Journal of Antimicrobial Chemotherapy (2002) **50**, 11–18
DOI: 10.1093/jac/dkf069

JAC

Original articles

Identification of PSE and OXA β-lactamase genes in *Pseudomonas aeruginosa* using PCR–restriction fragment length polymorphism

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Received 3 September 2001; returned 17 January 2002; revised 6 February 2002; accepted 27 March 2002

Objective: A method using PCR–restriction fragment length polymorphism was developed to identify *Pseudomonas aeruginosa* β-lactamase genes.

Methods: Two hundred and fifty-nine *P. aeruginosa* isolates were screened by PCR with 11 primer pairs designed to detect genes encoding PSE, OXA, TEM and SHV enzymes. PSE and OXA gene variants were distinguished by restriction of PCR products with endonucleases recognizing sites involved in point mutations. Nucleotide sequences were verified for a few isolates by sequencing the PCR products.

Results: Four isolates produced extended-spectrum β-lactamases (ESBLs) according to the double disc synergy test. PCR detecting bla_{PSE} genes was positive in 162 (62.5%) isolates: 151 carried $bla_{PSE\text{-}1}$ and 11 carried a variant encoding an enzyme differing from PSE-1 by a single amino acid substitution (Pro102 to Ser). PCR detecting sequences for enzymes of the OXA-10 group was positive in 68 (26.3%) isolates: 31 carried $bla_{OXA\text{-}10}$, one carried $bla_{OXA\text{-}14}$ and 36 carried a new variant intermediate between $bla_{OXA\text{-}13}$ and $bla_{OXA\text{-}19}$. The $bla_{OXA\text{-}2}$ gene was identified in 13 (5%) isolates. Two other isolates carried $bla_{OXA\text{-}2}$ variants encoding ESBLs differing from OXA-2 by a single amino acid substitution (Asp150 to Tyr and Trp159 to Cys, respectively). PCR detecting sequences for enzymes of the OXA-1 group was positive in 12 (4.6%) isolates. A bla_{TEM} gene was identified in five (1.9%) isolates (three $bla_{TEM\text{-}1}$, one $bla_{TEM\text{-}2}$, one $bla_{TEM\text{-}4}$).

Conclusion: This approach is effective for screening *P. aeruginosa* for β-lactamase gene carriage in epidemiological studies and for detecting new variants.

Introduction

Ticarcillin resistance in *Pseudomonas aeruginosa* can be mediated by derepression of class C chromosomal cephalosporinase production, overexpression of the MexA-MexB-OprM efflux system or acquisition of β-lactamases encoded by mobile genetic elements.[1] The enzymes encountered most frequently are carbenicillinases of the PSE (CARB) group (Ambler class A) and oxacillinases (Ambler class D). Classical PSE and OXA enzymes confer resistance to carboxypenicillins and ureidopenicillins, but not to ceftazidime. However, extended-spectrum β-lactamases (ESBLs) that hydrolyse third-generation cephalosporins have been reported with increasing frequency in this species in the past few years.[2] These ESBLs often result from amino acid substitutions in OXA enzymes or, less frequently, in TEM or SHV enzymes.

Four PSE enzymes have been reported in *P. aeruginosa*: PSE-1 (CARB-2), PSE-4 (CARB-1), CARB-3 and CARB-4. PSE-1, PSE-4 and CARB-3 are closely related, differing from one another by one or two amino acids, but share only 86.3% homology with CARB-4.[3–6] Five distinct groups of oxacillinases have been described by Sanschagrin *et al.*[7] OXA group I includes OXA-5, OXA-7, OXA-10 and its derivatives (OXA-11, OXA-14, OXA-16, OXA-17), and OXA-13 and its derivatives (OXA-19, OXA-28).[8–17] OXA group II includes

