Streptococcus pyogenes prtFII, but not *sfbI*, *sfbII* or *fbp54*, is represented more frequently among invasive-disease isolates of tropical Australia

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SUMMARY

Streptococcus pyogenes (group A streptococcus) strains may express several distinct fibronectin-binding proteins (FBPs) which are considered as major streptococcal adhesins. Of the FBPs, SfbI was shown *in vitro* to promote internalization of the bacterium into host cells and has been implicated in persistence. In the tropical Northern Territory, where group A streptococcal infection is common, multiple genotypes of the organism were found among isolates from invasive disease cases and no dominant strains were observed. To determine whether any FBPs is associated with invasive disease propensity of *S. pyogenes*, we have screened streptococcal isolates from bacteraemic and necrotizing fasciitis patients and isolates from uncomplicated infections for genetic endowment of 4 FBPs. No difference was observed in the distribution of *sfbII*, *fbp54* and *sfbI* between the blood isolates and isolates from uncomplicated infection. We conclude that the presence of *sfbI* does not appear to promote invasive diseases, despite its association with persistence. We also show a higher proportion of group A streptococcus strains isolated from invasive disease cases possess *prtFII* when compared to strains isolated from non-invasive disease cases. We suggest that *S. pyogenes* may recruit different FBPs for different purposes.

INTRODUCTION

Among the Aboriginal population living in the Northern Territory of Australia (NT), the incidence and prevalence of streptococcal infection and streptococcal diseases are high [1]. More than 100 different molecular types of *S. pyogenes* strains are circulating in the tropical region of the NT [2]. The incidence of acute rheumatic fever in Aboriginal populations is amongst the highest reported even though the isolation rate of *S. pyogenes* from throat swabs is low [3]. Skin is the major reservoir for *S. pyogenes* in this

population. The rate of streptococcal invasive diseases among the Aboriginal population is five times that of the general population and acute glomerulonephritis is endemic among the Australian Aborigines [1, 4, 5]. Interestingly, invasive *S. pyogenes* diseases in the indigenous population are not due to a dominant clone [4].

Adherence is the first step in colonization of hosts by bacterial pathogens, and bacterial adhesins may be useful vaccine antigens [6]. In *S. pyogenes*, adherence could be mediated by different surface exposed proteins, which include among others, fibronectin binding proteins (FBPs) [7–12]. Four FBPs (SfbI, OF, PrtFII and Fbp54) have been extensively studied.

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Among these, SfbI promotes intracellular invasion of group A streptococcus [13]. SfbI coated latex beads not only adhere to HEp2 cells but are also internalized into these cells, suggesting that SfbI by itself is sufficient for internalization of the bacteria into host cells. In some group A streptococcal strains, OF (opacity factor), a FBP responsible for causing opalescence of serum, was shown to be a major adhesin [8, 14, 15]. FBP54 was shown to be an adhesin for buccal epithelial cells but not for HEp2 cells [7]. Not all group A streptococcal strains have genetic endowment to express all the four FBPs. The differences in the repertoire of FBPs may reflect the propensity of the strains to colonize different tissues and thereby cause different patterns of diseases. To this end, a recent study [16] reported a high prevalence (92 %) of *sfbI* among the persisting *S. pyogenes* strains from the throats of patients with asymptomatic carriage after antibiotic therapy. This observation suggests that SfbI mediated internalization may be responsible for persistence in that population. Indeed using an *in vitro* assay, an association between persistent isolates and a high propensity to internalise bacterial cells was demonstrated [17]. However, a more recent German study [18] did not concur with the above observation of a positive association between sfbI-positive strains and persistence. In the NT, *sfbI* is present in 60 % of the *S. pyogenes* isolates [19]. Furthermore, in contrast to Connecticut [20] where throat is the primary site of infection for invasive diseases, in the NT the primary site of infection for group A streptococcus is the skin [4]. To understand the differences in epidemiology and the role of various FBPs, we have undertaken to screen group A streptococcal strains cultured from blood of patients with bacteraemia and necrotizing fasciitis, and wound and throat swabs for the genetic endowment of the four FBPs. Surprisingly, the results show that endowment for SfbI, which is the only FBP reported to mediate pathogen internalization of host cells, is not associated with invasive diseases nor is it overly represented in throat derived isolates. However, a trend showing a greater proportion of *prtFII*-positivity was observed among strains recovered from the invasive disease cases.

