

Molecular typing of *Streptococcus pyogenes* from remote Aboriginal communities where rheumatic fever is common and pyoderma is the predominant streptococcal infection

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SUMMARY

Aboriginal Australians in remote communities have high rates of rheumatic heart disease (RHD); yet pharyngitis is reportedly rare whilst pyoderma is common. Some strains of group A streptococci (GAS) have preference for the throat and others for the skin depending on M protein type. A study in three remote communities provided 350 GAS isolates for *emm* sequence typing, 244 were also *emm* pattern typed. There was 100% correlation between *emm* sequence and pattern type. Patterns D and E (non-throat tropic) made up 71% of throat and 87% of skin isolates although patterns A–C (throat tropic) were more common in the throat than the skin (RR 2.3, 95% CI 1.4–3.8) whilst the opposite was found for pattern D (RR 2.2, 95% CI 1.7–3.0). Pattern E favoured the throat (RR 1.4, 95% CI 1.1–1.8). Where environmental factors predispose to skin infection, *emm* pattern types D and E prevail, whatever the recovery site.

INTRODUCTION

The burden of disease due to group A streptococcus (GAS; *Streptococcus pyogenes*) varies widely from place to place and population to population. The relative incidence of GAS pharyngitis appears to be higher in temperate climate regions whilst pyoderma is less common [1–3]. In some tropical settings and minority indigenous populations, GAS skin infection predominates and symptomatic pharyngitis is relatively rare [4–6]. Nevertheless, these populations may still have high rates of acute rheumatic fever (ARF) and rheumatic heart disease (RHD) [7]. Aboriginal Australians in remote tropical communities have

some of the highest reported rates of ARF/RHD in the world [8] and it has been hypothesized that skin infection plays a pathogenic role in ARF in this population [9].

The variable potential of different strains of GAS to cause post-streptococcal sequelae was observed more than 60 years ago [10, 11]. In the 1950s, Rammelkamp and others observed that GAS with specific M serotypes appeared to cause acute post-streptococcal glomerulonephritis (APSGN) and these were not serotypes associated with ARF [12]. ARF was also seen to follow GAS throat infection alone whilst APSGN occurred after either throat or skin infection [13]. This gave rise to the concept of separate skin and throat GAS types.

In the early 1970s, Widdowson and others demonstrated the presence of an M-associated protein that separated GAS into two antigenic groups (I and II)

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matching the known skin and throat types [14]. This was subsequently shown to be due to a cell surface lipoproteinase on skin GAS types that binds fibronectin and causes opacity in horse serum [serum opacity factor (SOF) or streptococcal fibronectin binding protein II – SfbII] [15, 16]. Known rheumatogenic M types were predominantly SOF-negative. In 1989, Bessen and co-workers differentiated two distinct classes of M protein, based on the C repeat region. Class I correlated with SOF-negative strains and contained serotypes associated with outbreaks of ARF, whereas Class II strains were associated with skin tropism [17]. A molecular typing method for GAS was developed in the 1990s based on the nucleotides that determine the variable amino terminus of the M protein on the *emm* gene. M serotypes and *emm* types were shown to be concordant [18, 19]. Subsequently, a polymerase chain reaction (PCR)-based mapping technique further differentiated five patterns of *emm* and related genes; patterns A–C were associated with the throat, pattern D with the skin and pattern E was found in isolates from both throat and skin [20]. With few exceptions, GAS isolates of a given *emm* sequence type (*emmST*) were found to have a predictable *emm* pattern type [21]. An additional genetic marker for skin preference is plasminogen-binding group A streptococcal M protein (PAM) [22] that is found in many GAS isolates with pattern type D; it has also been associated with invasive infection [23].

The relationship between *emmST*, *emm* pattern type and site of GAS infection for the most part appears to hold in studies published from temperate climates and relatively low incidence settings for ARF [20, 24]. Yet, this is not the case for all tropical and high-incidence settings. For example, a study of GAS isolates from a remote Australian Aboriginal island community found that pattern types D and E were predominant, whatever the site of recovery [25]. The study was limited by the paucity of throat isolates (16/141) because GAS throat carriage was so uncommon.