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OXA-2, OXA-3, OXA-15 and OXA-20.[7,18–20] OXA group III includes OXA-1, OXA-4, OXA-30 and OXA-31, and OXA group IV is defined by OXA-9.[7,21–23] OXA group V currently consists of a single enzyme, LCR-1.[7,9] In addition, OXA-18 does not belong to any of these groups and has very low amino acid identity with other oxacillinases.[24]

In previous studies that examined β-lactam resistance mechanisms in a large number of *P. aeruginosa* isolates, characterization of β-lactamases was based solely on isoelectric point.[25,26] This method cannot identify these enzymes precisely, particularly those in the OXA group, since several enzymes have the same or very similar isoelectric points. We have therefore developed a method of identifying PSE and OXA β-lactamase genes using PCR–restriction fragment length polymorphism (PCR–RFLP) and applied it to characterization of the β-lactamases produced in 259 clinical isolates of *P. aeruginosa*.

Materials and methods

Theoretical aspects

The different DNA sequences of genes encoding PSE and OXA enzymes from *P. aeruginosa* were analysed using DNA software analysis package Oligo 6 (Molecular Biology Insights, Cascade, CO, USA).[3–24] Pairs of primers were designed to detect each group of β-lactamases. Restriction endonuclease sites affected by point mutations were analysed and primers designed to distinguish variants within the same group.

Bacterial isolates

A collection of 259 clinical isolates of *P. aeruginosa* producing class A or D β-lactamases was used for this study. The strains were isolated at Beaujon hospital during the periods 1984–1989 (128 isolates) and 1994–1999 (131 isolates), and were non-repetitive (only one isolate with a given antibiogram per patient was included in the set). Eighty-eight isolates belonged to serotype O12 and 171 to other serotypes. They were initially suspected of producing class A or class D β-lactamases on the basis of routine Mueller–Hinton disc diffusion tests performed in our laboratory (resistance to ticarcillin, increased susceptibility to ticarcillin when combined with clavulanic acid and/or increased susceptibility to piperacillin when combined with tazobactam). Two hundred and twenty-five isolates were found to be susceptible to ceftazidime, while 34 isolates had decreased susceptibility to this drug. β-Lactamase production was subsequently confirmed by detecting activity in sonicated extracts using the iodometric assay in an agar gel, with and without inhibitors (clavulanic acid, cloxacillin), according to Labia & Barthélémy.[27] Isolates whose β-lactamase activity was fully inhibited by cloxacillin, but not by clavulanic acid, were considered to produce only a derepressed class C cephalosporinase and were excluded from the study.

Detection of ESBLs

The 34 isolates with reduced susceptibility to ceftazidime were screened for the presence of ESBLs by a double disc synergy test. Three discs of ceftazidime were placed next to a disc of clavulanic acid at distances of 3, 2 and 1 cm. The test was considered positive when at least one of the three ceftazidime zones was expanded by the presence of clavulanic acid.

PCR screening

Bacterial DNA was prepared with InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions. DNA was used as template in PCR assays using 11 primer pairs: nine pairs, designed specially for this study, were based on the nucleotide sequences of bla_{PSE} and bla_{OXA} genes, and two pairs, reported previously by Rasheed *et al.*,[28] detect bla_{TEM} and bla_{SHV} genes (Table 1). Reaction mixtures (50 µL) contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.4 mM of each deoxynucleotide triphosphate (Boehringer), 2.5 U of *Taq* polymerase (Roche Diagnostics) and 8 µL of DNA. The DNA amplification programme consisted of an initial denaturation step (96°C, 5 min) followed by 30 cycles of denaturation (96°C, 30 s), annealing (55°C or 60°C, 30 s) and extension (72°C, 1 min), and a single final extension of 5 min at 72°C. Ten microlitres of reaction mix containing PCR product was analysed by electrophoresis in 1.5% (w/v) agarose (Sigma).

