the isolates was performed by conventional methods and 16S rRNA sequence analysis. *emm* typing and exotoxin profiling of the isolates were performed by polymerase chain reaction. 7.5% GAS was detected in 200 patients. All the GAS isolates were identified as *S. pyogenes*. *emm* typing can be carried out in 13 *S. pyogenes* isolates. *emm*89 (33.3%), *emm*44 (20%), *emm*6 (13.3%), *emm*84 (6.7%), *emm*1 (6.7%), and *emm*18.1 (6.7%) were found to be six *emm* types. The coverage rate of *S. pyogenes* strains for 26-valent vaccine was 61.5% and for the 30-valent vaccine 84.6%. The most common exotoxin was *spe*B (86.7%), followed by *spe*C (60%), *spe*F (33.3%), *ssa* (26.7%), *spe*A (20%), *spe*M (20%), *spe*J (13.3%), *spe*L (6.7%), and *spe*I (6.7%). As a result of determining the *emm* types of *S. pyogenes* species in Canakkale, it was concluded that the potential of 30-valent vaccine should be considered in Turkey and development of vaccines containing exotoxin types may be beneficial.

Introduction

Although GAS (especially *S. pyogenes*) has been in existence for many years, it remains as an important cause of global morbidity and mortality in resource-limited regions. *S. pyogenes*, which is a significant human pathogen, causes serious diseases, such as pharyngitis, skin infections, acute rheumatic fever (ARF), rheumatic heart disease (RHD), acute post-streptococcal glomerulonephritis (APSG), streptococcal toxic shock syndrome, and necrotizing fasciitis [1, 2]. In a study conducted in Turkey on the identification of GAS isolates at species level by 16S rRNA sequence analysis, *S. pyogenes* was found to be the most common when compared with gene bank data [3].

Many serious virulence factors play a role in the pathogenicity of *S. pyogenes*. The most notable factor is surface M protein encoded by the *emm* gene, showing its effect by facilitating adhesion, providing resistance to opsonophagocytosis and contributing to the overall burden of GAS infections [4, 5]. To date, more than 200 *emm* types and subtypes have been reported. The hyper-variable region of the *emm* gene forms the basis for *emm* typing in GAS. M protein is used as an epidemiological marker because of the different *emm*-type distribution in variable regions [6, 7]. *emm* gene sequence analysis is a useful tool to understand local transmission dynamics and geographical distribution of the *emm* types. Knowing the *emm*-type distribution of different patient populations in different regions of the same country can be used to understand local epidemiology and to determine local vaccine formulation. M protein, which is exhibited by all the strains of *S. pyogenes*, can be immunized in the host and is used in valuable vaccine development studies. Vaccines with 26-valves and 30-valves include streptococcal

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emm types that cause non-invasive (tonsillopharyngitis) and invasive infections [8, 9].

Superantigens (SAgs), containing mitogenic exotoxins, are produced by a small number of bacterial species and some viruses [10, 11]. GAS secretes several SAgs, e.g., streptococcal pyrogenic exotoxin (*spe*), streptococcal mitogenic exotoxin (*sme*Z), and streptococcal superantigen (*ssa*). These toxins are extracellular products and exhibit antigenic properties. SAgs, whose gene distribution and genomic heterogeneity have been determined, can be used as an additional epidemiological tool for further investigations of toxin-dependent diseases [12, 13].

The aim of the present study was to identify GAS isolates from 200 patients with tonsillopharyngitis between 2017 and 2018 in Canakkale and to detect *emm* types, exotoxin-gene profiles, and the coverage rate of the available vaccines.

Materials and Methods

Study Design and Sample Collection

All the experiments in this study were conducted in the Basic and Industrial Microbiology Laboratory of Biology Department at Canakkale Onsekiz Mart University. Each participant signed an informed consent form in accordance with the Declaration of Helsinki. Our study was approved by the Canakkale Onsekiz Mart University's Clinical Research Ethics Committee (Project Number: 27/2017-E.33577).

