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Molecular Subtyping of Prevalent M Serotypes of *Streptococcus pyogenes* Causing Invasive Disease

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Reproducible methodologies and a scheme for high-resolution genotyping of *Streptococcus pyogenes* were defined with respect to a study of six predominant M serotypes causing invasive group A streptococcal disease in the United Kingdom. Serotype reference strains were compared with nine clinical isolates of each serotype from patients with diseases such as pneumonia, puerperal sepsis, toxic shock-like-syndrome, cellulitis, or necrotizing fasciitis. Four enzymes were evaluated for their discriminatory power in 16S rRNA gene-specific ribotyping. Discriminatory power was greatest with *Eco*RI, which generated serotype-specific ribotypes, and with *Sac*I, which could subdivide strains of the same M serotype. Twenty-five combined ribotypes were found among the 60 strains, and the indices of discriminatory power (*D* values) of this method varied from 0.51 within serotype M1 to 0.98 within strains of serotype M5. Macrorestriction with the rarely cutting endonuclease *Sma*I and pulsed-field gel electrophoresis gave *D* values varying from 0.37 within serotype M1 to the maximal 1.0 within serotype M5. Comparison of macrorestriction profiles revealed various degrees of genetic heterogeneity within M serotypes. Strains of M1, M3, M6, and M11 exhibited clonally related macrorestriction profiles, while those of R28 and M5 strains were consistent with polyphyletic origin.

The Lancefield group A streptococci (*Streptococcus pyogenes*) (23) are major causative agents of human disease, ranging from pharyngitis to severe invasive diseases such as toxic shock-like syndrome and necrotizing fasciitis (9, 19, 24). Epidemiological surveillance has classically been based on phenotypic (serological) markers. The antiphagocytic M protein, encoded by the *emm* gene(s), is the antigenic basis of a serotyping scheme (7), constituting more than 80 M types. However, many (29% of current *S. pyogenes* isolates in the United Kingdom) isolates of *S. pyogenes* are nonserotypeable (10). There is considerable interest in the development of genotypic methods which can differentiate strains and/or subdivide M serotypes for the purposes of epidemiological typing.

Strains of *S. pyogenes* have been analyzed by several methods collectively termed genotypic typing (15). They include restriction endonuclease analysis of genomic DNA, rRNA gene polymorphism analysis (ribotyping), pulsed-field gel electrophoresis (PFGE), and multilocus enzyme electrophoresis. These studies have had diverse and sometimes conflicting implications. Restriction endonuclease digests of genomic DNA were shown to be M type specific (6) and were found to be more discriminatory than initial approaches to ribotyping (3, 8, 21). On the other hand, the choice of enzymes used by Bruneau et al. (4) in a study of pharyngeal isolates increased the discriminatory power of ribotyping and detected some correlation between M serotype and ribotype. Genomic DNA macrorestriction with a rarely cutting endonuclease, *Sfi*I, and resolution of large fragments by PFGE were used to detect polymorphisms between and within some M serotypes of *S. pyogenes* (14, 22). Population genetics studies by multilocus enzyme electrophoresis detected 33 electropherotypes among *S. pyogenes* isolates from patients with toxic shock syndrome or other invasive diseases. Although comprehensive M serotyping data were not available, all of those strains within a single M serotype belonged to a single electropherotype (16, 17). However, multilocus enzyme electrophoresis analysis of strains associated with rheumatic fever and acute poststreptococcal glomeruleronephritis subsequently indicated that single M types contained multiple electropherotypes and vice versa (11). Therefore, systematic studies of the relationship between genotype, M serotype, and disease symptomatology are required to resolve apparent anomalies in the literature, to establish a reproducible framework for molecular epidemiological typing, and to quantify the discriminatory power (13) of molecular epidemiological typing. The study described here addresses the question of genetic diversity and its degree of resolution for epidemiological purposes with respect to six of the predominant M serotypes causing invasive disease in the United Kingdom in 1993 and 1994 (Fig. 1).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Nine blood culture isolates of *S. pyogenes* (obtained in 1993 and 1994 from patients in different geographical regions of the United Kingdom) belonging to each of the M serotypes M1, M3, M5, M6, M11, and R28 were selected for study and are listed in Table 1. The type strains of each of these six M serotypes were obtained from the National Collection of Type Cultures (NCTC; Colindale, United Kingdom). Stock cultures were maintained in 16% blood glycerol broth at −70°C.

