Characterization of German penicillin non-susceptible serotype 23F pneumococci using multilocus sequence typing

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Three nationwide multicentre studies ($n = 5071$) showed an increase in antibiotic resistance in pneumococci in Germany. Serotype 23F was the predominant serotype ($n = 45$, 22.4%), followed by 6B ($n = 30$, 14.9%) and 9V ($n = 19$, 9.5%). Multilocus sequence typing was used to characterize 45 serotype 23F strains with reduced penicillin susceptibility. The Spanish^{23F}-1 clone [profile 4-4-2-4-4-1-1, sequence type (ST) 81] contributes significantly to the emergence of penicillin resistance in Germany ($n = 21$, 46.7% of all penicillin non-susceptible serotype 23F isolates). Isolates of ST 277 (profile 7-13-8-6-6-12-8), which has been found previously in the Netherlands, are also observed, particularly in western Germany ($n = 8$, 17.8%). A high proportion of strains ($n = 11$, 24.4%) have sequence types that have not been reported to date from other countries (STs 353–362). The major penicillin-resistant clones are present in Germany, a country with relatively low levels of β -lactam resistance.

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INTRODUCTION

Penicillin non-susceptible *Streptococcus pneumoniae* strains were first reported from Germany in 1992 (Reinert *et al.*, 1992). Within the last 5 years, an increase in both penicillin and macrolide resistance has been observed among *S. pneumoniae* in Germany, as documented by three surveillance studies performed by the National Reference Center for Streptococci. In a nationwide study of invasive pneumococcal disease in adults (study B), the proportion of strains with reduced susceptibility to penicillin ($\text{MIC} \geq 0.12 \mu\text{g ml}^{-1}$) increased from 1.8% in 1992 to 5.8% in 2000 (Reinert *et al.*, 2002). In an ongoing population-based nationwide surveillance study of invasive disease in children (study A), which started in 1997 and covered all paediatric hospitals and clinical microbiology laboratories in Germany, ten strains (3.3%) were found to be penicillin-intermediate ($\text{MIC } 0.12\text{--}1.0 \mu\text{g ml}^{-1}$), but no penicillin-resistant strains ($\text{MIC} > 2 \mu\text{g ml}^{-1}$) were detected (von Kries *et al.*, 2000). During

the same period, outpatient pneumococcal isolates were collected prospectively by 19 clinical microbiology laboratories in Germany between November 1998 and April 1999 (study C), with 6.8% ($n = 65$) of 961 pneumococcal strains showing reduced susceptibility to penicillin (Reinert *et al.*, 2001).

Multilocus sequence typing (MLST) is a recently developed technique that produces unambiguous molecular typing data that can be transmitted electronically via the Internet (Enright & Spratt, 1998; Maiden *et al.*, 1998). Using this procedure, nucleotide sequences of ~ 450 bp internal fragments of seven housekeeping genes are determined for each isolate. Different sequences at each locus are assigned as distinct alleles and, for each isolate, alleles at the seven loci define the allelic profile or sequence type (ST). The method is highly portable, as any laboratory can compare sequences at the seven loci in their isolates with those in a central database on the World Wide Web (<http://www.mlst.net>) and obtain the allelic profile of each isolate. The present study uses this technique to analyse clinical strains of penicillin non-susceptible, serotype 23F *S. pneumoniae* isolated in Germany between 1992 and 2000.

Abbreviations: MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing; NCCLS, National Committee for Clinical Laboratory Standards; SLV, single locus variant; ST, sequence type.

METHODS

Strains. Forty-five single patient isolates of serotype 23F pneumococci that were penicillin-intermediate or -resistant were collected between 1992 and 2000 from three national surveillance projects in Germany (Table 1). Twelve isolates were recovered from blood, three from cerebrospinal fluid, 17 from respiratory specimens and 13 from other or unknown sources. In addition, we scanned the MLST database, which includes the allelic profiles of over 700 pneumococci, for pneumococcal serotype 23F infections (Enright & Spratt, 1998).

