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Hydrolysis of cefazolin by enzymes produced by *Pseudomonas aeruginosa* after exposure to ceftazidime *in vitro*

Hidroliza cefazolina pomoću enzima proizvedenih od strane *Pseudomonas aeruginosa* posle *in vitro* izlaganja ceftazidimu

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Abstract

Background/Aim. Sometimes resistance of *Pseudomonas aeruginosa* (*Ps. aeruginosa*) is developed during antibiotic treatment, in spite of the initial susceptibility *in vitro*. The aim of this study was to use an *in vitro* model for the study of the development of resistant strains of *Ps. aeruginosa* after a short exposure to ceftazidime, and to study the hydrolysing capacity of β-lactamases produced by the resistant strains. **Methods.** Among 563 clinical strains of *Ps. aeruginosa*, 37 multisensitive strains were collected for the study. After being identified, strains with simultaneous sensitivity to 5 expanded spectrum cephalosporins were chosen. For each strain, the minimal inhibitory concentration (MIC) of the 5 expanded spectrum cephalosporins was determined, and the production of extended spectrum β-lactamases (ESBL) was excluded by the double-disc synergy diffusion test. Strains non producing ESBL were cultivated in concentrations of ceftazidime equal to MIC×2 and MIC×4. After 24 hours of culture, the development of resistant strains was estimated and the cephalosporinase activity of the produced β-lactamases was determined by their ability to hydrolyse cefazolin. Hydrolysis of cefazolin was studied by measuring the change of its absorbance on 272 nm using a Shimadzu 160A spectrophotometer. The hydrolyzing capacity of the enzymes was expressed as the percentage of the antibiotic, which was hydrolysed in 10 sec. **Results.** A total of 60% and 50% of strains developed resistant strains after exposure to ceftazidime in concentration MIC×2 and MIC×4, respectively. The hydrolyzing capacity of the original strains was 15-36% while the hydrolyzing capacity of the resistant strains was 10-73%. Totally 64% of the resistant strains expressed higher hydrolyzing capacity than the original strains. **Conclusion.** Regardless of the susceptibility test results, *Ps. aeruginosa* presented a high tendency to develop resistant strains after a short exposure to ceftazidime *in vitro*. In most cases the resistant strains expressed higher cephalosporinase activity than the original strains, suggesting derepression of chromosomal β-lactamases. Our model offers a simple, inexpensive and rapid method for detecting resistance of *Ps. aeruginosa* developed due to derepression of β-lactamases, and for discriminating resistant strains with derepressed β-lactamases from strains that developed other mechanisms of resistance.

Key words:
drug resistance, bacterial; anti-bacterial agents; cephalosporins; cefazolin; ceftazidime; pseudomonas aeruginosa.

Apstrakt

Uvod/Cilj. Tokom primene antibiotika ponekad dolazi do razvoja rezistencije *Pseudomonas aeruginosa* (*Ps. aeruginosa*) na njih bez obzira na početnu osetljivost *in vitro*. Cilj ove studije bio je da se primenom *in vitro* modela utvrdi razvijanje rezistentnog soja *Ps. aeruginosa* posle kratkog izlaganja ceftazidimu, kao i da se odredi hidrolitička aktivnost β-laktamaza koje proizvode rezistentni sojevi. **Metode.** Od 563 klinička soja *Ps. aeruginosa* za ovu studiju izdvojeno je 37 multisenzitivnih sojeva. Posle identifikacije, izdvojeni su sojevi istovremeno osetljivi na pet cefalosporina proširenog spektra. Za svaki soj izvršeno je određivanje minimalne inhibitorne koncentracije (MIC) pet cefalosporina. Sojevi koji ne produkuju β-laktamaze proširenog spektra (ESBL) određeni su pomoću *double-disc synergy diffusion* testa. Oni sojevi koji nisu proizvodili ESBL kultivisani su u prisustvu ceftazidima u koncentracijama koje su odgovarale MIC×2 i MIC×4. Posle 24 sata kultivisanja određeno je razvijanje rezistentnih sojeva i cefalosporinazna aktivnost produkovanih β-laktamaza na osnovu njihove sposobnosti da hidrolizuju cefazolin. Hidroliza cefazolina određena je merenjem njegove absorbance na 272 nm primenom spektrofotometra *Shimadzu* 160A. Hidrolizujući kapacitet enzima izražen je u procentima antibiotika hidrolizovanog tokom 10 sec. **Rezultati.** Ukupno 60% sojeva razvilo je rezistentne sojeve posle izlaganja ceftazidimu u koncentraciji MIC×2, dok je 50% sojeva razvilo rezistentne sojeve posle izlaganja antibiotiku u koncentraciji

