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# M genotyping and DNA fingerprinting of *Streptococcus pyogenes* isolates from an area of central Italy

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## SUMMARY

M protein gene typing was used to analyse *Streptococcus pyogenes* clinical isolates collected between 1983 and 1995 in an area of central Italy from patients presenting different types of infections; the same isolates were also characterized by means of DNA fingerprinting.

M type 1 was the most common (50% of study strains), followed by M types 4, 12 and 6. The proportion of M type 12 decreased with time, whereas M type 1 increased, in agreement with data obtained in many different areas. Most invasive strains belonged to types M1 (30%) and M12 (30%); on the other hand, the M1 type did frequently occur also among non-invasive isolates. DNA fingerprinting showed a correlation between M types and DNA patterns. This report provides epidemiological information from a geographic area not sampled recently, and further shows the usefulness of the M genotyping technique, which offers potential advantages over conventional serological typing methods.

## INTRODUCTION

The epidemiology and severity of group A streptococcus (GAS, *Streptococcus pyogenes*) infections changed remarkably starting from the mid-1980s when a resurgence of severe infections and their suppurative and non-suppurative sequele was recognized and documented. Outbreaks of rheumatic fever began to occur throughout the United States [1–3], and severe and suppurative systemic infections, including necrotizing fasciitis, myositis, malignant scarlet fever, bacteraemia and a streptococcal toxic shock syndrome were observed with increasing frequency in many areas of the USA, Europe and other parts of the world [2, 4–13]. Current available clinical and epidemiological data suggest that this trend is continuing [1].

During the same period in which this increase in the incidence of severe streptococcal infections was observed, a change in the most common M serotypes recovered from patients was also observed: the percentage of M3, M6 and M12 (which were prominent in the early 1980s) fell, whereas M type 1 rose [3, 14].

The increase in the proportion of M type 1 was observed in several countries such as the USA, Canada and Australia [2, 3, 10, 15]. In Sweden during 1988–9 it was the most predominant, representing almost 70% of all isolates and was associated with a higher fatality rate [11, 16].

Little is known about the epidemiology of *S. pyogenes* infections in Italy and available characterization data on streptococcal isolates are scarce [17].

Since M antisera are difficult and laborious to prepare and not commercially available, M serotyping is restricted to a few specialized reference laboratories in the world [18].

A new molecular approach based on the detection

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of the gene encoding M protein expression, the *emm* gene (*emm* typing or M genotyping) has recently been developed [18, 19].

In order to obtain information concerning the epidemiology of group A streptococcal infections in our geographic area, clinical isolates collected between 1983 and 1995 were subjected to M genotyping by using such an approach; strains were also characterized by DNA fingerprinting. To our knowledge, this study is the first of its kind in Italy.

## MATERIALS AND METHODS

### Cases

In 1983–95, 222 cases were observed at the Institute of Infectious Diseases of the University of Siena. The diagnosis of streptococcal infections was based on clinical criteria, the isolation of *S. pyogenes* and/or a two-fold or greater increase of anti-streptolysin O titres between acute and convalescent-phase sera.

Out of 222 patients, 122 were male and 100 female. The 210 patients ranged in age from 7 months to 14 years; 12 patients were aged 15–63 years. An additional 9 pediatric cases of non-suppurative sequele (8 acute glomerulonephritis and 1 rheumatic fever) were recognized.

Most cases were uncomplicated infections: pharingitis (149), scarlet fever (44), otitis (3), impetigo (3), vaginitis (1). Invasive infections were rare: bacteremia (3), abscess (2), cellulitis (5), adenitis (6), erysipelas (6). Cases always had a favourable clinical outcome. A report of the clinical features of the patients and temporal distribution of the cases has previously been published [20].