Susceptibility testing and serotyping. Susceptibility testing was performed by using the broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2000). Commercially manufactured plates (Micronaut-S; Merlin Diagnostics) that contained penicillin, cefotaxime, erythromycin, clindamycin, tetracycline and cation-adjusted Mueller–Hinton broth (Oxoid) plus 5% lysed horse blood (Oxoid) were utilized. MICs were determined following incubation at 35 °C for 20–24 h in ambient air. *S. pneumoniae* ATCC 49619 was used as a control strain. Isolates were stored at –70 °C on porous beads (Microbank; Mast Diagnostics). Susceptibility to cotrimoxazole was determined by means of E test strips (Viva Diagnostica). Current NCCLS interpretive criteria were used to define antimicrobial resistance (NCCLS, 2000).

Pneumococcal strains were serotyped by Neufeld’s Quellung reaction (Neufeld, 1902), using type and factor sera provided by the Statens Serum Institut, Copenhagen, Denmark. The serotype distribution of this collection of penicillin non-susceptible pneumococci was analysed with reference to all pneumococci collected in three German multi-centre studies (von Kries *et al.*, 2000; Reinert *et al.*, 2001, 2002).

DNA purification, PCR protocols and MLST. PCR amplification was carried out on chromosomal DNA by using an extension time of 30 s and an annealing temperature of 50 °C with *Taq* polymerase (Roche Diagnostics). DNA fragments were purified by using the QIAquick system (Qiagen) and sequencing reactions were carried out in each direction by using the primers that had been used for initial PCR amplification. After gel electrophoretic separation of 8 µl PCR product in 1% agarose, amplicons were visualized and their length determined by using UV transillumination. For gel documentation, the BioDoc-Analyze system (Biometra) was used.

MLST was carried out as described previously. Briefly, internal fragments of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl* genes were amplified by PCR from chromosomal DNA with the primer pairs described by Enright & Spratt (1998).

Assignment of alleles at each locus was carried out with software available at the pneumococcal MLST website (<http://www.mlst.net>). Alleles at each of the seven loci provide the allelic profile of each isolate and also define their ST. Allelic profiles are shown as the alleles at each of the seven loci, in the order *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, *ddl*. Allelic profiles of the German isolates were compared with each other, as well as with other isolates in the pneumococcal MLST database, by using software available at the MLST website.

Detection of resistance genes. For amplification of *tetM*, primers originally published by Provvedi *et al.* (1996) with the sequences 5'-TGGAATTGATTTATCAACGG-3' (positions 2496–2515) and 5'-TTCCAACCATAACAATCCTTG-3' (positions 3575–3556) were used. For amplification of *tetO*, the primers 5'-GGCACAGACCCGTA TACTGTT-3' (positions 442–462) and 5'-TTAAAGAGGGTCGC CATCTG-3' (positions 1230–1250) were used (Widdowson *et al.*, 1996; Widdowson & Klugman, 1998). For detection of *erm* and *mef*, primers described by Trieu-Cuot *et al.* (1990) and by Tait-Kamradt *et al.* (1997) with the sequences (*erm*) 5'-CGAGTGAAAAAGTACTCAACC-3' (positions 362–382) and 5'-GGCGTGTTTCATTGCTTGATG-3'

Table 1. Overview of studies on pneumococcal infections in Germany

Abbreviations: IPD, invasive pneumococcal disease; RTI, respiratory tract infections.

Study	Age group	Disease	Time	Centres	No. strains	No. strains pen-I or pen-R (%)	No. strains serotyped (%)	No. strains serotyped [pen-I or pen-R (%)]	No. serotype 23F (%)	No. strains (Pen I+R) serotyped as 23F (%)	Reference
A	Children	IPD	1997 ongoing	123	924	46 (5.0)	921 (99.7)	46 (100)	61 (6.6)	13 (28.3)	von Kries <i>et al.</i> (2000)
B	Adults*	IPD	1992 ongoing	86	3186	150 (4.8)	725 (22.8)	91 (60.7)	55 (7.6)	25 (27.5)	Reinert <i>et al.</i> (2002)
C	Children and adults	RTI	1998–1999	19	961	65 (6.8)	418 (43.5)	64 (98.5)	59 (14.1)	7 (10.9)	Reinert <i>et al.</i> (2001)
Total					5071	261 (5.1)	2064 (40.7)	201 (77.0)†	175 (8.5)‡	45 (22.4)	

*Until 1997, children were also included.

†Percentage of pen-I and pen-R strains (n = 261).