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MIC×4. Hidrolizujući kapacitet originalnih sojeva bio je 15–36%, a rezistentnih sojeva 10–73%. Ukupno 64% rezistentnih sojeva pokazalo je veći hidrolizujući kapacitet od originalnih sojeva. **Zaključak.** Bez obzira na rezultate testiranja osetljivosti, bakterija *Ps. aeruginosa* pokazala je visoku sklonost ka razvijanju rezistentnih sojeva posle kratkog *in vitro* izlaganja ceftazidimu. U većini slučajeva rezistentni sojevi pokazali su veću cefalosporinaznu aktivnost od originalnih sojeva, što ukazuje na derepresiju β-laktamaza. Prikazana metoda predstavlja jednostavan, jeftin i brz način određivanja rezistencije *Ps. aeruginosa* nastale usled derepresije β-laktamaza, kao i diskriminacije mutanata sa β-laktamazama od mutanata koji razvijaju druge mehanizme rezistencije.

Ključne reči:
lekovi, rezistencija mikroorganizama; antibiotici; cefalosporini; cefazolin; ceftazidim; pseudomonas aeruginosa.

Introduction

Clinical experience has shown that microbial resistance to antibiotics is sometimes developed during antibiotic therapy, in spite of the initial susceptibility of microbial pathogens before treatment [1]. This is observed very often in clinical strains of *Pseudomonas aeruginosa* (*Ps. aeruginosa*), an opportunistic pathogen involved in hospital infections. *Ps. aeruginosa* infections are common in patients with compromised immune system or chronic infections, and in patients treated in Intensive Care Units (ICU) [2–4]. Development of resistant strains of *Ps. aeruginosa* during antimicrobial therapy is a frequent problem with major clinical consequences in ICU, often resulting in therapeutic failure [5, 6].

Outbreaks of infection with strains of *Enterobacteriaceae* producing extended spectrum beta-lactamases (ESBL) revealed the necessity for screening for ESBL production, as strains producing ESBL may be found susceptible in antibiograms and appear resistant during antibiotic treatment [1, 7]. Although strains of *Ps. aeruginosa* producing ESBL have been reported to occur in Greek hospitals [8–13], the most common mechanism for development of resistance to β-lactams is selection of mutations leading to hyperproduction of the chromosomal AmpC β-lactamase [6, 14, 15]. The activity of the antipseudomonal penicillins and cephalosporins against *Ps. aeruginosa* is based on the fact that although these compounds are certainly hydrolyzed by AmpC, they are very weak inducers of this chromosomal β-lactamase [6, 16]. Nevertheless, during treatment with β-lactams, resistant mutants showing high levels of AmpC production are frequently selected, leading to therapeutic failure [16, 17]. Although there are modified double-disk tests for the detection of *Enterobacteriaceae* producing basal AmpC β-lactamases [7], there are no recommendations for the routine detection of these enzymes in *Ps. aeruginosa.*

The purpose of this study was to use an *in vitro* model for the study of the development of resistant strains of *Ps. aeruginosa* due to AmpC β-lactamases partially or stably derepressed, after a short exposure to ceftazidime, and to study the hydrolysing capacity of β-lactamases produced by the resistant strains. Ceftazidime was chosen because it is a 3rd generation cephalosporin with special activity against *Ps. aeruginosa*, and it is commonly used to treat pseudomonal infections. The tendency of *Pseudomonas* to develop resistant strains to ceftazidime *in vitro*, reflects a similar effect *in vivo*, which may lead to therapeutic failure in immunocompromised patients.

