

PCR M Typing: a New Method for Rapid Typing of Group A Streptococci

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A new approach for the M-typing of *Streptococcus pyogenes* is reported. Oligonucleotide primers were used in a PCR to amplify the N-terminal region of the *emm* gene. The presence of the PCR amplification product is associated with the corresponding M serotype. This technique offers potential advantages over other molecular typing methods.

Since the first description of the streptococcal M protein (8, 9), many studies have elucidated the function, immunochemistry, genetic structure, and antigenic variations of this bacterial molecule (3). This protein antigen, in fact, is one of the most important epidemiological markers used to identify and characterize group A streptococci (GAS). At present, more than 81 serologically different types of this protein are known. However, M-typing antisera are difficult to prepare and are not commercially available. Hence, after the publication of the N-terminal nucleotide sequence of the M protein-coding genes (*emm* genes) from different types, several laboratories have developed different molecular typing methods (1, 4, 5, 6, 7, 14).

In view of the fact that severe infections caused by GAS, including their suppurative and nonsuppurative sequelae, are increasing and that typing methods are essential tools for outbreak investigation and surveillance, we describe here a new approach based on application of the simple PCR technology. The procedure is inexpensive and easy to perform, even on a large number of samples. Experiments have been run to assess specificity, sensitivity, and reproducibility. To our knowledge, this technique, as it is here presented, has never been reported before.

The specificity of a given M serotype resides in the N-terminal portion of the mature GAS M protein. The corresponding 5' end of the *emm* gene is also type specific (3). Therefore, we used oligonucleotides annealing to this region as reverse primers in standard PCRs (Table 1). In all the reactions, the forward primer was represented by an oligonucleotide pairing to a highly conserved sequence internal to the *isp* locus (10). In the present study, we tested 33 clinical isolates of GAS strains belonging to serotypes M1 (two strains), M2 (four strains), M3 (three strains), M4 (eight strains), M5 (one strain), M6 (five strains), M8 (two strains), M12 (six strains), M18 (one strain), and M24 (one strain).

Grid experiments covering all the possible crosses between *emm*-specific oligonucleotides and available streptococcal strains

of known M serotype were performed. In brief, 2 μ l of chromosomal DNA embedded in agarose plugs, prepared as described by Ripa et al. (12), was added to a PCR mix containing 67 mM Tris (pH 8.8), 16 mM $(\text{NH}_4)_2\text{SO}_4$, 0.15 mg of bovine serum albumin per ml, 0.01% Tween 20, 2 mM MgCl_2 , 0.2 mM deoxynucleotide triphosphates, *emm*-specific reverse primer and *isp* forward primer (0.2 μ M each), and a 25:1 mixture of *Taq* and *Pfu* DNA polymerases (0.5:0.02 U), in a total volume of 30 μ l. It is not strictly required to prepare template DNA in agarose. We obtained comparable results using DNA released by cultured cells after freezing and thawing (2).

The amplification reaction included one cycle at 95°C for 1 min, followed by 25 cycles at 95°C for 15 s, 60°C for 2 min, and 68°C for 6 min. The reaction product was analyzed by agarose gel electrophoresis.

Experiments were performed in which every *emm*-specific oligonucleotide was used in an amplification reaction against the corresponding M type strain. In Fig. 1, the results obtained with a representative subset of strains are shown. All reactions

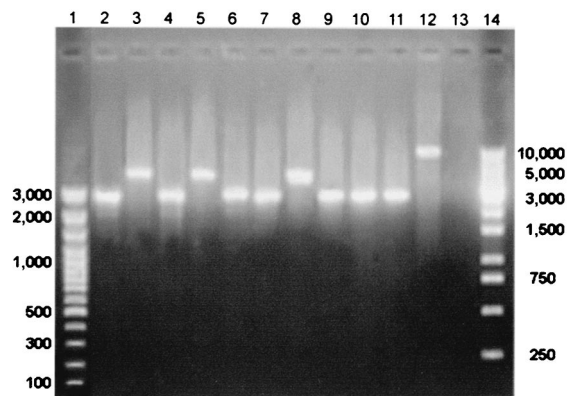


FIG. 1. PCR M typing sensitivity. M serotypes versus *emm*-specific oligonucleotides. Lanes 1 and 14, molecular weight standards (in base pairs); lane 2, M1–2 versus *emm*1; lane 3, M2 versus *emm*2; lane 4, M3 versus *emm*3; lane 5, M4 versus *emm*4; lane 6, M5 versus *emm*5; lane 7, M6 versus *emm*6; lane 8, M8 versus *emm*8; lane 9, M12 versus *emm*12; lane 10, M18 versus *emm*18; lane 11, M24 versus *emm*24; lane 12, internal control for forward primer, amplification of the *vir* regulon according to Gardiner et al. (3); lane 13, no-DNA control.