‡Percentage of serotyped strains (n = 2064).

§Percentage of serotyped pen-I and pen-R strains (n = 201).

(positions 978–958) and (*mef*) 5'-AGTATCATTAATCACTAGTGC-3' (positions 57–77) and 5'-GTAATAGATGCAATCACAGC-3' (positions 551–532) were selected (Reinert *et al.*, 2001).

RESULTS AND DISCUSSION

Serotyping data were available for 201 representative penicillin non-susceptible strains of pneumococci collected in three German multicentre studies (Table 1) (von Kries *et al.*, 2000; Reinert *et al.*, 2001, 2002). Serotype 23F was the predominant serotype ($n = 45$, 22.4%), followed by 6B ($n = 30$, 14.9%), 9V ($n = 19$, 9.5%), 19A ($n = 18$, 9%), 19F ($n = 17$, 8.5%), 14 ($n = 14$, 7%), 6A ($n = 9$, 4.5%), 15B ($n = 6$, 3%) and others ($n = 43$, 21.4%).

Table 2 shows demographic, antimicrobial susceptibility and MLST data for individual strains. The following sequence types were found: ST 81 ($n = 21$, 46.2%), ST 277 ($n = 8$, 17.8%), ST 242 ($n = 2$), ST 142 ($n = 2$), ST 323 ($n = 1$) and novel STs ($n = 11$, 24.4%).

ST 81 strains were isolated in Germany each year from 1992 to 1999. ST 81 (the ST of the Spanish^{23F}-1 clone; McGee *et al.*, 2001) appears to be geographically distributed throughout Germany and was recovered from both invasive and non-invasive disease sources. Seven strains among the group of German ST 81 strains were high-level penicillin-resistant ($\text{MIC} \geq 2 \mu\text{g ml}^{-1}$).

Most strains ($n = 15$, 71.4%) of ST 81 were susceptible to erythromycin ($\text{MIC} \leq 0.25 \mu\text{g ml}^{-1}$), but 90% of isolates (19 of 21) were resistant to tetracycline ($\text{MIC} \geq 4 \mu\text{g ml}^{-1}$, all *tetM*-positive). All German isolates of the Spanish^{23F}-1 clone had the typical allelic profile of this clone (ST 81).

The second most prevalent penicillin-resistant 23F clone in Germany is ST 277. Eight strains (17.8%) had ST 277 and another four were very closely related, differing from the allelic profile of this ST at only a single locus [single locus variants (SLVs)]. Only one isolate of ST 277 can be found in the MLST database; this is a penicillin-resistant serogroup 23 strain isolated in the Netherlands (strain JJ279-23). Strains of ST 277 were mostly isolated (four of eight ST 277 strains) in the extreme west of Germany, whilst the other strains were detected in central and southern Germany. No SLVs of ST 81 were detected, whereas four SLVs of ST 277 (STs 359 and 360, one each; 361, two strains) were found to be present in Germany. All SLVs showed alterations in the *xpt* gene, harbouring allele 6 (strains PS 275 and PS 278; ST 361), allele 20 (RKI 425; ST 359) or allele 65 (RKI 682; ST 360). All strains of ST 277 and its SLVs were intermediately penicillin-resistant and were erythromycin- and tetracycline-susceptible.

Two strains (PS 1953 and PS 2006) were identified as ST 242; one further strain was an SLV of this ST (RESP 95; ST 362). Interestingly, ST 242 has only been reported from Taiwan to date and is now designated the Taiwan^{23F}-15 clone (Shi *et al.*, 1998). The two ST 242 isolates in the German strain collection were intermediately penicillin-resistant and were erythromycin- and tetracycline-resistant, as are Taiwanese

isolates of this clone. The ST 242 strains showed the MLS_B type of resistance (*erm*-positive), whereas the SLV (ST 362) was *mef*-positive.

Two strains in the German strain collection (RKI 530 and RKI 531) belong to ST 142, which is defined by a single penicillin-resistant serotype 23F strain (M 402) that was isolated in 1998 in Spain. ST 142 is a SLV of ST 277, which is widespread in Germany. Interestingly, both ST 142 isolates were isolated in the same city, Dortmund, in 1999. Strains of ST 142 are susceptible to erythromycin and clindamycin; however, in contrast to the Spanish isolate, they are resistant to tetracycline.

