Identification, prevalence and population structure of non-typable *Streptococcus pneumoniae* in carriage samples isolated from preschoolers attending day-care centres

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The authors aimed to get insights into the population structure of non-(sero)typable pneumococci (NTPn), a specific group of natural atypical pneumococci whose identification is often difficult, and which has remained insufficiently studied. A total of 265 presumptive NTPn, isolated between 1997 and 2003 from the nasopharynx of children, were characterized. Strains were confirmed to be pneumococci on the basis of bile solubility, and PCR detection or Southern blotting hybridization of *lytA* and *psaA*, genes ubiquitous in this species. Multilocus sequence typing (MLST) was used to exclude two isolates that gave ambiguous results. Non-typability was confirmed by the Quellung reaction using Omniserum. A total of 213 isolates were considered to be true NTPn. The molecular analysis of the true NTPn by PFGE and MLST showed that this population was genetically diverse, although a dominant cluster, accounting for 66 % of the isolates, was identified. Antimicrobial resistance was observed in most genetic backgrounds, and multidrug resistance to penicillin, erythromycin, clindamycin, tetracycline and sulfamethoxazole-trimethoprim was associated with strains belonging to the dominant cluster. Comparison with PFGE fingerprints and sequence types of large collections of serotypable strains showed that the genetic backgrounds of all but two NTPn were different from those found in serotypable strains. In addition, we found that NTPn strains with similar genetic backgrounds to those identified in our study had been isolated from disease sources in other countries. These observations seem to indicate that NTPn have diverse genetic backgrounds and may have evolved as a distinct group of pneumococcal isolates.

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INTRODUCTION

Streptococcus pneumoniae is a respiratory pathogen that colonizes asymptotically the nasopharynx of humans. Although the mortality and morbidity associated with pneumococci are high, they are considered to be facultative pathogens: colonization is the rule, and disease the exception (Centers for Disease Control and Prevention, 2000).

Most pneumococci are shielded by a polysaccharide capsule, a major virulence factor that hinders phagocytosis. Up to now, at least 90 different capsular types have been described (Henrichsen, 1995). The capsule can be detected by reaction with specific antisera. However, although some isolates are

presumptively identified as pneumococci by routine identification tests, a negative result with type-specific antisera for the 90 serotypes is obtained. These atypical isolates are designated non-typable pneumococci (NTPn), and their correct discrimination from commensal viridans streptococci is often difficult (Carvalho *et al.*, 2003; Hanage *et al.*, 2005; Shayegani *et al.*, 1982; Whatmore *et al.*, 2000). The distinction between these closely related species is clinically important, since *S. pneumoniae* can cause serious disease and viridans streptococci are commensal organisms.

Some NTPn have been described as extremely contagious and have been associated with pneumococcal conjunctivitis, causing either large outbreaks (Centers for Disease Control and Prevention, 2002, 2003; Crum *et al.*, 2004; Ertugrul *et al.*, 1997; Martin *et al.*, 2003; Shayegani *et al.*, 1982) or sporadic cases of this disease (Barker *et al.*, 1999; Berron *et al.*, 2005). Occasionally, NTPn have been implicated in other types of

Abbreviations: MLST, multilocus sequence typing; NTPn, non-typable pneumococci; PMEN, Pneumococcal Molecular Epidemiology Network; SLV, single-locus variant; DLV, double-locus variant; SSI, Statens Serum Institute; ST, sequence type.

pneumococcal disease, such as otitis, respiratory infections, and invasive disease (Berron *et al.*, 2005; Finland & Barnes, 1977; Hathaway *et al.*, 2004). The frequency of NTPn among pneumococci isolated from sterile and non-sterile sites has been estimated as 0.5–2.2 % (Carvalho *et al.*, 2003; Finland & Barnes, 1977) and 10 % (Carvalho *et al.*, 2003), respectively.

Until 1997, no systematic study of the genetic relatedness of NTPn had been reported (Ertugrul *et al.*, 1997). Since then, a few studies have described the NTPn lineages associated with conjunctivitis episodes, and successful persistent lineages have been identified (Berron *et al.*, 2005; Martin *et al.*, 2003). In addition, a recent report from Hathaway and co-workers characterizes 27 NTPn isolates from various sources and provides important insights into the molecular nature of non-serotypeability in the lineages described (Hathaway *et al.*, 2004). Finally, Hanage and co-workers have applied multilocus sequence typing (MLST) to a collection of 121 presumptive NTPn and have found the technique useful to distinguish true NTPn from closely related species (Hanage *et al.*, 2005).

