

Molecular Epidemiology of Penicillin-Resistant Pneumococci Isolated in Nairobi, Kenya

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A total of 26% of the pneumococci isolated from an outpatient clinic in Nairobi, Kenya, during 1991 to 1992 had intermediate levels of penicillin resistance. Gene fingerprinting and DNA sequencing were used to distinguish the penicillin-binding protein (PBP) 1A, 2B, and 2X genes in 23 resistant isolates. Isolates were grouped into those that had identical forms of each of the three PBP genes (fingerprint groups) and those that had identical rRNA gene restriction patterns (ribotypes). Both methods divided the isolates into 11 groups. In a few cases, horizontal gene transfer appeared to have distributed an identical altered PBP gene into different pneumococcal lineages. Eight isolates were indistinguishable by ribotyping or multilocus enzyme electrophoresis and contained identical PBP 1A genes. Although these isolates were therefore members of the same clone, they were divided into two fingerprint groups which contained different PBP 2X and 2B genes. Presumably, members of this clone have acquired different altered PBP 2X and 2B genes on two separate occasions. One of these fingerprint groups contained isolates of serotype 14, whereas the other contained isolates of both serotypes 14 and 7. The identification of isolates in the latter group that are identical by all criteria, except serotype, implies the occurrence of a change in serotype. The predominant serotypes of the penicillin-resistant pneumococci from Nairobi were serotypes 14 and 19. In both cases, isolates of the same serotype which required the same MIC of penicillin were not members of a single clone, indicating that identity of serotype and MIC are not sufficient criteria for defining clones of resistant pneumococci even when the bacteria are isolated from a single clinic.

Penicillin resistance in *Streptococcus pneumoniae* (the pneumococcus) is entirely due to the development of altered forms of penicillin-binding proteins (PBPs) that have decreased affinity for β -lactam antibiotics (9, 30). In high-level penicillin-resistant pneumococci (requiring MICs of $>1 \mu\text{g}$ of benzylpenicillin per ml), there are reductions in the affinities of at least four of the five high-molecular-weight PBPs (1A, 1B, 2A, 2X, and 2B). Low-affinity forms of PBPs appear to have arisen by interspecies recombinational events, presumably mediated by genetic transformation, that have replaced parts of the pneumococcal PBP genes with the corresponding regions from the homologous PBP genes of closely related species (6). Consequently, the PBP genes of penicillin-resistant pneumococci have a mosaic structure, consisting of regions that are very similar to the corresponding regions in the genes from penicillin-susceptible pneumococci and regions that differ by as much as 20% in nucleotide sequence (6, 7, 14, 18).

Although penicillin-resistant or multiply antibiotic-resistant pneumococci have been reported from many countries (1, 13, 16, 19), there is little information on the relationships among resistant isolates recovered within countries or in different countries or continents. In most countries, resistance to penicillin is found predominantly in a small number of serotypes, e.g., serotypes 23F and 6B in Spain (16) and serotypes 19A, 6B, and 14 in South Africa (13). Analysis of resistant pneumococci by comparing the electrophoretic mobilities of their PBPs on sodium dodecyl sulfate-polyac-

rylamide gels suggested that they could be grouped into families (12). More detailed analyses have confirmed the existence of major clones of penicillin-resistant pneumococci that at present appear to be largely restricted geographically (4, 20, 21, 23, 29). An exception is the serotype 23F clone of multiply antibiotic-resistant pneumococci, which is very common in Spain, predominates in the United Kingdom, and has been identified recently in the United States (20, 21), South Africa (3, 29), and Mexico (3).

Pneumococci are naturally transformable. At least in the laboratory, transformation provides an efficient mechanism for the exchange of chromosomal genes, and in nature it might be expected to mediate the spread of altered PBP genes from resistant pneumococci into susceptible strains, to produce new resistant strains (4). Any attempt to understand the epidemiology of penicillin-resistant pneumococci must therefore be able to distinguish between the spread of resistant isolates (clonal spread) and the spread of resistance genes (horizontal spread). This can be achieved by using methods that can index both the overall genetic relatedness between isolates and the relatedness of their penicillin resistance (PBP) genes (4, 21). Resistant pneumococci that are not closely related overall but that contain identical altered PBP genes can then be proposed to have arisen by horizontal spread, whereas isolates that are indistinguishable in terms of both their overall relatedness and the relatedness of their altered PBP genes are the result of clonal spread.

DNA sequencing or, more conveniently, DNA fingerprinting with restriction enzymes that cut frequently provides suitable ways of distinguishing the PBP genes of penicillin-

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TABLE 1. Properties of penicillin-resistant pneumococci from Nairobi^a

Isolate	Patient no.	Specimen type	PBP 1A allele	PBP 2X allele	PBP 2B allele	Serotype	MIC (µg/ml)	Fingerprint group	Ribotype group
86014	1	SP	1*	1	1	14	0.5	I	1
100509	1	SP	1	1	1	14	0.5	I	1
100506	2	SP	1	1	1	14	0.25	I	1
132207	2	SP	1	1	1	14	0.5	I	1
132206	3	SP	1*	2*	2*	7	0.25	II	1
142145	4	SP	1*	2*	2	7	0.5	II	1
100511	5	BC	1*	2*	2*	14	0.5	II	1
100503	6	SP	1	2	2	14	0.5	II	1
85990	7	SP	2	3	3*	19	0.25	III	2
86022	8	TS	2	3	3	19	0.25	III	2
86012	9	TS	2	3	3	19	0.25	III	2
85997	10	SP	3	4	4	19	0.12	IV	3
132223	11	SP	3	4	4	19	0.12	IV	4
142162	12	BC	4	5	5	10	0.5	V	5
142163	12	BC	4	5	5*	10	0.5	V	5
100494	13	SP	5	6	6*	16	0.25	VI	6
100496	14	SP	5	6	6	16	0.25	VI	6
100512	15	SP	6	7	7	23	0.12	VII	7
100520	15	BC	6	7	7	23	0.25	VII	7
86016	16	SP	7*	8*	8	19	0.12	VIII	8
86007	17	SP	7*	8*	9	11	0.12	IX	9
85983	2	PNS	7*	8*	5*	9	0.25	X	10
86003	18	PNS	8	9	10*	15	0.25	XI	11
R6			S1	S1	S1	NE	0.008		

^a Asterisks indicate cases in which DNA sequencing was used to further investigate the identity or nonidentity of PBP genes that gave the same fingerprints (see text). SP, sputum; BC, blood culture; TS, throat swab; PNS, pernasal swab; NE, nonencapsulated. All isolates were from Kenya, with the exception of R6, which was from the United States.

resistant pneumococci (4), whereas multilocus enzyme electrophoresis (MLEE) or rRNA gene restriction pattern analysis (ribotyping) provides methods to assess the overall relatedness of isolates (11, 28). By these approaches, the identification of clones of penicillin-resistant pneumococci and the significance of horizontal spread of resistance genes and putative serotype changes in the spread of penicillin resistance have been demonstrated (4, 21).

