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RESEARCH ARTICLE

Distribution of *emm*, superantigen and other virulence genotypes and detection of phylogenetic relationships in group A streptococcal isolates

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ABSTRACT

Group A streptococci are important pathogens with various virulence factors, such as M protein, superantigens, hemolysins, deoxyribonuclease, and proteases. The aims of this study are to investigate the detection of *emm* genotypes and other virulence genes, such as SAgs, DNase, protease, antibiotic resistance, and phylogenetic relationships in GAS strains isolated from clinical samples.

Test strains were obtained from Çukurova University Balcalı Hospital and regional hospitals in Adana province. The M proteins were detected by sequence analysis of *emm* genes. SAgs and other virulence gene profiles were determined using the Multiplex-PCR method. The antibiotic susceptibility of the isolates was performed by the disc diffusion method and evaluated according to CLSI criteria. The PFGE method was used to determine the clonal relationship between the strains.

The *emm* gene was positive in 86 isolates. The most common *emm* genotypes were *emm*28 (22%), *emm*1 (18.6%), *emm*12 (13.9%), and e*mm*3 (11.6%). Also, the most common virulence genes were *speG* (58.1%), *speC* (56.9%), *sdaB* (53.4%), and *mac* (53.4%). The rates of resistance to erythromycin, clindamycin, levofloxacin, ciprofloxacin and telithromycin were 19.8%, 16.3%, 4.7%, 3.5%, and 3.5%, respectively.

As a result, additional regional studies on the detection and prevalence of GAS virulence factors in Turkey are required. We believe that this study will provide valuable information for epidemiological studies on *emm* sequences, Sags, and other virulence factors of *Streptococcus pyogenes* in Turkey.

KEYWORDS

M protein, superantigens, streptococci, PFGE

INTRODUCTION

Group A streptococci (GAS) may cause various diseases, such as pharyngitis, tonsilitis, scarlet fever, impetigo, necrotizing fasciitis, streptococcal toxic shock syndrome (STSS), acute rheumatic fever, and acute glomerulonephritis. The ability of GAS to cause invasive-systemic infections, as well as local tissue infections, is because it has a large number of virulence factors, such as M protein, pyrogenic exotoxins, capsule, streptolysin O and S, F protein, streptokinase, DNase [1–3].

M protein, located in the cell wall and encoded in *emm*, is an important virulence factor in antigenic structure with antifagocyte and anti-complementary properties [4]. The five

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parts of the *emm* that give immunological specificity to the M protein have been sequenced and all M serotypes have been determined. It has been shown that this protein, which is responsible for escape from the immune system, that is, for reinfection and reactivations, has more than 250 genotypes. Sequencing-based epidemiological studies have shown that different types of *emm* may be associated with both specific clinical pictures and geographic regions [5, 6]. Data obtained from *emm* genotyping studies worldwide are collected in the Centers for Disease Control and Prevention (CDC) and/or GeneBank databases. Within the scope of these studies, the verification of the newly detected subtypes is carried out by the CDC [7]. In the detection of M serotypes, direct sequencing of the high variable sequence that determines the M protein serospecificity in *emm* is accepted as the gold standard [8, 9].

Another putative virulence factor that affects prognosis in GAS infections is superantigens (Sags). It has been reported that they play a crucial role in the pathogenesis of high morbidity and mortality invasive involvements, such as pyrogenic and erythematous skin infections, STSS, bacteremia, sepsis, and peritonitis. Among the *S. pyogenes* species, 11 superantigen (Sag) genes could be characterized. Of these superantigens, *speG*, *speJ*, and *smeZ* are reported to be encoded in the chromosome, while *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, and *ssa* are also encoded in bacteriophages. In some clinical studies, an epidemiological relationship has been found between SAg gene profiles and some invasive infections [10–12].