Minipreparation of genomic DNA from plate cultures. A procedure based on that of Wilson (27) for gram-negative bacteria was developed for the preparation of streptococcal genomic DNA from a single plate culture. Enzyme concentrations and incubation times and temperatures were found to be critical for reproducible yield and quality of DNA. Strains were grown overnight on blood agar plates at 37°C. The cell growth was scraped off and was resuspended in 1 ml of TE glucose buffer (glucose, 50 mM; Tris, 25 mM [pH 8.0]; EDTA, 10 mM) in a microcentrifuge tube. The cells were spun down (2 min, 13,000 rpm, Jouan M14.11 microcentrifuge), resuspended in 100 μl of 40 μg of mutanolysin (Sigma) per ml in TE glucose buffer–50 μl of 50 mg of lysozyme (Sigma) per ml, and

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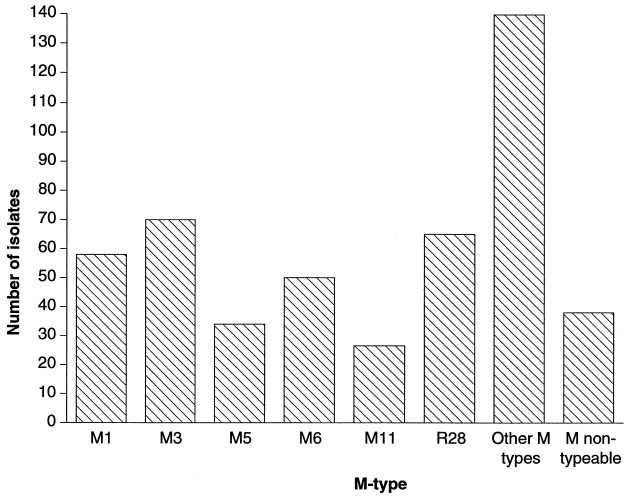

FIG. 1. Distribution of M serotypes causing invasive disease in the United Kingdom for the year 1994.

incubated at 37°C for 1 h. A total of 30 μl of 10% sodium dodecyl sulfate (SDS) and 3 μl of 20 mg of proteinase K (Sigma) per ml were added, and the combination was mixed and reincubated at 55°C for 30 min. Thereafter, 100 μl of 5 M NaCl was added with thorough mixing; this was followed by the addition of 80 μl of cetyltrimethylammonium bromide (10%) in 0.7 M NaCl. The solution was mixed and incubated for 10 min at 65°C, after which lysis was complete. An approximately equal volume of chloroform-isoamyl alcohol (24:1; vol/vol) was mixed in, and the mixture was spun in the microcentrifuge for 5 min. The viscous supernatant above the white interface was transferred to a fresh tube and was extracted with phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol). The aqueous supernatant was again transferred and precipitated by adding an equal volume of isopropanol at room temperature. The final pellet was resuspended in 100 μl of TE (Tris, 10 mM; EDTA, 1 mM [pH 7.5]).

Ribotyping with 16S rDNA. A 1,500-bp probe was generated from genomic DNA of the type strain of serotype M1, *S. pyogenes* NCTC 8198, by PCR with the primers 5′-AAGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′. The PCR products were separated from the primers with GeneClean (Bio 101, Inc., La Jolla, Calif.) and were labelled with biotin-16-dUTP by using a random-primed labelling kit (Boehringer Mannheim Biochemica GmbH, Mannheim, Germany). Restriction digests of genomic DNA (5 μg per lane) were electrophoresed in 0.7% agarose for 17 h at 55 V (*Pvu*II, *Eco*RI, and *Hin*dIII digests) or 24 h at 60 V (*Sac*I digests). Gels were vacuum blotted (LKB VacuGene apparatus; Pharmacia Biotech Ltd., Milton Keynes, United Kingdom) onto a Hybond N nylon membrane (Amersham Life Science, Bucks, United Kingdom). Hybridization was carried out under standard conditions (20) except that the membrane filters were washed twice at 57°C for 15 min. Reactions were visualized colorimetrically with BluGene reagent, and molecular weight markers were biotinylated *Hin*dIII fragments of phage lambda (Life Technologies, Paisley, Scotland, United Kingdom). The numerical index of discriminatory power, or the *D* value (13), was calculated for each enzyme and for each serotype.