Very little information is available on the frequency of penicillin-resistant pneumococci from African countries (other than South Africa) or other developing countries in which pneumococcal disease is common and in which antibiotics are often available without prescription. During a study of a cohort of human immunodeficiency virus-positive patients attending an outpatient clinic in Nairobi, Kenya, we have encountered a very high incidence of invasive pneumococcal disease (2). In the present paper, we report a 26% incidence of penicillin resistance in pneumococci from this group and demonstrate a considerable diversity in the penicillin-resistant pneumococci obtained in this single clinic over a short period of time that is not fully revealed by serotyping. We also provide further examples of the significance of the horizontal transfer of PBP genes and putative serotype changes in the spread of penicillin resistance in pneumococci.

MATERIALS AND METHODS

Growth media and strains. Pneumococci were grown in brain heart infusion broth plus 0.5% yeast extract or Todd-Hewitt broth or on brain heart infusion agar containing 5% defibrinated sheep's blood as described previously (22). Serotyping was performed by the Central Public Health Laboratories, Colindale, United Kingdom, and was kindly rechecked by the Department of Bacteriology, Statens Se-

ruminstitut, Copenhagen, Denmark. The pneumococcal isolates used in this work (Table 1) were clinically relevant strains obtained from blood, lung aspirate, sputum, or pernasal swabs from bacteriological investigations of patients studied at the Wellcome Trust-Kenya Medical Research Institute HIV Project, Nairobi, Kenya, during 1991 to 1992. Three pairs of strains (100506 and 132207, 142162 and 142163, and 100512 and 100520) were from samples taken during the course of a single episode of pneumococcal disease. The others were all from different patients or, in two cases (86014 and 100509 and 100506 and 85983), from the same patient but were obtained from different episodes of disease separated by at least 4 months.

PBP gene fingerprinting. The PBP 1A, 2B, and 2X genes were amplified from chromosomal DNA by the polymerase chain reaction (PCR), and gene fingerprints were obtained as described previously (4). Briefly, the amplified fragments were gel purified with GeneClean (Bio 101, Inc., La Jolla, Calif.) and were digested with restriction enzymes, end labeled with [³²P]deoxynucleoside triphosphate, fractionated on a 6% nondenaturing polyacrylamide gel, and autoradiographed to produce gene fingerprints. The enzymes *Hinf*I and *Dde*I plus *Mse*I were used for fingerprinting the PBP 1A and PBP 2X genes, and *Sty*I and *Hinf*I were used for the PBP 2B gene. pBR322 DNA digested with *Hpa*II and end labeled as described above was used as molecular size markers. The fingerprint patterns on the X-ray films were digitized and compared by using a Summasketch II Plus digitizer tablet (Summagraphics, Inc., Seymour, Conn.) and MolMatch software (Ultra-Violet Products Ltd., Cambridge, United Kingdom).

Direct sequencing of PCR-amplified DNA fragments. The PBP 1A, 2B, and 2X genes were amplified by the PCR as described above, except that biotin was incorporated at the 5' end of each of the upstream PCR primers, by using a

biotinylated phosphoramidite for the final nucleotide addition step during synthesis. The amplified fragments were gel purified with GeneClean, and the biotinylated and nonbiotinylated DNA strands were separated with avidin-coated paramagnetic beads (DynaL UK, Ltd., Wirral, Merseyside, United Kingdom) as described by Hultman et al. (10). DNA sequencing was carried out on the single-stranded DNA by using the Sequenase version 2 kit (U.S. Biochemicals Corp., Cleveland, Ohio). The regions of the PBP genes that were sequenced were those that were the most variable in unrelated penicillin-resistant pneumococci (6, 14, 18). The sequences of the primers (using the numbering found in references 8, 15, and 17) corresponded to nucleotides 2461 to 2477 and 3118 to 3097 for the PBP 1A gene; nucleotides 995 to 1015, 1270 to 1290, 1801 to 1781, and 2316 to 2292 for the PBP 2B gene; and nucleotides 959 to 980 and 2285 to 2251 for the PBP 2X gene.

MLEE. Pneumococci were grown at 37°C in 200 ml of brain heart infusion broth plus 0.5% yeast extract and were harvested in late exponential phase (about 8×10^8 CFU/ml), and cell extracts were prepared as described by Coffey et al. (4). Techniques for starch gel electrophoresis and enzyme staining have been described elsewhere (27). The enzymes analyzed were indophenol oxidase, alcohol dehydrogenase, leucyl-alanine peptidase, leucyl-glycyl-glycine peptidase, phenylalanyl-leucine peptidase, leucine aminopeptidase, nucleoside phosphorylase, 6-phosphogluconate dehydrogenase, esterase, glyceraldehyde-3-phosphate dehydrogenase (NADP dependent), carbamylate kinase, hexokinase, glutamate dehydrogenase, adenylate kinase, phosphoglucose isomerase, fructokinase, diaphorase, and lactate dehydrogenase. The differences between isolates were converted into a distance matrix, and the relatedness of the isolates was represented by a neighbor-joining tree (26).