### Bacterial strains

*Reference strains*

Fourteen reference strains of GAS belonging to different M serotypes and obtained from the C.C.U.G. Culture Collection of the Department of Clinical Bacteriology at the University of Goteborg, Sweden, and from Chiron Biocine in Siena, Italy, were used in this study: ISVT SF 130/13 (M1), ATCC 12344 (M1), T2/44/Rb4 (M2), ATCC 12345 (M2), ATCC 10389 (M3), CCUG 30915 (M3), ATCC 12385 (M4), ATCC 12347 (M5), ATCC 12348 (M6), ISVT SF/4 (M8), CCUG 12710 (M12), ATCC 12353 (M12), ISVT J17C (M18), ISVT C 98/97 (M24).

Table 1. *Source of 52* S. pyogenes *clinical isolates*

| Isolation site | Strains (*n*) |
|---|---|
| Throat*/nose | 35 |
| Ear | 4 |
| Skin | 3 |
| Blood | 3 |
| Pus | 5 |
| Soft tissue† | 2 |
| Total | 52 |

* Six strains from scarlet fever.
† One strain from necrotizing fasciitis.

*Clinical isolates*

A total of 52 GAS isolates were available for typing. Isolates were cultured from hospitalized patients presenting various infections and observed between 1983 and 1995 in the Institute of Infectious Diseases of the University of Siena (with the exception of one isolate from a case of necrotizing fasciitis, observed in the Division of Dermatology of the University of Siena). Isolation sites (throat, nose, ear, skin, blood, pus, soft tissue) are shown in Table 1. All the subjects (except for two) were epidemiologically unrelated.

*Identification*

Isolation and identification of each strain were performed according to standard procedures [21]. All strains were stored at −80 °C in Wilkins–Chalgren broth (Oxoid) supplemented with 20% (v/v) glycerol until analysis was performed.

### DNA isolation

Bacterial DNA was prepared according to the following procedure. *S. pyogenes* cultures were grown overnight at 37 °C in 10 ml of Todd–Hewitt broth. Cells were pelleted and resuspended in 1 ml of 50 mM Tris/HCl, 20% sucrose, 50 mM EDTA, pH 8. Lysozyme (50 mg/ml; Sigma) was added and allowed to react for 40 min at 37 °C. Harvested cells were resuspended in 0·5 ml of 10 mM Tris, 10 mM EDTA, pH 8·2. The cells were incubated with 20 $\mu$l of 40% SDS for 20 min at 65 °C, and then with 50 $\mu$l of proteinase K (50 mg/ml, Boehringer Mannheim GmbH, Germany) at 37 °C, overnight. After performing extraction twice with phenol and twice with chloroform, the nucleic acids were precipitated with the addition of 5 M NaCl and isopropanol and suspended in 10 mM Tris/HCl 1 mM EDTA pH 7·5. Purified DNA was digested with 4 $\mu$l DNase-free

RNase (0·5 mg/ml, Boehringer) at 37 °C for 30 min and stored at 4 °C.

### M protein genotyping

*Emm* typing (or M genotyping) consists of PCR amplification of the M protein gene (*emm* gene) of GAS strains and subsequent hybridization to a membrane carrying immobilized M type-specific probes, according to the method described by Kaufhold [19], with some modifications: in the reverse line blot hybridization assay, the *emm* gene-specific oligonucleotide solution was heat denatured (at 100 °C for 5 min) before use; washing after hybridization was performed twice at 54 °C for 5 min instead of once at 50 °C for 10 min.

#### *Oligonucleotide probes*

Oligonucleotide probes specific for M types 1–6, 8, 12, 18, 24 were used in this study. The sequences of the probes were described previously [18, 19]. The oligonucleotides were custom synthesized by Med Probe, Olso, Norway. Probes correspond to the N-terminal sequences of *emm* genes.

### Restriction digests

The restriction endonucleases *Hin*dIII and *Hae*III (Boehringher) were used for this study.

