Diagnostic Microbiology and Infectious Disease 88 (2017) 276–281

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mechanisms responsible for imipenem resistance among *Pseudomonas aeruginosa* clinical isolates exposed to imipenem concentrations within the mutant selection window

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ARTICLE INFO

Article history:
Received 20 February 2017
Received in revised form 30 March 2017
Accepted 7 April 2017
Available online 12 April 2017

Keywords:
P. aeruginosa
MPC
MSW
oprD
Imipenem-resistance

ABSTRACT

The aim of this study was to determine the propensities of imipenem to select for resistant *Pseudomonas aeruginosa* mutants by determining the mutant prevention concentrations (MPCs) for 9 unrelated clinical isolates and the accession of any relationship with mechanisms of resistance development. The MPC/MIC ratios ranged from 4 to 16. Detection of resistance mechanisms in the mutant derivatives of the nine isolates mainly revealed inactivating mutations in the gene coding for outer membrane protein OprD. Point mutations leading to premature stop codons or amino acid substitution S278P, ≥1bp deletion leading to frameshift mutations and interruption of the *oprD* by an insertion sequence, were observed. MPC and mutant selection window (MSW) are unique parameters that may guide the implementation of antimicrobial treatment, providing useful information about the necessary imipenem concentration needed in the infection area, in order to avoid the emergence of resistance, especially in clinical situations with high bacterial load.

1. Introduction

The opportunistic environmental bacterium *Pseudomonas aeruginosa* is one of the most important nosocomial pathogens, especially in intensive care units and among special patient populations such as neutropenic hosts, as well as those suffering from chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Intrinsic and acquired antibiotic resistance makes *P. aeruginosa* one of the most difficult organisms to treat.

In recent years, excessive clinical use of carbapenems has been incriminated for the increasing rates of acquired carbapenem resistance, which is associated with a variety of mechanisms, such as down-regulation of the carbapenem-specific porin OprD (responsible for imipenem resistance), overexpression of the mexAB-oprM efflux pump (responsible for meropenem and doripenem resistance with concomitant loss of OprD), and production of carbapenemases (Lister et al., 2009). Minor changes in susceptibility can be observed due to overexpression of AmpC, adding to the resistance potential (Lister et al., 2009).

It is accepted knowledge that inadequate dosing of antibiotics provides a selective pressure on bacteria and facilitates the emergence of resistance resulting from bacterial mutation. Several approaches have been proposed to restrict the emergence of resistance by optimizing the administration of antibiotics. One such approach is the implementation of the Mutant Prevention Concentration (MPC) in designing successful dosing strategies (Blondeau et al., 2004; Drlica and Zhao, 2007).

The MPC is a pharmacodynamic parameter, which was introduced in 1999 by Dong et al. as a tool to suppress the emergence of resistance during antimicrobial treatment (Dong et al., 1999). It is defined as the concentration that prevents selection of resistant mutants and corresponds to the MIC of the first-step mutant (Courvalin, 2008; Drlica and Zhao, 2007; Mouton et al., 2005; Olofsson and Cars, 2007; Zhao, 2003). Dosing schemes that exceed the MPC have been shown to successfully suppress the emergence of resistance for concentration-dependent antibiotics such as quinolones (Almeida et al., 2007; Homma et al., 2007; Liang et al., 2011; Zhao and Drlica, 2002). The Mutant Selection Window (MSW) is the antibiotic concentration range which extends from the MIC to MPC and facilitates mutations so that resistance accumulates in a stepwise manner and is selected for (Drlica and Zhao, 2007; Mouton et al., 2005).

Studies evaluating the MPC of time-dependent antibiotics such as carbapenems, for optimizing the use of this antibiotic class against relevant Gram-negative pathogens, are only a few. The same is true for studies testing the propensity of carbapenems to select for resistant mutants among susceptible pathogens.

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http://dx.doi.org/10.1016/j.diagmicrobio.2017.04.005
0732-8893/

The aim of this study was to investigate the MPC parameter of imipenem in susceptible non-carbapenemase producing *P. aeruginosa* clinical isolates and to elucidate the mechanisms of decreased susceptibility, in mutants obtained when the susceptible isolates are exposed to imipenem concentrations within the MSW.

2. Materials and methods

2.1. Bacterial strains

We studied *P. aeruginosa* clinical isolates that were obtained from single patients hospitalized in University General Hospital "Attikon", in Athens, Greece. The reference strain ATCC27853 was used as a control. Identification and MICs were determined using an automated system (BD Phoenix automated microbiology system, Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Imipenem, meropenem and doripenem MICs were further confirmed with Etest (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. MICs were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (EUCAST, 2017). Carbapenemase production was evaluated by phenotypic test with meropenem disks supplemented with 600 μg of 3-aminophenylboronic acid (APB), 1000 μg of dipicolinic acid (DPA) or 3000 μg of cloxacillin per disk (Pasteran et al., 2011). The genetic relatedness among studied isolates was evaluated with Pulsed Field Gel Electrophoresis (PFGE) analysis. DNA was prepared as per standard PFGE methods and chromosomal restriction fragments obtained after *Spe*I cleavage were visually compared (Tenover et al., 1995).

