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Rapid typing of group A streptococci by the use of DNA amplification and non-radioactive allele-specific oligonucleotide probes

A. Kaufhold *[,a], A. Podbielski [a], G. Baumgarten [a], M. Blokpoel [b], J. Top [b] and L. Schouls [b]

[a] *Institute of Medical Microbiology, Technical University (RWTH) Aachen, Pauwelsstrasse 30, 52057 Aachen, FRG, and*
[b] *National Institute of Public Health and Environmentral Protection, Bilthoven, the Netherlands*

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Abstract: Because of the allelic variations within the M protein gene (*emm* gene) of group A streptococci, reliable typing of this important human pathogen can be accomplished by the use of *emm* gene-specific oligonucleotide probes. Two technical modifications (a reverse dot blot and a reverse line blot hybridization assay) of a novel approach for the type-specific identification of *emm* genes have been developed. Both procedures involved amplification of an *emm* gene by polymerase chain reaction. The non-radioactively labeled amplicon was subsequently hybridized to a membrane carrying an array of immobilized *emm* gene-specific oligonucleotide probes, thus allowing the simultaneous analysis of the gene polymorphism in a single hybridization reaction. The feasibility of these rapid and easy to perform methods was shown for the unequivocal identification of reference strains and clinical isolates belonging to 16 different M serotypes.

Key words: Streptococcus pyogenes; M protein; Polymerase chain reaction; Hybridization assay; Gene polymorphism; Epidemiology

Introduction

Over the last few years there has been an increase in severe group A streptococcus (GAS; *Streptococcus pyogenes*) infections, including their suppurative and non-suppurative sequelae [1]. An improved understanding of the epidemiology and pathogenesis of these infections requires reliable laboratory techniques for the precise identification and characterization of GAS. Thus far, most epidemiological studies have relied on the serological identification of certain protein antigens. Classical techniques, including opacity factor (OF) typing and T agglutination typing are relatively easy to perform but have limited specificity [2]. The most important serological marker is the M protein and, at present, there are approximately 74 recognized M serotypes [2]. Since M typing antisera are difficult and laborious to prepare and not commercially available, comprehensive M typing is restricted to a few specialized reference laboratories in the world. Several alternative methods to improve the means of strain identification have been reported, e.g. typing by multilocus enzyme electrophoresis [3,4] and the application of various DNA fingerprinting techniques

* Corresponding author. Tel.: (0241) 8089510; FAX: (0241) 876140

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[5–7]. Recently, we have shown that another approach, namely the identification of M protein genes (*emm* genes) by oligonucleotide probes corresponding to the N-terminal regions of several *emm* genes, provides a highly sensitive and specific tool for an unequivocal strain identification [8–10]. The main purpose of the present investigation was to further improve the probe technology by establishing two different formats of an elegant hybridization assay, hereafter referred to as reverse dot blot and reverse line blot hybridization assays. The technical procedures are described in detail.

Materials and Methods

Bacterial strains

Reference strains and clinical isolates of GAS investigated in the present study were obtained from the strain collections of the World Health Organization Collaborating Centers for Reference and Research on Streptococci, University of Minnesota, Minneapolis, and Prague, Czech Republic or were from the strain collection of the Institute of Medical Microbiology, Aachen, FRG. Altogether, 64 epidemiologically unrelated strains belonging to 16 serotypes (M1, M2, M3, M4, M5, M6, M8, M11, M12, M18, M19, M24, M41, M49, M52, and M57) were selected for this investigation. All isolates had been confirmed as GAS by identification of their group carbohydrate antigen and serologically classified according to their M antigen using the standard immunodiffusion method and absorbed type-specific antisera raised in rabbits [11]. In addition, 77 GAS strains recently isolated from clinical specimens from Dutch patients were investigated (see below).