Genomic DNA (10 $\mu$g) was separately digested with 30 U of each enzyme tested, under conditions recommended by the supplier. The resulting digests were analysed with gel electrophoresis (70 V for 30 min and then 35 V for 8 h) on a 1% agarose gel using Tris-acetate-EDTA buffer (containing 0·5 $\mu$g of ethidium bromide per ml) and then photographed under u.v. illumination with a Polaroid camera. *Hin*dIII restriction fragments of lambda phage DNA were used as size markers.

### Statistical evaluation

Fisher's exact test was used for comparison of proportions.

## RESULTS

In the present study, *S. pyogenes* isolates from an area of central Italy were subjected to M genotyping by analysing the allelic variations within the *emm* gene, according to the method described by Kaufhold and colleagues [19].

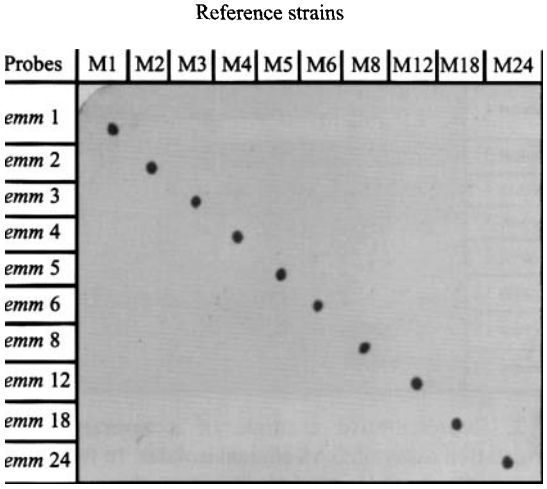


**Fig. 1.** Reverse line blot hybridization assay of GAS reference strains belonging to different M serotypes. The *emm* gene-specific oligonucleotide probes were applied in horizontal lines on a membrane; the amplified *emm* genes of reference strains were applied in vertical lines perpendicularly. Each probe specifically hybridized with the corresponding reference strains.

Table 2. *M genotyping of 52* S. pyogenes *clinical isolates*

| *emm* gene | Number (%) of strains |
|---|---|
| *emm* 1 | 26 (50) |
| *emm* 2 | 0 |
| *emm* 3 | 0 |
| *emm* 4 | 6 (11) |
| *emm* 5 | 0 |
| *emm* 6 | 3 (6) |
| *emm* 8 | 0 |
| *emm* 12 | 5 (10) |
| *emm* 18 | 0 |
| *emm* 24 | 0 |
| Non typeable* | 12 (23) |
| Total | 52 (100) |

* No hybridization with any of the 10 available probes.

Ten probes, specific for some M types which are presently known to be of major clinical and epidemiological significance were used, after having shown their unequivocal identification of reference strains belonging to the corresponding M serotypes (Figure 1).

Fifty-two *S. pyogenes* isolates obtained in 1983–95 from subjects displaying various types of infections were analysed using M genotyping. The results are shown in Table 2; an example of data obtained is shown in Figure 2.

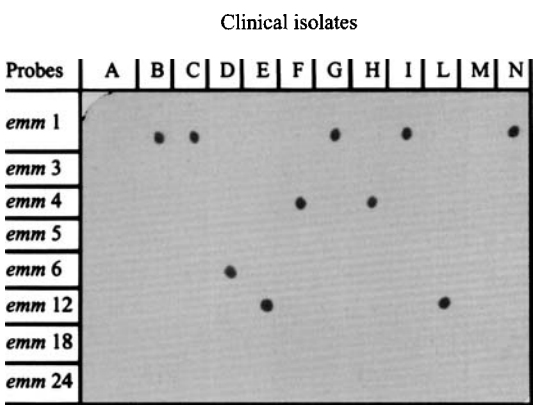


**Fig. 2.** Representative example of a reverse line blot hybridization assay of GAS clinical isolates. In this example 5 strains (B, C, G, I, N) hybridized to the *emm* 1 gene-specific probe, 2 strains (F, H) hybridized to the *emm* 4 gene-specific, 2 strains (E, L) hybridized to the *emm* 12 gene-specific probe, 1 strain (D) hybridized to the *emm* 6 gene-specific probe; 2 strains (A, M) were not typeable by the currently employed probes.