Detection of restriction fragment length polymorphisms around the rRNA genes (ribotyping). Chromosomal DNA was prepared essentially as described by Pitcher et al. (25), with the addition of mutanolysin (20 µg/ml) to aid lysis. Approximately 5 µg of *EcoRI*-digested DNA was fractionated on an 0.8% agarose gel and, after transfer to nylon membranes (Hybond N) by vacuum blotting (Vacu-aid; Hybaid, Ltd., Teddington, United Kingdom), was probed with a digoxigenin-labeled cDNA made by reverse transcription of 16S plus 23S rRNA from *Escherichia coli* (Boehringer-Mannheim, Lewes, United Kingdom) by using a modification of the method for making biotinylated probes described by Pitcher et al. (24). Membranes were prehybridized, hybridized at 50°C, and washed at the same temperature, and hybridizing DNA fragments were detected under the conditions recommended in the digoxigenin luminescence detection kit (Boehringer-Mannheim).

Nucleotide sequence accession numbers. The sequences have been submitted to the EMBL data library under the accession numbers Z21799 to Z21814.

RESULTS

Incidence of penicillin-resistant pneumococci from Nairobi.

Isolates were from a collection of 223 clinically relevant pneumococci obtained from patients studied at the Wellcome Trust-Kenya Medical Research Institute HIV Project, Nairobi, during 1991 to 1992. Overall antimicrobial resistance rates were as follows: penicillin, 26%; tetracycline, 35%; erythromycin, 0%; and chloramphenicol, 0.7%. The MICs of penicillin ranged from <0.008 to 0.5 µg/ml. A total

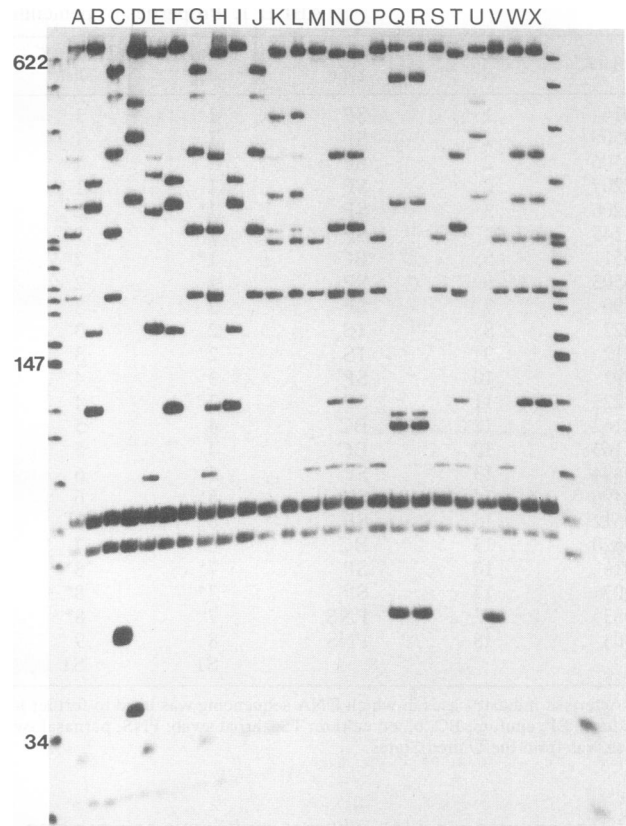


FIG. 1. *Hin*I fingerprints of the PBP 2X gene. The 32 P-labeled *Hin*I fragments obtained from the PBP 2X genes of resistant isolates were fractionated on a polyacrylamide gel and were detected by autoradiography. The PBP 1A genes were from isolates 85983 (lane A), 85990 (lane B), 85997 (lane C), 86003 (lane D), 86007 (lane E), 86012 (lane F), 86014 (lane G), 86016 (lane H), 86022 (lane I), 100494 (lane J), 100496 (lane K), 100503 (lane L), 100506 (lane M), 100509 (lane N), 100511 (lane O), 100512 (lane P), 100520 (lane Q), 132206 (lane R), 132207 (lane S), 132223 (lane T), 142145 (lane U), 142162 (lane V), 142163 (lane W), and R6 (lane X). pBR322 DNA digested with *Hpa*II was used as molecular size markers (left and right lanes). The sizes of the visible pBR322 *Hpa*II fragments are 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, and 26 bp.

of 23 of the penicillin-resistant pneumococci (MICs of 0.1 to 0.5 µg/ml) were chosen for further analysis (Table 1).

Analysis of PBP genes of penicillin-resistant pneumococci. The PBP 1A, 2B, and 2X genes of each of the 23 resistant strains were amplified by PCR and were digested with restriction enzymes that cut frequently, and the sizes of the resulting fragments were analyzed on a polyacrylamide gel (gene fingerprints). The restriction enzymes used were those that were expected to give the largest number of DNA fragments on the basis of the sequences of the PBP genes from the penicillin-susceptible strain R6 (6, 14, 18). The resolution of the fingerprinting method was increased by analyzing the fragments produced from each gene using at least two restriction enzymes (see Materials and Methods). Figure 1 shows the PBP 2X gene fingerprints of the resistant pneumococci obtained with *Hin*I.

PBP genes that gave identical fragments with each of the restriction enzymes were considered very similar, and probably identical, in sequence. In a few cases, PBP genes that