2.2. Mutant prevention concentration determination

For each *P. aeruginosa* isolate, approximately 10^{10} fresh growing cells were applied onto a series of imipenem- containing agar plates, each differing by two-fold dilutions (drug concentrations ranged from 0.5 mg/L to 128 mg/L). Plates were incubated at 37 °C in closed plastic bags for a total of 48 h and examined every 24 h for the appearance of colonies. Minimum imipenem concentrations that prevented growth were confirmed by transferring the surface growth of each plate via cotton swab to a second plate containing the same drug concentration, followed by incubation. MPC was recorded as the lowest antibiotic concentration at which no colonies grew on an agar plate. For each strain, MPC of imipenem was determined in at least three independent experiments (Blondeau, 2009). The variation between experiments was not more than one concentration step. For each strain, colonies that grew in the highest imipenem concentration were subcultured, submitted to imipenem MIC evaluation by Etest (AB Biodisk) to check for mutants and stored for further testing.

The MSW is the concentration range bordered by the MIC (lower boundary) and MPC (upper boundary) values in which selective enrichment and amplification of resistant mutant isolates most likely occurs (Zhao and Drlica, 2008). The width of MSW is expressed as a ratio (MPC/MIC), which is also termed as mutation prevention index (MPI) (Mouton et al., 2005).

2.3. Quantitative real-time PCR (QRT-PCR)

The expression of genes encoding the three major *P. aeruginosa* efflux pumps, MexAB-OprM (*mexB*), MexCD-OprJ (*mexD*), and MexXY-OprM (*mexY*), porin OprD (*oprD*) and β-lactamase AmpC (*ampC*) was determined by real-time reverse transcription-PCR (RT-PCR) for all pairs of susceptible and after the MPC strains following previously described primers (Table 1) (Rodríguez-Martínez et al., 2009; Quale et al., 2006). Briefly, total RNA was extracted with innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany) in the late logarithmic phase of the bacterial culture. Reverse transcription (RT) of 1 μg of total RNA was performed with the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, USA) according to manufacturer instructions. QRT–PCR was performed in an MX3005P instrument (Stratagene, La Jolla, CA, USA) with Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies, USA). The single-copy housekeeping gene *rpsL* was used for normalization and cycling parameters for all tested genes were one cycle of 95 °C for 10 min followed by 45 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 90 s. The threshold cycle (Ct) of each sample, which identified the PCR cycle at which the fluorescence exceeded a threshold value, was calculated by the MX3005 software. The relative expression of examined genes was assessed using the DDCt method as described previously (Livak and Schmittgen, 2001). Gene expressions of clinical isolates were compared to that of standard isolate ATCC27853 and of mutants retrieved after exposure to imipenem, were compared to that of the susceptible parent clinical isolate. An isolate was characterized as MexAB-OprM or MexCD-OprJ overproducer, if the expression of *mexB* or *mexD* was at least two times higher and as a MexXY-OprM overproducer if the expression of *mexY* was at least four times higher than the standard isolate ATCC27853 or the parent isolate (Hocquet et al., 2006; Quale et al., 2006). Mutants were considered as *ampC* overproducers if the corresponding mRNA level was at least 10-fold higher than that of the standard isolate ATCC27853 or parent clinical isolate and borderline if the level was 5–10-fold higher (Cabot et al., 2011).

2.4. Gene amplification and sequencing

The *oprD* gene was amplified by PCR as previously reported (Rodríguez-Martínez et al., 2009). Primers used for PCR and sequencing

Table 1
Primers used in this work.

Primer	Sequence (5′ → 3′)	PCR product size (bp)	Use	Reference
MxB-U	CAAGGGCGTCGGTGACTTCCAG	272	Quantitative real-time PCR of *mexB*	Rodríguez-Martínez et al. (2009)
MxB-L	ACCTGGGAACCGTCGGGATTGA			
MxY-U	GGACCACGCCGAAACCGAACG	522	Quantitative real-time PCR of *mexY*	Rodríguez-Martínez et al. (2009)
MxY-L	CGCCGCAACTGACCCGCTACA			
MxD-For	GGACGGCTCGCTGGTCCGGCT	236	Quantitative real-time PCR of *mexD*	Rodríguez-Martínez et al. (2009)
MxD-Rev	CGACGAAGCGCGAGGTGTCGT			
OprD-For	GCTCGACCTCGAGGCAGGCCA	242	Quantitative real-time PCR of *oprD*	Rodríguez-Martínez et al. (2009)
OprD-Rev	CCAGCGATTGGTCGGATGCCA			
AmpC-F	CGCCGTACAACCGGTGAT	113	Quantitative real-time PCR of *ampC*	Quale et al. (2006)
AmpC-R	CGGCCGTCCTCTTTCGA			
Rspl-1	GCTGCAAAACTGCCCGCAACG	249	Quantitative real-time PCR of *rpsL*	Rodríguez-Martínez et al. (2009)
Rspl-2	ACCCGAGGTGTCCAGCGAACC			
OprDSEQF1	CTACGCAGATGCGACATGC	1586	PCR/Sequencing	Fowler and Hanson (2014)
OprDSEQR1	CCTTTATAGGCGCGTTGCC			
OprDRTF2	GAAAGTGATGAAGTGGAGCG		Sequencing	Fowler and Hanson (2014)
OprDRTF3	GAAGCCAAGTACGTGGTCCAG			
OprDRTR3	CAGGATCGACAGCGGATAGTC			