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Pseudomonas aeruginosa Serotype O12 Outbreak Studied by Arbitrary Primer PCR

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A total of 16 colonizing and infecting ofloxacin-resistant *Pseudomonas aeruginosa* strains and two strains isolated from ventilation equipment fluids, all with similar colonial morphologies and with minor but distinct susceptibility differences, were suspected of belonging to a single outbreak and were studied by arbitrary primer (AP) PCR. Thirteen nonrelated strains were included to evaluate the discriminatory capacity of the technique. AP PCR fingerprinting was compared with serotyping, phage typing, and antibiotic susceptibility testing. AP PCR was performed independently with three different primers. The different AP PCR typing systems yielded almost identical patterns for the epidemic strains and enabled us to differentiate most of the nonrelated strains from each other and from the outbreak strains. The combination of AP PCR typing and the phenotyping techniques that we used enabled us to conclude that an outbreak was occurring. In general, the typeability of AP PCR was greater than those of phage typing and serotyping, while the discriminatory powers of the three methods were comparable.

Rapid and highly discriminating typing methods, i.e., methods which enable the identification of bacterial clones, are needed in a hospital environment. Phenotyping methods may be poorly reproducible or may detect transient, unstable differences between clonally related strains because of culture conditions and/or environmental influences (e.g., antibiotic treatment) or may be applied to only a few species. During the past few decades, different molecular biology typing methods which may circumvent these problems because they study differences at the genetic level have been developed. Restriction endonuclease analysis (REA) (4), low-frequency REA (3, 16, 20), and REA combined with hybridization with probes complementary to repetitive genomic sequences, e.g., ribotyping (2, 4, 5, 16) and M13 typing (17, 21) or the use of species-specific probes (13, 18, 19), are among the best known and are widely applied. However, these established genotyping techniques may yield complex fingerprints resulting in interpretation problems (REA) or may be laborious and technically demanding (low-frequency REA and REA combined with hybridization).

Recently, the possibility of differentiating between eukaryotic or prokaryotic organisms by using arbitrary primers in a PCR was described independently (22, 25) and was called arbitrary primer (AP) PCR (22) or randomly amplified polymorphic DNA analysis (25). Subsequently, others have confirmed that AP PCR is a universally applicable, technically less demanding, rapid, and highly discriminatory typing method (1, 8–11, 23).

In the study described here, we evaluated the suitability of AP PCR as a typing technique for hospital epidemiological purposes by comparing it with serotyping, phage typing, and antibiotic susceptibility testing and by including strains suspected of belonging to a single outbreak as well as nonrelated strains from different sources.

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MATERIALS AND METHODS

Organisms. Table 1 summarizes the epidemiological data for the *Pseudomonas aeruginosa* strains studied. The organisms studied were from the following different sources: (i) suspected outbreak organisms, 16 of which infected and colonized 14 patients from three different wards during a 3-month period (strains OB1 to OB16), and 2 of which were isolated from ventilation equipment (strains OB17 and OB18); (ii) nonoutbreak strains from culture collections (strains CC1 to CC4); (iii) nonoutbreak strains which were isolated at the same hospital during previous years (strains HP1 to HP5); (iv) and nonoutbreak strains isolated at the same hospital during the same period (strains HS1 to HS4), but with phenotypic characteristics which clearly differentiated these strains from the outbreak strains. Finally, a fifth group consisted of strains which were obtained after subculture from strains OB3, OB4, OB5, and HP3 in the presence of different antibiotics. Derivative strains were obtained by reculturing colonies from the inner margin of the inhibition zone around the antibiotic disk on a Kirby-Bauer antibiogram (12) until resistance was complete (inhibition zone diameter, 0 mm). Derivative strains are designated as the parent strain designation plus the antibiotic designation. The antibiotics were chosen to mimic the susceptibility transitions which might have occurred during the outbreak.

