JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1997, p. 2689–2691 Vol. 35, No. 10
0095-1137/97/$04.00+0

PCR–Enzyme-Linked Immunosorbent Assay and Sequencing as an Alternative to Serology for M-Antigen Typing of *Streptococcus pyogenes*

N. A. SAUNDERS,[1]* G. HALLAS,[2] E. T. GAWORZEWSKA,[2] L. METHERELL,[1] A. EFSTRATIOU,[2] J. V. HOOKEY,[1] AND R. C. GEORGE[2]

HRL[1] and RSIL,[2] CPHL, London NW9 5HT, United Kingdom

Received 13 March 1997/Returned for modification 16 May 1997/Accepted 15 July 1997

A rapid PCR–enzyme-linked immunosorbent assay for identification of 10 important *emm* gene types of *Streptococcus pyogenes* was developed. The *emm* genotypes of a coded panel of strains of known M serotype were determined, and in 144 of 149 cases (97%) the results were congruous. Strains of types that were not included in the panel of capture probes were *emm* genotyped by sequencing.

Infections with *Streptococcus pyogenes* (group A streptococci [GAS]) are common and various. Accurate typing methods are essential tools for outbreak investigation and surveillance of GAS. Traditionally, typing depends upon the serological detection of cell wall antigens by a combined system of T- and M-antigen typing (12) and, for opacity-factor-positive cultures, the inhibition of the opacity reaction with specific antisera (6). These are the classical techniques against which other typing systems must be evaluated. M-antigen typing provides the greatest discrimination between strains, with at least 74 types currently recognized (3). However, M typing reagents are not available commercially, and maintenance of a comprehensive system is expensive. Consequently, classical M typing is restricted to a few reference centers.

The M proteins promote the survival of invading *S. pyogenes* cells within the host by preventing opsonization. The different M protein types are encoded by the *emm* gene family. M type specificity is conferred by the variable N terminus of the protein. PCR primers for the *emm* gene that amplify the variable N-terminal encoding domain have been described previously (11). The amplicons produced encompass sequences determining type specificity, and their analysis can identify the M type.

Here we report a rapid and convenient genotypic method for typing *S. pyogenes* based on the hybridization of labelled *emm* gene PCR amplicons to type-specific capture oligonucleotides.

Clinical strains of *S. pyogenes* from the collection of the Streptococcus Reference Unit were selected for analysis and cultured overnight on blood agar. All strains were subjected to M typing with a panel of antisera as described previously (2). Cells were harvested and washed twice in sterile distilled water (1 ml) to remove traces of blood agar. The cell pellet (approximately 10 μl) was resuspended in 200 μl of water, and then DNA was released by heating the mixture at 99°C for 10 min. Extracts were stored at −20°C. Prior to PCR, the extracts were thawed and then centrifuged briefly to sediment particulates.

The PCR primers employed were those described by Podbielski and colleagues (11). The PCR mix in a final volume of 50 μl comprised 1× PCR buffer (Gibco), 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 100 ng of 5′-fluorescein-labelled forward primer 5′-ATAAGGAGCATAAAAATGG CT, 100 ng of reverse primer 5′-AGCTTAGTTTTCTTCTTT GCG, 1 U of *Taq* polymerase (Gibco), and 1 μl of DNA extract. The temperature cycle conditions were 1 cycle at 94°C for 3 min; 35 cycles at 93°C for 30 sec, 50°C for 30 sec, and 72°C for 2 min; and 1 cycle at 72°C for 5 min (Omnigene; Hybaid Ltd.). Upon electrophoresis, the PCR products from most strains of *S. pyogenes* comprised a single major amplicon of 800 to 1,500 bp, corresponding to the full-length *emm* gene.

The principle of the PCR–enzyme-linked immunosorbent assay (ELISA) is illustrated in Fig. 1. The DNA sequences encoding the N-terminal hypervariable region of strains of types M1, M3, Arp4 (M4), M5, M6, M11, M12, R28 (M28), M76, and M78 (GenBank accession no. X07933, U11945, S82054, X15198, M20374, U11986, U11938, U11937, U11948, U11992, and U11990) were aligned, and probes were selected (Table 1) from the region employed by Kaufhold et al. (4, 5). All capture probes were synthesized in 5′ biotinylated form and were immobilized to microtiter plates as described previously (9).

The PCR products to be analyzed were denatured by heat at 99°C for 10 min, chilled on ice, and then diluted to 600 μl with ice-cold hybridization buffer (9). An aliquot (50 μl) of the diluted mixture was added to each microtiter plate test well and then diluted to 100 μl with hybridization buffer. Hybridization was effected by incubation of the microtiter plate at 37°C for 10 min and then at 50°C for 20 min by floating the plate in a covered water bath. Following hybridization the fluorescein-labelled PCR-derived target sequence was detected as described previously (9). The hybridization and detection steps may be completed in under two hours.