200 patients with tonsillopharyngitis, who applied to Emergency, Family Medicine, Ear, Nose, Throat, and Child Health and Diseases Departments of Health Application and Research Hospital in Canakkale, from October 2017 to May 2018, were enrolled in this study. The throat swab samples were collected by the doctors. They were stored at 4 °C and transported to the microbiology laboratory within 2 h. Amies was used as a transport medium.

Identification and DNA Extraction of GAS

The throat swabs were streaked on 5% sheep blood agar plates (Bes-Lab, Turkey) and incubated under 5% CO_2 at 37 °C overnight. β-hemolytic colonies were chosen and then subcultured. The isolates were identified using conventional methods (Gram staining, catalase, L-pyrrolidonyl β-NAPHTHYLAMİDE, bacitracin-SXT). All the isolates were stored at − 70 °C in Triptic Soy Broth (TSB; Merck, German) supplemented with 20% glycerol until further analysis. For DNA extraction from the GAS isolates, a commercial extraction and purification kit (Biospeedy, Turkey) was used according to the manufacturer's instructions.

Identification of GAS Species by 16S rRNA

In order to amplify a 1390-bp fragment of the target 16S rRNA gene, the primers listed in Table 1 were used [14]. PCR amplification was performed in a final volume of 25 µL containing 1× PCR buffer, 0.8 mM dNTP, 0.4 µM of each primer, 2 µL of template DNA, and 1 U/25 µL Taq polymerase. All the reagents were purchased from Geneon (German) and Biospeedy (Turkey). The amplification was performed on a thermocycler nexus gradient (Eppendorf) and the cycling program consisted of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 55 °C for 45 s, annealing at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were electrophoresed through 1.7% agarose gel.

emm Typing of *S. pyogenes*

The *emm* types of *S. pyogenes* isolates were determined by the Centers for Disease Control and Prevention (CDC, USA) website (https://www.cdc.gov/ncidod/biotech/strep/doc.htm) [15]. The set of primers, annealing temperature (T_m), and amplicon size (bp) are listed in Table 1. The products were run on 1.5% agarose gel.

Detection of Toxin Genes

Toxin genes of *S. pyogenes*, including *spe*A, *spe*C, *spe*G, *spe*M, *spe*F, *spe*B, *spe*H, *spe*I, *spe*J, *spe*L, *sme*Z, and *ssa*, were detected by simplex and multiplex PCR with published primers as shown in Table 1 [16–18].

The amplification of all the SAg genes was performed using the following conditions: denaturing at 94 °C for 7 min, 30 cycles at 94 °C for 45 s, annealing at the appropriate temperature for 45 s standardized in the laboratory for each gene (Table 1), and 80 s of extension at 72 °C with a final elongation step at 72 °C for 7 min. PCR products were separated by electrophoresis on 2% agarose gel.

A 100-bp DNA ladder was used as a size marker (Biospeedy, Turkey). The PCR products were purified using PCR cleaning kit (Geneon, German) and then sequence analyses were conducted. The 16S rRNA, *emm*, and SAg gene sequences were searched for ≥ 99 homology by BLAST program, National Center for Biotechnology (https://www.ncbi.nlm.nih.gov/BLAST/).

Statistical Analysis

SPSS 22 version was used for statistical analysis. Gender, age, *emm* types of GAS, and superantigen genes

Table 1 Primers, primers' annealing temperatures (°C) and amplicon sizes (bp) used for amplification of 16S rRNA, *emm*, and SAg genes