AP PCR typing. Target DNA was prepared from cells grown overnight at 37°C on Mueller-Hinton agar II (MHAII; BBL Microbiology Systems, Cockeysville, Md.). A 1-μl volume of cells was scraped from the plates with a calibrated inoculation needle. Cells were washed in saline, resuspended in 300 μl of distilled water, and heated at 100°C for 10 min. The boiled

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TABLE 1. Overview of epidemiologic data and typing results for 38 *P. aeruginosa* strains

Strain type and designation[a]	Ward[b]	AP PCR type with primer[c]			Serotype[d]	Phage type[e]	Colony type	Antibiogram type[f]
		M13	OPM1	PH1				
Suspected outbreak-related strains								
Colonizing and infecting strains								
OB1, OB2, OB3, OB8, OB9	2	1	1	1	12	NT	2	A
OB4	6	1	1	1	12	NT	2	A+CIP
OB5	2	1	1	1	12	NT	2	A+IPM
OB16	2	1	1	1	12	NT	2	A+CAZ/PIP
OB6, OB7, OB14	2	1	1	1	12	119X	2	A+CIP
OB15	7	1	1	1	12	119X	2	A+CIP
OB10, OB13	2	1	1	1	12	68.119X	2	A
OB11	7	1	1	1	12	68.119X	2	A
OB12	6	1	1	1	12	68.119X	2	A+CIP
Strains isolated from ventilation equipment fluids								
OB17	2	1	1	1	12	68.119X	2	A+CIP
OB18	2	1	1	1	12	119X	2	A+CIP
Nonoutbreak-related strains								
Culture collection strains								
CC1		2	2	2	6	31.44	3	1
CC2		3	3	3	6	31.+	4	1
CC3		3	3	3	NT	16.21+31.44.68	5	2
CC4		4	4	4	10	44.68.1214.+	6	1
Strains isolated at the same hospital during previous years (hospital previously)								
HP1	3	5	5	5	3	24.31.44.F8.109.119X.352.M4	7	3
HP2	2	5	5	5	3	24.31.44.F8.109.119X.352.M4	7	3
HP3	1	1′	1	1	12	NT	3	A-OFL+PIP
HP4		6	6	6	16	16.21.31.44.68.F8.119X.+	8	4
HP5	2	7	7	7	11	31.44.68.+	6	5
Strains isolated at the same hospital during the outbreak period (hospital simultaneously)								
HS1	4	8	8	8	PA	NT	9	6
HS2	2	9	9	9	NT	7.31.73	10	7
HS3	2	10	10	10	4	NT	11	A′
HS4	5	1″	11	1″	PA	31	1	A+CIP
Strains subcultured in the presence of antibiotics (parent strain+antibiotic)								
OB3+CIP	2	1	1	1	12	NT	2	A+CIP
OB3+PIP	2	1	1	1	12	NT	2	A+CAZ/PIP
OB3+IPM	2	1	1	1	12	NT	2	A+IPM
OB3+CAZ	2	1	1	1	12	NT	2	A+CAZ/PIP
OB4+IPM	6	1	1	1	12	NT	2	A+IPM/CIP
OB5+CIP	2	1	1	1	12	NT	2	A+IPM/CIP
HP3+OFL	1	1′	1	1			3	A+PIP

[a] Culture collection strain abbreviations: CC1, ATCC 27853; CC2, ATCC 10145; CC3, ATCC 19712; CC4, ATCC 17831 (ATCC, American Type Culture Collection, Rockville, Md.).

[b] Wards: 1, hematology; 2, medical intensive care A; 3, nephrology; 4, pneumology; 5, rehabilitation center; 6, neurosurgery; 7, medical intensive care B.

[c] AP PCR typing result: 1′, pattern lacks 260-bp fragment observed for pattern 1; 1″, observed differences from pattern 1 may be due to overloading of strain HS4.

[d] Serotyping result: NT, nontypeable; PA, polyagglutinating.

[e] Phage typing result: identification numbers of phages capable of lysing the cells are indicated; NT, nontypeable.

