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Resistance to antibiotics in clinical isolates of *Pseudomonas aeruginosa*

Résistance aux antibiotiques dans des souches cliniques de *Pseudomonas aeruginosa*

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Abstract

Objectives. – To analyse the global resistance to some antibiotics used to treat nosocomial infections by *Pseudomonas aeruginosa*, specially to carbapenems, and its relationship with the presence of carbapenemases, OXA, VIM and IMP.

Methods. – The study included 229 *P. aeruginosa* isolates from a Hospital in Northern Spain (year 2002). Susceptibility to antimicrobial agents was determined by the analysis of the MIC. Genetic typing was carried out by RAPD-PCR fingerprinting with primer ERIC-2. Genetic experiments to detect class-1 integrons were performed by PCR with primers 5′CS and 3′CS. Detection of carbapenemases was done by phenotypic (Hodge test and DDST) and genotypic methods (PCR with primers for *imp*, *vim1*, *vim2* and *oxa40* genes).

Results. – 23.9% of isolates were resistant to ceftazidime, 35.9% to cefotaxime, 5.3% to amikacin, 54.9% to gentamicin, 14.6% to imipenem and 6.6% to meropenem. Isolates resistant to imipenem (33) were furtherly tested. Genetic typing didn't show clonal relatedness among the most of the isolates. Class-1 integrons were present in most isolates (sizes 600–1700 bp). Phenotypic methods for carbapenemases showed 5 positive isolates. Genotypic methods showed the presence of two isolates with the *oxa40* gene.

Conclusions. – Meropenem, amikacin and imipenem were the most active agents to treat infections caused by *Pseudomonas aeruginosa.* In our study, the presence of carbapenemase enzymes wasn't high. Phenotypic tests cannot be considered as accurate screening tool to detect carbapenemases. This is the fist report of the oxa40 gene in *Pseudomonas aeruginosa* isolates.

Résumé

But de l'étude. – L'analyse de la résistance globale aux quelques antibiotiques employés dans le traitement des infections nosocomiales par *Pseudomonas aeruginosa*, particulièrement aux carbapénèmes, et la relation avec la présence de carbapénèmases, OXA, VIM et IMP.

Méthodes. – L'étude inclut 229 isolats obtenus d'un hôpital du nord d'Espagne (année 2002). La sensibilité aux antibiotiques a été réalisée par la détermination de la MIC; le typage moléculaire des souches résistantes à l'imipénème par PCR (amorce ERIC-2); la détection d'intégrons par PCR (amorces 5′CS et 3′CS) ; et l'analyse de carbapénèmases par des techniques phénotypiques (Hodge et DDST), et génotypiques (PCR-amorces pour les genes *imp*, *vim1*, *vim2* et *oxa40*).

Résultats. – 23,9 % des souches étaient résistantes à la ceftazidime, 35,9 % à la céfotaxime, 14,6 % à l'imipénème, 6,6 % au méropénème, 5,3 % à l'amikacine, et 54,9 % à la gentamycine. Le typage moléculaire n'a pas montré de relation génotypique entre les souches résistantes à l'imipénème. La plupart des souches portaient des intégrons de classe 1 (600–1700 pb). Cinq isolats ont montré un résultat positif avec les techniques phénotypiques pour la détection de carbapénèmases. Les techniques génotypiques ont montré deux isolats avec le gène *oxa40*.

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Conclusion. – Le méropénème, l'amikacine et l'imipénème sont les agents les plus actifs pour traiter des infections par *Pseudomonas aeruginosa*. Dans cette étude la présence de carbapénèmases n'a pas été haute. Les techniques phénotypiques ne peuvent pas être considérées comme une bonne méthode pour la détection de carbapénèmases. C'est la première notification du gène *oxa40* dans des isolats de *Pseudomonas aeruginosa*.

Keywords: Pseudomonas aeruginosa; Antibiotic resistance; Carbapenems; Carbapenemases

Mots clés : Pseudomonas aeruginosa ; Antibiotic resistance ; Carbapenems ; Carbapenemases

1. Introduction

Pseudomonas aeruginosa and its close relatives are important opportunistic pathogens which play an important role in hospital intensive care units, causing a wide spectrum of nosocomial infections. The spread of this organism in healthcare settings is often difficult to control, due to the presence of multiple intrinsic and acquired mechanisms of antimicrobial resistance [1].

Carbapenem antibiotics are considered agents of choice to treat serious infections caused by *Pseudomonas aeruginosa*. Progressive antimicrobial resistance to most classes of antibiotics, including carbapenems, has made treatment of infection caused by this bacteria particurlarly difficult, even with combination therapy.

Patients in intensive care units, oncology departments, burn units and surgery wards frequently show multiresistant isolates, which contributes to a high morbidity and mortality [2].