Restriction of PCR products

The bla_{PSE} genes were identified by digestion of PCR products with *Apo*I and *Mbo*II (Figure 1). Genes encoding enzymes of OXA group I were differentiated by digestion of PCR products with *Bst*EII, *Nde*I, *Sau*3AI, *Hae*III, *Hha*I, *Bbv*I and *Hph*I (Figure 2). *Bbv*I, *Hin*dIII and *Sau*96I were used to identify sequences encoding enzymes within the OXA group II (Figure 3). Ten microlitres of reaction mix containing PCR product was digested as instructed by the endonuclease supplier (New England Biolabs). The DNA fragments generated were analysed by electrophoresis in 2–4% (w/v) Metaphor agarose gel (FMC Products, Rockland, ME, USA), according to the expected fragment size.

Nucleotide sequence determination

For at least one isolate corresponding to each type of PCR–RFLP pattern observed, the identification of the β-lactamase gene was verified by determining its sequence. Pairs of primers were used to amplify two overlapping fragments covering the full-length gene (Table 2) and the nucleotide

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Table 1. Primer pairs used for β-lactamase screening

Primer pairs	Sequence	β-Lactamase detected	Product size (bp)
1	5′ ACC GTA TTG AGC CTG ATT TA 3′ 5′ ATT GAA GCC TGT GTT TGA GC 3′	PSE group (PSE-1, PSE-4, CARB-3)	321
2	5′ TAA TAG AAA AGC AAG TAG GA 3′ 5′ AAC TAT GAT TGG GGA TTG AG 3′	CARB-4	435
3	5′ TCA ACA AAT CGC CAG AGA AG 3′ 5′ TCC CAC ACC AGA AAA ACC AG 3′	OXA group I (OXA-10 group)	276
4	5′ AAG AAA CGC TAC TCG CCT GC 3′ 5′ CCA CTC AAC CCA TCC TAC CC 3′	OXA group II (OXA-2 group)	478
5	5′ TTT TCT GTT GTT TGG GTT TT 3′ 5′ TTT CTT GGC TTT TAT GCT TG 3′	OXA group III (OXA-1 group)	427
6	5′ AGC CGC ATA TTT AGT TCT AG 3′ 5′ ACC TCA GTT CCT TTC TCT AC 3′	OXA-5	664
7	5′ CGA TTA CGG CAA CAA GGA 3′ 5′ TTA GGC GGG CGA AGA CGA 3′	OXA-18	322
8	5′ AGA GCG GTG ACT ACT GGA TA 3′ 5′ AAA GCA TTG ACG GAT TGA AG 3′	OXA-20	308
9	5′ CCT TTG GTC TCT TTA TTG CG 3′ 5′ CGT CTT TGG CTA TCT GCG TT 3′	LCR-1	706
10	5′ ATG AGT ATT CAA CAT TTC CG 3′ 5′ CTG ACA GTT ACC AAT GCT TA 3′	TEM group	867
11	5′ GGT TAT GCG TTA TAT TCG CC 3′ 5′ TTA GCT TTG CCA GTG CTC 3′	SHV group	867

Table 2. Primer pairs used for DNA sequencing

Primer pairs	Sequence	β-Lactamase detected
12	5′ TAG GTG TTT CCG TTC TTG 3′ 5′ TCA TTT CGC TCT TCC ATT 3′	PSE group
13	5′ CAG CAG TTG TGT GGA GTG 3′ 5′ CTT GTT AGC CTT ATC AGC 3′	
14	5′ ATG GCA ATC CGA ATC TTC GC 3′ 5′ CCT TCT ATC CAG TAA TCG CC 3′	OXA-2 and derived ESBL
15	5′ GAC CAA GAT TTG CGA TCA GC 3′ 5′ ATA GAG CGA AGG ATT GCC CG 3′	
16	5′ TTT CGA GTA CGG CAT TAG CT 3′ 5′ GAC ACC AGG ATT TGA CTC AG 3′	OXA-10 and derived ESBL
17	5′ GGC GGC ACC TGA ATA TCT AG 3′ 5′ GAA TGG ATT TTC TTA GCG GC 3′	
18	5′ ATT ACT GCG TGT CTT TCA 3′ 5′ CTC TTT CCC ATT GTT TCA 3′	OXA-13 and derived ESBL
19	5′ TCC CCA ACG CAA TTA TCG GC 3′ 5′ TAG CCA CCA ATG ATG CCC TC 3′	
20	5′ CAC AAT ACA TAT CAA CTT CG 3′ 5′ GCC ATA AGT GAT AAT GC 3′	OXA-1 group
21	5′ CCA TTA TTT GAA GGA ACT GA 3′ 5′ TAG TGT GTT TAG AAT GGT GA 3′	