[f] Antibiogram result: A, inhibition zones for NET, TM, GM, and OFL on Kirby-Bauer antibiogram, 0 mm; A+antibiotic, the inhibition zone for the A antibiogram type and the indicated antibiotic, 0 mm; A′, identical to A, but smaller zones for CAZ, CIP, IPM, and PIP; A-OFL+PIP, inhibition zones for NET, TM, GM, and PIP, 0 mm; 1 to 11, other susceptibility patterns.

suspensions of each strain were diluted 10- and 100-fold, and each of the three preparations (log 10^0, log 10^{-1}, log 10^{-2}) was used as the target DNA in the AP PCR. After observation of the electrophoresis patterns obtained after amplification of the different dilutions, the amplified products of each strain were pooled and rerun. Reaction mixtures of 25 μl contained 0.025 U of *Tth* polymerase per μl; 200 μM (each) the four deoxynucleotide triphosphates (Pharmacia Biotech, Uppsala, Sweden); 1% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.); 8 μM primer PH1 (TCA CGA TGC A), 0.8 μM primer M13 (GAG GGT GGX GGX TCT), or 1 μM primer OPM1 (GTT GGT GGC T); and 2.5 μl of sample in 3 mM $MgCl_2$–50 mM KCl–10 mM Tris-HCl (pH 9.0). Primers PH1 and M13 were synthesized by Pharmacia, and primer OPM1 was purchased from Operon Technologies (Alameda, Calif.).

The sequence of primer M13 was chosen to correspond to a consensus repeat sequence which was first described in the M13 bacteriophage (21) and which has been shown to be present in bacteria as well as in plants and animals (17). Therefore, PCR typing with primer M13 is not really AP PCR typing, since the sequence of this primer is not arbitrary. Single-primer PCR typing or M13 PCR typing would be more suited descriptions of PCR typing with primer M13.

After 10 min of denaturation at 95°C, reaction mixtures were run through three cycles of denaturation for 1 min at 95°C, annealing for 30 s at 30°C, and extension for 20 s at 65°C and through 27 cycles of denaturation for 30 s at 95°C, annealing for 20 s at 36°C, and extension for 1 min at 72°C. Finally, a 7-min extension at 72°C was carried out. Electrophoresis was carried out for 3 hours at 150 V on a 2% nucleic acid agarose (Pharmacia) gel containing 0.5 μg of ethidium bromide per ml in 0.1 M Tris–0.1 M borate–0.02 M EDTA buffer (pH 8.0). Gels were photographed, and the patterns were interpreted without densitometry or similarity calculations.

Phenotyping. Serotyping was carried out as described previously (6, 7), and phage typing was performed as described by Pitt (14). Testing of the susceptibilities to the chemotherapeutic agents ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GM), imipenem (IPM), netilmicin (NET), ofloxacin (OFL), piperacillin (PIP), and tobramycin (TM) was carried out on MHAII by the disk diffusion antibiogram method (12). Colonial morphology types were assigned by taking into account the color, shape, and consistency of the colonies grown on MHAII.

RESULTS AND DISCUSSION

Setup of the study. The strains used in the study enabled (i) comparison of most probably nonrelated strains from different origins with a group of most probably related strains, (ii) comparison of strains isolated at the same place and the same time but with different phenotypic characteristics, and (iii) evaluation of the stabilities of AP PCR fingerprints after subculture and after altering the susceptibility patterns of the strains. The applicability of AP PCR fingerprinting for epidemiological purposes was further evaluated by comparison of AP PCR with phage typing and serotyping, which are two of the most widely used techniques for phenotyping *P. aeruginosa* strains.

