MOLECULAR IDENTIFICATION AND TYPING

Virulence regulon polymorphism in group A streptococci revealed by long PCR and implications for epidemiological and evolutionary studies

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A method based on long PCR amplification and restriction endonuclease analysis of the virulence regulon was developed for a rapid (2 days), simple differentiation of group A streptococci. The PCR product size varied from 12.3 kb for serotypes M1 (NCTC 8198) and M12 (NCTC 10085) to 7.8 kb for serotype M6 (NCTC 8302). The fragment patterns formed on *Hae*III digestion of the products were unique and this allowed the differentiation of each of the M-type strains (M1, M3, M4, M5, M6, M11, M12, M28, M76 and M78) studied. Contemporary M1 isolates all gave the same fragment pattern but differed from the prototype strain (NCTC 8198) in not having a 1.25-kb fragment. Isolates of serotypes M1 and M3 each had similar patterns, an indication of their clonality and global dispersion. In contrast, more than one restriction fragment length polymorphism (RFLP) pattern was detected among clinical isolates of serotypes M5, M6, M12, M4, M(R)28 and M78. Two strains that were M-protein non-typable by serological means were provisionally classified as M6 by comparisons of *Hae*III long PCR fragment patterns.

Introduction

Streptococcus pyogenes (Lancefield group A β -haemolytic streptococcus; GAS) is a ubiquitous and versatile human pathogen. Typical clinical presentations include pharyngitis, scarlet fever, impetigo and, more rarely, necrotising fasciitis and myositis. The non-suppurative sequelae, glomerulonephritis and acute rheumatic fever, are leading causes of morbidity and mortality in many areas of the developing world [1, 2]. Recently, a toxic shock-like streptococcal syndrome has been associated with invasive strains [3, 4]. Despite the availability of effective antibiotics and supportive therapy, GAS continues to cause serious invasive diseases with significant mortality [5–8]. Recent publicity generated in response to cases of necrotising fasciitis and myositis also served to focus attention on diseases caused by GAS [9].

The most commonly accepted method of characterisation of streptococcal strains is by serological identification of the T and M proteins [10, 11]. Characterisation may also be accomplished by the detection of serum opacity factor (OF), consistently associated with specific M proteins [12–15]. Absorbed typing antisera against cell-surface M proteins divide GAS into *c.* 100 distinct types. However, because M

antisera are unavailable commercially and their preparation is laborious and costly, M typing has become restricted to a few specialised laboratories. Furthermore, *c.* 10% of strains in the UK are M protein non-typable, either because of diminution or loss of M-protein expression, in-vitro proteinase digestion or the lack of availability of appropriate antisera [6, 7].

To overcome these limitations alternative typing methods have been developed. These have included phenotypic methods such as bacteriophage and bacteriocin typing and multilocus enzyme electrophoresis [16–19]. More recently, several genotypic DNA-based methods have been applied with varying degrees of success. These have included restriction endonuclease analysis, pulsed-field gel electrophoresis (PFGE) and ribotyping [20–23], all of which are technically demanding and time-consuming.

The main virulence determinants of GAS, including M proteins, immunoglobulin Fc-receptors and C5a peptidase, are located within a virulence regulon [24]. The *emm* and *emm*-like genes (*c.* 1.5 kb in size) encoding the M and M-like proteins are flanked (5'–3') by the *vir* (*c.* 1.5 kb) and the *scpA* (*c.* 4.4 kb) genes [25–28].

The application of PCR to the typing of micro-organisms is well documented but there have been few reports of the amplification of DNA targets > 4–5 kb. However, Barnes recently reported the amplification of

DNA up to 45 kb ('long' PCR) with an enhanced proof-reading DNA polymerase [29]. This study presents a protocol for the characterisation and differentiation of GAS that is technically easy to perform and can be done in 2 days, based on PCR amplification of up to 12.3 kb and restriction endonuclease analysis of the virulence regulon.

Materials and methods

Strains

Ten M-type strains, 67 contemporary clinical isolates from different geographical areas within the UK and nine strains that were M-protein non-typable were analysed. Two duplicate strains were included (Table 1). Stock clinical cultures were maintained in blood glycerol broth (16% v/v) at -70°C .

