Journal of Antimicrobial Chemotherapy (2002) **50**, *Suppl. S2*, 75–81
DOI: 10.1093/jac/dkf511

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From ecological reservoir to disease: the nasopharynx, day-care centres and drug-resistant clones of *Streptococcus pneumoniae*

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Several lines of epidemiological and microbiological studies point to the multiple and critical roles of the nasopharynx of children—particularly those of pre-school age and attending day-care centres (DCCs)—in the emergence and spread of drug-resistant *Streptococcus pneumoniae* (DRP). A systematic yearly surveillance of the nasopharyngeal flora of children attending DCCs has been carried out in Lisbon since 1996. Molecular typing of several hundred DRP isolates showed that the great majority of DRP were represented by a relatively few clonal types that were frequently carried by many children in geographically distant DCCs and over several years of surveillance. The same epidemic DRP clones were also frequent among pneumococci causing both paediatric and adult disease worldwide. Penicillin-resistant pneumococci carry sequences of heterologous origin in their *pbp* genes and also in the recently identified *murM*: a gene essential for expression of penicillin resistance and for the unique cell wall structure of penicillin-resistant pneumococci. Virtually all DRP express only a limited number (five or six) of the very large genetic repertoire (up to 90) of serotypes available for this bacterial species and the serotypes of drug-resistant strains happens to be the same as the serotypes of drug-susceptible pneumococci that most frequently colonize pre-school age children. These observations strongly suggest that the nasopharynx of children is an important global ecological reservoir of DRP and may also play a critical role as the favoured anatomical site for the evolution of DRP.

Introduction

One of the intriguing findings that emerged from the study of antibiotic-resistant *Streptococcus pneumoniae* is the surprisingly limited number of clonal lineages, identified by molecular typing techniques, that are responsible for a disproportionately large fraction of resistant pneumococcal disease in virtually all parts of the world where appropriate surveillance studies have been carried out. Particularly striking is the huge contribution of two specific clones to resistant pneumococcal disease: the first of these is a pneumococcal lineage with high-level resistance to penicillin (P), tetracycline (T), chloramphenicol (C) and occasionally also to erythromycin (E) and co-trimoxazole (SXT). This particular lineage, Spain^{23F}-1, is often referred to as the Spanish/USA pandemic clone;[1] it most frequently expresses serotype 23F or 19. Disease-causing strains belonging to this clone have now been identified in most states in the USA,[2,3] in Latin America, Asia, and Western and Southern Europe, as well as in South Africa and Israel.[4] In a recent study more than half of all antibiotic-resistant *S. pneumoniae* recovered in 12 New York City hospitals during a 6 month period in 1998 belonged to the Spanish/USA clone Spain^{23F}-1 plus a second pandemic lineage called Spain^{9V}-3 often referred to as the French/Spanish clone or France^{9V}-3.[5] Strains belonging to this second lineage are resistant to penicillin and often to tetracycline and trimethoprim–sulfamethoxazole, and this clone which usually expresses capsular type 9V or 14 has disseminated as widely as the Spanish/USA clone.[4] The existence of such clones with stability in time and over geographical distance is surprising, and is in sharp contrast to the established enormous genetic diversity of drug-susceptible pneumococcal isolates.[6,7]

Studies in children's day-care centres

The massive global spread of a few epidemic lineages of drug-resistant pneumococci (DRP) strongly suggests the existence

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of an ecological reservoir for these particular bacterial clones. We are going to summarize briefly results from an ongoing molecular epidemiological study, the Lisbon Day-Care Centre (DCC) Initiative, which was designed to sample one of the suspected ecological reservoirs of DRP: the nasopharynx of pre-school age children.