Methods

A total of 563 clinical strains of *Ps. aeruginosa* were collected from clinical specimens in the AHEPA General Hospital, Thessaloniki, Greece, and Agios Loukas General Hospital, Thessaloniki, Greece. The isolates were non repetitive (one per patient). All multisensitive strains, which showed sensitivity to β-lactams, aminoglycosides and quinolones were chosen for the study. After being identified with Vitek I (bioMerieux) and confirmed by the agar dilution method in Iso-Sensitest agar medium (Oxoid, Basingstoke, UK), strains with simultaneous sensitivity to the following expanded spectrum cephalosporins: ceftazidime, cefotaxime, ceftriaxone, cefixime, cefepime, were collected for the study:

The following commercial forms of cephalosporins were used: ceftazidime (SOLVETAN, Glaxo Wellcome, dr.pd.inj. 1g/vial), cefotaxime (CLAFORAN, Hoechst Marion Roussel, dr.pd.inj. 1g/vial), ceftriaxone (ROCEPHIN, Roche, dr.pd.inj. 1g/vial), cefixime (CEFTORAL, Vianex, coated tablets 400 mg), cefepime (MAXIPIME, Bristol Myers Squibb, dr.pd.inj. 1g/vial), cefazolin (KEFZOL, Pharmaserve Lilly, dr.pd.inj. 1g/vial).

The Iso-Sensitest agar medium (Oxoid, Basingstoke, UK) was used for microbial cultures.

The susceptibility of the isolates to antibiotics was determined by the automated identification system Vitek I (bioMerieux) and was confirmed by the agar dilution method in Iso-Sensitest agar medium (Oxoid, Basingstoke, UK). Susceptibility tests were performed and interpreted according to the recommendations of the British Society for Antimicrobial Chemotherapy (BSAC) [18, 19]. For each strain, the minimal inhibitory concentration (MIC) of the 5 expanded spectrum cephalosporins was determined, and the production of ESBL was excluded by the double-disc synergy diffusion test (DDST) [6, 16].

Strains with simultaneous sensitivity to the 5 expanded spectrum β-lactams were suspended in distilled water, adjusted to a 0.5 McFarland standard and diluted in distilled water (1:100), to obtain a suspension containing 10^6 cfu/mL. A sterile cotton-wool swab was dipped into the suspension and the inoculum was spread evenly over the entire surface of plates containing Iso-Sensitest agar medium and ceftazidime in concentrations equal to MIC×2 and MIC×4. The plates were incubated at 37 °C for 24 hours, and were checked for the development of resistant strains.

β-lactamases produced by the original and the resistant strains were separated from microbial cells and their cepha-

losporinase activity was tested by their ability to hydrolyse cefazolin. Hydrolysis of cefazolin was studied by measuring the change of its absorbance in 272 nm, using a Shimadzu 160A spectrophotometer, as described in the British Pharmacopoeia [20]. The hydrolysing capacity of the enzymes was expressed as the percentage of the antibiotic which was hydrolysed in 10 sec.

Results

Out of 563 collected clinical strains of *Ps. aeruginosa*, 37 strains showed sensitivity to β-lactams, aminoglycosides and quinolones. From these multisensitive strains, ten strains showed simultaneous sensitivity to the expanded spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone, cefixime, cefepime), confirmed by determination of the relative MICs. The production of ESBL was further excluded by the DDST. The MIC of ceftazidime for each of these strains ranged from 0.5 to 1.0 μg/mL.

