



The Place of Group A Streptococci in Moroccan Children with Pharyngitis and Emm Type Distribution

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Abstract

Background: *Streptococcus pyogenes* is responsible for a wide variety of diseases, including noninvasive and severe invasive infections. The *emm* gene encodes the M protein that is the virulence factor and immunological determinant of group A streptococci. *Emm* typing is the group A Streptococci (GAS) standard molecular typing method based on the amplification of the N terminal hypervariable region of the *emm* gene.

Objectives: The aim of the present study was to determine the prevalence of GAS in children with pharyngitis and determine different types of *emm* gene in the GAS isolates using *emm* typing.

Methods: The study was carried out over a period of 14 months (from February 2017 to March 2018). Throat samples were collected from cases aged ≤ 18 years with pharyngitis referring to a primary health care center in Fez, Morocco. GAS isolates were subjected to conventional tests to confirm species identification. Antimicrobial susceptibility testing was performed using the standard disk diffusion method. We researched *emm* gene by a polymerase chain reaction (PCR). *Emm* types were determined by a sequence-based protocol. Demographic and clinical data were recorded from each patient.

Results: From a total of 177 throat samples, 11 isolates (6.2%) were identified as GAS in children with pharyngitis. Antibiotic sensitivity testing revealed that all the GAS isolates were sensitive to penicillin. The sequencing of the PCR products of the *emm* gene revealed that *emm90* was the most obtained *emm* type (30,77%); while *emm75* was the least type observed (7.7%).

Conclusions: The *emm90* is the most prevalent type detected from patients with tonsillitis. Penicillin and erythromycin are still the foremost effective antibiotics to treat GAS pharyngitis.

Keywords: *Streptococcus pyogenes*, Children, Pharyngitis, *emm* Typing

1. Background

Streptococcus pyogenes Group A *Streptococcus* (GAS) is one of the foremost clinically important Gram-positive pathogens causing a wide spectrum of human infections. It causes diseases ranging from relatively benign infections, such as sore throats, to severe invasive diseases, like streptococcal toxic shock syndrome and acute rheumatic fever (ARF) (1, 2). The rate of mortality of invasive GAS infections, especially those with ARF, is high in developing countries, and the majority of them develop rheumatic heart disease (RHD) (1, 3, 4). In Morocco, two-thirds of children with ARF have a history of sore throat, of whom 53.1% de-

velop RHD and 2.7% die (4).

GAS is responsible for 15 - 30% of acute pharyngitis cases in children (5, 6). This elevated prevalence may be due to the changes in specific virulence factors of GAS. Several outbreaks of a rare serotype of GAS pharyngitis and invasive GAS infections have been reported (7-9). Cell-surface M protein is the main determinant of the virulence of GAS (2). The M protein is encoded by the *emm* gene and has a hypervariable region of 40 to 50 amino-terminal acid residues (10-12). The sequencing of this region of the M protein constitutes the GAS typing system and it is named *emm* typing. It is the currently accepted reference standard methodology used for characterizing GAS isolates

and identifying the different *emm* types (13).

Nowadays, more than 170 *emm* types and 750 *emm* subtypes of GAS have been documented (14). The distribution of *emm* types is different depending on the countries and regions (2, 15). Therefore, typing of *streptococcus pyogenes* isolates in each region is essential as part of the epidemiological surveillance for the disease (6, 16).

Regarding the treatment of GAS infections, it is based on beta-lactams, especially the penicillin G that presents the treatment of choice for doctors because of its effectiveness, cost, and narrow spectrum of activity. Erythromycin and first-generation cephalosporin are recommended for patients with penicillin allergies (17, 18). However, inadequate treatment of pharyngitis and specially GAS pharyngitis can cause severe diseases. This is why it is important to know the pathogens responsible for pharyngitis and their virulence in order to reduce the risk of ARF and other post-streptococcal infections.

2. Objectives

To our knowledge, this is the first study in Morocco that gives an idea about the different types of M protein in GAS isolated from children with pharyngitis. The main aim of this exploration was to determine the prevalence of GAS pharyngitis in children with throat infections and know the different types of their M proteins.

3. Methods

3.1. Patients and Samples

This cross-sectional descriptive study was done for 14 months (from February 2017 to March 2018). The samples were taken from the throats of children with pharyngitis, consulting a primary health care center in Fez, Morocco.

The patients who participated in this study were diagnosed by their doctors with throat infections. We included all children who were presented with an erythematous or erythematous pultaceous sore throat, fever, and headache. Patients with other respiratory tract symptoms and those with prior antibiotic therapy (in the last seven days) were excluded. A throat swab was considered the technique of sampling in this study. The swab collects a sample of the secretions produced in the back of the throat. To decrease technical bias, the back of the throat should be clearly seen. If the tongue and/or the cheeks are touched, the throat swab is remade. The tube should be sealed tightly.