Macrorestriction and PFGE. Streptococci were grown overnight on blood agar plates at 37°C, and the cells were resuspended in 2 ml of solution I (1 M NaCl, 10 mM Tris-HCl [pH 7.6]) to a density equivalent to that of a McFarland no. 5 standard. A total of 400 μl of this suspension was added to 400 μl of molten low-melting-point agarose dissolved in solution I, dispensed into block formers, and cooled to 4°C. The solidified blocks were added to 2 ml of solution II (solution I plus 100 mM EDTA, containing 1 mg of lysozyme per ml and 7 μg of mutanolysin per ml), and the mixture was incubated overnight at 37°C. The solution described above was replaced with 2 ml of solution III (0.5 M EDTA [pH 9.5], 1% Sarkosyl NL-30, 0.5 mg of proteinase K per ml), and the blocks were incubated at 56°C for 6 h. After exchange for fresh solution III, the blocks were reincubated overnight at the same temperature. The blocks were washed three times (30 min each time) with 2 ml of TE buffer (10 mM Tris, 10 mM EDTA [pH 7.5]) prior to storage at 4°C. Macrorestriction with *Sma*I was carried out as described previously (1, 18). PFGE was carried out in a CHEF DRII apparatus (Bio-Rad) for 22 h at 200 V. Ramping times were 10 to 35 s (for strains of serotypes M1, M3, M5, and R28) or 1 to 20 s for strains of serotypes M6 and M11). Molecular mass markers were concatamers of phage lambda DNA (size range, 48.5 to 1018.5 kb; New England Biolabs, Hitchin, United Kingdom). *D* values (13) were calculated for each serotype.

RESULTS

Ribotyping with 16S rDNA. Genomic DNA was prepared as described in Materials and Methods and was digested with the enzymes *Pvu*II, *Hin*dIII, *Sac*I, and *Eco*RI. Southern blots were hybridized with the labelled intragenic 16S rRNA gene probe. The first three of these endonucleases lack a restriction site within the published sequence of the 16S rRNA gene of *S. pyogenes* (GenBank accession no. X59029), while *Eco*RI has one restriction site at nucleotide 576.

The 60 strains, comprising the type strain and nine clinical isolates of each of the six M serotypes, were all typeable. We found 16S rRNA gene polymorphisms among the 60 strains with each of the four enzymes. In the case of *Pvu*II (coded P) or *Hin*dIII (coded H), strains belonging to several different M serotypes sometimes shared a single 16S ribotype. Five to six bands from 4.3 to about 9.5 kbp in size were detected in *Hin*dIII blots. All strains of serotypes M1, M3, M11, and R28 shared a single *Hin*dIII ribotype, ribotype H1 (Fig. 2A, lane 1). The type strain of serotype M6 had a unique *Hin*dIII ribotype, ribotype H3 (Fig. 2A, lane 3). The nine M6 clinical isolates shared a unique H ribotype, ribotype H4 (Fig. 2A, lane 4), while serotype M5 was composed of two H ribotypes (Fig. 2A, lanes 1 and 2). *Hin*dIII failed to discriminate between isolates within four of the six serotypes and yielded low *D* values for isolates of the other serotypes (0.47 for M5 and 0.2 for M6).

Five to six bands from 2.3 to 9.3 kbp in size were detected in *Pvu*II blots. All strains of serotypes M1, M3, and M11 shared ribotype P1 (Fig. 2B, lane 1), as did all but one strain of serotypes M6 and R28. Serotypes M5, M6, and R28 displayed diversity with respect to the *Pvu*II ribotype, but *D* values were useful only in the first case (serotype M5; $D = 0.65$), in which three P types occurred among 10 strains (Fig. 2B, lanes 1 through 3). A unique P type was found for the type strain of M6 (Fig. 2B, lane 4). In *Eco*RI blots (coded E), from 8 to 11 bands ranging in size from 2 to about 23 kbp were detected. The E ribotypes of the six type strains were different from each other, but they did not differ from those of strains of the same serotype(s) except in the case of M6 (Fig. 2C). Four additional E ribotypes were found (Fig. 2C, lanes 4, 8, 9, and 11). Serotypes M1, M3, and M11 had E ribotypes which were distinctive and which were shared by all of the strains of those serotypes (Fig. 2C, lanes 1, 2, and 5). Serotype R28 comprised three E ribotypes. There was considerable diversity of the E ribotype (six) within strains of the serotype M5. The E ribotypes of 55 of the 60 strains were serotype specific, while the 5 strains that were exceptions were found in serotype M5. *Eco*RI failed to discriminate between strains for serotypes M1, M3, and M11, had low *D* values (0.2 and 0.38) for strains of serotypes M6 and R28, and had a relatively high *D* value (0.85) only for strains of serotype M5.