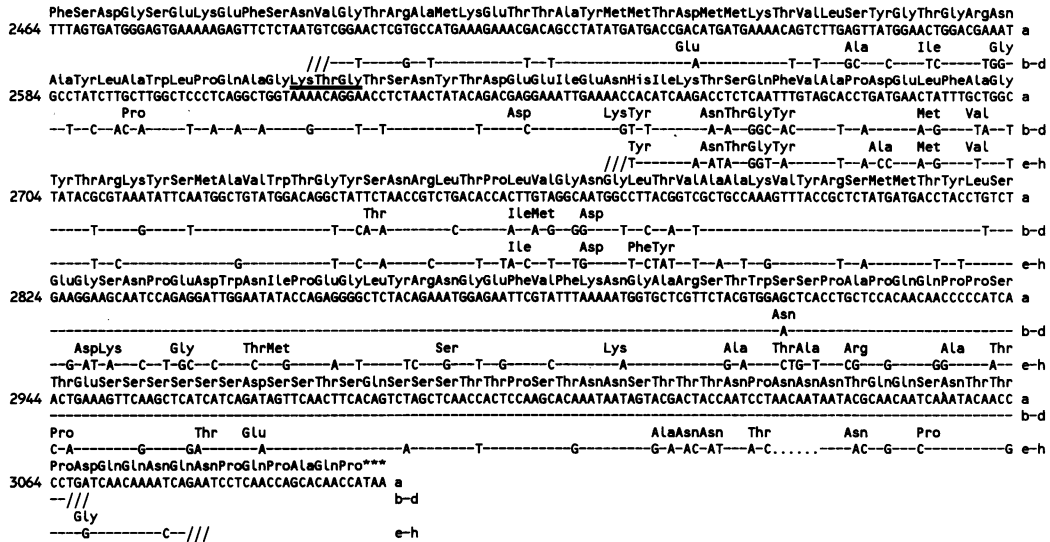


FIG. 2. Sequences of part of the PBP 1A gene of penicillin-resistant pneumococci. The sequence shown in line a is that of the penicillin-susceptible strain R6. The nucleotide and amino acid sequences are numbered at the end of each line as by Martin et al. (17). The other lines show the positions where the nucleotide and amino acid sequences differ from those of strain R6. The sequences are those from strains 85983 (b), 86007 (c), 86016 (d), 86014 (e), 100511 (f), 132206 (g), and 142145 (h). The position of the Lys-Thr-Gly conserved sequence motif is underlined. ///, the limits of the sequence that was determined.

appeared to be identical by fingerprinting were found in isolates of different serotypes. In all of these situations (see asterisks in Table 1), regions of the PBP gene from an isolate of each serotype were sequenced (see Materials and Methods) to establish whether the genes were identical or just sufficiently similar to give the same fingerprints. The sequences of the regions of the PBP genes that were determined are shown in Fig. 2 to 4.

In all but two cases, PBP genes with identical fingerprints were confirmed as being identical by DNA sequencing. The exceptions were the PBP 2B genes of isolates 132206, 142145, 100511, and 100503 and 85990, 86022, and 86012 and the PBP 2B genes of isolates 86003 and 100494 and 100496. In these two cases, the PBP 2B genes could not be distinguished by fingerprinting, but DNA sequencing showed that they differed slightly in sequence (compare lines d and e with f and line g with h in Fig. 3). The PBP 2B genes were therefore given different allele numbers in Table 1.

Among the 23 penicillin-resistant pneumococci, 8 different alleles of the PBP 1A gene, 10 different alleles of the PBP 2B gene, and 9 different alleles of the PBP 2X gene were distinguished. The digitized fingerprint patterns of each of the different alleles of the three PBP genes are shown in Fig. 5 to 7.

Classification of penicillin-resistant pneumococci into fingerprint groups and ribotype groups. Isolates were grouped into those that appeared to have identical forms of each of the three PBP genes (fingerprint groups). The 23 resistant isolates fell into 11 fingerprint groups (Table 1). Classification into fingerprint groups was carried out blind, in the absence of any knowledge of serotypes. As expected, three pairs of isolates obtained from patients during a single infection (100506 and 132207 from patient 2, 142162 and 142163 from patient 12, and 100512 and 100520 from patient 15) were indistinguishable. One further isolate (85983) from patient 2 was clearly distinguishable from the two other isolates from this patient. This was not surprising, since isolate 85983 was from an episode of pneumococcal disease

that occurred over 7 months before the other strains were isolated. Isolates 86014 and 100509 from patient 1 were indistinguishable, even though they were isolated from separate episodes of pneumococcal disease occurring over 4 months apart.

The grouping of the resistant strains obtained by ribotyping correlated well with the grouping achieved by PBP gene fingerprinting. The 11 different ribotype patterns found among the 23 resistant isolates are shown in Fig. 8. With the exception of fingerprint group IV, isolates in the same fingerprint group had the same ribotype. The two isolates of fingerprint group IV (85997 and 132223) differed in ribotype but were shown to be extremely closely related, since they expressed identical isoenzymes for each of 18 enzymes analyzed by MLEE (Table 2). Isolates in different PBP fingerprint groups gave distinct ribotypes, with the exception of the isolates in fingerprint groups I and II, which could not be distinguished by ribotyping. The isolates of fingerprint groups I and II contained identical forms of PBP 1A but different forms of both PBP 2B and 2X and were extremely closely related since they were indistinguishable by MLEE (18 enzymes examined) as well as by ribotyping.

Except in one case, isolates classified as being members of the same fingerprint group were of the same serotype and required very similar MICs of penicillin. Fingerprint group II, however, consisted of two isolates of serotype 7 and two isolates of serotype 14. These four isolates were kindly reserotyped by both Colindale and the Statens Serum Institut and were confirmed to be serotypes 7 (7B) and 14. Two regions of the PBP 1A, 2X, and 2B genes were sequenced from at least one serotype 7B and one serotype 14 isolate (see asterisks in Table 1). No differences between the PBP gene sequences of the isolates of serotype 7B and 14 were found. The isolates of fingerprint group II were indistinguishable by either MLEE or ribotyping (see above), possessed identical PBP 1A, 2B, and 2X genes, and were therefore identical by all criteria except serotype.