Table 3. *Variation with time of M types of* S. pyogenes *clinical isolates*

| | Number (%) of strains | |
|---|---|---|
| *emm* gene | 1983–9 | 1990–5 |
| *emm* 1 | 5 (36) | 21 (55) |
| *emm* 4 | 1 (8) | 5 (13) |
| *emm* 6 | 0 | 3 (8) |
| *emm* 12 | 4 (28) | 1 (3) |
| Non-typeable | 4 (28) | 8 (21) |
| Total | 14 (100) | 38 (100) |

Among the 52 isolates examined, 4 different *emm* gene types were identified: *emm*1, *emm*4, *emm*6, *emm*12. The *emm*1 type was the most common, being found in 26 (50%) of study strains. *Emm*4 type was exhibited by 6 strains (11%), *emm*12 type by 5 strains (10%) and *emm*6 type by only 3 strains (6%). Twelve strains (23%) did not hybridize with any of the probes used (Table 2).

The distribution of *emm* types over time and in different clinical conditions was analysed.

The temporal variation of *emm* types is shown in Table 3, which compares the *emm* genotypes detected in 1983–9 and those observed in 1990–5.

Although the number of isolates belonging to the first group is small, it may be noted that the proportion of *emm*12 type decreased significantly over time ($P = 0{\cdot}0048$), whereas *emm*1 type increased ($P = 0{\cdot}34$).

Table 4. *Distribution, by source, of M type in* S. pyogenes *clinical isolates*

| *emm* gene | URT* | Skin | Pus | ST† | Blood | Total |
|---|---|---|---|---|---|---|
| *emm* 1 | 21 | 2 | 2 | | 1 | 26 |
| *emm* 4 | 5 | | | 1 | | 6 |
| *emm* 6 | 2 | | | 1 | | 3 |
| *emm* 12 | 2 | | 1 | | 2 | 5 |
| Untypeable | 9 | 1 | 2 | | | 12 |

* URT, upper respiratory tract.
† ST, soft tissue.

Table 5. *M type distribution in* S. pyogenes *clinical isolates from invasive and non-invasive infections*

| | Number (%) of strains | |
|---|---|---|
| *emm* gene | Invasive | Non-invasive |
| *emm* 1 | 3 (30) | 23 (55) |
| *emm* 4 | 1 (10) | 5 (12) |
| *emm* 6 | 1 (10) | 2 (5) |
| *emm* 12 | 3 (30) | 2 (5) |
| Non-typeable | 2 (20) | 10 (23) |
| Total | 10 (100) | 42 (100) |

Table 6. *Genomic DNA RF patterns and M types of 33* S. pyogenes *isolates*

| Strains (*n*) | M type | RF pattern |
|---|---|---|
| 18 | M1 | a |
| 4 | M4 | b |
| 2 | M6 | c |
| 4 | M12 | d |
| 2 | Untypeable | e |
| 1 | Untypeable | f |
| 1 | Untypeable | g |
| 1 | Untypeable | h |

Analysis of the association between *emm* types and isolation sites is summarized in Table 4.

Among isolates from the upper respiratory tract, *emm*1, *emm*4, *emm*6 and *emm*12 types were identified. Six throat strains associated with scarlet fever belonged to the *emm*4 type (4 strains) and *emm*1 type (2 strains). Strains isolated from blood belonged to *emm*1 and *emm*12 types. Among isolates from soft tissues, one strain associated with a case of necrotizing fasciitis belonged to the *emm*6 type (Table 4).