Four to five bands with large molecular sizes, most greater than 23 kb, were detected in *Sac*I (coded S) blots (data not shown). If serotype M5 is not considered, the S ribotypes of the other serotypes were specific. For example, ribotypes S1, S2, and S3 were associated with serotype M1, ribotypes S4 to S6 were associated with serotype M3, ribotypes S13 to 15 were associated with serotype M6, and ribotypes S16 to S19 were associated with serotype M11. Serotype M5 contained nine different S ribotypes, with four of them overlapping those found in other serotypes (S2, S3, S4, and S10). However, even in serotype M5 there were five specific ribotypes (S7, S8, S9, S11, and S12). The majority of the serotype R28 strains (8 of 10) shared a unique ribotype, ribotype S20, while two serotype R28 strains had ribotypes that overlapped with other serotypes. The *D* values for *Sac*I within individual serotypes were lowest

TABLE 1. Epidemiology and genotype markers of *S. pyogenes* strains

Serotype and strain no.	Disease[a]	Place of isolation[a]	Yr of isolation	16S rRNA gene profiles[b]				Combined ribotype	mrp *Sma*I[c]
				*Pvu*II	*Hin*dIII	*Eco*RI	*Sac*I		
Serotype M1									
NCTC 8198			1950	P1	H1	E1	S1	Sp-R1	M1.1
R3102	Pneumonia	Stoke	1993	P1	H1	E1	S2	Sp-R2	M1.2
R2365	Fatal TSLS[d]	Bedford	1993	P1	H1	E1	S2	Sp-R2	M1.3
R2025	Cellulitis, TSLS	London, hospital A	1993	P1	H1	E1	S2	Sp-R2	M1.3
R1037	Scarlet fever, fatal septicemia	Kettering	1993	P1	H1	E1	S2	Sp-R2	M1.3
R0528	Fatal pneumonia	Bishop Auckland	1993	P1	H1	E1	S2	Sp-R2	M1.3
R0002	Cellulitis	Barnstaple	1994	P1	H1	E1	S2	Sp-R2	M1.3
R2082	Cellulitis	Nottingham	1994	P1	H1	E1	S2	Sp-R2	M1.3
R1020	Infected pressure sore	Croydon	1994	P1	H1	E1	S3	Sp-R3	M1.3
R1301	Septicemia[e]	Gateshead	1994	P1	H1	E1	S3	Sp-R3	M1.3
Serotype M3									
100064			1958	P1	H1	E2	S4	Sp-R4	M3.1
R2594	Septicemia[e]	Nottingham	1993	P1	H1	E2	S5	Sp-R5	M3.2
R0671	Streptococcal gangrene	Exeter	1993	P1	H1	E2	S5	Sp-R5	M3.2
R1806	Puerperal sepsis	London, hospital B	1993	P1	H1	E2	S4	Sp-R4	M3.3
R2461	Gangrenous cellulitis	Southend	1993	P1	H1	E2	S5	Sp-R5	M3.2
R2022	Cellulitis	High Wycombe	1993	P1	H1	E2	S5	Sp-R5	M3.1
R2074	Urinary tract infection, septicemia	Stoke on Trent	1994	P1	H1	E2	S6	Sp-R6	M3.2
R1824	Pneumonia	Belfast	1994	P1	H1	E2	S5	Sp-R5	M3.4
R1308	Fatal TSLS	Canterbury	1994	P1	H1	E2	S4	Sp-R4	M3.2
R1463	Infected eczema	Plymouth	1994	P1	H1	E2	S4	Sp-R4	M3.2
Serotype M5									
100065			Pre-1963	P2	H2	E6	S7	Sp-R7	M5.1