In this work, we aimed to obtain further insights into the population structure of NTPn. We have used PFGE and MLST to systematically characterize over 200 isolates of NTPn collected between 1997 and 2003 from the nasopharynx of day-care centre attendees. To our knowledge, this is the largest study conducted so far on the molecular characterization of NTPn, and the second (Hathaway *et al.*, 2004) that has focused on strains recovered from their natural ecological niche, the nasopharynx.

METHODS

Bacterial strains. Pneumococcal strains used in this study were isolated between 1997 and 2003 from the nasopharynx of preschool children attending day-care centres in Lisbon, Portugal. Identification of pneumococci was routinely done on the basis of: (i) selective growth in tryptic soy agar containing 5 % defibrinated sheep blood supplemented with 5 mg gentamicin l⁻¹ (BBL) incubated overnight in anaerobic jars at 37 °C; (ii) colony morphology; (iii) α -haemolytic activity; and (iv) optochin susceptibility after incubation in a 5 % CO₂ atmosphere (Rouff *et al.*, 2003). If decreased optochin susceptibility was observed, bile solubility was determined.

Bile solubility. This assay was performed according to standard procedures (Rouff *et al.*, 2003).

Detection of *lytA* and *psaA*. PCR detection of the genes encoding the major pneumococcal autolysin (*lytA*) and pneumococcal surface adhesin A (*psaA*), both ubiquitous in pneumococci, was carried out using primers and conditions previously described (Messmer *et al.*, 1997; Morrison *et al.*, 2000). Briefly, primers A750 (5'-GGC TAC TGG TAC GTA CAT TC-3') and A1145 (5'-AAT CAA GCC ATC TGG CTC TA-3') were used to amplify a 395 bp internal fragment of *lytA* (Messmer *et al.*, 1997), and primers P1 (5'-CTT TCT GCA ATC ATT CTT G-3') and P2 (5'-GCC TTC TTT ACC TTG TTC TGC-3') were used to amplify an 838 bp internal fragment of *psaA* (Morrison *et al.*, 2000).

Immunological serotyping. Capsular typing was done by using the chessboard system (Sorensen, 1993) using specific antisera from the Statens Serum Institute (SSI, Copenhagen). Omniserum (SSI,

Copenhagen), a serum that contains antibodies to all known pneumococcal types, was used to confirm non-typeability. In addition, a representative of each non-typable clone identified by PFGE (see below) was sent to Dr Margit Kaltoft at the SSI to confirm non-typeability.

Antimicrobial susceptibility testing. Testing of susceptibility to chloramphenicol, erythromycin, clindamycin, tetracycline and sulfamethoxazole-trimethoprim was performed using the Kirby–Bauer technique, according to the National Committee for Clinical Laboratory Standards recommendations and definitions (National Committee for Clinical Laboratory Standards, 2004). MICs of penicillin were determined with the Etest (AB Biodisk) according to the manufacturer's instructions.

PFGE. Preparation of chromosomal DNA, digestion with *Sma*I endonuclease and separation of DNA fragments by PFGE were carried out as previously described (Sá-Leão *et al.*, 2000a). PFGE patterns were analysed with Bionumerics software (version 3.0, Applied Maths, Gent, Belgium). Patterns were clustered by UPGMA, and a dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 1.0 % and a tolerance of 1.5 %. PFGE clusters were defined as isolates with a similarity of 80 % or higher on the dendrogram (Gertz *et al.*, 2003).

PFGE patterns were compared with patterns of serotypable pneumococcal strains isolated between 1996 and 2003 in the same settings (Nunes *et al.*, 2005; Sá-Leão *et al.*, 2000b; Sousa *et al.*, 2005), and with PFGE patterns of the 26 reference strains included in the Pneumococcal Molecular Epidemiology Network (PMEN) (McGee *et al.*, 2001), (<http://www.sph.emory.edu/PMEN/>, last accessed September 2005).