Strains that had been subjected to classical M typing were coded by one of us (A.E.) and, in a blind investigation, subjected to genotyping. The strains were selected to include 102 isolates of the 10 M types in the genotyping scheme and 48 isolates that belonged to other types. The latter group included strains that were difficult to serotype due to their cross-reactivities and strains belonging to serotypes related to the ten in the genotyping system.

For 149 of the 150 strains, analysis of the PCR products by gel electrophoresis showed an amplicon within the anticipated size range (800 to 1,500 bp). The reason for the repeated failure of *emm* gene amplification of a single strain of serotype M4 is unclear. It is possible that this strain, which was excluded from the study, had a mutation affecting one of the primer binding sites. Approximately 10% of the amplifications gave an additional, truncated product of between 150 and 250 bp. Se-

* Corresponding author. Mailing address: HRL, CPHL, 61 Colindale Ave. London NW9 5HT, United Kingdom. Phone: 44 181 200 4400, ext. 3072. Fax: 44 181 200 1569. E-mail: nsaunder@phls.co.uk.

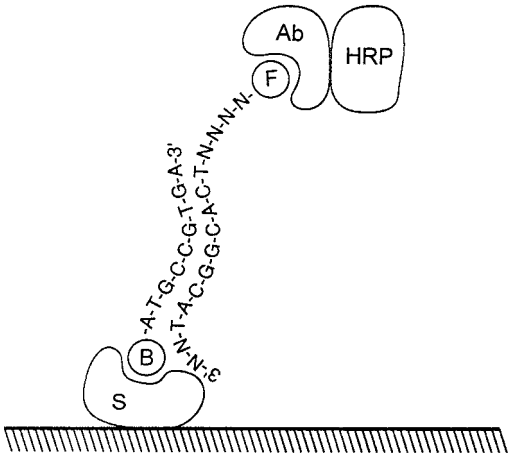

FIG. 1. Principle of the capture probe ELISA method. The capture probe has a 5′ biotin moiety (B) which binds to streptavidin (S) that has been attached to the surface of a microtiter plate well. The complementary sequence from the denatured PCR amplicon, which is labelled with fluorescein (F) recognized by the antibody (Ab), is hybridized to the capture probe. Finally, any hapten remaining bound to the well following washing is detected by anti-hapten–horse radish peroxidase (HRP) with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. A positive probe reaction is defined as having at least four times the absorbance (at 620 nm) of the average value for the other probes. At least 75% of the positive reactions were in the range of 10 to 50 times higher than the average.

quence analysis showed that this amplicon corresponded to the 5′ hypervariable end of the *emm* gene (results not shown). A DNA homology search revealed a sequence that showed some similarity to the reverse primer situated at the expected location within some *emm* genes. Amplifications that gave primarily the truncated PCR product corresponding to the 5′ end of the *emm* gene were relatively unreactive with the M-all capture probe but allowed identification of the specific *emm* gene type. The M-all probe gave a positive signal for 138 of the 149 strains which yielded a PCR amplicon.

Strains belonging to the serotypes included on the capture probe plate were identified to the expected type in 98 of 101 cases (97%). The strains of related M types were not captured by any probe in 46 of 48 cases (96%) (Table 2). Strains discordant by serotyping and PCR-ELISA were subjected to repeat M serotype and *emm* genotype testing. This resulted in the serotypes of several strains being reclassified. In one case (strain 46) the repeat tests revealed reactivity with both the M1 and M4 probes. It was confirmed by serological typing of single colonies that this culture was a mixture of M type 1 and M type 4.

Automated sequencing was performed as described previously (9). The template was prepared by PCR as described above except that the forward primer was unlabelled. The partial sequence of the N termini of the *emm* gene amplicons of four strains was determined by using the forward primer. The results are shown in Table 3. On repeat genotyping of two strains (23 and 45), the initial probe reaction could not be reproduced. The sequence determined for the serotype M5 strain 74 had 94% similarity over 165 bp to the sequence (GenBank accession no. M20374) determined by Miller and

TABLE 1. Capture probes and primers

Serotype or primer designation	Label and sequence
M-all	Biotin-TTAGTTTWGCAAGTTCTTCAGCTTGTTT
M1	Biotin-TTCTATAACTTCCCTAGGATTACCATC
M3	Biotin-ATGTCTAGGAAACTCTCCATTAACACT
M4	Biotin-GAATCAGCCTGAGGCTTTTTAATCTC
M5	Biotin-CGGGTCATTTATTGTACCCCTAGTC
M6	Biotin-GCTTTGTCCGGGTTTTCTACCGTC
M11	Biotin-AGCGCTTTGCCCCGCAGCCTTAA
M12	Biotin-GTAGAGTTCTGAACGCTGTTTCAG
M28	Biotin-AAGTCTCAGTACTTTTTGGAGACTCC
M76	Biotin-TTAGAAACGCTTTTAGAGTTCGCGTC
M78	Biotin-TTAGTAATACTACGAGAGTTCTGAGAC
Primers	
MF	Fluorescein-ATAAGGAGCATAAAAATGGCT
MR	AGCTTAGTTTTCTTCTTTGCG