One strain (RESP 798; ST 355) is an SLV to the high-level cephalosporin-resistant serotype 23F clone from the US (Tennessee^{23F}-4; pneumococcal network, accession no. ATCC 51916). In contrast to the Tennessee^{23F}-4 clone, the German isolate remained susceptible to cephalosporins. Five of the German strains had allelic profiles that were not related closely (SLVs) to any isolates in the MLST database (STs 353, 354, 356, 357, 358).

Penicillin resistance in pneumococci is now an infectious disease problem of international significance. Penicillin resistance rates vary even between neighbouring European countries (Klugman *et al.*, 1997; Pradier *et al.*, 1997). An extremely high level of penicillin resistance is reported from southern and south-eastern Europe, whereas the level of pneumococcal β -lactam resistance remains low in Germany (Reinert *et al.*, 1995, 2001, 2002; von Kries *et al.*, 2000), the Netherlands (Hermans *et al.*, 1997), the northern European countries (Nielsen & Henriksen, 1992; Henriques *et al.*, 2000) and the UK (Johnson, 1998; Andrews *et al.*, 1999).

MLST now offers an excellent tool for unambiguous characterization of pneumococci by sequencing approximately 450 bp fragments of seven housekeeping loci (Enright & Spratt, 1998). MLST is particularly useful for comparing penicillin- and multiresistant isolates recovered in one geographical region with those described elsewhere (Zhou *et al.*, 2000).

The present study showed serotype 23F to be the most predominant penicillin non-susceptible serotype isolated in Germany. Strains from three nationwide surveys were included in the analysis. As study A (population-based study covering all laboratories in Germany) and study B (Table 1) are nationwide studies, the penicillin non-sensitive serotype 23F pneumococcal strains included in the present analysis are representative isolates for invasive pneumococcal disease in Germany. In contrast, representativeness of the present analysis for pneumococcal respiratory tract infections may be limited.

Among all serotype 23F pneumococci that were recovered in Germany from 1992 to 2000, MLST unambiguously demonstrated that the Spain^{23F}-1 clone (ST 81) contributed significantly to the spread of penicillin resistance in Germany. Representatives of ST 277 and SLVs of this ST are also observed in Germany. A few isolates were indistinguishable

Table 2. Characteristics of 45 penicillin non-susceptible pneumococcal serotype 23F strains isolated in Germany in 1992–2000, in comparison to international reference strains

Strains from the MLST strain collection are marked with bold face. Abbreviations: F.S., federal state; CLI, clindamycin; CTX, cefotaxime; ERY, erythromycin; PEN, penicillin G; SXT, cotrimoxazole; TET, tetracycline; ST, sequence type; ND, no data; B, Berlin; BA, Bavaria; BW, Baden-Württemberg; HE, Hesse; HH, Hamburg; LS, Lower Saxony; NRW, North Rhine–Westphalia; SA, Saxony; TU, Thuringia; BS, bronchial secretion; conj., conjunctiva; CSF, cerebrospinal fluid; Nasop., nasopharynx; PF, pleural fluid; f, female; m, male.

