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An adaptive response of *Enterobacter aerogenes* to imipenem: regulation of porin balance in clinical isolates

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ABSTRACT

Imipenem (IPM) is a carbapenem antibiotic frequently used in severe hospital infections. Several reports have mentioned the emergence of resistant isolates exhibiting membrane modifications. A study was conducted between September 2005 and August 2007 to survey infections due to *Enterobacter aerogenes* in patients hospitalised in a French university hospital. Resistant *E. aerogenes* clinical isolates obtained from patients treated with IPM and collected during the 3 months following initiation of treatment were phenotypically and molecularly characterised for β-lactamases, efflux pumps activity and outer membrane proteins. Among the 339 patients infected with *E. aerogenes* during the study period, 41 isolates (12.1%) were resistant to extended-spectrum cephalosporins and 17 patients (5.0%) were treated with IPM. The isolates from these 17 patients presented TEM-24 and basal efflux expression. Following IPM treatment, an IPM-intermediate-susceptible (IPM-I) isolate emerged in 11 patients and an IPM-resistant (IPM-R) isolate in 6 patients. A change in the porin balance (Omp35/Omp36) was observed in IPM-I isolates exhibiting ertapenem resistance. Finally, a porin deficiency (Omp35 and Omp36 absence) was detected in IPM-R isolates associated with efflux pump expression. This study indicates that the alteration in porin expression, including the shift of porin expression and lack of porins, contribute to the *E. aerogenes* adaptive response to IPM treatment.

1. Introduction

In clinically important enterobacteria, the major mechanisms of resistance to β-lactams involve enzymatic degradation due to overexpressed chromosomal cephalosporinases and/or plasmid-mediated extended-spectrum β-lactamases (ESBLs), associated or not with a decrease in outer membrane permeability [1–3]. In *Enterobacter aerogenes*, the most prevalent clone producing ESBL contained plasmids carrying a gene coding for TEM-24 [3,4]. Moreover, the change in membrane permeability is mainly due to the alteration of porins located in the outer membrane, conferred by downregulation of synthesis and/or porin modifications [5–10]. In addition, overexpression of efflux pumps, the other side of the mechanical barrier, has been demonstrated in various multidrug-resistant (MDR) *E. aerogenes* isolates [9,11,12].

In some *E. aerogenes* isolates collected from infected patients treated with imipenem (IPM), a correlation between antibiotic resistance and the presence of porins has been reported previously [5,6,9,10]. This indicates the involvement of a complex regulation process that is able to detect the toxic molecule, or its noxious activity (e.g. metabolites), and respond to IPM [7]. Interestingly, modification of outer membrane permeability, e.g. lack of porins observed in MDR clinical isolates, creates a physiological disadvantage with regard to the involvement of porins in nutrient uptake that are required for bacterial life and growth. Antibiotic pressure promotes the selection of isolates exhibiting alterations in membrane permeability and consequently may induce an important bacterial fitness cost [13–17].

In a recent study, we showed that the virulence of *E. aerogenes* in the *Caenorhabditis elegans* model is tightly linked to alteration of *E. aerogenes* membrane physiology, which is reflected by different levels of resistance to IPM [14].

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The objectives of this work were (i) to define the existence of a step-by-step adaptation of clinical isolates being faced with IPM, (ii) to define the involvement of IPM in the selection of membrane modifications (porin balance) in the isolates becoming resistant to ertapenem during treatment and (iii) to compare the outcome of patients treated with IPM because of infections due to *E. aerogenes* resistant to extended-spectrum cephalosporins (ESCs).

2. Materials and methods

2.1. Patient population

A prospective surveillance programme on resistant *E. aerogenes* isolates was initiated on 1 September 2005 and carried out until 31 August 2007 in Nîmes University Hospital (Nîmes, France). The study was approved by the local research ethics committee (Comité d'éthique Sud Méditerranée III, Nîmes, France) and was conducted according to the principles expressed in the Declaration of Helsinki. Patients provided verbal informed consent as the ethics committee had decided that verbal consent was sufficient because no additional samplings were required to carry out the study.

