Distribution of epidemic antibiotic-resistant pneumococcal clones in Scottish pneumococcal isolates analysed by multilocus sequence typing

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Sequence types of pneumococci isolated in Scotland between 1996 and 2003 were compared with those of globally prevalent antibiotic-resistant clones. Multilocus sequence typing was performed on 252 invasive pneumococcal isolates referred to the Scottish Meningococcus and Pneumococcus Reference Laboratory. Isolates were not preselected for antimicrobial resistance, patient age or disease caused. Sequence types were compared with globally significant antimicrobial-resistant clones identified by the Pneumococcal Molecular Epidemiology Network (PMEN). Sequence types identical with three of the 26 PMEN clones were present in the Scottish collection; the clones were the Spain^{9V}-3 clone (sequence type 156, seven isolates), the England¹⁴-9 clone (sequence type 9, eight isolates) and the Utah^{35B}-24 clone (sequence type 377, one isolate). Many Scottish isolates related to PMEN clones had lower antimicrobial MICs than those described for the corresponding PMEN type strain. A number of single- (SLVs) and double-locus variants (DLVs) were present. Fifteen SLVs related to PMEN sequence types 37, 67, 90, 81, 156, 236 and 377 were detected. The collection contained 10 DLVs related to PMEN sequence types 37, 156, 173 and 338. The majority of SLVs and DLVs were penicillin- or erythromycin-sensitive variants of the resistant PMEN type strains. Capsule switching in isolates related to the PMEN clones was also detected. The highest levels of penicillin resistance were detected in sequence type 320 (serotype 19F), which is not a PMEN clone. These data suggest that PMEN clones are not widely distributed in disease-causing isolates in Scotland.

Received 9 August 2005

Revised 2 September 2005

Accepted 5 September 2005

INTRODUCTION

Since the earliest report of penicillin resistance in pneumococci from Boston, USA, in the 1960s (Kislak *et al.*, 1965), antibiotic resistance in this organism has continued to emerge. Levels of antibiotic resistance have expanded to include a wider range of antimicrobials, and an increased number of affected geographical locations (Klugman, 1990; Schreiber & Jacobs, 1995). DNA-sequencing techniques have led to the identification of pneumococcal antimicrobial-resistant clones. This phenomenon is due to the acquisition

of resistance genes within specific clonal lineages (Munoz *et al.*, 1992). In particular, clones originating from Spain, South Africa, Hungary and the USA have achieved a global distribution (Coffey *et al.*, 1996; McDougal *et al.*, 1995; Smith & Klugman, 1997). The understanding of the epidemiology of the emergence and spread of resistance within the pneumococcal population has been facilitated by the Pneumococcal Molecular Epidemiology Network (PMEN) (McGee *et al.*, 2001). The PMEN system takes advantage of multilocus sequence typing (MLST), a DNA-sequence-based typing method, which uses data from a number of housekeeping genes to provide a sequence type (ST). MLST data are highly discriminatory and electronically portable between laboratories via the internet, making the technique ideal for investigating the clonal spread of antibiotic-resistant pneumococci (Enright & Spratt, 1998a). Criteria

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Abbreviations: DLV, double-locus variant; MLST, multilocus sequence typing; PMEN, Pneumococcal Molecular Epidemiology Network; SLV, single-locus variant; ST, sequence type.

for inclusion of a clone in the PMEN database include widespread geographical distribution, persistence over a number of years, and resistance to one or more antibiotics (McGee *et al.*, 2001).

Previous work (Jefferies *et al.*, 2003) using MLST and serotyping has constructed a phylogenetic framework for a collection of pneumococci isolated in Scotland. We describe an analysis of these isolates in terms of their antimicrobial susceptibility, ST and relationship to the globally significant PMEN clones.

METHODS

Isolates. Isolates were selected from a collection referred to the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) over the period 1996–2003. Isolates for this study ($n=252$) comprised 190 blood isolates, 8 cerebrospinal fluid isolates, 22 eye isolates, 9 ear isolates, 7 sputum isolates, and 16 isolates from miscellaneous sources. Isolates were selected initially on the basis of serogroup to include at least five members where available, and to represent a proportional number from the wide range of serotypes found in Scotland (Denham & Clarke, 2005); they included rare serotypes, and isolates well distributed with respect to geographical location within Scotland, type of disease caused, and age of patient. Isolates were not preselected according to antimicrobial resistance.