PFGE letter assignments were given arbitrarily, and two or three capital letters were assigned to each PFGE clone. If a clone had been identified in a previous study, the same letter assignment was maintained to enable direct comparison with previous publications.

MLST. MLST was performed using primers and conditions described elsewhere (Enright & Spratt, 1998), except for two primers that were designed by us in order to obtain better amplification results: 5'-GTT CCA TTT TCA ACC AAG GC-3' (rec2-dn), which was used instead of rec-dn, and 5'-AGA GTG GGG ATT ATT CCT CC-3' (spi2-up), which was used instead of spi-up. These two new primers were used to overcome the difficult PCR amplifications observed for some isolates, in which DNA did not anneal adequately with the original rec-dn and spi-up primers. Sequencing was conducted at MacroGen, Inc. (Seoul, Korea).

In the interpretation of results, isolates sharing at least five of the seven loci that define the allelic profile were assumed to be genetically related and thus to belong to the same group (Brueggemann *et al.*, 2003; Sá-Leão *et al.*, 2001). Correlation between the sequence types (STs) of novel clones with STs deposited in the *S. pneumoniae* database was performed using eBURST version 2 (<http://eburst.mlst.net/2.asp>) (Feil *et al.*, 2004).

RESULTS

Presumptive isolation of non-typable *S. pneumoniae*

Between 1997 and 2003, 6274 pneumococcal-positive cultures were obtained from nasopharyngeal samples of children attending day-care centres in the Lisbon area. Routinely, only one colony of *S. pneumoniae* was purified from each nasopharyngeal sample. The culture obtained was used for antibiotic determination and was frozen

for further studies. However, if more than one type of pneumococcus-like colony was present on the primary-isolation agar plate, the different types were isolated in pure cultures, characterized by antibiotype, and frozen. During these studies, serotyping by the Quellung reaction was performed for 3585 isolates, 2496 of which were drug resistant. We found that 7.4 % of the isolates serotyped could not be assigned a capsular type using the commercially available sera from the SSI. These strains were thus identified as presumptive non-typable pneumococci, and their characterization was the subject of this study.

Confirmation of presumptive non-typable pneumococci (NTPn) as true pneumococci

All 265 presumptive NTPn were assayed for bile solubility and by PCR for the detection of *lytA* and *psaA* genes. Of these, eight strains were bile-solubility-test negative and PCR internal fragments of *lytA* and *psaA* could not be obtained (group I in Table 1). These strains were excluded from the study. They probably belong to other streptococcal species. Two-hundred and twenty four strains were bile soluble, *lytA*⁺ and *psaA*⁺, and were considered to be true pneumococci (Table 1, group II). The remaining 33 isolates gave ambiguous results, and Southern hybridizations with *lytA*- or *psaA*-specific probes were performed as appropriate. These enabled the additional confirmation of 31 isolates as pneumococci (Table 1, groups III and IV). The two isolates described in groups V and VI were excluded from the study, since, in addition to the atypical results obtained, by MLST all alleles were novel and had low sequence similarities (ranging from 93 to 97 %) compared to the ones described to date in the MLST database.

Confirmation of non-serotypeability

The 255 true pneumococci were then retested for non-serotypeability using Omniserum, a serum that contains antibodies to all known pneumococcal capsular types. Eighty-four percent of the true pneumococci were confirmed to be non-typable, i.e. in the presence of Omniserum, no capsule could be visualized in a phase-contrast

microscope and no agglutination of the bacteria occurred. However, 42 isolates were positive in this assay. Indeed, we were able to assign a capsular type to each of them by using the chess board system proposed by Sorensen (1993). The capsular types detected were: 3 (13 strains), 11A (two strains), 14 (six strains), 16F (three strains), 19F (four strains), 21 (one strain), 23F (three strains), 29 (two strains), 37 (one strain), 38 (six strains) and 39 (one strain).

With these data, we were able to estimate the true prevalence of NTPn among total pneumococci: it was on average 5.9 %, and ranged between 3.8 % in 1997 and 11 % in 1999.

Molecular analysis of true NTPn

Of the initial 265 presumptive NTPn, 213 were confirmed as true NTPn and these were all analysed by PFGE. A total of 17 PFGE clusters were identified, five of which were represented by single isolates (Fig. 1). A major cluster designated NNN accounted for 48 % of all NTPn isolates; the remaining ones had prevalences ranging from 0.5 to 13.1 %.