R1024	Fatal endocarditis	Blackburn	1994	P1	H1	E10	S8	Sp-R8	M5.2
R1563	Fatal septicemia[e]	London, hospital C	1994	P2	H1	E1	S2	Sp-R9	M5.3
R0321	TSLS	Kettering	1994	P2	H2	E6	S9	Sp-R10	M5.4
R1390	Pneumonia	London, hospital D	1994	P1	H1	E7	S10	Sp-R11	M5.5
R1111	Cellulitis, intravenous drug user	Lanarkshire	1994	P2	H2	E6	S9	Sp-R10	M5.6
R0145	Puerperal sepsis	High Wycombe	1994	P1	H1	E7	S11	Sp-R12	M5.7
R1479	Cellulitis	Leicester	1994	P2	H1	E6	S12	Sp-R13	M5.8
R1906	Septicemia[e]	Glasgow	1994	P3	H1	E9	S3	Sp-R14	M5.9
R0581	TSLS	Wigan	1994	P1	H1	E2	S4	Sp-R4	M5.10
Serotype M6									
NCTC 8302			1918	P4	H3	E3	S13	Sp-R15	M6.1
R2095	Septicemia[e]	Newcastle	1994	P1	H4	E4	S14	Sp-R16	M6.2
R2544	Septicemia[e]	London, hospital D	1993	P1	H4	E4	S15	Sp-R17	M6.3
R1521	Necrotising fasciitis	Worthing	1994	P1	H4	E4	S14	Sp-R16	M6.2
R1411	Cellulitis, intravenous drug user	Coventry	1994	P1	H4	E4	S14	Sp-R16	M6.2
R1431	Endocarditis	Nottingham	1993	P1	H4	E4	S13	Sp-R18	M6.4
R0828	Septicemia[e]	South Shields	1994	P1	H4	E4	S14	Sp-R16	M6.2
R1006	Septicemia[e]	Bishop Auckland	1994	P1	H4	E4	S14	Sp-R16	M6.2
R1047	Septic arthritis	Burnley	1994	P1	H4	E4	S14	Sp-R16	M6.5
R2094	Septicemia[e]	Ashford	1994	P1	H4	E4	S14	Sp-R16	M6.2
Serotype M11									
100068			Pre-1963	P1	H1	E5	S16	Sp-R19	M11.1
R0152	Septicemia[e]	Newcastle	1994	P1	H1	E5	S17	Sp-R20	M11.2
R1045	Septic arthritis	Burnley	1994	P1	H1	E5	S18	Sp-R21	M11.2
R2666	Abscess, intravenous drug user	Manchester	1994	P1	H1	E5	S18	Sp-R21	M11.2
R1629	Septic arthritis	Truro	1994	P1	H1	E5	S17	Sp-R20	M11.3
R1554	Septicemia[e]	Wolverhampton	1994	P1	H1	E5	S18	Sp-R21	M11.4
R0181	TSLS	Glasgow	1994	P1	H1	E5	S18	Sp-R21	M11.3
R1129	Wound infection	Taunton	1994	P1	H1	E5	S18	Sp-R21	M11.2
R2444	Septicemia[e]	Barnsley	1994	P1	H1	E5	S18	Sp-R21	M11.2
R2342	Chest infection	Manchester	1994	P1	H1	E5	S19	Sp-R22	M11.5
Serotype R28									
NCTC 8308			Pre-1936	P1	H1	E7	S20	Sp-R23	R28.1
R1896	Fatal TSLS	Cambridge	1994	P3	H1	E9	S20	Sp-R24	R28.2
R2719	Septicemia[e]	Audley	1993	P1	H1	E7	S20	Sp-R23	R28.3
R2929	Fatal TSLS	Nottingham	1993	P1	H1	E7	S20	Sp-R23	R28.3
R3007	Septicemia[e]	Stoke on Trent	1993	P1	H1	E7	S10	Sp-R11	R28.4
R0133	Fatal septicemia[e]	York	1993	P1	H1	E7	S20	Sp-R23	R28.5
R1983	Septic arthritis	Norwich	1994	P1	H1	E7	S20	Sp-R23	R28.5
R1713	Cellulitis	Nottingham	1994	P1	H1	E7	S20	Sp-R23	R28.3
R1958	Septicemia[e]	London, hospital E	1994	P1	H1	E8	S19	Sp-R25	R28.6
R0022	Septicemia[e]	London, hospital F	1993	P1	H1	E7	S20	Sp-R23	R28.3

