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Lacking of efflux mechanism in clinical isolate of *Pseudomonas aeruginosa* highly resistant to β-Lactams And imipenem

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Abstract

In this investigation, the isolate of *Pseudomonas aeruginosa* from cystic fibrosis was highly resistant to β-lactams and β-lactamase inhibitors. The resistant determinants of clinical isolate to imipenem, ceftazidime, ceftriaxone and cefepime were conjugally non-transfer. The slow or non-enzymatically mediated breakdown of imipenem and other broad spectrum β-lactams suggest the resistance of *P.aeruginosa* isolate to these drugs might be attributed to either permeability or efflux. Impaired penetration of imipenem and other β-lactams through the membrane was detected by a diminished expression of outer membrane (OM) proteins of approximate weight of 46 and 39 Kdal, matched to Opr D and OprF, respectively. Efflux resistance mechanism for meropenem and β-lactams has been ruled out since the isolate failed to express outer membrane protein of about 50 Kdal, which is matched to Opr M protein channel. Thus, reduced permeability in the clinical isolate may be the main mechanism conferring resistance against β-lactams including imipenem.

Key words

P.aeruginosa, imipenem, β-lactams, resistance, permeability barriers, Lacking efflux, β-lactamase.

Introduction

Pseudomonas aeruginosa infections are a major clinical problem since this organism exhibits natural and acquired resistance to many structurally and

functionally diverse antibiotics **[1].** Mutations can confer further resistance to antipseudomonal drugs.

Since 1982, ceftazidime, imipenem and ciprofloxacin have increasingly replaced penicillins and aminoglycosides in antipseudomonal chemotherapy, altering the selection pressures on the species **[2].**

The potency of β-lactams in *P.aeruginosa* is limited by several barriers. First, impermeable outer membrane significantly decreases the access of most hydrophilic β-lactams to their targets, the penicillin-binding proteins (PBPs). Second, the plasmid-coded or chromosomal coded β-lactamases, which hydrolyse β-lactams in periplasmic space. Finally, active efflux systems extrude β-lactams **[3].**

Imipenem is anomalous β-lactams in antipseudomonal behaviour, as well as in β-lactamase stability. MICs of imipenem for *P.aeruginosa* isolates are unrelated to those of otherβ-lactams, whereas a strong relationship exists between the MICs of different penicillins and cephalosporins **[4].** Basically, imipenem MICs are independent of derepression of chromosomal AmpC β-lactamase, which strongly codetermines the MICs of penicillins and cephalosporins **[4].** No description of cross-resistance between imipenem and other β-lactam antibiotics has been reported **[5].**

The transferability of resistant determinants to imipenem and other β-lactams was variable **[6-7].**

This study was claimed to investigate the susceptibility of a clinical isolate of *P.aeruginosa* obtained from a patient with cystic fibrosis against imipenem and other broad-spectrum β-lactams. Transferability of resistant factor(s) to imipenem and /or other β-lactam antibiotics was investigated. In an attempt to explore the mode(s) of resistance of cystic fibrosis isolate to imipenem and other β-lactam antibiotics, hydrolysis of imipenem and other β-lactams by the crude lysates of clinical isolate

were studied. In addition, the reduced penetration of molecules of these drugs across the membranes was also under focused.

Experimental

Bacterial strains

A clinical isolate of *P.aeruginosa* was obtained from a patient with cystic fibrosis as well as two imipenem-susceptible clinical isolates were of our collection. The isolates were identified by the standard methods **[8].** A standard strain of *P.aeruginosa* (ATCC 27853) was included in this study.

Susceptibility testing

The MICs of a variety of β-lactams including carbapenems for the clinical isolates as well as the standard strain (ATCC 27853) were determined by E-test according to the manufacture's procedure and the standard techniques **[9].** Briefly, Mueller-Hinton agar plates were surface inoculated by swabs dipped in 0.5 McFarland turbidity of microorganisms. Allow excess moisture to be absorbed for about 10-15 minutes so that surface is completely dry then apply the E-test strips.

Effect of OM permeabilizer on MICs

Susceptibility testing of imipenem and ceftazidime with or without 2.5 mM EDTA **[10]** was carried out to ascertain the effect of permeabilizer on MICs. The test was performed by two-fold serial broth dilution method with an inoculum of $5X10^5$ cells/ml. Data were reported as MICs, which reflected the lowest concentration of antibiotic inhibiting visible growth after 18h incubation at 37^0C.

Conjugal transfer of resistance

The method of conjugal transfer of antibiotic resistance in mixed culture of donor (resistant) and recipient (susceptible) strains of *P.aeruginosa* was performed according to **Krcmery *et al* [11].** Briefly, 0.5 ml of a 6-hour shaken culture of the donor strain of *P.aeruginosa* in nutrient broth (Difco) with KNo_3 was mixed with 0.5 ml of recipient strain cultured identically, and statically incubated at 32^0C overnight. Then, 0.05 ml of the mating mixture was spread on the surface of nutrient agar containing an antibiotic for selection plus 200 mg/L of rifampicin to which the recipient strain was resistant and the donor was susceptible. The agar plates were incubated at 35^0C overnight. Colonies that eventually grew on the selective media for transconjugants were then picked up and analyzed for the presence of non-selected resistance determinants.