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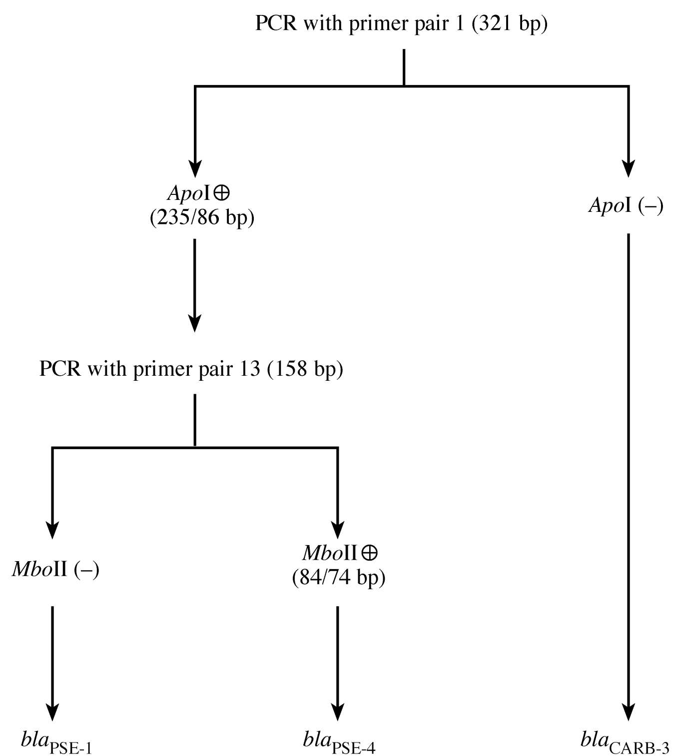

Figure 1. Identification of genes encoding β-lactamases of the PSE group: circled plus sign, digestion by the enzyme indicated; (–), no digestion by the enzyme indicated.

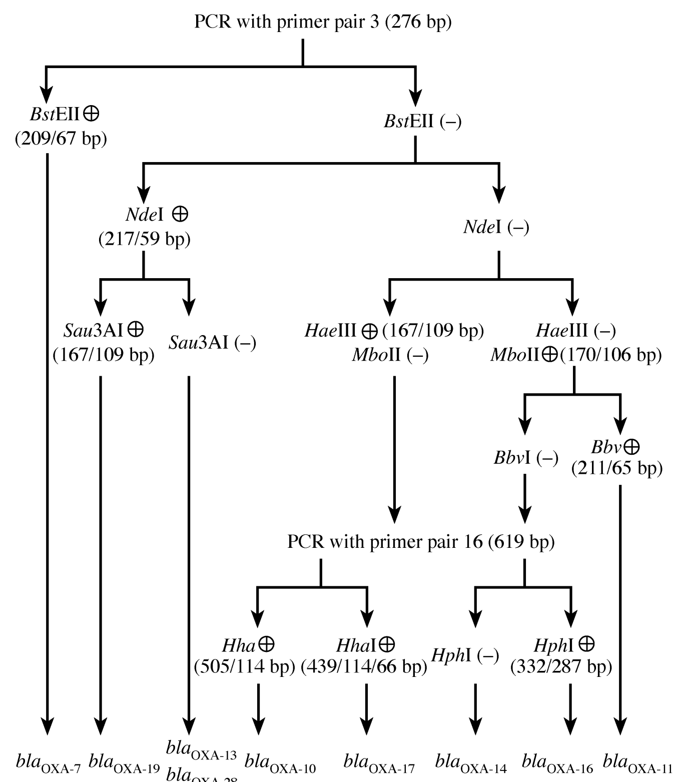

Figure 2. Identification of genes encoding β-lactamases of OXA group I: circled plus sign, digestion by the enzyme indicated; (–), no digestion by the enzyme indicated.