DNA isolation

Two different protocols were used. For the reverse dot blot hybridization format, streptococcal genomic DNA was prepared by the method of Bollet et al. [12]. Briefly, a 10-ml Todd Hewitt broth culture (37°C, 18 h) was centrifuged and washed once in TE buffer (10 mM Tris · HCl, pH 7.6, 1 mM EDTA). The pellet was resuspended in 100 μl TE buffer and 50 μl 10% SDS. Following incubation (65°C, 30 min) and centrifugation, the sediment was thoroughly resuspended in a few μl of the remaining fluid. The lysate was heated five times for 1 min in a microwave oven (600 W). The pellet was resuspended in 200 μl TE buffer, and after one phenol/chloroform extraction and centrifugation cycle, 1/10 volume of 3 M sodium acetate was added to the supernatant. Finally, after ethanol precipitation, the DNA was dissolved in 100 μl double-distilled water. Template DNA for the genotyping by the reverse line blot hybridization assay was obtained from GAS strains grown on Columbia agar horse blood plates for 16–24 h at 37°C. One loop of cells was resuspended in 100 μl of 150 mM NaCl and heated for 30 min at 60°C. Cells were then centrifuged and the pellet resuspended in 1 ml of 150 mM NaCl. The cells were spun down again and the pellet resuspended in 100 μl lysing buffer (200 U/ml mutanolysin, 10 mM Tris, 1 mM EDTA, pH 8.0), incubated at 37°C for 30 min and subsequently boiled for 10–30 min. This crude lysate was used without further purification as source of template DNA in the PCR.

Construction of oligonucleotide probes

The sequences of the majority of the probes used in this study were described previously [9]. Furthermore, the nucleotide sequences of the 5′ ends of more than 30 different *emm* genes were determined in our laboratories (unpublished data) with the aid of an automated DNA sequencer (Applied Biosystems, Weiterstadt, FRG, and Gouda, the Netherlands) in order to design additional *emm* gene-specific probes. The sequences of the oligonucleotide probes newly evaluated in the present study are shown in Table 1. The oligonucleotides were either prepared on a model 200A DNA synthesizer (Beckman Instruments, Munich, FRG) and further processed according to the instructions of the manufacturer or were custom synthesized by Applied Biosystems (PE ABI division, Gouda, the Netherlands).

Amplification of DNA by polymerase chain reaction (PCR)

For PCR amplification of *emm* genes the 'all M' primers described by Podbielski et al. [13] were used.

Amplification of emm genes and digoxigenin labeling by PCR for use in the dot blot hybridization assay. DNA (0.5–1 μg) was amplified in a 100 μl total reaction including 10 × PCR buffer (500 mM KCl, 100 mM Tris · HCl, pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin), 200 μM each of dATP, dCTP, dGTP, dTTP, 0.5 μl digoxigenin-11-dUTP (Boehringer Mannheim, FRG), 0.5 μM of each primer and 2.5 U *Taq* DNA polymerase. The cycling reaction was done in a programmable heat block (Trio-Thermoblock, Biometra, Göttingen, FRG). After starting the reaction using the hot start protocol [14], each assay was subjected to 30 cycles of 1 min at 94°C, 1 min at 50°C and 2.5 min at 72°C per cycle, followed by a final extension of 7.5 min at 72°C. To confirm the successful amplification of the target DNA, 10-μl samples of the total reaction volume were mixed with 1 μl loading dye, loaded onto 1% agarose gels, electrophoresed, stained with ethidium bromide and photographed.

Amplification of emm genes and biotin labeling by PCR for use in the reverse line blot hybridization assay. In order to label the PCR fragment during the amplification reaction the downstream primer carried a 5′ biotin label, *emm* genes were amplified by adding 4 μl of crude GAS lysate to a final 50-μl reaction volume containing *Tth* buffer (10 mM Tris · HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 9.0), 200 μM of each dNTP, 20 pmol of each primer and 0.5 U of *Tth* DNA polymerase (HT Biotechnology Limited, Cambridge, UK). After layering the mixture with 25 μl of paraffin oil, the reaction mixture was heated for 4 min at 94°C, subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C and 2.5 min at 72°C for each cycle, followed by a final extension of 7 min at 72°C. The cycling reaction was done in a programmable heating block (Hybaid Omnigene, Biozyme, Landgraaf, the Netherlands). Successful amplification was confirmed by analyzing 2-μl samples of the PCR mixture on ethidium bromide-stained agarose gels. If insufficient PCR product was obtained, 1 μl of the PCR mixture was reamplified using the above described protocol.

Hybridization assays

Basically, both procedures involved amplification of an *emm* gene by PCR. The PCR product (already labeled during the PCR cycles) was subsequently hybridized to a membrane carrying immobilized *emm* gene-specific oligonucleotide probes.

