emm Gene Polymorphism among *Streptococcus pyogenes* Isolated from Throat Culture

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Abstract- DNA sequencing is the most conclusive method for *emm* (M protein gene) typing of *Streptococcus pyogenes*. This method is not a feasible approach in developing countries where streptococcal infection is widespread among adults and children. Alternatively the PCR-RFLP has the potential for rapid screening of different types of *S. pyogenes*. To document the *emm* type distribution of *S. pyogenes* in a group of patients suffering from pharyngitis, the restriction fragment length polymorphism (RFLP) profile of 50 isolates were analyzed. By using Hae III+ HincII (double digestion) and Dde I restriction enzymes and based on RFLP, the profile patterns of the isolates were compared. The analysis of data identified 15 distinct RFLP patterns for Hae III+ Hinc II and 13 patterns for Dde I. They differ from each other by at least one band. Although the number of isolates was not sufficient to make any epidemiological conclusion, but the finding demonstrated that the *S. pyogenes* population among pateints was heterogeneous. Regarding the PCR method, we managed to improve the results by modification of CDC protocol in three different ways. This study was conducted in normal circumstances when pharyngitis was at the peak seasonal incident. However *emm* amplicon restriction digest analysis is a valuable tool for rapid analysis of *S. pyogenes* infection in more important situation like outbreaks and in selected type of study like consideration of nosocomial infection.

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Key words: *Streptococcus pyogenes*, PCR- RFLP, *emm gene*, Pharyngitis

Introduction

Infections caused by *Streptococcus pyogenes* - group A strepeococci (GAS), may lead to a spectrum of diseases ranging from benign condition such as impetigo to sever invasive diseases and serious non-suppurative sequelae (1).

GAS surface structure (including its M protein) accounts for several bacterium determinants of virulence (2). For more than 70 years, serotyping of M antigens has been the primary basis for strain classification, epidemiological data interpretation and information on pathogenesis (3). The problems associated with the M serotyping are the limitation of different M type antisera and high rate of non typeable strains (4-8). Advances in DNA sequencing technology in the late 20th century have resulted in developing of a method for determination of M genotype (*emm* gene) (9). *emm* amplicon sequence analysis is a valuable tool for rapid analysis of outbreaks and other significant situations (8). However, performance of *emm* gene sequencing is not a feasible approach especially in developing countries (5). To circumvent sequence analysis, the *emm* specific amplicon can be subjected to restriction fragment profile (8).

In present study, the *emm* typing was settled for 50 strains of *S. pyogenes.* During conducting the experiment, we came up with three methods resulting in lower costs and better outcome.

Materials and Methods

Bacterial stains

A total of 50 *S. pyogenes* strains were analyzed. These were recovered from throat cultures of 519 patients with pharyngitis in a cross sectional study during 2003-2004 in Kerman, south-east of Iran (10). The *S. pyogenes* strains were identefied as being β-hemolytic, catalase neg., VP negative, BC sensitive, PYR positive, Gram positive cocci (10,11). Few β-hemolytic streptococci, namely group C-*equisimilis*, *anginosus* and group G were tested concurrently. They were isolated from throat

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cultures of some other patients with acute pharyngitis too.

Standard strains

S. Pyogenes, ATCC: 8668 was incorporated as the positive control and *Enterococcus feacalis*, NCTC: 8213 as the negative control. Two M known serotypes, M1 and M3 strains (kindly provided by Pasteur Institute, Tehran-Iran) were tested too.

***emm* typing**

The *emm* analysis was a modified procedure of that conducted by Beal et al (12). For DNA extraction, 10-20 colonies from an overnight culture on Sheep blood agar (SBA) were picked up and suspended in ~300 µL sterile 0.85% NaCl. Then the suspension was heated at 70º C for 15 min and spun down for 2 min by micro-fuge technique. The pellets were re-suspended in 50 µL sterile TE (Tris-EDTA) and incubated at 37ºC with Mutanolysin (10 µL) and Hyaloronidase (2 µL) (Sigma-USA) for 30 min (12).