MLST analysis was done for at least two isolates of each PFGE cluster that had two or more isolates and for the five isolates which represented single genetic lineages. Overall, 49 isolates were typed by MLST. Eight novel alleles were identified (*aroE*, 70 and 71; *gdh*, 98; *gki*, 114; *spi*, 107; *xpt*, 151; *ddl*, 164 and 170) and submitted for approval to the MLST database, and eight novel STs (1540, 1541, 1617, 1618, 1619, 1703, 1704 and 1705) were assigned.

MLST divided the strains into 17 STs, which belonged to ten groups, as defined in Methods (Fig. 1). With a single exception (one strain classified as PFGE NB, ST1705), all STs assigned to strains belonging to a given PFGE cluster were either identical, or single-locus variants (SLVs) or double-locus variants (DLVs) of each other, and thus MLST confirmed the PFGE relatedness of those strains. However, MLST was much less discriminatory than PFGE, since only ten groups of strains were defined, compared to the 17 clusters obtained by PFGE. For example, in the most extreme case, a single sequence type, ST344, was associated

Table 1. Properties of presumptive non-typable pneumococci

+, Positive; –, negative; ND, not determined.

Group	No. of isolates	Bile solubility	PCR result		Southern hybridization result	
			<i>lytA</i>	<i>psaA</i>	<i>lytA</i>	<i>psaA</i>
I	8	–	–	–	ND	ND
II	224	+	+	+	ND	ND
III	30	+	+	–	ND	+
IV	1	+	–	+	+	ND
V	1	+	–	–	+	+
VI	1	–	–	+	–	ND

*Weak result.

with as many as six PFGE clusters: NNN, DV, BH, BF, NI and EA (Fig. 1). Based on MLST classification alone, the most prevalent group (group 1, associated with STs 344, 897, 1541 and 1619) accounted for 66 % of all isolates. The prevalence of other groups ranged from 0.5 to 13.6 %.

Comparison of NTPn PFGE fingerprints with those of serotypable strains

PFGE patterns of all NTPn isolates were then compared with the entire in-house PFGE database deposited in Bionumerics, which contains patterns of over 2500 carriage strains

collected between 1996 and 2003, and includes both drug-resistant and drug-susceptible isolates of 50 different serotypes. In addition, our database also includes the PFGE patterns of the 26 PMEN clones currently available.

Of the 17 PFGE clusters identified among the 213 NTPn, only PFGE type B (detected in a single strain) was also identified among serotypable strains; the remaining 16 clusters were unique to NTPn. Interestingly, the solitary strain of PFGE type B was associated with ST156, and both by PFGE and MLST was identified as a non-typable representative of clone Spain^{9V}-3.

Fig. 1. PFGE dendrogram and ST information for non-typable pneumococci. Dice coefficient values (percentages) are indicated in the scale above the dendrogram. An 80% cut-off was used to define PFGE clones. Capital letters refer to PFGE nomenclature. Numbers and triangles at the right-hand end of branches indicate the number of isolates with 100% similar PFGE pattern. STs are indicated as well as clonal group assignments.

International dissemination of NTPn identified in Portugal

We searched the MLST database (www.mlst.net, last accessed September 2005) for information on all strains

deposited that had the same STs as those identified in our study or had been assigned SLVs or DLVs of those STs. The data were then plotted using eBURST version 2 and relevant epidemiological data were retrieved (Table 2). This comparison also supported the observation that the genetic

Table 2. Groups and STs of non-typable pneumococcal strains

The table shows the relationship between nasopharyngeal Portuguese isolates and other epidemiologically unrelated strains. Group assignment is as defined in Methods. Data from countries other than Portugal were obtained from the MLST database (www.mlst.net) and from references cited in the text. NT, non-typable.