Owing to a variety of social and cultural trends primarily in countries in the developed world, an increasing proportion of children of pre-school age have attended day-care since the beginning of the 1980s. Children of this age group are known to have the highest rate of carriage of respiratory pathogens such as *S. pneumoniae*, possibly related to an immature immune system, which is sub-optimal for mounting an antibody response against capsular polysaccharides. Cohorting this age group, with its known predisposition for viral and bacterial respiratory diseases and behavioural traits that favour person-to-person contact, combined with the enormous amounts of antibiotics used for respiratory diseases of children, has promoted DCCs as environments where the selection and amplification of antibiotic-resistant *S. pneumoniae* are highly favoured. Several studies have shown that attendance in day-care is a risk factor for carriage of resistant strains of pneumococci.[8]

The Lisbon DCC Initiative

For the reasons described above, a large number of published studies have concentrated on DCCs and their role in the epidemiology of DRP. However, only a few of these have used molecular typing techniques. In contrast, the Lisbon DCC Initiative was organized as a continuously operating surveillance system capable of identifying clonal types of pneumococci through their molecular fingerprints and also as being able to recognize trends and changes in the nasopharyngeal flora of pre-school age children attending centres in Lisbon.[9,10] The purpose of this brief review is to summarize some relevant observations from this surveillance. No attempt has been made to review the extensive literature on DCCs and other aspects of DRP.

In the Lisbon Initiative the participating DCCs were selected to represent a variety of geographical areas with different demographic and social backgrounds. The last census in 1991 indicated that there were 7500 children aged 6 months to 6 years attending DCCs. The DCC Initiative began in 1996. By the end of the third year of surveillance in 1998, 16 DCCs and 1617 children participated in the study, which generated 2111 nasopharyngeal samples, 1096 yielding *S. pneumoniae*. Thus, our study examined a significant proportion (8–10%) of the target population. From most of the samples a single bacterial colony was picked from each positive sample and was characterized by serotyping, susceptibility testing (antibiotype) and molecular typing, the latter using pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and DNA hybridization with a probe specific for the *lytA* gene (a method that can provide information about the carriage of prophages). Antibiotic-resistant isolates were also tested by the appropriate DNA probes to identify the particular resistance determinant. In a more recent surveillance, 6–11 individual colonies were picked from each nasopharyngeal sample in order to test for possible carriage of multiple *S. pneumoniae* strains.[11]

Table 1 provides some basic figures for the operation of the Lisbon DCC Initiative. The numbers of children tested, the participating DCCs, the numbers of *S. pneumoniae* carriers and the numbers and percentages of DRP during the 3 year period of surveillance between 1996 and 1998 are given.

DRP clones in colonization and disease. The first question that the data from the Lisbon DCC study allowed us to answer was related to the presence in the nasopharyngeal flora of DCC attendants of DRP clones identified previously among disease-causing isolates. During the 3 years of surveillance, the percentage of DRP has increased from 40% (1996) through 45% (1997) to 50% (1998) of all isolates (see Table 1). Molecular fingerprinting of the total of 413 DRP isolates identified as many as 57 distinct clonal types by PFGE. Most interestingly, however, the great majority of the DRP isolates (284 of 413 or 68%) were represented by as few as eight epidemic clones. The rest of the 129 DRP (32%) were more genetically heterogeneous and were represented by 49 different clonal types (Figure 1). Table 2 summarizes the serotypes, antibiotypes and MLST profiles of the eight epidemic DRP clones, and Table 3 identifies countries in which the same eight clonal types were detected among disease-causing

Table 1. Carriage rate of *S. pneumoniae* and of DRP in the Lisbon DCC Initiative, during a 3 year surveillance period

Year	DCCs	Children tested	*S. pneumoniae* carriers (%)	DRP (%)	DRP used for molecular testing
1996	7	586	277 (47.3)	111 (40)	91
1997	12	745	353 (47.4)	161 (45)	131
1998	12	780	465 (59.6)	231 (50)	191

Data from de Lencastre *et al.*[9,10]

Table 2. Clonal types, serotypes and antibiotypes of epidemic DRP isolates identified in the Lisbon DCC Initiative