Deoxyribonuclease (DNase), one of the enzymes secreted by GAS, depolymerizes DNA and ensures the spread of bacteria. Proteases encoded by *speB*, *spyCEP*, and *scpA* genes are enzymes that cause tissue destruction. *spe*B, a previously misnamed pyrogenic exotoxin, is a cysteine protease that can inactivate the C3b factor of complement and many host factors, such as IL-1 and immunoglobulins, which play a role in the immune response. C5a peptidase is a protease that allows the bacteria to escape from the complement system and consequently, the phagocytosis of neutrophils [13]. Studies are increasing, targeting diversity in other putative virulence factor genes, such as GAS *emm*, SAgs, protease inhibitors, and DNases. However, in our country, there are few studies investigating limited gene diversity. In addition, when considering all studies related to the determination of *emm* genotypes in Turkey, genotypes *emm1* and *emm12* (approximately one-third of all samples) are reported to be more prevalent [14].

Penicillin group antibiotics are the first choice in the treatment of GAS infections. Erythromycin or macrolide antibiotics are used in patients with penicillin allergies [13].

This study aims to investigate the *emm* genotypes of GAS strains isolated from clinical samples in our region, detect the presence of some virulence genes and investigate the phylogenetic relationship between the strains. The data obtained through this study will form local epidemiological knowledge and contribute to the determination of national gene diversity. This study will also shed light on the correlation between some involvement and genotypes in GAS infections.

METHODS

This study was conducted with the ethical approval of the Çukurova University Faculty of Medicine Non-interventional Clinical Research Ethics Committee (Date: 10.10.2014 and Decision No: 04). GAS strains were isolated from patients with upper respiratory tract, lower respiratory tract, skin-soft tissue infections (impetigo, wound infections), and urinary tract infections (kidney transplant, undefined urinary tract infections) followed in various clinics were included in this study. Between May 2015 and February 2017, a total of 86 *S. pyogenes* isolates were isolated from clinical samples throat swabs (n:54), sputum (n:14), skin-soft tissue swab (n:12), and urine (n:6). These isolates were identified using the VITEK-II. The samples included in this study were obtained from the Laboratory of the Balcalı Hospital of the Çukurova University and Adana Regional hospital.

The test samples delivered to our laboratory were first inoculated on 5% Sheep Blood Colombia Agar media and incubated at 37°C for 18–24 h to reproduce and obtain pure colonies These bacterial colonies were stored at −20°C in media containing 10% glycerol for further investigations.

emm genotypes were determined in accordance with the CDC website's technique and instructions [15]. CDC database was used for *emm* typing [7]. SAgs and other virulence gene profiles (*spd3*, *sdc*, *sdaB*, *sdaD*, *speB*, *spyCEP*, *scpA*, *mac*, *sic*, *speL*, *speK*, *speM*, *speC*, *speI*, *speA*, *speH*, *speG*, *speJ*, *smeZ* and *ssa*) of the isolates were determined by Multiplex- Polymerase Chain Reaction (PCR) method [16].

Penicillin G, gentamicin, linezolid, chloramphenicol, telithromycin, levofloxacin, ciprofloxacin, erythromycin, and clindamycin susceptibilities were investigated by the Kirby-Bauer disk diffusion method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [13].

Pulsed-Field Gel Electrophoresis (PFGE) was used to investigate the phylogenetic relationships among streptococcal isolates. The *Listeria monocytogenes* PFGE protocol from PulseNet was used for this [17]. DNA-agarose patterns prepared for the PFGE method were electrophoresed with 1% PFGE agarose after restriction with *Sma*I. The electrophoresis procedure used in the CHEF-DR II system included the following parameters: start stroke time of 4.6 s, end stroke time of 40 s, stroke angle of 120°, and the electric current density of 6 V/cm^2, the temperature of 10°C, and a total time of 20 h. Band profiles were analyzed using the GelCompar II software system (version 4.0; Applied Maths, Sint-Martens-Latem, Belgium). Dice similarity coefficient was used to determine the relationship between strains, and the similarity rate was assumed to be greater than or equal to 95% [18].