Although the antibiotic resistance is caused by multiple mechanisms that this bacteria presents, such as impermeability of its outer membrane and the presence of efflux pumps, one growing factor leading to carbapenem resistance is the presence of carbapenemases [3,4].

The number of isolates with acquired carbapenemases and metallo-β-lactamases emerged and spread during the early 1990s, and the detection of a considerable number of OXA, IMP and VIM-type carbapenemases has been reported in many countries. In addition, these genes are easily transferable because, in many times, they are inserted in movile structures, such as integrons. Due to this ability to spread, β-lactamase production has become a serious concern.

The most important clinically-significant carbapenemases in *Pseudomonas aeruginosa* are class B metallo-β-lactamases such as VIM and IMP-type [5]. In fact, the presence of *Pseudomonas aeruginosa* producing IMP enzymes was firstly described in Japan, and different IMP type enzymes have been described in Japan, China, Canada, Italy, Brazil and USA. With regard to VIM enzymes, they were firstly identified in Italy, and different types of *vim* genes have been reported from other european countries and other regions like Asia and America. [6,7].

There are few reports of carbapenem-hydrolising OXA enzymes in *Pseudomonas aeruginosa* isolates, being the OXA-50 enzyme the only one identified in *Pseudomonas aeruginosa* [8]. However, OXA-type enzymes are more frequently found in other non fermenting bacilli like *Acinetobacter baumannii*.

The aim of this study was to analyse the global resistance to some of the most frequently used antibiotics for the treatment of nosocomial infections caused by *Pseudomonas aeruginosa*, with a special remark to carbapenems, and its relationship with the presence of carbapenemases, VIM, IMP and OXA-type.

2. Materials and methods

2.1. Clinical isolates

229 clinical isolates of *Pseudomonas aeruginosa* were obtained from chronic bronchiectasis patients attending a Hospital of the Basque Health Public Service (Osakidetza). The isolates were obtained following conventional bacteriological culture techniques from a range of specimens: 80% sputa, 9% urines, 6% skin ulcer swabs, and others (5%), and were initially identified with the API 32 system (Biomerieux, Marcy l'Etoile, France) and stored at -80 °C in nutrient broth containing 50% glycerol (vol/vol).

2.2. Genetic Typing

Clonal relatedness of the isolates was achieved by RAPD-PCR fingerprinting. Amplification was performed in a final volume of 25 µl containing the following: 25 ng of template DNA, 200 µM (each) dNTPs, 25 pmol of primer and 0.6 U of Taq polymerase. The experiments were done with ERIC2 (5′-AAGTAAGTACCTGGGGTGAGCG-3′) with the following amplification conditions: 1x (5 min at 94 °C , 5 min at 35 °C and 5 min a 72 °C) , 28x (1 min at 94 °C, 2 min at 52 °C and 2 min at 72 °C) and 1x (10 min at 72 °C).

2.3. Susceptibility test

The susceptibility of isolates to antimicrobial agents was determined by the analysis of the MIC following the NCCLS (National Comitee for Clinical Laboratory Standards) recommendations [9]. *Pseudomonas aeruginosa* strain ATCC 27853 was used as a control strain. The antibiotics tested were cefotaxime (Hoescht Marion Roussel), ceftazidime (Glaxo Wellcome), imipenem (Merck Sharp & Dohme), mer-

openem (Zeneca Pharmaceutical), amikacin (Bristol Myers Squibb) and gentamicin (Schering Plough).

2.4. Phenotypic detection of carbapenemases

Phenotypic detection of carbapenemase activity was done by the Hodge test with zinc sulfate previously described [10]. The surface of a Mueller-Hinton agar plate with zinc sulfate (to a final concentration of 70 μg/ml) was inoculated with a culture suspension of control strain *E. coli* ATCC 25922 adjusted to a one tenth turbidity of a 0.5 McFarland. Then, an imipenem disc was placed at the centre and the strains to be tested were streaked from the edge of the disc to the periphery. After overnight incubation, the presence of a distorted inhibition zone was interpreted as a positive result for carbapenemases. Positive isolates were then tested by double-disc synergy test to differentiate metalloenzymes from other carbapenemases.

DDST (double disc synergy test). Test strains were adjusted to the McFarland 0.5 standard and used to inoculate Mueller-Hinton agar plates. A 10 μg IMP disc was placed on the plate and a 10 μl-SMA solution disc was placed at a distance of 10 mm (edge to edge). SMA solution was made by a mixture of 4 volumes of O.5 M EDTA plus 6 volumes of a 300 mg/ml sodium mercaptoacetic acid (SMA) solution. After overnight incubation, the presence of a synergistic inhibition zone between both dics was interpreted as a positive result.