DNA preparation

Bacteria were grown on blood agar at 37°C , in an aerobic atmosphere overnight. The bacteria were harvested and suspended into 1 ml of 0.2 M sodium acetate buffer (pH 6.0) and centrifuged at 7500 *g* for 10 min. The pellet was resuspended in 100 μl of mutanolysin (40 $\mu\text{g}/\text{ml}$ in 980 μl of 1 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.0) and 50 μl of fresh lysozyme (50 mg/ml) and incubated at 37°C for 4 h. Chromosomal DNA was then extracted after lysis with freshly prepared guanidium isothiocyanate reagent [30].

Long PCR amplification of the virulence regulon

Chromosomal DNA (A_{260} c. 500–250 ng) was amplified by PCR with an XLTM PCR kit (Perkin Elmer, Applied Biosystems) with primers designed for the entire virulence regulon (PrimerTM computer program, C. W. Dieffenbach, Department of Surgery and Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA): 5'-CTA AAC CGT ATC TTT GAC GCA CT3' (339–363, nucleotide sequence numbering 720 base pairs upstream from the start of the *vir* gene ORF [26]) and 5'-TTG ATG CTT TTG TAG CTA AAG CAC3' (4189–4213 numbering of the *scpA* gene [27]). Each amplification mixture in sterile thin-walled PCR tubes (Perkin Elmer, Applied Biosystems) comprised template DNA, 12.5 pmol of each primer, 50 μM of each dNTP (dATP, dCTP, dGTP and TTP), 1 $\times$ XL buffer, 0.75 mM Mg (OAc)₂, (Analar, BDH) 2.5% v/v glycerol and *rTth* DNA polymerase 1.5 units. Filter-sterilised (0.22 μm) water was added to a final volume of 50 μl and the reaction was overlaid with 75 μl of mineral oil (Sigma).

Thermal cycling was done on a Perkin Elmer model 480 cyclotherm following a 'hot start' at 80°C . Guidelines for amplification were given by the manufacturers. Two linked programmes were used: firstly, denaturation at 94°C for 10 s and annealing extension at 62°C

for 6.5 min for 20 cycles; secondly, 94°C for 10 s and annealing extension at 62°C for 7.5 min with a 13-s auto-segment extension at each step for 12 cycles. A final step at 72°C for 10 min was used. The aqueous PCR product was removed by the addition of chloroform (100 μl) and stored at 4°C .

DNA restriction endonuclease analysis of the PCR amplified virulence regulon

Approximately 500 ng (5–7 μl) of PCR product was digested with 2 units of restriction endonucleases (Boehringer Mannheim) at 37°C for 1.5 h. The enzymes *Asp*700, *Bam*HI, *Dra*I, *Eco*RI, *Hind*III, *Hpa*I and *Hae*III were used. Twenty μl of digested PCR product was analysed by gel electrophoresis in molecular biology grade agarose (BioRad) 0.8%, w/v in Tris-acetate-EDTA buffer, pH 8.0, at 35 mA for 20 h. The fragment sizes were computed from their migration distances against the reference standards *Hind*III/Lambda bacteriophage DNA and ϕ X 174 replicative form (RF) DNA/*Hae*III fragment markers (BioRad). For schematic representations, fragment patterns were plotted on a log₁₀ scale.

Results

Oligonucleotide primers were designed for long PCR amplification of the entire virulence regulon, including the *vir*, *emm* and *scpA* genes, from reference M types, contemporary isolates and M-protein non-typable strains (Table 1). The largest product size was 12.3 kb from types M1 and M12 and the smallest was 7.8 kb for M type 6 (Fig. 1).