Strain	Isolation year	Town	F.S.	Source	Sex	Age	MIC (µg ml ⁻¹) of:						Allelic profile							ST
							PEN	CTX	ERY	CLI	TET	SXT	<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>	
ST 81																				
PS 16	1992	Göttingen	LS	Conj.	m	2	1	1	0.06	0.06	32	16	4	4	2	4	4	1	1	81
PS 62	1992	Munich	BA	BS	m	62	2	1	0.06	0.06	32	8	4	4	2	4	4	1	1	81
PS 91-D305	1992	ND	ND	PF	m	ND	2	1	0.06	0.06	32	8	4	4	2	4	4	1	1	81
PS 184-D306*	1992	Munich	BA	CSF	m	ND	1	1	0.06	0.06	16	8	4	4	2	4	4	1	1	81
PS 376	1992	Berlin	B	BS	m	65	1	1	0.06	0.06	32	4	4	4	2	4	4	1	1	81
PS 453	1992	Hanover	LS	BS	m	5	2	2	32†	32	32	8	4	4	2	4	4	1	1	81
PS 570	1992	Munich	BA	ND	f	85	1	1	0.06	0.06	32	6	4	4	2	4	4	1	1	81
PS 853	1993	Freiburg	BW	ND	f	2	1	1	0.06	0.06	32	6	4	4	2	4	4	1	1	81
PS 999	1993	Hanover	LS	Sputum	m	66	1	0.5	0.06	0.06	32	8	4	4	2	4	4	1	1	81
PS 1005	1993	Frankfurt‡	HE	Blood	ND	ND	1	0.5	0.06	0.06	32	32	4	4	2	4	4	1	1	81
PS 1058	1993	Hanover	LS	BS	m	66	1	1	0.125	0.06	32	16	4	4	2	4	4	1	1	81
PS 1271	1994	Aachen	NRW	BS	m	64	1	1	32†	16	0.5	32	4	4	2	4	4	1	1	81
PS 1702	1995	Aachen	NRW	Ear	ND	6	2	1	32§	0.25	8	4	4	4	2	4	4	1	1	81
PS 1762	1995	Hanover	LS	Blood	m	50	2	1	0.06	0.06	4	4	4	4	2	4	4	1	1	81
PS 1923	1995	Bonn	NRW	ND	ND	ND	1	1	0.06	0.06	8	4	4	4	2	4	4	1	1	81
PS 2352	1996	Bonn	NRW	Sputum	m	59	1	1	0.06	0.06	4	4	4	4	2	4	4	1	1	81
PS 2361	1996	Hanover	LS	Nasop.	m	4	1	1	32†	32	16	4	4	4	2	4	4	1	1	81
PN 958	1997	Dillingen	BA	BS	f	72	1	1	0.06	0.06	32	4	4	4	2	4	4	1	1	81
RESP 203	1998	ND	ND	Nasop.	m	2	2	0.25	32†	32	16	8	4	4	2	4	4	1	1	81
RKI 277	1998	Stuttgart	BW	Blood	m	2	0.5	0.5	2§	0.06	0.25	4	4	4	2	4	4	1	1	81
RESP 950	1999	Weiden	BA	Sinus	m	55	2	0.25	0.06	0.06	32	4	4	4	2	4	4	1	1	81
ST 277																				
PS 335-D310	1992	Aachen	NRW	Conj.	m	1	0.25	0.06	0.06	0.06	0.125	2	7	13	8	6	6	12	8	277
PS 2188	1996	Aachen	NRW	Blood	m	ND	0.25	0.06	0.06	0.06	0.5	1	7	13	8	6	6	12	8	277
PS 2211	1996	Münster	NRW	Blood	m	53	0.25	0.25	0.06	0.06	0.5	0.125	7	13	8	6	6	12	8	277
PS 2216	1996	Cologne	NRW	Wound	m	35	0.25	0.06	0.06	0.06	0.5	0.25	7	13	8	6	6	12	8	277

