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Clonal structure of invasive *Streptococcus pyogenes* in Northern Scotland

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SUMMARY

We have used molecular techniques to characterize 51 group A streptococci from Scotland and 17 'serious disease' isolates from other countries, in order to establish the clonal structure of invasive *Streptococcus pyogenes* strains circulating between 1986 and 1993. Strains were grouped by restriction endonuclease analysis, pulsed field gel electrophoresis and ribotyping patterns, and were examined for the presence of alleles of the *speA* gene by polymerase chain reaction and DNA sequence analysis. Serious and fatal infections in Scotland were caused by several clones. One clone (9 of 51 strains) was M type 1 and possessed the *speA* gene allele 2. This was the clone previously identified as causing severe infection in the USA. Another clone (5 of 51 strains) was M type 3 and had *speA* gene allele 3. In view of the clear association of more than one clone with severe, invasive and fatal infections, horizontal gene exchange between genotypes merits further investigation.

INTRODUCTION

Reports in the mid 1980s from the United States, Europe and Australia of aggressive fulminant *Streptococcus pyogenes* infections in previously healthy persons have indicated that we are experiencing a resurgence of severe infections and intoxications by this organism [1]. The recently reported cases in Gloucester and elsewhere in the UK resulting in substantial media attention have highlighted the ability of *S. pyogenes* to cause such severe infection. Common features include bacteraemia and septicaemia [2, 3], disseminated intravascular coagulation [4], a toxic shock-like syndrome (TSLS) [5, 6] and severe skin, soft tissue, joint and visceral infections [7, 8]. Mortality rates of 30% have been recorded.

In the USA invasive infections have been predominantly (81%) caused by type M1 and M3 *S. pyogenes*: before 1979 M1 strains were isolated from only 18% of such patients [9]. The association of several M types with serious and invasive infections indicates that invasive potential is not serotype specific. Multilocus enzyme electrophoresis [10] and restriction enzyme analysis [11] have been used

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to study the genotypes of strains causing TSLS and serious sepsis. Of 108 isolates recovered from patients in the USA with TSLS or other invasive diseases nearly half the disease episodes, including more than two thirds of the TSLS cases, were caused by strains of two related multilocus genotypes (ET1 and ET2). Ninety percent of TSLS isolates expressed exotoxin A. Likewise 17 of 19 serious disease isolates examined by restriction enzyme analysis had a characteristic fragment profile (termed the invasive or I profile) and all were positive for the pyrogenic exotoxin A gene, *speA* [11].

These results strongly suggest that a clone – or a group of closely related clones – of *S. pyogenes* expressing exotoxin A is responsible for the majority of cases of serious or invasive infections caused by this bacterium. Most of the strains examined in these studies were isolated from patients in the United States. Isolation of organisms belonging to ET1 and ET2 from Yugoslavian patients with systemic infection and scarlet fever [10], and organisms with the type I restriction profile from Norway and New Zealand [11] suggests that these invasive and virulent strains are geographically widespread.

Forty-seven percent of deaths associated with *S. pyogenes* infections in the United Kingdom in recent years have been caused by M type 1 strains [12]. The close correlation between DNA restriction fragment profile and serotype [13] suggests that these strains may belong to the ET1/ET2/I clone or group of clones. a conclusion strengthened by the work of Musser and colleagues [14].

We report here the results of restriction endonuclease analysis (REA). pulsed field gel electrophoresis (PFGE) and ribotyping to determine directly the genomic relationship of *S. pyogenes* strains with the aim of determining whether a clonal group exists amongst clinically significant isolates and whether strains isolated from cases of invasive infection in Northern Scotland are related to the strains causing serious disease in the USA. The occurrence of the *speA* gene and its allelic variants was also examined.

