International Journal of Antimicrobial Agents 47 (2016) 77–83

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Increase of efflux-mediated resistance in *Pseudomonas aeruginosa* during antibiotic treatment in patients suffering from nosocomial pneumonia☆

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ARTICLE INFO

Article history:
Received 13 August 2015
Accepted 4 November 2015

Keywords:
Pseudomonas aeruginosa
Resistance
Efflux
MexAB–OprM
MexXY–OprM
EUCAST breakpoints

ABSTRACT

Increases in antibiotic minimum inhibitory concentrations (MICs) for *Pseudomonas aeruginosa* during treatment are commonly observed but their relationship to efflux overexpression remains poorly documented. In this study, pairs of first [at time of diagnosis (D0)] and last [during treatment (DL)] *P. aeruginosa* isolates were obtained from patients treated for suspicion of nosocomial pneumonia. Pair clonality was determined by repetitive extragenic palindromic PCR. Overexpression of *mexA* and *mexX* was assessed by real-time PCR, and expression of *mexC* and *mexE* was assessed by PCR. Antibiotics received by patients before and during treatment were determined from clinical charts. For D0 isolates, 24% were from patients without antibiotics for 1 month and 64% were negative for *mexA*/*mexX* overexpression and *mexC*/*mexE* expression. For DL isolates, approximately one-half of the patients had received piperacillin/tazobactam, amikacin, meropenem and/or cefepime, and 17% had received ciprofloxacin (alone or in combination); 38% did not show changes in expression of the four genes, whereas 38% showed increased expression for one gene (mainly *mexA* or *mexX*), 19% for two genes (mainly *mexA* and *mexX*) and 5% for three or four genes. Isolates overexpressing *mexA* or *mexX* had median MICs above EUCAST clinical resistance breakpoints for ciprofloxacin, cefepime and meropenem, or for ciprofloxacin, amikacin, cefepime and meropenem, respectively. *mexA* or *mexX* overexpression was statistically significantly associated with patients' exposure to ciprofloxacin and meropenem or cefepime and meropenem, respectively. Overexpression of genes encoding antibiotic transporters in *P. aeruginosa* during treatment is frequent and is associated with increases in MICs above EUCAST clinical susceptibility breakpoints.

1. Introduction

With its large genome, *Pseudomonas aeruginosa* shows a remarkable adaptability for surviving under antibiotic selection pressure [1]. Although the emergence of resistance in *P. aeruginosa* during antibiotic treatment has been described since the mid 1980s [2], documentation of its mechanisms remains scarce. In this context, we collected successive clonally related isolates of *P. aeruginosa* from patients hospitalised in intensive care units (ICUs) with clinically suspected nosocomial pneumonia and receiving one or several commonly used antipseudomonal antibiotics [piperacillin/tazobactam (TZP), cefepime, meropenem, amikacin and ciprofloxacin]. We previously reported a markedly decreased susceptibility occurring during therapy for each of these antibiotics

☆ Portions of this work were presented at the 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria, 10–13 April 2010 (abstract no. P780).

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http://dx.doi.org/10.1016/j.ijantimicag.2015.11.004
0924-8579/

that was correlated in a statistically significant manner with the proportion of their use when examined at the whole population level [3].

The present report builds on the observation that efflux-mediated resistance can be upregulated in vitro upon incubation of bacteria with substrates of the corresponding transporters (see, e.g., [4]). Efflux can decrease the activity of all commonly used antipseudomonal drugs, namely β-lactams (substrates of MexAB–OprM; cefepime also being a substrate of MexCD–OprJ and MexXY–OprM, and piperacillin and meropenem are also marginally transported by MexCD–OprJ), aminoglycosides (substrates of MexXY–OprM) and ciprofloxacin (substrate of all three abovementioned transporters and of MexEF–OprN) (see [5] for a review). Up to now, overexpression of antibiotic efflux transporters in *P. aeruginosa* (mainly MexXY–OprM) during treatment has been only clearly demonstrated in clinical isolates collected from cystic fibrosis patients receiving repeated courses of antibiotics (see, e.g., [6] and references cited therein). Using clonally related pairs of *P. aeruginosa* isolates, we show here that overexpression of efflux is frequent and is associated with increases in minimum inhibitory concentrations (MICs) to levels above the current European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) clinical susceptibility breakpoints for the antibiotics that are substrates of the corresponding transporters.