Reverse dot blot hybridization assay. The oligonucleotide probes were dot-blotted onto a nylon membrane (Pall Biosupport, Dreieich, FRG) with the aid of a dot blot manifold (Hybri Dot Manifold, BRL, Eggenstein, FRG) and fixed to the membrane by UV cross-linking (Stratalinker 1800, Stratagene, Heidelberg, FRG). In some cases, the oligonucleotides were tailed prior to their attachment to membranes. For poly dT-tailing, 200 pmol of each oligonucleotide probe was incubated for 60 min at 37°C in a 100-μl total reaction including 4 μl 25 mM $CoCl_2$, 100 nmol dTTP, 60 U terminal deoxyribonucleotidyltransferase (Boehringer, Mannheim) and 20 μl 5 × enzyme buffer provided by the manufacturer. The reaction was stopped by adding 100 μl of 10 mM EDTA and 100 μl of TE buffer. An aliquot of this total volume of 300 μl was dot-blotted onto a nylon membrane. The membrane was incubated at 55°C in hybridization solution (0.5 ml per cm^2

Table 1
Oligonucleotide probes corresponding to N-terminal sequences of *emm* genes

Probe designation	Nucleotide sequence of the probe										
emm4	5′	CCA	CGC	TGA	ATC	AGC	CTG	AGG	CTT	TTT	AAT	3′
emm8	5′	TCG	TTA	TTA	GAA	ATA	CTA	TGA	GAT	TTT	GGG	3′
emm11	5′	CGC	TCA	CGT	TTG	TAC	CTT	TAG	GAG	CGC	TTT	3′
emm41	5′	GTA	AAG	CTT	CTT	CTT	GGG	CTT	GTG	CTA	ATC	3′
emm52	5′	TTC	GGT	ATA	TCG	ATG	GTG	ATC	AAC	AGG	TTG	3′
emm57	5′	CAA	AAT	AGG	CGT	CAT	CGA	AGT	AAT	ATC	GCT	3′

membrane) containing 6 × SSC, 0.5% SDS, 5 × Denhardt's solution and 100 μg/ml salmon sperm DNA. Subsequently, 10 μl of the PCR-amplified DNA was denatured by addition of an equal volume of 400 mM NaOH/10 mM EDTA and added to the hybridization solution. After incubation for at least 1 h at 50°C, the membrane was washed at room temperature in 2 × SSC, 0.5% SDS for 5 min, in 2 × SSC, 0.1% SDS for 10 min, and in 0.1 × SSC, 0.1% SDS at 50°C for 20 min. Chemiluminescent detection of the capture-bound digoxigenin-labeled PCR product was performed in accordance with the instructions given by the manufacturer of the 'DIG Luminescent Detection Kit' (Boehringer, Mannheim). Visualization of light signals was accomplished by exposure of the membrane to an X-ray film (Cronex 4, Du Pont de Nemours, Bad Homburg, FRG) for 2–30 min (depending on the signal intensity).

Reverse line blot hybridization assay. In this format, *emm* gene-specific oligonucleotides carrying a 5′ amino group were covalently linked to the carboxyl groups of the negatively charged, activated Biodyne C membrane [15]. For this purpose, a Biodyne C membrane was activated by a 10-min incubation in 16% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. After activation, the membrane was rinsed with water and placed into a miniblotter system (Immunetics, ITK Diagnostics, Uithoorn, the Netherlands). Each slot of the miniblotter was filled with a different *emm* gene-specific oligonucleotide solution (150 μl, 0.3 μM probe in 500 mM $NaHCO_3$, pH 8.4), thus creating a pattern of parallel lines. After a 1-min incubation period at room temperature, excess oligonucleotide solution was removed by aspiration. The blot was then removed from the miniblotter, treated with 100 mM NaOH for 10 min to inactivate the membrane and washed in 2 × SSPE (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA, pH 7.2), 0.1% SDS for 5 min at 50°C. Again the blot was placed into the miniblotter; however, compared to the oligo blotting step it was mounted in a 90° rotated position. This positioned the slots of the miniblotter perpendicularly to the lines containing the probes. Individual slots of the miniblotter were filled with heat-denatured biotin-labeled PCR products (30 μl PCR product in 120 μl 2 × SSPE, 0.1% SDS) and hybridized for 45 min at 50°C. After removing excess PCR solution by aspiration, the blot was washed in 2 × SSPE, 0.5% SDS at 50°C for 10 min and incubated with streptavidin–peroxidase conjugate (Boehringer, Mannheim; diluted 1:4000 in 2 × SSPE, 0.5% SDS) for 30 min at 42°C. Subsequently, the blot was washed in 2 × SSPE, 0.5% SDS for 10 min at 42°C and rinsed briefly at room temperature in 2 × SSPE. Chemiluminescent detection was performed using the ECL detection liquid (Amersham, 's Hertogenbosch, the Netherlands) and visualized by a 5-min exposure of the membrane to an X-ray film (Hyperfilm, Amersham).