Because of low DNA product and according to personal experiences, the Mutanolysin was replaced by magnetic energy of microwave (defrost power: 2 min for three cycles with 2 min gap between each cycle). After heating in 100ºC for 10 min, the supernatant of lysate was used immediately for PCR (12).

Polymerase chain reaction-PCR

The *emm* genes were amplified by using the following primers (12). tattc(c/g)gcttagaaattaa, and gcaagttcttcagcttgtttt (Primm, Italy) 0.2 µL of each. The PCR reaction mixture (20µL) was consisted of 10 µL 10X buffer containing 15mMol MgCl2 (Sinagen, Iran), 2 µL of d NTP (Bioron, GmbH), 0.5 µL Taq (3 u/µL) (FAZA, Iran), 8.2 µLdH_2o and 0.5 µL of lysate supernatant (12).

Again due to insufficient or absence of product, the above mixture was replaced by lyophilized PCR premix kit (BioNeer, Korea) and the amount of template was increased from 0.5 to 4 µL. The modified selected condition led to much better results.

Amplification of gene was performed in a PCR thermal cycler (MWG, Germany) using the cycle parameters given on CDC website: Denaturation at 94ºC, annealing at 46.5ºC, and extension at 72ºC (11, 12).

The PCR products were analyzed by gel electrophoresis in a 2.5-3% agarose gel (Eurobio, MBG) using a 100 bp ladder (bioLabs, New England). According to our trial they were run at 80 volts for 5 min, 70 volts for 20-30 min and 60 volts for 60 min. At the end The gel was rinsed with tap water and photographed under UV in Gel Doc (UV I doc- France).

RFLP of *emm* amplicons

The *emm* amplicons in 15-20 µL of PCR product were double digested by 0.25-0.5 µL of Hae III + Hinc II (BioLab, New England) and Dde I (Biolab, New England) seperately. The period of digestion was ~14 hours at 37ºC (11).

Results

The size of *emm* amplicons were in the range of 800-1500 bp (Figure 1). Using one pair of CDC recommended primers (mentioned before) could not yield any amplicon for 2 (4%) of isolates, n23 and p6. These two isolates were documented as *S. pyogenes* by PYR and other standard biochemical tests before.

The analysis of double digestion with HaeIII+ HincII, identified 15 distinct RFLP patterns. They differed from one another by at least single band. (Figure 2). All strains were sorted up according to comparison of detected bands by gel electrophoresis (Table 1). Among 50 isolates, the most prevalent *emm* type was group 1 with sixteen members. Their RFLP patterns were resembled to the standard strain, followed by group 2 with nine members. Group 2 were resembled to M3 serotype. The HaeIII+ HincII restriction enzymes could not digest three amplicons related to isolates d60, b70 and b89. All tests (with exceptional results) were repeated at least for 3 times. Thirteen RFLP patterns were the outcome of using Dde I restriction enzyme (Figure 3) According to the same categories they were set in table 2. Again the most prevalent was group 1 (similar to standard strain) with eighteen members.

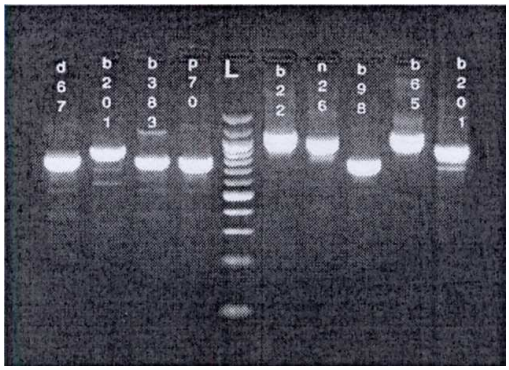

Figure 1. PCR product of emm gene of some S. pyogenes strains.
The size of amplicons were ~800-1500 bh. L:ladder