Clone	Serotype	No. DRP isolates	Antibiotype	MLST allelic profile							
				aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	ST
A	23F	69	P, C, T, Sxt	4	4	2	4	4	1	1	81
B	14/9V	38	P, Sxt	7	11	10	1	6	8	1	156
E	6A/6B	22	E, Cc, T	32	28	1	1	15	52	14	386
M	6B	64	E, Cc, T, Sxt	7	65	1	2	6	1	14	385
R	14	28	E	1	5	4	5	5	1	8	9
DDD	15	15	E,Cc,T,p	2	5	36	12	17	21	14	63
H	19	25	E, Cc	7	14	4	12	1	1	14	177
FF	23F	23	p	7	13	8	6	6	12	8	277
C	6B	–	–	5	6	1	2	6	3	4	90

Data from Sá-Leão *et al.*[12–14] Resistance to: P (penicillin, high level), C (chloramphenicol), T (tetracycline), Sxt (co-trimoxazole), Cc (clindamycin), E (erythromycin), p (penicillin, low level). ST, sequence type.

isolates. The data clearly show that DRP clones frequently identified in disease are also present in the nasopharynx of healthy children.[12,13]

Epidemic behaviour of pneumococcal clones in DCCs. The second question that the DCC data have allowed us to answer relates to the frequency of representation of these epidemic clones: did these clones exhibit epidemic behaviour in the DCC setting also? We defined this as the capacity of a clone to colonize several children in one DCC and also to disseminate and colonize children in different DCCs distantly located from one another. We showed that each of the DCCs surveyed in 1996 had a unique clonal profile with respect to the *S. pneumoniae* recovered from the nasopharynx of the children. However, representatives of the epidemic clones were typically carried by more than one child in most DCCs.[12] Figure 2 illustrates the wide geographical spread of two penicillin-resistant clones among the 16 Lisbon area DCCs.[12] In a striking parallel of geographical dispersal, Figure 3 illustrates the recovery of the same two penicillin-resistant clones from disease in 12 New York area hospitals.[5] Clearly, the capacity for geographical spread is shared by epidemic clones in the setting of colonization as well as causing pneumococcal disease.

Table 3. International spread of disease by epidemic clones of DRP

Clone	Lisbon DCC serotypes	Disease
A	23F	pandemic
B	14/9V	pandemic
E	6A/6B	Bulgaria, Poland
M	6B	Italy, Greece, Israel, UK, USA, Iceland
R	14	Argentina, UK, USA
DDD	15	Sweden, Spain
H	19	Spain, Denmark
FF	23F	Brazil, Columbia, The Netherlands, Iceland, Denmark, UK
C	6B	Spain, Iceland

Data from Sá-Leão *et al.*[14]

Role of the nasopharynx in the epidemiology of DRP

Can the nasopharynx of children attending DCCs be considered an ecological reservoir for drug-resistant clones? The criterion for such a proposition would be the enrichment of the nasopharyngeal flora for epidemic clones, i.e. high frequency of carriage, over prolonged time periods. Figure 4 shows that this may indeed be the case: the combined representation of the eight widely disseminated clones was quite high in most of the DCCs during each one of the three surveillance periods, approaching in some cases 80% of the isolates or more.[13,14]

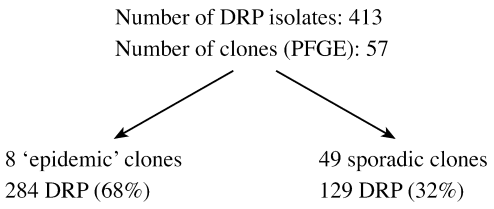

Figure 1. Molecular typing of DRP isolates recovered between 1996 and 1998 in the Lisbon DCC Initiative. Data from Sá-Leão *et al.*[12,13]

