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Clonal diversity of *Streptococcus pyogenes* within some M-types revealed by multilocus enzyme electrophoresis

A. M. HAASE[1], A. MELDER[1], J. D. MATHEWS[1], D. J. KEMP[1] AND M. ADAMS[2]

[1] *Menzies School of Health Research, PO Box 41096, Darwin NT 0811, Australia*
[2] *Evolutionary Biology Unit, South Australian Museum, North Terrace, Adelaide SA 5000, Australia*

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SUMMARY

Twenty-two reference isolates and 30 local isolates of group A Streptococci were classified into 36 electrophoretic types (ET) on the basis of allozyme variation at 27 enzyme loci. Local isolates were characterized by a high frequency of M-non typable strains. M-type and ET were more closely associated in local isolates from an endemically-infected population; nevertheless, amongst the local isolates there were also strains of the same ET type with different M-types. A possible explanation is that genetic exchange between strains may introduce different M-types into strains of defined ET when these are exposed to strong selection in the presence of heavy loads of infection.

In contrast to the reported clustering of strains associated with toxic shock-like syndrome into two closely related ET clones, we found no relationship of ET phenotype to acute poststreptococcal glomerulonephritis or rheumatic fever.

INTRODUCTION

Severe streptococcal infections occur in many countries [1–7]. Important sequelae of streptococcal infections include toxic shock-like syndrome (TSLS), rheumatic fever (RF) and acute poststreptococcal glomerulonephritis (APSGN). APSGN is now rare in many developed countries, but outbreaks are still reported from slums of big cities, and in developing countries [8]. Both rheumatic fever (RF) and outbreaks of APSGN are frequent in children in disadvantaged populations. RF is still prevalent with occasional epidemics, amongst Aboriginal communities in Northern Australia where streptococcal infections are endemic and streptococcal impetigo is particularly frequent [9]. APSGN is more usually epidemic in these communities, and in an environment where streptococcal impetigo is ubiquitous, this strongly suggests that APSGN can be induced only by certain genetic strains of group A streptococci (GAS). However, very little is known about the genetic relationships of GAS with regard to their disease associations.

Strain characterization and studies of disease transmission have almost entirely focused on the analysis of cell surface proteins. The M protein, a major cell surface protein used for serotyping, is one marker for the pathogenic potential of GAS.

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Table 1. *Group A streptococcal strains used in this study*

Strain*§¶	Source	Disease association**	M-type†	ET
PL-7††	Skin, fam 1¶¶	Impetigo	M53/80	1
PL-11††	Fam 4¶¶	Impetigo	M53/80	1
PL-12††	Skin, fam 1¶¶	Hematuria	M53/80	1
PL-13††	Fam 1¶¶	Hematuria	M53/80	1
PL-14††	Fam 1¶¶	Impetigo	M53/80	1
NS 70††	Blood	Renal failure	MNT	2
NS 14††	Blood	—	MNT	3
NS 2††	Throat swab	Tonsilitis	MNT	4
2031*§	SF 130/13	Rheumatic fever/APSGN*	M1	5
PL-1††	Fam 1¶¶	Impetigo	—	6
PL-3††	Fam 1¶¶	Impetigo	M55	6
PL-10††	Fam 3¶¶	Impetigo	M55	6
PL-6††	Fam 5¶¶	Impetigo	MNT	7
NS 54††	Skin	APSGN	MNT	7
Petal*§	B737/71/1 Prague	APSGN*	M49	8
	1/64	—	—	—
PL-5††	Skin, fam 2¶¶	Impetigo	M55	9
PL-8††	Skin, fam 1¶¶	Impetigo	M55	9
2077§	3890-V-Ramkisson	APSGN*	M57	10
NS 22††	Scabies	APSGN	M57	10
NS 38††	Lesion	APSGN	M55	10
2042§	T14/46/6	—	M14	11
NS 10*††	Throat	APSGN	M53	12
2033*§	B930/24	—	M3	13
2097§	100063 Colindale	—	M3	13
Dorothy 55††	Skin	APSGN	M55	14
NS 81††	—	APSGN	M55	15
2073§	Hauson-CV-382	APSGN*	M53	16
Darlene	Sore swab, infected scabies	—	M1	17
Patrick§	—	—	M59	18
2317§	R75/2681 Colindale	APSGN*	M80	19