[a] All strains were isolated from blood cultures in the United Kingdom. Data are not applicable to reference strains.
[b] Arabic numbers following the enzyme code (P, *Pvu*II; H, *Hin*dIII; E, *Eco*RI; S, *Sac*I) indicate different patterns of hybridization with the probe.
[c] Macrorestriction profile with *Sma*I.
[d] TSLS, streptococcal toxic shock-like syndrome.
[e] Portal of entry unknown.

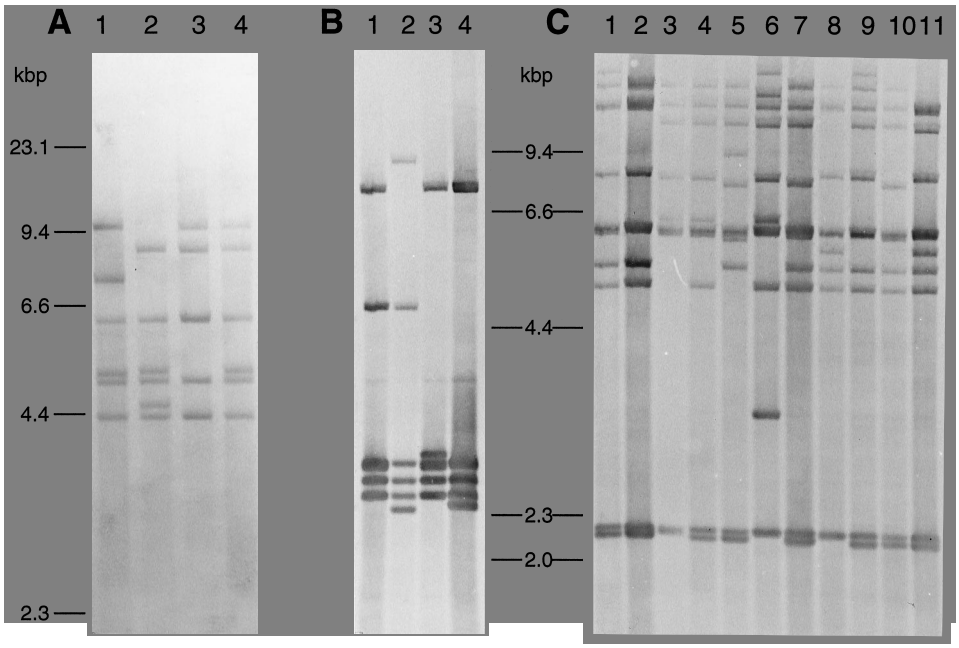

FIG. 2. The 16S ribotypes of *S. pyogenes*. Genomic Southern blots were probed with an intragenic 16S rRNA gene probe as described in Materials and Methods. (A) *Hin*dIII ribotypes. Lanes 1 through 4, ribotypes H1 to H4, respectively. (B) *Pvu*II ribotypes. Lanes 1 through 4, ribotypes P1 through P4, respectively. (C) *Eco*RI ribotypes. Lanes 1 through 9, ribotypes E1 through E9, respectively; lane 10, ribotype E7; lane 11, ribotype E10.

for serotype R28 (0.38) and highest for serotype M5 (0.98). The *D* value for *Sac*I for all six serotypes together was 0.95. Combined ribotypes, designated Sp-R1 to Sp-R25, were derived by combining the 16S gene polymorphisms with all four enzymes (Table 1).

Macrorestriction and PFGE. Since the G+C content of *S. pyogenes* is low (35 to 39 mol%), the following endonucleases whose restriction sites are GC-rich were evaluated for macrorestriction: *Apa*I, *Bgl*I, *Bss*HII, *Eag*I, *Kpn*I, *Nae*I, *Ngo*MI, *Ngo* AIV, *Sac*II, *Sal*I, *Sfi*I, *Sma*I, and *Sst*II. Of these, *Sma*I, which generated 10 to 13 fragments between 40 and 500 kbp in size, yielded macrorestriction patterns (mrps) which were considered to be the most suitable and discriminatory. A protocol was modified for the preparation of DNA from *S. pyogenes* (see Materials and Methods) and gave 100% typeability for all strains in the study. Differential pulse times were used to improve the resolution of fragments between 40 and 150 kbp in size which were found to be characteristic of serotypes M6 and M11 (Fig. 3B).

The discriminatory power of macrorestriction and PFGE was considerable. The six type strains (other than that of serotype M3) had unique mrps (Fig. 3A, lane 2; Fig. 3B, lanes 2 and 8; Fig. 3C, lane 2; Fig. 3D, lane 2). The least discrimination ($D = 0.37$) was found within serotype M1, within which 8 of 10 clinical isolates shared mrp M1.3 (Fig. 3A, lane 4). The greatest discrimination was found within serotype M5, in which each isolate had a unique mrp (Fig. 3C, lanes 2 through 11). Serotypes M1, M3, M6, and M11 were each found to contain characteristic sets of mrps, within which only minor band variations were found (Fig. 3A and B). In each case, one of these mrps, e.g., M1.3, M3.2, M6.2, or M11.2, was the mrp for half or more of the strains of that serotype. *D* values within M6 and M11 were 0.66 and 0.76, respectively. Three of the six R28 mrps (Fig. 3D, lanes 2 through 4) were related and contained 7 of 10 strains, while three unrelated R28 mrps (Fig. 3D, lanes 5 through 7) were found in single strains only, yielding a *D* value of 0.64 within this serotype. None of the 10 M5 mrps had any obvious relationship to those of the other M5 strains, yielding the maximal *D* value of 1.0 within this serotype. These mrps were also unique in the study as a whole.

