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Identification of AmpC β-lactamases in Clinical *Pseudomonas Aeruginosa* Strains

Identyfikacja β-laktamaz typu AmpC u szczepów klinicznych *Pseudomonas aeruginosa*

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Abstract

Background. β-lactamase production is the major mechanism of resistance of clinical *P. aeruginosa* strains to β-lactams. Class C β-lactamases are cephalosporinases whose expression is inducible by certain β-lactams, especially cefoxitin and imipenem. Extended-spectrum cephalosporins, such as ceftazidime and cefotaxime, and ureidopenicillins are very weak inducers of AmpC β-lactamases, but they serve as substrates of β-lactamases. Mutations occurring in the regulatory components of AmpC can lead to constitutive hyperexpression of chromosomal cephalosporinase with concomitant high-level antipseudomonal β-lactam resistance.
Objectives. The purpose of the study was to evaluate the induction and derepression of AmpC β-lactamases of *P. aeruginosa* strains.
Material and Methods. Sixty-six *P. aeruginosa* clinical isolates were evaluated for AmpC β-lactamase production using a variety of inducer-substrate antibiotic combinations in a disk approximation format and 3-aminophenylboronic acid (APB), a specific inhibitor of class C β-lactamases, in the disk potentiation test. The examined combinations included cefoxitin/ceftriaxon, cefoxitin/ceftazidime, imipenem/cefotaxime, imipenem/ceftazidime, and imipenem/piperacillin-tazobactam.
Results. 90.9% of the strains were shown to be inducible for the production of AmpC β-lactamases by different inducer/substrate combinations and 7.6% of all isolates were stably derepressed for the expression of AmpC. The combinations cefoxitin/ceftriaxon and imipenem/piperacillin-tazobactam provided the greatest sensitivity (94% and 90.9%, respectively). Detection of AmpC by the disk potentiation test was based on the enlargement of the growth-inhibitory zone diameter (by ≥ 5 mm) around a disk containing a ceftazidime or a cefotaxime disk in combination with APB.
Conclusions. The methods used will be the source of information concerning the mechanisms of *P. aeruginosa* resistance (**Adv Clin Exp Med 2008, 17, 5, 519–523**).

Key words: *Pseudomonas aeruginosa*, AmpC β-lactamases, 3-aminophenylboronic acid.

Streszczenie

Wprowadzenie. Głównym mechanizmem odpowiedzialnym za narastanie oporności na antybiotyki β-laktamowe wśród szczepów szpitalnych *P. aeruginosa* jest wytwarzanie β-laktamaz. Klasę C β-laktamaz stanowią cefalosporynazy, których ekspresja jest indukowana przez niektóre β-laktamy, głównie cefalotynę i imipenem. Cefalosporyny o szerokim spektrum, takie jak: ceftazydym i cefotaksym oraz ureidopenicyliny są słabymi induktorami β-laktamaz AmpC, stosuje się je natomiast jako substraty tych enzymów. Mutacje zachodzące w genach kontrolujących wytwarzanie β-laktamazy AmpC mogą prowadzić do trwałego odblokowania ich wydzielania, w wyniku czego dochodzi do wzrostu oporności szczepów na anty-*Pseudomonas* β-laktamy.
Cel pracy. Badano występowanie indukcji i derepresji β-laktamaz AmpC u szczepów *P. aeruginosa*.
Materiał i metody. Zbadano zdolność 66 klinicznych szczepów *P. aeruginosa* do wytwarzania β-laktamaz AmpC, stosując liczne połączenia induktor/substrat (cefoksytyna/ceftriakson, cefoksytyna/ceftazydym, imipenem/cefotaksym, imipenem/ceftazydym, imipenem/piperacylina-tazobaktam) oraz kwasu 3-aminofenyloborowego, jako specyficznego inhibitora tych enzymów.
Wyniki. U 90,9% testowanych szczepów *P. aeruginosa* stwierdzono indukcję β-laktamaz AmpC po zastosowaniu różnych kombinacji induktor/substrat, a u 7,6% izolatów całkowitą derepresję genu kodującego wytwarzanie AmpC. Antybiotyki cefoksytyna/ceftriakson i imipenem/piperacylina-tazobaktam charakteryzowały się najwięk-

szą aktywnością (odpowiednio 94 i 90,9%). Wykrycie enzymów AmpC za pomocą testu krążka z APB polegało na obserwacji powiększenia strefy zahamowania wzrostu (większej lub równej 5 mm) wokół krążka zawierającego ceftazydym lub cefotaksym w połączeniu z inhibitorem.
Wnioski. Użyte metody dostarczają informacji na temat mechanizmów oporności występujących u *P. aeruginosa* (**Adv Clin Exp Med 2008, 17, 5, 519–523**).