MATERIALS AND METHODS

Strains: Group A streptococcus isolates from patients across the tropical top end of the NT between 1990–8 were characterized by a molecular typing procedure, called vir typing [21]. Vir typing is based on polymerase chain reaction (PCR) and subsequent restriction profiling of the *mga* locus, which comprises genes for M and M-like proteins (*emm*), C5a peptidase (*scpA*) and a regulator (*mga*). The isolates were from (a) blood culture representing 35 bacteraemic and 2 necrotizing fasciitis cases (invasive diseases), and (b) 30 pyoderma and 8 throat infections. The details of the site of isolation, vir types and other characteristics are shown in Table 1. In all, 75 distinct vir types were obtained from 73 subjects. One patient had bacteraemia episodes 11 months apart with 2 distinct vir types (20.1 and 20.2) infecting, and another patient had bacteraemia with vir type 5 isolated from blood culture and 5 days later vir type 12.1 was isolated from skin. In this study, vir types 20.1, 20.2 and 5 were treated as strains from invasive disease cases and vir type 12.1 was treated as a strain from uncomplicated infection. In all, 37 vir types were from the invasive diseases and the remaining 38 were from uncomplicated infections (Table 1). In this study Aborigines account for 73 % of the invasive disease cases.

DNA manipulations and Southern blots: Probes of *sfbI* and *prtFII* were generated by PCR using the following primer and template combinations: for *sfbI*: 5′-TATCAAAATCTTCTAAGTGCTGAG-3′ and 5′-AATGGAACACTAACTTCGGACGGG-3′ with DNA template from JRS145; and for *prtFII*: 5′-GAAGAAAAGCTTCCAGACGAGCAAGG-3′ and 5′-GGAATCTCAGAGTTACTTTCTGGTTC-C-3′ with DNA template from NS1140. Composition of PCR reaction mix was according to Hartas et al. [21] and the cycling conditions were: 95 °C for 15 s, 50 °C (for *sfbI*) or 60 °C (for *prtFII*) for 60 s, and 72 °C for 60 s for 30 cycles. Genomic DNA from group A streptococcus was prepared as described by Chassy [22]. Briefly, cells from overnight cultures (5 ml) were washed in 10 mM Tris, 1 mM EDTA (TE) and resuspended in 300 μl TE. To this suspension, 700 μl of 24 % polyethylene glycol 20 000 in TE was added. After treatment with lysozyme (20 mg/ml, 300 μl), lysates were prepared by the addition of sodium dodecylsulphate (1 % in TE) and incubation at 65 °C for 15 min. The lysates were treated with RNase A (10 mg/ml) for 30 min and proteinase K (20 mg/ml) for 30 min at 37 °C. DNA was extracted by phenol/chloroform and precipitated by ethanol. The DNA pellet was dissolved in TE for further manipulations. The genomic DNA was digested with *SpeI* and Southern blots hybridized with the radio-

