

# Evaluation of emm gene types, toxin gene profiles and clonal relatedness of group A streptococci

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## ABSTRACT

The aim of this study is to evaluate antibiotic susceptibilities, *emm* gene types, toxin gene profiles and clonal relatedness of group A streptococci (GAS) isolates obtained from patients and carriers. A total of 79 clinical isolates from patients and 60 isolates from carriers were included in the study. *Emm* typing, toxin gene detection for *speA*, *speB*, *speC*, *speG* and *smeZ* genes and pulsed-field gel electrophoresis (PFGE) was performed. Twenty-one distinct *emm* types were detected; the most common types were *emm12*, *emm89*, *emm1*, *emm77*, *emm4* and *emm3*. The detection rates of both *emm* types and the toxin genes didn't differ significantly between patients and carriers. The presence of *speA* and *smeZ* was significantly higher in *emm1* and *speG* was significantly lower in *emm4* when compared to the other *emm* types. The rate of clustering obtained with PFGE wasn't significantly different in patients and carriers. As a result, twelve of the 21 *emm* types detected in this study were covered by the 26-valent vaccine, constituting 77.7% of the *emm* typeable isolates; however the *emm4* type which is one of the most common types in the present study is not among this coverage.

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KEY WORDS: Group A streptococci, *emm* typing, toxin genes, *speA*, *speB*, *speC*, clonal relation, PFGE

## INTRODUCTION

*Streptococcus pyogenes* (group A streptococci GAS) cause a wide spectrum of infections ranging from pharyngitis and skin infections to necrotising fasciitis and toxic shock syndrome [1]. Increase in the rate of invasive infections and related morbidity and mortality in the last few decades besides the presence of GAS strains in asymptomatic carriers has gained attention to the virulence factors and have put forward the requirement to investigate the genetic characteristics of this microorganism including toxin gene profiles and *emm* types [1, 2]. M protein, encoded by *emm* gene, is particularly important both as a virulence factor and as the most important candidate for vaccine studies [1]. The 5' portion of *emm* gene which is critical for the achievement of immunological specificity has been sequenced for all of known M sero-

types forming 124 *emm* types and the data are available at the CDC databases (<http://www.cdc.gov/ncidod/biotech/strep/emmtypes.html>). The most common *emm* types reported are *emm1*, *emm6*, *emm18*, *emm48*, *emm49*, *emm75*, *emm95* and *emm103* [3]. A 26-valent vaccine covering the estimated most common M types is on the agenda [4, 5]. Streptococcal pyrogenic toxins, which behave as superantigens, are also important in virulence of the bacteria and reported to be linked to infections with high morbidity and mortality [6, 7]. *SpeA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *smeZ* and *smeZ2* are the known pyrogenic toxins of GAS defined until recently [6, 7]. Phylogenetic analyses give rise to the thought that the toxin genes are spread by horizontal transfer and the production of the toxic enzymes differ with varying *emm* types [1]. In this respect, it is crucial to know about the *emm* types, pyrogenic toxin gene profiles, clonal relatedness of the isolates in particular regions and to manifest any relation between them. It is also essential to determine the distribution of these parameters in the isolates from asymptomatic carrier population for all the better insight into GAS infections and virulence. The study in this area will also be useful for effective vaccine development.

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The aim of this study is to evaluate and to compare *emm* gene types, toxin gene profiles and clonal relatedness of group A streptococci (GAS) obtained from patients with indicated GAS infections and from GAS carriers.

## MATERIALS AND METHODS

This research has been performed with the approval of Zonguldak Karaelmas University, Training and Research Hospital Ethics Committee.

### Patient isolates

GAS isolates were collected from patients admitted to our hospital with indicated GAS infections between January 2009 and April 2010.

### Carrier isolates

GAS isolates were obtained from students aged between 7-14 years from three schools (School F, School A, School K) between November 2009 and June 2010. Informed consent forms from parents were taken for each student included. Three screening studies were performed with at least one month interval in each school. An accompanying otorhinolaryngologist was present in each of the screening studies for the physical examination of the students to eliminate respiratory tract infections and for the sampling. The students with at least two positive GAS cultures at the end of three screening studies without recent history and signs of infection were included in the study as carriers.