Group	eBURST graphic representation	ST	Country	Year	Source or diagnosis	Serotype
1		344	Norway	1996	Blood	NT
		344	Australia	1998	Blood	NT
		344	Poland	2001	Nasopharynx	NT
		344	Spain	Not available	Eye	NT
		344	Switzerland	Not available	Nasopharynx	NT
		344	Portugal	1997–2003	Nasopharynx	NT
		449	UK	2000	Nasopharynx	NT
		897	Portugal	1999	Nasopharynx	NT
		1054	Finland	Not available	Nasopharynx	NT
		1270	USA	2003	Conjunctivitis	NT
		1541	Portugal	1998	Nasopharynx	NT
		1619	Portugal	2001	Nasopharynx	NT
2	1540	1540	Portugal	2001	Nasopharynx	NT
3		941	Spain	2001	Eye	NT
		941	Portugal	1999	Nasopharynx	NT
		942	Spain	2000	Eye	NT
		943	Spain	1998	Eye	NT
		1704	Portugal	2001	Nasopharynx	NT
4		448	UK	2000	Nasopharynx	NT
		448	Switzerland	Not available	Blood/nasopharynx	NT
		448	Portugal	2002	Nasopharynx	NT
		1229	Finland	Not available	Not available	NT
		1290	Finland	Not available	Not available	NT
5	1618	1618	Portugal	2001	Nasopharynx	NT
6		196	Spain	1998	Meningitis	19A
		202	Spain	1997–1998	Meningitis	19A
		888	Portugal	2001	Nasopharynx	NT
		890	Portugal	2002	Nasopharynx	19A
7 (clone Spain ^{9V} -3)		156 and at least 35 other STs	Worldwide dissemination	Has been in circulation for over 25 years	Several	Originally 9V but several others described
8		1153	Portugal	2002	Nasopharynx	NT
		1156	Portugal	2002–2003	Nasopharynx	NT
		1617	Portugal	2001	Nasopharynx	NT
		1703	Portugal	2001	Nasopharynx	NT
		1278	Portugal	2001–2003	Nasopharynx	NT
9	1278	1278	Portugal	2001–2003	Nasopharynx	NT
10		1182	UK	2003	Not available	NT
		1705	Portugal	2002	Nasopharynx	NT

backgrounds of the Portuguese NTPn are exclusive to non-typable strains and by PFGE and MLST have no close genetic relationship with serotypable strains. The two exceptions were the strain of ST156 (already described above) and a single additional strain of ST888: STs related to the latter

were found to be associated with strains of capsular type 19A isolated in Portugal and Spain. In addition, five out of the ten MLST groups identified in our study have also been described among NTPn strains recovered in other countries in the last decade. The clinical sources and diagnoses

indicate that these NTPn strains have not only been isolated from carriage but have also been implicated in episodes of conjunctivitis, otitis and invasive disease (Table 2).

Epidemiology of Portuguese NTPn isolated from carriers

Integration of the molecular data with the antimicrobial-resistance pattern of the isolates and their geographic and temporal origin has shown that isolates belonging to MLST group 1 have been isolated since 1997 in all day-care centre studies conducted by us (Table 3). This dominant group, particularly strains of PFGE clone NNN, has been detected in several day-care centres, and the majority of the isolates were found to be multidrug resistant to penicillin (intermediate resistance), erythromycin, clindamycin, tetracycline and sulfamethoxazole-trimethoprim. Interestingly, in 2001, the number of genetic lineages of NTPn increased substantially, although the prevalence of NTPn among total pneumococci was not particularly high (4.9%). A few of these genetic lineages have persisted over time.

DISCUSSION

In this study, we aimed to get insights into the population structure of non-(sero)typable pneumococci, a specific group of atypical pneumococci that has remained insufficiently studied, so that its molecular nature, origin, epidemiology and importance are poorly understood. The few

studies described so far that have looked at the genetic backgrounds of these strains have focused on strains isolated from the conjunctiva (Berron *et al.*, 2005; Carvalho *et al.*, 2003; Crum *et al.*, 2004; Ertugrul *et al.*, 1997; Martin *et al.*, 2003), the exceptions being the work of Hathaway *et al.* (2004), which characterized 20 nasopharyngeal and seven blood isolates, and a recent study from Hanage *et al.* (2005). The latter study included 121 presumptive NTPn isolated either from the nasopharynx or from the middle ear fluid, and used MLST to unambiguously identify the true pneumococci. In these studies, strains that are highly transmissible have been identified, and at least two genetic lineages, defined by ST448 and ST344, were detected over several years or were isolated from patients in different countries (Berron *et al.*, 2005; Hanage *et al.*, 2005; Hathaway *et al.*, 2004; Martin *et al.*, 2003). In our study, we characterized 213 true NTPn, isolated over a period of seven years from the nasopharynx of healthy children attending day-care centres in the Lisbon area. We believe this is the largest collection of NTPn characterized so far.

