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Original Article

Isolation of *Streptococcus pyogenes* from children with pharyngitis and *emm* type analysis

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Abstract

Background: The group A streptococcus (GAS) M protein, encoded by the *emm* gene, acts as a major virulence factor. *Emm*-typing is the GAS gold standard molecular typing and is based on the DNA sequence of the nucleotides of the *emm* gene. The aim of the present study was to isolate GAS from patients and to detect the *emm* types of the isolates using *emm* typing.
Methods: A total of 1000 throat samples were collected from patients with pharyngitis referred to Aboozar Children's Hospital in Ahvaz, Iran. We performed antimicrobial susceptibility testing on all isolates using the Kirby–Bauer disk diffusion method. Additionally, amplification of the *emm* gene was performed using polymerase chain reaction using the standard primers and described protocol.
Results: From all throat samples screened, 25 isolates (2.5%) were identified as GAS. Antibiotic susceptibility testing revealed that all the GAS isolates were susceptible to penicillin and erythromycin, but 44% showed resistance to vancomycin. Based on polymerase chain reaction for the *emm* gene, the obtained *emm* types were: *emm-3*, observed in 20 isolates (80%); *emm-1* observed in four isolates (16%); and *emm-75* observed in one isolate (4%).
Conclusion: The result of the present study showed that penicillin and erythromycin are still the most effective antibiotics against the organism. The *emm* typing revealed that *emm* type-3 was detected in most of the isolates from patients with purulent pharyngitis. On the basis of the findings of this study, we may conclude that *emm* typing provides new insights on the genetic diversity of the M proteins, and is of demonstrable value for molecular studies of GAS.

Keywords: antibiotic resistance; *emm* typing; *Streptococcus pyogenes*

1. Introduction

Group A Streptococcus (GAS) or *Streptococcus pyogenes* is a gram-positive cocci with humans as its specific host. It is capable of causing a large variety of infections ranging from simple benign infections like sore throats and impetigo to fatal diseases like streptococcal toxic shock syndrome and necrotizing fasciitis, acute rheumatic fever, and acute glomerulonephritis.[1,2] The mortality rate of severe GAS infections remains high, both in developed and developing countries.[3,4] Additionally, there are reports of outbreaks of invasive GAS infections in the community and in hospitals.[5,6]

Due to an elevated global prevalence of GAS disease, epidemiological surveillance is necessary to detect changes in disease distribution in various populations. Typing of a collection of GAS isolates is important as part of the epidemiological surveillance for the disease. There are several

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typing methods available for screening GAS isolates. Among them, typing based on the M protein, a cell-surface protein that is the major virulence and immunological determinant of GAS, has been the most widely used method.[7] The M protein which is encoded by the *emm* gene possesses a hypervariable region of the amino-terminal with 40–50 amino acid residues.[8] A GAS typing system based on sequencing of this N-terminal hypervariable region of the M protein (*emm*) gene is known as *emm* typing and is the "gold standard" method used to characterize GAS isolates.[9] This method has been used for identification of different *emm* types. The surface proteins are not only a suitable substrate for typing and studying the molecular epidemiology of GAS isolates, but also represents choice candidates for the development of an effective vaccine against GAS-related serious diseases due to their critical role in host–bacteria relationships.[10,11] Currently, more than 170 *emm* types and 750 *emm* subtypes of GAS are known.[12] The distribution of *emm* types reportedly varies among different countries and regions.[13]

Due to the lack of comprehensive information about different types of M protein among GAS isolates in Ahvaz city, the current study was proposed to isolate GAS from the patients' throat suffering from pharyngitis and typing their M proteins. This undertaking is the first molecular epidemiologic analysis of GAS strains associated with children's pharyngitis in south western Iran.

2. Methods

2.1. Sampling

Our study reviewed a total of 1000 throat samples obtained from children with pharyngitis ranging in age from 2 years to 14 years, who were referred to Aboozar Children's Hospital in Ahvaz, southwestern Iran, from November 2012 to June 2013. The preliminary proposal of the work was reviewed and approved by the hospital's Institutional Review and Ethics Board, and the necessary permission to collect the requisite samples and initiate the work was obtained.

