

## Molecular epidemiology of penicillin-non-susceptible *Streptococcus pneumoniae* isolates from children with invasive pneumococcal disease in Germany

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### ABSTRACT

A population-based nationwide surveillance of antibiotic resistance associated with invasive pneumococcal disease (IPD) in children and adolescents (aged <16 years) was performed in Germany between 1997 and 2004. In total, 1517 isolates were collected, of which 5.1% and 1.1% were intermediately- or fully-resistant, respectively, to penicillin G. During the 8-year study period, an increase in resistance to both penicillin G and erythromycin A was observed, and the frequency of isolates exhibiting reduced susceptibility to penicillin G or erythromycin A increased from 1.4% and 11.1%, respectively, in 1997, to 8.7% and 29.0%, respectively, in 2004. Among the penicillin non-susceptible pneumococcal isolates, serotypes 14 (24.5% of isolates), 23F (16.0%) and 6B (16.0%) were found most frequently. Multilocus sequence typing of 58 (62%) penicillin G non-susceptible isolates revealed that sequence type (ST) 156 (Spain<sup>9V</sup>-3 clone) and its single-locus variant ST 557 were widespread in Germany. Moreover, 17 new penicillin G non-susceptible STs were defined for the first time. The study illustrated the genetic heterogeneity of antibiotic-resistant pneumococcal isolates in Germany.

**Keywords** Antibiotic resistance, Germany, multilocus sequence typing, penicillin-non-susceptible, *Streptococcus pneumoniae*, surveillance

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### INTRODUCTION

*Streptococcus pneumoniae* continues to be a significant cause of morbidity and mortality in humans [1]. The worldwide increase in antibiotic resistance has become a serious problem within the last 20 years [2]. In Europe, rates of resistance to penicillin G vary substantially among countries. For example, high rates of penicillin G resistance have been reported from France and Spain, whereas significantly lower resistance rates are documented in Germany, Austria and Switzerland [3–5].

Among children aged <2 years, the incidence of invasive pneumococcal disease (IPD) ranges from c. 14 cases/100 000 in Germany and The Netherlands to >90 cases/100 000 in Spain. The vulnerability of children to *S. pneumoniae* can also be demonstrated by the high rate of sequelae (>20% in Germany) and the high mortality rate (7.5%) for pneumococcal meningitis. Furthermore, in addition to penicillin G, antibiotic resistance among *S. pneumoniae* isolates is increasing in Europe, particularly in France, Spain and eastern European countries, whereas Germany and northern European countries appear to be less affected. A seven-valent pneumococcal conjugate vaccine (7vPCV), which has been shown to be highly efficacious in preventing IPD among infants in the USA, was licensed for use in Europe during 2001. It is expected that widespread use of the vaccine will

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reduce the incidence of invasive pneumococcal disease and the levels of pneumococcal resistance significantly [6].

Multilocus sequencing typing (MLST) is a molecular typing method that provides unambiguous data concerning seven housekeeping genes that can be transmitted electronically [7,8]. MLST has the major advantage that any laboratory can compare the sequences of the seven loci of isolates with those in a central database (<http://www.mlst.net>), and thereby obtain the corresponding allelic profile. The present study used this technique to analyse the genetic relatedness of penicillin G-resistant *S. pneumoniae* isolates from a population-based study of IPD among children in Germany.

## MATERIALS AND METHODS

### Study design

The study was based on active surveillance in Germany of IPD in the population aged <16 years. Children were enrolled in the study if they had been admitted to a paediatric hospital and if *S. pneumoniae* had been isolated from at least one culture of blood, cerebrospinal fluid or a sample from any other normally sterile body site. Cases were identified through two independent surveillance systems: a hospital-based system (including all paediatric hospitals in Germany) and a laboratory-based system (including all microbiological laboratories cooperating with paediatric hospitals). The paediatric hospitals and microbiological laboratories were asked, on a monthly basis, for data concerning cases of IPD; case reports from both systems were validated by questionnaires. The participating laboratories were requested to send pneumococcal isolates to the German National Reference Centre for Streptococci (NRCS), where the species diagnosis was confirmed and serotyping and antimicrobial susceptibility testing were performed [9].