### Isolate identification

Identification of GAS isolates was performed using bacitracin (0.04 U) and trimethoprim sulfamethoxazole (1.25/23.75 µg) discs (Becton Dickinson, USA) and L-pyrrolidonyl arylamidase (PYR) test (Becton Dickinson, USA) as well as latex agglutination test (Plasmatec Lab. Products Ltd., UK) and BBL system (Becton Dickinson, USA) when needed.

### DNA extraction

The DNA extraction was done by a commercial extraction kit (EZ-10 Spin Column Genomic DNA MiniPreps Kit, Bio Basic, Canada) as described by the manufacturer.

### Typing of isolates

The *emm* typing was performed by DNA sequencing using the protocol recommended by Centers for Disease Control (CDC) (<http://www.cdc.gov/ncidod/biotech/strep/doc.html>). ABI Prism 310 Genetic Analyser (Applied Biosystems, USA) and ABI Prism 310 Data Collection Software were used. The reference DNA sequences from CDC website ([ftp://ftp.cdc.gov/pub/infectious\\_diseases/biotech/tsemm](ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm)) were used. The global alignment was done by CLC Bioinformatics Software (Aarhus, Denmark) and the local alignment was performed by National Centre for Biotechnology Information (NCBI) Gene Bank and Basic Local Alignment Tool (BLAST) server.

### Determination of toxin gene profiles

The presence of *speA*, *speB*, *speC*, *speG* and *smeZ* genes were tested by polymerase chain reaction (PCR) as described before [7-10]. The primers used for each gene is shown in Table 1.

### Determination of clonal relatedness

The clonal relatedness of the isolates obtained from the patients and carriers was assessed using pulsed-field gel electrophoresis (PFGE) of the genomic DNA as described before with some modifications [11]. Restriction of the genomic DNA was achieved with 25U of *SmaI* (Sigma, Steinham, Germany) for 2h at 30°C. The DNA fragments were electrophoresed by the CHEF-DR II system (BioRad Laboratories, Nazareth, Belgium) at 10°C at 6V/cm for 20h with initial and final switch times of 4,6 and 40 seconds, respectively. The PFGE profiles were analysed by GelCompar II Software (version 3.0; Applied Maths, Belgium) based on Dice coefficient. The dendrogramme was generated by unweighted pair group method with mathematical averaging (UPGMA). According to the interpretative criteria of Tenover et al., [12] the isolates revealing more than 80% similarity were considered to be "closely related". Indistinguishable isolates were shown by a common "numeral". Closely related isolates were demonstrated by addition of a "letter" next to the numeral.

### Statistical analysis

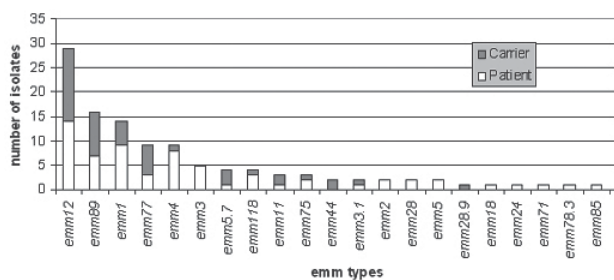
Statistical analyses were performed with SPSS for Windows, release 13.0 (SPSS Inc. Chicago, IL, USA). Distribu-

**TABLE 1.** The primers used for determination of the presence of toxin genes.

Toxin gene	Primer F (5'→3')	Primer R (5'→3')	Size of amplification product (bp)	Reference
<i>speA</i>	ACTTAAGAACCAAGAGATGG	CCTTATTCTTAGGTATGAAC	309	Weeks et al. [10]
<i>speB</i>	GGATCCCAACCCAGTTGTAAATCTCT	AACGTTCTAAGGTTTGATGCCCTACAA	764	Mollick et al. [9]
<i>speC</i>	AAGTGACTCTAAGAAAAGACA	TTGAGTATCAATGTTTAAATG	130	Goshorn et al. [8]
<i>speG</i>	AGAACTTATTGCCC	TAGTAGCAAGGAAAAGG	155	Schmitz et al. [7]
<i>smeZ</i>	TAACCTCTGAAAAGAGGC	CATTGGTTCTCTTGATAAG	391	Schmitz et al. [7]