TABLE 2. Summary of results of genotyping a panel of strains

Serotype	No. of strains	No. correctly identified	No. reacting with M-all probe
M1	9	9	9
M3	10	9	10
M4	8	7	8
M5	12	11	8
M6	11	11	6
M11	11	11	10
M12	10	10	10
M28	7	7	7
M76	9	9	9
M78	11	11	11
M1-M4 mix	1	1	1
M22	4	4	4
M48	3	3	3
M60	2	2	1
M61	3	3	3
M62	3	3	3
M63	4	4	4
M66	3	2	3
M77	5	5	5
M81	2	2	2
P180	1	1	1
P2110	2	1	2
P2841	4	4	4
P4245	4	4	4
P4854	2	2	2
P4931	3	3	3
P5757	3	3	3
M-ive, T28	1	1	1
M-ive, T3	1	1	1
Total	149	144	138

TABLE 3. Sequence analysis of strains giving discordant serotyping and genotyping results

Strain no.	Serotype	Capture probe genotype	Sequence homology[a] (GenBank accession no.)
23	M66	*emm-28*[b]	98% Homology to *emm-66* (U11999)
25	M3	None (no reaction)	97% Homology to *emm-2* (X56608)
40	M4	*emm-1*	97% Homology to *emm-1* (X07933)
45	PT2110	*emm-5*[b]	
74	M5	None (no reaction)	94% Homology to *emm-5* (M20374)

[a] Sequence homology over at least 157 bases within the hypervariable domain.
[b] No reaction with capture probe on repeat testing.

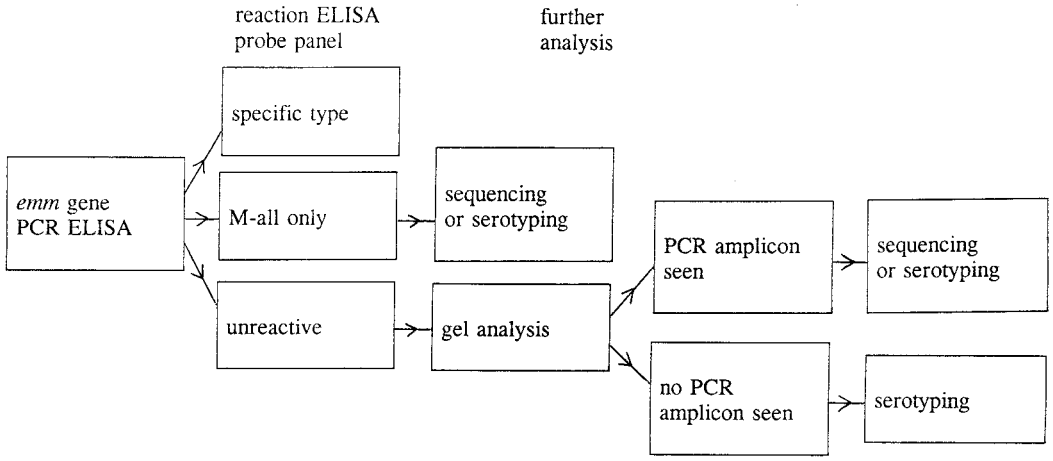

FIG. 2. PCR–ELISA-based scheme for *emm* typing of *S. pyogenes* strains. Three possible results of the initial PCR-ELISA test are considered. Within this scheme, strains may be identified to the *emm* genotype by either PCR-ELISA or sequencing. Serotyping is the only alternative for the small number of strains which fail to give a PCR amplicon.

colleagues (7). However, it differed by four bases within the sequence corresponding to the capture probe. In the remaining two cases the sequence was consistent with the result obtained by the capture probe method. Strain 40 (serotype M4) gave an amplicon hybridizing to the M1 capture probe, and the sequence was 97% homologous with the gene encoding M1 (GenBank accession no. X07933). The sequence of the amplicon derived from strain 25 (serotype M3), which did not hybridize to a specific capture probe, was homologous to the M2-encoding gene. PCR mixture contamination is an unlikely explanation of these discordant results, since the large quantity of the starting template prevents significant amplification of contaminants by competition. Beall and colleagues (1) have also reported a strain of one serotype (M77) which apparently carried the gene for a different serotype (M27). It is possible that these results are due to strains carrying more than one *emm* gene, only one of which is amplified.