Serotyping. Isolates were serotyped by co-agglutination, as described by Smart (1986).

MLST. MLST (Enright & Spratt, 1998a) was performed on *Streptococcus pneumoniae* strains using a semi-automated protocol (Jefferies *et al.*, 2003, 2004). Single-locus variants (SLVs) and double-locus variants (DLVs) are thought to be sufficiently related to be considered members of the same clonal group of the parental ST clone [based upon related sequence types (BURST), <http://eburst.mlst.net/>].

Antimicrobial susceptibility testing. MICs for Scottish and PMEN isolates were determined using Etest strips (AB Biodisk), in accordance with the manufacturer's instructions. Breakpoints for susceptibility were those given by the British Society for Antimicrobial Chemotherapy (<http://www.bsac.org.uk>; penicillin resistance ≥ 2 mg l⁻¹, intermediate resistance 0.12–1.0 mg l⁻¹; erythromycin resistance >1 mg l⁻¹; clindamycin resistance >1 mg l⁻¹; ceftriaxone resistance ≥ 2 mg l⁻¹).

RESULTS

Demographic data

The 252 isolates in our collection are from 39 different serotypes, and they include eight non-typable isolates (Jefferies *et al.*, 2004). The isolates were assigned to 109 STs. The mean age of patients was 45 years (range <1 month to 103 years). The range of penicillin susceptibility was 0.004–2 µg ml⁻¹, with the majority of isolates penicillin susceptible (MIC <0.12 µg ml⁻¹, $n=214$). One isolate, an SLV of the Taiwan^{19F}-14 clone, was resistant to ceftriaxone, and the collection contained just two isolates with a ceftriaxone MIC of 2 µg ml⁻¹. There were 26 erythromycin-resistant isolates, and these were found in STs 9, 90, 143, 156, 180, 814, 860, 271, 285, 320, 377 and 1000.

Isolates with identical STs to PMEN clones

There were 16 isolates with STs identical to three PMEN clones (Spain^{9V}-3, England¹⁴-9 and Utah^{35B}-24) (Table 1). Although members of the Spain^{9V}-3 clone are penicillin resistant, and erythromycin sensitive, all of the seven isolates in our collection with an identical ST to the Spain^{9V}-3 PMEN clone had lower penicillin MICs than the corresponding type strain. One of these isolates was an erythromycin-resistant, penicillin-intermediate variant of the Spain^{9V}-3 clone. Three of the isolates related to the Spain^{9V}-3 clone had different capsular types (serotypes 8 and 14, and a non-typable isolate). In contrast, the eight isolates with an ST identical to the erythromycin-resistant England¹⁴-9 clone were erythromycin resistant, and had an identical serotype to the type strain. The remaining isolate, which had an identical ST and serotype to the penicillin-resistant Utah^{35B}-24 clone, was an erythromycin resistant, but penicillin intermediate, variant of this clone.

SLVs and DLVs related to PMEN clones

Analysis of our 252 isolates for SLVs and DLVs of the PMEN clones (Table 2) revealed seven SLV groups related to seven PMEN clones, and four DLV groups related to four PMEN clones. For 14 of the 15 SLVs of the PMEN clones, the penicillin MIC was less than the corresponding PMEN type strain (intermediate resistance).

Analysis of the PMEN SLVs for erythromycin susceptibility revealed two variants that were erythromycin resistant, and penicillin intermediate or sensitive, one belonging to the Spain^{23F}-1 clone and one to the Spain^{9V}-3 clone. When comparing erythromycin-resistant PMEN clones, only one SLV (related to the Taiwan^{19F}-14 clone) demonstrated high-level erythromycin resistance, although the type strain demonstrates low-level resistance. Many of the SLVs of the PMEN clones demonstrated capsule switching (Table 2). Of interest was the observation that the SLVs occurred at the same loci for a particular clonal group; for example, the *ddl* locus for SLVs of Utah^{35B}-24 and Spain^{9V}-3, and *gki* for SLVs of Spain^{6B}-2.