This study characterizes GAS isolates recovered from throat and skin swabs as part of a prospective surveillance project conducted in remote mainland Aboriginal communities with exceptionally high rates of ARF/RHD [26]. The aim was to confirm the relationship between *emmST*, *emm* pattern type, PAM and SOF in these remote settings and to assess whether *emm* pattern type correlates with the site of GAS recovery. Finding a predominance of *emm*

pattern types D and E would also lend support to the skin infection hypothesis in ARF.

MATERIALS AND METHODS

Source of GAS isolates

GAS isolates came from a longitudinal surveillance study of streptococcal skin infection, pharyngitis and throat carriage in three remote mainland Aboriginal communities in the tropical Top End of the Northern Territory, Australia. The communities (1, 2 and 3) were separated by at least 400 km. The aim of the study was to investigate the association of GAS isolates with ARF in these settings [4]. The study protocol was approved by the local ethics committee and informed consent was obtained. Researchers then endeavoured to visit enrolled households on a monthly basis, for 2 years in the case of community 1 and for 1 year each in communities 2 and 3. Each individual was asked about sore throat (in the local language) and each throat was inspected for erythema, pus and tonsillar enlargement. Throat swabs were then taken from every person present according to a throat swab protocol, regardless of throat symptoms. Children and parents were asked about the presence of skin sores before limbs, exposed areas and the trunk were carefully inspected. Pyoderma was defined as one or more pustular, crusted, or vesicular lesions or dry and inflamed skin ulcers >1 cm in diameter. Non-infected insect bites and non-inflamed partly healed pyoderma lesions were excluded [4]. Swabs were taken from any skin sores. Additional clinical and demographic information was also collected.

Because we were unable to obtain blood samples for serological testing, individuals were considered to be carrying GAS in the throat if GAS was recovered from the throat swab and they were asymptomatic at the time.

Recovery and identification of GAS isolates

Culture methods have been described in more detail elsewhere [26]. Plates were incubated at 37 °C in 5% CO₂ and examined after 24 h and 48 h. Large colony-forming beta-haemolytic streptococci (BHS) were subcultured for identification using a Streptococcal Grouping kit (Oxoid Diagnostic Reagents, England, UK). Isolates were then stored in tryptone soya broth (Oxoid) with 15% glycerol at –70 °C.

***emm* sequence type determination**

The procedure for *emm* sequence typing followed that recommended on the Centers for Disease Control and Prevention (CDC) website [27] with minor modifications. We used InstaGene™ matrix (Bio-Rad, Hercules, CA, USA) for DNA sample preparation; 20 colonies were harvested directly from culture plates and re-suspended in 200 µl. All other steps were according to manufacturer's instructions. PCR was conducted in 50 µl reaction volumes with 5 µl of the InstaGene™ preparation, 10 nmol dNTPs, 25 pmol each of primers 1 and 2, 2.5 U *Taq* (Qiagen, Hilden, NRW, Germany), 5 µl 10× *Taq* buffer, sdH₂O to 50 µl. PCR clean-up was done using QIAquick 96 PCR purification kit (Qiagen), eluting in 60 µl sdH₂O.

For the sequencing reaction undiluted BigDye terminator version 3.1 (Applied Biosystems, Foster City, CA, USA) premix in half volume (10 µl) with 2 µl purified PCR product, 12.5 pmol *emmseq2*, 4 µl premix and 3.5 µl sdH₂O was used and the reaction program was as per protocol. Clean up was achieved by adding 10 µl sdH₂O and using DyeEx96 (Qiagen). The product was desiccated and sent to a commercial sequencing facility for separation. For sequence analysis, electropherograms were viewed with DNAstar Seqman software (Madison, WI, USA) and sequences were blasted against the CDC *Streptococcus pyogenes emm* sequence database [27]. Novel *emm* sequences were submitted to the administrator for assignment.