Gene	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)	References
16S rRNA	F 5′AGA GTT TGA TCC TGG CTC AG3′ R 5′GAC GGG CGG TGT GTA CAA3′	1390	55	Edwards et al. [14]
emm	F 5′TAT TSG GCT TAG AAA ATT AA3′ R 5′GCA AGT TCT TCA GCT TGT TT3′	914	46	CDC
*spe*A	F 5′CCA AGC CAA CTT CAC AGA TC3′ R 5′CTT TAT YCT TAG RTA TGA AC3′	523	50.1	Rivera et al. [18]
*spe*C	F 5′TGT CTT ATG AGG CCT CTC3′ R 5′ATC TGA TCT AGT CCC TTC3′	386	50.1	Rivera et al. [18]
*spe*G	F 5′GAT GAA AAT TTA AAA GAT TTA A3′ R 5′GGG GGG AGA ATA GCA CTA GT3′	648	50.1	Chatellier et al. [16]
ssa	F 5′GTG CAC AAT TAT TAT CGA TTA GTG3′ R 5′GGT GAA CCT CTA TAG CTA TAG CTG AAG3′	723	60.1	Igwe et al. [17]
*spe*L	F 5′TTA GGA TGG TTT CTG CGG AAG AGA C3′ R 5′TTC CTC TTT CTC GCC TGA GCC GTG3′	596	60.1	Igwe et al. [17]
*spe*H	F 5′CAC ATA TTG ATA AGA AAA TCT ACA GC3′ R 5′GAA ATT GAG TTG AGT CTA TTC TCT CG3′	666	59.1	Igwe et al. [17]
*spe*I	F 5′CTT TGG AGT ATT CTC CTC CC3′ R 5′CTC TCT CTG TCA CCA TGT CC3′	382	59.1	Rivera et al. [18]
*spe*J	F 5′GTT ATA ATA ATC TTT CAT GGG TAC GG3′ R 5′CTT TCA TGT TTA TTG CCA TTG ATC GC3′	545	59.1	Igwe et al. [17]
*spe*B	F 5′CAA CCA GTT GTT AAA TCT CT3′ R 5′CTA AGG TTT GAT GCC TAC AA3′	762	58.4	Chatellier et al. [16]
*spe*F	F 5′CGA AAT TAG AAA AGA GGA C3′ R 5′GGC TGA GCA AAA GTG TGT G3′	1193	57.2	Rivera et al. [18]
*spe*M	F 5′GCT CTA TAC ACT ACT GAG AGT GTC3′ R 5′CAT ATC AAT CGT TTC ATT ATC TG3′	612	56.2	Igwe et al. [17]
*sme*Z	F 5′TAG AAG TAG ATA ATA ATT CCD3′ R 5′TTA GGA GTC AAT TTC TAT ATD3′	629	48.3	Chatellier et al. [16]

were computed as data and analyzed by χ^2 test. P value of < 0.05 was considered statistically significant.

Results

The DNA sequences of the 16 S rRNA gene region of the 15 GAS isolates were compared with the gene bank data and all were identified as *S. pyogenes.*

emm Types of GAS

According to the blast analysis results, six *emm* types, *emm*89 (5 isolates, 33.3%), *emm*44 (3 isolates, 20%), *emm*6 (2 isolates, 13.3%), *emm*84 (1 isolate, 6.7%), *emm*1 (1 isolate, 6.7%) and *emm*18.1 (1 isolate, 6.7%), were identified from *13 S. pyogenes* isolates. *emm* typing could not be performed in two isolates.

Determination of Exotoxin-Gene Profiles by PCR

In this study, *spe*G, *spe*H, and *sme*Z were not detected among the 15 *S. pyogenes* isolates. *spe*B (13 isolates, 86.7%) was the most prevalent, followed by *spe*C (9 isolates, 60%), *spe*F (5 isolates, 33.3%), *ssa* (4 isolates, 26.7%), *spe*A (3 isolates, 20%), *spe*M (3 isolates, 20%), *spe*J (2 isolates, 13.3%), *spe*L (1 isolate, 6.7%), and *spe*I (1 isolate, 6.7%). The exotoxin-gene sequences, which were found in the *S. pyogenes* isolates, were aligned with the multiple sequence aligner program at https://www.ebi.ac.uk/Tools/msa/Clustal Omega. The phylogenetic tree was drawn with NCBI clustal omega program and shown in Fig. 1.

There was no statistically significant relationship between *S. pyogenes emm* types, exotoxin genes, and the genders and ages ($P > 0.05$) of the patients with tonsillopharyngitis (Tables 2 and 3). *emm* types and virulence gene distributions of the *S. pyogenes* isolates are presented in Tables 3 and 4.