MATERIALS AND METHODS

Bacterial isolates

Sixty-eight stains of group A streptococci were studied (Table 1). Seventeen serotype M1 *speA* positive strains (GPA 242-258), from cases of systemic disease or severe wound infection, were provided by D. Johnson. University of Minnesota. USA and have been described previously [11]. A letter was sent to all medical microbiology departments in Scotland in March 1992 inviting them to submit GAS strains from cases of invasive infections for molecular studies. Fifty-one isolates were received, including all the strains (15) isolated from blood cultures. or from other sites in cases of serious disease at the Department of Medical Microbiology. Foresterhill, Aberdeen between December 1991 and October 1993. all surviving blood culture isolates (21) made at the Glasgow Royal Infirmary between August 1986 and June 1992, and five strains from a cluster of cases of severe and fatal infections (with two asymptomatic contact strains) that occurred in Nairn in October and November 1986 and one from a fatal case of necrotizing fasciitis in Strathpeffer in 1993. Four of the Nairn cases had severe invasive infections of skin and soft tissues: two had a marked myositis and were fatal.

Nairn is a rural town 15 miles east of Inverness on the Moray coast with a population of 8500, and Strathpeffer (population 1400) is situated 25 miles north west of Inverness.

DNA extraction

Confluent growth from quarter to half of a chocolate agar plate was suspended in 300 μl TE8 (10 mM-Tris, pH 8·0: 10 mM-EDTA). Cell lysis was achieved by the addition of 80 μg Mutanolysin (Sigma; Poole, Dorset, UK), 100 μg Proteinase K (Sigma) and 300 μl lysis mix (10 mM-EDTA, 2% v/v Triton-X 100, 50 mM-Tris, pH 8·0) followed by a 3 h incubation at 37 °C. DNA was extracted once with phenol and again with equal amounts of phenol and chloroform:isoamyl alcohol (24:1) and precipitated at −70 °C for 15 min with absolute alcohol. Air dried pellets were resuspended in 100 μl TE.

M genotyping

M typing was performed as described by Kaufhold and colleagues [15] using oligonucleotide probes specific for M types 1 and 3. DNA (20 μg) was blotted onto a nitrocellulose membrane (Hybond-N, Amersham) and hybridized overnight at 50 °C with digoxygenin (DIG: Boehringer Mannheim, UK, Ltd.) labelled oligonucleotide probes that had been synthesized using an Applied Biosystems 391A DNA synthesiser. The probes correspond to the N-terminal sequences of M protein genes: M type 1 – 5′ TTC TAT AAC TTC CCT AGG ATT ACC ATC ACC 3′; M type 3 – 5′ CAT GTC TAG GAA ACT CTC CAT TAA CAC TCC 3′. Hybridized probe DNA was reacted with lumigen PPD and catalyst from the Boehringer Mannheim DNA DIG-Labelling Kit as per manufacturers instructions and autoradiography was carried out using Kodak X-O MAT S100 film for 3 h at room temperature.

Detection of speA *gene*

Strains were tested for the presence of the *speA* gene by polymerase chain reaction (PCR) amplification of target DNA sequences using the SPA 1 and SPA 2 oligonucleotide primers described by Musser and colleagues [10]. PCR reactions (20 μl) were carried out using 40 ng of target DNA, 0·25 μM of each primer, deoxynucleotide triphosphates (0·2 mM; Boehringer Mannheim, UK), Taq polymerase (0·025 units/μl: Cambio, Cambridge, UK) and Taq polymerase buffer under the following amplification conditions: denaturation at 94 °C for 4 min: 30 cycles of denaturation, annealing and extension at 94 °C (1 min), 55 °C (1 min) and 72 °C (1·5 min) respectively: and a final single cycle of 94 °C (1 min), 55 °C (1 min) and 72 °C (8 min). PCR products were visualized by staining with ethidium bromide (10 mg/ml) following electrophoresis at 100 V for 2 h on 2·0% agarose gels (type II, low EEO: Sigma) in 0·5 × TBE (10 × – per l, 45 mM-Tris base, 45 mM boric acid, 1 mM-Na.EDTA).

Allelic variation of speA *gene*

DNA sequencing of *speA* positive PCR reactions was carried out to determine the distribution of *speA* alleles within the streptococcal populations. The sequenced fragments, of approximately 700 bp in length, contained the variable

Table 1. *Details of molecular characterization of group A streptococci from Scotland and elsewhere*