PN 1003	1997	Erlangen	BA	Blood	ND	ND	0-25	0-06	0-06	0-06	0-125	2	7	13	8	6	6	12	8	277
PN 962	1997	Erlangen	BA	Conj.	ND	1 m	0-25	0-06	0-06	0-06	0-5	4	7	13	8	6	6	12	8	277
RESP 4	1998	Hamburg	HH	Nasop.	f	5	0-25	0-125	0-125	0-125	0-25	1	7	13	8	6	6	12	8	277
RKI 629	2000	Heidelberg	BW	Blood	f	5	0-25	0-06	0-125	0-06	0-5	8	7	13	8	6	6	12	8	277
ST 242																				
PS 1953	1995	Aachen	NRW	ND	ND	ND	1	1	32†	32	32	1	15	29	4	21	30	1	14	242
PS 2006	1995	Frankfurt‡	HE	ND	ND	ND	0-25	0-25	32†	32	32	0-5	15	29	4	21	30	1	14	242
ST 142																				
RKI 530	1999	Dortmund	NRW	Blood	m	1	0-25	0-125	0-125	0-06	8	0-125	7	5	8	6	6	12	8	142
RKI 531	1999	Dortmund	NRW	Blood	m	1 m	0-25	0-06	0-06	0-06	8	0-125	7	5	8	6	6	12	8	142
ST 323																				
PS 943	1993	Hanover	LS	Sputum	m	34	0-125	0-125	0-06	0-06	4	2	2	13	8	12	6	6	18	323
New STs																				
PS 1374	1994	Jena	TU	ND	m	5	0-5	0-5	0-06	0-06	0-5	0-5	1	5	9	5	6	3	65	353
PS 2031	1996	Frankfurt‡	HE	CSF	m	9	0-25	0-125	0-06	0-06	0-5	4	1	5	9	5	15	3	8	354
RESP 798	1999	Weiden	BA	Nasop.	m	4	0-125	0-06	0-06	0-06	0-25	0-125	1	8	4	2	6	4	6	355¶
RESP 801	1999	Neukirchen	ND	Eye	m	1	0-125	0-06	0-06	0-125	0-125	1	1	13	27	20	6	1	1	356
RKI 243	1998	Stuttgart	BW	Blood	ND	1	0-25	0-125	0-06	0-06	0-06	0-125	7	13	8	6	15	20	14	357
PS 2260	1996	Erlangen	BA	Conj.	m	4 m	0-25	0-25	0-06	0-06	0-5	0-125	7	13	8	6	15	74**	6	358
RKI 425	1999	Glauchau	SA	Blood	m	3	0-25	0-06	0-06	0-06	0-5	0-125	7	13	8	6	6	20	8	359
RKI 682	1999	Munich	BA	Blood	m	2	0-25	0-06	0-125	0-06	0-5	0-125	7	13	8	6	6	65**	8	360
PS 275-D307	1992	Erlangen	BA	CSF	f	70	0-25	0-06	0-06	0-06	2	3	7	13	8	6	6	6	8	361
PS 278-D308	1992	Berlin	B	Wound	m	86	0-25	0-25	0-06	0-06	2	8	7	13	8	6	6	6	8	361
RESP 95	1998	Leverkusen	NRW	Nasop.	f	32	0-125	0-125	8§	0-125	32	1	15	13	4	21	30	1	14	362††

*Identical to strain D306-W-23F in MLST database.

†*erm*-positive.

‡Frankfurt am Main.

§*mef*-positive.

||SLV of ST 277.

¶SLV of ST 37 and ST 33.

**New allele seen to date only in Germany.

††SLV of ST 242.

by MLST (or, in some cases, differed at a single locus) from multiply resistant strains from Taiwan or the USA. Only five strains were not closely related to any in the MLST database.

Data on the epidemiology of pneumococcal disease in Germany are scant to date. Reichmann *et al.* (1995) compared penicillin-resistant *S. pneumoniae* strains isolated in different parts of Germany between 1982 and 1992 with penicillin-resistant isolates, mainly of serogroups 6, 9, 14, 19 and 23, from other European countries. Strains were characterized by their serotypes, antibiotic resistance patterns, penicillin-binding protein properties and multilocus enzyme electrophoresis (MLEE). Eleven of the 14 penicillin-resistant German isolates were assigned to five genotypes that were also isolated in other countries. The study by Reichmann *et al.* (1995) included five serotype 23F strains of *S. pneumoniae*, which are included in the collection of strains in this investigation. Serotype 23F strains PS 91-D305 and PS 184-D306, classified as MLEE type 2, were shown by MLST to be equivalent to ST 81 (the Spain^{23F}-1 clone). MLEE types 21 and 22 were demonstrated by MLST to be closely related to and to correspond to ST 277 (strain PS 335-D310) and an SLV of ST 277 (ST 361, strains PS 275-D307 and PS 278-D308), respectively. These observations underscore the high discriminative power of both MLEE and MLST and document that both methods lead to identical results when investigating clonal relatedness of pneumococcal isolates.

MLST was used by Zhou *et al.* (2000) to characterize isolates of the major Spanish clones of penicillin-resistant and multiply antibiotic-resistant *S. pneumoniae* strains, including previously characterized isolates of the Spanish^{23F}-1 clone (Coffey *et al.*, 1996). Fifteen out of 16 of these isolates had an identical allelic profile (ST 81, profile 4-4-2-4-4-1-1), which was considered to be the typical allelic profile of the Spanish^{23F}-1 clone.