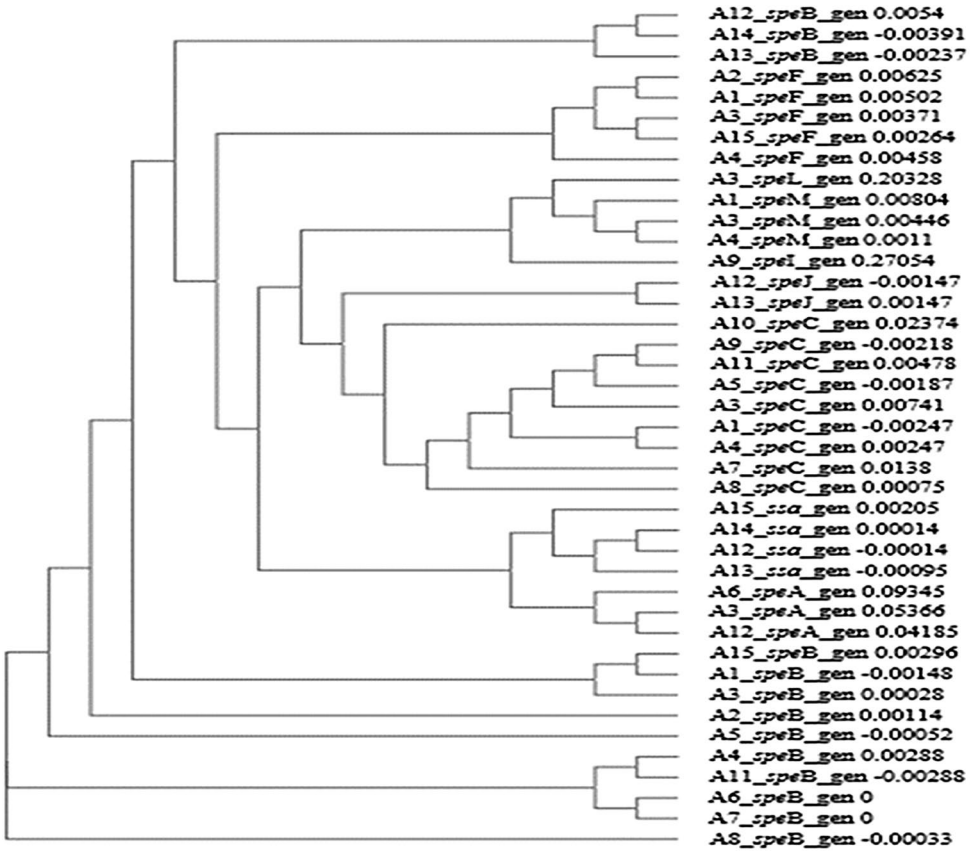

Fig. 1 Comparative phylogenetic tree of exotoxin gene species belonging to the 15 *S. pyogenes* isolates (A1–A15)

Table 2 The distribution of *emm* types according to gender and age groups (< 15, 15 and > 15)

emm type	Patients number (%)	Male	Female	< 15	15	> 15
*emm*1	1 (% 6.7)	–	1 (% 6.7)	1 (% 6.7)	–	–
*emm*6	2 (% 13.3)	–	2 (% 13.3)	1 (%6.7)	–	1 (% 6.7)
*emm*18.1	1 (% 6.7)	1 (% 6.7)	–	–	–	1(% 6.7)
*emm*44	3 (% 20)	2 (% 13.3)	1 (% 6.7)	2 (% 13.3)	–	1 (% 6.7)
*emm*84	1 (% 6.7)	1 (% 6.7)	–	1 (% 6.7)	–	–
*emm*89	5 (% 33.3)	1 (% 6.7)	4 (% 26.7)	3 (% 20)	1 (% 6.7)	1 (% 6.7)
Tip yok	2 (% 13.3)	–	2 (% 13.3)	1 (% 6.7)	1 (% 6.7)	–