A total of 18 *P. aeruginosa* strains collected during a 3-month period from three different wards at University Hospital, Ghent, Belgium (outbreak-related strains designated OB [Table 1]), could be differentiated from other strains of this species by using phenotypic characteristics. Colonial morphologies were very typical, since colonies were dark green. Also, the antibiotic susceptibility patterns were rather typical, since all of the strains were resistant to OFL, GM, TM, and NE (designated antibiogram type A). Therefore, these strains were suspected of belonging to a single outbreak. However, while all strains were completely resistant to the four antibiotics mentioned above, 8 (strains OB4, OB6, OB7, OB12, OB14, OB15, OB17, and OB18) were also resistant to CIP (A+CIP), OB5 was also resistant to imipenem (A+IPM), and OB16 was also resistant to CAZ and PIP (A+CAZ/PIP). On the other hand, strains HS3 and HS4, which were isolated during the same period as the OB strains but with different colonial morphologies and from another ward, and strain HP3, which was isolated 2 years earlier at the same hospital, had rather similar antibiotic susceptibility characteristics (designated A′, A+CIP, and A-OFL+PIP, respectively). Thus, it was difficult to determine whether all outbreak-related strains were clonally related and whether strains HS3, HS4, and HP3 were related to the outbreak strains.

Therefore, we first evaluated whether the observed susceptibility differences could be due to in vivo selection during treatment by culturing some of the strains in the presence of CAZ, CIP, IPM, OFL, or PIP, i.e., the antibiotics to which the strains revealed different susceptibilities. This also enabled us to study the influence of repeated subculture and of altered susceptibility on AP PCR fingerprints and phenotypes. Second, we tried to determine which of these strains did cluster when studied by serotyping, phage typing, and AP PCR typing.

Selection of antibiotic resistance and influence on typing results. It was found that resistance to the additional antibiotics mentioned above was easily selected. Strains were resistant after 5 to 12 subcultures in the presence of these antibiotics. This suggests that if outbreak-related strains and strains HP3, HS3, and HS4 belonged to the same clone, the observed susceptibility differences could be explained by selection as a result of different in vivo chemotherapeutic treatments. Subculture and alteration of susceptibility patterns did not influence AP PCR type, colony type, serotype, or phage type (Table 1).

Technical remarks on AP PCR. The use of boiled cell suspensions avoided the need for DNA extraction. The fingerprints that we observed were identical to those obtained when phenol-chloroform-extracted DNA was used as the target (data not shown). Similar results have been reported previously (10). DNA standardization was avoided by using different DNA concentrations, i.e., dilutions of the boiled suspensions, as the targets. Pooling of the products obtained after AP PCR with the different dilutions enhanced the reproducibility of the tests, probably because this approach ensures that all of the fragments that can possibly be generated during AP PCR are present; the final pool yielded the same fingerprints for related strains, although the AP PCR fingerprints of the unpooled products were identical for each of the three dilutions for some of the strains but different for some or all of the dilutions for other strains (data not shown).

Because the use of *Tth* polymerase resulted in a higher yield of amplicon compared with that from the use of *Taq* polymerase (data not shown), we preferred to use *Tth* polymerase, although this resulted in more background "noise," i.e., the presence of multiple lower-intensity fragments.

By using boiled cell suspensions and dilutions of these suspensions, we could type 10 to 15 strains with one primer within 9 h (of which only 3 h was labor-intensive) when starting from pure cultures.

Reproducibility of AP PCR typing and interpretation of fingerprints. Although the patterns obtained for the outbreak strains were highly similar, minor differences were observed because of the absence or presence of weak-intensity fragments. Still, the overall similarity between the fingerprints of