Data from this study clearly document that most penicillin non-susceptible strains are closely related to those found in Spain. Results of our study also underscore that travelling may contribute to the spread of resistance. Germans spent 82.6 billion DM (38.4 billion US\$) on travel in 1998. For many years, the main holiday destination for the German population has been Spain; a total of 5.7 million Germans flew to Spain (19.7% of all destinations) in 1998. It is therefore likely that resistant strains are continuously imported into Germany, presumably mainly from Spain. Nevertheless, it remains unclear why these strains do not spread within Germany to a greater extent.

Differing antibiotic consumption in European countries may largely account for these differences. Our group studied the relationship between penicillin and erythromycin resistance among isolates of *S. pneumoniae*, relative to macrolide consumption, in Germany over 9 years (1992–2000) (Granizo *et al.*, 2000; Reinert *et al.*, 2002). A significant relationship was observed between erythromycin resistance and macrolide consumption. Although that ecological analysis was not able to demonstrate a causal relationship between

antibiotic consumption and development of resistance, it suggested that overuse of certain specific antibiotics is more likely to be related to the increase in resistance. High prevalence of the Spanish 23F-clone in Germany may also be due to the relatively high consumption of tetracycline, which was widely used in Germany in the mid-1990s, as this 23F clone is also tetracycline-resistant. A study by Cars *et al.* (2001) has demonstrated that sales of antibiotics varied more than fourfold within Europe; France and Spain had the highest sales, whereas the Netherlands, Denmark, Sweden and Germany had the lowest (Cars *et al.*, 2001). In addition, the relatively high frequency of pre-school children staying at home and not at day-care centres may contribute to the lower level of antibiotic resistance in Germany.

In conclusion, penicillin non-susceptible serotype 23F isolates in Germany can be assigned to two major clones, but other strains also contribute significantly to resistance. Some isolates appear to have not yet been identified outside Germany. The findings of the present study underscore that, despite the low level of β -lactam resistance, penicillin-resistant serotype 23F ST81, which is responsible for high resistance rates reported from other European countries, is present in Germany. Consequently, to preserve the currently low antimicrobial resistance levels among clinical isolates of *S. pneumoniae*, prudent usage of antimicrobials in Germany is warranted.

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REFERENCES

- Andrews, J., Ashby, J., Jevons, G., Lines, N. & Wise, R. (1999). Antimicrobial resistance in gram-positive pathogens isolated in the UK between October 1996 and January 1997. *J Antimicrob Chemother* **43**, 689–698.
- Cars, O., Molstad, S. & Melander, A. (2001). Variation in antibiotic use in the European Union. *Lancet* **357**, 1851–1853.
- Coffey, T. J., Berrón, S., Daniels, M., Garcia-Leoni, M. E., Cercenado, E., Bouza, E., Fenoll, A. & Spratt, B. G. (1996). Multiply antibiotic-resistant *Streptococcus pneumoniae* recovered from Spanish hospitals (1988–1994): novel major clones of serotypes 14, 19F and 15F. *Microbiology* **142**, 2747–2757.
- Enright, M. C. & Spratt, B. G. (1998). A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* **144**, 3049–3060.
- Granizo, J. J., Aguilar, L., Casal, J., Dal-Ré, R. & Baquero, F. (2000). *Streptococcus pyogenes* resistance to erythromycin in relation to macrolide consumption in Spain (1986–1997). *J Antimicrob Chemother* **46**, 959–964.