We have found that presumptive identification of NTPn based on colony morphology, optochin susceptibility and a negative capsular serotype reaction led to the misclassification of 19% of the 265 presumptive NTPn initially identified. Ten isolates were not pneumococci, and 42 were serotypable strains. Misidentification of bacteria as NTPn may result from the detection of strains with atypical properties, the modulation of capsular expression,

Table 3. Epidemiology of Portuguese non-typable pneumococci isolated from carriers

Abbreviations: Pen, penicillin; Em, erythromycin; Cm, clindamycin; Tet, tetracycline; SXT, sulfamethoxazole-trimethoprim; I, intermediate resistance; R, resistant.

Clone (PFGE)	Group (MLST)	Prevalence of clone among total NTPn (%)	Most common antimicrobial resistance pattern	No. of samples recovered in each year						No. of day care centres
				1997	1998	1999	2001	2002	2003	
NNN	1	47.9	Pen (I), Em, Cm, Tet, SXT	2	9	28	39	16	8	19
BF	1	6.1	Pen (I), Em, Cm, Tet, SXT					1	12	3
BE	1	3.8	Pen (I), Em, Cm, Tet, SXT			7	1			2
DV	1	3.3	Pen (I), Em, Cm, Tet, SXT					6	1	1
NI	1	2.8	Pen (I), Em, Cm, Tet, SXT				2	4		2
BH	1	1.9	Pen (I), Em, Cm, Tet, SXT	3			1			2
EA	1	0.5	Pen (I), Em, Cm, Tet, SXT				1			1
CJ	2	0.5	Susceptible to all				1			1
CE	3	2.8	Susceptible to all			4	2			4
DZ	3	0.5	Susceptible to all			1				1
CB	4	13.1	Susceptible to all				8	16	4	6
NM	5	2.3	Pen (I), Em				5			1
Y	6	0.5	Pen (I), SXT				1			1
B	7	0.5	Pen (R), SXT				1			1
NC	8	8.0	Pen (I/R)				4	7	6	5
AI	8	1.4	Pen (I), SXT				3			2
NB	9	3.8	Pen (I), SXT				5	1	2	1
NB	10	0.5	Pen (I), SXT					1		1

or technical and experimental errors. In any case, additional tests should be performed to correctly identify NTPn.

In our study, the bile solubility assay correctly identified all but one of the strains as pneumococci. PCR detection of genes ubiquitous in pneumococci, particularly *lytA* (Messmer *et al.*, 1997), *psaA* (Morrison *et al.*, 2000) and *ply* (Cima-Cabal *et al.*, 1999), has been used as an alternative method for species identification. We found that, with the primers and conditions used, *lytA* detection performed better than *psaA*, in agreement with previous findings (Messmer *et al.*, 2004). However, since homologues of both genes have been detected in strains of closely related streptococcal species (Dowson, 2004; Jado *et al.*, 2001), we advocate, for each atypical strain, the detection of both genes, since the odds of having a non-pneumococcal isolate carrying *lytA* and *psaA* simultaneously will be smaller. In addition, to clarify negative results obtained by PCR, Southern blotting with specific probes has worked well.

MLST is a good technique to define pneumococci (Dowson, 2004; Hanage *et al.*, 2005), but is more expensive and time-consuming than any of the other methods that we evaluated. Even so, we found it useful to exclude two isolates that gave ambiguous results (groups V and VI of Table 1).

To confirm that true pneumococci are non-typable, re-serotyping after growth under conditions that favour capsular expression should be performed, since we found that on retesting the isolates, 16 % were actually serotypable. Similar results have been reported by others (Hathaway *et al.*, 2004). Omniserum, a polyvalent serum that contains antibodies to the 90 capsular types, was a fast alternative to confirm negative results. All positive results were later assigned a capsular type when the Quellung reaction was used with specific type sera.