2. Materials and methods

2.1. Patient selection and bacterial isolates

The study protocol was approved by the Ethical Committee of the Faculty of Medicine of the Université catholique de Louvain (Brussels, Belgium) within the context of a grant application to the Belgian Fonds de la Recherche Scientifique Médicale (F.R.S.M.). The patient selection criteria have been described in detail previously [3]. In brief, samples were obtained from 59 patients hospitalised in the ICUs of five teaching hospitals in Belgium with a diagnosis (or suspicion) of nosocomial pneumonia and for whom *P. aeruginosa* could be isolated from endotracheal aspirate, bronchoalveolar lavage, pleural fluid, empyema or blood and was considered as the most likely cause of the disease (see [3] for detailed diagnostic criteria). A first sample (D0 sample) was obtained at the time of initial diagnosis and additional samples were collected during the course of therapy. In the present study, we only considered this first sample and the last usable sample of each patient (DL sample) (no. of days after collection of D0 sample: range, 1–123 days; mean, 23 days; median, 17.5 days) [3]. The methods used for identification of the isolates and for assessment of clonal relatedness of strains within paired isolates (D0 vs. DL samples) have been described previously [3]. This allowed for the identification of 62 clonal pairs of strains in the 59 patients. For three patients, we observed the presence of two different strains both in D0 and DL samples that were clonally distinct from each other in both samples but were clonally related when comparing the same strain between the D0 and the DL samples [3]. *Pseudomonas aeruginosa* ATCC 27853 (American Tissue Culture Collection, Manassas, VA) and PAO1 [7] reference strains were used as quality control for susceptibility testing and as reference for standardisation of gene expression levels, respectively. All strains were kept at −80 °C in 20% glycerol–80% cation-adjusted Muller–Hinton broth (CA-MHB) until analysis and were grown overnight at 37 °C on tryptic soy agar plates (Becton Dickson, Franklin Lakes, NJ) before being used for experiments.

2.2. Antibiotic prescription data

Data on antibiotics prescribed to the patients at the time of pneumonia diagnosis and up to 1 month before the onset of pneumonia were collected from clinical charts. The doses, schedules and mode of administration were checked against the corresponding standards of care and were, for most of the patients, those recommended for severe infections based on the applicable Summary of Product Characteristics [3].

2.3. Minimum inhibitory concentration determination and susceptibility interpretive criteria

MICs were determined by geometric microdilution in CA-MHB according to CLSI recommendations [8]. Susceptibility was assessed according to both the 2014 CLSI [8] and 2015 EUCAST (http://www.eucast.org/) interpretive criteria.

2.4. Preparation of cDNA

2.4.1. Culture of strains

Two to three colonies of each overnight culture were suspended in 10 mL of sterile MHB, were diluted to obtain an optical density at 620 nm (OD_{620}) of 0.1, and were incubated at 37 °C under aerobic conditions and shaking at 150 rpm until reaching an OD_{620} of 1.0 ± 0.1. Two aliquots of 1.5 mL were taken and were centrifuged at $5000 \times g$ for 5 min at 4 °C. After discarding the supernatants, pellets were stored at −80 °C until analysis.

2.4.2. Extraction of total RNA

After sample thawing, RNA was extracted using an RNeasy® Kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. Bacterial pellets (ca. 5×10^8 CFU/100 μL) were re-suspended in 100 μL of TE buffer [10 mM Tris–HCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8] containing 0.4 mg/mL lysozyme (Sigma–Aldrich, Saint Louis, MO) and were incubated at room temperature for 10 min. Lysis was achieved by adding 350 μL of commercial lysis Buffer RLT (QIAGEN) supplemented with 1% β-mercaptoethanol (Bio-Rad, Hercules, CA) and vigorous shaking, after which 250 μL of ethanol (96–100%) was added to the lysate, followed by shaking through pipetting. The suspension (usually 700 μL) was put on an RNeasy® Mini Spin Column (QIAGEN) and was treated exactly as recommended by the manufacturer. The extracted RNA was stored at −80 °C before use.

2.4.3. RNA purification, quantification and retrotranscription

After thawing, extracted RNA was treated with Turbo DNase I (Ambion, Austin, TX) to eliminate possible contamination with DNA. A mix containing 2 μL of enzyme, 10 μL of RNA, 5 μL of ad hoc buffer and 33 μL of water was incubated for 30 min at 37 °C, after which an additional 2 μL of enzyme was added to the samples, which were then incubated for another 30 min at 37 °C. The reaction was stopped by incubation with 10 μL of DNAse Inactivation Reagent (Ambion) for 2 min, after which samples were centrifuged at 14 000 rpm for 2 min and supernatants were collected and stored at −80 °C. Absence of contaminating DNA was checked by performing a control PCR amplification of the *rpsL* housekeeping gene using the primers rpsL-F (CGGCACTGCGTAAGGTATGC) and rpsL-R (CGTACTTCGAACGACCCTGCT). The PCR programme consisted of 10 min at 95 °C, 40 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 10 min 72 °C. The absence of an amplicon after gel electrophoresis was checked for. RNA was quantified using a Qubit® RNA BR Assay Kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was then amplified using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions, except that only 500 ng of RNA was used as starting material instead of 1 μg for optimisation reasons.