Epidemiological relationships among penicillin non-susceptible *Streptococcus pneumoniae* strains recovered in the Czech Republic

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Since 1986, penicillin non-susceptible pneumococci (PNSP) have been found in the Czech Republic. As documented by a nationwide study, the proportion of invasive strains with reduced susceptibility to penicillin has fluctuated around 5% in the past decade. Although the level of resistance to penicillin remains stable, the contribution of different capsular serotypes among the PNSP population varies. Whereas serotype 19A was predominantly associated with penicillin resistance until 1997, serotype 9V became most common among PNSP strains in 1998. In a collection of PNSP strains ($n=225$) isolated from 2000 to 2002, the most frequent serotype was 9V ($n=91$, 40.4%), followed by 19F ($n=30$, 13.3%) and 14 ($n=25$, 11.5%). PFGE and multilocus sequence typing were used to characterize a set of PNSP of the currently predominant serotypes 9V ($n=42$), 14 ($n=15$) and 19F ($n=14$). The Spain^{9V}-3 clone [sequence type (ST) 156] was responsible for a large proportion (100% of serotype 9V strains, $n=42$; 93.3% of serotype 14 strains, $n=14$) of the analysed strains. A representative of the Taiwan^{19F}-14 clone (ST 236) was also recovered in the Czech Republic (a single isolate of serotype 19F). These findings confirm the spread of the major penicillin-resistant clones in the Czech Republic.

INTRODUCTION

The global occurrence of penicillin non-susceptible pneumococci (PNSP) has become a serious problem in the treatment of pneumococcal infections. A steady increase in penicillin resistance and the worldwide spread of penicillin-resistant clones have been documented for the past two decades (Soares *et al.*, 1993; Baquero, 1995; Coffey *et al.*, 1995). In 2003, the mean proportion of PNSP isolates reported through the European Antimicrobial Resistance Surveillance System (EARSS) was 10%; however, a wide variation between countries in the prevalence of PNSP, from <1% in Estonia and Germany, to 36% in Romania, has been observed (European Antimicrobial Resistance Surveillance System, 2003). In the Czech Republic (formerly part of Czechoslovakia until 1992), the first PNSP strains were documented in 1986 (Janečková *et al.*, 1989). Between 1996 and 2002, the prevalence rates of invasive *Streptococcus pneumoniae* strains with reduced susceptibility to penicillin oscillated around 5%, as shown in a nationwide study performed by the National Reference Center for Antibiotics (European Antimicrobial Resistance Surveillance System, 2003; Urbášková *et al.*, 2004). Out of 666 invasive pneumococci collected during this period, only one isolate was

penicillin resistant ($\text{MIC} \geq 2 \mu\text{g ml}^{-1}$), and 25 strains tested penicillin-intermediate ($\text{MIC } 0.125\text{--}1.0 \mu\text{g ml}^{-1}$). From 1992, all consecutive isolates with reduced susceptibility to penicillin from child carriers and children with acute otitis media (AOM) were collected by 31 clinical microbiology laboratories distributed throughout the Czech Republic. From 1992 to 2002, 419 PNSP carriage strains and 236 PNSP strains isolated from middle-ear fluid (MEF) were referred to the National Institute of Public Health. In both of these sets, the proportion of strains highly resistant to penicillin was almost 20%. Although the level of resistance to penicillin has remained stable in the past decade, changes in the contribution of different capsular serotypes in the PNSP population have been noted. Since its first isolation in 1994, serotype 9V has gradually replaced serotype 19A, which was highly prevalent in the Czech Republic until 1997 (Urbášková & Motlová, 1999; Urbášková *et al.*, 2004). Among non-invasive PNSP strains, the proportion of serotype 9V increased from 0% in 1992 to 46.5% in 2002, while that of serotype 19A decreased from 58.5% to 16.3% during the same period. Since 1998, serotype 9V has become predominant among the PNSP serotypes, followed by 19F and 14 (Urbášková *et al.*, 2004).