After correct identification of NTPn, we concluded that the prevalence of NTPn in the pneumococcal-positive nasopharyngeal samples of children attending day-care centres was 5.9 %, when all collections (from 1997 to 2003) were pooled together. However, we believe that this value is probably an underestimate. In fact, in most surveillance studies such as ours, a single pneumococcal-like colony is routinely picked from a primary culture for further purification and characterization, although it is well known that mixed cultures are frequently found in nasopharyngeal samples (Hodges *et al.*, 1946; Sá-Leão *et al.*, 2002; St Sauver *et al.*, 2000). Since many NTPn exhibit rough and small colonies resembling those of viridans streptococci, they may be neglected when smooth and bigger colonies typical of capsulated pneumococci are also present. In our studies, when different colony morphologies were noted on the plates, one colony of each type was purified. However, these differences may not be obvious. Even so, 52 isolates of the current NTPn collection were isolated along with a capsulated strain from the same agar plates. In all cases, this mixed culture contained two different strains as determined by

PFGE analysis (data not shown). Whether this co-existence is of ecological significance is not currently known.

The molecular analysis of the 213 true NTPn by PFGE and MLST showed that this population is genetically diverse, although a dominant clone (named NNN) accounting for 48 % of the isolates was identified by PFGE. In addition, by MLST, this PFGE clone clustered together (group 1 in MLST analysis) with other PFGE clones, increasing its prevalence to 66 %. Interestingly, this dominant cluster has also been detected in several other countries during the last decade. Antimicrobial resistance was observed in 13 of the 17 genetic backgrounds (defined by PFGE), and accounted for 86 % of the strains. Multidrug resistance to penicillin, erythromycin, clindamycin, tetracycline and sulfamethoxazole-trimethoprim was associated with the dominant clonal group 1.

A recent study has suggested that some NTPn, including those of ST344 (clonal group 1), might have evolved from capsulated strains of serotypes 33 and/or 37 (Hathaway *et al.*, 2004), based on the observation that a DNA fragment homologous to *cpsN* of these serotypes was detected in these NTPn strains. In addition, Lull and co-workers observed that spontaneous non-typable variants of strains of serotypes 33F and 37 appeared upon *in vitro* cultivation (Lull *et al.*, 2000). However, in our work, comparison with PFGE fingerprints and STs of large collections of serotypable strains (which included serotypes 33F and 37) showed that the genetic backgrounds of all but one NTPn were different from those found in serotypable strains. Our results suggest that the Portuguese NTPn are not closely related to serotypable pneumococci, but rather may have evolved through time as a distinct group of isolates.

MLST analysis allowed us to conclude that five of the ten groups defined, representing 87 % of the NTPn collection, had STs associated with NTPn isolated in other countries and from a variety of clinical sources. These observations seem to indicate that the population structure of NTPn isolated from carriage is the same as the one isolated from disease, regardless of the specific genetic background.

The molecular mechanisms leading to the non-serotypability of the strains described in this study are currently being investigated. However, preliminary results suggest that at least part of the capsular polysaccharide operon (*cps*) may be absent from these strains, since repeated attempts to detect *cpsA* (a conserved gene of the *cps* operon) and other *cps* genes by a multiplex PCR technique (Brito *et al.*, 2003) have failed for all 213 strains described here. These results are in agreement with the finding that in NTPn strains identified by STs 344 and 448, a homologue of *aliB* (a peptide-binding molecule) has been found in the capsule region leading to loss of capsular expression (Hathaway *et al.*, 2004). Although the lack of capsule, considered by many the most important virulence factor, may appear a major disadvantage, *in vitro* studies have shown that strains lacking the capsule have higher adherence to epithelial cells

and higher transformability efficiencies (Weiser *et al.*, 1994; Weiser & Kapoor, 1999), two key features for colonization and microevolution.

The true role of NTPn in the wider framework of pneumococcal ecology is unknown, but recent evidence has highlighted that due to their increased transformability, these strains may be privileged vectors of horizontal gene transfer between viridans streptococci and typable pneumococci (Hauser *et al.*, 2004).

In summary, NTPn in Portugal have diverse genetic backgrounds which are not the same as the ones found among serotypable strains. NTPn are often drug resistant, and some clusters have been isolated over several years in different countries and from a variety of colonization and disease sources, including human sterile sites. Monitoring of such strains appears to be of importance. Further insights into the role of NTPn in pneumococcal ecology should be explored.

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