RESULTS

In this study, as a result of *emm*-PCR analysis, *emm* was found positive in 86 isolates. All *emm*-positive isolates were

evaluated in terms of GAS *emm* genotyping. Using the CDC database, the typing of *emm* positive isolates revealed 22 different *emm* genotypes/subtypes, of which 13 were *emm* genotypes (*emm*28, *emm*1, *emm*12, *emm*3, *emm*89, *emm*2, *emm*244, *emm*212, *emm*219, *emm*163, *emm*245, *emm*241, *emm*196), and nine were *emm* subtypes (*emm*118.5, *emm*11.1, *emm*1.65, *emm*203.1, *emm*89.31, *emm*108.1, *emm*6.19, *emm*28.5, and stG7882.3). The most prevalent genotype was *emm28* (22%), followed by *emm1* (18.6%), *emm12* (13.9%), *emm3* (11.6%), and *emm89* (3.4%). The distribution of *emm* genotypes according to the origin of the samples, 17 different genotypes of *emm* were detected in the throat swab samples obtained from 54 patients with upper respiratory tract infection. Eight different genotypes of *emm* were found in 14 sputum samples provided from patients with lower respiratory tract infections. Seven different genotypes of *emm* were identified in 12 soft tissue samples from patients with soft tissue infections. In the urine samples collected from patients with urinary system infection, six different genotypes of *emm* were detected (Table 1).

We investigated the prevalence of virulence genes in GAS strains, and we detected that the most prevalent Sag type was *speG* (58.1%), followed by *speC* (56.9%). In contrast, the *speL* gene was not in any isolates. Also, while the *sdaB* (53.4%) was the most common type of the DNase enzyme, the *sdaD* was not identified in any isolates, and the *mac* (53.4%) was one of the most prevalent protease factors. However, *sic* (16.2%) was the least detected (Table 2).

Table 2. The frequency of Sag, DNase and protease genes in GAS isolates

Virulence genes	Total isolates	Percent %	Virulence genes	Total isolates	Percent %
speG	50	58.1	*speL*	0	0
speC	49	56.9	*sdaB*	46	53.4
speJ	25	29.0	*spd3*	24	27.9
speA	18	20.9	*sdc*	22	25.5
speI	15	17.4	*sdaD*	0	0
speM	10	11.6	*mac*	46	53.4
speH	9	10.4	*scpA*	34	39.5
speK	5	5.8	*spyCEP*	26	30.2
smeZ	5	5.8	*speB*	21	24.4
ssa	4	4.6	*sic*	14	16.2

In 85 (98.8%) of the isolates, there were at least one and a most four Sag types. It was determined that one isolate had the DNase and protease genes but lacked the Sag gene. A minimum of one and a maximum of three isolates had DNase genes, which were found in 52 (60.4%) isolates. On the other hand, 34 isolates lacked the DNase gene. In 48 (55.7%) isolates, there were one to four protease genes. However, protease genes could not be detected in 38 isolates.

One of the significant findings in our study was the distribution of *emm* genotypes and virulence factors in GAS isolates isolated from the samples of two different patients. In the episode of the patient whose wound swab was taken

Table 1. Distribution of *emm* types in GAS isolates isolated from clinical samples

emm Types	Samples				Total (86)	
	Throat swab	Sputum	Skin-soft tissue swab	Urine	Number	Percent %
28	6	7	5[a], 1[b]	–	19	22.0
1	14	1	1[b]	–	16	18.6
12	10	–	1[a]	1[c]	12	13.9
3	7	1	1[a]	1[c]	10	11.6
89	1	1	1[b]	–	3	3.4
118.5	3	–	–	–	3	3.4
2	2	1	–	–	3	3.4
241	2	–	–	–	2	2.3
28.5	1	–	1[b]	–	2	2.3
163	1	1	–	–	2	2.3
212	1			1[c]	2	2.3
245	1		1[b]		2	2.3
244	1				1	1.1
203.1	1				1	1.1
89.31	1				1	1.1
6.19	1				1	1.1
stG 7882.3	1				1	1.1
11.1				1[d]	1	1.1
1.65				1[d]	1	1.1
108.1				1[d]	1	1.1
219		1			1	1.1
126		1			1	1.1
Total	54	14	12	6	86	100

a: Samples taken from patients with impetigo, b: Samples taken from patients with wound infections, c: Samples urine samples taken from patients with undefined urinary tract infections, d: Urine samples taken from kidney transplant patients.