DISCUSSION

Recent evidence is suggestive of a global upsurge in the number of cases of invasive disease caused by group A streptococci, often with fatal outcomes (11, 12, 25). It is imperative for infection control in the community and in hospitals to establish discriminatory subtyping methods which reproducibly subdivide common M serotypes. Such molecular methods should be cross-referenced to classical (phenotypic) M typing, but they should also be capable of standing alone for the comparison of data between laboratories concerning defined epidemiological situations.

Protocols for the preparation of DNA from group A streptococci commonly use liquid cultures, whose handling may be hazardous. The method reported here for the rapid extraction of genomic DNA from overnight agar plate cultures gave reproducible cell lysis, with a good yield of high-quality genomic DNA. Such minipreparative methods offer significant advantages for molecular epidemiological studies.

The discriminatory power of 16S ribotyping was seen to depend on the choice of restriction enzyme used, and three levels of discrimination were evident, with serotype M5 being an exception to this generalization. Although their use was a feature of earlier studies (3, 21), the least discriminatory enzymes were *Hin*dIII or *Pvu*II: these enzymes generated polymorphisms which either overlapped several M serotypes or were at best only moderately discriminatory within certain serotypes (such as M5 or M6). The second level, that of serotype-specific ribotypes, was found with *Eco*RI: each M serotype except serotype M5 had a distinct set of E ribotypes; for the reasons considered below, serotype M5 was exceptional. At the third level, 16S ribotyping could also be used to subtype isolates within an M serotype. Here, *Sac*I was the only enzyme