Słowa kluczowe: *Pseudomonas aeruginosa*, AmpC β-laktamazy, kwas 3-aminofenyloborowy.

P. aeruginosa is an opportunistic pathogen that is able to cause severe invasive diseases in critically ill and immunocompromised patients. Because of its ubiquitous nature, ability to survive in most environments, and innate resistance to many antibiotics and antiseptics, *P. aeruginosa* is a common pathogen in hospitals, particularly in intensive care units (ICUs) [1]. *P. aeruginosa* exhibits intrinsic resistance to several β-lactams and may acquire additional resistance mechanisms, including decreased outer-membrane permeability, penicillin binding protein modifications, production of β-lactamases, increased expression of efflux pump systems, and decreased porin expression [2, 3].

The production of β-lactamases is the major mechanism of resistance to β-lactams in chronic *P. aeruginosa* infections. Class C β-lactamases, which belong to group 1 according to the classification of Bush et al. [4], are cephalosporinases, which are poorly inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. They are chromosomally encoded by the ampC gene commonly found among members of the family *Enterobacteriaceae* and the genus *Pseudomonas* and other nonfermenting Gram-negative bacilli [5, 6]. With the exception of *Escherichia coli* and *Shigellae*, AmpC β-lactamase is produced at low basal levels, but its expression is inducible by certain β-lactams, especially cefoxitin and imipenem [7]. Extended-spectrum cephalosporins, such as ceftazidime and cefotaxime, and ureidopenicillins are very weak inducers of AmpC but can serve as substrates of this chromosomal β-lactamase [8]. Unfortunately, mutations can occur in the regulatory components of AmpC, leading to constitutive hyperexpression of chromosomal cephalosporinase with concomitant high-level antipseudomonal β-lactam resistance [9].

The purpose of the study was to evaluate clinical *P. aeruginosa* strains for AmpC β-lactamase production using several inducer/substrate combinations. The induction or derepression of AmpC for all strains was confirmed using 3-aminophenylboronic acid (APB) as an inhibitor of this β-lactamase.

Material and Methods

Bacterial Strains

Sixty-six strains of *P. aeruginosa* were originally isolated from a variety of clinical specimens: feces (28), urine (12), blood (1), bronchial washings (10), sputum (1), wound swabs (9), pharyngeal swabs (2), an ulceration swab (1), a swab from skin around a tracheotomy (1), and from the ear (1). The bacteria were obtained from an outpatient clinic, a municipal hospital, and the main hospital in Siedlce. All clinical isolates were non-mucoid oxidase-positive, pyocyanin and pyoverdin producers and were able to grow at 42°C. The strains were identified as *P. aeruginosa* according to the reaction patterns in the Api 20NE system (bioMérieux). Stock cultures were stored in TSB (trypticase soy broth, Difco) containing 20% glycerol at –80°C. All strains were maintained on trypticase soy agar (Difco) slants at room temperature for up to 48 h prior to testing.

Induction of AmpC Synthesis

The induction of AmpC synthesis was based on the disk approximation (D-test) assay [5, 10] using cefoxitin/ceftriaxon (FOX/CRO). Several other inducer/substrate combinations, including cefoxitin/ceftazidime (FOX/CAZ), imipenem/ /cefotaxime (IPM/CTX), imipenem/ceftazidime (IPM/CAZ), and imipenem/piperacillin-tazobactam (IPM/TZP), were added for comparison. All disks were obtained from BBL (Becton Dickinson) and Bio-Rad at the following potencies: TZP (100 and 10 μg), IPM (10 μg), CAZ (30 μg), FOX (30 μg), CTX (30 μg), and CRO (30 μg).

The disk diffusion susceptibility testing was performed according to the CLSI guidelines [11]. A colony of a test strain was suspended in and diluted with MH broth medium to 10^8 CFU/ml and spread on a Mueller-Hinton (MH) agar plate (BBL) with a cotton swab. Inducer/substrate disks were placed on the surface of the MH agar plates at a distance of 25 mm center using a template. After overnight incubation the zones of inhibition were measured on both the induced (adjacent to the inducer disk) and uninduced sides of the substrate disk from the disk edge to the zone edge.

A test was considered positive if the zone of inhibition was reduced by ≥ 2 mm on the induced side of the substrate disk (Figs. 1–3). The *P. aeruginosa* ATCC 27853 reference strain was used as a control for procedure standardization.

The tested strains were considered noninducible if none of the substrate/inducer combinations produced a positive test and the isolate was not stably derepressed for AmpC. Strains were considered inducible if a positive test was obtained with any of the inducer/substrate combinations. Strains were considered to be stably derepressed if resistance was observed to all substrate drugs and when it could be confirmed by inhibition of AmpC activity, without evidence of metallo-β-lactamase production.