on different dates due to skin-soft tissue infection, it was determined that the genotypes of *emm* were different (*emm28-emm28.8*) and some virulence factors were similar (Table 3). In the first sample, *emm*28.5 (Isolate no:70), *speC* and *mac*, *scpA*, *and spyCEP* protease genes were detected. In the second sample (Isolate no:11) taken two months after the same patient, *emm28* and *speC*, *sdaB*, *mac*, *scpA*, *and spyCEP* genes were detected. In the two-sample (wound swab and throat swab) types taken from different regions belonging to the other patient, *emm* genotypes and defined virulence factors were different from each other. However, only the *mac* gene was common (Table 3). *emm*245, *sdaB*, *spd3*, *mac*, scpA, *spyCEP*, *and speB* genes were detected in the strain isolated from the skin-soft tissue sample (Isolate no:76). e*mm*203.1, *speC*, *speG*, s*peJ*, *mac*, *and sic* genes were identified in the strain isolated from the throat sample (Isolate no: 78).

According to the results of our antibiotic suspectility testing, while all GAS isolates were susceptible to penicillin G, gentamicin, linezolid, and chloramphenicol, 19.8%, 16.3%, 4.7%, 3.5%, and 3.5% of GAS isolates were resistant to erythromycin, clindamycin, levofloxacin, ciprofloxacin, and telithromycin, respectively. Erythromycin resistance was observed in *emm*28, *emm*3, *emm*12, clindamycin resistance in *emm*28, *emm*2, *emm*12, *emm*241, levofloxacin resistance in *emm*28, *emm*28.5, *emm*3 ciprofloxacin resistance in *emm*212, *emm*3, *emm*11.1 and telithromycin in *emm*1, *emm*89, genotypes.

In evaluating phylogenetic relationships of the isolates with PFGE analysis, two isolates belonging to the same patient's wound culture isolates on different dates were 95% similar. Furthermore, the other isolates were separated into unrelated clusters, and there was no significant phylogenetic relationship between the isolates (Fig. 1).

DISCUSSION

The number of studies affecting the prognosis of infections of GAS strains that target genetic features, such as virulence factors, toxin structures, and *emm* types, is increasing.

Like our study, several studies investigated the distribution and prevalence of genotyping of GAS strain [14, 19–25]. The numbers of *emm* types and common genotypes obtained in these studies have been different or similar to our findings. However, as with epidemiological reasons, differences in the incidence rates could be expected in different periods and geographic regions. It is also possible to get different genotype incidence results even in studies performed in the same geographical area in different periods. If we discuss the matter in more detail, in terms of studies conducted in our country, compared with our study, there were differences in the distribution of *emm* genotypes; differences may be due to the number of samples studied, the type of infection, and regional differences. It is also the most common *emm*28 genotype in our study differed from the studies conducted in our country. In general, the common *emm* genotypes in the studies conducted in Turkey, including this work, are *emm*1, *emm*12, *and emm*89. However, studies carried out at different times in different regions are needed to have more detailed information and insights about *emm* genotypes in Turkey.

The knowledge about the role of Streptococcal Sags in infection and pathogenesis has increased over the years. Therefore, the distribution of Sag genes has been an epidemiological tool for further investigating the relationship between genomic heterogeneity, toxin gene and infection. In our study, while the most common Sag types were *speG* and *speC*, no *speL* gene was found in any isolates, among virulence genes such as SAgs, DNase, and protease in GAS strains. Among the DNase, the most common gene was *the sdaB*, whereas the *sdaD* was not in any isolates. In addition, we observed that the *mac* was the most common and the *sic* the least common among protease factors. The Sags, which we defined as more prevalent in our study, were consistent with the study done by Imöhl et al. [10] and were compatible with the studies conducted by Strus et al., Friaes et al., Mengeloğlu, and Karaky et al. [26–29]. In addition, the frequency of Sags, DNase, and protease genes we found in the present study, was similar to a work conducted by Dağı et al. [13].