The patterns generated by DNA restriction endonuclease digestion with *Hae*III were unique for each type strain studied (Figs. 2 and 3). The number of fragments produced varied from 10 for type M1 to four for types M3 and M6. Fragment sizes varied from 4.4 kb (M6) to c. 400 bp (M1, M5, M6, M4 and M11; Fig. 3). The mean values (SD) for within-gel errors ($n = 3$) for type M5 (NCTC 100065) were 2.11 (50 bp) kb 1.90 (50), 1.80 (20), 1.20 (50), 1.15 (10) and 0.385 (10 bp) kb for the six products. For strain M12-2681 they were 2.20 (50 bp) kb 2.00 (50), 1.60 (50), 1.30 (50), 1.16 (40), 0.86 (20) kb and 0.58 (20 bp) kb for the seven bands (Fig. 3). Other enzymes specific for A-T rich DNA such as *Asp*700, *Bam*HI, *Dra*I, *Eco*RI, *Hind*III and *Hpa*I were less discriminatory (data not shown). There was no *Eco*RI cutting site within the regulon. All strains examined shared a common 1.15-kb 'core' fragment, the significance of which is not clear (Fig. 3).

The fragment patterns for contemporary clinical isolates from different geographical regions within the UK were examined (Table 1; Fig. 3). Twenty-five distinct

Table 1. Strains of GAS examined and their RFLP pattern on *Hae*III digestion of the virulence regulon

M-type strain	Place of isolation	Disease*	RFLP pattern
M1, NCTC 8198	London	Scarlet fever	1
M1-0002	Barnstable	Cellulitis	1A
M1-0528	Bishop Auckland	Fatal pneumonia	
M1-1037	Kettering	Scarlet fever	
M1-2025	London, hospital A	Cellulitis, TSLS	
M1-2365	Bedford	Fatal TSLS	
M1-3102	Stoke	Pneumonia	
M1-1020	Croydon	Pressure sore	
M1-1301	Gateshead	Septicaemia	
M1-2082	Nottingham	Cellulitis	
M3, NCTC 100064	—	—	3
M3-0671	Exeter	Gangrene	3
M3-1806	London, hospital B	Puerperal sepsis	
M3-2022	High Wycombe	Cellulitis	
M3-2461	Southend	Cellulitis	
M3-2594	Nottingham	Septicaemia	
M3-1463	Plymouth	Infected eczema	
M3-1824	Belfast	Pneumonia	
M3-2074	Stoke-on-Trent	Septicaemia	
M5, NCTC 100065	—	—	5
M5-1024	Blackburn	Fatal endocarditis	5A
M5-1563	London, hospital C	Fatal septicaemia	5B
M5-0321	Kettering	TSLS	5C
M5-1111	Lanarkshire	Cellulitis	
M5-1390	London, hospital D	Pneumonia	5D
M5-0145	High Wycombe	Puerperal sepsis	
M5-1479	Leicester	Cellulitis	5E
M5-1906	Glasgow	Septicaemia	
M5-0581	Wigan	TSLS	5F
M6, NCTC 8302	—	—	6
M6-1006	Bishop Auckland	Septicaemia	6
M6-1411	Coventry	Cellulitis	
M6-2544	London, hospital D	Septicaemia	6A
M6-1521	Worthing	Necrotising fasciitis	
M6-2095	Ashford	Septicaemia	6B
M6-1431	Nottingham	Endocarditis	
M6-0828	South Shields	Septicaemia	12
M12, NCTC 10085	—	—	
M12-0753	Harrogate	Septicaemia	12
M12-1628	Sheffield	Leukaemia/Rash	
M12-2629	Wolverhampton	Septicaemia	
M12-2681 ^b	Cambridge	Septicaemia	
M12-0180	Dundee	None given	
M12-1564	Taunton	None given	12A
M12, NG	Wolverhampton	Septicaemia	
M4, NCTC 8326	—	—	4
M4-1352	Oxford	Endocarditis	4
M4-0115	Reading	None given	
M4-2099	Birmingham	Cellulitis	4A
M4-2591	Crawley	Septicaemia	
M4-0112	Devon	TSLS	
M4-0190	Livingston	Cellulitis	
M4-1785	Portsmouth	Septicaemia	
M4-2737	Devon	Arthritis	4B
M11, NCTC 100068	—	—	11
M11-0152	Newcastle	Septicaemia	11
M11-0181	Glasgow	TSLS	
M11-1129	Taunton	Wound infection	
M11-1554	Wolverhampton	Septicaemia	
M(R)28, NCTC 8308	—	—	28
M(R)28-0133	York	Septicaemia	TSLS
M(R)28-2719	Audley	Septicaemia	
M(R)28-2929	Nottingham	TSLS	