We recorded the demographic and clinical data of patients, including the age of children, gender, and disease

onset. The symptoms were also registered. The data were collected after an unwritten agreement and explanation about the purpose of the study. Microbial analysis was performed at the laboratory of microbiology and molecular biology, Fez Medicine School. For quality control, we used a collection of GAS strains already isolated in our laboratory.

3.2. Phenotypic GAS Characterization

After the collection of the throat swabs from the children included in the study, it was immediately placed in brain heart infusion broth (BHI) (Oxoid, UK). Later, the samples were incubated at 37°C for 24 hours in the laboratory oven.

The next day, a subculture was carried out on blood agar plates with 5% CO₂ at 37°C overnight (Biomérieux, France). To identify the colonies isolated, different characteristics of morphology or growth were considered. The beta hemolysis on blood agar medium, catalase test, Gram staining, and latex agglutination test were the used (Oxoid, UK). The bacitracin susceptibility was determined by the absence of an inhibition zone around the disk of bacitracin (0.04 IU). The sulfamethoxazole-trimethoprim (SXT) resistance was determined by the presence of an inhibition zone around the disk of SXT.

3.3. Antibiogram

To determine the sensitivity/resistance of GAS colonies against different antibiotics, we used the standard disk-diffusion method on Mueller Hinton agar with 5% sheep blood (Biomérieux, France). The colonies were incubated for 24 hours at 37°C with 5% CO₂ Jar according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017). The commercial antibiotic discs (Oxoid, France) were as follows: penicillin G (1 µg), Vancomycin (5 µg), clindamycin, erythromycin (15 µg), gentamicin (10 µg), tetracycline, and rifampicin.

3.4. Detection of the *emm* Gene

Total DNA was extracted from the GAS isolated and the broth of the throat respectively using a commercial extraction and purification kit (Prep Man) (Applied Biosystems, USA). In order to amplify the *emm* gene, primers 1 and 2 (<http://www.cdc.gov/streplab/protocol-emm-type.html>), were used for amplifying the N-terminal region of the *emm* gene: primer 1: 5-TAT TCG CTT AGA AAA TTA A-3 and primer 2: 5-GCA AGT TCT TCA GCT TGT TT-3.

The PCR reaction mixture included PCR buffer, 1.5 mM MgCl₂, 10 mM deoxynucleotide, 70 pM of each primer, 3 U Taq polymerase, and 1 µL of the template DNA (Promega,

Madison, USA). The final volume was 25 μ L. Amplification was performed on a thermocycler Hybaid's PCR Express™. The amplification program for PCR reactions composed of an initial denaturation (94°C, 1 minute), 30 cycles of denaturation (94°C, 15 seconds), annealing (47°C, 30 seconds), extension (72°C, 1 min 15 seconds), and a final extension (72°C, 7 minutes). PCR products were run on 2% agarose gel (FMC BioProducts, Rockland, ME) containing 0.5 mg/mL ethidium bromide (Qiagen) followed by ultraviolet illumination, and were photographed with an Olympus digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany). The PCR products were sequenced with the same primers used for PCR amplification to determine the *emm* sequence type (3130-1 Genetic Analyzer, Applied Biosystems, Foster City, CA). The sequences of *emm* gene were analyzed using the BLAST program (<http://www.ncbi.nlm.nih.gov>).

3.5. Data Management and Analysis

Descriptive statistics were used to characterize the respondent's demographic and clinical characteristics. Frequencies were used for qualitative variables. Means and standard deviations were used for quantitative variables. Statistical analyses were performed using EPIinfo7.

3.6. Ethics Statement

The research protocol was approved by the Ethics Committees of the Hassan II University Hospital Center, Fez, Morocco. Written informed consent was obtained from each child's parent or legal guardian. The data were analyzed anonymously.

4. Results

The present study was carried out on 177 children with pharyngitis. The age range of the participants was between 5 and 17 years. The mean age was 9 ± 3 years and the sex ratio (male/female) was 1.05. The majority of the cases belonged to a middle-class family (72.3%) and were admitted in autumn and winter (43.5% and 39%, respectively) (Table 1).

The majority of children with pharyngitis were presented with a sore throat and pain in swallowing and fever greater than 38°C (91.5% and 90.4%, respectively). The tonsillar exudate was present in 35.6% of children (Table 2). None of the children had developed ARF.