In most cases, particular alleles of each PBP gene were

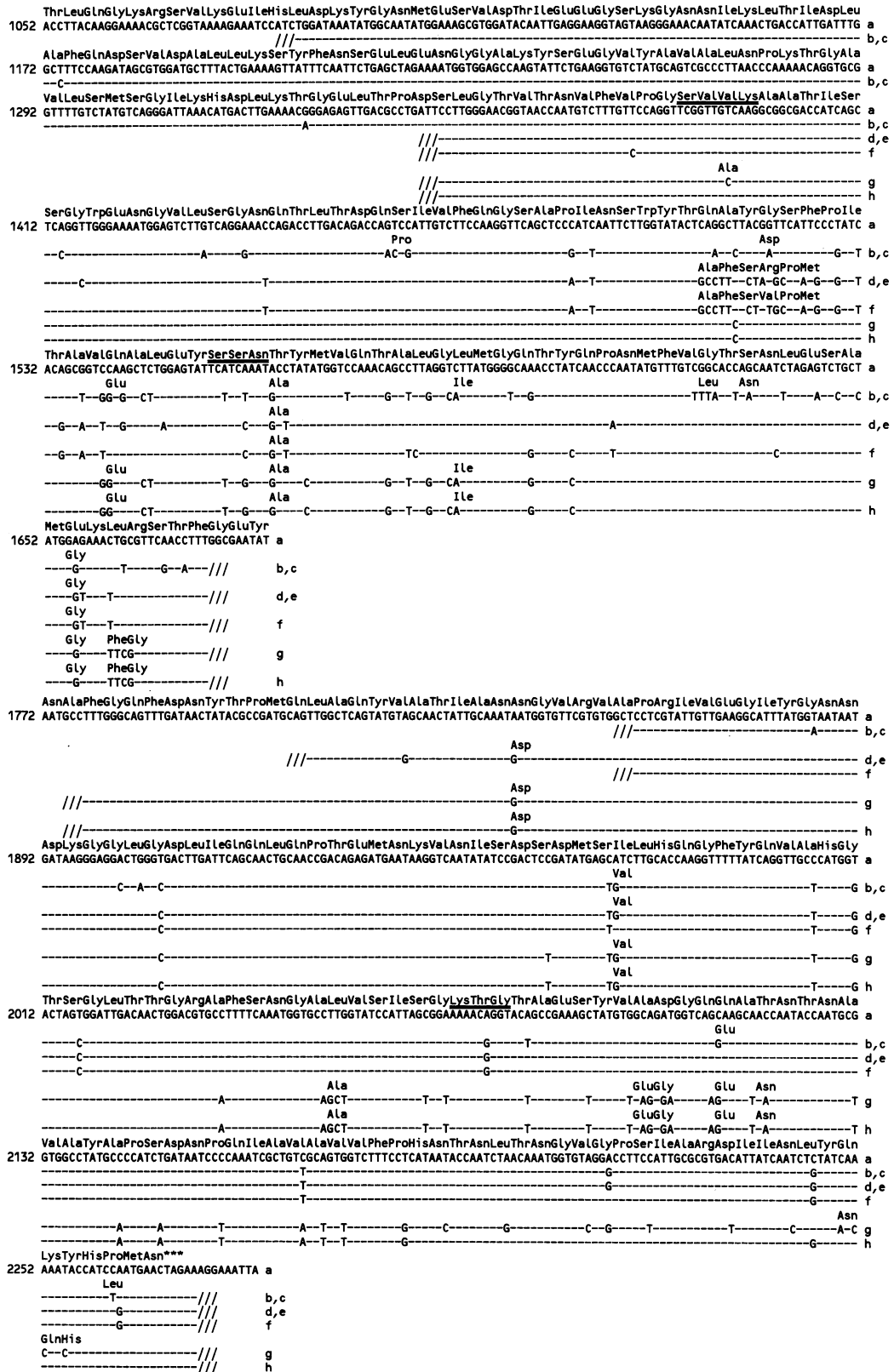


FIG. 3. Sequences of parts of the PBP 2B gene of penicillin-resistant pneumococci. The sequences of two regions of the gene of strain R6 are shown in line a. The sequences are numbered as by Dowson et al. (7). The other sequences are from strains 85983 (b), 142163 (c), 100511 (d), 132206 (e), 85990 (f), 86003 (g), and 100494 (h). The positions of the Ser-X-X-Lys, Ser-X-Asn, and Lys-Thr-Gly conserved sequence motifs are underlined.

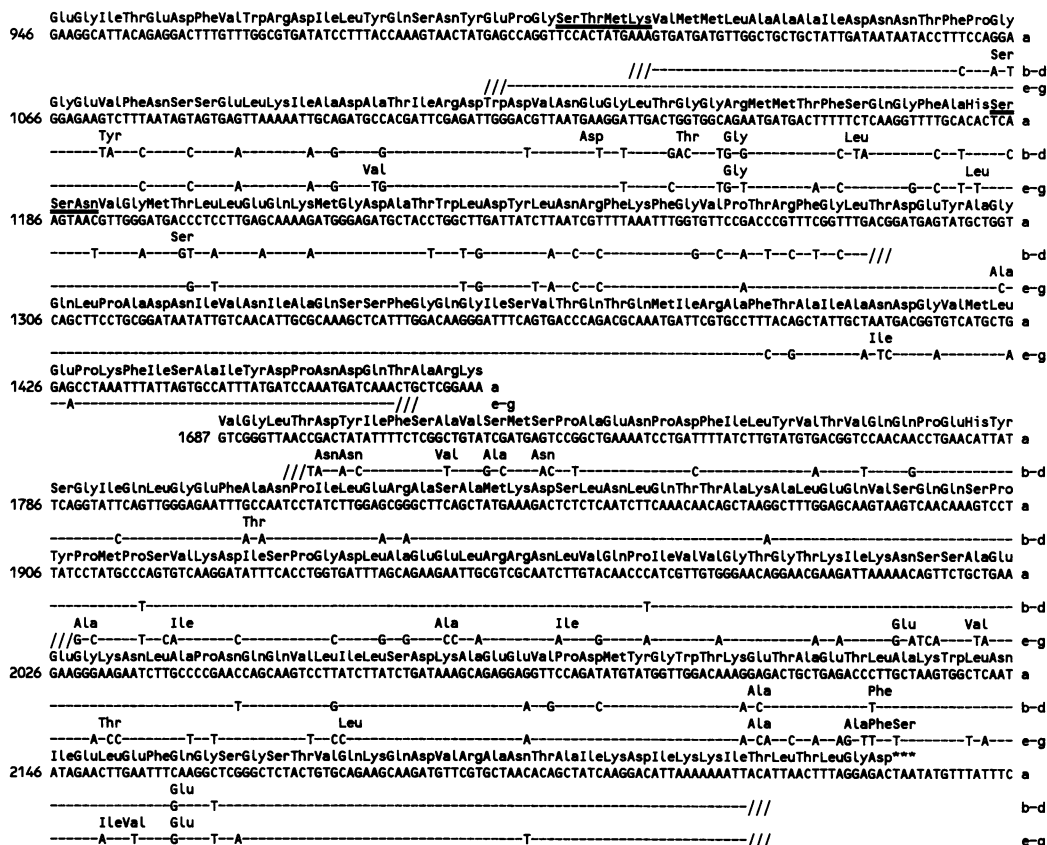


FIG. 4. Sequences of parts of the PBP 2X gene of penicillin-resistant pneumococci. The sequence of two regions of the gene of strain R6 are shown in line a. The sequences are numbered as by Laible et al. (14). The other sequences are those from strains 100511 (b), 132206 (c), 142145 (d), 85983 (e), 86007 (f), and 86016 (g). The positions of the Ser-X-X-Lys and the Ser-X-Asn conserved sequence motifs are underlined.