### Susceptibility testing

MICs were determined using the broth microdilution method recommended by the CLSI [10]. Microtitre plates containing penicillin G, cefotaxime, erythromycin A and clindamycin were used with cation-adjusted Mueller–Hinton broth (Oxoid, Wesel, Germany) containing lysed horse blood (Oxoid) 5% v/v and a final inoculum of  $5 \times 10^5$  CFU/mL. MICs were determined following incubation at 35°C for 24 h in ambient air. *S. pneumoniae* ATCC 49619 was used as a control strain. Current CLSI interpretive criteria were used to define antimicrobial resistance [10]. Isolates were stored at -70°C on porous beads (Microbank; Mast Diagnostics, Rheinfeld, Germany).

### Serotyping

Pneumococcal isolates were serotyped by Neufeld's Quellung reaction using type and factor sera provided by the Statens Serum Institut (Copenhagen, Denmark).

### MLST

A subgroup ( $n = 58$ ) of all ( $n = 94$ ) penicillin G non-susceptible isolates was selected by randomisation of the sequential database entry number of penicillin G non-susceptible isolates received between 1997 and 2004. MLST was performed as described previously. In brief, 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 and Spratt [8]. The alleles at each of the seven loci provide the allelic profile for each isolate, and also define their sequence type (ST). Allelic profiles are reported as the alleles at each of the seven loci, in the order *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*. The allelic profiles of the German isolates were compared with each other and with other isolates in the pneumococcal MLST database (<http://www.mlst.net>). Additional MLST data for control strains were also obtained from the database.

### Phylogenetic analysis

For phylogenetic analysis, the sequences of the *aroE*, *gdh*, *gki*, *recP*, *spi* and *xpt* gene fragments were concatenated using the concatenation tool available at <http://www.mlst.net>. As recommended, the *ddl* gene was excluded from analysis, since it appears to be highly variable in penicillin-resistant isolates [11]. The clonal relatedness of the 37 different MLSTs, as well as the relatedness of these 37 MLSTs to all other MLSTs in the database, was investigated using the eBURST program (<http://spneumoniae.mlst.net/eburst/>) [12]. The concatenated sequences (2715 bp) were also aligned with the multiple sequence alignment tool ClustalW. The PUZZLE program was used to create a phylogenetic tree from the multiple alignment by maximum-likelihood analysis [13]. All programs were obtained from the HUSAR program package of the Biocomputing Service Group (<http://genius.dkfz-heidelberg.de>).

### Statistical analysis

Linear regression analysis was performed using SPSS v.14.0 (SPSS Inc., Chicago, IL, USA). Chi-square tests were performed using the chi-square webtool of Georgetown University (<http://www.georgetown.edu/>). All serotypes represented by four or fewer non-susceptible isolates were grouped together in the 'other' category. In addition, the resistance ratio was calculated by dividing the number of non-susceptible isolates of each serotype by the total number of isolates of the same serotype. This resistance ratio can generate values between 0% and 100%; if there is no association between serotype and susceptibility to penicillin, all resistance ratio values should be similar.

## RESULTS

Between 1997 and 2004, 1517 isolates of *S. pneumoniae* were collected consecutively by 188 of 245 laboratories participating in the study. These isolates were from blood ( $n = 966$ ; 63.7%), cerebrospinal fluid ( $n = 505$ ; 33.3%) and other normally sterile body sites ( $n = 46$ ; 3.0%). Antibiotic resistance data are summarised in Table 1. Of the

**Table 1.** Antibiotic resistance of 1517 *Streptococcus pneumoniae* isolates from children with invasive pneumococcal disease in Germany between 1997 and 2004

Antibiotic	MIC (mg/L)			Susceptible (S) n (%)	Intermediate (I) <sup>a</sup> n (%)	Resistant (R) <sup>a</sup> n (%)
	Range	50%	90%			
Penicillin G	0.008–4	0.016	0.03	1423 (93.8)	77 (5.1)	17 (1.1)
Cefotaxime	0.008–2	0.016	0.03	1505 (99.2)	9 (0.6)	3 (0.2)
Erythromycin A	0.016–32	0.06	8	1155 (76.1)	3 (0.2)	359 (23.7)
Clindamycin	0.016–32	0.06	0.125	1424 (93.9)	4 (0.3)	89 (5.9)

<sup>a</sup>Breakpoints according to the CLSI [10]: penicillin G, 0.12–1 mg/L (I), ≥2 mg/L (R); cefotaxime (meningitis), 1 mg/L (I), ≥2 mg/L (R); cefotaxime (non-meningitis), 2 mg/L (I), ≥4 mg/L (R); erythromycin A, 0.5 mg/L (I), ≥1 mg/L (R); clindamycin, 0.5 mg/L (I), ≥1 mg/L (R).