Results and Discussion

Since the N terminus of a mature GAS M protein is specific for a given M serotype, this region is a suitable candidate to serve as a target sequence for a type-specific hybridization assay. Using oligonucleotide probes corresponding to the N-terminal sequences of several *emm* genes, we have recently demonstrated that such an 'oligotyping' system provides an excellent tool for reliable strain identification [8,9]. In the present study, the previously evaluated panel of oligonucleotide probes was expanded by the addition of probes directed to type-specific sequences of *emm4, emm8, emm11, emm41, emm52,* and *emm57*. In terms of specificity and standardization, the molecular approach is superior to the serological M protein typing using polyclonal antibodies. Also, with respect to hands-on time, the recently described conventional dot blot hybridization assay already compared favorably with the serological technique [9]. However, the hybridization assays described here clearly provide a further substantial technical improvement because they avoid time-consuming sequential hybridization procedures. These hybridization formats allow the simultaneous analysis of gene polymorphisms at a PCR-amplified DNA locus in a single hybridization reaction. First described by Saiki et al. [16], the reverse dot blot hybridization technique has been primarily applied to HLA

genotyping [16–20], but has also been used for the detection of microorganisms such as *Listeria monocytogenes* [21]. In the present investigation, the feasibility of this method to specifically identify allelic variations within a bacterial gene has been shown for the first time. In the first part of this collaborative study, 64 well-defined strains of GAS belonging to 16 different M types were unequivocally identified by the reverse dot blot hybridization format. A representative blot is shown in Fig. 1. Providing the oligonucleotides with homopolymer dT-tails was not necessary for unambiguous hybridization results; however, occasionally the tailed probes tended to give somewhat stronger hybridization signals. The reverse line blot hybridization assay was established additionally, because of its particularly elegant performance, i.e. its capacity to theoretically type 40 different isolates using 40 different *emm* gene-specific oligonucleotide probes on just one single blot. The covalent binding of an amino-linked oligonucleotide to a negatively charged membrane pushes the probe outwards, making it easy to react with the target DNA during the hybridization procedure. As part of an ongoing nationwide surveillance study, thus far the latter approach has been applied to 77 GAS strains isolated from specimens of Dutch patients suffering from severe streptococcal infections. Fig. 2 displays an example of a typical result obtained by this technique. As shown in Table 2, 78% of the strains were typeable using the 16 currently available *emm* gene-specific probes. Of note, 35% of these GAS strain recently isolated from invasive infections belonged to the T1 serotype and harbored the *emm1* gene. In conclusion, we have presented in detail two different hybridization protocols which, in combination with non-radioactive detection systems, proved to be a highly convenient means by which typing of GAS can be performed. Accurate typing of GAS is an essential tool in studies of the epidemiology, pathogenesis and therapy of streptococcal infections, and the novel methods described can be readily established in every laboratory interested in performing such studies. In the present investigation, up to 16 oligonucleotide probes were used simultaneously, but including even more probes should not be difficult as long as one important prereq-

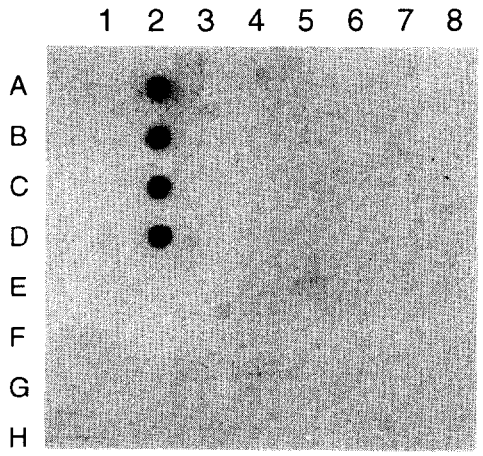

Fig. 1. Representative example of a reverse dot blot hybridization assay. The oligonucleotide probes (here all probes were provided with homopolymer dT-tails) were dot-blotted onto a membrane in a grid-like pattern. Each oligonucleotide solution was applied in four different concentrations in four vertically arranged dots, i.e. the dots in columns 1–8, rows A–D, and the dots in columns 1–8, rows E–H represent the 16 different oligonucleotide probes. In this example the PCR-amplified *emm* gene of a serotype M2 strain was hybridized to the membrane containing the array of probes. Note that hybridization signals were specifically obtained with the *emm2* gene-specific probe (dots in column 2, rows A–D).