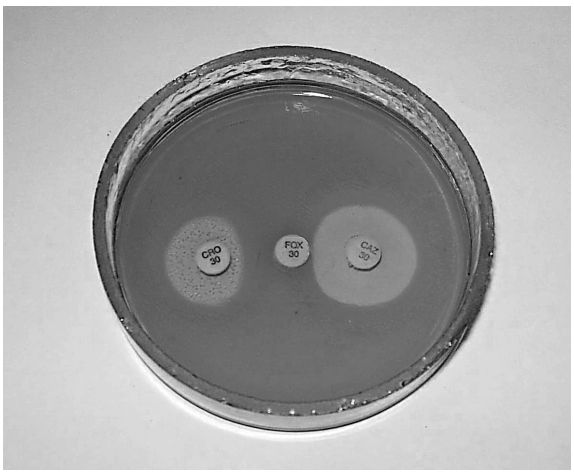

Fig. 1. Identification of AmpC β-lactamase with the use of cefoxitin/ceftriaxon and cefoxitin/ceftazidime

Ryc. 1. Identyfikacja β-laktamazy AmpC z zastosowaniem cefoksytyny/ceftriaksonu i cefoksytyny/ceftazydymu

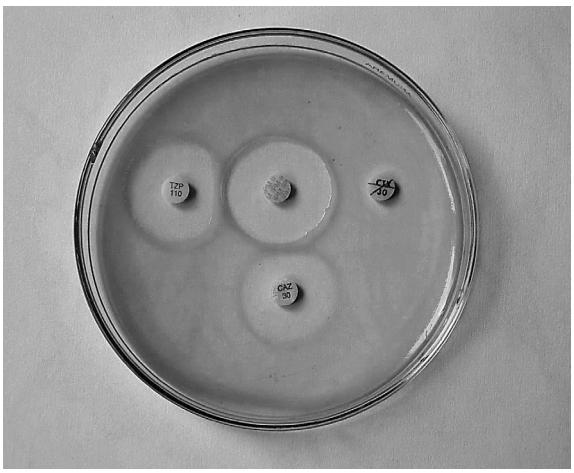

Fig. 2. Identification of AmpC β-lactamase with the use of imipenem/piperacillin-tazobactam, imipenem/ceftazidime and imipenem/cefotaxime

Ryc. 2. Identyfikacja β-laktamazy AmpC z zastosowaniem imipenemu/piperacyliny-tazobaktamu, imipenemu/ceftazydymu i imipenemu/cefotaksymu

Fig. 3. Identification of AmpC β-lactamase with the use of 3-aminophenyloboronic acid (APB)

Ryc. 3. Identyfikacja β-laktamazy AmpC z zastosowaniem kwasu 3-aminofenyloborowego (APB)

The induction or derepression of AmpC for all strains was confirmed with a disk potentiation test [6, 12] using 3-aminophenylboronic acid (APB; Sigma-Aldrich) as an inhibitor of AmpC β-lactamases (Fig. 3). Disks containing boronic acid were prepared as follows: 120 mg of 3-aminophenylboronic acid was dissolved in 3 ml of dimethyl sulfoxide (DMSO, Sigma-Aldrich). Three milliliters of sterile distilled water was added to this solution. Twenty microliters of the stock solution was dispensed onto disks containing 30 μg of cefotaxime and ceftazidime disks. The disks were allowed to dry for 2 h at the room temperature and used immediately. To perform the assay, a disk diffusion susceptibility testing was performed according to the CLSI guidelines [11]. A disks containing 30 μg of CTX/CAZ with or without APB were placed on the MH agar plate with a center-to-center distance of 30 mm. The agar plates were incubated at 37°C overnight. An organism that demonstrated a zone of inhibition diameter of 5 mm or greater around the disk containing CTX/CAZ and APB than the zone of inhibition diameter around the disk containing CTX/CAZ was considered to be an AmpC producer.

Results

Of the 66 strains of *P. aeruginosa*, 60 (90.9%) were shown to be inducible for AmpC by different substrate/inducer combinations through the use of the affirmative test. The sensitivity of each screening combination was: FOX/CAZ 37.9% (2–3% of strains did not produce a zone of inhibition surrounding the CAZ disk), IPM/CTX 40% (7–10.6% of strains had no zone of inhibition sur-

rounding the CTX disk), IPM/CAZ 84.9% (3–4.5% of strains had no zone of inhibition surrounding the CAZ disk), IPM/TZP 90.9% (2–3% strains had no zone of inhibition surrounding the TZP disk), and FOX/CRO 94% (1–1.5% of strains had no zone of inhibition surrounding the CRO disk). Six (9.1%) strains of *P. aeruginosa* demonstrated a positive test for all the substrate/inducer combinations and 22 (33.3%) for four combinations without FOX/CAZ. The best substrate/inducer combination was FOX/CRO. Only 3 (4.5%) strains did not give a positive result; in this case the bacteria generated a zone of inhibition of < 2 mm on the uninduced side of the CRO disk.