1517 isolates, 94 showed reduced susceptibility to penicillin G (6.2%), of which 17 (1.1%) were highly-resistant. Erythromycin A and clindamycin resistance was observed in 23.7% and 5.9% of isolates, respectively. During the study period, a significant increase in both penicillin G (MIC ≥2 mg/L; *p* 0.008) and erythromycin A (MIC ≥1 mg/L; *p* 0.002) resistance was observed. The rate of penicillin G non-susceptible isolates (MIC ≥0.1 mg/L; *p* 0.073) rose from 1.4% in 1997 to 8.7% in 2004. It is of note that 3.5% of strains were penicillin G-resistant (≥2 mg/L) in 2004, whereas no resistant isolates were obtained in 1997.

Serotyping of the penicillin non-susceptible isolates identified 13 different serotypes, with serotypes 14 (24.5%), 6B (16.0%) and 23F (16.0%) being predominant. The coverage of the seven-valent pneumococcal conjugate vaccine was 77% for penicillin non-susceptible isolates (Table 2). Comparison of the calculated resistance ratios for all serotypes showed that the values differed

**Table 2.** Serotype distribution of 94 penicillin non-susceptible and 1423 penicillin-susceptible *Streptococcus pneumoniae* isolates from invasive pneumococcal disease in Germany between 1997 and 2004

Serotype	Penicillin non-susceptible n (%) of non-susceptible isolates	Penicillin-susceptible n (%) of all susceptible isolates	Total (n)	Resistance ratio <sup>a</sup> (%)
14	23 (24.5)	370 (26.0)	393	6
23F	15 (16.0)	98 (6.9)	113	13
6B	15 (16.0)	89 (6.3)	104	14
19F	11 (11.7)	89 (6.3)	100	11
19A	9 (9.6)	41 (2.9)	50	18
9V	8 (8.5)	49 (3.4)	57	14
9A	4 (4.3)	17 (1.2)	21	19
15A	2 (2.1)	13 (0.9)	15	13
29	1 (1.1)	0	1	100
15C	1 (1.1)	8 (0.6)	9	11
23B	1 (1.1)	3 (0.2)	4	25
6A	1 (1.1)	60 (4.2)	61	2
7F	1 (1.1)	91 (6.4)	92	1
NT	1 (1.1)	3 (0.2)	4	25
Rough	1 (1.1)	0	1	1
Others	0	492 (34.6)	492	0
Total	94 (100)	1423 (100)	1517	6

NT, non-typeable.

<sup>a</sup>Number of non-susceptible isolates of a serotype/total number of isolates belonging to the same serotype.

enormously, implying that there is an association between serotype and penicillin resistance (Table 2). Analysis using chi-square tests showed that serotype and antibiotic susceptibility were associated at a highly significant level (chi-square 66.6; degree of freedom 6; *p* ≤0.001).

MLST was performed on 58 randomly selected penicillin G non-susceptible isolates and identified 38 different STs. Interestingly, one serotype 14 isolate (RKI 1158) showed an atypical ST, with unusual mutations in all seven alleles investigated. ST 557 (a single-locus variant of the Spain<sup>9V</sup>-3 clone; 8.6% of isolates) and the closely related ST 156 (Spain<sup>9V</sup>-3 clone; 8.6%) were the STs found most commonly among the group of 58 selected isolates (Table 3). The allelic profiles of 17 new penicillin G-resistant 'German' isolates are listed in Table 4.