***emm* pattern type determination**

The *emm* pattern type was determined according to the protocol described by Bessen *et al.* [25], with a restricted set of primers as shown in Table 1.

Presence of PAM and SOF

PAM-positive *emmST* were differentiated by identifying sequences with homology to the plasminogen-binding A repeats of PAM within the deduced amino-acid sequence of the translated *emm* sequence data [22, 23]. Information regarding the presence of the SOF phenotype for each *emm* sequence type was obtained from the CDC website and published sources [27, 28].

Data analysis

Data were analysed using STATA 8 (Stata Corporation, College Station, TX, USA). Relative risks (RR) for

Table 1. *Primer set used for emm pattern typing* [25]

Primer set	Approximate size of product (kb)	<i>emm</i> pattern type(s)
UP-2 and SF1-R	1.0–2.5	A–C
SF4-A and G3-R	2.8	D or E
	1.0	E
SF4-A and SF1-R	1.2–1.8	D

emm pattern types were calculated by comparing the proportions for each site of GAS recovery and confidence intervals (CI) were calculated by standard methods.

RESULTS

Surveillance and recovery of GAS isolates

Forty-nine households and 1173 people were enrolled. There were 531 household visits and 4842 consultations. Some of the epidemiological data have been documented in a previous report; symptomatic pharyngitis was extremely rare in study children and pyoderma was common [4]. Three hundred and fifty GAS isolates were available for molecular typing, 222 from the throat and 128 from pyoderma lesions. All isolates were *emm* sequence typed. The first 244 GAS isolates, 150 throat and 94 skin isolates, were also *emm* pattern typed (Fig. 1).

Long-term throat carriage of a specific GAS *emmST* was uncommon and there were only six people in the study who had the same *emmST* recovered from the throat on more than one occasion (providing an extra nine isolates). If GAS of a specific *emmST* was recovered from multiple skin sites from a person on the same day, they were not counted as multiple isolates. However, one child had *emm100* recovered from skin sores for two consecutive months (providing one extra isolate). Otherwise we did not recover GAS from skin sores in a serial manner from anyone else. As a result 10 GAS isolates were removed from the analysis (Fig. 1). There were eight instances where GAS was recovered from the skin and the throat from one individual at the same visit; on four occasions they had a different *emmST* in the throat from that on the skin and four had the same *emmST* at both sites. These were left in the analysis. On 61 occasions more than one person in a household was colonized with a specific *emmST* at the same time or at a subsequent visit.

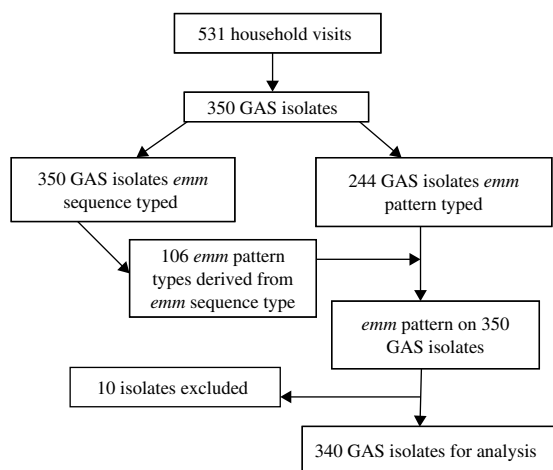


Fig. 1. Flow diagram of GAS isolates: determination of *emm* pattern type.