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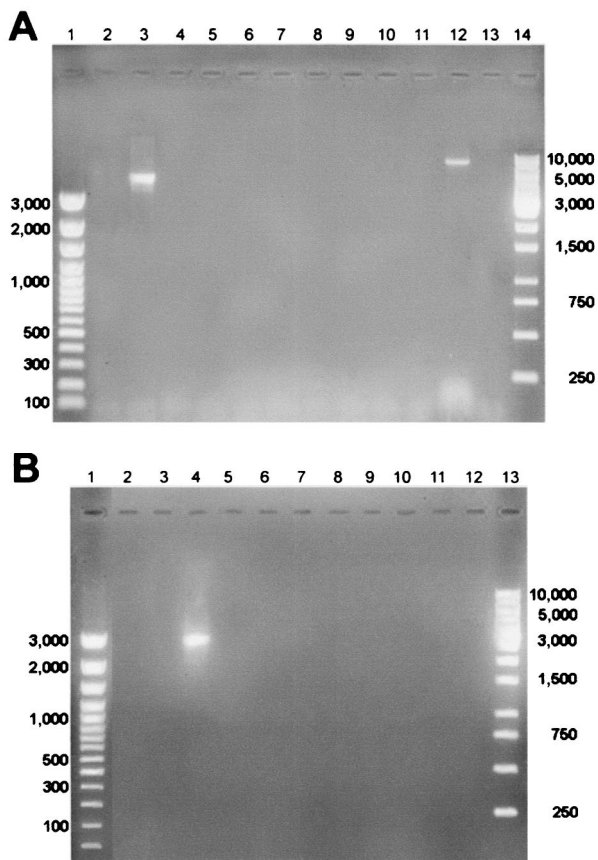


FIG. 2. PCR M typing specificity. (A) *emm*-specific oligonucleotides in reaction containing M2 serotype template chromosomal DNA. Lanes 1 and 14, molecular size standards (in base pairs); lane 2, M2 versus *emm*1; lane 3, M2 versus *emm*2; lane 4, M2 versus *emm*3; lane 5, M2 versus *emm*4; lane 6, M2 versus *emm*5; lane 7, M2 versus *emm*6; lane 8, M8 versus *emm*8; lane 9, M2 versus *emm*12; lane 10, M2 versus *emm*18; lane 11, M2 versus *emm*24; lane 12, internal control for forward primer, amplification of the *vir* regulon according to Gardiner et al.; lane 13, no-DNA control. (3). (B) *emm*3 oligonucleotide in amplification reactions with different M serotype chromosomal DNAs. Lanes 1 and 14, molecular size standards (in base pairs); lane 2, *emm*3 versus M1; lane 3, *emm*3 versus M2; lane 4, *emm*3 versus M3; lane 5, *emm*3 versus M4; lane 6, *emm*3 versus M5; lane 7, *emm*3 versus M6; lane 8, *emm*3 versus M8; lane 9, *emm*3 versus M12; lane 10, *emm*3 versus M18; lane 11, *emm*3 versus M24; lane 12, no-DNA control.

gave a positive amplification signal. Reactions containing M1, M3, M5, M6, M12, M18, and M24 strains DNA produced a \approx 3-kb amplicon, while for M2, M4, and M8, a \approx 5-kb fragment was obtained. This difference can be explained by the well-documented hypervariability of this portion of the *Streptococcus pyogenes* chromosome.

In particular, the template chromosomal DNA amplified by our PCRs may contain the *fcrA* gene, which encodes an immunoglobulin G (IgG)-Fc binding protein (11). The fact that the region included between the *vir* and *emm* genes has an estimated length of about 1.7 to 1.9 kb (11) and that the *isp* locus maps upstream to the *vir* gene can fully explain the difference of our PCR product length (5 kb versus 3 kb). Moreover, the *fcrA* gene has been previously reported to be present in M2, M4, and M8 strains (11) and not in the other M type strains used in the present work.

In parallel experiments, the whole set of oligonucleotides were used in PCRs with template DNA isolated from a selected M serotype strain. In Fig. 2A, results of a typical experiment in which M2 strain DNA was added are shown; only the PCR containing *emm*2 oligoprimers gave an amplification product. Subsequently, a counter-test was performed in which a selected *emm*-specific oligonucleotide was used as the reverse primer in reactions where chromosomal DNA isolated from different M serotype strains was added as the template. Results