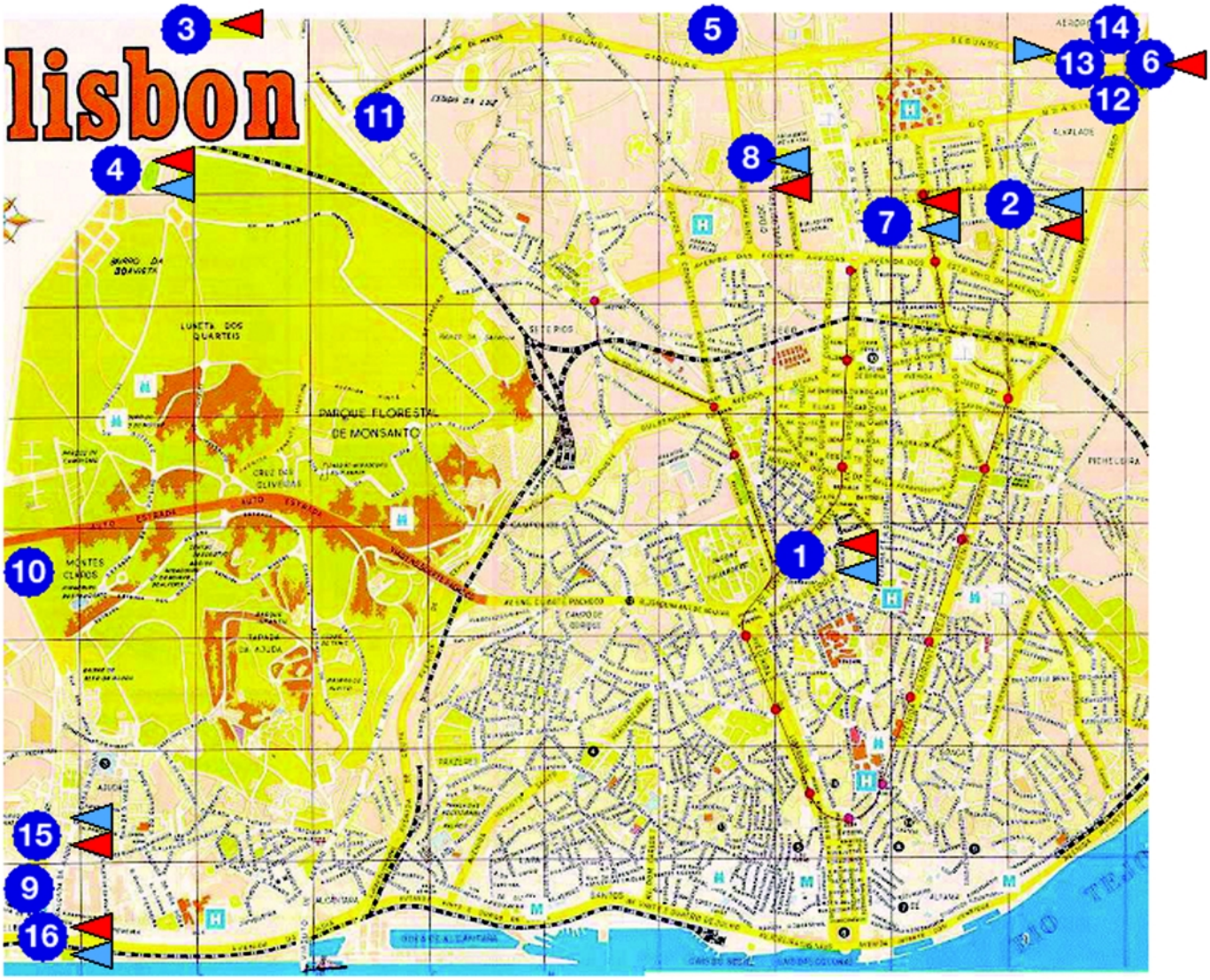

Figure 2. Location of the 16 DCCs participating in the Lisbon DCC Initiative. Reproduced with permission from de Lencastre *et al.*[9] Red arrows, clone Sp-1; blue arrows, clone Sp-3.