Correlation of *emm* sequence type, *emm* pattern type and site of recovery

Consistent with published data [21, 25], we found 100% concordance between *emm* sequence type and *emm* pattern type in the first 244 GAS isolates from this study. We were therefore confident about predicting the *emm* pattern type for the remaining 106 GAS isolates where we knew the *emm* sequence type (Fig. 1). Summary data for *emm* sequence type, *emm* pattern type and site of recovery are shown in Table 2. The combined *emm* pattern data for skin and throat isolates are shown in Figure 2. Of 213 isolates recovered from the throat, 61 were pattern types A–C (29%) whilst 16/127 skin isolates were types A–C (13%) (RR for throat 2.3, 95% CI 1.4–3.8). Pattern type D accounted for 67/127 (53%) skin isolates and 51/213 throat isolates (24%) (RR for skin 2.21, 95% CI 1.7–3.0). Pattern type E was also more common in throat isolates (101/213, 47%), than skin isolates (44/127, 35%) (RR for throat 1.4, 95% CI 1.1–1.8). GAS was recovered from the throat of only two people in the study with symptomatic pharyngitis (both adults); the *emm* sequences types were 11 and 87 (both *emm* pattern type E).

Outbreak of *emm55*

The data were skewed by an outbreak during February 2005 when the prevalence of pyoderma in children of community 3 jumped to 18% compared to the median point prevalence of 6.1%. It coincided with the sudden appearance of GAS *emm55* (pattern

Table 2. *emm* sequence types and *emm* pattern types from three remote communities

<i>emm</i> type	No. throat	%	No. skin	%	<i>emm</i> pattern type
1	16	100	—	—	A–C
12	4	100	—	—	A–C
12:1	1	100	—	—	A–C
18:12	1	100	—	—	A–C
19:4	1	50	1	50	A–C
55	33	70	14	30	A–C
stG653.1	5	83	1	17	A–C
33	3	25	9	75	D
42	2	67	1	33	D
53	—	—	2	100	D
56	—	—	1	100	D
71	11	55	9	45	D
81:2	2	67	1	33	D
91	4	29	10	71	D
91:1	2	67	1	33	D
97:1	2	50	2	50	D
98	2	50	2	50	D
99:5	—	—	6	100	D
100	5	38	8	62	D
100:5	—	—	1	100	D
101	2	100	—	—	D
105	4	100	—	—	D
116:1	—	—	2	100	D
123	4	57	3	43	D
stNS1033	8	47	9	53	D
11	16	73	6	27	E
15:1	3	75	1	25	E
13L	—	—	5	100	E
22	13	100	—	—	E
25:1	3	100	—	—	E
44/61	6	55	5	45	E
58	1	100	—	—	E
58:8	8	100	—	—	E
75:1	8	89	1	11	E
76:6	3	43	4	57	E
77	1	100	—	—	E
78	10	100	—	—	E
87	2	50	2	50	E
103	12	63	7	37	E
110	2	67	1	33	E
114:2	2	40	3	60	E
st2904.1	9	60	6	40	E
stNS554	2	40	3	60	E

types A–C) in both skin sores (*n*=5) and throat (*n*=18). The strain had not previously been seen in the study. There was no discernible increase in the incidence of sore throat or proven GAS pharyngitis, but there was a subsequent community outbreak of APSGN [29]. The same *emm* sequence type (*emm55*) then appeared in community 1 during April 2005

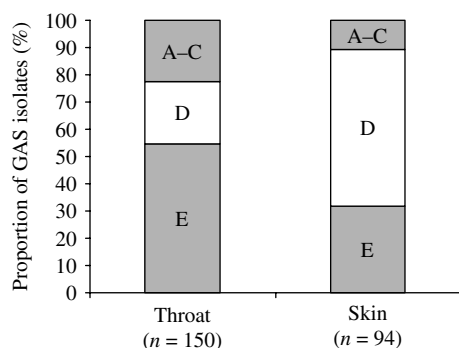


Fig. 2. Percentage of *emm* pattern types by site of recovery.