Isolate number	Date of isolation	Geographic source	Clinical sample	Clinical presentation	M genotype	*speA* PCR	*speA* allele	REA type	Ribotype	PFGE type
Minnesota collection										
(1 strain)	1990	Canada	Lymph node	Not known	1	+	2	1	1	1
(16 strains)	1989 1990	Various	Various	Various	1	+	2	1	1	2
Scottish collection										
GPA 193	02/04/92	Elgin	Ear swab	Not known	1	+	2	1	1	1
GPA 201	01/04/92	Elgin	High vaginal swab	Now known	1	+	2	1	1	1
GPA 196	14/01/92	Aberdeen	Blood	Skin blisters	1	+	2	1	1	2
GPA 202	22/04/92	Aberdeen	Blood	Erysipelas	1	+	2	1	1	2
GPA 211	30/01/92	Aberdeen	Blood	Cellulitis	1	+	2	1	1	2
GPA 212	29/03/92	Aberdeen	Blood	Pyrexia	1	+	2	1	1	2
GPA 262	02/01/93	Aberdeen	Blood	Pyrexia	1	+	2	1	1	2
GPA 269	05/07/93	Aberdeen	Blood	Myositis	1	+	2	1	1	2
GPA 207	00/00/86	Nairn	Necrotic tissue	Necrotising fasciitis	1	+	2	1	1	2
GPA 210	00/00/86	Nairn	Throat	Asymptomatic contact of GPA 205	1	+	2	1	1	2
GPA 211	16/04/93	Strathpeffer	Necrotic tissue	Fatal Necrotising fasciitis	1	+	2	1	1	2
GPA 213	28/05/92	Aberdeen	Sputum	Pneumonia/rash	1	+	2	1	1	2
GPA 271	08/07/93	Aberdeen	Throat	Pharyngitis	3	+	3	2	1	4
GPA 204	00/00/86	Nairn	Blood	Fatal myositis	3	+	3	2	1	4
GPA 209	00/00/86	Nairn	Throat	Asymptomatic contact of GPA 204	3	+	3	2	1	4
GPA 205	00/00/86	Nairn	Blood	Septicaemia	3	+	3	2	1	4
GPA 200	27/12/91	Aberdeen	Blood	Septicaemia	3	+	3	2	1	4
GPA 206	00/00/86	Nairn	Blood	Fatal septicaemia	3	+	3	2	1	4
GPA 226	00/12/89	Glasgow	Blood	Cellulitis	3	+	ND	4	1	17
GPA 238	00/06/92	Glasgow	Blood	Drug abuse	ND*	+	ND	4	1	17

GPA 221	00/11/86	Glasgow	Blood	Post-partum	Non-M1/M3	+	ND	10	2	13
GPA 218	00/04/86	Glasgow	Blood	Obstetric	ND	+	ND	9	6	11
GPA 224	00/05/89	Glasgow	Blood	Lung infection	Non-M1/M3	+	ND	6	7	15
GPA 275	29/06/93	Aberdeen	High vaginal swab	Thrush	ND	—	—	15	2	4
GPA 215	10/06/92	Aberdeen	Wound swab	Wound	ND	—	—	14	2	9
GPA 214	09/06/92	Aberdeen	Post A-V shunt	Not known	1	—	—	18	2	6
GPA 208	00/00/86	Nairn	Necrotic tissue	Necrotising fasciitis	Non-M1/M3	—	—	8	2	5
GPA 241	15/11/92	Aberdeen	Blood	Septic abortion	Non-M1/M3	—	—	3	3	2
GPA 239	22/02/92	Aberdeen	Blood	Thigh abscess	Non-M1/M3	—	—	4	3	3
GPA 261	01/01/93	Aberdeen	Blood	Pyrexia	Non-M1/M3	—	—	5	3	6
GPA 263	08/01/93	Aberdeen	Blood	Jaundice	Non-M1/M3	—	—	5	3	6
GPA 267	03/07/93	Aberdeen	Blood	Post-partum pyrexia	Non-M1/M3	—	—	3	3	7
GPA 264	05/02/93	Aberdeen	Blood	Post-partum pyrexia	Non-M1/M3	—	—	6	4	8
GPA 216	21/06/92	Aberdeen	Blood	Pyrexia	Non-M1/M3	—	—	7	4	9
GPA 276	14/10/93	Aberdeen	Blood	Rash	Non-M1/M3	—	—	6	4	10
GPA 222	00/01/87	Glasgow	Blood	Stroke	1	—	—	1	1	2
GPA 220	00/11/86	Glasgow	Blood	Drug abuse	Non-M1/M3	—	—	17	2	8
GPA 227	00/02/90	Glasgow	Blood	Drug abuse	Non-M1/M3	—	—	4	2	9
GPA 219	00/08/86	Glasgow	Blood	Burns	1	—	—	11	2	12
GPA 223	00/05/87	Glasgow	Blood	Amputation	Non-M1/M3	—	—	2	2	14
GPA 225	00/08/89	Glasgow	Blood	Lung infection	1	—	—	10	2	16
GPA 234	00/11/90	Glasgow	Blood	Burns	Non-M1/M3	—	—	18	2	20
GPA 235	00/11/90	Glasgow	Blood	Burns	Non-M1/M3	—	—	16	2	20
GPA 236	00/01/92	Glasgow	Blood	Burns	Non-M1/M3	—	—	16	2	20
GPA 237	00/04/92	Glasgow	Blood	Burns	Non-M1/M3	—	—	16	2	20
GPA 229	00/04/90	Glasgow	Blood	Venflon site	Non-M1/M3	—	—	12	3	19
GPA 230	00/09/90	Glasgow	Blood	Septic toe	1	—	—	13	4	5
GPA 231	00/10/90	Glasgow	Blood	Wound	1	—	—	13	4	5
GPA 232	00/04/90	Glasgow	Blood	Uterine infection	1	—	—	13	4	5
GPA 233	00/08/90	Glasgow	Blood	Cellulitis	1	—	—	5	5	16
GPA 228	00/03/90	Glasgow	Blood	Surgery	1	—	—	12	8	18