restricted to a single fingerprint group. However, there were three cases in which PBP genes with identical fingerprints were found in isolates from different fingerprint groups. Allele 1 of the PBP 1A gene was found in fingerprint groups I and II, allele 5 of the PBP 2B gene was found in isolates of fingerprint groups V and X, and allele 7 of the PBP 1A gene and allele 8 of the PBP 2X gene were found in isolates of fingerprint groups VIII, IX, and X. In all cases, the identities of the PBP genes in different fingerprint groups were confirmed by partial DNA sequencing.

MLEE. The overall relatedness of one isolate from each ribotype group was assessed by MLEE. Of the 18 enzymes that were assayed in these isolates, the following 10 enzymes were monomorphic: 6-phosphogluconate dehydrogenase, hexokinase, leucyl-glycyl-glycine peptidase, fructokinase, glyceraldehyde-3-phosphate dehydrogenase, leucine aminopeptidase, esterase, lactate dehydrogenase, indophenol oxidase, and alcohol dehydrogenase. The differences among strains in the mobilities of the 18 enzymes (Table 2) were converted to a distance matrix, and a neighbor-joining tree was constructed (Fig. 9).

DISCUSSION

Penicillin-resistant pneumococci isolated in the outpatient clinic in Nairobi were most commonly of serotype 14 or 19. However, resistance was also found in isolates of serotypes 7, 9, 10, 11, 15, 16, 19, and 23. At present, approximately 26% of the pneumococci from this Nairobi clinic have

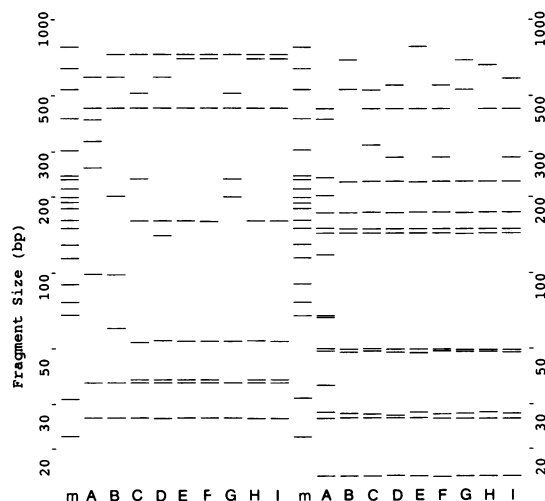


FIG. 5. Fingerprints of the PBP 1A genes of penicillin-resistant pneumococci. The digitized patterns of the *Hind*III (left part of figure) and *Dde*I plus *Mse*I (right part) fragments obtained from each of the different allelic forms of the PBP 1A gene are shown. m, pBR322 × *Hpa*II size markers. The fingerprints are from strains 100509 (A), 85990 (B), 85997 (C), 142163 (D), 100494 (E), 100512 (F), 86016 (G), 86003 (H), and R6 (I).

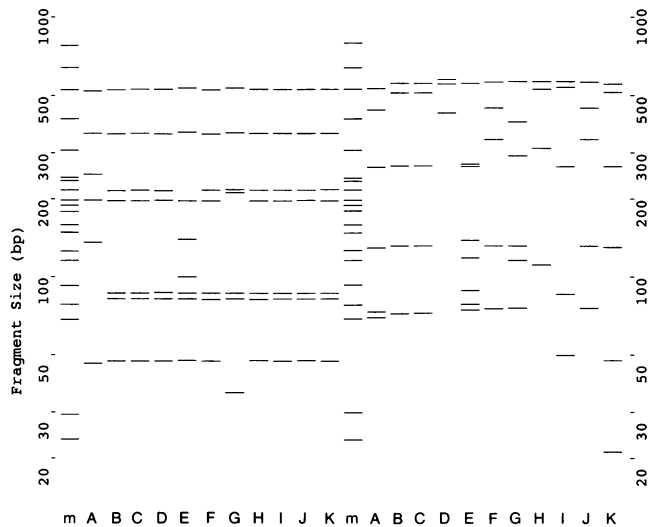


FIG. 6. Fingerprints of the PBP 2B genes of penicillin-resistant pneumococci. The digitized patterns of the *Hinfl* (left part) and *SfiI* (right part) fragments obtained from each of the allelic forms of the PBP 2B genes are shown. The 2 and 3 alleles and the 6 and 10 alleles cannot be distinguished by fingerprinting with these enzymes but were shown to differ by sequencing (see text). m, size markers. The fingerprints are from strains 100509 (A), 132206 (B), 85990 (C), 85997 (D), 142163 (E), 100494 (F), 100512 (G), 86016 (H), 86007 (I), 86003 (J), and R6 (K).

intermediate-level resistance to penicillin (MICs, 0.1 to 1 µg/ml). The maximum MIC of benzylpenicillin for the pneumococcal isolates was 0.5 µg/ml, and no isolates with high-level resistance to penicillin were identified.