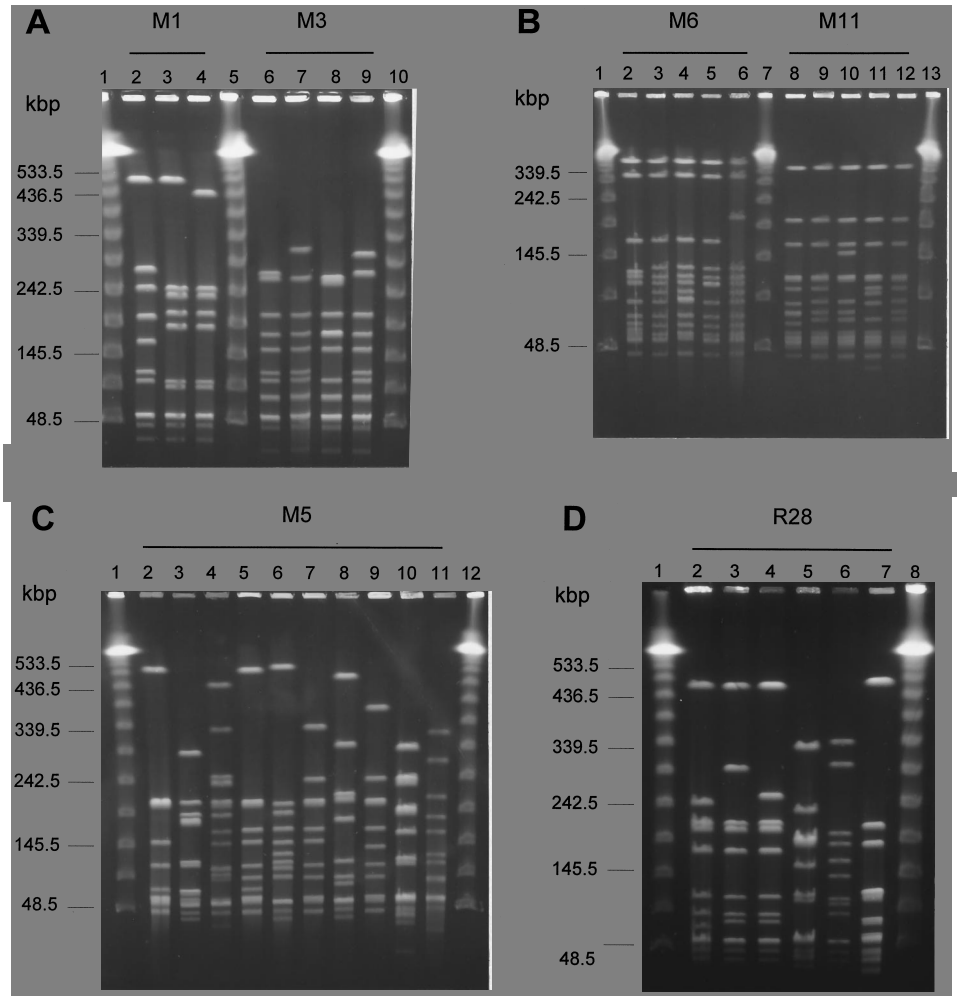

FIG. 3. Macrorestriction profiles of *S. pyogenes* resolved by PFGE. (A) Lanes 1, 5, and 10, molecular mass markers (concatamers of phage lambda); lanes 2 through 4, mrps M1.1 through M1.3, respectively; lanes 6 through 9, mrps M3.1 through M3.4, respectively. (B) Lanes 1, 7, and 13, molecular mass markers; lanes 2 through 6, mrps M6.1 through M6.5, respectively; lanes 8 through 12, mrps M11.1 through M11.5, respectively. (C) Lanes 1 and 12, molecular mass markers; lanes 2 through 11, mrps M5.1 through M5.10, respectively. (D) Lanes 1 and 8, molecular mass markers; lanes 2 through 7, mrps R28.1 through R28.6, respectively.

to yield intraserotype *D* values (varying from 0.38 for R28 to 0.98 for M5). Our results parallel those of Bruneau et al. (4), who recently reported the use of this enzyme for ribotyping pharyngeal isolates. We noted that the large molecular sizes of the *Sac*I bands are consistent with the sites concerned being distant from the rRNA operons themselves, and also that their sizes led to difficulty with resolution by standard agarose gel electrophoresis. Intraserotype *D* values were 0.51 (within serotype M1), 0.53 (within serotypes M6 and R28), 0.64 (within serotypes M3 and M11), and 0.98 (within serotype M5). They did not differ between combined ribotyping analysis with all four enzymes or with the two most discriminatory ones. Hence, *Eco*RI and *Sac*I provide an adequate basis for 16S ribotyping of *S. pyogenes* isolates.

Comparison of M serotypes with 16S ribotypes or with mrps indicated that various degrees of genetic heterogeneity existed within the six serotypes studied. The two methods gave parallel results. Serotype M1 exhibited the least heterogeneity (three combined ribotypes and three mrps). NCTC 8198, the type strain of *S. pyogenes* and the reference strain for M1, was distinct in both combined ribotype and mrp. Our results thus concur with those of earlier studies (5, 21), which attributed clonality to recent isolates of this serotype, which has often been associated with invasive disease. Serotypes M3, M6, and M11 exhibited somewhat more heterogeneity than serotype M1 (three to four combined ribotypes or four to five mrps), but the single-band-shift differences in mrps again suggest a common ancestry of isolates within these serotypes. The significance of minor mrp band shifts is still a matter of interpretation. Maslow et al. (15) consider strains with one or two mrp band shifts consistent with a single genetic event to be clonally related. Nonetheless Baquar et al. (1) showed that outbreak and background strains of *Salmonella* could be reproducibly distinguished by a single mrp band shift, which was stable over many years. Our interpretation of the large numbers of common bands within a serotype (Fig. 3A and B) is that M3, M6, and M11 resemble M1 in having a clonal basis.

Serotype R28, for which four combined ribotypes were found, was distinctive in that 6 of 10 strains appeared to be clonally related by their mrps (Fig. 3D, lanes 2 to 4), while three unrelated mrps were found among the other four strains. Serotype M5 was the most heterogeneous in the study. The 10 strains could be subdivided into nine combined ribotypes or 10 mrps. The latter appeared to be distantly related. These results

are consistent with polyphyletic origins for serotypes M5 and R28.

The *emm* gene cluster lies within a chromosomal virulence regulon consisting of one to three tandemly arranged and related genes, which have been categorized into four subfamilies according to sequence divergences at their 3′ ends. The evolution of *emm* gene diversity is thought to involve intragenic recombination and gene duplication, but evidence that the horizontal spread of *emm* genes could lead to homologous recombination within the cluster has also been provided (2). The vehicle for this is unknown, but it might be a plasmid, conjugative transposon, or bacteriophage. The unusual diversity of our M5 isolates suggests that the horizontal spread of an *emm* gene among genetically diverse clones might account for the heterogeneity observed within isolates of that serotype. This deserves to be further studied. At least one *emm*-like gene from an M5 strain has been shown to have a mosaic structure consisting of segments from *emm*-like genes of different strains (26).

In summary, we have described here methods which can be used for high-resolution genotyping of *S. pyogenes* isolates and have delineated a scheme for subtyping isolates within the M serotypes. The combination of ribotypes with mrps could distinguish four genotypes within 10 M1 strains, five genotypes within M6 strains, six genotypes within M11 and R28 strains, eight genotypes within M3 strains, and one genotype in each of the 10 M5 strains. Such data provide a framework for molecular epidemiological studies as well as tools for investigating the evolutionary genetics of these important pathogenic bacteria.

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