* ND, Not determined.

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region differentiating alleles *speA* 1, 2, 3 and 4 [14]. PCR products were purified using Centricon C-100 columns (Amicon, UK) and approximately 150 ng of DNA sequenced using the following primers and a Taq dideoyx terminator kit as per the manufacturers instructions (ABI, Warrington, UK). Sequencing primers: SPA 1 – 5′ ATG GAA AAC AAT AAA AAA GTA TTG 3′; SPA 2 – 5′ GAA GTC TAC CTA ACA ACC AAG TAA 3′; SPA 3 – 5′ CAA GAA CCG AGA GAT GTC A 3′; SPA 4 – 5′ CCT CCA TAA ATA CAC GCT C 3′. Sequence analysis was performed using an ABI 373A automated DNA sequencer.

Restriction endonuclease analysis and electrophoresis of DNA

Approximately 5 μg of DNA was digested with *Cfo* I (18 h at 37 °C; Boehringer Mannheim) and *Hin*d III (3 h at 37 °C; Boehringer Mannheim) restriction enzymes at 2 U enzyme per μg DNA following the manufacturers instructions.

Following separation of the fragments by electrophoresis at 40 V for 20 h on 0·8% agarose gels (type II, low EEO; Sigma) in 0·5 × TBE. DNA was visualized by staining with ethidium bromide.

Ribotyping

Following electrophoresis of DNA fragments generated by *Hin*d III digestion, the gels were washed and blotted by the methods of Southern [16]. A reverse transcriptase DIG labelled *Escherichia coli* 16 + 23S rRNA probe was hybridized overnight at 68 °C to nitrocellulose membrane (Hybond-N, Amersham) bound DNA and autoradiography carried out using Kodak X-O MAT S100 film for 3 h at room temperature.

Pulsed field gel electrophoresis

Preparation of genomic DNA for PFGE was carried out as described by Single and Martin [17]. DNA was digested at 50 °C for 24 h with 40 U *Sfi* I restriction endonuclease. Digestion reactions were stopped by washing inserts in TE at 4 °C for 1 h. Restriction fragments were separated on 1·0% agarose gels in 0·5 × TBE using a CHEF Mapper (Bio-rad, Hemel Hempstead, UK) and the following run conditions: 19 h and 12 min at 14 °C with an initial switch time of 18·76 s, rising on a linear ramp to 57·48 s at 6 V/cm, with an included angle of 120°. DNA fragments were visualized under short wave UV after staining with ethidium bromide and migration compared with that of a λ 50 kb ladder (Bio-rad).

RESULTS

Table 1 summarizes the clinical details and temporal and geographic characteristics of the strains, and outlines the results of the molecular studies carried out on them.