One of our most striking findings was the virulence factors profiles of GAS isolates isolated from the same tissue samples taken two times on different dates (between two months) from a patient who could not respond to treatment with skin-soft tissue infection. The *emm* genotypes of these isolates were different (*emm28–emm28.8*) and some virulence factors were similar (Table 3). Within two months, it was evaluated as an important finding that shows how important the modifications or gains seen in virulence factors in escaping from the immune system with the acquisition of the *sdaB* gene through the M protein modification and bacteriophage demonstrated by the first isolate. In the PFGE analysis of these two isolates, it was determined that

Table 3. Distribution of virulence genes of GAS isolated from samples from the same patients from different times/regions

Isolate No	Sample	*emm* types	Sag genes	DNase genes	Protease genes
11*	Wound swab	28	*speC*	*sdaB*	*mac, scpA, spyCEP*
70*	Wound swab	28.5	*speC*		*mac, scpA, spyCEP*
76+	Wound swab	245		*sdaB,spd3*	*mac, scpA, spyCEP, speB*
78+	Throat swab	203.1	*speC, speG, speJ*		*mac, sic*

* Virulence gene profiles of GAS isolates isolated from the same wound samples taken two times on different dates from a patient.
+ Virulence gene profiles of GAS isolates collected from different body parts from the other patient.