**TABLE 2.** The distribution of *speA*, *speC*, *speG* and *smeZ* genes in carriers and patients

Isolate	<i>speA</i>			<i>speC</i>			<i>speG</i>			<i>smeZ</i>		
	n	%	p	n	%	p	n	%	p	n	%	p
Carriers (n=60)	21	35.0		29	48.3		46	76.7		8	13.3	
Patients (n=79)	21	26.6	0.377	40	50.6	0.788	66	83.5	0.424	19	24.1	0.172
Total	42	30.2		69	49.6		112	80.6		27	19.4	



**FIGURE 1.** The distribution of *emm* types in patients and carriers.

tion of numerical data was assessed by using one-sample Kolmogorov-Smirnov test. Differences between the groups according to the categorical variables and the association between the variables were assessed by Pearson's chi-square test and the Fisher's Exact test. The results were evaluated in 95% confidence interval and the value of statistical significance was accepted as: " $p < 0.05$ ".

## RESULTS

### Patient isolates

A total of 79 clinical GAS isolates were obtained during the study period. A total of 66 of them were isolated from nasopharyngeal specimen.

### Carrier isolates

A total of 60 GAS isolates were obtained from carriers in three schools after screening of 680 students of whom 538 were noted as healthy; 142 students were excluded from the study as they had particular signs of infection during the physical examination by the otorhinolaryngologist. The average rate of GAS carriage was found as 11.2%.

### emm types

Of the 139 isolates included in the study, 112 were typeable by DNA sequencing for *emm* types (80.6%). A total of 21 distinct *emm* types (1, 2, 3, 3.1, 4, 5, 5.7, 11, 12, 24, 28, 28.9, 44, 71, 75, 77, 78.3, 85, 89 and 118) were detected; the most common types being *emm12*, *emm89*, *emm1*, *emm77*, *emm4* and *emm3* constituting 73.2% of the typeable isolates. The rates of detection of *emm* types was not significantly different in patients and carriers ( $p > 0.05$ ) (Figure 1).

**TABLE 3.** Distribution of toxin gene profiles in the groups.

Toxin gene profiles					Carriers (n=60)	Patients (n=79)	Total (n=139)
<i>speA</i>	<i>speB</i>	<i>speC</i>	<i>speG</i>	<i>smeZ</i>			
-	+	+	+	-	13	22	35
-	+	-	+	-	15	19	34
+	+	-	+	-	7	8	15
-	+	+	-	-	5	6	11
+	+	+	+	-	6	2	8
+	+	-	+	+	0	7	7
+	+	+	+	+	2	4	6
+	+	-	-	-	5	0	5
-	+	-	-	-	2	3	5
-	+	-	+	+	3	1	4
-	+	+	+	+	1	3	4
-	+	+	-	+	0	3	3
-	+	-	-	+	0	1	1
+	+	+	-	-	1	0	1
+	+	-	-	+	0	0	0
+	+	+	-	+	0	0	0

The "++++" profile was found in seven of the patients / none in carriers and "+ + - -" profile was found in five of the carriers / none in patients.

### Toxin gene profiles

PCR demonstrated the presence of *speB* in all of the isolates. The distribution of *speA*, *speC*, *speG* and *smeZ* genes in carriers and patients is shown in Table 2. The detection rates of the toxin genes did not differ significantly between patients and carriers ( $p > 0.05$ ). The isolates formed 14 out of the 16 probable toxin profiles when all five of the genes tested, *speA*, *speB*, *speC*, *speG*, *smeZ* in order, were marked as positive/negative. The distribution of toxin gene profiles in patients and carriers is shown in Table 3. The average number of toxin genes (out of the five genes tested) was 2.78 ( $\pm 0.88$ ) for the total of the isolates while it was 2.70 ( $\pm 0.85$ ) for carrier isolates and 2.85 ( $\pm 0.91$ ) for patient isolates; the difference was not statistically significant ( $p > 0.05$ ).