Table 1. (continued)

M-type strain	Place of isolation	Disease*	RFLP pattern
M(R)28-0022	London, hospital F	Septicaemia	28
M(R)28-1713	Nottingham	Cellulitis	
M(R)28-1983	Norwich	Septic arthritis	
M(R)28-0211	Birmingham	Cellulitis	
M(R)28-0215	Devon	TSLS	
M(R)28-0217	Oxford	Septicaemia	
M(R)28-3007	Stockton-on-Tees	Septicaemia	28A
M76, NCTC 12056	—	—	76
M76-0966	Oxford	Septicaemia	76
M76-2592	Nottingham	Pneumonia	
M76-2593	Nottingham	Pneumonia	
M78, NCTC 12058	—	—	78
M78-1131	Clwyd	None given	78A
M78-1636	Harrow	None given	78B
<i>M protein non-typable strains (NT-)</i>			
NT 318	Derry	Pneumonia	6
NT 406	Derry	Pneumonia	
NT 204	Uxbridge	None given	NE
NT 205	Uxbridge	None given	
NT 357	Oxford	None given	NE
NT 358	Oxford	None given	
NT 360	Oxford	None given	
NT 466	London, hospital A	Fatal TSLS	NE
NT 1136	London, hospital B	Not given	NE

TSLS, toxic shock-like syndrome.

*Strains isolated from blood cultures in the UK, 1993–1994.

Fig. 1. Agarose gel analysis of long PCR amplified virulence regulon from representative GAS M type strains. Lanes 1, 7, 13, *Hind*III/lambda bacteriophage DNA marker; 2, M1 NCTC 8198; 3, M3 NCTC 100064; 4, M5 NCTC 100065; 5, M6 NCTC 8302; 6, M12 NCTC 10085; 8, M4 NCTC 8326; 9, M11 NCTC 100068; 10, M(R)28 NCTC 8308; 11, M76 NCTC 12056; 12, M78 NCTC 12058. OF+, Production of serum opacity factor.

restriction fragment length polymorphism (RFLP) patterns were found among the 89 strains studied (Fig. 3). The M-protein non-typable strains gave distinct patterns. The nomenclature used for distinguishing patterns was the M-protein type followed by an RFLP pattern subtype. Thus, RFLP 5 corresponded to M-protein type 5 strains and 5A, 5B, 5C, 5D, 5E, 5F indicated subtype patterns within the M5 strains (Fig. 3).

The M1 isolates each showed identical *Hae*III RFLP profiles, although they differed from the prototype M1 strain (NCTC 8198) in not having a 1.25-kb fragment (Table 1; Fig. 3). Some isolates from a single M type shared identical patterns, including those from types M1, M3, M11 and M76. In contrast, isolates of some types (M5, M6, M12, M4, M(R)28 and M78) gave dissimilar RFLP patterns (Fig. 3). The M5 isolates

Fig. 2. Agarose gel electrophoresis of long PCR amplified virulence regulon from representative GAS type strains digested with the restriction endonuclease *Hae* III. Lanes **1, 8, 14**, *Hind* III/lambda bacteriophage DNA marker; **7, 15**, OX174 RF DNA/*Hae* III marker; **2**, M1 NCTC 8198; **3**, M3 NCT 100064; **4**, M5 NCTC 100065; **5**, M6 NCTC 8302; **6**, M12 NCTC 10085; **9**, M4 NCTC 8326; **10**, M11 NCTC 100068; **11**, M(R)28 NCTC 8308; **12**, M76 NCTC 12056; **13**, M78 NCTC 12058.

gave six RFLP subtypes (5A–5F), whereas those from types M12 and M(R)28 gave only two.