sequences of the PCR products were determined by Genome Express on an Abiprism model 377 DNA sequencer (Perkin Elmer). The GenBank database was searched for homologous nucleotide sequences.

Results

ESBL detection

Among the 34 isolates with decreased susceptibility to ceftazidime, 29 were considered to overproduce the *P. aeruginosa* chromosomal class C cephalosporinase, in addition to producing a class A or D enzyme, since their β-lactamase activities were inhibited only in the presence of both clavulanic acid and cloxacillin.[25,27] Four isolates displayed a positive double disc synergy test; each of these was presumed to produce an ESBL. A synergy pattern was visible between the discs placed 3 cm apart for strain 606, 2 cm apart for strains 617 and 893, and only when placed 1 cm apart for strain 98. For the remaining isolate, which did not produce a derepressed cephalosporinase or an ESBL, ceftazidime resistance was presumed to be due to a non-enzymic mechanism.

PCR screening

Two hundred and fifty-eight of the 259 isolates tested gave positive PCR results with a single primer pair (161 with pair 1, 67 with pair 3, 13 with pair 4, 12 with pair 5, five with pair 10) and one isolate yielded PCR products with two primer pairs (pairs 1 and 3). In every case, the size of the PCR product was consistent with the predicted amplified fragment size (Table 1). None of the isolates gave positive PCR results for sequences encoding CARB-4, OXA-5, OXA-18, OXA-20, LCR-1 or the SHV enzyme group.

Identification of PSE group β-lactamases

PCR with primer pair 1, which detects sequences for PSE-1, PSE-4 and CARB-3, was positive for 162 (62.5%) isolates. In all cases, *Apo*I cleaved the 321 bp amplification product, thus excluding CARB-3 (Figure 1). PSE-4 differs from PSE-1 by a C to A change at position 942, leading to the appearance of an *Mbo*II restriction site.[4] Fragments generated by amplification with primer pair 13 were therefore subjected to *Mbo*II restriction. No cleavage of any of the 162 PCR products was observed, as expected for PSE-1. The nucleotide sequences of the β-lactamase genes of three of these isolates were analysed and confirmed to be $bla_{PSE\text{-}1}$ in two of them. The third gene differed from $bla_{PSE\text{-}1}$ by a single C to T mutation at nucleotide 401 causing a Pro102–Ser change in the amino acid sequence of the enzyme. This mutation generates a *Tsp*509I restriction site at position 398. The *Tsp*509I restriction patterns of the

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PCR products generated with primer pair 12 were analysed for the 162 isolates. The new *Tsp*509I restriction site was present in 11 of them. These 11 isolates were therefore assumed to carry a new bla_{PSE} gene encoding a variant of PSE-1.

Identification of β-lactamases of OXA group I

PCR with primer pair 3 detects genes encoding OXA group I enzymes.This reaction was positive in 68 (26.3%) isolates, including the ESBL-producing strain 98. *Bst*EII did not cleave the 276 bp fragment obtained from any of the isolates, thereby excluding carriage of bla_{OXA-7}. In 36 isolates, PCR products were cleaved to two fragments by *Nde*I but were not cleaved by *Sau*3AI, as expected for the bla_{OXA-13} PCR product (Figure 2). However, analysis of the nucleotide sequences of PCR fragments from three of these isolates showed that the *bla* genes differed from bla_{OXA-13} by a G to A mutation at position 1157, causing the replacement of Ser73 by asparagine in the β-lactamase. This mutation involves the disappearance of a *Hha*I site. Accordingly, the *Hha*I restriction profiles of the PCR products generated with primer pair 18 were analysed. All 36 PCR products lacked the *Hha*I site, so it was concluded that these *bla* genes encode a variant of OXA-13.