Epidemiological data suggest that certain M-types are more often associated with APSGN or RF [1, 9–15]. However, some outbreaks of a 'nephritogenic' M-type infection do not result in APSGN. The rarity of repeat attacks of APSGN suggests that a first attack is sufficient to induce immunity to all nephritogenic strains and that disease induction depends on one or more molecules common to all such nephritogenic strains; several candidate molecules have been identified [16–19].

New techniques are needed to provide more understanding of the genetic diversity of GAS and how it relates to disease induction. One such technique, multilocus enzyme electrophoresis, has shown that the majority of streptococcal isolates responsible for TSLS world-wide over the past decade are members of two closely related clones [20]. We describe here the results of a study, using this technique, of the molecular population genetics of streptococcal strains, some of which have been associated with RF or APSGN.

MATERIALS AND METHODS

Bacterial strains

Nineteen reference strains were kindly provided by Dr Diana Martin of the New Zealand Communicable Disease Centre; 30 isolates were taken from patients at

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Table 1. (*cont.*)

Strain*§¶	Source	Disease association**	M-type†	ET
X4276	—	APSGN*	M80	19
2040*§	R53/1077	APSGN*	M12	20
NS 100††	Throat	APSGN	—	21
Phylis§	—	—	M60	22
NS 17††	Blood	Hematuria	—	23
2034*§	SS 241	—	M4	24
2049*§	C98/97	Rheumatic fever*	M24	25
2054§	D24/46	—	M30	25
2051§	J17F/38/13/W2	—	M26	26
2053	—	—	M29	26
PL-4††	Skin, fam 1¶¶	Impetigo	MNT	27
2045§	J17C	—	M18	28
PL-2††	Skin, fam 2	Proteinuria	M3	29
Paul§			M28	30
NS 49††	Skin	TSLS	MNT	31
PL-9††	Skin, fam 3¶¶	Impetigo	MNT	32
2036§	S 24/75/8/W14	Rheumatic fever*	M6	33
2035*§	T5/B/PS	Rheumatic fever*	M5	34
NS 76††	Skin	APSGN	M53/80	35
2072*	10870 Colindale	—		36
2075*	Trinidad-A-75	APSGN*	M55	—
NS 52††	Chorea	—	MNT	—

* M type has been confirmed by DNA sequencing.
§ M type reference strain. Original strain designation is quoted under 'source'.
¶ any names used in strain designation are artificial and do not identify a patient.
† MNT with M-nontypable.
** reported disease association.
†† isolates from the Northern Territory of Australia.
¶¶ numbers stand for families in different households.

the Royal Darwin Hospital and from Aboriginal communities in the Northern Territory of Australia. Samples were usually taken as swabs from skin or throat, or, in cases of invasive disease, isolated from blood or joints (see Table 1). Bacteria were cultured on horse blood agar. Table 1 summarises the 52 isolates used in this study. For ten of the isolates, the M-type was confirmed by DNA sequencing.

Cell extracts

Bacterial cultures were grown from a single colony in 20 ml Todd–Hewitt broth medium for 20 h. Cells were spun down at 7000 rpm for 5 min in a Beckman centrifuge using a JA-20 rotor. Pellets were washed twice in TE buffer and resuspended in an equal pellet volume of 30 mM-Tris pH 7·4, 0·1% mercaptoethanol. Cells were then freeze-thawed twice and sonicated for 90 sec using a Branson sonicator and a microtip.