Most (78%) of the M-protein non-typable strains showed unique patterns. Similar profiles were observed for NT 204, NT 205, NT 357, NT 358 and NT 360 (Fig. 3). Two strains, NT 318 and NT 406, had *Hae* III virulence regulon patterns similar to type M6 (Fig. 3).

Discussion

Recent cases of necrotising fasciitis and myositis within the UK have highlighted the need for surveillance of streptococcal infections and for identification and differentiation of strains of GAS [5, 9]. Genotyping by ribotyping and PFGE augments M-protein typing but both are technically complex and time-consuming [21–23].

Fig. 3. See footnote opposite

The antigenically variable M (encoded in the *emm* gene) and M-like (*emm*-L gene) proteins confer resistance to phagocytosis and the binding of immunoglobulins [31]. Transcription of *emm*, *emm*-L and *scpA* have been shown to be regulated by the virulence gene (*vir*), at a locus called the virulence regulon [24, 26, 32–34].

Amplification by PCR with *Taq* DNA polymerase is mostly limited to DNA templates <4–5 kb, due partly to the absence of proof-reading (3'–5' exonuclease) activity in the *Taq* enzyme. PCR primers specific for the entire *vir* regulon combined with a DNA polymerase with proof-reading activity have been used successfully in this study to amplify the entire virulence gene of GAS.

While this work was in progress, Gardiner *et al.* reported the amplification of a 5–6.5-kb fragment of the virulence regulon with primers that did not fully encompass the virulence regulon [35]. The size of the *vir* regulon, excluding spacer sequences between the *vir*-*emm* (or *emm*-like) and *scpA* genes, is estimated at c. 7.5 kb [25–27]. In this report, similar-sized PCR products were observed for types M3 and M6 (Fig. 1). However, in other M types, such as M1 and M12 there were products of up to 12.3 kb (Fig. 1). Products >7.5 kb in size indicated that a regulon was either multi-allelic (two or three *emm* or *emm*-L genes) or had regions of spacer sequence between the three genes.

In this work, RFLP within the virulence regulon for GAS was determined with seven DNA restriction endonucleases. The enzyme of choice, that gave the maximum discrimination among strains, was *Hae*III. To investigate the variation within the regulon of UK predominant M types, clinical isolates were examined (Table 1). Those representing types M1 and M3 had similar RFLP patterns, in support of the general concept of their clonality and global dispersion [18, 36–38]. Furthermore, strains of M-type 11 may also be regarded as clonal within the UK on the basis of this study and PFGE [22]. Recent M1 strains differed from the prototype strain NCTC 8198 in not having a 1.25-kb fragment (Table 1; Fig. 3). It is not clear why this fragment was lost and whether or not it encoded a 'virulence' factor. The RFLP patterns for isolates representing the M5, M6, M12, M4, M(R)28 and M78 subtypes were heterogeneous. This is consistent with previous findings that some populations of invasive GAS are genetically diverse rather than of clonal origin. Similar variation within the same M5 and M(R)28 strains was demonstrated by PFGE [22]. The data presented here, when compared with those from PFGE, suggest that long PCR typing of the virulence regulon from GAS may be more discriminatory. For example, M6 isolates gave closely related profiles on PFGE yet they could be further categorised into subtypes 6A and 6B on the basis of

their virulence gene polymorphism (Table 1; Fig. 3) [22].

Two strains, NT 318 and NT 406, serologically M-protein non-typable, were provisionally identified as M-type 6 by both long PCR and solid-phase *emm* 6 gene-specific oligonucleotide capture probe analysis. The presence of an *emm* gene in serologically M-protein non-typable strains, as detected by the *emm* gene capture probe (N. A. Saunders, unpublished observation), suggests that M-protein antigen expression is either attenuated or suppressed. It is expected that as more M-type strains are examined by long PCR, more M-non-typable GAS may be seen to have a virulence regulon profile characteristic of a particular M type.

The data presented here show that long PCR amplification and *Hae*III digestion of the virulence regulon from GAS can be used for the rapid and technically straightforward differentiation of M-type strains and of clinical isolates.

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