Only fragmentary data are available about the genetic background of PNSP strains isolated in the Czech Republic to date. PFGE analysis (Sa Figueiredo *et al.*, 1995) of 72 PNSP strains collected from clinical specimens in the Czech and Slovak Republics between 1990 and 1992 showed high clonal

Abbreviations: AOM, acute otitis media; MEF, middle-ear fluid; MLST, multilocus sequence typing; PMEN, Pneumococcal Molecular Epidemiology Network; PNSP, penicillin non-susceptible pneumococci; SLV, single-locus variant; ST, sequence type.

uniformity of highly penicillin-resistant strains of serotypes 14 and 19A. Isolates of serotype 14 belonged to a major clonal group later included in the Pneumococcal Molecular Epidemiology Network (PMEN) as the CSR¹⁴-10 clone (McGee *et al.*, 2001). Isolates of serotype 19A were divided into two subgroups, one of which was indistinguishable from the multiresistant Hungary^{19A}-6 clone, and the other of which was unique: its representative isolates were later accepted in the PMEN as the CSR^{19A}-11 clone (McGee *et al.*, 2001). Low-resistance isolates of serotypes 6A/B, 14, 15B, 19F, 19A and 23F showed a high degree of variation in the chromosomal background, with 20 different PFGE patterns found for 32 isolates. In the late 1990s, clinical isolates of serotypes 9V ($n=10$), 19F ($n=1$) and 23F ($n=7$) referred to Brian Spratt, St Mary's Hospital, London, UK, were identified by multilocus sequence typing (MLST) as members of the Spain^{9V}-3 and Spain^{23F}-1 clones. Also, clonal relatedness of 19A strains ($n=4$) with the Hungary^{19A}-6 clone was proved: they were found to be a single-locus variant (SLV) of this clone (www.mlst.net).

The present study combined the PFGE and MLST methods to characterize PNSP strains of the currently predominant serotypes 9V, 19F and 14 collected in the Czech Republic from 2000 to 2002.

METHODS

Isolates. A collection of 71 PNSP of serotypes 9V ($n=42$), 19F ($n=14$) and 14 ($n=15$), referred to the National Institute of Public Health by 31 microbiology labs in 21 cities between 2000 and 2002, was studied (Table 1). The study isolates were randomly selected to be as heterogeneous as possible in terms of susceptibility to antibiotics and geographical origin. Regarding the site of isolation, three sets of PNSP strains from three national surveillance projects were used: 37 carriage strains from nasopharyngeal swabs of healthy children, 22 isolates from MEF of children aged 5 years and younger, and 12 invasive isolates from blood ($n=10$) and cerebrospinal fluid ($n=2$) of patients regardless of age.

Antibiotic susceptibility testing. MICs of penicillin, erythromycin, clindamycin, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole were determined by the broth microdilution method, as described by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards. To read the results, the CLSI interpretation criteria were used, except for tetracycline, erythromycin and clindamycin, for which the CLSI intermediate values were considered to constitute resistance (National Committee for Clinical Laboratory Standards, 2000).

Serotyping. The strains were serotyped by Quellung reaction, using type and factor sera provided by the Statens Institut, Copenhagen, Denmark (Sorensen, 1993).

PFGE. PFGE of chromosomal DNA was performed as previously described (Soares *et al.*, 1993). Reference strains of epidemic clones Spain^{9V}-3 (ATCC 700671), CSR¹⁴-10 (ATCC 700677) and Taiwan^{19F}-14 (ATCC 700905), kindly provided by Herminia de Lencastre, were used for comparison. Isolates with identical profiles belonged to the same type. Isolates that differed by one to six bands were assigned to different subtypes, and were considered to be members of the same clonal group.