The nasopharynx as the 'birthplace' of DRP

Is the evolutionary origin of DRP clones the nasopharynx of children, from which they continue to emerge to cause both paediatric and adult disease? The similarity of the clonal types of DRP among disease-causing isolates and among strains colonizing children in DCCs does not itself give an unequivocal answer to this question. However, the unique serotypes of DRP clones strongly suggest that the direction of movement of clones is from the ecological reservoir (nasopharynx) to sites of disease. The rank order of serotypes of drug-susceptible *S. pneumoniae* causing invasive disease is known for several countries. In Spain, in the 1990s, the most frequent serogroups/types of *S. pneumoniae* among blood isolates from adult patients in order of decreasing frequency was 3, 9, 14, 8, 4, 19, 6, 23, 1 and 18. The same rank order of blood isolates from children was 19, 6, 14, 18, 23, 1, 5, 6, 12 and 3.[15] In sharp contrast, the distribution of the most frequent serogroups/types among penicillin-resistant isolates from the same country was 6, 14, 23, 19 and 9 and these five serogroups/types accounted for 92.2% of all the resistant isolates.[15] Interestingly, the same relatively few capsular types are expressed by DRP strains irrespective of their geographical or clinical sites of isolation in all parts of the world. The serotypes of drug-resistant strains represent the capsular polysaccharides typical of pneumococci colonizing pre-school age children. Several studies have documented the dominance of these few polysaccharide types in this age group, which rapidly declined in frequency by the age of 3–5.[16] The striking similarity of serotypes of drug-resistant clones to

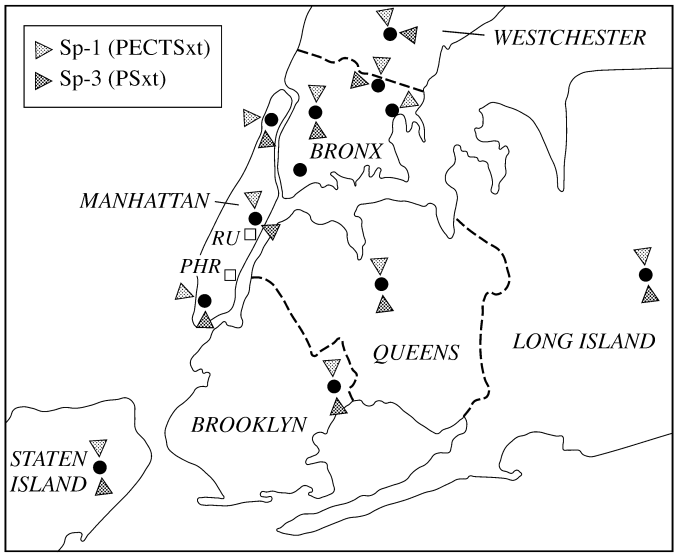

Figure 3. Dissemination of two epidemic penicillin-resistant clones of *S. pneumoniae* in 12 hospitals in New York City. Reproduced with permission from Roberts *et al.*[5]