Standard patient demographics and clinical data were recorded, including age, sex, and disease onset, and patient symptoms were recorded. All patients who presented with fever and sore throat were entered into the study, and those with prior antibiotic therapy or patients with other respiratory tract symptoms such as rhinorrhea or nasal congestion were excluded from the study by the available pediatrics infectious diseases specialist at the time of admission.

2.2. Phenotypic identification of GAS

A single throat swab was taken from each patient and immediately placed in a thioglycolate broth and transferred to the microbiology laboratory, where the broth was incubated at 37°C for 24 hours, with subsequent subculture on a sheep blood agar plate (HiMedia, Mombai, India) the next day. The identities of the colonies were confirmed based on morphological and growth characteristics, including gram staining, beta-hemolysis on blood agar medium, bacitracin susceptibility, pyrrolidonyl arylamidase test, and resistance to trimethoprim-sulfamethoxazole.[14]

2.3. Antimicrobial susceptibility testing

For confirmed GAS isolates, antimicrobial susceptibility testing was done using the standard disk-diffusion method on Müeller–Hinton agar with 5% sheep blood, incubated overnight at 37°C in air enriched with 5% CO_2 according to the Clinical and Laboratory Standard Institute (CLSI) guidelines.[15] The commercial antibiotic discs (MAST Co., London, UK) were as follows: penicillin G, ampicillin, ceftriaxone, vancomycin, azithromycin, chloramphenicol, clindamycin, and erythromycin. The interpretation criteria of the susceptibility testing were in accordance to the CLSI recommendations.

2.4. Polymerase chain reaction amplification for the detection of the emm gene

For DNA extraction from the isolates, a commercial extraction and purification kit (Roche, Berlin, Germany) was used according to the manufacturer's instructions. The extracted DNA purity was measured with a photobiometer (Eppendorf, Hamburg, Germany) in 260/280 nm UV long waves. In order to amplify the *emm* gene, the set of primers of *emm1* (5′-TATTCGCTTAGAAAATTAA-3′) and *emm2* (5′-GCAAGTTCTTCAGCTTGTTT-3′) were used,[16] which amplifies a 914 bp fragment of the target gene. Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 μL containing 1 × PCR buffer, 1.5mM $MgCl_2$, 200μM deoxynucleotide, 0.4mM of each primer, 1.5 U *Taq* polymerase, and 1 μL of template DNA. All the reagents were purchased from Qiagen, Hilden, Germany. Amplification was performed on a thermocycler nexus gradient (Eppendorf) and the cycling program consisted of initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 46°C for 45 seconds, extension at 72°C for 59 seconds, and a final extension at 72°C for 7 minutes. A control positive for *S. pyogenes* ATCC 8668 and a control negative for *S. pyogenes* ATCC 8668 were included in each PCR run. The products were run on 1.5% agarose gel (w/vol.) containing 0.5 μg/mL ethidium bromide (Qiagen). Results were recorded using the gel documentation system (Protein Simple, San Jose, CA, USA). A 100-bp DNA ladder was used as a size marker (Roche). The PCR products were sent for sequence analysis (Bioneer Co., Daejeon, South Korea). The *emm* sequences were blasted against the *emm* database at the BLAST program, National Center for Biotechnology (www.ncbi.nlm.nih.gov/BLAST/) to determine the *emm* sequence type.

The data were analyzed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). In the univariate analysis, continuous and categorical data were analyzed using Student *t* test and the Mantel–Haenszel test, respectively.

Table 1
Antimicrobial susceptibility testing results for group A streptococcus isolates.