After culture in the presence of ceftazidime in concentrations equal to MIC×2 and MIC×4, eleven resistant strains were developed: six out of ten strains developed resistant strains in the presence of a concentration of ceftazidime equal to MIC×2 (60%) and five out of ten strains developed resistant strains in the presence of a concentration of ceftazidime equal to MIC×4 (50%).

Hydrolysis of cefazolin by the enzymes produced by the original and the resistant strains of *Ps. aeruginosa* is presented in Figure 1. The hydrolyzing capacity of the original strains was 15–36% while the hydrolyzing capacity of the resistant strains was 10-73%. Seven out of eleven resistant strains (64%) presented a higher hydrolysing capacity than original strains and four out of eleven mutant strains (36%) presented a lower hydrolysing capacity than original strains (Table 1).

β-lactamases (AmpC) can be upregulated by subinhibitory concentrations of certain β-lactam antibiotics [6, 21, 22]. Further, mutations can occur in the regulatory components of AmpC leading to a stable hyperproduction of AmpC with concomitant high-level resistance to many classes of β-lactam antibiotics [22, 23]. Induction is a transient phenotypic response to a β-lactam; stably-derepression, on the other hand, is the permanent hyperproduction of the enzyme, regardless of antibiotic presence. Derepression may be partial, such that the organism produces an unusually high uninduced level of enzyme but retains inducibility, or total, such that β-1actamase expression is constitutive (i.e. entirely unregulated by antibiotic presence) [14, 24]. Stably derepressed mutants occur at frequencies from 10^{-5} to 10^{-8} in β-1actamase inducible populations [25–27].

In our study we investigated *in vitro* development of resistant strains of *Ps. aeruginosa*, due to derepressed AmpC β-lactamases after exposure to ceftazidime in concentrations much higher than MIC (MIC×2 and MIC×4). A double screening test was used for the collection of strains, in order to exclude the production of ESBL: phenotype and DDST [16]. Thus, the collected strains produced only AmpC β-lactamases.

AmpC is a group I class C chromosomally encoded β-lactamase present in *Ps. aeruginosa* and in most *Enterobacteriaceae* [17]. In a wild-type cell, Amp*C* production is expressed at constitutively low levels due to the binding of UDP-MurNAc-pentapeptide to Amp*R*. Mutations associated with Amp*R* and Amp*D* can result in Amp*C* overproduction, which has been termed derepression [17, 28, 29]. Phenotypically, derepressed mutants can be resistant to expanded-spectrum cephalosporins, due to overproduction of AmpC [30].

In our study the collected strains were incubated in the presence of ceftazidime, in concentrations higher than MIC (MIC×2 and MIC×4). In the presence of these concentrations of ceftazidime, no colonies of the initial strains should be developed [6, 21, 22]. Nevertheless, an unexpectedly high number of resistant strains was observed. In 20 cultures, 11 clones of resistant strains of *Ps. aeruginosa* were developed (a total of 55%); 6 clones of resistant strains were developed in the presence of a concentration of ceftazidime equal to MIC×2 (60%) and 5 clones of resistant strains were developed in the presence of a concentration of ceftazidime equal to MIC×4 (50%).

Table 1

Hydrolysis of cefazolin (% in 10 sec) by original strains of *Pseudomonas aeruginosa* and by strains that developed resistance in the presence of concentrations of ceftazidime equal to MIC×2 and MIC×4

Original strain of *Pseudomonas aeruginosa*	Strain developed in the presence of ceftazidime MIC×2	Strain developed in the presence of ceftazidime MIC×4
20	28	26
36	33	73
23	10	10
21	29	28
21	–	–
25	–	–
20	–	–
18	–	–
15	23	26
28	23	–

*MIC – Minimal Inhibitory Concentration

Discussion

Development of resistance to the antipseudomonal penicillins and cephalosporins, mediated by hyperproduction of the chromosomal cephalosporinase AmpC, is a major problem in the treatment of *Ps. aeruginosa* infections [15]. Inducible AmpC