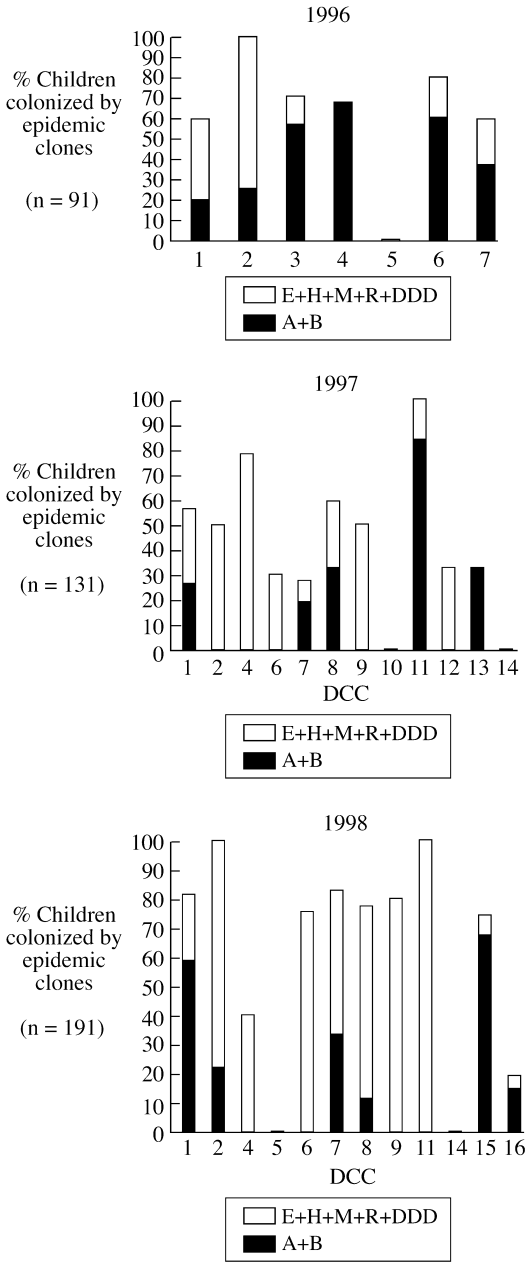

Figure 4. Frequent carriage of epidemic DRP clones in DCCs in Lisbon. Data reproduced from de Lencastre *et al.*[10]

these paediatric serotypes presents the strongest evidence that the origin of the drug-resistant clones may indeed be in children, specifically during prolonged carriage of the paediatric serotypes.

It is intriguing to consider that the initial evolutionary constraint on DRP clones may actually be the immunological status of the human host, namely, the immature immune system of young children, which restricts persistence in the nasopharynx to strains of only five or six pneumococcal serotypes. The capacity of *S. pneumoniae* to undergo spontaneous switch in serotype *in vivo* has been demonstrated repeatedly.[17] Table 4 lists such serotype switching events observed in epidemiological studies conducted in our laboratories.[18] Importantly, in all but one of the cases listed, the genetic event involved switching from one paediatric capsular type to another. Also, importantly, in the two cases where appropriate data were available, the serotype switch could be localized to the nasopharynx, in one case to a child attending a DCC,[19] and in the other case to an HIV-positive patient in a hospice.[20] It was in this latter case that the serotype switch took *S. pneumoniae* out of the paediatric serotypes: a strain belonging to the pandemic serotype 23F Spanish/USA clone, and colonizing a number of patients in the hospice, acquired the highly virulent serotype 3. Testing the virulence potential of the original clone expressing serotype 23F against that of the strain with the newly acquired serotype 3 demonstrated a massive (10^6-fold) increase in the virulence of the type 3 strain in the mouse peritoneal model.

Some other unusual properties of drug-resistant clinical isolates of *S. pneumoniae* may also have originated at the primary site of habitation and evolution of these clones. It was demonstrated that the production of low-affinity penicillin binding proteins in penicillin-resistant isolates is associated with the acquisition of mosaic DNA sequences in *pbp* genes, the likely source of which were other, heterologous microbial species that inhabit the nasopharynx and/or oropharynx of humans.[21] Recent studies have identified the *murMN* operon of *S. pneumoniae*, involved in the synthesis of branched structured cell wall components, which are frequent among some clones of penicillin-resistant pneumococci. Functioning *murMN* was found to be essential for the expression of penicillin resistance.[22] Different clones of penicillin-resistant pneumococci were shown to carry distinct alleles of the *murM* gene, which differed from the *murM* gene of most *S. pneumo-*

Table 4. *In vivo* switch of the capsular type in drug-resistant clones of *S. pneumoniae*