Table 1. Pneumococcal strains

Collection	Time period	No. of laboratories (no. of cities)	No. of strains	No. of PNSP strains (%)	No. of PNSP of serotype 9V (%) / no. of analysed strains (%)	No. of PNSP of serotype 19F (%) / no. of analysed strains (%)	No. of PNSP of serotype 14 (%) / no. of analysed strains (%)
Carriage PNSP isolates from children*	2000–2002	31 (23)	120	120 (100)†	45 (37.5)/22 (48.9)	22 (18.3)/11 (50)	10 (8.3)/4 (40)
PNSP isolates from children* with AOM	2000–2002	29 (23)	85	85 (100)†	30 (35.3)/8 (26.7)	8 (9.4)/3 (37.5)	16 (18.8)/11 (68.7)
All invasive isolates	2000–2002	47 (38)	421	20 (4.7)	16 (80)/12 (75)	0	0
Total			626	225 (35.9)	91 (40.4)/42 (46.1)	30 (13.3)/14 (46.7)	26 (11.5)/15 (57.7)

*Children aged under 6 years.

†Only PNSP isolates were collected.

MLST. The sequences of the ~450 bp internal fragments from the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl* genes were amplified by PCR using primers, as described by Enright & Spratt (1998). Alleles and sequence type (STs) were assigned using the data on *S. pneumoniae* available at the MLST web site (www.mlst.net). Allelic profiles of new STs were electronically submitted to the *S. pneumoniae* MLST database. Isolates with identical allelic profiles or differing in a single locus were considered as closely related; isolates differing in two loci were assumed to be possibly genetically related.

RESULTS AND DISCUSSION

PFGE analysis

Out of the 71 isolates analysed by PFGE, 69 had either identical or related PFGE patterns generated by *Sma*I digestion (Table 2). Comparison of the electrophoretic patterns revealed eight related PFGE subtypes of this dominant clone, assigned as clone A. PFGE subtypes A1, A5 and A8 were shared by the pairs of serotypes 9V and 19F, 14 and 19F, and 9V and 14, respectively. Subtype A2 was common to isolates of all study serotypes. Most strains ($n=64$, 92.7%) of clone A were intermediately penicillin-resistant (MIC 0.125–1.0 $\mu\text{g ml}^{-1}$), and five strains only were highly resistant to penicillin (MIC $\geq 2 \mu\text{g ml}^{-1}$). All strains of clone A were resistant to trimethoprim-sulfamethoxazole (MIC $\geq 4/76 \mu\text{g ml}^{-1}$); some strains ($n=18$, 25.3%) were also resistant to erythromycin (MIC $\geq 0.5 \mu\text{g ml}^{-1}$), clindamycin ($n=14$, 19.7%, MIC $\geq 1 \mu\text{g ml}^{-1}$), chloramphenicol ($n=13$, 18.8%, MIC $\geq 8 \mu\text{g ml}^{-1}$) or tetracycline ($n=6$, 8.7%, MIC $\geq 4 \mu\text{g ml}^{-1}$). Resistance to these antibiotics was predominantly associated with serotype 14 strains belonging to subtype A5, all isolates of this serotype ($n=12$) being resistant to erythromycin, clindamycin and chloramphenicol, and five to tetracycline also. In contrast, isolates of serotype 19F belonging to subtype A5 were erythromycin, clindamycin, chloramphenicol and tetracycline susceptible. The PFGE patterns of clone A subtypes were compared with that generated by a Spain^{9V}-3 isolate. The profiles were identical

(subtype A1) or very similar to that of the international Spain^{9V}-3 clone (maximum difference of six bands), which indicates that the isolates are genotypically identical or related, and can be considered to be members of the same clone. Only two strains of serotype 19F were not related to clone A; moreover, they differed from each other by seven bands in PFGE patterns and were assigned to types C1 and D1. Susceptibility profiles of strains are shown in Table 3. The comparison of PFGE patterns with that generated by a representative isolate of the Taiwan^{19F}-14 international clone showed high similarity (four-band difference) of the isolate to type D1, and therefore the relatedness of type D1 to the Taiwan^{19F}-14 clone; the PFGE pattern of a C1 type isolate differed by seven bands from that of the Taiwan^{19F}-14 clone, and consequently the isolate was considered to be unrelated to this clone.