DLVs were found for four clonal groups (Spain^{9V}-3, Tennessee^{23F}-4, Poland^{23F}-16 and Columbia^{23F}-26). One isolate (ST 176 03-1562) was a DLV of the Poland^{23F}-16 and Columbia^{23F}-26 clones. The DLV isolates related to both the Poland^{23F}-16 clone and the Columbia^{23F}-26 clone, and a DLV related to the Tennessee^{23F}-4 clone demonstrated capsule switching. The two DLV clonal groups related to penicillin-resistant PMEN clones (ST 163 and 176) had lower penicillin MICs than the corresponding PMEN type strain; both of these isolates had different *ddl* loci compared with the corresponding PMEN type strain. For the erythromycin-resistant PMEN clone (Tennessee^{23F}-4), the DLV isolates in our collection were erythromycin-sensitive variants, and all varied from the type strain at the *gki* and *recP* loci.

Table 1. Selected Scottish pneumococci sharing an identical MLST with a representative strain from the PMEN

PEN, penicillin; ERY, erythromycin; CLI, clindamycin; CRO, ceftriaxone. The strain identification number indicates the year of isolation, e.g. 01-3648 was isolated in 2001. Type strains are shown in bold.

Strain	Serotype	MLST	aroE	gdh	gki	recP	spi	xpt	ddl	MIC (mg l ⁻¹)			
										PEN	ERY	CLI	CRO
Spain^{9V}-3	9V	156	7	11	10	1	6	8	1	2	0·12	0·12	1·0
00-1153	9V	156	7	11	10	1	6	8	1	0·38	0·016	0·125	0·5
00-2292	NT	156	7	11	10	1	6	8	1	0·5	0·016	0·125	0·38
00-4477	14	156	7	11	10	1	6	8	1	0·5	0·016	0·19	0·5
01-1752	8	156	7	11	10	1	6	8	1	0·75	0·047	2·0	0·5
00-5049	9V	156	7	11	10	1	6	8	1	0·5	2	0·19	0·5
00-1139	9V	156	7	11	10	1	6	8	1	0·38	0·023	0·125	0·75
01-1668	9V	156	7	11	10	1	6	8	1	0·5	0·023	0·19	0·5
England¹⁴-9	14	9	1	5	4	5	5	1	8	0·03	32	0·12	0·03
03-1138	14	9	1	5	4	5	5	1	8	0·002	12	0·125	0·047
02-1309	14	9	1	5	4	5	5	1	8	0·012	6	0·125	0·08
00-4850	14	9	1	5	4	5	5	1	8	0·006	3	0·094	0·016
03-1600	14	9	1	5	4	5	5	1	8	0·012	2	0·125	0·012
00-3674	14	9	1	5	4	5	5	1	8	0·008	1·5	0·25	0·032
03-3091	14	9	1	5	4	5	5	1	8	0·016	2	0·094	0·012
01-3648	14	9	1	5	4	5	5	1	8	0·016	3	0·125	0·023
03-3121	14	9	1	5	4	5	5	1	8	0·016	6	0·19	0·016
Utah^{35B}-24	35	377	18	12	4	44	14	77	93	2	1	0·25	0·38
00-3991	35	377	18	12	4	44	14	77	93	0·25	2	0·125	0·75

STs of antibiotic-resistant isolates not related to epidemic clones

Analysis of our collection of 252 pneumococci for the presence of resistance to penicillin or erythromycin revealed six isolates not found in the PMEN database (Table 3). Two of these isolates were resistant to penicillin, erythromycin and clindamycin, and belong to a multi-drug-resistant clone isolated in Australia, China and Hong Kong (ST 320). There were four isolates that were erythromycin-resistant variants of previously described clones. All these clones are not assigned to the PMEN database. In addition, we noted a serotype 6 isolate with high-level erythromycin resistance belonging to ST 814; to our knowledge, this is the first description of this ST.

DISCUSSION

This study provides useful information on the spread of international clones within the Scottish pneumococcal population. It is necessary to understand whether antibiotic resistance in the pneumococcal population in Scotland is driven by expansion of the PMEN clones, or the horizontal donation of locally acquired resistance genes. Studies that have focussed on resistant pneumococcal isolates have represented a biased view of the pneumococcal population, and tended to support a clonal relationship amongst resistant isolates. The present study includes a population consisting of both sensitive and intermediate resistant isolates, which is more representative of the pneumococcal

population (although it excludes the carried population), and it suggests that the PMEN clones are not widely distributed amongst disease-causing isolates in Scotland, with many failing to display a fully resistant phenotype. However, it is difficult to obtain a precise picture of the molecular epidemiology of resistance in this cohort of pneumococcal isolates without data on the penicillin-resistance genes.