Table 3 The distribution of virulence genes according to gender and age

SAgs	Patient number *n* (%)	Male	Female	< 15	15	> 15
*spe*A	3 (% 20)	2 (% 13.3)	1 (% 6.7)	1 (% 6.7)	–	2 (% 13.3)
*spe*C	9 (% 60)	3 (% 20)	6 (% 40)	5 (% 33.3)	2 (%13.3)	2 (% 13.3)
*spe*M	3 (% 20)	1 (% 6.7)	2 (% 13.3)	2 (% 13.3)	–	1 (% 6.7)
*spe*F	5 (% 33.3)	1 (% 6.7)	4 (% 26.7)	4 (% 26.7)	–	1 (% 6.7)
*spe*B	13 (% 86.7)	4 (% 26.7)	9 (% 60)	8 (% 53.3)	1 (% 6.7)	4 (% 26.7)
*spe*I	1 (% 6.7)	1 (% 6.7)	–	1 (% 6.7)	–	–
*spe*J	2 (% 13.3)	1 (% 6.7)	1 (% 6.7)	2 (% 13.3)	–	–
*spe*L	1 (% 6.7)	1 (% 6.7)	–	–	–	1 (% 6.7)
ssa	4 (% 26.7)	2 (% 13.3)	2 (% 13.3)	3 (% 20)	–	1 (% 6.7)

Table 4 Distribution of virulence genes in *emm* types

Isolates	*emm* type	*spe*A	*spe*C	*spe*M	*spe*F	*spe*B	*spe*I	*spe*J	*spe*L	*ssa*
17 (A1)	*emm*1	−	+	+	+	+	−	−	−	−
20 (A2)	−	−	−	−	+	+	−	−	−	−
10 (A3)	*emm*18.1	+	+	+	+	+	−	−	+	−
45 (A4)	*emm*89	−	+	+	+	+	−	−	−	−
52 (A5)	*emm*89	−	+	−	−	+	−	−	−	−
1 (A6)	*emm*89	+	−	−	−	+	−	−	−	−
96 (A7)	*emm*89	−	+	−	−	+	−	−	−	−
193 (A8)	*emm*89	−	+	−	−	+	−	−	−	−
101 (A9)	*emm*84	−	+	−	−	−	+	−	−	−
153 (A10)	−	−	+	−	−	−	−	−	−	−
164 (A11)	*emm*6	−	+	−	−	+	−	−	−	−
118 (A12)	*emm*44	+	−	−	−	+	−	+	−	+
127 (A13)	*emm*44	−	−	−	−	+	−	+	−	+
136 (A14)	*emm*44	−	−	−	−	+	−	−	−	+
130 (A15)	*emm*6	−	−	−	+	+	−	−	−	+

Discussion

S. pyogenes infection is observed in approximately 2–4% of 100,000 populations in developed countries and 12–83% in developing countries and in domestic populations of developed countries such as the USA and Australia [19]. 20.05% *S. pyogenes* were reported in Turkey, 29.5% in Iran, 9.2% in Nepal, and 7.5% in this study [20–22].

Molecular epidemiology studies report that there are significant differences in *emm*-type distribution at global level, especially between high-income countries and resource-poor countries and tropical regions. Although a small number of *emm* types are observed in developed countries, a wide variety of *emm* types are more common in disease-related strains in low-income countries. Previous studies have reported that socioeconomic factors have a considerable influence on the diversity of *emm* types and circulation of *S. pyogenes* [23].

In order to prevent the spread of GAS infections and post-streptococcal diseases, especially in individuals not receiving antibiotic treatment, two types of anti-GAS vaccines have been developed. These vaccines consist of peptides in the aminothermal region of the M protein. Based on the serotypes of GAS infections and the current epidemiological data in North America and Europe, the 26-valent and 30-valent recombinant multivalent vaccine, which contains 26 and 30 different *emm* types, has been designed. Epidemiological studies have shown that 26-valent vaccine is more effective in industrialized countries than in developing countries, accounting for an efficacy rate of 72% and 24%, respectively [9, 24].