MLST analysis

Two representative isolates, if available, of each PFGE subtype of each serotype were chosen for MLST analysis (Table 2). A total of 24 strains were characterized by MLST. The results of MLST were assumed to be shared by other isolates of the same serotype within a PFGE subtype. Data on serotype, demographic characteristics, antibiotic susceptibility profile and results of PFGE and MLST for individual isolates are given in Table 3.

Within clone A, three different STs were found: ST 156 ($n=15$), ST 380 ($n=6$) and ST 143 ($n=1$). Allelic profiles identical to that of the international Spain^{9V}-3 clone (ST 156) were found in all of the analysed isolates of serotype 9V ($n=11$), and in four out of five isolates of serotype 14, indicating that they were serotype variants of this clone. Both ST 143 and ST 380 are double-locus variants of ST 156; therefore, strains possessing either ST 143 or ST 380 were considered to be possibly related to ST 156. The ST 143 allelic profile was found in a single isolate of serotype 14, which was the only strain of PFGE pattern A6. Isolates of

Table 2. MLST results and PFGE patterns of isolates of selected serotypes

PFGE pattern	No. of isolates of each serotype (ST of representative isolates)			Total (no. of strains analysed by MLST)
	9V	14	19F	
A1	26 (ST 156)		2 (ST 380)	28 (4)
A2	7 (ST 156)	1 (ST 156)	7 (ST 380)	15 (5)
A3	1 (ST 156)			1 (1)
A4	4 (ST 156)			4 (2)
A5		12 (ST 156)	3 (ST 380)	15 (4)
A6		1 (ST 143)		1 (1)
A7	2 (ST 156)			2 (2)
A8	2 (ST 156)	1 (ST 156)		3 (3)
C1			1 (ST 1657)	1 (1)
D1			1 (ST 236)	1 (1)
Total	42	15	14	71 (24)

Table 3. Characteristics of 24 invasive PNSP strains isolated in the Czech Republic