Control strains for metallo-β-lactamase assays were: *P. aeruginosa*A327 (IMP-1); *P. aeruginosa*3/P/10586 (VIM-1) and *P. aeruginosa*P4824 (VIM-2), kindly sent by Dr. Kevin J. Towner (Nottingham, UK). *Acinetobacter baumannii* SM 28 the control strain for carbapenemase OXA-40 was also included in all experiments as reference strain [11].

2.5. Genotypic detection of IMP, VIM, and OXA-40 carbapenemases by PCR

Genetic experiments for detection of *bla*OXA-40, *bla*IMP, *bla*VIM-1 and *bla*VIM-2 genes were carried out by PCR experiments with the following primers: OXA40-P2: 5′-TTCCCCTAACATGAATTTGT-3′, OXA40-P1: 5′-GTAC TAATCAAAGTTGTGAA-3′; IMP-F: 5′-CTACCGCAGCA GAGTCTTTG-3′, IMP-R: 5′-AACCGATTTTGCCTTAC CAT-3′; and VIM-Diar: 5′-AGGTGGGCCATTCAGCCAGA-3′, VIM-1upv: 5′-GTCGCAAGTCCGTTAGCCCAT-3′ and VIM-2upv: 5′-GATTCTAGCGGTGAGTATCCG-3′. All amplifications were carried out following the corresponding conditions [12]. The expected size of the fragments were 1023, 610, 583 and 587 bp respectively.

2.6. Detection of class 1 integrons

Class 1 integrons were detected by PCR with the primers 3′ CS: 5′-AAAGCAGACTTGACCTGA-3′ and 5′CS: 5′-GGCATCCAAGCAGCAAG-3′ following the corresponding conditions [13].

2.7. Hybridization experiments

DNA in electrophoresis gels was transferred to a nylon membrane by the Southern technique. The DNAs were then UV-cross-linked for 2 min. A 17 nucleotide sequence (Invitrogen) corresponding to an internal region of the OXA-40-like genes digoxigenine-labelled was used as a probe. Dectection of hybrids was done by colorimetric detection using an anti-digoxigenin antibody coupled to alkaline phosphatase following the manufacturers indications (Roche).

3. Results

Percentage of non-susceptible isolates (including resistant and intermediate isolates) was as follows: 32,7% to cedtazidime, 75,7% to cefotaxime, 21,7% to imipenem, 11,5% to meropenem, 15,5% to amikacin and 73% to gentamicin. The highest MIC 50 and MIC 90 were for cefotaxime (32 > 128) and gentamicin (16 > 128) (Table 1).

Isolates resistant to imipenem were also resistant to other antibiotics: 55% ceftazidime, 58% to cefotaxime, 9% to amikacin, 72% to gentamicin, 30% to meropenem.

Isolates resistant to imipenem (33) were genetically typed to analyse the clonal relatedness, and furtherly tested with phenotypic and genotypic methods in order to detect the presence of carbapenemases.

The number of clones identified was 24, among 22 patients, being clone I the most predominant with 5 isolates. There were 2 clones with 2 isolates each, genetically related to the clone I. This clone gave positive results with the phenotypic test to detect carbapenemases, and this was the genotype where one of the isolates boring the *oxa40* gene was identified.

Among these 33 imipenem resistant isolates tested with the modified Hodge method, 5 gave a positive result, 20 were negative and 8 gave equivocal results. Our results showed 8

Table 1
Susceptibility to antibiotics of clinical isolates of *Pseudomonas aeruginosa*

	MIC 50	MIC 90	% Susceptible	% Intermediate	% Resistant
Ceftazidime	8	64	67,3	8,8	23,9
Cefotaxime	32	> 128	24,3	39,8	35,9
Amikacin	8	32	84,5	10,2	5,3
Gentamicin	16	> 128	27	18,1	54,9
Imipenem	2	16	78,3	7,1	14,6
Meropenem	< 1	8	88,5	4,9	6,6

Table 2
Results of phenotypic and genotypic tests for carbapenemase detection among imipenem-resistant isolates

P. aeruginosa $N = 33$	Phenotype			Genotype			
	HODGE	DDST	Total	OXA	*imp*	*vim1*	*vim2*
	+	-	5	2	0	0	0
-	-	20	0	0	0	0	
nt	nt	8	0	0	0	0	

non-tipable isolates (24,2%). All Hodge positive isolates were synergy negative by DDST test.

Since the phenotypic test did not reveal the nature of the β-lactamases present in the isolates, they were analysed by molecular methods. PCR amplification with primers derived from the most usual carbapenemases (OXA, IMP and VIM) was used as first attempt to identify the genes responsible of the detected carbapenemase activity. We did not obtain the amplification of any IMP and VIM carbapenemase genes. However, two isolates, named P15 and P23, produced a fragment around one kb when subjected to amplification with the primers for *oxa40* gene. These 2 isolates were not clonally related.