- Henriques, B., Kalin, M., Örtqvist, Å. & 8 other authors (2000). Molecular epidemiology of *Streptococcus pneumoniae* causing invasive disease in 5 countries. *J Infect Dis* **182**, 833–839.
- Hermans, P. W. M., Sluiter, M., Elzenaar, K. & 8 other authors (1997). Penicillin-resistant *Streptococcus pneumoniae* in the Netherlands: results of a 1-year molecular epidemiologic survey. *J Infect Dis* **175**, 1413–1422.
- Johnson, A. P. (1998). Antibiotic resistance among clinically important gram-positive bacteria in the UK. *J Hosp Infect* **40**, 17–26.
- Klugman, K., Goldstein, F., Kohno, S. & Baquero, F. (1997). The role of fourth-generation cephalosporins in the treatment of infections caused by penicillin-resistant streptococci. *Clin Microbiol Infect* **3** (Suppl. 1), S48–S60.
- Maiden, M. C. J., Bygraves, J. A., Feil, E. & 10 other authors (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**, 3140–3145.
- McGee, L., McDougal, L., Zhou, J. & 8 other authors (2001). Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. *J Clin Microbiol* **39**, 2565–2571.
- NCCLS (2000). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically (M7-A4); Approved Standard (M100-S8)*, 4th edn. Wayne, PA: NCCLS.
- Neufeld, F. (1902). Über die Agglutination der Pneumokokken und über die Theorie der Agglutination. *Z Hyg Infektionskr* **34**, 54–72 (in German).
- Nielsen, S. V. & Henriksen, J. (1992). Capsular types of *Streptococcus pneumoniae* isolated from blood and CSF during 1982–1987. *Clin Infect Dis* **15**, 794–798.
- Pradier, C., Dunais, B., Carsenti-Etessé, H. & Dellamonica, P. (1997). Pneumococcal resistance patterns in Europe. *Eur J Clin Microbiol Infect Dis* **16**, 644–647.
- Provvedi, R., Manganelli, R. & Pozzi, G. (1996). Characterization of conjugative transposon Tn5251 of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **135**, 231–236.
- Reichmann, P., Varon, E., Günther, E., Reinert, R. R., Lütticken, R., Marton, A., Geslin, P., Wagner, J. & Hakenbeck, R. (1995). Penicillin-resistant *Streptococcus pneumoniae* in Germany: genetic relationship to clones from other European countries. *J Med Microbiol* **43**, 377–385.
- Reinert, R. R., Kaufhold, A. & Kierdorf, H. (1992). Penicillin-resistant pneumococcus in community-acquired bacteremic pneumonia in Germany. *Infection* **20**, 238–239.
- Reinert, R. R., Queck, A., Kaufhold, A., Kresken, M. & Lütticken, R. (1995). Antimicrobial resistance and type distribution of *Streptococcus pneumoniae* isolates causing systemic infections in Germany, 1992–1994. *Clin Infect Dis* **21**, 1398–1401.
- Reinert, R. R., Simic, S., Al-Lahham, A., Reinert, S., Lemperle, M. & Lütticken, R. (2001). Antimicrobial resistance of *Streptococcus pneumoniae* recovered from outpatients with respiratory tract infections in Germany from 1998 to 1999: results of a national surveillance study. *J Clin Microbiol* **39**, 1187–1189.
- Reinert, R. R., Al-Lahham, A., Lemperle, M., Tenholte, C., Briefs, C., Haupts, S., Gerards, H. H. & Lütticken, R. (2002). Emergence of macrolide and penicillin resistance among invasive pneumococcal isolates in Germany. *J Antimicrob Chemother* **49**, 61–68.
- Shi, Z.-Y., Enright, M. C., Wilkinson, P., Griffiths, D. & Spratt, B. G. (1998). Identification of three major clones of multiply antibiotic-resistant *Streptococcus pneumoniae* in Taiwanese hospitals by multilocus sequence typing. *J Clin Microbiol* **36**, 3514–3519.
- Tait-Kamradt, A., Clancy, J., Cronan, M., Dib-Hajj, F., Wondrack, L., Yuan, W. & Sutcliffe, J. (1997). *mefE* is necessary for the erythromycin-resistant M phenotype in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **41**, 2251–2255.
- Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C. & Courvalin, P. (1990). Nucleotide sequence of the erythromycin resistance gene of the conjugative transposon Tn1545. *Nucleic Acids Res* **18**, 3660.
- von Kries, R., Siedler, A., Schmitt, H. J. & Reinert, R. R. (2000). Proportion of invasive pneumococcal infections in German children preventable by pneumococcal conjugate vaccines. *Clin Infect Dis* **31**, 482–487.
- Widdowson, C. A. & Klugman, K. P. (1998). The molecular mechanisms of tetracycline resistance in the pneumococcus. *Microb Drug Resist* **4**, 79–84.
- Widdowson, C. A., Klugman, K. P. & Hanslo, D. (1996). Identification of the tetracycline resistance gene, *tet(O)*, in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **40**, 2891–2893.
- Zhou, J., Enright, M. C. & Spratt, B. G. (2000). Identification of the major Spanish clones of penicillin-resistant pneumococci via the Internet using multilocus sequence typing. *J Clin Microbiol* **38**, 977–986.