All of the penicillin-resistant pneumococci had PBP 1A, 2B, and 2X gene fingerprints that differed from those of the penicillin-susceptible strain R6. This is consistent with the

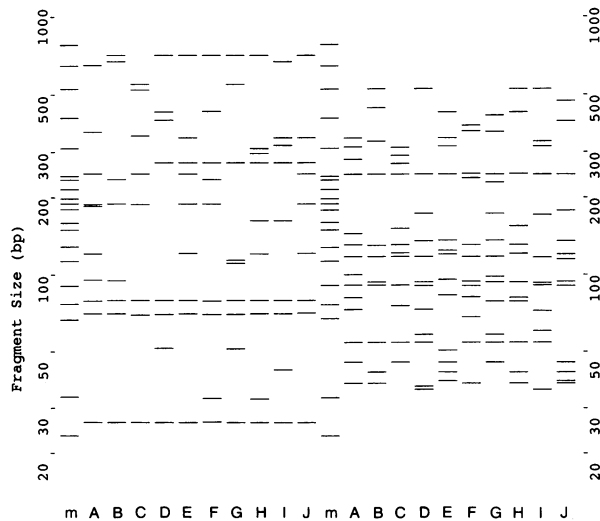


FIG. 7. Fingerprints of the PBP 2X genes of penicillin-resistant pneumococci. The digitized patterns of the *Hinfl* (left part) and *DdeI* plus *MseI* (right part) fragments obtained from each of the allelic forms of the PBP 2X gene are shown. m, size markers. The fingerprints are from strains 100509 (A), 132206 (B), 85990 (C), 85997 (D), 142163 (E), 100494 (F), 100512 (G), 86016 (H), 86003 (I), and R6 (J).

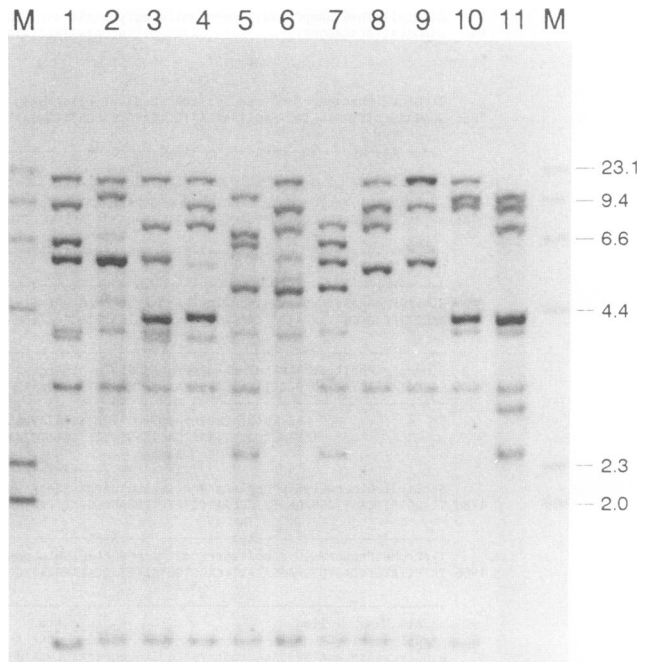


FIG. 8. Ribotyping patterns of penicillin-resistant pneumococci. The lanes correspond to the ribotyping patterns 1 to 11 shown in Table 1. A digoxigenin-labeled *HindIII* digest of bacteriophage lambda was used to provide molecular size markers (M). Sizes are shown in kilobase pairs.

finding that pneumococci with benzylpenicillin MICs of ≥ 0.1 µg/ml produce altered forms of PBPs 1A, 2B, and 2X that have sequences very different from those of penicillin-susceptible pneumococci. In most cases, PBP gene fingerprinting with multiple restriction enzymes provides a reliable method for identifying PBP genes that are identical in sequence. The utility of this method depends on the fact that the PBP genes of resistant pneumococci are very highly variable in sequence. Different fingerprints clearly imply distinct PBP genes (differences in DNA modification will not occur, since all of the DNA is unmodified because of the use

TABLE 2. Allele profiles of penicillin-resistant pneumococci^a

Isolate	Allele							
	GD2	NSP	ADK	CAK	PLP	PGI	LGP	DIA
86014	2	1	2	2	3	2	3	3
100503	2	1	2	2	3	2	3	3
86022	1	1	3	3	3	2	3	3
85997	1	1	2	2	3	2	3	3
132223	1	1	2	2	3	2	3	3
142163	1	2	2	2	3	2	3	1
100494	3	2	2	2	2	2	3	3
86016	1	1	2	2	2	2	2	3
86007	2	1	2	2	3	2	3	1
85983	1	1	2	2	2	2	2	3
100520	1	2	1	1	1	1	1	1
86003	1	3	2	2	2	2	2	2

^a GD2, glyceraldehyde-3-phosphate dehydrogenase (NADP dependent); NSP, nucleoside phosphorylase; ADK, adenylate kinase; CAK, carbamylate kinase; PLP, phenylalanyl-leucine peptidase; PGI, phosphoglucose isomerase; LGP, leucyl-glycyl-glycine peptidase; DIA, diaphorase. The other 10 enzymes that were assayed in these isolates (see Materials and Methods) were monomorphic.

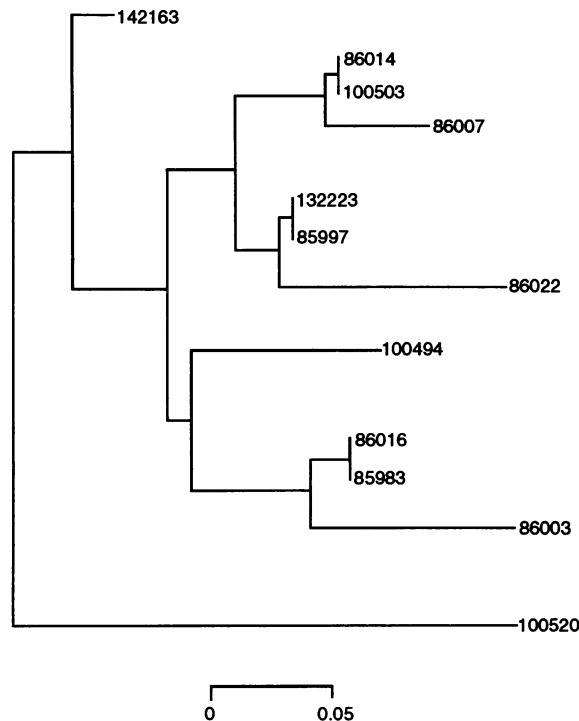


FIG. 9. Overall relatedness of penicillin-resistant pneumococci. A neighbor-joining tree (26) was constructed from the differences among isolates in the electrophoretic mobilities on starch gels of 18 enzymes. The bar at the bottom of the figure corresponds to a genetic distance of 0.05, corresponding to a difference at 1 of the 18 loci. The most distantly related isolates (e.g., 86014 and 100520) are separated by a genetic distance of 0.44 and differ at all of the 8 polymorphic loci.