Hybridization experiments with an internal nucleotide probe for *oxa40* like genes were positive, and the sequencing experiments revealed the sequence of the *oxa40* gene (data not shown).

Table 2 summarises the results of the different assays used to detect carbapenemases in the 33 isolates of *Pseudomonas aeruginosa* resistant to imipenem.

Class 1 integrons were present in most of the imipenem resistant isolates (27), ranging in size between 600 and 1700 bp. The most frequent integrons were 600 bp (15 isolates), 1700 bp (7 isolates) and 850 bp (7 isolates). Several isolates bored combinations of 2 or 3 bands (8 and 2 respectively).

4. Discussion

Our results showed that the most active agents to treat infection caused by *Pseudomonas aeruginosa* were meropenem and amikacin, with a percentage of of 88,5 and 84,5% respectively, followed by imipenem and ceftazidime (78,3% and 67%); showing the highest rates of resistances gentamicin and cefotaxime, with percentages of 54,9 and 35,9 respectively. These findings were in concordance with the trend found in other regions of Spain, where the most active agents were amikacin and meropenem [14,15]. In a multicentric study carried out in 136 hospitals in Spain, where 1014 isolates of *Pseudomonas aeruginosa* were analysed, the susceptibility rates were higher for all antibiotics, compared with our results, being the most active one meropenem, (92,2%), followed by amikacin (91,4%), imipenem (86,2%) and ceftazidime (85,2%) [16]. This can be explained by the progressive increase in antibiotic resistance over the years; these data were collected from strains isolated in 1998 while isolates from our study were collected in 2002.

When comparing our results with those obtained in other european coutries, we found similar susceptibility rates for meropenem, amikacin and imipenem to those obtained in a multicentric study carried out in 25 european university hospitals during 1997 and 1998. The most remarkable difference is the susceptibility rates of gentamicin, that are much higher in Europe (72,9%) than in our environment. This could be explained for the geographical differences in suspectibility patterns [17].

In comparison with a worldwide study carried out by the SENTRY Antimicrobial Surveillance Program during 1997 and 1999, in 4 major world regions: Asia-Pacific, Europe, Latin America and the United States/ Canada amikacin was, as well as in our study, the antibiotic which highest susceptibility rates. Amikacin was followed by meropenem and imipenem, in all regions except Latin America. In fact Latin America presents the lowest susceptibiliy rates to all antimictobial agents tested, followed by European and Asia-Pacific isolates. Canada showed the best global susceptibility results. Europe was the only region in which there was a significant decline in the beta-lactam and aminoglycoside susceptibility rates over the 3 monitored years [18,19].

We didn't find clonal relatedness among most of the imipenem-resistant isolates. These matches with the results found previously in other studies carried out by our group [20] and in Europe, where imipenem-resistant isolates were not generally clonally related [17]. Generally, isolates belonging to the same clone were obtained from the same patient, except for isolates belonging to clone I, that belonged to 3 different patients.

The presence of class 1 integrons is of great concern as they are one of the most efficient genetic elements responsible of the spread of antibiotic resistance genes (specially to aminoglycosides) in the hospital environment.

Phenotypic test for the detection of carbapenemases showed that some isolates produced carbapenemases (5 out of 33), but these enzymes were not metallo-β-lactamases, because all isolates were negative for the DDST. Our results showed a percentage of 24,24% of non tipable isolates, mainly due to the mucoid phenotype of the isolates that produced invasive growth all over the plate. Different phenotypic tests have been developed to detect the production of carbapenemases; however, they don't give satisfactory results to identify these enzymes in these isolates of *Pseudomonas aeruginosa*.

Our experience has demonstrated that the Hodge test is easy to perform and interpretate. However it is not a good tool for the screening of clinical isolates expressing frequently a mucoid phenotype.

Genotypic test for carbapenemases didn't give any positive results for the metallo-β-lactamases IMP and VIM-like. Although these enzymes are being described in different countries from Asia, Europe and America, they seem not to be frequent in our environment. [21,22].

However, this is the fist report of the *oxa40* gene in *Pseudomonas aeruginosa* isolates, a gene first described in carbapenem resistant *A. baumannii* isolates from the same hospital

[11] and a gene widely disseminated in the Iberian Peninsula [23]. More studies are being developed to determine the exact genetic location of this carbapenemase gene.

5. Conclusions

The most active agents to treat infection caused by *Pseudomonas aeruginosa* were meropenem and amikacin, followed by imipenem. In our study, the presence of carbapenemase enzymes wasn't high. Our results showed that phenotypic tests performed on mucoid isolates cannot be consider as accurate screening tool to detect carbapenemases. This is the first report of the *oxa40* gene in *Pseudomonas aeruginosa* isolates a gene previously reported from isolates of *A. baumannii* from our environment.

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