The incidence of invasive pneumococcal disease in Scotland is similar to that reported in most studies from the USA, Sweden, and other parts of the UK, with serotypes/groups 14, 23, 9, 6 and 19 predominating among pneumococci that cause invasive disease (Kyaw *et al.*, 2000, 2002; Clarke *et al.*, 2003). Of interest is the relatively low prevalence of high-level penicillin resistance among pneumococci in Scotland; over the period 1992–1999, only two isolates were reported with MICs ≥ 2 mg l⁻¹ (0·2% of isolates) (Kyaw *et al.*, 2000). More recent data for the year 2000 found no isolate ($n=360$) with a penicillin MIC > 2 mg l⁻¹ (Clarke *et al.*, 2003). Levels of erythromycin resistance were higher, at 10% over the period 1994–1999, rising to 13% in 2000 (Kyaw *et al.*, 2000; Clarke *et al.*, 2003).

Our collection contained a small number of isolates identical to the Spain^{9V}-3 clone, but some of these displayed different capsular types (non-typable, 8 and 14), and had lower penicillin MICs than the corresponding PMEN type strain; the strains were tested using Etest methodology. Of interest was the sequence changes in the D-alanine,

Table 2. SLVs and DLVs of the PMEN clones in Scotland

Strain	Serotype	MLST	aroE	gdh	gki	recP	spi	xpt	ddl	MIC (mg l ⁻¹)			
										PEN	ERY	CLI	CRO
Spain^{23F}-1	23F	81	4	4	2	4	4	1	1	2	0·06	0·06	0·5
01-4363 (SLV)	19F	285	4	4	2	4	38	1	1	0·38	1·0	0·064	0·38
Spain^{6B}-2	6B	90	5	6	1	2	6	3	4	2	0·12	0·12	1·0
00-2529 (SLV)	6B	96	5	6	39	2	6	3	4	0·5	0·032	0·25	0·38
01-5034 (SLV)	6B	96	5	6	39	2	6	3	4	1·5	0·064	0·094	0·5
01-2517 (SLV)	6B	96	5	6	39	2	6	3	4	0·75	0·023	0·094	0·38
Spain^{9V}-3	9V	156	7	11	10	1	6	8	1	2	0·12	0·12	1·0
03-1599 (SLV)	9V	162	7	11	10	1	6	8	14	0·016	0·064	0·125	0·016
03-3058 (SLV)	9V	162	7	11	10	1	6	8	14	0·012	0·064	0·38	0·012
00-2496 (SLV)	19F	162	7	11	10	1	6	8	14	0·008	0·023	0·064	0·016
00-5092 (SLV)	9V	162	7	11	10	1	6	8	14	0·008	0·032	0·125	0·016
03-3105 (SLV)	19F	162	7	11	10	1	6	8	14	0·032	0·064	0·19	0·125
00-5197 (DLV)	9V	860	40	11	10	1	6	8	14	0·03	2·0	0·094	0·125
03-1184 (DLV)	9V	163	7	11	10	12	6	8	14	0·012	0·094	0·19	0·016
Tennessee^{23F}-4	23F	37	1	8	6	2	6	4	6	0·12	32	0·12	0·016
03-2025 (SLV)	23F	33	1	8	1	2	6	4	6	0·012	0·047	0·19	0·12
00-2683 (DLV)	23	311	1	8	9	1	6	4	6	0·047	0·023	0·125	0·19
00-1225 (DLV)	23F	311	1	8	9	1	6	4	6	0·047	0·023	0·125	0·125
01-3045 (DLV)	23F	311	1	8	9	1	6	4	6	0·064	0·064	0·125	0·19
01-5455 (DLV)	23F	311	1	8	9	1	6	4	6	0·016	0·032	0·064	0·016
03-2042 (DLV)	23F	311	1	8	9	1	6	4	6	0·064	0·064	0·19	0·064
00-1842 (DLV)	3	311	1	8	9	1	6	4	6	0·094	0·032	0·125	0·19
Taiwan^{19F}-14	19F	236	15	16	19	15	6	20	26	2	4	0·12	1·0
02-1201 (SLV)	19F	271	4	16	19	15	6	20	26	1	256	256	2·0
Poland^{23F}-16	23F	173	7	13	8	1	10	6	36	8	>8	0·19	8·0
03-1562 (DLV)	6B	176	7	13	8	6	10	6	14	0·016	0·047	0·125	0·016
Tennessee¹⁴-18	014	67	2	8	7	4	6	1	1	8	>8	>8	8
03-1121 (SLV)	9N	66	2	8	2	4	6	1	1	0·016	0·032	0·125	0·016
00-2050 (SLV)	9V	66	2	8	2	4	6	1	1	0·006	0·016	0·125	0·016
Utah^{35B}-24	35B	377	18	12	4	44	14	77	93	2	1	0·25	0·38
01-5218 (SLV)	29	558	18	12	4	44	14	77	97	0·25	0·064	0·094	0·25
01-4089 (SLV)	35B	558	18	12	4	44	14	77	97	0·19	0·032	0·094	0·38
Columbia^{23F}-26	023F	338	7	13	8	6	1	6	8	0·12	0·06	0·12	0·125
03-1562 (DLV)	6B	176	7	13	8	6	10	6	14	0·016	0·047	0·125	0·016