Multilocus enzyme electrophoresis

Electrophoresis and selective enzyme staining were performed as described [21]. The following enzymes displayed bands of sufficient activity and resolution to permit reliable genetic interpretation: alcohol dehydrogenase (ADH), fructose-bisphosphate aldolase (ALD), diaphorase (DIA), enolase (ENOL), esterase (EST), glyceraldehyde-3-phosphate dehydrogenase (GAPD-1), glyceraldehyde-3-phosphate dehydrogenase, NADP phosphorylating (GAPD-2), lactoylglutathione

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lyase (GLO), glucose-6-phosphate isomerase (GPI), glutathione reductase (GSR), 3-hydroxybutyrate dehydrogenase (HBDH), hexokinase (HK), cytosol aminopeptidase (LAP), malate dehydrogenase (MDH), mannitol-1-phosphate dehydrogenase (MPDH), nucleoside-diphosphate kinase (NDPK), purine-nucleoside phosphorylase (NP, two loci), dipeptidase (PEP-A, two loci), tripeptide aminopeptidase (PEP-B), phosphoglycerate mutase (PGAM), phosphoglucomutase (PGM, two loci), superoxide dismutase (SOD), triose-phosphate isomerase (TPI), and an unidentified kinase.

Distinctive electromorphs of each enzyme were designated a–h in order of increasing anodal migration and were equated with alleles at the corresponding structural gene locus. Three instances were found where an enzyme was encoded by two loci, and in these cases the loci were designated numerically in order of increasing electrophoretic mobility. Each isolate was characterized by its combination of alleles and allocated an electrophoretic type (ET). A dendrogram based on the UPGMA approach [21] was used to summarize the genetic relationships amongst ETs, with equal weighting given to each allozyme polymorphism.

RESULTS

In the 52 strains, 21 of the 27 loci tested were polymorphic. The number of alleles for each locus was between 2 and 8 with an average of 3 loci. Analysis of enzyme allele distribution demonstrated that the strains could be divided into 36 different electrophoretic types (ETs) (Fig. 1). Five isolates fall into ET 1 whereas most other ETs are only represented by 1 or 2 isolates (Table 1). Exceptions are ET 6 and ET 10, each with three isolates; ET 1, ET 6 and ET 10 isolates were all obtained from samples collected recently in the Northern Territory.

Estimates of the degree of genetic relatedness of these isolates are shown in a dendrogram (Fig. 1). The most divergent isolate (45% allozyme difference from other isolates) was 2036, an M6 type reference strain associated with rheumatic fever. Two other clusters of 16 ETs each are 30% apart. Each of these clusters falls into two subclusters at a distance of about 25%. Overall however, there appear to be no obvious subdivisions present, with between-electrotype genetic differences ranging in an almost continuous spread from 4–45%. This conclusion is further reinforced by the results of a parsimony analysis on the data using the computer program PAUP [22], which indicates that amongst the 200 shortest trees, ET 2/ET 3 and ET 14/ET 15 are the only two robust clusters (analysis not shown).

In striking contrast to the clustering of TSLS strains observed previously [20], strains associated with APSGN or RF are dispersed all over the dendrogram. Some strains associated with RF are at least 22% different from others but this is no greater difference than between two strains from APSGN patients. Most notably the M6 reference strain 2036 associated with RF is clearly distinct from all other strains known to be associated with RF.

Table 2 quantifies the relationship of ET-type to M-type. For 47 isolates with complete typing results, there were $47 \times 46/2 = 1081$ possible pairs of isolates (for 47 isolates there are $n(n-1)/2$ possible pairings), of which 20 pairs were of identical ET, 71 pairs identical for M-type, and 16 pairs were identical for both M-type and ET. (If M-non-typable strains are excluded, there are 39 isolates, 741