### The clonal relatedness of the isolates

One isolate from each of the carriers was included in PFGE studies. A total of 61 clonotypes were established for the 133 isolates available for PFGE (Figure 2). Of the 133 isolates, 105 formed 33 clusters with 2-9 members while 28 had unique profiles. The clustering rate for the total of isolates was 78.9% (77.9% in patients and 80.4% in carriers); there was no significant difference between patients and

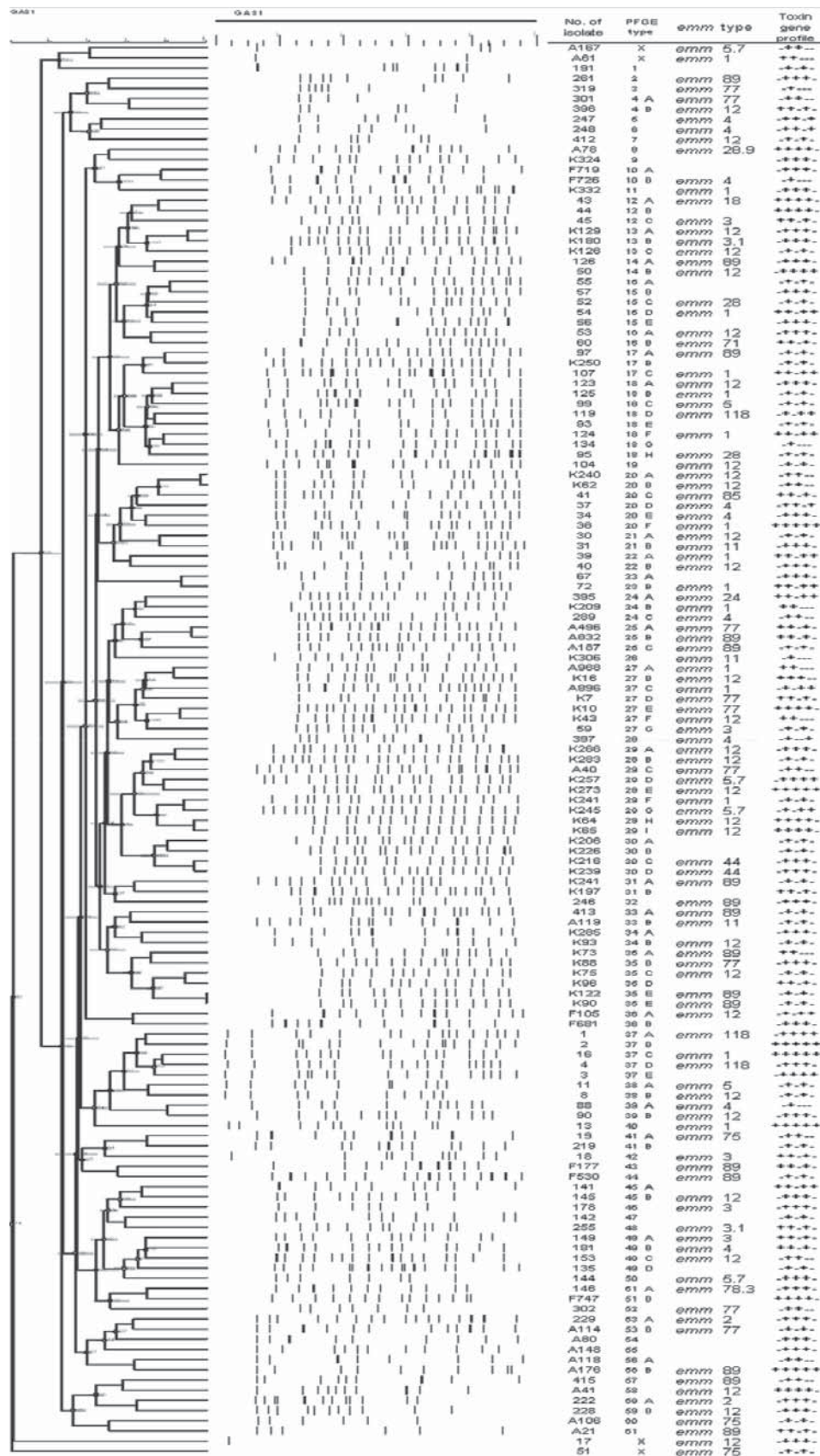


FIGURE 2. The dendrogramme of PFGE profiles generated by unweighted pair group method with mathematical averaging (UPGMA) based on Dice coefficient. Indistinguishable isolates were shown by a common "numeral". Closely related isolates were demonstrated by addition of a "letter" next to the numeral. +/- for toxin gene profiles represents presence/absence of *speA*, *speB*, *speC*, *speG*, *smeZ* in order. A, F, K in front of the isolate numbers represent the different schools where the carrier isolates were obtained; the isolates without letters are the patient isolates.

**TABLE 4.** Distribution of toxin genes in the most common emm types in respect to the isolate groups.

emm type	Total	n		Mean number of the toxin genes		speA		speC		speG		smeZ	
		P <sup>1</sup>	C <sup>2</sup>	P	C	P	C	P	C	P	C	P	C
emm12	29	14	15	2.7	3.1	1	6	9	9	13	11	1	2
emm89	16	7	9	2.6	2.7	0	5	5	2	6	7	0	2
emm1	14	9	5	4.1	2.2	8	3	3	1	9	2	8	0
emm77	9	3	6	1.3	2.8	0	3	2	3	0	5	0	1
emm4	9	8	1	2.5	1.0	1	0	5	0	2	0	4	0
Total	77	41	36	3.4	2.7	10	17	24	15	30	25	13	5

Legend: <sup>1</sup> Patients' group, <sup>2</sup> Carriers' group

carriers ( $p > 0.05$ ) (see in Figure 2 K90/ K122, K62/K240, A496/A832, K64/K65, K216/K239, K75/K98, K129/K180 ).