In Taiwan, the most common *emm* type was *emm*12, followed by *emm*1 and *emm*4 [25]. The study reported that *emm*3 (80%), *emm*1 (16%), and *emm*75 (4%) were detected from throat cultures in Iran [26]. *emm*89 (16%), *emm*12 (10%), *emm*2 (9%), and *emm*1 (8%) types were common in Lebanon [27]. In Greece (2007–2013), *emm* genes were investigated in isolates collected from 1080 pharyngitis and 22 tonsillitis. In the pharyngitis *emm*12 (15.7%), *emm*1 (15.6%), *emm*4 (11.8%), *emm*77 (11.7%), *emm*28 (10.7%), *emm*3 (6.8%); and in the tonsillitis *emm*3 (18.2%), *emm*89 (13.6%), *emm*1, *emm*2, *emm*28, and *emm*4 types were detected. It has been reported that the recommended 30-valent GAS vaccine covers 97.2% of these *emm* types [28]. 7 of the 10 different *emm* types (*emm*1, *emm*5, *emm*14, *emm*18, *emm*19, *emm*29, and *emm*89) were detected in the 26-valent vaccine and the coverage rate was 50% [3]. In a study in our country, *emm*1 (30.9%), *emm*12 (14.6%), *emm*89 (8.1%), *emm*118 (7.3%), and *emm*4 (5.7%) were reported [20]. In the present study, 6 *emm* types were recovered from 13 *S. pyogenes* isolates. The most common *emm* type was *emm*89 (33.3%), followed by *emm*44 (20%), *emm*6 (13.3%), *emm*84, *emm*1, and *emm*18.1 (6.7%). As in the other studies, *emm*1 and *emm*89 types have also been detected in this study. *emm*1, *emm*6, and *emm*89 are available in 26-valent; and *emm*89, *emm*44, *emm*6, and *emm*1 types in the 30-valent vaccine. The isolates obtained from Canakkale were found to cover the 26-valent vaccine at a rate of 61.5% and the 30-valent vaccine at a rate of 84.6%.

Superantigens are toxins that can react in the host by activating T cells non-conventionally [29]. *spe*A (17.2%), *spe*B (72.4%), *spe*C (13.8%), and *spe*F (69.0%) exotoxin genes were detected in 29 GAS isolates in Taiwan [25]. In Lebanon, *spe*B (87%), *ssa* (36%), and *spe*G (30%) superantigens were commonly found [27]. In India, *spe*B (100%), *sme*Z (100%), *spe*C (28%), *spe*H (28%), *spe*I (28%), *spe*L (22%), *ssa* (17%), *spe*M (11%), and *spe*J (11%) were recovered from 18 GAS, isolated from throat cultures [30]. In Beijing, 13 SAg gene profiles were investigated in GAS, isolated from

patients with pharyngitis. *spe*B (99.2%), *spe*C (99.2%), *sme*Z (99.2%), *spe*F (98.8%), *spe*G (98.5%), *ssa* (98.5%), *spe*J (49%), *spe*A (48.6%), *spe*I (46.3%), and *spe*H (43.6%) were observed; *spe*K, *spe*L, and *spe*M genes were not detected [31]. In a study in Turkey, *spe*A (8.2%), *spe*C (8.9%), and both genes (1.5%) were found in two isolates [20]. In the present study, *sme*Z, *spe*G, and *spe*H exotoxin genes were not found in any of the *S. pyogenes* isolates, differently from other studies. The most common virulence gene was found to be *spe*B (86.7%), which was followed by *spe*C (60%), *spe*F (33.3%), *ssa* (26.7%), *spe*A (20%), *spe*M (20%), *spe*J (13.3%), *spe*L (6.7%), and *spe*I (6.7%). These rates were similar to other studies.

emm typing studies have been conducted on various populations to determine the epidemiology of *S. pyogenes* isolates and to provide information about the biology, pathogenesis, and genetic structure of bacteria. The absence of licensed vaccine and the increased resistance to antibiotics are a concern for public health. Several measures must be taken to prevent outbreaks caused by this pathogen [32].

The scope of studies should be expanded to include many regions in Turkey by increasing the number of samples and including invasive and non-invasive diseases caused by *S. pyogenes*.

Author Contribution BMY: Data collection and statistical analysis. MA: Collection of the throat swabs, laboratory studies, and writing of the article.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval Our study was approved by the Canakkale Onsekiz Mart University's Clinical Research Ethics Committee (Project Number: 27/2017-E.33577).

Informed Consent Each participant signed an informed consent form in accordance with the Declaration of Helsinki.

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