Hydrolysis of imipenem and other β-lactams by crude lysate

Hydrolysis of imipenem and β-lactams by crude lysate of cystic fibrosis clinical isolate was measured by microbiological method **[12].** In brief, overnight shaken cultures were centrifuged and then disrupted with ultrasound. Protein concentration was adjusted to 4 g/l. One ml of this lysate was then mixed with 5 ml of 6 mM substrate in a phosphate buffer, pH 6 **[7].** In control (flasks) experiments (contain heat-inactivated enzyme; water in place of the enzyme; and buffer instead of the antibiotic) and in the experimental ones, the amount of non-hydrolysed β-lactam antibiotics was estimated by microbiological assay. The percentages of hydrolysis by the crude lysate of clinical isolate after various time intervals (30, 60, 240, and 480 min) were estimated.

Separation of outer membrane (OM)

The isolates were grown in Mueller-Hinton broth in shaking water bath overnight at 35^0C. Cultures were centrifuged at 4500 rpm for 15 min. The cells were

resuspended in cold membrane buffer at 4^0C and sonicated in ice six times at 30-second intervals. The unbroken cells were removed by centrifugation at 5200 rpm for 5 min at 4^0C. The supernatants were centrifuged at 14,500 rpm for 1 h. The pellets were resuspended in 1 ml of 1% sodium-lauryl sarcosinate (Sarkosil, Sigma), kept for 20 min at room temperature and then centrifuged again at 14,500 rpm for 1 h. The pellets (with OM) were frozen at -80^0C to be subsequently used for electrophoretic run **[13].**

Electrophoresis

Electrophoresis run was done at 25 mA of constant current with 12% SDS-polyacrylamide gel (PAGE). Membrane protein inoculum was solubilized in the sample solution: Tris-Hcl pH 6.8; Sodium dodecyl sulfate 2%; glycerol 10%; bromothymol blue 0.001%, 2-mercaptoethanol 5% and boiled for 5 min. The gel was stained with Coomassie blue for 3-4 h and destained with a solution of methanol, acetic acid and water overnight.

Results and Discussion

The susceptibility of the clinical isolates of *P.aeruginosa* to imipenem as well as to other antipseudomonal β-lactams is listed in table1. The interpretation of the data shown in table 1 by **NCCLS [9]** revealed that, only cystic fibrosis clinical isolate was highly resistant to all antibiotics under investigation, except to meropenem.

Table 1. MICs of β-lactams against the clinical isolates of *P.aeruginosa* by Etest

Antibiotic	MIC (μg/ml)			
	Cystic fibrosis isolate	Isolate no. 87	Isolate no. 94	Standard strain ATCC 27853

Imipenem	32	3	2	1
Meropenem	4	2	1.5	0.5
Ceftazidime	32	8	4	1.5
Ceftriaxone	>32	8	6	8
Cefepime	>256	4	2	2
Cefaclor	>256	N	N	N
Cephalothin	>256	N	N	N
Piperacillin/ Tazobactam	>256	16	12	6
Ticarcillin/ clavulanicacid	>256	24	16	16

N= Not tested

The results of conjugal transfer of resistance determinants from cystic fibrosis (CF) isolate to a susceptible *P.aeruginosa* isolate revealed that; resistance to imipenem, ceftazidime, ceftriaxone, and cefepime were non-transferable. This consistent with that reported by **Knothe *et al* [7]** and not consistent with that reported by **Hupkova *et al* [6].** Thus, resistance to these antibiotics in CF isolate seems to be chromosomal in origin. On the other hand, determinants of resistance to benzylpenicillin, cefaclor and cephalothin were conjugally transferred, and probably were plasmid mediated.

Bacteria utilize several ingenious mechanisms to develop resistance. These include degradation of the drug, inactivation of the drug by enzymatic modification, and alteration of the drug target. These mechanisms are all quite specific for a single drug or a single class of drugs. However, there are more general mechanisms of drug resistance in which access of the unaltered agent to the target is prevented by the barrier and active transport functions of biological membranes **[14].**

The resistance of *P.areuginosa* to β-lactams could be attributed mainly to the selection of resistant variants **[15].** Inactivation of antipseudomonal β-lactams by β-

lactamases has been investigated. The data (table 1) revealed that, β-lactamase inhibitors; i.e. clavulanic acid, and tazobactam, in combination with other β-lactams have no effect against the cystic fibrosis clinical isolate. This suggests that other resistance mechanisms than β-lactamase may have been operating in this isolate.

The conclusion that β-lactams resistance is independent of β-lactamase production is substantiated by the results obtained from the hydrolysis by crude lysates (table 2). The data in table 2 revealed that, there was slow hydrolysis of imipenem by lysate from CF isolate. The table also shows that crude lysate has no effect on ceftazidime, cefepime and ceftriaxone. This result was consistent with that reported by **Knothe *et al* [7].** The highest rates of hydrolysis by the clinical isolate were observed with benzylpenicillin and cephalothin, which showed 100% and 89% hydrolysis, respectively.