Analysis of the association between *emm* types and different (invasive and non-invasive) infections is shown in Table 5. Although the two groups are

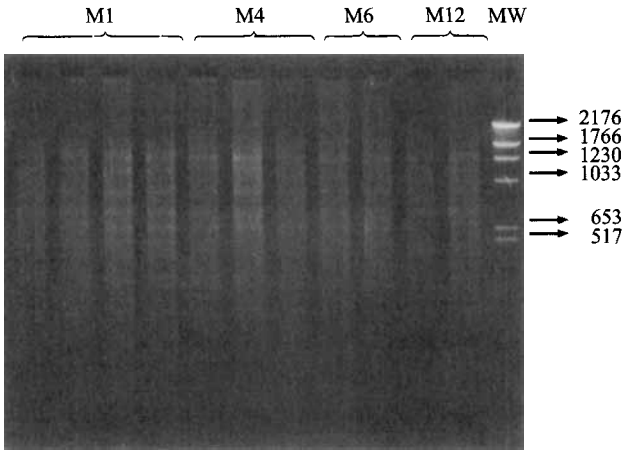


**Fig. 3.** Restriction endonuclease (*Hin*dIII) cleavage patterns of chromosomal DNA from *S. pyogenes* clinical isolates representing different M types. Four distinct DNA patterns, associated with different M types, can be identified: pattern a (M1 strains), pattern b (M4 strains), pattern c (M6 strains), pattern d (M12 strains). MW, molecular weight markers noted in kilobases.

numerically heterogeneous, it may be observed that most invasive strains belonged to *emm*1 type (30%) and *emm*12 type (30%); interestingly, *emm*12 invasive strains were among the earliest sampled isolates in our collection. On the other hand, the *emm*1 type did frequently occur also among non-invasive isolates (Table 5).

Thirty-three strains were also characterized using DNA fingerprinting. The restriction endonuclease *Hin*dIII was used for each of the 33 isolates; additionally, 22 isolates were also cut by *Hae*III.

Results obtained with the different tested enzymes were similar, with a single exception: *Hae*III revealed some differences in the DNA pattern of one strain, which *Hin*dIII was unable to distinguish (data not shown).

Results of RFLP analysis are shown in Table 6 and Figure 3.

Among the 33 isolates examined, 8 distinct DNA patterns were identified; these patterns are designated with the letters a–h (Table 6).

Comparison of DNA fingerprints and *emm* types is also shown in Table 6 and Figure 3.

DNA were conserved among strains of the same *emm* type, yet were easily distinguished from those of different *emm* types.

Strains which were not typed with the probes used yielded unique fingerprints, with the exception of two strains from two epidemiologically related subjects, displaying identical DNA patterns (Table 6).

## DISCUSSION

By means of M genotyping we analysed 52 *S. pyogenes* isolates obtained over the course of approximately 12 years in an area of central Italy from subjects with various infections.

With the set of probes used, it was possible to type 77% of isolates, with the *emm*1 type mainly dictating the degree of typability, followed by *emm*4, *emm*12 and *emm*6 types.

Although our findings cannot be directly compared with those of others due to difference in the methods employed, the strain sources and the isolation times, they are in accordance with many data obtained from various parts of the world, which indicate the wide distribution of the M types we observed, and the present predominance of the M1 types [3, 12, 14, 21].

It is commonly held that particular M types of group A streptococci tend to be frequently associated with specific diseases or anatomic sites of infection, although the reason is still not understood [18].

The heterogeneous *emm* types we detected among isolates from the upper respiratory tract are well known 'throat strains' [18]. The association between the *emm*4 type and scarlet fever was also reported in earlier surveys [12].

Notably, the invasive strain sample we analysed, although not large, was associated with *emm*1 and *emm*12 types, as is well documented in large-scale studies [3, 9–12, 14, 15, 17, 21, 22].

One strain from a case of necrotizing fasciitis belonged to the *emm*6 type: this was one of the most commonly isolated *emm* types, along with *emm*1 and *emm*3, in several studies [13].

The distribution of *emm* types showed variations from year to year. During the study period the proportion of isolates of the *emm*12 type fell; conversely, the percentage of the *emm*1 type isolates increased.