M genotyping

Sixty-four of the strains were examined for hybridization to M type 1 and M type 3 oligonucleotide probes (Table 1). The strains from the Minnesota collection had previously been classified as M type 1 using standard methods [11] and gave positive reactions with the M type 1 probe validating the genotyping method. Nearly half (21 isolates) of the 51 strains from Scotland were also M type 1 and 7

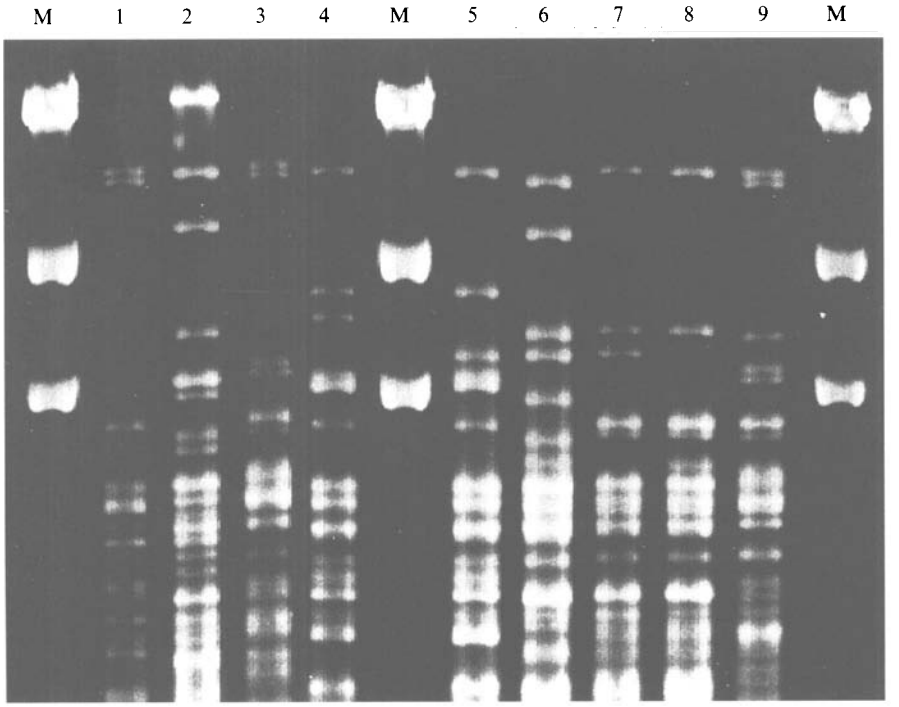

Fig. 1. Restriction patterns of *S. pyogenes* DNA digested with *Cfo* I endonuclease, separated by gel electrophoresis and stained with ethidium bromide. Lane M, *Hin*d III digested λ size standard (Biorad, 23·1 kb, 9·4 kb, 6·6 kb, 4·4 kb); Lane 1, GPA 200; Lane 2, GPA 208; Lane 3, GPA 216; Lane 4, GPA 239; Lane 5, GPA 241; Lane 6, GPA 249*; Lane 7, GPA 261; Lane 8, GPA 218; Lane 9, GPA 264. *Strain from Minnesota collection.

strains were M type 3. Nineteen isolates gave negative reactions with both the M type 1 and 3 probes and were classed as non-M1/M3.

Three of the strains causing severe and fatal infections in Nairn were M type 3, one was M type 1 and another was non-M1/M3. The strain that caused a fatal case of necrotising fasciitis in Strathpeffer in 1993 was genotyped as M1.

Allelic variation of speA *gene*

Amplification of target DNA with *speA* gene primers SPA 1 and SPA 2 resulted in a single PCR product of approximately 700 bp, the expected size. All isolates with *speA* allele 2 and REA type 1 were of M type 1, and those with allele 3 and REA type 2 were M type 3 (Table 1).

Only *speA* alleles 2 and 3 were present in the group of isolates studied, with *speA* 2 predominating. All strains from the Minnesota collection had *speA* allele 2 as did 12 Scottish isolates, including the strain from a case of fatal infection in Strathpeffer. *SpeA* allele 3 was present in six Scottish isolates, including those from the two fatal cases in Nairn.