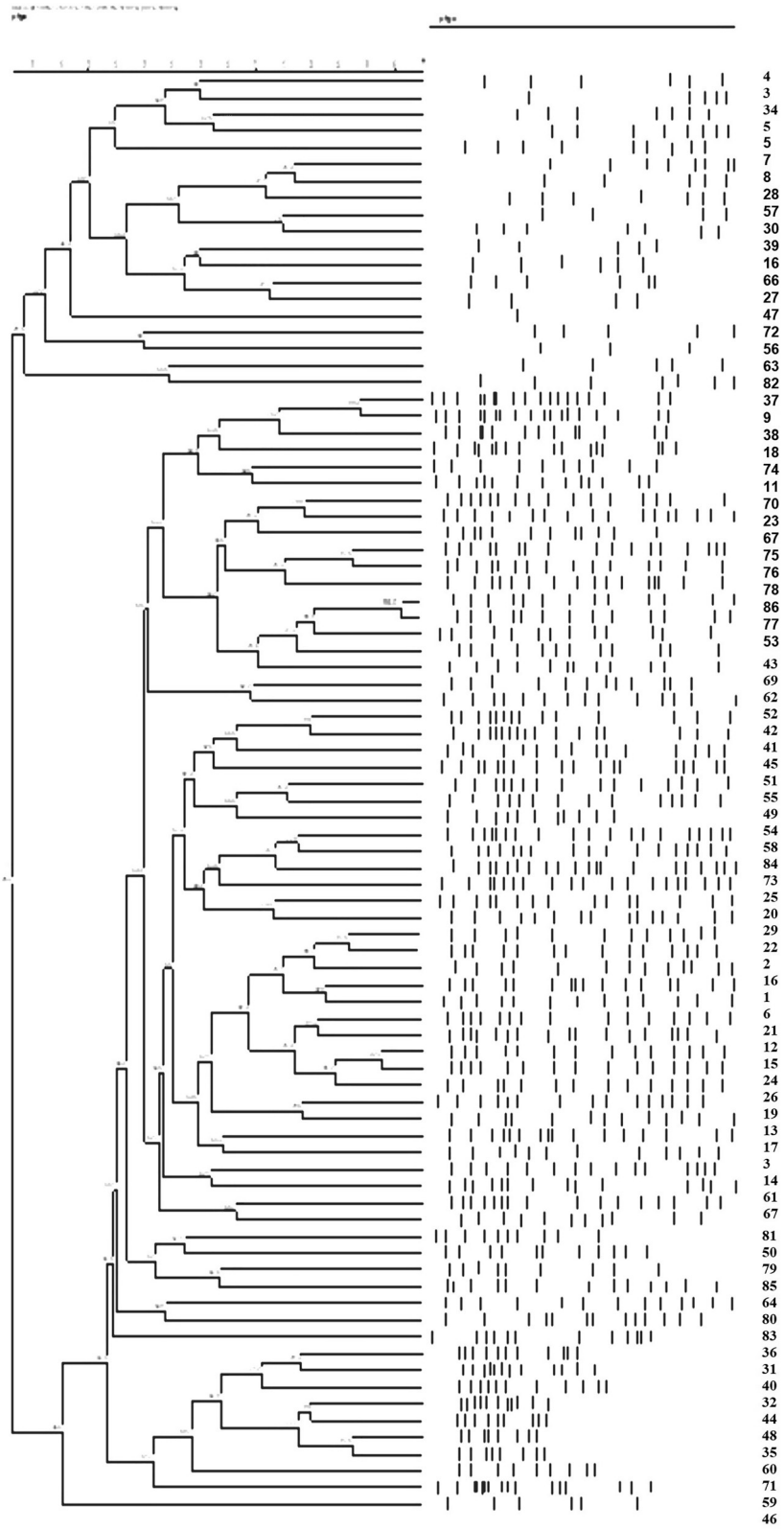

Fig. 1. Dendrogram of PFGE clusters and phylogenetic relationships of GAS isolates

they were closely related with a 95% similarity rate. PFGE patterns suggest that these isolate likely descended from a common ancestor. In other words, this patient was considered a reactivation, not reinfection.

Another remarkable result was that the isolates isolated from the upper respiratory tract and wound region of a patient showed different types of *emm* and other virulence factors profiles. The strain isolated from the wound sample had *emm*245, *sdaB*, *spd3*, *mac*, *scpA*, *spyCEP*, *and speB* genes, while the strain isolated from the throat sample had *emm*203.1, *speC*, *speG*, *speJ*, *mac*, *sic* genes. This result is one of the most critical proofs that different strains with different *emm* genotypes and virulence factors profiles can be seen in different localizations in the same patient.

In our study, GAS isolates were sensitive to penicillin G, gentamicin, linezolid, and chloramphenicol. Consistent with the findings obtained in our study, no resistance was observed against these antibiotic groups in other studies reported in our country [13, 25]. These antibiotic options can be used in the treatment of GAS infections. However, it should not be forgotten that the isolates have many virulence genes and that resistance may be seen against these antibiotic groups over time. The resistance of GAS isolates to erythromycin (19.8%), clindamycin (16.3%), levofloxacin (4.7%), ciprofloxacin (3.5%), and telithromycin (3.5%) in our study may be due to the preference of these antibiotics groups in empirical treatment. In addition, erythromycin, clindamycin, levofloxacin, ciprofloxacin, and telithromycin resistance showed a heterogeneous distribution according to *emm* genotypes.

In our study, when the phylogenetic relationships of the isolates were evaluated by PFGE analysis, it was observed that two isolates of the same patient's wound culture isolated from different dates were 95% similar. Also, the other isolates were separated into unrelated clusters, and there was no significant phylogenetic relationship between the isolates. PFGE analysis showed no significant phylogenetic relationship between the isolates in our study, which was consistent with the studies by Karaky et al. and Mengeloğlu [28, 29].

CONCLUSION

The findings obtained in this study suggest that it is necessary to evaluate the patient's clinical history and virulence factors together by determining the profiles of GAS virulence factors and their genetic relationships in our country, which is important regarding vaccine studies, predictive guides, and epidemic analysis. Also, it will be helpful to continue this preliminary study with larger case groups and a number of patients.

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Authors' contributions: SK collected samples, cultured the isolates, and SK performed DNA extraction, PCR, *emm* sequencing, PFGE. FK and SK designed this study. FK and SK supervised the practical work and data management. SK, CÖG and FK wrote the manuscript. All authors approved the final version of this manuscript.

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