Temporal fluctuations in the proportions of M types have been well documented. Carefully conducted prospective studies have shown that the introduction and spread of specific M types through a population can be a very dynamic and rapid process [1].

The temporal changes of *emm* types we observed are in accordance with findings from many other European countries and the USA [3, 12, 14, 16]. Although marked geographic variations in M type distribution have been found, in all countries studied until now, the M1 type has been found to increase and

to predominate during recent years, representing 20–80% of study strains [3, 9, 10, 15, 16, 23, 24]. The present study, concerning isolates from a geographic area not sampled recently, provides epidemiological information and further extends the geographical range over which the M1 type at present is predominating.

In most studies from various parts of the world, the increased proportions of the M1 type tend to be associated with the increased incidence of severe streptococcal infections, suggesting that those M1 strains which are currently being isolated are particularly virulent [1, 3, 11, 12, 16, 25].

We observed a trend toward an increased number of cases during the second half of the 1980s, but not a change in the severity of diseases [20]. This might be due, in part, to the fact that our cases are mainly pediatric [20]; however, increased incidences of bacteraemia and necrotizing fasciitis (particularly in subjects with varicella infections) have been reported even during paediatric age [1, 2, 4–6].

Such findings suggest that virulence may not be simply related to specific M types, but there is the likelihood that specific strains with enhanced virulence potential may exist within a given serotype.

Further characterization of study strains with DNA fingerprinting showed a correlation between M types and DNA patterns, as already reported [26], and did not allow the differentiation of strains of the same M type obtained in different years from different types of infections.

However, by using the same and different genetic techniques, others have found strain heterogeneity within the same M type [27–32] and some authors have also suggested that such differences may be associated with a different type of virulence potential [27, 30–32]. This discrepancy between those studies and our data could be partly due to the similar geographical origin of all our study strains, to the small number of invasive strains available for typing and to differences in the methods employed.

On the other hand, the reported detection of genotypically identical M1 strains either in bacteraemic patients or those with uncomplicated infections and healthy carriers suggests the importance also of host factors in determining the type and severity of the infection, as addressed by several studies reporting a link between lower antibody levels to the M1 antigen and to erythrogenic toxins and development of fatal infections [1, 27, 29].

Further work is in progress to characterize a larger number of isolates using these and other techniques and to analyse the host's immune response.

The genetic M typing technique employed, which is already used in GAS surveillance in the Netherlands [20], proved to be a very useful and reliable method for strain characterization.

Serological M protein typing has several limitations. It is technically demanding and requires a large number of typing sera which are not commercially available and are very difficult and laborious to prepare and to maintain. In addition, the process of obtaining unambiguous M typing results can be notoriously difficult. Therefore, M serotyping is restricted to a few specialized reference laboratories in the world.

Many GAS isolates are currently M non-typeable, due to either a lack of availability of appropriate M antisera or a scarce or completely lacking M protein expression [10, 14, 17, 32, 33].

Since *emm* genotyping detects the genetic determinant of the M protein, the expression of the gene is irrelevant for the successful application of this method. Thus, for epidemiological investigations, *emm* genotyping may circumvent the potential obstacles set at the level of M gene expression.

Moreover, *emm* genotyping presents some relevant technical advantages.

Probes may be reliably synthesized in a short time and may be stored easily for long time without a loss of quality.

The molecular technique does not require elaborate preparation of DNA and allows the simultaneous analysis of several isolates using several *emm*- gene specific probes on a single blot, thus avoiding time-consuming sequential hybridization procedures: the entire typing method as performed in this study can be accomplished within 2 days and can be further shortened to 1 day by using a more rapid method for the isolation of DNA, as reported [19].

Another important technical point is that the hybridization protocol, which makes use of a non-radioactive DNA detection system, does not require sophisticated equipment and particular skills.

This reliable, feasible, rapid and relatively easy to perform technique may therefore enable more laboratories to do this typing.

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