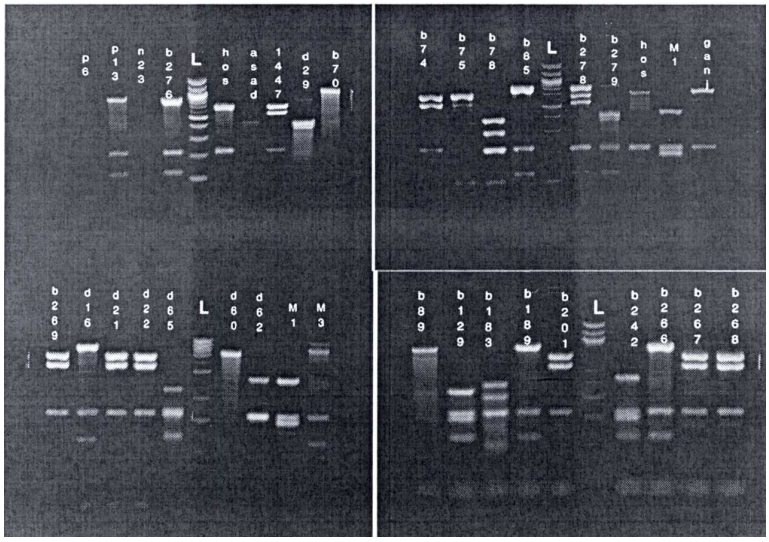

Figure 2. PCR-RFLP patterns of emm gene, extracted from S.pyogenes after HaeIII+HincII double digest. L:ladder

The second most prevalent was group 2 with ten members resembling the M3 serotype. Dde I also was not able to digest the amplicons of p6 and n23.

The distribution of 40 out of 50 (80%) digested amplicons by Hae III+ Hinc II and Dde I between categoris were similar to each other.

Discussion

More than 90% of all streptococcal infections are caused by *S. pyogenes* (group A streptococci -GAS) (8). Strains differentiation of *S. pyogenes*, has been based on the serological test of M protein antigens (12). The standard serotyping methods indicate that the majority of GAS isolates are M non-typeable (MNT). More confidently, the PCR-RFLP analysis has the potential for rapid screening of different GAS *emm* genes that encode M surface proteins (5). We performed *emm* typing of streptococcal isolates by PCR-RFLP. According to the results, no amplicon was detected from *emm* gene PCR of PYR negative (group C or G) β-hemolytic streptococci. However, Allon and Nchnitzer have gotten *emm* amplicons from some group G streptococci by PCR (13, 14).

Table 1. The category of PCR-RFLP pattern of isolates, double digested with HaeIII+HincII. Numbering of each group does not show any special character. The code of each isolate was chosen by using the first letter of patient's physician name+chain number.

Group	No. of Detected bands	Range size of band (bp)	Members
1	3	~220→700	Standarde strain, d21, d22, d25, b64, b74, b87, b106, b168, b201, b267, b268, b293, b296, b299, b300
2	3	~130→900	Control stain M3, p13, d14, d16, d30, b85, b189, b266, b276
3	2	< 400, 400	p7, d29, d69, b72
4	2	200, 800	Blood, synovial, n16
5	No digestion	---	d60, b70, b89
6	3	150→350	d65, b129
7	4	100→~400	b78, b183
8	No amplication	---	p6, n23
9	3	200-450	Control strain M1, b242
10	2	700,800	b54, b75
11	3	~180→320	d72
12	5	~120→800	b278
13	4	~120→400	b279
14	3	~180→1000	n54
15	4	100→900	d17

Table 2. The category of PCR-RFLP pattern of isolates, digested with Dde I. Numbering of each group does not show any special character. The code of each isolate was chosen by using the first letter of patient's physician name+chain number.