At baseline, all patients infected with an ESC-resistant *E. aerogenes* and treated with IPM/cilastatin (3 g/day by intravenous injection) were pre-included. Clinical samples were checked regularly during IPM treatment to monitor for possible emergence of clinical isolates less susceptible to IPM. Patients were definitively included if they presented an IPM-intermediate-susceptible (IPM-I) or IPM-resistant (IPM-R) isolate during the 3 months following initiation of IPM treatment. The following clinical data were collected prospectively: demographic data; clinical ward; diagnosis at admission; isolation site of bacteria; monomicrobial or polymicrobial infection; clinical outcome; underlying diseases following the Charlson co-morbidity index [18], with severity according to the McCabe score; hospitalisation or surgical treatment; transfer from another hospital; hospitalisation in the Intensive Care Unit (ICU) or nursing home in the last 12 months; antimicrobial treatment in the previous month; hospital-acquired or imported *E. aerogenes* causing infection; and exposure to various catheters (urinary or venous catheters) during the current hospitalisation and before the *E. aerogenes* infection. Patients were deemed to have an imported *E. aerogenes* isolate if the first culture positive for ESC-resistant *E. aerogenes* was obtained within 48 h of admission. In this patient category, patients with recurrent stays in healthcare settings (2 days during a 1-month period) and those who had never been in contact with such settings were differentiated.

2.2. Bacterial identification and typing

The genus and species were determined biochemically using a VITEK® 2-AST N017 identification card (bioMérieux, Marcy l'Étoile, France).

Macrorestriction analysis of chromosomal DNA was performed by pulsed-field gel electrophoresis (PFGE) according to published procedures following *Xba*I restriction (New England Biolabs, Evry, France) using a CHEF system (Bio-Rad SA, Ivry-sur-Seine, France). PFGE patterns were analysed using GelCompar software (Applied Math, Kortrijk, Belgium) and an unweighted pair-group method with the Dice coefficient of similarity. Isolates were considered to be within a cluster if the coefficient of similarity was >80%. The different isolates were compared with strain EAI_A belonging to the French *E. aerogenes* clone [3].

2.3. Antibacterial susceptibility

Susceptibility to IPM, ertapenem, cefepime, chloramphenicol, ofloxacin, ciprofloxacin, tetracycline and tigecycline was determined by the standard broth dilution method in triplicate as previously described [11,12]. Minimum inhibitory concentrations (MICs) were also determined in the presence of the efflux pump inhibitor phenylalanyl-arginyl-β-naphthylamide (PAβN) (Sigma-Aldrich, Saint-Quentin-Fallavier, France) at 26.3 μg/mL under conditions for which no intrinsic effect has been observed [11]. *Enterobacter aerogenes* strain ATCC 13048 and strain EA27, a clinical isolate that was previously characterised as overexpressing the AcrAB efflux system, were used as controls [11,12,19]. Isolates were classified as susceptible (S), intermediate (I) or resistant (R) to the antibiotics tested according to the recommendations of the Antibiogram Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org).

2.4. Phenotypic and molecular detection of extended-spectrum β-lactamase/AmpC

ESBL expression was checked by the double-disk synergy test using Mueller–Hinton (MH) agar containing 250 mg/L cloxacillin, and AmpC hyperproduction was detected by measuring MICs of ceftazidime, cefepime and imipenem by the Etest method using MH agar without and with 250 mg/L cloxacillin (http://www.sfm-microbiologie.org). The genes bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ were screened by PCR and were identified by sequencing the PCR products as previously reported [3,13,20].

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunodetection of outer membrane proteins

Exponential-phase bacteria in Luria–Bertani broth were pelleted and solubilised as previously described [11]. Samples (bacterial amount corresponding to 0.02 optical density units at 600 nm) were loaded onto SDS-PAGE gels (10% or 14% polyacrylamide, 0.1% SDS) and then electrotransferred to nitrocellulose membranes [11]. Membranes were saturated using 4% milk in Tris-buffered sodium (50 mM Tris–HCl, 150 mM NaCl, pH 8). Polyclonal antibodies directed against outer membrane proteins (porins, OmpX and OmpA) and against the efflux pump components (AcrA and TolC) were used for detection [11,19,21]. Detection of antigen–antibody complexes was performed with alkaline phosphatase-conjugated AffiniPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove PA) using 5-bromo-4-chloro-3-indolylphosphate p-toluidine (BCIP) and nitro blue tetrazolium chloride (NBT) (Sigma-Aldrich) according to the manufacturer's instructions.

E. aerogenes strains ATCC 13048, EAEP289 (a mutant of strain EA27 exhibiting norfloxacin efflux) and EAEP294 (a mutant of EA27 that does not express AcrA) were used as controls [19].

2.6. Outer membrane extraction and protein identification

The method for extracting outer membranes has been described previously [9]. The band corresponding to a new major protein was excised from the SDS-PAGE gels and was sequenced by mass spectrometry [22]. Determination of the degrees of identity and similarity with known proteins was carried out using BLASTx, BLASTP and FASTA from the Genetics Computer Group suite of programs.

2.7. Statistical analysis

Comparison of antibiotic resistance between IPM-S and IPM-I/R *E. aerogenes* strains, mortality and other clinical parameters in patients harbouring IPM-I or IPM-R strains was assessed using the Fisher's exact test (for continuous variables) and the χ^2 test (for