Table 2
Phenotypic and genotypic identification of 77 GAS strains isolated during July to December 1992 from patients with severe streptococcal infections

T serotype	*Emm* gene	Number (%) of strains
T1	*emm1*	27 (35.1)
T3	*emm3*	6 (7.8)
T6	*emm6*	6 (7.8)
T11	*emm11*	5 (6.5)
T other [a]	*emm* other [b]	16 (20.7)
T other [a]	*emm* non-typeable [c]	17 (22.1)
Total		77 (100)

[a] T other: T serotype determined, but not belonging to T1, T3, T6 or T11.
[b] *emm* other: genotype determined by the reverse line blot hybridization assay, but no hybridization with the oligonucleotide probes directed against *emm1, emm3, emm6* or *emm11*.
[c] *emm* non-typeable: no hybridization with any of the 16 currently evaluated oligonucleotide probes.

uisite is taken into consideration: all of the membrane-bound oligonucleotides have to be sequence-specific under identical hybridization conditions. This requirement can often be met by adjusting the length of the probes. Until the optimum balance between the length of individual probes and the hybridization condition is experimentally proven, at present it would be prudent to perform hybridization assays in duplicate, i.e. one at a temperature of high and the other at a temperature of lower stringency. Advantages of both of the hybridization techniques also include that membranes with immobilized probes can be prepared in advance and stored until needed. Besides sensitivity and specificity, the methods are reproducible, easy to perform and rapid. The entire typing procedure, including the preparation of the DNA by either of the two protocols described, can easily be accomplished within 1 day.

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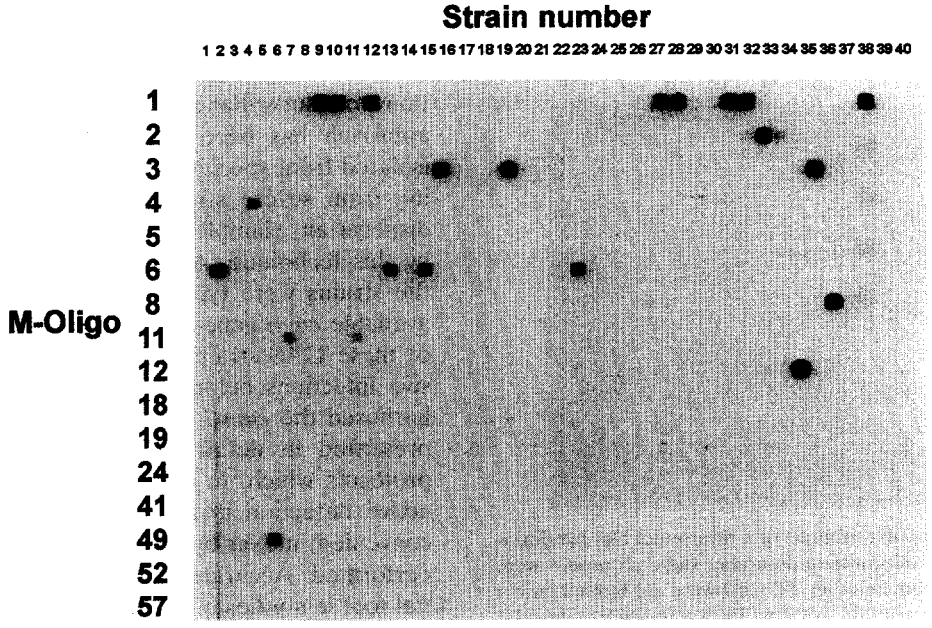

Fig. 2. Representative example of a reverse line blot hybridization assay. Sixteen *emm* gene-specific oligonucleotide probes (here designated as 'M-Oligo' 1–57) were applied in horizontal lines onto a Biodyne C membrane. Biotin-labeled PCR amplicons of the *emm* genes of 40 GAS strains were applied in vertical lines perpendicularly to the lines containing the probes. The amplified *emm* gene of each strain hybridized either specifically to one of the probes or did not hybridize to any of the available probes. For example, *emm* genes of strains nos. 9, 10, 12, 27, 28, 31, 32, and 38 hybridized to the *emm1* gene-specific probe, while strains nos. 39 or 40 were not typeable for their *emm* genes by the currently evaluated probes. The fainter signals for strains nos. 4, 6 and 11 could be due to a lower optimum hybridization temperature neccessary for the *emm4*- and *emm11*-specific probes. For future optimization, the length of these probes will be adjusted to obtain equal annealing temperatures for all probes.

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