(skin = 2 and throat = 15); it was not accompanied by a detectable increase in pyoderma or pharyngitis, but it did appear to precipitate another outbreak of APSGN (Northern Territory Centre for Disease Control, Darwin, unpublished data). Up to the time of the appearance of *emm55*, pattern types A–C had accounted for only 12/179 (6.7%, 95% CI 4–11) of GAS isolates. In the first 6 months of 2005, types A–C accounted for 65/162 (40%, 95% CI 33–48) of GAS isolates, 72% (47/65) of those were *emm55*. Overall, this sequence type accounted for 47/61 (77%) of pattern A–C throat isolates and 14/16 (88%) of A–C skin isolates.

Gradation of tissue tropism

Thirteen of the most common *emm* sequence types (with >10 isolates) and site of recovery are shown in Figure 3, together with PAM and SOF patterns. A transition from pattern types A–C, through pattern type E, and then to pattern type D can be seen as the GAS isolates move from throat tropic to more skin tropic. One exception is the outbreak strain, *emm55*. Three of the five most common pattern D isolates were PAM positive and SOF was positive in all six of the common pattern E types. Pattern A–C types (*emm1* and *emm55*) were negative for both PAM and SOF.

DISCUSSION

The findings of this study confirm that *emm* pattern type can be predicted by *emm* sequence type in GAS isolates from these remote communities. This matches the findings of McGregor *et al.* who studied 495 GAS isolates collected from many parts of the world [21] including several tropical countries. The

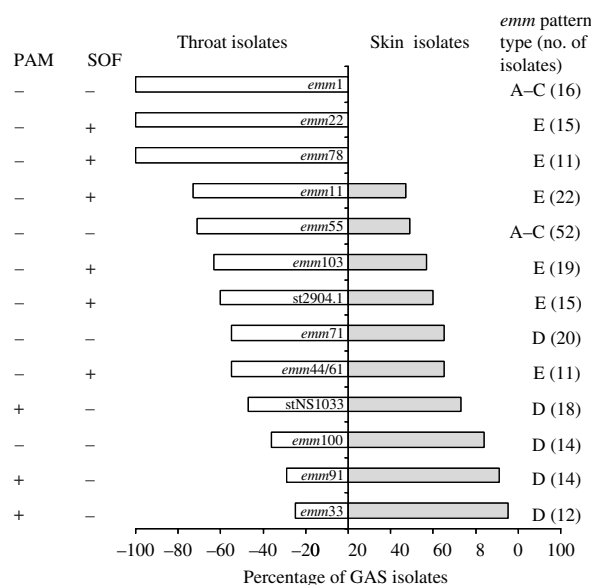


Fig. 3. The most common GAS *emm* sequence types (>10 isolates) showing the percentages recovered from the throat and from the skin together with *emm* pattern type. PAM, Presence of the plasminogen-binding group A streptococcal M protein genotype; SOF, serum opacity factor.

emm gene codes for M protein, a key anti-phagocytic virulence factor that forms the basis of M serological typing and is responsible for evoking serotype-specific host immunity. For the most part, specific *emm* types are clonally related, when compared by multi-locus sequence typing (MLST) using bacterial house-keeping genes, although there are some exceptions where one MLST sequence type is associated with more than one *emm* sequence type [21]. This mainly occurs in skin types (*emm* pattern D) and suggests recombinatorial replacement of *emm* type. Such recombination may be most likely to occur at skin sites, such as in pyoderma, where multiple GAS strains can be present together and accompanied by selective pressure from the host immune response.

The distinction between types associated with throat and skin appears to be most marked in temperate regions of the northern hemisphere. M serotypes or *emm* sequence types corresponding to pattern types A–C or E predominate in throat isolates from the United Kingdom, continental Europe and the United States [2, 24, 30, 31]. By contrast, pyoderma was found to be the dominant manifestation of GAS infection in a remote Aboriginal island community and was mainly attributable to pattern types D (43%) and type E (40%); one reason may be that skin isolates outnumbered throat isolates by 10:1

[25]. A–C was the most abundant pattern type in throat isolates, but the numbers were small with only three *emmST* identified. This study covered a much larger population and produced a high proportion of throat isolates, providing the GAS *emm* pattern type distribution in widely separated mainland Aboriginal communities that was similar to that described in the remote island population. It is reasonable to suggest that this *emm* pattern type distribution is likely to be the norm rather than the exception across the region.