**Table 3.** Distribution of multilocus sequence types (STs) of 58<sup>a</sup> penicillin non-susceptible *Streptococcus pneumoniae* isolates in Germany between 1997 and 2004

ST	Serotype	Clone designation <sup>b</sup>	n	%
13	14	SLV England <sup>14</sup> -9 clone	1	1.7
15	14	SLV England <sup>14</sup> -9 clone	1	1.7
30	23F		1	1.7
63	15A	Major global clone Sweden <sup>15A</sup> -25	1	1.7
81	9V, 23F	PMEN global clone Spain <sup>23F</sup> -1	3	5.2
135	6B, 23F		3	5.2
142	23F		2	3.4
143	14		1	1.7
156	9A, 14	Pen-R Spain <sup>9V</sup> -3 clone	5	8.6
230	19A	PMEN global clone 'Clone 32'	2	3.4
276	19A, 19F		2	3.4
277	6B, 23F		3	5.2
315	6B	PMEN global clone Poland <sup>6B</sup> -20	3	5.2
334	9A	SLV of penR Spain <sup>9V</sup> -3 clone	1	1.7
337	23F		1	1.7
339	19F		2	3.4
357	23F		1	1.7
359	23F		1	1.7
440	23F		1	1.7
550	14		1	1.7
557	9V, 14	SLV of Pen-R Spain <sup>9V</sup> -3 clone	5	8.6
New			17	29.3
Total			58	100

SLV, single-locus variant.

<sup>a</sup>The 58 isolates were selected randomly for MLST from among the total of 94 penicillin G non-susceptible isolates (see Table 3) and reflect the serotype distribution among the 94 isolates.

<sup>b</sup>See homepage of the Pneumococcal Molecular Epidemiology Network (PMEN) (<http://www.sph.emory.edu/PMEN>).

**Table 4.** New multilocus sequence types (STs) found among 58 penicillin non-susceptible *Streptococcus pneumoniae* isolates from invasive pneumococcal disease in German children between 1997 and 2004

Isolate	ST	Serotype	Alleles						
			<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>
RKI 381	1087	19F	43	19	9	17	6	22	14
RKI 852	1190	6A	7	25	4	4	15	20	14
RKI 810	1444	23F	15	29	4	21	30	1	8
RKI 1205	1545	19F	2	5	36	16	1	21	14
RKI 372	1546	19A	2	13	2	5	6	26	8
RKI 627	1548	6B	2	22	4	5	27	4	14
RKI 830	1549	14	2	29	36	12	13	21	14
RKI 1110	1556	14	7	11	10	12	6	8	1
RKI 1117	1557	9A	7	11	10	17	6	8	14
RKI 1019	1565	19A	8	5	29	12	9	3	6
RKI 271	1576	15A	10	8	8	8	6	28	14
RKI 1157	1583	19F	15	16	19	15	6	20	71
RKI 176	1587	19A	8	19	2	17	25	5	168
RKI 662	1588	29	69	25	1	4	15	1	28
RKI 1083	1589	7F	8	9	2	1	6	1	167
RKI 1162	1590	6B	7	101	54	16	6	1	14
RKI 1158 <sup>a</sup>	ND	14	4V	20V	49V	40V	55V	51V	12V

<sup>a</sup>Atypical *S. pneumoniae* isolated from blood of a child aged 11 months. The isolate was an optochin-susceptible and bile-soluble isolate of serotype 14, but showed variations in all seven alleles.  
V, variant; ND, no data.

An eBURST analysis of all isolates available in the MLST database revealed that five isolates (ST 143, ST 156, ST 334, ST 557 and ST 1557) were members of a large clonal complex of 97 STs, of which ST 156 is the predicted founder. Of the clones described in the complex, ST 143, ST 156, ST 334, ST 557 and ST 1557 were all found in the present study. In addition, ST 30, ST 63, ST 81, ST 135, ST 230 and ST 315 were also predicted founders of clonal complexes. ST 142, ST 277, ST 337, ST 339 and ST 359 were revealed to be members of a clonal complex of 89 STs, with ST 176 as the predicted founder. The other STs found among the isolates in this study were members of smaller complexes or existed as singletons.

The above observations were confirmed by analysis of a dendrogram constructed using maximum likelihood and the PUZZLE program (data not shown). Analysis showed a large genetic diversity of isolates and confirmed that isolate RKI 1158, with the atypical ST, was an outlier.