For the remaining 32 isolates producing OXA group I enzymes (including strain 98), no digestion of the PCR products with *Nde*I was observed, as expected for bla_{OXA-10} and its derivatives. Digestion of PCR products with *Hae*III and the absence of digestion with *Mbo*II identified the 31 non-ESBL genes as bla_{OXA-10}. This was confirmed by determining the nucleotide sequence of the *bla* genes of two isolates. *Mbo*II digested the PCR product obtained with strain 98 whereas *Hae*III did not. The PCR product also lacked the *Bbv*I site typical of bla_{OXA-11} and the *Hph*I site typical of bla_{OXA-16}; so the *bla* gene was assumed to be bla_{OXA-14}. This was confirmed by nucleotide sequence analysis.

Identification of OXA group II β-lactamases

PCR analysis with primer pair 4, which detects genes encoding OXA group II β-lactamases, gave products with 13 (5%) isolates, including the two ESBL-producing strains 617 and 893. In each case, the PCR product was cleaved by *Bbv*I, but not by *Hin*dIII or *Sau*96I. The β-lactamase genes in the 11 non-ESBL isolates were therefore presumed to be bla_{OXA-2} (Figure 3). This conclusion was confirmed by sequencing three of the PCR products.

The *Sau*96I recognition site typical of bla_{OXA-15} was not found in the PCR products from strains 617 and 893, suggesting the carriage of a bla_{OXA-2} variant. Analysis of the nucleotide sequences of these genes showed that the *bla* gene of strain 617 differs from bla_{OXA-2} by a G to T mutation at nucleotide 575 and from bla_{OXA-15} by a G to A mutation at nucleotide 576, resulting in a tyrosine at amino acid 150 instead of the aspartate found in OXA-2 and the glycine in OXA-15. The nucleotide sequence of the *bla* gene in strain 893 differs from bla_{OXA-2} by a G to T mutation at position 604, leading to the replacement of tryptophan by cysteine at amino acid 159.

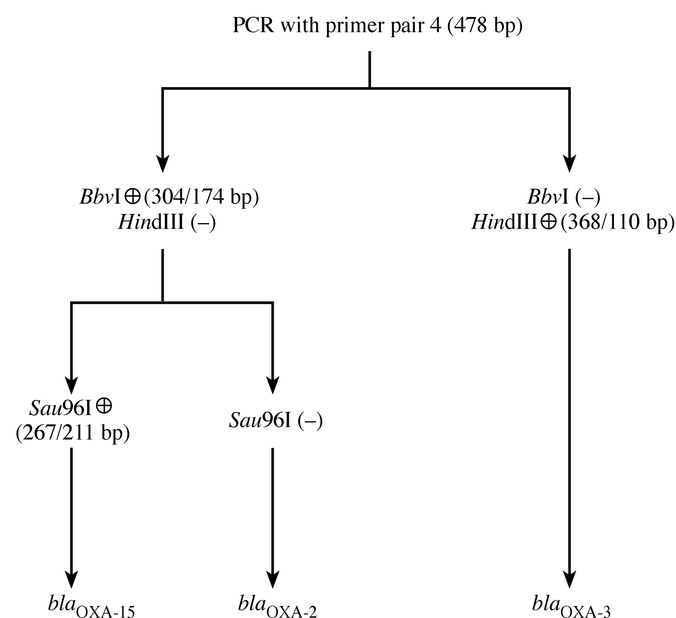

Figure 3. Identification of genes encoding β-lactamases of OXA group II: circled plus sign, digestion by the enzyme indicated; (–), no digestion by the enzyme indicated.

Identification of OXA group III β-lactamases

PCR analysis with primer pair 5, which detects genes encoding OXA group III enzymes, was positive for 12 (4.6%) isolates. Analysis of the nucleotide sequences of the PCR products from two of these isolates showed that in both cases the enzyme encoded was OXA-31.