#### Association of emm types, toxin genes profiles and PFGE profiles

The distribution of toxin genes for the most common emm types in the present study in patients' and carriers' groups is shown in Table 4, Figure 2. The mean number of genes detected was significantly high in emm1 type patient isolates compared to carrier isolates. ( $p < 0.05$ ). The presence of speA (11/14,  $p = 0.005$ ) and smeZ (8/14,  $p = 0.004$ ) was significantly higher in emm1 and the presence of speG was significantly lower in emm4 ( $p = 0.005$ ) when compared to the other emm types. All of the smeZ positive-emm1 type isolates belonged to patient group (Figure 2).

#### Vaccine coverage

Twelve of the 21 emm types detected in this study were covered by the 26-valent vaccine, constituting 77.7% of the emm typeable isolates.

## DISCUSSION

Recent studies on GAS have focused on the bacterial cell structures and toxins related with severe infections. The presence of nasopharyngeal carriers besides high morbidity and mortality related to GAS infections have led researchers to the evaluation of the molecular characteristics of patient and carrier isolates. The emm types are known to show variance in different regions and time periods [13, 14]. While emm1, emm3 and emm12 have gained predominance in the USA recently [6], various emm types are in advance in different European countries [13]. Previously reported predominant emm types are emm1, emm12, emm4, emm28 and emm3 in the USA [15]; emm1, emm12, emm3, emm75 and emm4 in Mexico [3]; emm12, emm1, emm4 and 28 in Chile [16]; emm77, emm1, emm28, emm89 and emm12 in Finland [17]; emm11, emm12, emm4 and emm1 in Taiwan [18]; emm1 and emm12 in Romania [19]; emm75, emm1, emm6, emm12,