Geographical origin	Strain	Isolation year	Antibiotype	Clone	Serotype		Direction of capsular switch
					expected	found	
South Korea	K30	1991	PETCSxt	A	23F	19F	23F→19F
Italy	Gen11R	1995	PETCSxt	A	23F	19F	23F→19F
USA (NC)	Noc211	1991	PETCSxt	A	23F	14	23F→14
USA (NY)	SV36	1995	PETCSxt	A	23F	3	23F→3
Spain	SP19	1995	PETCSxt	A	23F	19B	23F→19B
USA (CA)	Ca17	1996	PE	A	23F	19	23F→19
USA (CA)	Ca53	1996	PE	A	23F	14	23F→14
Iceland	Ic233	1995	PTCSxt	A	23F	19	23F→19
Hungary	MA40	1994	PETCSxt	Hun	19A	11	19A→11
Argentina	AR647	1995	PSxt	B	14	19F	14→19F
Colombia	COLA189	1995	PSxt	B	14	9V	14→9V
Mexico	24HIM	1994	PESxt	B	14	9A	14→9A

Reproduced with permission from Ramirez & Tomasz.[18]
Resistance to: P (penicillin), E (erythromycin), T (tetracycline), C (chloramphenicol), Sxt (co-trimoxazole).

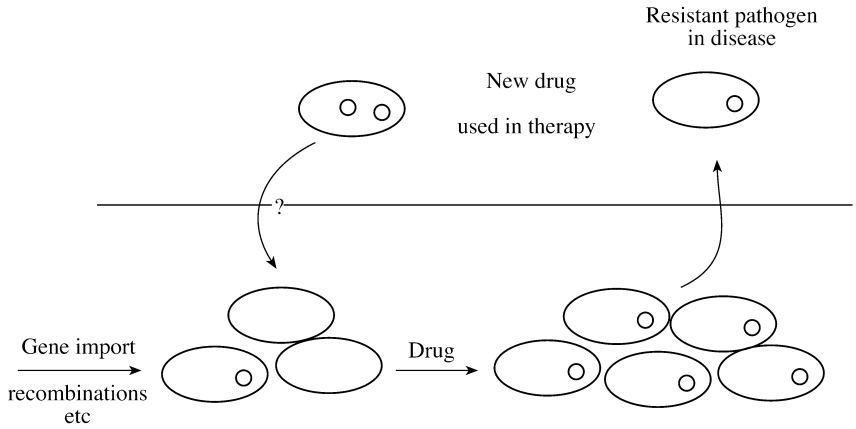

Figure 5. Acquisition of drug resistance genes and selection of drug-resistant clones of *S. pneumoniae* in the nasopharynx. Large ellipsoid structures symbolize bacteria; small circles represent resistance genes. The horizontal line separates bacteria at disease sites (above) and bacteria at colonization sites (below).

niae strains by the presence of mosaic sequences that may also have originated from heterologous microbial cohabitants of the human upper respiratory tract.[22] Specifically, the nasopharynx of young children, where genetic exchange events with other microbial species, acquisition and dispersal of resistance and virulence-related determinants are assumed to take place. Whether or not resistance traits selected during therapy by agents (e.g. fluoroquinolones) with restricted use in adult disease also find their way into the nasopharyngeal reservoirs remains to be established (Figure 5).

The nasopharynx of pre-school age children appears to serve both as the anatomical site of origin and also as a sanctuary for DRP. Having children in DCCs has created social structures in which child-to-child transmission of DRP and other respiratory pathogens can readily occur. Thus, DCCs may also represent powerful amplifiers contributing to the spread of disease-causing DRP clones. It was the recognition of the role of DCCs in the epidemiology of DRP disease that prompted the European Union to initiate the project 'European Resistance Intervention Study—Reducing Resistance in Respiratory Tract Pathogens in Children' (Contract n QLK2-CT-2000-01020), a multi-national effort to find ways to reduce the carriage rate of DRP in European DCCs.

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