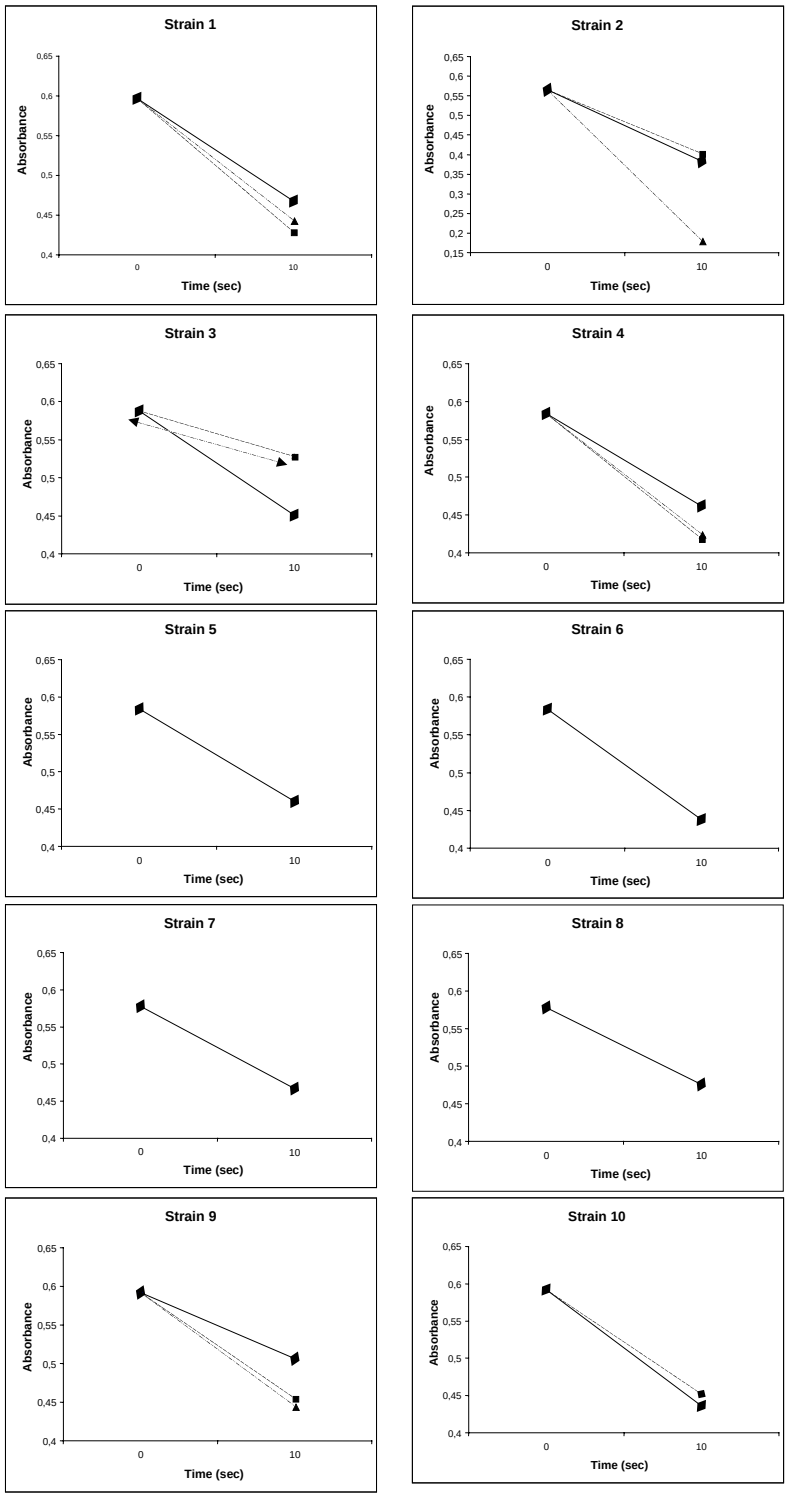

Fig. 1 – Hydrolysis of cefazolin by enzymes produced by the original and resistant strains of *Pseudomonas aeruginosa*
Hydrolysis of cefazolin was estimated by measuring the change of its absorbance on 272 nm using a *Shimadzu* 160A spectrophotometer. —♦— Hydrolysis of cefazolin by enzymes produced by the original strains of *Pseudomonas aeruginosa*; ---■--- hydrolysis of cefazolin by enzymes produced by resistant strains of *Pseudomonas aeruginosa,* which were developed during culture in the presence of ceftazidime in concentration equal to MIC×2; ---▲--- hydrolysis of cefazolin by enzymes produced by resistant strains of *Pseudomonas aeruginosa*, which were developed during culture in the presence of ceftazidime in concentration equal to MIC×4

This number of resistant strains seems to be extremely high but it is consistent to the rapidity with which bacteria produce mutants (frequency 10–5 to 10–8) [25–27]. This means that commonly used treatments such as the currently popular expanded spectrum 3rd generation cephalosporins are, in turn, compromised. The consequences of this type of antibiotic nullification are highly important if we consider that: 1) the clinical strains of *Ps. aeruginosa*, which were used, were not only highly sensitive to ceftazidime but also multisensitive to the most potent and highly effective cephalosporins,

the strains of *Ps. aeruginosa* were cultivated in concentrations of ceftazidime considerably higher than MIC (MIC×2 and MIC×4).

In our study seven out of eleven resistant strains presented a higher hydrolysing capacity than original strains and four out of eleven resistant strains presented a lower hydrolysing capacity than original strains. This means that in four out of eleven strains, resistance was developed by a mechanism not associated with derepressed expression of chromosomal AmpC. Other mechanisms of developing resistance must be implicated in these cases, like decreased permeability and upregulation of the efflux system [31–37].