Among one hundred seventy-seven throat samples obtained from children with pharyngitis, 11 were identified as *S. pyogenes* by the conventional method (6.2%). The GAS

Table 1. Demographic Characteristics of the Study Population

Characteristics	No. of Cases	Percentage of Total
Gender		
Male	90	50.8
Female	87	49.2
Season		
Autumn	77	43.5
Winter	69	39
Spring	30	16.9
Summer	1	0.6
Education level		
Unschooling	21	11.9
Koranic	128	72.3
Primary	23	13
Secondary	5	2.8
Social level		
Low	21	11.9
Middle	156	88.1
High		0

isolated from children belonged to 4 male (36.4%) and 7 female (63.6%) patients. Five out of the eleven children with GAS pharyngitis had tonsillar exudates (45.5%) and the others had pharyngeal erythema. Ten (90.9%) isolates of GAS were sensitive to bacitracin and resistant to SXT. All GAS isolates were sensitive to penicillin and rifampicin, whereas one isolate expressed resistance to erythromycin and clindamycin (9.1%). The results of the antibiogram are presented in Table 3.

PCR generated 13 different bands of the *emm* gene: 11 present the GAS isolated previously by the conventional method and 2 obtained from the broth of the throat (Figure 1). In 11 GAS isolates in culture, the BLAST analysis of sequence similarities identified 5 types of *emm* gene. Two other *emm* sequence types were identified from the DNA of 2 broths of the throat. The frequency of *emm* types were as follows: *emm* type 1, one isolate (7.69%); *emm*12, two isolates (15.38%); *emm*22, one isolate (7.69%); *emm*75, one isolate (7.7%); *emm*82, one isolate (7.69%); *emm*89, three isolates (23.08%); and *emm*90, four isolates (30.77%). Table 4 presents the distribution of *emm* types according to patients' age and sex. All children with pharyngitis and that presented the type *emm* 90 were admitted in autumn. The other types of *emm* were presented in autumn and winter. The distribution of *emm* types according to the season is presented in Table 5.

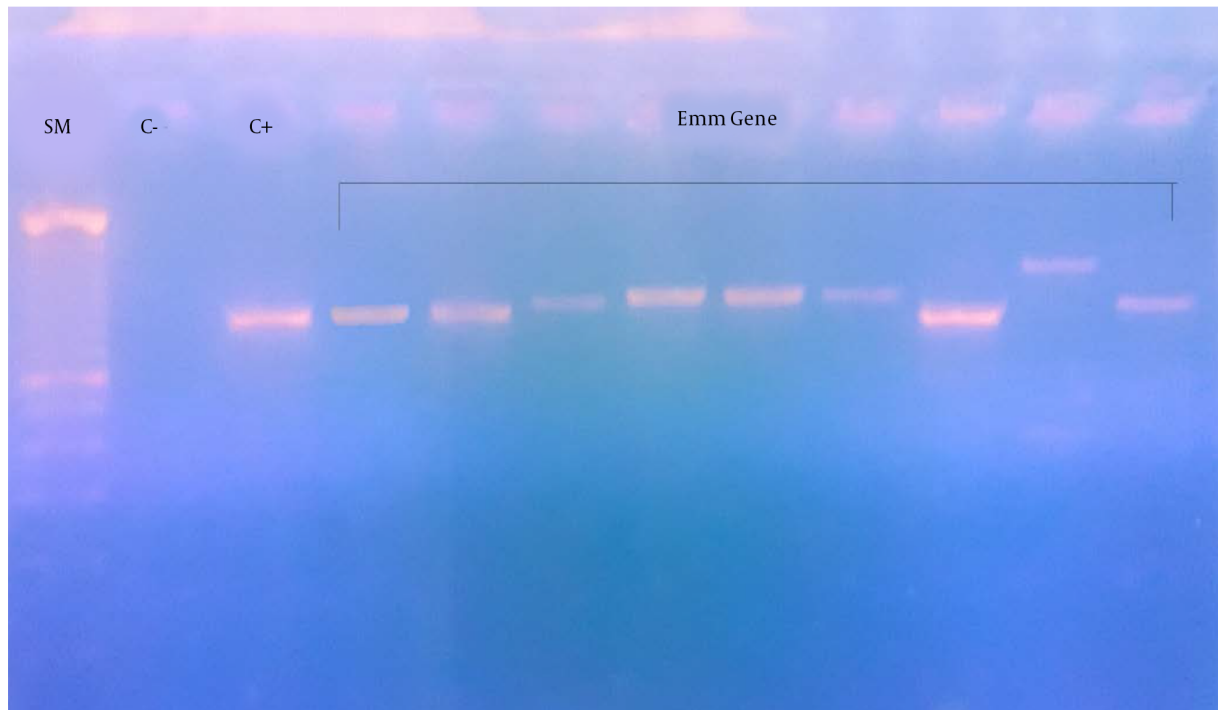


Figure 1. Polymerase chain reaction (PCR) amplification of M protein in group A *Streptococcus* (GAS). SM: DNA size marker, C-: negative control, C+: positive control *Streptococcus pyogenes*; Lanes 4-12, *emm* gene with the length ranging from 900 to 1400-bp.