Serotype	Strain no.	Year of isolation	City	Sex*	Age†	Source‡	MIC (mg l ⁻¹) of:§							PFGE type	ST	Allelic profile							
							PEN	CTX	CMP	TET	ERY	CLI	SXT			<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>rec</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>	
9V	4405	2001	Č. Budějovice	M	68 y	Blood	0·5	0·5	2	0·25	0·063	0·125	>32	A1	156	7	11	10	1	6	8	1	
	7085	2001	Praha	F	10 m	MEF	4	1	4	0·25	0·125	0·125	>32	A1	156	7	11	10	1	6	8	1	
	3677	2001	Pardubice	M	52 y	Blood	0·125	0·125	4	0·25	0·25	0·125	>32	A2	156	7	11	10	1	6	8	1	
	7197	2001	Brno	M	1 y	NP	0·5	0·5	4	0·25	0·125	0·125	>32	A2	156	7	11	10	1	6	8	1	
	1278	2000	Most	M	1 y	NP	1	0·5	4	0·25	0·032	0·063	>32	A3	156	7	11	10	1	6	8	1	
	3831	2002	Plzeň	M	32 y	Blood	1	1	0·5	0·5	0·125	0·25	>32	A4	156	7	11	10	1	6	8	1	
	205	2000	Praha	M	79 y	Blood	0·125	0·063	1	0·25	0·063	0·063	32	A4	156	7	11	10	1	6	8	1	
	2427	2000	Ostrava	F	11 m	CSF	1	1	4	0·5	>32	0·125	>32	A7	156	7	11	10	1	6	8	1	
	3885	2002	Ostrava	F	2 m	NP	1	1	4	0·25	>32	0·25	>32	A7	156	7	11	10	1	6	8	1	
	7447	2001	Praha	M	1 y	MEF	4	1	2	0·25	0·063	0·125	>32	A8	156	7	11	10	1	6	8	1	
3884	2002	Ostrava	F	53 y	CSF	1	1	4	0·5	0·125	0·125	>32	A8	156	7	11	10	1	6	8	1		
14	5734	2001	Náchod	M	1 y	NP	1	1	2	0·25	0·125	0·063	>32	A2	156	7	11	10	1	6	8	1	
	1241	2000	Nový Jičín	F	1 y	MEF	1	1	16	0·5	>32	>32	>32	A5	156	7	11	10	1	6	8	1	
	6966	2001	Příbram	M	4 y	MEF	2	1	32	0·25	16	>32	>32	A5	156	7	11	10	1	6	8	1	
	2207	2000	Plzeň	M	1 m	MEF	1	0·25	4	>32	>32	>32	>32	A6	143	7	5	10	18	6	8	1	
	293	2000	Plzeň	M	5 y	NP	0·25	0·125	16	0·5	>32	>32	>32	A8	156	7	11	10	1	6	8	1	
19F	1267	2000	Kladno	F	11 m	MEF	0·125	0·125	4	0·25	0·063	0·063	>32	A1	380	7	11	19	1	6	20	1	
	889	2000	Praha	M	1 y	NP	0·25	1	2	0·5	0·032	0·125	32	A1	380	7	11	19	1	6	20	1	
	6088	2001	Praha	M	1 y	NP	0·125	0·25	2	0·125	0·125	0·063	>32	A2	380	7	11	19	1	6	20	1	
	172	2000	Ostrava	F	1 y	NP	0·125	0·25	2	0·25	0·063	0·125	>32	A2	380	7	11	19	1	6	20	1	
	1947	2000	Praha	M	1 y	NP	0·125	0·125	4	0·25	0·063	0·063	32	A5	380	7	11	19	1	6	20	1	
	198	2000	Brno	F	2 y	MEF	0·25	0·25	4	0·25	0·063	0·063	>32	A5	380	7	11	19	1	6	20	1	
	4257	2001	Praha	M	3 y	NP	0·125	0·125	2	2	2	0·125	>32	C1	1657II	15	16	19	15	6	20	97	
	4401	2001	Praha	M	5 y	MEF	0·5	0·25	4	32	8	0·125	32	D1	236	15	16	19	15	6	20	26	

*F, female; M, male.

†y, Year; m, month.

‡CSF, cerebrospinal fluid; NP, nasopharynx.

§PEN, penicillin; CTX, cefotaxime; CMP, chloramphenicol; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, trimethoprim-sulfamethoxazole.

||New ST.

penicillin-resistant serotype 14 belonging to ST 143 have been found in Poland (year of isolation: 1994) and Hungary (year of isolation: 2002) (www.mlst.net). ST 380, the other identified double-locus variant ST of the Spain^{9V}-3 clone, was only identified in serotype 19F isolates. According to the MLST database, a single isolate of ST 380 has been included in the *S. pneumoniae* dataset: a Czech strain isolated in 1997. The remaining two strains of serotype 19F were of ST 236 and ST 1657. ST 236 was assigned to the Taiwan^{19F}-14 clone. A novel ST 1657 was found to be an SLV of ST 236.

As documented by other authors (Sá-Leão *et al.*, 2001; Gertz *et al.*, 2003; Serrano *et al.*, 2005), the results obtained by PFGE and MLST may not always overlap. A possible explanation is the difference in discriminatory power between these molecular techniques. In our study, discrepancies were also noted. For example, serotype 19F isolates had PFGE subtype A profiles indistinguishable from those generated by isolates of serotypes 9V and 14, while their allelic profiles differed in two of the seven loci when characterized by MLST. Notwithstanding these variations, all of the isolates belonging to clone A appeared to be genetically related by MLST also, when a less stringent approach (five of seven shared alleles) to define a clonal group (Feil *et al.*, 2004) was applied. On the other hand, strains 4257 and 4401 of serotype 19F had different PFGE types, but shared six of seven loci when characterized by MLST. This could be explained by the classification of isolates into PFGE types being based on a seven-band difference in profiles.