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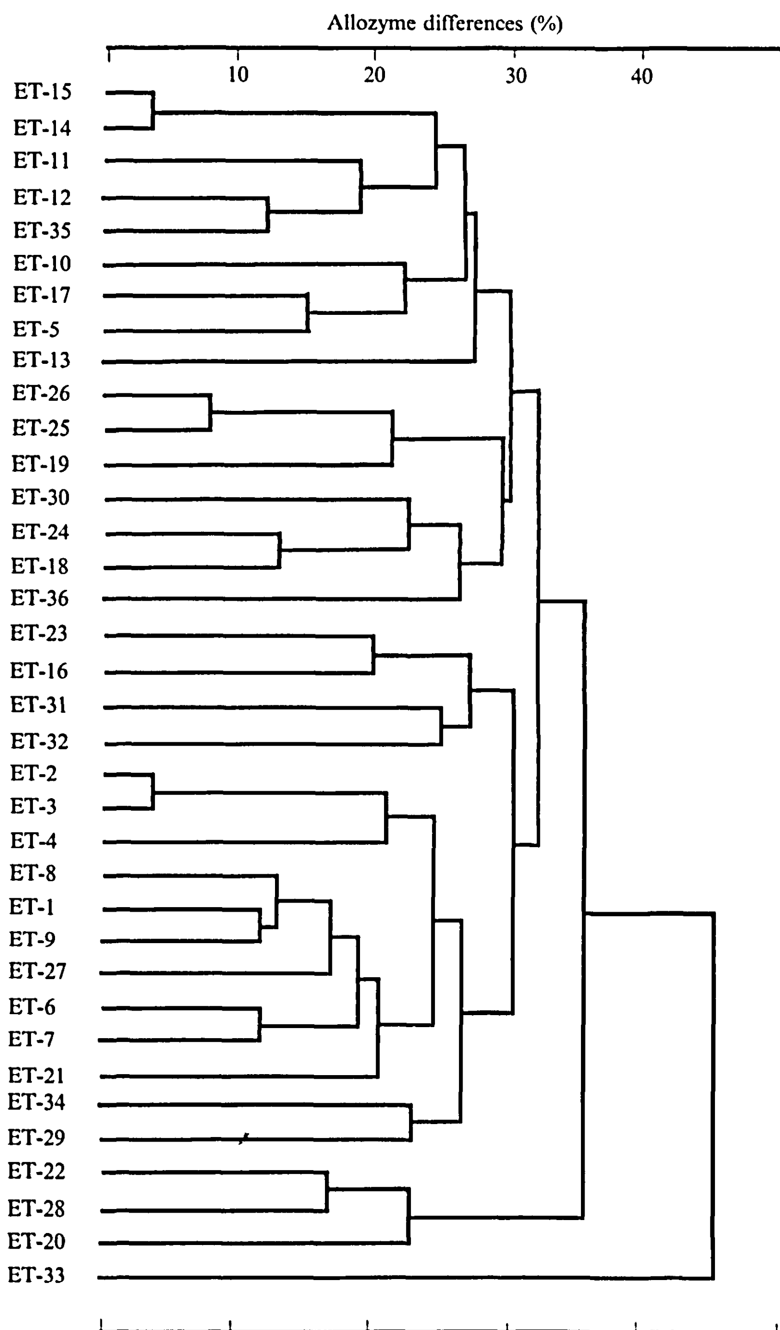

Fig. 1. Dendrogram depicting genetic distance between the isolates. The dendrogram was constructed using data from multilocus enzyme electrophoresis as described in the text. ET numbering is not uniform with the ET numbers in MLEE studies published by others.

possible pairs, with 19 that are of identical ET, 43 of identical M-type, and 15 pairs that are identical for both). Table 2 shows that ET is more predictive of M-type than M-type is predictive of ET. Table 1 shows that the association of M-type and ET is largely due to the local rather than to the reference strains.

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Table 2. *Association of M-type and ET-type across 47 pairs of typed isolates*

	Number of pairs M-type identical	M-type non-identical	Sum
ET identical	16	4	20
ET non identical	55	1006	1061
	71	1010	1081

Observed identical for both = 16.
Expected = 1·31 $P < 0{\cdot}001$.