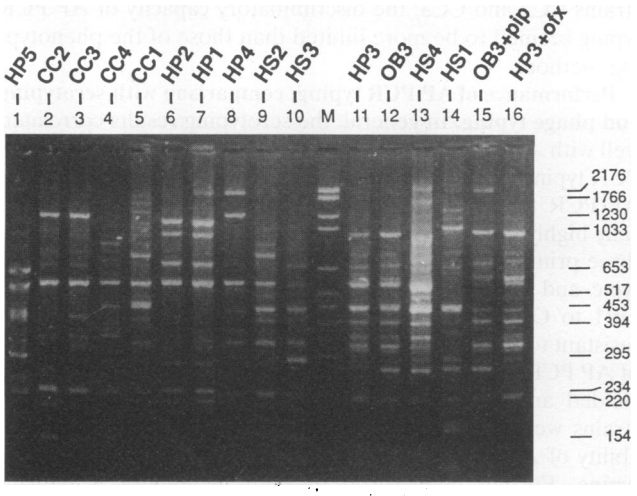

FIG. 1. Overview of fingerprints obtained after AP PCR with primer M13. Strain designations are indicated above the lanes: CC, culture collection strain; HS, strain isolated at the same hospital during the outbreak; HP, strain isolated at the same hospital during previous periods; OB, strain suspected of belonging to a single outbreak; strain + antibiotic, parent strain subcultured in the presence of the antibiotic until no inhibition zone was observable. Molecular sizes are indicated in base pairs. M, molecular mass marker. The AP PCR fingerprints of the other 17 suspected outbreak-related strains were similar to the fingerprints shown in lanes 12 and 15.

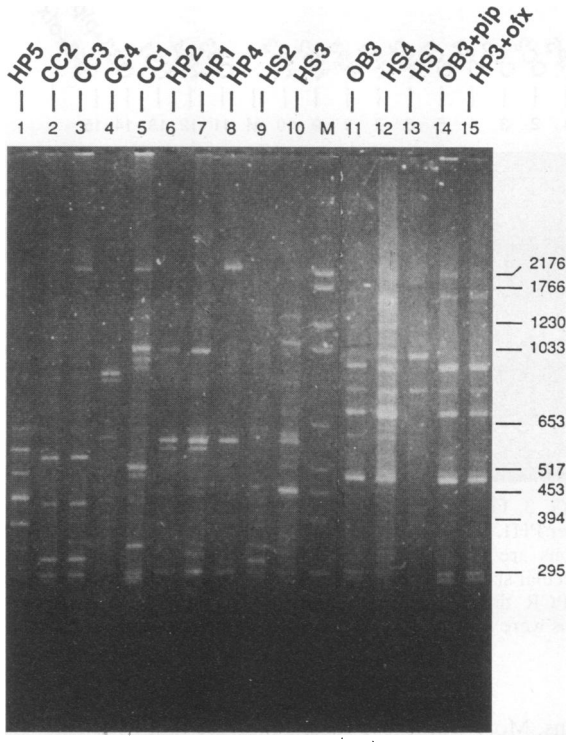

FIG. 2. Overview of fingerprints obtained after AP PCR with primer OPM1. Strain designations are indicated above the lanes. Abbreviations are identical to those described in the legend to Fig. 1. Molecular sizes are indicated in base pairs. M, molecular mass marker. The AP PCR fingerprints of the other 17 suspected outbreak-related strains were similar to the fingerprints shown in lanes 11 and 14.

the outbreak-related strains was high and enabled easy differentiation from most of the nonoutbreak-related strains, regardless of which of the three primers was used. The presence of these minor-intensity fragments, the run-to-run reproducibilities of which varied, may be explained by the strong amplification results that we obtained with *Tth* polymerase. Therefore, only the major amplification fragments were taken into account in interpretation of the patterns, and this resulted in easy interpretation of the fingerprints. For example, although the patterns for strains CC2 and CC3 or for strain HP3 and strain HP3 plus OFL obtained with primer M13 were not identical, their overall similarities were clear and allowed us to easily differentiate these strains from other strains.

The reproducibility of the technique was also supported by the fact that the same typing results were obtained with different primers.

Typing results. Table 1 lists the AP PCR types, serotypes, phage types, colony types, and antibiotic susceptibility patterns for 38 strains (7 of which were derived from clinical strains by subculture in the presence of different antibiotics). Figures 1, 2, and 3 represent the different AP PCR patterns obtained for suspected outbreak-related strains and for nonrelated strains after performing AP PCR with primers M13, OPM1, and PH1, respectively.