Identification of TEM group β-lactamases

PCR analysis with primer pair 10, which detects bla_{TEM} genes, was positive for five (1.9%) isolates, including the ESBL-producing strain 606. Sequencing the PCR products revealed that three isolates harbour bla_{TEM-1}, one harbours bla_{TEM-2} and strain 606 harbours bla_{TEM-4}.

Distribution of β-lactamases according to O serotype and study period

Assuming that the genes detected reflect the β-lactamases produced, PSE-1 was identified in all isolates of serotype O12. Among isolates of other serotypes, 62 (36.3%) produced PSE-1, 108 (63.2%) produced another enzyme and one (0.5%) produced both PSE-1 and an OXA enzyme.

The comparative distribution of β-lactamases during the two study periods is shown in Table 3. PSE-1 was predomin-

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Table 3. β-Lactamases identified in 259 *P. aeruginosa* isolates

β-Lactamase	No. (%) of isolates	
	1984–1989	1994–1999
PSE (CARB) group	77 (59.7)	85 (64.9)
PSE-1	74	77
PSE variant	3	8
OXA group I	41 (31.8)	27 (20.6)
OXA-13 group	25	11
OXA-10	16	15
OXA-14	0	1
OXA group II	0	13 (9.9)
OXA-2	0	11
OXA-2-derived ESBL	0	2
OXA group III	11 (8.5)	1 (0.8)
TEM group	0	5 (3.8)
TEM-1	0	3
TEM-2	0	1
TEM-4	0	1

ant over both periods, followed by enzymes of OXA group I. OXA group II and TEM β-lactamases and ESBLs were identified exclusively in isolates from 1994–1999, whereas most OXA group III β-lactamases were found in isolates from 1984–1989.

Discussion

Because of the large number of new β-lactamases described in *P. aeruginosa* in the past few years, some with identical or very similar isoelectric points, isoelectric focusing can no longer be considered an effective method of identifying β-lactamases in this species. Epidemiological studies should use PCR-based detection tests followed by analysis of the PCR products by sequencing or restriction with endonucleases chosen to detect restriction site changes generated by point mutations. PCR–RFLP analysis has been successfully applied to the identification of TEM β-lactamases in Enterobacteriaceae.[29] In *P. aeruginosa*, its use has been limited to the detection of OXA-10-derived ESBLs without fully differentiating variants within this group.[30] The main difficulty in applying PCR–RFLP to the characterization of oxacillinases is that they constitute a heterogeneous group of enzymes, including subgroups with large evolutionary distances between them.[7] Therefore, in contrast to TEM or SHV enzymes, all oxacillinases cannot be detected by PCR with a single primer pair. In this study, seven PCR primer pairs were designed to detect oxacillinases, and two further pairs were used for carbenicillinases. It has been suggested that this approach may be hampered by the presence of multiple β-lactamases in the same isolate.[2] In this study, only one isolate gave PCR products with two primer pairs. Furthermore, the restriction patterns were unambiguous, indicating that the PCR products did not contain mixtures of sequences from closely related β-lactamases, which would have been amplified simultaneously. Thus, in this study, the presence of multiple class A or D enzymes in a single isolate was rare. However, overproduction of the class C cephalosporinase was detected in 29/259 (11.2%) isolates.

As reported previously,[26] so in this study PSE enzymes were the most common β-lactamases, followed by oxacillinases. TEM enzymes were uncommon and none of the isolates produced a SHV enzyme. Within the PSE group, PSE-1 was by far the most common enzyme, and it was strongly associated with serotype O12. Thus, $bla_{\text{PSE-1}}$ was found in all O12 isolates but in only 36.3% of other serotypes. Other carbenicillinases were not identified, but 11 isolates produced a variant of PSE-1 differing from it by a single amino acid (Ser102 instead of proline).