DISCUSSION

The association between ET's and M-types (Fig. 1 and Table 1) was weaker than in a previous study where M-type was a clear indicator of ET [20]. However in that study strains were pre-selected for a particular phenotype over a relatively short time frame, in contrast to this study of a wider range of reference strains and local isolates. Seven of the eight M-non type able (MNT) isolates were of different ET's, confirming that there must be many distinct MNT isolates.

Our findings (Fig. 1 and Table 1) show that one ET can be represented by two or more strains of different M-type and conversely some strains of the same M-type were of different ET's. Table 2 suggests that ET is more predictive of M-type than M-type is of ET; this could mean that ET diverges more rapidly because of the multiple loci subject to evolutionary change, or it could mean that the same M-type, subject to more selective pressure, can be introduced into different ET backgrounds as a result of genetic exchange between strains. A precedent for such an exchange is the occurrence of M12 from a group A streptococcus in a group G streptococcus [23]. Such exchanges may be common within the sample studied here. For instance strains with M-type 55 occur with ET 6, 9, 10, 14, 15; although ET 14 and 15 are closely related (Fig. 1), the others are quite distant. Similarly M1 occurs with ET 5 and 17, M53 with ET 12 and 16, and M3 strains with ET 13 and 29; the latter two are only very distantly related. All these strains have been isolated from patients in the Northern Territory where there are very heavy loads of streptococcal infection with multiple strains circulating at any one time. In this situation there may be an unusually high rate of immunological selection against GAS, and thus a high rate of recombination.

Less frequently, strains of different M-type have the same ET. For instance ET 10 is associated with strain 2077, an M57 reference strain, NS22, a clinical isolate from the Northern Territory with M57, and NS38, an M55-type clinical isolate from the Northern Territory collected at about the same time as NS22. ET 26 and ET 25 are both represented by two reference strains of different M-types each: M26/M29 and M24/M30 respectively. Strains of M24 have been strongly associated with rheumatic fever in the literature [10]. The other strains have not been reported to be consistently associated with any such sequelae. Yet ET 25 and 26 show only about 8% genetic divergence.

Two separate M55 isolates (Dorothy and NS81) proved to have similar ET's (ET 14 and 15 respectively); both were isolated from APSGN patients (Dorothy in May 1980 and NS81 in June 1992), suggesting that this nephritogenic strain has changed little over 12 years. Earlier findings from Minnesota identified one M-type with epidemics of APSGN 10 years apart [24].

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NS54 (M-non-typable, ET 7) is a local isolate collected around November 1991 from a patient with APSGN, while PL6 (MNT, ET 7) was collected during an outbreak of APSGN in a local Aboriginal community in September 1992. Surprisingly other strains collected concurrently with PL6 had very different ET's, indicating a very high infection load with unrelated strains. For instance although all isolates of ET 1 (PL7, PL12, PL13, PL14) were collected from one family, this family also had strains of ET 6, 9 and 27. Another family yielded ET 9, 6, 29 and 32. Strains typed as ET 1 and 9, are closely related (12% allozyme differences) to Petal 49 (ET 8), a classical isolate causing APSGN. This pattern is in agreement with data demonstrating that TSLS outbreaks are usually caused by two or three distinct clones [25] rather than by a single clone as in the case of meningitis [26]. Further support comes from the results of pulsed field electrophoresis and DNA fingerprinting studies which showed different RFLP patterns for streptococci of the same M-type. These studies suggested that a limited series of streptococcal clones is predominant at any one time and place [27, 28].

These results suggest that the classical serological M-typing may no longer be appropriate for strain classification because there appears to be considerable variation among strains of a particular M-type and M-type is not necessarily indicative of disease association. In this study electrophoretic typing has not usefully categorized streptococcal isolates according to rheumatogenicity or nephritogenicity.

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