A previous US study of serological response had shown that patients with ARF have an elevated response to the specific class I epitope but little response to class II epitope [32]. In Australian Aboriginal populations, raised levels of antibodies to class I and class II epitopes have not been associated with any specific disease but probably indicate the burden of GAS exposure [33]. In this setting, it has been suggested that the high rates of impetigo in childhood engender non-type specific protective immunity against later throat colonization and pharyngitis. This has yet to be proven.

A similar picture to that seen here has been observed in a study from Ethiopia where 78 different *emm* sequence types were found in 217 GAS isolates [34]. Pattern type E was responsible for 38% of isolates, pattern D for 28% and patterns A–C for 18%. Patterns A–C accounted for 3% of skin isolates and 24% of throat isolates. Pattern D was also responsible for 28% of GAS pharyngitis and 4/7 of the isolates associated with ARF. Likewise pattern types D and E dominate in populations of north and south in India, although the rates of throat and skin infection are markedly different; the south Indian population has high rates of pyoderma and the north Indian has high rates of pharyngitis [35, 36].

Despite prevalence rates of RHD >25/1000 in the three communities of this study (Northern Territory Rheumatic Heart Disease Prevention Program, unpublished data), pyoderma was the outstanding manifestation of GAS infection in children and *emm* pattern types D and E of GAS predominated. This study had a much higher proportion of throat isolates (222/350, 63%) than did a previous study conducted among Aboriginal people living on a remote island (16/141, 11%) [25] and provides a broader view. The data suggest that the background rate of pattern types A–C in the three mainland communities was <7% but the proportion was subsequently inflated

by outbreaks in the two large communities of *emm55* having *emm* pattern type A–C. Ironically, the outbreaks involved GAS skin infections (in community 3), not pharyngitis, and were also accompanied by outbreaks of APSGN rather than ARF. GAS M serotype 55 has previously been associated with similar outbreaks of APSGN in the Top End of the Northern Territory, Trinidad and elsewhere [37–39]. Another *emm* pattern type A–C, M/*emm* 12, has been associated with outbreaks of APSGN [40] and raises additional questions about the specificity of *emm* pattern as a predictor of disease.

It has previously been pointed out that *emm* pattern type is an imperfect marker of tissue tropism and does not preclude infection at other sites [25]. Indeed, the factors that determine tissue preference seem to be complex; they include SOF and PAM for the skin [15, 22] and maltodextrin utilization conferring the ability to survive in saliva for the throat [41]. Much remains a mystery. In this study (Fig. 3), PAM provided some indication of tissue preference; SOF provided none. The three most throat tropic *emmST* were exclusively isolated from the throat, whereas even the most skin tropic *emmST* were able to colonize the throat. Colonization of the throat following skin infection is not uncommon [42]. These results suggest that throat colonization is the default condition and that the ability to cause skin infection is probably due to the presence of one or more skin tropic factors that are absent in throat strains.

Our study reaffirms that where ecological and epidemiological factors predispose to skin infection, as occurs in overcrowded tropical environments with poor hygiene conditions [43], *emm* pattern types D and E prevail and even account for the majority of throat GAS isolates. Pyoderma is likely to remain the predominant GAS infection in remote tropical Australian Aboriginal communities in the absence of effective interventions [43]. There is evidence for extensive genetic recombination between GAS strains and this is more likely to occur where there is a high burden of GAS colonization and infection, especially where more than one strain occupies the same site [44]. Despite this recombination, our study confirms concordance between *emm* sequence type and *emm* pattern type when compared with the published data [5, 21, 25]. We therefore also conclude that *emm* pattern typing in these tropical settings, which is labour intensive and difficult to interpret, offers no useful information in addition to

that provided by *emm* sequence typing when investigating the epidemiology of GAS.

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DECLARATION OF INTEREST

None.

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