Restriction endonuclease analysis

Restriction enzyme digestion of extracted DNA with *Cfo* I yielded between 10 and 17 distinguishable bands in the 4·4–23·1 kb size range resulting in the

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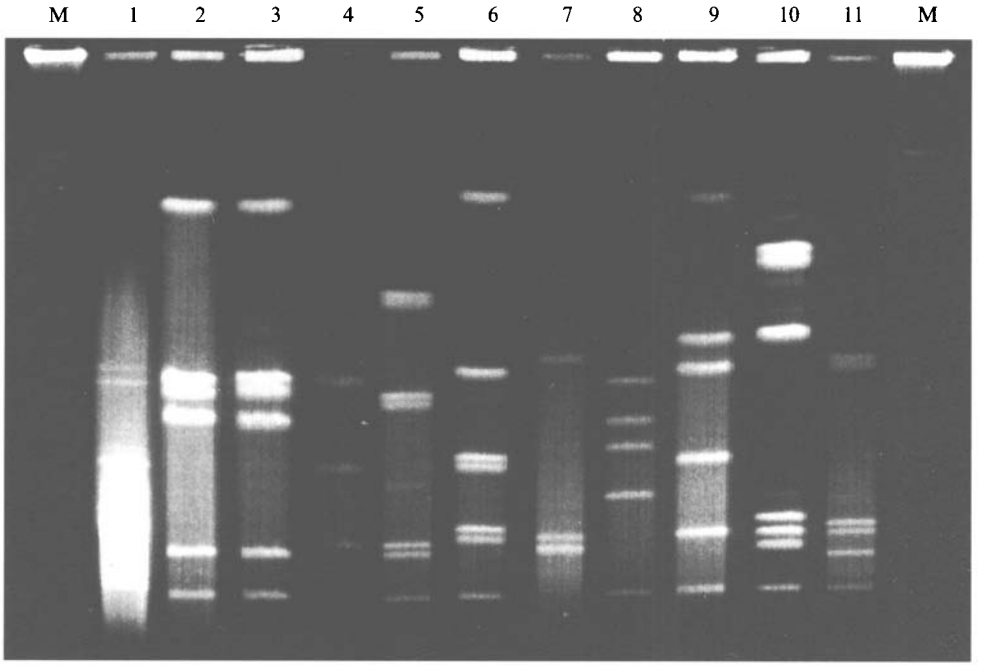

Fig. 2. Pulsed field gel electrophoresis patterns of *S. pyogenes* DNA digested with *Sfi* I endonuclease and stained with ethidium bromide. Lane M, 50 kb Ladder size standard (Biorad; 970 kb to 48·5 kb); Lane 1, GPA 216; Lane 2, GPA 245*; Lane 3, GPA 269; Lane 4, GPA 239; Lane 5, GPA 200; Lane 6, GPA 261; Lane 7, GPA 267; Lane 8, GPA 249*; Lane 9, GPA 264; Lane 10, GPA 276; Lane 11, GPA 208. *Strain from Minnesota collection.

recognition of 18 reproducible profiles (Fig. 1; Table 1). All the Minnesota strains were assigned to type 1. The Scottish isolates formed an heterogeneous group but the type 1 profile was observed for 12 isolates, including a case of necrotizing fasciitis in Nairn (GPA 207) and the fatal case from Strathpeffer (GPA 211). Four of the Nairn strains, including those from the two fatal cases, were found to belong to type 2.

Ribotyping

Eight reproducible and clearly distinguishable ribotypes were identified, each with 8 or 9 bands in the 9·4–2·0 kb size range. All the Minnesota isolates were assigned to type 1 and a large proportion of the Scottish organisms were of type 1 (22 of 51 strains), type 2 (14 of 51 strains) or type 3 (5 of 51 strains; Table 1). With the exception of isolate GPA 208, which was assigned to type 2, all Nairn and Strathpeffer strains displayed the type 1 profile.

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis of genomic DNA cut with *Sfi* I gave 20 distinct banding patterns with 4–7 fragments visible in the 50–750 kb size range (Fig. 2: Table 1). Profile 2 occurred in 16 out of 17 in the Minnesota collection, including those from Canada, New Zealand and Norway, and was observed in 12 out of 51

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Scottish isolates. Isolates causing severe and fatal infections in Nairn and Strathpeffer were of PFGE type 2 (GPA 207, 211), 4 (GPA 204, 206) and 5 (GPA 208).