The use of capture probes for *emm* gene typing was first demonstrated by Kaufhold et al. (4, 5). However, those authors suggested that it would be desirable to modify the capture oligonucleotides so that they would hybridize at a single temperature. The M1 capture probe described by Kaufhold and colleagues (5) does not capture amplicons derived from some variant M1 types, due to nonmatching bases within the probe sequence (10). However, the variant strains showed an unusual T8M1 serotype (10) and, in addition, electrophoretic typing indicated that they belonged to clonal lines that are clearly distinct from the classical M1 strain (8). In the present study it was not considered worthwhile to include multiple probes for subtypes of type M1, since strains of the T8M1 serotype are uncommon.

The 10 type-specific capture probes used in this study should identify over 60% of current strains submitted to the PHLS Streptococcus Reference Unit, which handles all GAS M serotyping in the United Kingdom. The addition of more probes, particularly for serotypes of emerging importance, would increase the typeability of strains. Strains that cannot be identified by the probe panel may be analyzed by direct sequencing. Comparison of DNA sequences with the growing number of *emm* gene sequences available in sequence databases would determine the M type of a high percentage of strains (1). In this scheme, any strain not assigned to a particular serologically defined type would be termed an *emm* genotype on the basis of its unique sequence.

We suggest that the PCR-ELISA be used as the initial step in an M typing scheme (Fig. 2). Isolates that reacted only with the M-all probe would then be typed by sequence analysis. PCR amplicons that gave no signal with either an *emm* gene-specific probe or M-all would be retested by PCR-ELISA. Persistently unreactive PCR amplicons could be typed by sequencing. For the small number of strains that give no amplification product, serological typing would be necessary.

The use of expensive serological or sequencing reagents would be minimized by the introduction of PCR-ELISA to screen all isolates. We are currently testing additional capture probes for M serotypes of *S. pyogenes* that are of increasing importance.

REFERENCES

1. **Beall, B., R. Facklam, and T. Thompson.** 1996. Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. J. Clin. Microbiol. **34:**953–958.
2. **Gaworzewska, E., and G. Colman.** 1988. Changes in the pattern of infection caused by *Streptococcus pyogenes*. Epidemiol. Infect. **100:**257–269.
3. **Johnson, D. R., and E. L. Kaplan.** 1993. A review of the correlation of T-agglutination patterns and M-protein typing and opacity factor production in the identification of group A streptococci. J. Med. Microbiol. **38:**311–315.
4. **Kaufhold, A., A. Podbielski, G. Baumgarten, M. Blokpoel, J. Top, and L. Schouls.** 1994. Rapid typing of group A streptococci by the use of DNA amplification and non-radioactive allele-specific oligonucleotide probes. FEMS Microbiol. Lett. **119:**19–26.
5. **Kaufhold, A., A. Podbielski, D. R. Johnson, E. L. Kaplan, and R. Lutticken.** 1992. M protein gene typing of *Streptococcus pyogenes* by nonradioactively labeled oligonucleotide probes. J. Clin. Microbiol. **30:**2391–2397.
6. **Maxted, W. R., J. P. Widdowson, C. A. M. Fraser, L. C. Ball, and D. C. J. Bassett.** 1973. The use of the serum opacity reaction in the typing of group-A streptococci. J. Med. Microbiol. **6:**83–89.
7. **Miller, L., L. Gray, E. Beachey, and M. Kehoe.** 1988. Antigenic variation among group A streptococcal M proteins: nucleotide sequence of the serotype 5 M protein gene and its relationship with genes encoding types 6 and 24 M proteins. J. Biol. Chem. **263:**5668–5673.
8. **Musser, J. M., V. Kapur, J. Szeto, X. Pan, D. S. Swanson, and D. R. Martin.** 1995. Genetic diversity and relationships among *Streptococcus pyogenes* strains expressing serotype M1 protein: recent intercontinental spread of a subclone causing episodes of invasive disease. Infect. Immun. **63:**994–1003.
9. **Patel, S., M. Yates, and N. A. Saunders.** 1997. PCR–enzyme-linked immunosorbent assay and partial rRNA gene sequencing: a rational approach to identifying mycobacteria. J. Clin. Microbiol. **35:**2375–2380.
10. **Penney, T. J., D. R. Martin, L. C. Williams, S. A. de Malmanche, and P. L. Bergquist.** 1995. A single *emm* gene-specific oligonucleotide probe does not recognise all members of the *Streptococcus pyogenes* M type 1. FEMS Microbiol. Lett. **130:**145–150.
11. **Podbielski, A., B. Melzer, and R. Lütticken.** 1991. Application of the polymerase chain reaction to study the M protein(-like) gene family in beta-hemolytic streptococci. Med. Microbiol. Immunol. **180:**213–227.
12. **Williams, R. E. O., and W. R. Maxted.** 1953. The type classification of *Streptococcus pyogenes*. Atti Congr. Int. Microbiol. Roma **1:**46–49.