D-alanine ligase (*ddl*) locus of SLV and DLV variants of the Spain^{9V}-3, Poland^{23F}-16, Utah^{35B}-24 and Columbia^{23F}-26 PMEN clones. The *ddl* gene is known to be closely linked to

the penicillin-binding protein *pbp2b* gene, particular alleles of which confer resistance to β -lactam antibiotics. These changes may account for the differing susceptibilities of

Table 3. Scottish antibiotic-resistant isolates unrelated to PMEN clones

Strain	Serotype	MLST	aroE	gdh	gki	recP	spi	xpt	ddl	MIC (mg l ⁻¹)			
										PEN	ERY	CLI	CRO
01-5062	15	1000	5	12	29	12	9	39	18	0·094	256	256	0·094
00-3709	14	143	7	5	10	18	6	8	1	0·5	256	256	0·75
01-1512	3	180	7	15	2	10	6	1	22	0·064	256	256	0·094
02-2319	19F	320	4	16	19	15	6	20	1	2·0	256	256	0·75
02-2871	19F	320	4	16	19	15	6	20	1	2·0	256	256	0·5
01-4625	6A	814	8	5	4	15	10	20	6	0·047	256	256	0·047

these isolates to β -lactam agents (Beall *et al.*, 2002). The *pbp2b* genes may have been acquired as part of a hitchhiking effect, along with the DNA fragments acquired during alterations in the *ddl* locus (Enright & Spratt, 1998b).

Most of the Scottish erythromycin-resistant isolates related to the PMEN clones were related to the England¹⁴-9 clone, which displays the most frequently identified serotype in Scotland (Kyaw *et al.*, 2000). This clone displays the drug efflux erythromycin-resistance pattern (MIC 2–12.0 mg l⁻¹), which is thought to be due to carriage of the *mefA* gene (Amezaga *et al.*, 2002). Of the erythromycin-resistant isolates not related to the PMEN clones, all demonstrated the high-level erythromycin-resistance phenotype (MLS_B).

A unique feature of the global spread of drug-resistant pneumococci during the last four decades is the emergence of relatively few genetic lineages that have achieved unusually wide geographic spread. The mechanisms of dissemination and successful establishment of these clones are unknown. At present, the incidence of penicillin- and multi-drug-resistant pneumococci is relatively low in Scotland. Our collection of pneumococci from Scotland, which was not selected on the basis of antimicrobial susceptibility, has shown that isolates with an identical genotype to PMEN clones are circulating in the Scottish population; however, many of these isolates do not display the same phenotypic traits of high-level antimicrobial resistance or serotype as the parental clone. Further work is required to elucidate whether future changes in the antibiotic resistance pattern of Scottish pneumococci are driven by the clonal expansion of PMEN clones, and/or horizontal recombination of locally induced resistance genes.

ACKNOWLEDGEMENTS

This study was funded by the Scottish Executive Health Department, Chief Scientist Office. We acknowledge the use of the pneumococcal MLST database, which is located at Imperial College London, and is funded by the Wellcome Trust.

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