Table 1. *Characteristics of NT study isolates*

Isolate	VT	Date*	I/N†	*sfbI*	*prtFII*	OF	Site‡	Isolate	VT	Date*	I/N†	*sfbI*	*prtFII*	OF	Site‡
NS 730	2·2	24/2/96	I	+	+	+	N	NS 282	17·2	5/3/95	N	−	−	−	W
NS 210	34	3/6/94	I	+	+	+	B	NS 836	46	12/5/96	N	−	−	−	W
NS 931	57	13/9/96	I	+	−	−	N	NS 1033	3·22	14/1/97	N	+	−	−	W
NS 192	3·2	12/4/94	I	+	+	+	B	NS 50·1	12·1	22/10/91	N	−	−	−	W
NS 13	24	19/2/91	I	−	+	−	B	NS 179	7·2	22/3/94	N	+	+	+	W
NS 101	33·1	11/12/92	I	+	+	−	B	NS 53	29·1	6/11/91	N	−	+	−	W
NS 1140	101	27/4/97	I	−	+	−	B	NS 265	11	1/3/95	N	−	+	−	W
NS 235	118	14/11/94	I	−	+	−	B	NS 351	22	20/4/95	N	+	−	+	W
NS 1	23	16/7/90	I	−	+	−	B	NS 436	3·3	19/6/95	N	−	+	+	W
NS 178	26	21/3/94	I	−	+	−	B	NS 32	29·2	21/6/91	N	−	−	−	W
NS 43	9	15/8/91	I	−	−	+	B	NS 1096	32	24/3/97	N	−	−	+	W
NS 88·2	17·4	29/7/92	I	−	−	−	B	NS 506	134	22/8/95	N	+	+	−	W
NS 125	20·1	29/6/93	I	−	+	−	B	NS 1045	117	21/1/97	N	+	−	+	W
NS 240	116	30/1/95	I	+	+	+	B	NS 564	119	2/10/95	N	+	+	−	W
NS 225	115	13/10/94	I	−	+	−	B	NS 581	42	13/10/95	N	−	+	−	W
NS 90	52	19/8/92	I	+	+	−	B	NS 611	3·7	19/11/95	N	+	−	−	W
NS 223	4	30/9/94	I	−	+	−	B	NS 671	133	18/12/95	N	−	+	+	W
NS 1133	17·1	29/4/97	I	−	+	+	B	NS 684	132	2/1/96	N	+	+	+	W
NS 83	37·1	29/6/92	I	+	+	+	B	NS 803	131	13/4/96	N	−	+	−	W
NS 50	5	17/10/91	I	−	+	−	B	NS 804	130	15/4/96	N	+	−	−	W
NS 226	14·1	13/10/94	I	+	+	−	B	NS 980	129	12/11/96	N	+	−	+	W
NS 8	25	1/1/90	I	+	+	+	B	NS 1030	77	10/1/97	N	+	+	−	W
NS 80	69	23/6/92	I	−	+	−	B	NS 1095	128	25/3/97	N	+	−	+	W
NS 344	127	15/4/95	I	+	−	−	B	NS 1099	126	1/4/97	N	−	+	−	W
NS 476	39	17/7/95	I	−	+	−	B	NS 1122	65	22/4/97	N	+	−	+	W
NS 6	12·2	1/1/90	I	−	+	−	B	NS 1185	135	7/7/97	N	−	−	−	W
NS 20	3·4	7/5/91	I	+	−	+	B	NS 1210	125	17/8/97	N	+	−	+	W
NS 25	18	12/6/91	I	+	−	−	B	NS 1216	49·1	10/8/97	N	+	−	+	W
NS 66	41·2	17/3/92	I	+	−	+	B	NS 1353	44	7/1/98	N	+	+	−	W
NS 195	120	19/4/94	I	−	+	−	B	NS 180	110	22/3/94	N	−	+	−	T
NS 78	30	23/6/92	I	+	−	+	B	NS 182	60	24/3/94	N	−	+	+	T
NS 204	121	18/5/94	I	+	−	+	B	NS 199	55	5/5/94	N	+	+	+	T
NS 205	20·2	23/5/94	I	−	+	−	B	NS 236	111	16/11/94	N	−	+	+	T
NS 216	122	14/6/94	I	−	−	−	B	NS 672	112	19/12/95	N	+	+	+	T
NS 878	124	1/6/96	I	+	+	+	B	NS 678	113	23/12/95	N	+	−	+	T
NS 909	123	20/8/96	I	+	+	+	B	NS 696	114	16/1/96	N	−	−	−	T
NS 567	38	10/10/95	I	+	−	−	B	NS 1107	33·3	2/4/97	N	+	−	+	T
NS 14	96	26/1/91	N	+	+	+	W								

* Collection date of isolates.
† I and N refer to classification of isolates from invasive and non-invasive cases respectively.
‡ Site or disease from which the organism was isolated: N, necrotizing fasciitis; B, bacteraemia; W, wound skin; T, throat.

labelled probes to screen for *sfbI* and *prtFII* as described earlier [19]. A similar approach to screen for *sfbII* often gave ambiguous results as the hybridization signals were weak, possibly due to sequence variation in *sfbII*. Therefore, for this study the *sfbII* status was inferred using opacity factor assays, which correlates well with the genotype; in our earlier study, 24 of 25 *sfbII* positive strains are OF-positive [19]. *Assay for opacity factor*: This assay was performed as described previously [23]. Briefly, overnight cultures (5 ml) in Todd Hewitt broth containing 2% neopep-

tone were centrifuged and 10 μl of the supernatant mixed with 100 μl horse serum in microtitre plate wells. The plates were then incubated at 37 °C overnight in a humid chamber. One hundred microlitres of saline was mixed with the contents of each well and then each well scored for opalescence.

Screening for fbp54 *by PCR*: Specific primers (5′-CCGGATCCATGATTATGCGTAAATACCTCC-AG-3′ and 5′-CCCTGCAGTGCTTATCAGAAT-AATTTCATAGAGAGAATTTTGGC-3′) were used

(underlined sequence contained restriction sites not present in the *fbp54* gene). PCR conditions employed were as described above with an initial cycle of 94 °C for 2 min, 57 °C for 2 min and 72 °C for 2 min. Amplification consisted for 35 cycles at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1·5 min. The reaction was completed with a final cycle of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 7 min.

RESULTS AND DISCUSSION

Our previous work showed that many commonly circulating group A streptococcal strain in the Northern Territory could cause invasive disease and there is no one dominant strain [4]. Given this observation, the demarcation between strains associated with invasive and uncomplicated infections is less defined. Nevertheless, it is possible that some strains have greater predilection for invasion. Specific virulence determinants that are required by strains to cause invasive diseases are unknown. Given that group A streptococcus may contain up to four genes encoding FBP's (*sfbI*, *sfbII*, *prtFII* and *fbp54*) and that SfbI has been shown to be preferentially associated with persistent cases of infection in one study [16], the carriage of FBP genes by group A streptococcus isolates from non-invasive and invasive diseases was determined.