Only 1 (1.5%) strain of *P. aeruginosa*, isolated from feces, showed no induction of AmpC after using any of the substrate/inducer combinations. The strain was susceptible to IPM, CTX, CAZ, CRO, and TZP.

Of the 66 strains of *P. aeruginosa*, 5 (7.6%) were stably derepressed for the production of AmpC. These strains gave a positive result for all the substrate/inducer combinations. They were resistant to CTX, CRO, and CAZ. None of the strains were resistant to IPM and had evidence of metallo-β-lactamases or extended-spectrum β-lactamases (data not shown). The reference strain *P. aeruginosa* ATCC 27853 was shown to be inducible for AmpC synthesis by all of the inducer/substrate combinations.

The induction or derepression of AmpC for all strains was confirmed with a disk potentiation test using 3-aminophenylboronic acid (APB) as the specific inhibitor of class C β-lactamases. In 65 (98.5%) strains of *P. aeruginosa*, enlargement was observed of the growth-inhibitory zone diameter around the disk containing CAZ or CTX in combination with the inhibitor. Enlargement of the growth-inhibitory zone diameter by ≥ 5 mm around disk containing CTX with APB was frequently observed. The *P. aeruginosa* strains were inhibited less by APB when CAZ was used. The strains which were stably derepressed for the production of AmpC were strongly inhibited (the inhibitory zone diameter around a disk containing CTX and CAZ was greater than 10 mm) by APB.

Discussion

The purpose of the study was to evaluate AmpC β-lactamase activity in *P. aeruginosa* strains. Using several inducer/substrate combinations and 3-aminophenylboronic acid (APB), a specific inhibitor of class C β-lactamases, it was found that 90.9% of clinical *P. aeruginosa* strains were capable of inducing the synthesis of AmpC. Only one strain, isolated from a feces sample, showed no induction of AmpC. Other authors [5], using the same methods, found 85.8% of clinical *P. aeruginosa* isolates producing an AmpC enzyme.

IPM and FOX are considered to be strong inducers of but poor substrates for AmpC activity in *P. aeruginosa* [7]. Extended-spectrum cephalosporins, such as ceftazidime and cefotaxime, and ureidopenicillins are very weak inducers of AmpC but can be used as substrates of this chromosomal β-lactamase [8]. Dune and Hardin [5] reported that the combination IPM/TZP provided excellent sensitivity (97.1%) for the detection of inducible AmpC in *P. aeruginosa*. Lister et al. [10] demonstrated 100% sensitivity of FOX/PIP for the detection of inducible AmpC among clinical isolates of *P. aeruginosa*. In the present study the IPM/TZP combination provided 90.9% sensitivity and the combination FOX/CRO presented 94% sensitivity.

The selection of an antimicrobial agent for infection caused by *P. aeruginosa* should be based on the assumption that certain drugs, such as antipseudomonal penicillins and cephalosporins, might increase the risk of selecting stably derepressed AmpC-producing strains during therapy [8, 13]. Of the 66 studied strains, 5 (7.6%) were stably derepressed for AmpC production. They were resistant to cefotaxime, ceftriaxone, ceftazidime, mezlocillin, and carbenicillin. Three of the 5 strains were multiresistant and showed resistance to penicillins, cephalosporins, aminoglycosides, and fluoroquinolones. The remaining strains presented moderate susceptibility only to meropenem, imipenem, and colistin (data not shown). In a study by Deplano et al. [1], epidemic multiresistant *P. aeruginosa* showed overexpression of AmpC chromosomal cephalosporinase combined with decreased expression of porin OprD and absence of metallo-β-lactamases or extended-spectrum β-lactamases. The frequency of the strains of the present study demonstrating stable derepression of AmpC is much less that the 38% and 18% reported, respectively, by Pfaller et al. [14] and Tam et al. [15] for *P. aeruginosa* strains isolated from the bloodstream. The presented numbers, however, are much smaller and reflect bacteria recovered from many sources.

The induction or derepression of AmpC for all strains was confirmed by a disk potentiation test [6, 12] using 3-aminophenylboronic acid (APB). In 65 (98.5%) strains of *P. aeruginosa*, enlargement of the growth-inhibitory zone diameter around the disks containing CAZ or CTX in combination with the inhibitor was observed. This method could also detect *E. coli* [6], *Hafnia alvei* [16], and *Klebsiella pneumoniae* [6, 16] as AmpC producers.

According to these results, the disk approximation test and the disk enhancement test were very simple, highly sensitive, and specific to the identification of *P. aeruginosa* producing class C β-lactamases. Thus they are fully applicable for routine use in clinical microbiology laboratories.

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