Antibiotic	Sensitive no (%)	Intermediate no (%)	Resistant no (%)
Penicillin	25 (100)	0 (0)	0 (0)
Ampicillin	25 (100)	0 (0)	0 (0)
Erythromycin	21 (84)	3 (12)	1 (4)
Azithromycin	17 (68)	6 (24)	2 (8)
Ceftriaxone	17 (68)	5 (20)	3 (12)
Vancomycin	14 (56)	0 (0)	11 (44)
Clindamycin	17 (68)	7 (28)	1 (4)
Chloramphenicol	12 (48)	9 (36)	4 (16)

3. Results

From 1000 throat samples obtained from children with sore throat of different severities (mild to severe), 25 samples were positive (2.5%) in which the isolates were identified as GAS on the basis of phenotypic identification criteria. The GAS isolates belonged to 14 (56%) male and 11 (44%) female patients. The results from susceptibility testing are presented in Table 1, showing fully and 84% sensitivity to penicillin and erythromycin, respectively. Chloramphenicol accounted for the least effective antibiotic (48%).

The presence of the *emm* gene was analyzed using PCR, which generated a 914-bp band for all GAS isolates (Fig. 1). Blast analysis of sequence similarities for the 25 GAS isolates represented three different *emm* sequence types. The frequency of *emm* types among the GAS isolates were as follows: *emm* type 1, four isolates (16%); *emm*3, 20 isolates (80%); and *emm*75, one isolate (4%).

All of our GAS strains were isolated from patients with purulent pharyngitis and it seems that the *emm*3 type is mainly associated with severe pharyngitis in the region of study. The association between antimicrobial profile and *emm* type was also investigated in this study, where no significant correlation was observed (results not shown). In Table 2, the distribution of *emm* types are presented according to patients' age and sex. The *emm*75 and *emm*3 types sequences were confirmed by GenBank with accession numbers LM999955 and LM999956, respectively.

4. Discussion

Streptococcal infections are a major problem in medical and health care centers. Typing of GAS is an important part of the epidemiological and pathogenetic studies of streptococcal diseases. Most of the understanding regarding GAS epidemiology is based on the M typing system including *emm* typing.[17]

In our study, low strain diversity was noticed by *emm* typing, where *emm*3 was the most prevalent type with a frequency of 80%. This lower *emm* distribution is similar to a recent study from Northern Iran, in which four sequence types of *emm*5, *emm*12, *emm*79, and *emm*86 were reported with *emm*5 as the most prevalent.[18] None of the types are similar to ours, which could be explained by the difference in climate and geographical conditions which may affect the GAS strain diversity; their study was conducted in a distinctly opposite geographical area, with a considerably different climate. In a similar study from China, 13 different *emm* types were identified among 185 GAS isolates from