DISCUSSION

The mid to late 1980s and the early 1990s saw a world-wide resurgence of severe infections caused by *S. pyogenes*. Although the standard M and T typing systems correlate closely with REA groupings of organisms [13], no detailed genomic information on the population structure of *S. pyogenes* can be gleaned from them. Multilocus enzyme electrophoresis (MLEE) and REA studies in the USA have demonstrated that between 50 and 90% of isolates from serious infection have the ET1/ET2/I genotype and are M type 1 [10, 11]. Further work on streptococci from New Zealand, Norway and Yugoslavia suggested that this clone is widespread [10, 11]. It was also proposed that in the USA, development of TSLS is intimately associated with carriage and expression of the *speA* gene [11].

No strong trend in the incidence of serious infection with group A streptococci has been evident in Scotland during the decade 1985–94, the annual mean number of bacteraemias and isolates from CSF and brain abscesses being 59·6, with a median of 58 and a standard deviation of 11·97 (Scottish Centre for Infection and Environmental Health, personal communication). Seventy or more infections of this type were reported in 1986 (75), 1991 (70) and 1993 (80).

The results of our genotyping study on strains isolated from invasive infections during this period showed that the ET1/ET2/I *speA* positive clone was also active in Scotland (10 of 51 strains), being isolated from one of the cluster of cases of serious streptococcal infection in Nairn in 1986, from a fatal infection in Strathpeffer in 1993, from a case of myositis in Aberdeen in 1993, from five blood cultures in Aberdeen in 1992 and 1993 and from two trivial infections in Elgin in 1992. All strains had the genotype of ribotype 1, PFGE type 2 and REA type 1. However, only a minority of the invasive isolates from Northern Scotland belonged to this clone. M type 3 strains with identical genotypes (ribotype 1, PFGE type 4, REA type 2) being isolated from the four remaining cases of severe streptococcal infection in Nairn, including two fatal streptococcal infections (myositis and septicaemia). A strain with identical characteristics was isolated in blood culture in Aberdeen in 1991. The remaining Scottish organisms fell into an heterogeneous group of clones.

Of the 51 Scottish isolates, 23 were positive for the *speA* gene by PCR. Although this technique demonstrates the presence, and not expression, of a gene, the results indicate that nearly half of the isolates from Scotland have the potential to produce streptococcal exotoxin A. All isolates with the *speA* allele 2 were of REA type 1 and, with the exception of one organism (GPA 249), PFGE type 2. Strains with *speA* allele 3 were REA type 2 and PFGE type 4.

Six of the 51 strains from Scotland had been isolated from fatal (3 strains) or serious soft tissue (1 myositis, 3 necrotising fasciitis) infections. All but one had the *speA* gene and were either M type 1, PFGE clone 2 with *speA* allele 2 (2 strains), or M type 3, PFGE clone 4 with allele 3 (3 strains).

The genetic heterogeneity seen in the isolates from Glasgow can be taken to indicate the diversity present in populations of invasive group A streptococci

circulating in Scotland between 1986 and 1992. This genotypic diversity contrasts with our finding that the majority of severe disease syndromes with major tissue destruction during the same period were caused by organisms belonging to only two clones. Further work will be needed to determine the frequency of these clones in the group A streptococcal population as a whole and their association with other clinical syndromes. This work is now in progress.

Our results support the view that a clear association exists between the carriage of the *speA* gene and the ability of a strain to cause serious soft tissue or fatal infections. However, as isolates from several cases of septicaemia in Scotland did not possess the *speA* gene, presence of this gene does not appear to be a necessary prerequisite for invasive disease.

The outcome of the investigation of the strains isolated from the temporal and geographical cluster of severe and fatal cases of infection in Nairn in 1986, which showed that three genotypes were involved, was unexpected. Its statistical improbability underlines the need for studies to test the hypothesis that hitherto undetected important streptococcal virulence factors may be common to both genotypes, perhaps being transferred from genotype to genotype by horizontal gene exchange. It is interesting to note that the only contact between the Nairn cases that could be traced was a social one between a patient with necrotising fasciitis caused by an M type 1, ribotype 1, PFGE 2, REA 1 strain and a fatal case caused by an M3, ribotype 1, PFGE 4, REA 2 strain.

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