The international spread of a restricted number of multi-resistant clones has been documented and the propensity to horizontal gene recombination in these clones has been verified (Coffey *et al.*, 1991, 1999; Munoz *et al.*, 1991; Gasc *et al.*, 1995). The present study demonstrated the Spain^{9V}-3 clone (ST 156) to make a major contribution to the PNSP population in the Czech Republic. Apparently, the genetic background of this clone is not restricted to serotype 9V, but is also disseminated within serotype 14 and 19F. In contrast, none of the current isolates of serotype 14 was related to the clone CSR¹⁴-10 described previously in the former Czechoslovakia.

The genetic background of the Spain^{9V}-3 clone in serotypes 11A 14, and 19F has been reported previously (Coffey *et al.*, 1999; Enright *et al.*, 1999; Porat *et al.*, 2004). The ability of pneumococci to undergo capsular transformation could compromise the efficacy of the conjugate pneumococcal vaccine, as the number of serotypes contained in the vaccine is limited (Obaro & Adegbola, 2002; McEllistrem *et al.*, 2003). The available 7-valent vaccine includes conjugates derived from polysaccharides of types 4, 6B, 9V, 14, 18C, 19F and 23F. Porat *et al.* (2004) have documented non-vaccine serotype 11A variants of the international Spain^{9V}-3 clone, one of the major antibiotic-non-susceptible clones, among carriage and MEF isolates obtained from Israeli children. The authors imply that although the increased prevalence of serotype 11A may be of minor consequence, since this

serotype has a relatively low potential to cause invasive disease, the conjugate vaccine would open a reservoir for this serotype as a cause of AOM. In our study, isolates of serotype 11A were not included for analysis, as only a single penicillin-intermediate strain of this serotype was referred to the National Institute of Public Health between 2000 and 2002. Nevertheless, continued surveillance of pneumococcal serotype distribution will be of concern when the pneumococcal vaccine is widely used in the Czech Republic.

Representatives of the Taiwan^{19F}-14 clone (ST 236) and SLVs of this ST were also detected in the Czech Republic. Multidrug-resistant strains of ST 236 have been found in Taiwan since at least 1993 (Shi *et al.*, 1998). The Taiwan^{19F}-14 clone is highly predominant among penicillin-resistant strains in far-eastern countries (Song *et al.*, 1997; Shi *et al.*, 1998); however, it appears already to have spread intercontinentally, as two penicillin-resistant isolates of serotype 19F with allelic profiles similar to that of Taiwan^{19F}-14 have been identified in the UK (Enright & Spratt, 1998). Richter *et al.* (2002) have provided evidence of the global spread of this clone, as penicillin-resistant isolates of serotype 19F collected in the USA are grouped into the same cluster with this clone.

Despite stable low rates of resistance to penicillin in the Czech Republic, a significant shift in serotype distribution has been reported (Urbášková & Motlová, 1999; Urbášková *et al.*, 2004). The shift could be explained by the introduction of the Spain^{9V}-3 clone into the country from high-prevalence western European regions after the political changes in 1989, with the subsequent sharp rise in international travel. Interestingly, in results parallel to those from the Czech Republic, Buxbaum *et al.* (2004) found serogroup 9 to have become predominant among the penicillin non-susceptible serogroups in Austria in 1998. In contrast to the Czech Republic, this change was accompanied by a dramatic rise in penicillin resistance, which almost doubled the rate in Austria to nearly 10%. Due to geographic proximity, resistant strains of serogroup 9 may have been imported into the Czech Republic from Austria; nevertheless, this hypothesis still remains to be confirmed, as detailed information on the genetic background of Austrian strains is not yet available. Moreover, other unknown factors might have been involved in the expansion of the Spain^{9V}-3 clone, given that it is not clear why other pandemic multiresistant clones did not spread in the country with the same impact.

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