of PCR), but genes with very similar but not identical sequences may produce identical fingerprints. After extensive use of this procedure, together with DNA sequencing, to analyze the PBP genes of penicillin-resistant pneumococci isolated worldwide, we have uncovered a few examples of PBP genes that are indistinguishable by fingerprinting but that are different in sequence (5). In the present study, the PBP 2B genes of fingerprint groups II and III and of groups VI and XI were shown to be different in sequence although they were not distinguished by fingerprinting. Direct sequencing of highly variable regions of each of the PBP genes provides a convenient method that can be used to check the identity of PBP genes when required.

Although isolated from a small region over a limited time period, the 23 penicillin-resistant pneumococci were very diverse and could be divided into 11 different PBP fingerprint groups and 11 different ribotype groups. There was a good correlation between the groupings of the isolates obtained by PBP fingerprinting and ribotyping, with the exception of the two isolates in fingerprint group IV which had distinct ribotypes. However, MLEE suggested that the latter isolates were members of the same clone which presumably differ slightly around the rRNA genes. It is, therefore, likely that the fingerprint groups in Table 1 define distinct penicillin-resistant clones. It should be stressed that this will not always be the case, since we have described examples of penicillin-resistant pneumococci from Spain that have identical alleles of each of the three PBP genes (i.e., the same fingerprint group) but which can be divided into two groups,

of different serotypes, that are not closely related when analyzed by MLEE (4). In this case, horizontal gene transfer is believed to have distributed the same resistance genes into two genetically diverse pneumococcal lineages to produce new penicillin-resistant clones (4).

Isolates in each fingerprint group were of the same serotype and required similar MICs of benzylpenicillin, except for fingerprint group II, in which two isolates were serotype 7B and two isolates were serotype 14. DNA sequencing confirmed that these four isolates possessed identical altered forms of PBPs 1A, 2B, and 2X. The occurrence of indistinguishable isozymes at each of the 18 enzyme-encoding loci that were examined indicates that the serotype 7B and 14 isolates are members of the same clone. This view is supported by ribotyping, which also failed to distinguish among these four isolates. A similar example of penicillin-resistant pneumococci that are identical by all criteria, except serotype, has been described previously. In that case, a multiply antibiotic-resistant isolate of serotype 19F from Spain was identical by all criteria (except serotype) to the members of the multiply antibiotic-resistant serotype 23F clone that is common in Spain (4). Subsequently, multiply antibiotic-resistant serotype 19F isolates that are members of the serotype 23F clone were identified in a second Spanish hospital (29) and in California (20). In the absence of information about the genetic basis of capsular biosynthesis in pneumococci, it is not possible to investigate further the reason why isolates that are apparently indistinguishable have different serotypes. However, it appears that some type of serotype change has occurred in both of these examples, perhaps mediated by the horizontal transfer and recombinational replacement of genes specifying capsular type (4).

The isolates of fingerprint group II could not be distinguished from those of fingerprint group I by either ribotyping or MLEE. The isolates of these two fingerprint groups are therefore members of the same clone. In addition, all of these isolates possess PBP 1A genes that are identical by both fingerprinting and DNA sequencing (although they have clearly different PBP 2X and 2B genes) and are mostly serotype 14. It seems likely that the isolates of fingerprint groups I and II are derived from a recent common ancestor, possibly of serotype 14, which acquired an altered PBP 1A gene to result in a slightly increased MIC for penicillin. Subsequently, the latter isolate may have gained higher-level penicillin resistance on two separate occasions by the acquisition of different altered PBP 2X and PBP 2B genes to produce isolates that we separate into fingerprints I and II. As discussed above, a change of serotype appears additionally to have occurred within fingerprint group II.

We found three examples among the Kenyan isolates in which the same altered PBP gene was found in isolates of different fingerprint groups. In all three cases, the identities of the PBP genes in each fingerprint group were established by DNA sequencing. The presence of allele 1 of the PBP 1A gene in isolates from fingerprint groups I and II may not represent an example of horizontal spread of the PBP 1A gene, since these isolates are members of a single clone (see above). The other two cases probably do involve horizontal transfer of PBP genes. Thus, the isolates of fingerprint groups V and X possessed the same PBP 2B allele but differed both by ribotype and by serotype and were shown by MLEE to be members of distantly related pneumococcal lineages (Fig. 9). Isolates of fingerprint groups VIII, IX, and X, which possessed the same PBP 1A allele, also differed by both ribotype and serotype, although the former and latter

strains could not be distinguished by MLEE. However, horizontal transfer of the PBP 1A gene is likely to have occurred, since isolates of fingerprint groups VIII and X were not closely related to the isolate of fingerprint group IX (Fig. 9).

Serotyping, or phage typing, is often used to distinguish bacterial isolates for epidemiological studies. However, it is well established that these methods are inadequate, since they are based on differences in cell surface structures that evolve rapidly and which may be subject to convergent evolution (28). In contrast, ribotyping and, particularly, MLEE provide much more satisfactory methods for estimating the relatedness of different isolates of the same species, since directly or indirectly they examine genetic diversity that is mostly believed to be selectively neutral. The heterogeneity among both the serotype 14 and the serotype 19 penicillin-resistant pneumococci from a single clinic confirms the inadequacy of serotyping alone for epidemiological studies of penicillin-resistant pneumococci.

In conclusion, our results demonstrate the diversity that can exist within penicillin-resistant pneumococci isolated within a short time period from a single clinic. They also illustrate the complex relationships between resistant pneumococcal isolates, probably arising from the horizontal transfer between lineages of PBP genes and genes specifying capsular type, which can be revealed only by methods that can assess both the relatedness of individual PBP genes and the overall genetic relatedness of isolates.

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