First, we evaluated whether the outbreak-related strains were clonally related. All outbreak-related strains (OB1 to OB16), including the two strains from the suspected source (OB17 and OB18), had a typical colonial morphology, had highly similar AP PCR fingerprints, belonged to serotype O12, were phage nontypeable (8 strains), or were lysed by phage 119X (5 strains) or by phages 119X and 68 (5 strains). The AP PCR fingerprints of the outbreak-related strains were clearly different from those obtained from all nonoutbreak-related strains except strain HP3 (see below). The serotypes of the outbreak-related strains were different from those of the other strains included in the study except strain HP3. Phage types except those for nontypeable strains allowed differentiation of all nonoutbreak-related strains. Phage types 119X and 68 have been shown to be typical for serotype O12 strains (24).

These typing results and the fact that we could show that the observed differences in antibiotic susceptibilities could have been caused by different antibiotic treatments of the patients allowed us to conclude that the suspected outbreak-related strains belonged to a single epidemic and that this epidemic was caused by the contamination of automated ventilation equipment. It should be stressed that, since serotype O12 strains are considered to be part of a single clone and since these strains cannot be differentiated from each other by genotyping techniques like ribotyping, REA, or protein electrophoresis profile analysis (4, 15), the possibility that some of the outbreak-related strains were from another origin cannot be ruled out.

On the other hand, strain HP3, which was isolated 2 years earlier at another ward in the same hospital and which also belonged to serotype O12, had a colony type different from those of the outbreak-related strains. It was the only serotype O12 strain that was susceptible to OFL, although OFL resistance could readily be selected (strain HP3+OFL), and it could be differentiated from the outbreak-related strains by single-primer typing with primer M13 by the absence of a major-intensity fragment of approximately 260 bp (Fig. 1, lanes 11 and 16) which was present in the outbreak-related strains (Fig. 1, lanes 12 and 15). Thus, these combined results allowed us to differentiate strain HP3 from the outbreak-related

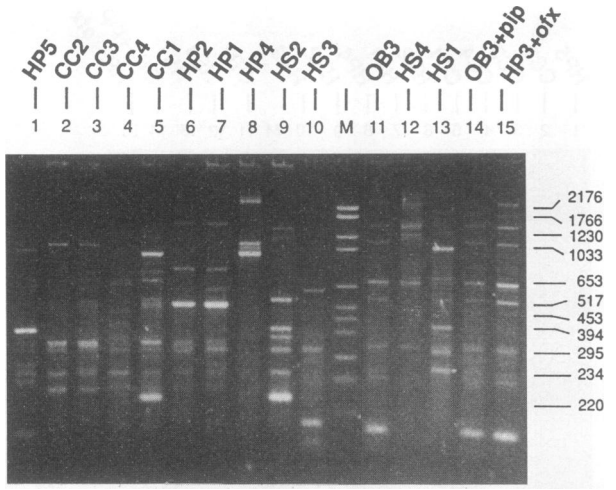

FIG. 3. Overview of fingerprints obtained after AP PCR with primer PH1. Strain designations are indicated above the lanes. Abbreviations are identical to those described in the legend to Fig. 1. Molecular sizes are indicated in base pairs. M, molecular mass marker. AP PCR fingerprints of the other 17 suspected outbreak-related strains were similar to the fingerprints shown in lanes 11 and 14.

strains. Moreover, these results indicate that M13 PCR typing can differentiate between O12 strains.

The next question was whether strains HS4, HS3, and HP3, which had antibiotic susceptibility patterns that were very similar to those of the outbreak-related strains, were part of the outbreak. The differences between strain HP3 and the outbreak-related strains were discussed above.

Serotyping results revealed that strain HS3 was serotype O4 and that strain HS4 was polyagglutinable. The AP PCR fingerprints of strain HS3 clearly differed from those of outbreak-related strains. Strain HS4 had AP PCR types for primers M13 and OPM1 (Fig. 1, lane 13, and Fig. 2, lane 12) which were very similar (AP PCR type 1″) to those observed for the outbreak-related strains (AP PCR type 1). The observed differences were not considered significant because they could have been caused by overloading of strain HS4, but AP PCR with primer PH1 provided clear differentiation between strain HS4 and the outbreak-related strains (Fig. 3, lanes 12 and 11, respectively). Moreover, strain HS4 differed from the outbreak-related strains by its colony type; it was isolated at another ward, and differed in phage type from the phage types which are commonly present among O12 strains.