emm22, emm4 and emm5 in Italy [20] and emm1 and emm28 in Greece [13] while those are reported to be emm1, emm3 and emm28 in general [7, 13]. There are two studies in literature investigating emm types of nasopharyngeal isolates in our country; revealing predominance of emm12, emm3 and emm1 in Ankara [21], and emm1, emm3 and emm4 in Istanbul [22]. The most common types in our region were emm12, emm89, emm1, emm77, emm4 and emm3 (73.2% of the isolates) generating substantial concordance with the literature. It was remarkable that the rate of emm3 type was significantly higher in patients than in carriers while no significant difference was found for the other emm types. The superantigen gene prevalence in GAS can vary in particular regions and time periods besides the relation of these genes to particular emm types (the surface M protein is suggested influencing the entry of bacteriophage gene into the bacteria) [23]. The number and distribution of toxin genes and association with emm types is the subject of recent studies. In the present study, we investigated five genes and the isolates were found to reveal a mean of 2.78 genes in the total of the population creating no difference between patients and carriers. In our studied population, the presence of speB was 100% and that of speG was 81% in line with the previous reports [24-27]. The most variable rates are reported for speA and speC probably owing to the fact that they are not chromosomally coded and to intense research of these genes [25]. In our study, the rate of speA presence was 30.2% in total, creating no difference between patients and carriers. Schmitz et al. [7] suggested that investigating the toxin gene profiles formed by the presence/absence of the total of the toxin genes instead of individual toxin genes may be more crucial to understand the infection dynamics and reported the most common profiles found in their large population of isolates. When the profiles were adapted to the genes that were investigated in our study, ++++, +--- profiles (defined by the presence/absence of speA, speB, speC, speG and smeZ in order) were among the most common profiles in concordance with Schmitz's data [7]. Besides, the "++++" profile was found in seven of the patients / none in carriers and "+-

--" profile was found in five of the carriers/none in patients. The rate of clustering was not significantly different in patients and carriers. The clustering rate was higher in the school where the rate of carriers was higher when compared to the other schools screened. When the clonal relation was evaluated including the *emm* types and toxin genes, it was observed that some clonally related isolates had the same *emm* types and toxin genes as well (Figure 2, K64/K65, K90/K122, K216/K239, K62/K240), however, there were some other isolates with clonal relatedness yielding different *emm* types and toxin genes (Figure 2, K50/K126). It was also worth attention that some of the clusters consisted of solely carrier isolates (PFGE profile 27, 29, 35) while others included patient isolates only (PFGE profile 15, 18, 37).

In our study, among the most common *emm* types detected, *emm1* had the highest mean number of toxin genes among the five genes tested. The mean number of genes detected was significantly higher in *emm1* type patient isolates compared to carrier isolates. The presence of *speA* and *smeZ* was significantly higher in *emm1* and the presence of *speG* was significantly lower in *emm4* when compared to the other *emm* types. All of the *smeZ* positive-*emm1* type isolates belonged to patient group. These findings are in concordance with previous reports by Darenberg et al. who found higher number of genes in *emm1* [26] and Luca-Harari et al. who found association between *emm1* and *speA* and lower rates of *speG* in *emm4* and *emm77* type isolates [13, 24]. Others studies also reported significant presence of *speA*, *speG* and *smeZ* in *emm1* type isolates [2, 25]. The 26-valent vaccine covers proteins of the *emm* types of 24, 5, 6, 19, 29, 14, 1, 12, 28, 3, 1.2, 18, 2, 43, 13, 22, 11, 59, 33, 89, 101, 77, 114, 75, 76 and 92 types [4]. Studies on *emm* types included in the 26-valent vaccine have noted coverage rates of 86% from the USA [15] and 69% (16 *emm* types) from Europe [13]. There are two studies on *emm* types completed from our country reporting 76.9% coverage from Ankara [21] and 77% coverage from Istanbul [22]. Twelve of the 21 *emm* types detected in this study were covered by the 26-valent vaccine, constituting 77.7% of the *emm* typeable isolates. The types *emm4*, which is one of the most common types in the present study, and *emm118* are reported in the three studies from our country; however, they are not among vaccine coverage. On the whole, *emm* types reported until recently from our country are in 70-80% coverage of the 26-valent vaccine.

## CONCLUSION

There are a number of subjects for further investigation on GAS such as the relation of *emm1* type with increased number of toxin genes, the presence of certain *emm* types and toxin profiles in patients rather than in carriers, to put for-

ward relations between the clonotypes, *emm* types and toxin gene profiles as well as vaccine coverage for particular regions, to which the present study has made some contributions.

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## DECLARATION OF INTEREST

None to declare.

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