5. Discussion

Diseases caused by *S. pyogenes* are a worldwide problem. The determination of GAS prevalence and *emm* typing is an added value to studies of streptococcal diseases. The M typing system, including *emm* typing, plays a significant role in the understanding of the GAS epidemiology. As far as we know, this research is the first attempt at *S. pyogenes* characterization through *emm* typing in Morocco.

A global estimate of cases of GAS infections was carried out in 2005. It was found that over 616 million incident cases per year of GAS pharyngitis are in developing countries and GAS was considered the 9th leading cause of death due to infection (1). In a study done from March 2006 to February 2007 in four primary health care centers in Rabat and Sale cities in Morocco, the overall prevalence of GAS was 9.3% and it was 9.1% in children (19). In our study, the prevalence of GAS was 6.2%, lower than that estimated by the work of Barbosa and al. (23%) (20). A meta-analysis study found that the prevalence of GAS in 14 studies analyzed was 37%. However, the authors highlighted a high heterogeneity in these studies and the prevalence was ranged from 17% to 58% (21). Another study in Northern India found a prevalence of 2.8% of children with GAS, which

is lower than our results (22).

In addition, *emm* typing revealed 7 *emm* profiles and *emm90* was the most prevalent type with a frequency of 30.77%, followed by *emm89* (23.08%) and *emm12* (15.38%). Studies done in Sweden and Brazil reported that just one child with pharyngitis presented the type *emm90* (3.1% and 0.92%, respectively), which is lower than our result (23, 24). The studies done by the French National Reference Center for Streptococci and Chen and al. linked this type of *emm* to invasive GAS infections (25, 26). However, other studies showed that *emm90* was involved in a severe epidemic of GAS pharyngitis (7, 16). Few studies found that *emm90* is related to GAS pharyngitis because it is a new M type and it is rarely isolated (27).

The distribution of the *emm* types varies depending on the region. In studies done in Canada, India, and Italy, it was found that *emm12* was the most prevalent *emm* type among children with GAS pharyngitis (28-30). In a study done in Lebanon, Bahnan and al. reported that the most prevalent *emm* types were *emm1*, *emm22*, and *emm28* (31). Moreover, in Tunisia, it was shown that *emm118*, *emm42*, and *emm28* were the most predominant types (32). There were no similarities in the types of *emm* between different studies and ours. This could be explained by the specific ge-

Table 2. Distribution of the Children with Acute Throat Infection According to Their Presenting Symptoms and Signs

Symptoms	No. of Cases	Percentage
Presented Symptoms		
Sore throat and pain in swallowing		
Yes	162	91.5
Total	177	100
Fever		
Yes	160	90.4
Total	177	100
Headache		
Yes	140	79.1
Total	177	100
Nausea and vomiting		
Yes	60	33.9
Total	177	100
Chills and sweats		
Yes	79	44.6
Total	177	100
Presented Sign		
Tonsillar exudates		
Yes	63	35.6
Total	177	100
Earache		
Yes	24	13.6
Total	177	100
Lymphadenopathy		
Yes	46	26
Total	177	100
Rash		
Yes	2	1.1
Total	177	100

ographic conditions and differences in climate in different regions of the world that may affect the diversity of *emm* gene (2). We also found that *emm* 75 had the lowest frequency. This Result is similar to the study in Iran and Tokyo that revealed that *emm* 75 was the least prevalent isolated *emm* type (33, 34).

The distribution of the *emm* types varies depending on the season. In our study, *emm* 90 and *emm*75 peaked in autumn. However, in the study done in the US, *emm*90 (cluster E2) and *emm*75 (cluster E6) peaked in summer (35, 36). Other studies done in Western Norway and Finland indicated that a peak of GAS incidence occurred during win-

Table 3. Antibigram Results for Group A Streptococcus

Antibiotics	Sensitive, No. (%)	Resistant No. (%)
Bacitracin	10 (90.9)	1 (9.1)
Sulfamethoxazole-trimethoprim	1 (9.1)	10 (90.9)
Penicillin	11 (100)	0 (0)
Gentamicin	10 (90.9)	1 (9.1)
Erythromycin	10 (90.9)	1 (9.1)
Clindamycin	10 (90.9)	1 (9.1)
Tetracycline	9 (81.8)	2 (18.2)
Vancomycin	9 (81.8)	2 (18.2)
Rifampicin	11 (100)	0 (0)

ter and summer. The most presented *emm* types were *emm*1 and *emm*28, while the *emm* 75 was less presented in this series (37, 38)

Due to the small sample size, the distribution of the *emm* types according to age was not examined. This is similar to the observation of the study that was done in Japan (34).