## DISCUSSION

An increasing level of both penicillin G and erythromycin A resistance was observed in the present study of 1517 *S. pneumoniae* isolates from children with IPD in Germany. During the 8-year study period there was a greater than six-fold increase in penicillin G resistance, and an almost

three-fold increase in macrolide resistance. Although the development of resistance among IPD isolates from children parallels that found in adults and for non-invasive disease isolates in Germany, the overall level of resistance among children seems to be higher [5,14]. However, the resistance level is still markedly lower than that reported in southern and eastern European countries [4].

MLST is now used increasingly to analyse the clonal relatedness of pneumococci [8,15–17]. The present study is the first in Germany to apply this new technique to a relatively complete and representative group of penicillin non-susceptible pneumococcal isolates, collected as part of a population-based nationwide surveillance programme [9]. ST 557 (single-locus variant of Spain<sup>9V</sup>-3 clone), the closely related ST 156 (Spain<sup>9V</sup>-3 clone) and ST 81 (Spain<sup>23F</sup>-1 clone) were the predominant STs among these isolates. Strains belonging to ST 156 were first isolated during the late 1980s in Spain, and are now distributed worldwide (<http://www.mlst.net>). In addition to being a carriage strain, ST 156 has also been isolated from cases of bacteraemia and meningitis in many countries, including Spain, the UK, Denmark, Poland, Uruguay, Canada, The Netherlands, France, the Czech Republic, Israel and, recently, New Zealand [18]. Using eBURST analysis, the results of the present study predicted that ST 156 was the founder of a clonal group of pneumococcal isolates prevalent in Germany. However, Spratt *et al.* [19] suggest that ST 162 is most probably the founder of this clonal complex, since single-locus variants of ST 156 have been greatly over-sampled.

In contrast, ST 557, which was the clone found most commonly in the present study, has been isolated previously only in Sweden (serotype 19F) and Sri Lanka (serotype 6B, penicillin G-susceptible). In addition, the Spain<sup>23F</sup>-1 clone, which contributes significantly to the worldwide spread of penicillin and multiple antibiotic resistance in *S. pneumoniae* [16,20–22], is also found in 5.2% of penicillin non-susceptible invasive infections among children in Germany. However, the present study revealed 38 different clones among the 58 isolates examined, including 17 clones that have been reported, to date, only in Germany. Analyses of the clonal relatedness by eBURST and construction of the phylogenetic tree by maximum-likelihood analysis highlighted the clonal

diversity of penicillin G non-susceptible *S. pneumoniae* isolates in Germany, and suggested that these strains probably resulted from multiple independent selection events. Such variability seems to be typical for *S. pneumoniae* isolates, with the exception of some countries, e.g., Spain, where clones Spain<sup>9V</sup>-3 and Spain<sup>23F</sup>-1 constitute nearly all antibiotic non-susceptible pneumococcal isolates [15,17,23].

Pneumococcal conjugate vaccines now offer the possibility of preventing IPD in children. A seven-valent pneumococcal conjugate vaccine (containing serotypes 4, 6B, 9V, 14, 18C, 19F and 23F), which has been reported to be immunogenic and efficacious in infants, was licensed in Europe during 2001 [6,24]. In Germany, this vaccine is currently recommended only for children with an increased risk of IPD, and <10% of children aged  $\leq 2$  years are vaccinated. The present study revealed a high coverage (77%) of penicillin non-susceptible isolates by this vaccine. Moreover, recent data from the USA have shown that use of this vaccine can be a powerful strategy for reducing antibiotic resistance in a community [25]. However, the present study also found that a significant amount of penicillin resistance in Germany was associated with serotype 19A, a serotype that is covered only partially by the seven-valent pneumococcal conjugate vaccine, suggesting that the inclusion of serotype 19A in future vaccine formulations is required.

In summary, the present study revealed a significant increase in antibiotic resistance among IPD isolates from German children. MLST proved to be a powerful tool for analysing the molecular background to the development of resistance, and for documenting the genetic diversity of pneumococcal clones. Restricted use of antibiotics and wider use of the seven-valent pneumococcal conjugate vaccine may be an effective approach for combating the future spread of antibiotic resistance among *S. pneumoniae* strains in Germany.

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