The present findings, which were observed in our study *in vitro*, reflect also the *in vivo* tendency of *Ps. aeruginosa* to develop resistant strains during chemotherapy with ceftazidime, and finally the probability of therapeutic failure. This probability seems to be higher than expected, and can be developed in a very short time. Fortunately, our immune system protects us against resistant strains developed during chemotherapy, and so, results of this phenomenon may not be dramatic in all cases. However, in immunocompromised patients and in severely ill patients of ICU, the danger of therapeutic failure is very high and the risk of death is not infrequent. In fact, the rapidity with which *Ps. aeruginosa* produces resistant strains ensures that commonly used treatments such as the currently popular antipseudomonal cephalosporin, ceftazidime, may be proved ineffective. Although there are modified double-disk tests for the detection of *Enterobacteriaceae* producing basal AmpC β-lactamases [7], there are no recommendations for the detection of these enzymes in *Ps. aeruginosa*. On the other hand, there is no available test to predict which strains carry inducible AmpC enzymes [22] or which mutations will be developed during treatment.

Conclusion

Our study indicates that *Ps. aeruginosa* producing basal AmpC β-lactamases presents a high tendency to develop resistant strains after exposure to ceftazidime *in vitro*, regardless of the susceptibility test results. This tendency reflects a similar effect *in vivo*, which may lead to therapeutic failure in immunocompromised patients. In clinical practice, it is probable that development of resistance during treatment may occur more rapidly and more frequently than we expected. Special care should be taken for ICU patients, as the kind of mutations that are developed during treatment cannot be predicted.

Resistant strains that were developed in our study expressed higher cephalosporinase activity than original strains, suggesting derepression of chromosomal β-lactamases in most cases. Our model offers a simple, inexpensive and rapid method for detecting resistance of *Ps. aeruginosa* due to derepression of β-lactamases, and for discriminating mutants with derepressed β-lactamases from mutants that developed other mechanisms of resistance.

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