As reported by several studies, all our tested isolates were sensitive to penicillin G (18, 28, 31-33). Hence, penicillin remains the drug of choice for treating GAS infections. In case of allergy to penicillin, researchers recommend erythromycin as the best therapeutic alternative for streptococcal infection in the oral cavity. However, over the last few years, some countries reported resistance to erythromycin because of the overuse of the drug. In our study, 9.1% of cases were found resistant to erythromycin, which is lower than the percentage that was found in Brazil, Lebanon, and Spain (15.4%, 10%, and 10.6%, respectively) (18, 31, 39). This percentage of resistance to erythromycin is higher than the observations of the studies that were done in India, Tunisia, Iran, and Turkey (5.6%, 4.8%, 0%, and 3.8%, respectively) (28, 32, 33, 40). Moreover, the studies conducted in Germany and Italy reported an incremental increase of resistance to erythromycin in GAS strains (24% and 35.8%, respectively) (41, 42). GAS still has good sensitivity to penicillin and can be applied in the treatment of bacterial pharyngitis.

Although the ARF cases were not developed in our study, and the complications of tonsillitis are rare, it still presents a risk to acquire the post-streptococcal infections.

5.1. Limitations

This study has some limitations. Firstly, children were diagnosed in just one health center, which indicates that the number of cases included in our study was too small.

Table 4. Distribution of *emm* Types According to Patients' Sex and Age

<i>Emm</i> Type	Age (< 15 y), No. (%)	Sex, No. (%)	
		Male	Female
<i>Emm90</i>	4 (100)	3 (75.0)	1 (25.0)
<i>Emm89</i>	3 (100)	1 (33.3)	2 (66.7)
<i>Emm12</i>	2 (100)	0 (0)	2 (100)
<i>Emm1</i>	1 (100)	1 (100)	0 (0)
<i>Emm22</i>	1 (100)	0 (0)	1 (100)
<i>Emm75</i>	1 (100)	1 (100)	0 (0)
<i>Emm82</i>	1 (100)	1 (100)	0 (0)

Table 5. *Emm* Types Distribution According to Season

<i>Emm</i>	Autumn	Winter	Spring
<i>Emm 22</i>	0	1	0
<i>Emm 1</i>	1	0	0
<i>Emm 12</i>	1	1	0
<i>Emm 75</i>	1	0	0
<i>Emm 82</i>	1	0	0
<i>Emm 89</i>	1	1	1
<i>Emm 90</i>	4	0	0
Total	9	3	1

Secondly, we tested 200 children with sore throats and we tried to exclude 23 those with antibiotic consumption. Finally, some children or their parents refused to make a throat swab sample. Because of these reasons, the number of GAS recovered from this study population was low and we could not find any significant relationship between *emm* types, and age and antibiotic resistance.

5.2. Conclusions

Despite the fact that the positive GAS number in our work was low, we can conclude that *emm90* is the most prevalent type in the region. Also, penicillin and erythromycin are still the treatment choices for GAS pharyngitis cases. The *emm* typing is useful for molecular studies of GAS and provides new information on the diversity of the *emm* gene. A higher number of isolates could be considered in the future to validate this genetic diversity. Although this study revealed no complications of GAS pharyngitis, the majority of cases of ARF, mentioned in the last study in Morocco, had a history of acute pharyngitis. Thus, this result indicates the importance of considering GAS pharyngitis in the future and its virulence to prevent children who have many episodes of pharyngitis to

develop post-streptococcal complications.

Footnotes

Authors' Contribution: Study concept and design: B.O and S. H; Acquisition of data: S. H; Analysis and interpretation of data: S. H, B.O, B.A and K.M; Drafting of the manuscript: S. H; Critical revision of the manuscript for important intellectual content: B.O, S.A and S.E; Statistical analysis: B.D and S.H; Administrative, technical, and material support: B.O, S.A; Study supervision: S.E and B.O.

Conflict of Interests: None

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Informed Consent: Written informed consent was obtained from each child's parent or legal guardian.

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