The OXA group I β-lactamases described by Sanschagrin *et al.*[7] included OXA-5, OXA-7 and OXA-10 and its variants. More recently, a new OXA-10-related subgroup, which includes OXA-13 and the ESBLs OXA-19 and OXA-28, has been identified.[14,15,17] OXA-19 differs from OXA-13 by two amino acid substitutions (Ser73–Asn and Gly157–Asp). An aspartate at position 157 is also found in all OXA-10-derived ESBLs and appears to be important for ceftazidime hydrolysis.[10,11,13,15] The amino acid at position 73 seems to influence the level of resistance to penicillins conferred. The presence of Ser73 in OXA-13 is unusual since asparagine is found at this position in most enzymes of the group.[15] In the present study, analysis of the nucleotide sequences of the *bla* genes of three isolates revealed sequences encoding asparagine at position 73 and glycine at position 157, defining a new variant, intermediate between OXA-13 and OXA-19.

The OXA group III β-lactamases include OXA-1 and its derivatives OXA-4, OXA-30 and OXA-31, which differ from OXA-1 by two, one and four amino acid substitutions, respectively.[21–23] Because the mutations responsible for the allelic variation do not create changes in restriction pattern that can be easily differentiated with commercially available endonucleases, the *bla* genes within this group could not be distinguished by PCR–RFLP. Determination of the nucleotide sequences of the *bla* genes of two of the 12 isolates revealed $bla_{\text{OXA-31}}$, encoding an enzyme described recently in a single isolate of *P. aeruginosa*.[23]

ESBLs were found in four isolates from 1994–1999. The double disc synergy test was positive when discs were placed 3 cm apart for the TEM-producing isolate, but the discs had to be brought closer to 2 cm or even 1 cm, to detect production of OXA-derived ESBLs. Thus, three of the four ESBLs would have been undetected by the standard disc test with a separation of 3 cm. One ESBL was correctly identified by PCR–RFLP as OXA-14, an enzyme described previously in a

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P. aeruginosa isolate from Turkey. OXA-14 differs from OXA-10 by the replacement of Gly157 by aspartate.[11] Two other ESBLs were OXA-2-derived enzymes. Prior to this report, OXA-15 was the only known extended-spectrum variant of OXA-2,[19] differing from it by an Asp150–Gly substitution. The mutation generates a *Sau*96I restriction site in the gene. Neither gene for the OXA-2-derived ESBLs had this site and were therefore presumed to have unknown point mutations, which were subsequently identified by nucleotide sequencing. The fourth ESBL was TEM-4, which has already been reported in a single isolate of *P. aeruginosa*.[31]

The limitation of this PCR–RFLP approach is that it can only detect mutations at known positions. Analysing the nucleotide sequences of PCR products obtained from a few isolates fortuitously discovered point mutations at previously unknown positions, identifying new alleles encoding new enzyme variants. Some mutations may not involve the appearance or disappearance of a recognition site for commercially available endonucleases. These would not be detected by the current approach. Lastly, the method is rather time consuming and labour intensive because of the large number of PCRs and restriction digests needed to identify all β-lactamases unambiguously. However, since a few enzymes are widespread in *P. aeruginosa* while others are very uncommon, in the majority of isolates, β-lactamases can be detected and identified using four primer pairs (for the PSE and the three main OXA groups) and a limited number of endonucleases. This method can be augmented by sequence analysis of PCR products for ESBLs or enzymes conferring unusual resistance patterns.

In conclusion, PCR–RFLP is an effective method of screening *P. aeruginosa* β-lactamases in epidemiological studies. It gave results consistent with previous reports, which used isoelectric point analysis and indicated that PSE-1 is the most common acquired β-lactamase in *P. aeruginosa* isolates, and revealed the distribution of various OXA enzymes in our hospital. Moreover, it was instrumental in the detection of four new β-lactamase variants, including two OXA-2-derived ESBLs. Further studies are needed to clone and sequence the genes and describe the characteristics of these new β-lactamases.

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