Gram-negative bacteria are surrounded by a permeability barrier, the outer membrane (OM). β-lactams were shown to utilize predominantly the porins pathway to cross the OM **[16].** Outer membrane preparations of clinical isolates were electrophoretically separated and stained according to **Giordano *et al* [13].**

Table 2. Hydrolysis of β-lactams by cystic fibrosis clinical isolate

Strain	Time of incubation with crude lysate (min)	Hydrolysis (%) of the substrate					
		PEN-G	CF	IP	TZ	PM	TX
Imipenem resistance isolate of *P.aeruginosa*	30	100	48	7	0	0	0
	60	100	63	10	0	0	0
	240	100	75	16	0	0	0
	480	100	89	23	0	0	0

PEN-G = Benzylpenicillin CF = Cephalothin IP = Imipenem
TZ = Ceftazidime PM = Cefepime TX = Ceftriaxone

As figure 1 shows, only cystic fibrosis isolate diminished the expression of some bands, with special emphasis on those corresponding to apparent molecular weight of 39, 46 and 50 Kdal. The figure also represents the OM patterns of imipenem-sensitive clinical isolates (87 and 94) as well as the standard strain (ATCC 27853).

The significantly diminished expression of outer membrane protein of approximate molecular weight of 46 Kdal on SDS-PAGE (Fig 1), suggesting an impaired uptake of imipenem. This protein,Opr D, forms outer membrane pores (D2 porin) that are permeable to imipenem but not to penicillins and cephalosporins **[4,15].** Thus, impermeability of imipenem, mediated through the lack of Opr D protein, leads to the reduction of active antibiotic molecules able to reach the target PBPs. This data was consistent with that previously reported **[17-20].**

The lack of enzymatically mediated breakdown of cefepime, ceftriaxone and ceftazidime (table 2) indicated that the resistance to these

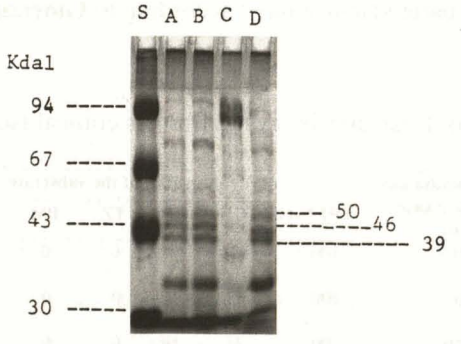

FIG. 1. Electrophoretic profiles of outer membrane preparations of: sensitive isolates No. 94 and 87. respectively (A and B). imipenem-resistant isolate (C). and standard strain. ATCC 27853 (D). S: standard mole. wt. Markers.

agents might be attributed to an impaired penetration through the membrane proteins. As figure 1 revealed, outer membrane protein of around 39 Kdal was highly diminished in only imipenem-resistant CF isolate. This protein is probably matched to porin F, which is involved in crossing of β-lactam antibiotics other than carpabenems **[13].** OM impermeability, as resistant mechanism, has been substantiated by the effect of OM permeabilizer (EDTA) on the susceptibility of CF isolate to the tested antibiotics. Permeabilization of OM with 2.5 mM EDTA decreased the MIC values of imipenem and ceftazidime from 32 and 32 μg/ml to 16 and 8 μg/ml, i.e. 2- to 4-fold decrease, respectively.

The potency of β-lactams in *P.aeruginosa* might also be limited by active efflux systems, which extrude β-lactams **[3].** Several multidrug efflux systems have been described in *P.aeruginosa*, including Mex AB-Opr M, Mex CD-OprJ, Mex EF-Opr N, and Mex XY- (Opr-M) systems **[10,17].** The Mex AB-Opr M system is the major pump contributes to intrinsic antibiotic resistance **[10].** Upregulation of Mex AB-Opr M raises the MICs of pencillins, cephalosporins, quinolones, tetracyclines, chloramphenicol, and meropenem but not those of imipenem **[3,4,17,21,22].** In this investigation, the role of Opr M protein of outer membrane, as efflux transporter, was ruled out. Since a protein band matched Opr M with approximate molecular weight of 50 Kdal **[23]** was diminished only in imipenem-resistant CF isolate (fig.1). The correlation between resistance and efflux may not be simple, because the influx of carbapenems is affected by the levels of Opr-D. Since the cystic fibrosis isolate was resistant to imipenem but not to meropenem (table 1), moreover, the isolate was deficient in Opr-M protein (fig 1). Thus, the results substantiate the suggestion that meropenem is a substrate of MexAB-Opr-M system, while imipenem is not **[3].** The role of impermeability in pseudomonal resistance was confirmed by **Li *et al*[10].**

Conclusively, intrinsic resistance of *P.aeruginosa* is depending largely on interplay of impermeability with multi-drug efflux **[4].** But in this study, CF clinical

isolate expresses a diminished level of efflux resistance mechanism; meanwhile it produces an outer membrane of exceptionally low permeability.

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