Combination of these results allowed us to conclude that neither HS3, HS4, nor HP3 was part of the outbreak.

All of the other nonrelated strains were different from the outbreak-related strains with regard to serotype, phage type, colony type, antibiogram, and AP PCR type. The AP PCR types of the 10 randomly picked nonrelated strains were different except for those for two pairs of strains. The AP PCR fingerprints obtained with each of the three primers for strains HP1-HP2 and CC2-CC3 could not be differentiated from each other according to the interpretation criterion described above. Strains HP1 and HP2 were found to have the same colonial morphology, serotype, susceptibility pattern, and phage type, while strains CC2 and CC3 had different susceptibilities, colonial morphologies, and phage types (and an unknown serotype because of the nontypeability of strain CC3). In the case of strains HP1 and HP2, the usefulness of AP PCR in recognizing closely related strains was confirmed. For strains CC2 and CC3, the discriminatory capacity of AP PCR typing seemed to be more limited than those of the phenotyping methods.

Performance of AP PCR typing: comparison with serotyping and phage typing. In general, the serotyping results correlated well with AP PCR results. The discriminatory capacities of AP PCR typing and serotyping seemed to compare well. However, AP PCR typing differentiated between strains of the genetically highly homogeneous O12 serotype clone (with one of the three primers), and this result corresponded well with phenotypic and epidemiologic observations. In contrast to strains OB1 to OB18, strain HP3 was susceptible to OFL and was resistant to PIP and was isolated 2 years earlier. The typeability of AP PCR was greater than that of serotyping, since all strains yielded amplification fragment length patterns, while some strains were polyagglutinable or seronontypeable. The typeability of AP PCR typing was also greater than that of phage typing. Further comparison of both techniques is difficult because of the many phage-nontypeable strains examined in the present study.

Stability of AP PCR fingerprints. Subcultivation, whether or not the susceptibility pattern was altered deliberately during subcultivation, did not influence the AP PCR pattern; identical fingerprints were seen for, for example, strains HP3 and HP3+OFL (Fig. 1, lanes 11 and 16) and strains OB3 and OB3+PIP for primers OPM1 and PH1 (Fig. 2 and 3, lanes 11 and 14), while minor differences between OB3 and OB3+PIP were present when primer M13 was used (Fig. 1, lanes 12 and 15). In addition, the high degree of similarity between strain HP3 and the outbreak-related strains, all of which belonged to (clonal) serotype O12, illustrates that AP PCR patterns can be conserved over long periods of time, since HP3 was isolated 2 years earlier.

Applicability of AP PCR typing in the clinical microbiology laboratory. AP PCR typing has several advantages over phenotyping and other genotyping techniques. AP PCR is rapid (10 to 15 strains can be typed with one primer in 9 h, only 3 h of which is labor-demanding). AP PCR is universally applicable, while the application of most phenotyping techniques is limited to one or a few species. AP PCR is technically less demanding than ribotyping and low-frequency REA and is a low-cost method in comparison with the other genotyping techniques, especially because of the decreased workload. Thus, the combination of the advantages mentioned above with the high degree of typeability and discriminatory capacity of AP PCR typing provides us with a technique that can be applied in nonspecialized laboratories and which yields fast and reliable results at low cost.

Conclusion. AP PCR supported the clonal relatedness of strains isolated from the same outbreak and differentiated between the outbreak-related strains and a collection of nonrelated strains, including a nonrelated strain belonging to the same serotype (O12). AP PCR patterns were not influenced by repeated subculture, even when the susceptibilities of the strains during subcultivation were altered. AP PCR typing confirmed the clonal relatedness of two strains randomly picked from a large collection but did not differentiate between two clearly different culture collection strains. AP PCR typing had a higher degree of typeability than serotyping and phage typing, and the discriminatory capacity of AP PCR typing was comparable to that of phenotyping. Single-primer typing with primer M13 suggests that serotype O12 strains, which are phenotypically and genotypically very similar, could be differentiated from each other.

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