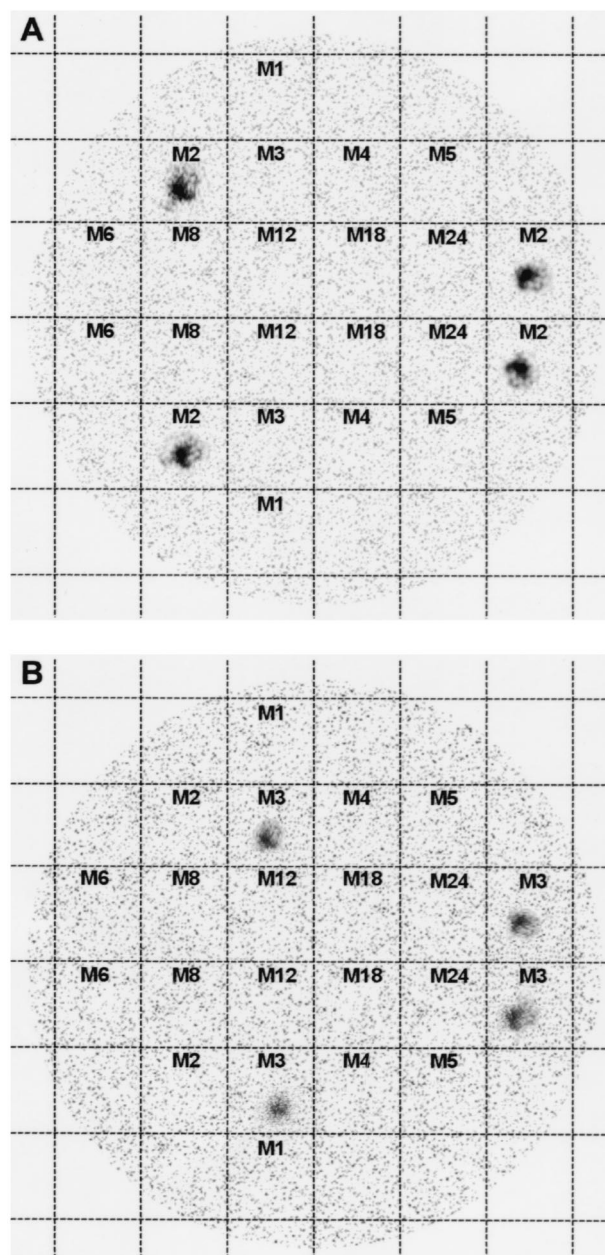


FIG. 3. Colony hybridization. The numbers in the grids correspond to the M serotypes. (A) Hybridization experiment with the ^{32}P -labeled *emm*2-specific probe (corresponding to the *emm*2 oligonucleotide reported in Table 1). (B) Hybridization experiment with the ^{32}P -labeled *emm*3 oligonucleotide (corresponding to the *emm*3 oligonucleotide reported in Table 1).

TABLE 1. Oligonucleotide probes used in this study

Probe	Nucleotide sequences ^a	Reference
<i>emm1</i>	5' TTC TAT AAC TTC CCT AGG ATT ACC ATC ACC 3'	5
<i>emm2</i>	5' TGC TTC TTT TTT GAC AGG GAC AGG GTT CTT 3'	5
<i>emm3</i>	5' CAT GTC TAG GAA ATC CTC CAT TAA CAC TCC 3'	5
<i>emm4</i>	5' CCA CGC TGA ATC AGC CTC AGG CTT TTT AAT 3'	6
<i>emm5</i>	5' CGG GTC ATT TAT TGT ACC CCT AGT CAC GGC 3'	5
<i>emm6</i>	5' TGC TTT GTC CGG GTT TTC TAC CGT CCC CCT 3'	5
<i>emm8</i>	5' TCG TTA TTA GAA ATA CTA TGA GAT TTT GGG 3'	6
<i>emm12</i>	5' ACG TTG TTT TTC TGC GAC TAA ATC ACT ATG 3'	5
<i>emm18</i>	5' CGT CTT TAT TGT CTG CTG TAG CTC GAG TAA 3'	5
<i>emm24</i>	5' TTC TTG TAC TTT TTC CAG AGT ATC TGT CTG 3'	5
<i>isp</i>	5' CCG TAT CTT TAG CGC ACT CGA GGA CAA TTT GCG AGA TTA G 3'	3

^a For *emm* oligonucleotide probes, the sequences are complementary to the coding strand of the N-terminal region of the corresponding *emm* gene sequences.

for experiments conducted using the *emm3* primer are presented in Fig. 2B, where the only PCR positive for amplification was that containing M3 strain chromosomal DNA. Identical analyses were performed on the other M type strains (M1, M4, M5, M6, M8, M12, and M18), with comparable results (not shown). Taken together, these data support the high grade of specificity of the technique.

To definitely rule out nonspecificity of the PCR, we conducted colony blot hybridization experiments. Following described protocols (13), DNA immobilized on a nylon filter by UV light was hybridized to the same set of ³²P-labeled *emm* gene-specific probes used in the PCR experiments (Table 1). The results of this set of experiments are shown in Fig. 3, where it can be seen that the *emm2* oligonucleotide hybridizes only with M2 strain DNA (Fig. 3A). The same relative result was obtained when the *emm3* oligonucleotide was used (Fig. 3B).

With this simple protocol, typing of the M protein could be attempted by any laboratory in which PCR is routinely in use. Results are not as informative as those obtainable with the sequencing approach; nevertheless, the technique offers high specificity, since it is based on sequencing data. We think that with a representative set of *emm*-specific oligonucleotides and a good experimental design, it is possible to

carry out good molecular epidemiology studies without the need to adopt any expensive and/or time-consuming techniques. For example, one could start by ranking M types in potential order of frequency and testing common types first. All typed isolates could then be excluded from subsequent analyses.

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