Since these *S. pyogenes* isolates belong to distinct vir types, they are epidemiologically unrelated isolates. Thirty-seven and 38 distinct vir types represented isolates from invasive and the uncomplicated infections respectively. Overall they comprise about two thirds of reported strains circulating in this region. All isolates were positive for *fbp54* without exception. For OF, 38% of strains from invasive and 50% strains from uncomplicated infections were positive ($P = 0\cdot288$) suggesting that this protein may not have a role in promoting invasive diseases. Since SfbI is sufficient for GAS invasion of host cells, we anticipated that a greater proportion of strains representing isolates from invasive diseases might be positive for this gene than those from uncomplicated infection. Contrary to this expectation, we did not find a difference in the distribution of *sfbI* between the two sets of strains ($P = 0\cdot7342$). This result suggests that SfbI-mediated internalization of host cells is not likely to be a necessary characteristic of group A streptococcus for invasive diseases. This interpretation is consistent with the lack of *sfbI* among M types 1 and 18 that are known to be associated with severe invasive diseases elsewhere [24].

There are a few minor disconcordant observations between this study and our earlier report [19]. Firstly, unlike in the previous study, we found a few (7 strains) OF-positive strains that are sfbI-negative. Nonetheless, a majority of OF-positive strains are sfbI-positive (26 strains). Secondly, of the 32 vir types common to both the studies, 7 strains showed different distributions for sfbI and sfbII/OF. Reasons for this could be due to different isolates representing the same vir types used in these studies. Despite these minor differences, there is overall agreement in the *sfbI* and OF distribution in both the studies.

Forty-five of the 75 NT strains (60%) possess *prtFII*. A larger proportion (70%) is represented among the strains from the blood than among the strains from uncomplicated infections (50%) ($P = 0\cdot073$). Although this is not statistically significant, the trend is important given high endemicity of group A streptococcus infection in this area ('passenger' strain hypothesis, see below).

S. pyogenes may utilize different adhesins for attachment and colonization of different tissues and for invasion. For instance, M protein may mediate adherence to keratinocytes in the epidermis, whereas SfbI may be necessary for adherence of group A streptococcus to pharyngeal cells and Langerhans' cells, a component of the basal layer of the epidermis [25]. Different repertoires of adhesins and their regulators, and the interactions of the adhesins with each other may determine tissue tropism and pathogenicity of *S. pyogenes*. In Table 1, 21% of the strains from uncomplicated infections are from the throat. Comparison of the distribution of the FBP genes between these throat isolates and the wound/skin isolates revealed no significant difference ($P = 1\cdot000$ for *sfbI*; $P = 0\cdot232$ for OF; $P = 0\cdot698$ for *prtFII*). Calculations on the permutations of association of OF, *sfbI* and *prtFII* were also investigated but did not reveal any significant result. These observations suggest that the primary site of infection cannot be attributed to any of the four FBPs.

Several reports describe that a limited number of clones are responsible for group A streptococcal invasive diseases [26–29]. These studies were carried out in non-endemic regions for streptococcal infection where the *per capita* diversity of the organism is likely to be low. Our recent studies with isolates from the tropical NT where the *per capita* diversity of group A streptococcus is high [2] reveal that many strains may

cause invasive disease and that there is no clonal spread of a dominant strain among both Aboriginal and non-Aboriginal residents. This raises questions regarding the identity of common features of the group A streptococcal strains, which render them more invasive. While *prtFII* is present in a greater proportion of invasive vir types, 30 % of the vir types that were isolated from the invasive disease patients did not harbour the gene. This suggests that factors other than PrtFII, such as NAD glycohydrolase [30], may also increase the invasive propensity of group A streptococcus. Alternatively, in a population with a high pathogen load where infection with multiple types is common and where multiple types have been recovered from an impetigo lesion [31], a co-infecting *prtFII*-positive strain might somehow promote a less benign *prtFII*-negative 'passenger' strain to cause invasive disease.

Our finding, that the strains with genetic endowment of *prtFII* are more likely to be recovered from invasive disease cases than strains that lack the gene, has implications for an FBP-based vaccine. The knowledge of distribution of FBP genes among *S. pyogenes* isolates from defined geographical areas may provide a basis for selection of appropriate antigens. Finally, despite inference of an association between SfbI-mediated cellular uptake and persistent infection, our data failed to show greater distribution of *sfbI* among strains from invasive disease cases.

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