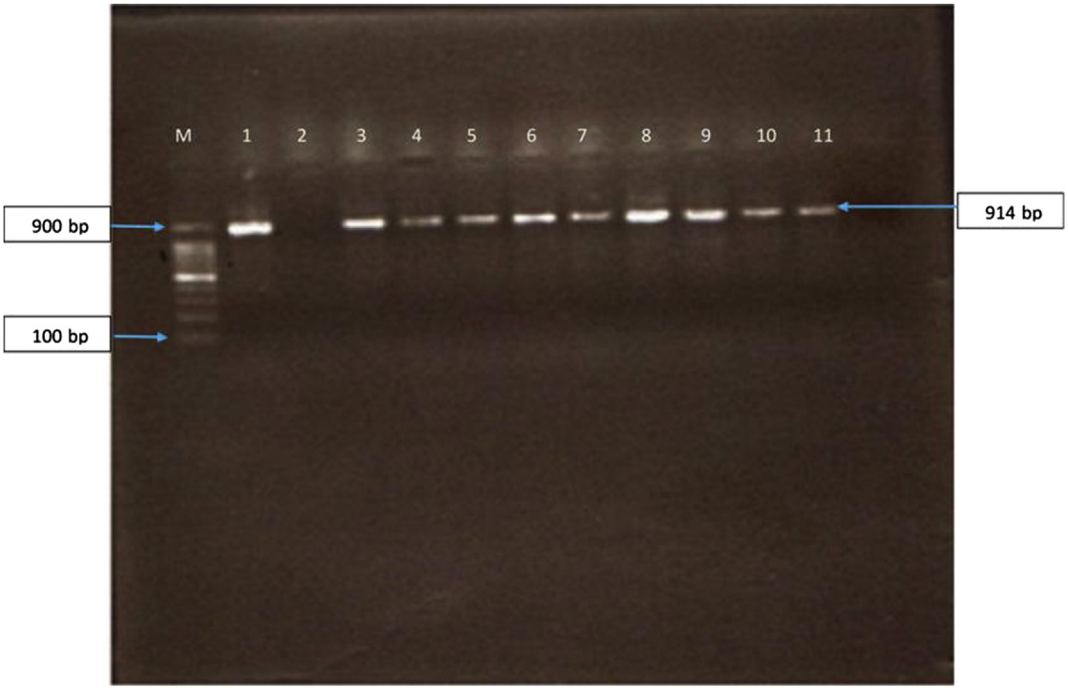

Fig. 1. Polymerase chain reaction amplification of emm gene in streptococcus pyogenes. Lane 1, DNA size marker; Lane 2, positive control *Streptococcus pyogenes* ATCC 8668; Lane 3, negative control; Lanes 4–12, *emm* gene with length 914 bp.

Table 2
emm type distribution according to patients' age and sex.

Sex no (%)		Age yr (%)			*emm* type
Male	Female	>10	5–10	<5	
8 (40)	12 (60)	5 (25)	12 (60)	3 (15)	*emm*3
1 (25)	3 (75)	0	1 (25)	3 (75)	*emm*1
1 (100)	0	0	1 (100)	0	*emm*75

pharyngitis cases with *emm*1 and *emm*12 as the more prevalent types.[12] However, in the study of Bahnan et al,[19] from Lebanon, 33 different *emm* types were discovered in 103 GAS strains examined and the most prevalent types were reported as *emm*1 and *emm*22. Also, the reported prevalent types from Taiwan were *emm*1, *emm*4, and *emm*12, discovered in noninvasive streptococcal disease.[20] Steer et al[7] in a detailed study reported the similarities in *emm* type distribution between the developing and developed countries. For instance, the most common pharyngeal types reported in Asia, were *emm*44, *emm*12, and *emm*75, respectively. This variation in distribution of *emm* type profiles from different parts of the world shows that specific geographic conditions affect the *emm* diversity.

In our study *emm*75 showed the lowest frequency and was different from the study of Sagar et al,[21] in which they reported *emm*75 as the major type in isolates from patients' throats.

According to the susceptibility testing results, all our tested isolates were sensitive to penicillin and ampicillin which agrees with the findings of Jafarpour et al,[18] Behnan et al,[19] Le Hello et al,[9] and Wu et al.[22] Most researchers know erythromycin to be the best alternative drug for cases of penicillin allergy in infections caused by GAS in the oral cavity. Although the resistance to erythromycin is low in most countries, in recent years some local resistance to the drug has been reported due to overuse. In our study, a 4% resistance to erythromycin was noticed which is lower than the 15.6% in the study of Rijal et al,[23] and 10% resistance in the study of Bahnan et al.[19] Moreover, an incremental increase in GAS strains with resistance to erythromycin has been reported in Taiwan, Europe, and the USA.[22]

Based on these results, it can be concluded that GAS still has good sensitivity to penicillin, and there is no obvious penicillin-resistant GAS at the present time in the studied region. Therefore, penicillin can be applied in the treatment of bacterial pharyngitis with or without susceptibility testing.

4.1. Limitations

This study had several limitations. Firstly, the number of cases included in our study was too small. Although we tested 1000 children with sore throats of different severities, and we tried to exclude those with antibiotic consumption or other respiratory complications, since this hospital is a referral children hospital for the whole province, the exact control of this exclusion criterion was difficult and perhaps this explains the low number of GAS recovered from this study population. Due to the same reason, we could not find any significant relationship between *emm* types and severity of the disease and antibiotic resistance.

In conclusion, although the number of positive samples in our work was low, this study is the first report on *emm* typing from the southern part of Iran. Based on our findings, *emm* type 3 was the most prevent type in the region, and we still have no problem in the treatment of GAS pharyngitis cases with penicillin and erythromycin. From this study we may conclude that *emm* typing provides new insights on the genetic diversity of the M proteins and is of value for molecular studies of GAS. This is more valid when extended studies with higher numbers of isolates are conducted in the future.

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