Group	No. of Detected bands	Range size of band (bp)	Members
1	8	< 100→~250	Standard strain, d17, d21, d22, d25, n54, b64, b74, b87, b106, b168, b201, b267, b268, b293, b296, b299, b300
2	6	< 100 →~280	Control strain M3, p13, d14, d16, d30, b85, b189, b266, b276, b279
3	7	< 100→~300	p7, d29, d69, b54, b129
4	8	< 100→400	Blood, n16
5	5	< 100→~220	d60, b72
6	5	< 100→300	d89
7	4	~80→200	b78, b183
8	No amplification	---	p6, n23
9	3	~70→300	Control strain M1, d65, b242, synovial
10	5	< 100→300	b75
11	6	< 100→~280	d72
12	7	< 100→~250	b278
13	5	< 100→~220	b70

Further more, we did not obtaine any amplicon from 2 (4%) of *S. pyogenes* isolates even after 4 times repetition of PCR. In CDC protocol a pair of alternative primers (MF2 and MR1) have been recommended for this rare situation (11). They were not available in our lab. For the first time in Iran, this molecular study demonstrated a heterogeneous population consisting of different *emm* genotypes of *S. pyogenes* among patients with pharyngitis. The PCR-RFLP categories status of about 80% of isolates in double digestion with HaeIII+ HincII and Dde I were similar.

The present study was performed in a normal situation, when pharyngitis was at the peak seasonal incident. However GAS infections may be associated with introduction or re-appearance of individual M types within a population (13-15). To date a total of 117 validated *emm* types have been documented (16).

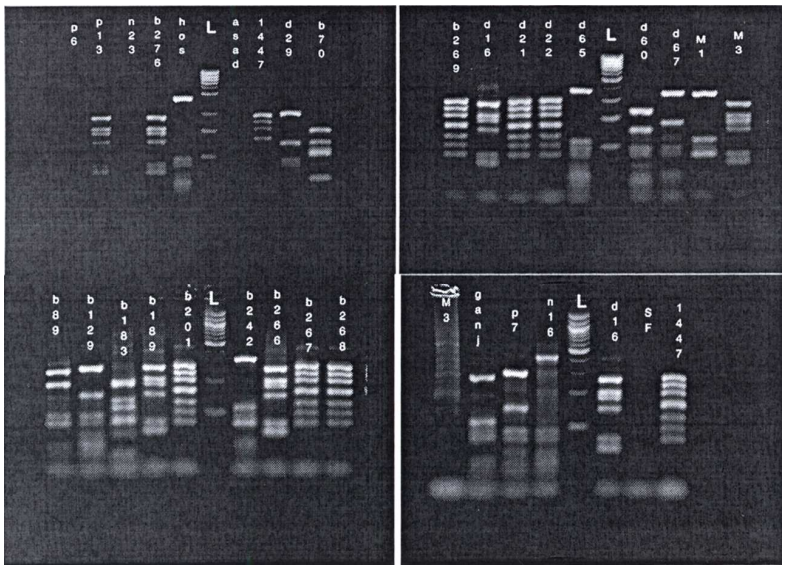

Figure 3. PCR-RFLP patterns of emm gene, extracted from S.pyogenes after Dde diges. L:ladder

Basically, the *emm* amplicon restriction digest analysis is a valuable tool for rapid analysis of outbreak situation or selected types of study such as consideration of nosocomial infections. The PCR-RFLP can differentiate outbreak-related strains from contemporaneous background strains of the same M serotype (17).

On the subject of amplification of *emm* gene, because of poor PCR products (13-18), we had to make some modification on CDC protocol after about 100 tests. These changes were the enhancement of quantity of template amount, use of PCR premix Kit instead of master mixture, and replacing of Mutanolysin by electrical power of microwave instrument. The latter also provides a cheaper cost of examination. In our lab the microwave is used for melting the agarose. Three above mentioned changes led to much better results.

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