Microbial Pathogenesis 112 (2017) 122–125

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Collateral sensitivity between aminoglycosides and beta-lactam antibiotics depends on active proton pumps

Leila Azimi [a], Abdolaziz Rastegar Lari [b, *]

[a] Pediatric Infections Research Center, Research Institute for Children Health, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 29 April 2017
Received in revised form
12 September 2017
Accepted 24 September 2017
Available online 25 September 2017

Keywords:
Collateral sensitivity
Antibiotic resistance
Aminoglycoside
Beta-lactam antibiotics

ABSTRACT

Background: Selection inversion is the hypothesis for antibiotic resistant inhabitation in bacteria and collateral sensitivity is one of the proposed phenomena for achievement of this hypothesis. The presence of collateral sensitivity associated with the proton motivation pump between the aminoglycosides and beta-lactam group of antibiotics is one of the examples of collateral sensitivity in some studies. The aim of this study was to demonstrate that collateral sensitivity between aminoglycosides and beta-lactam antibiotics associated with proton motivation pump may not be true in all cases.
Methods: In this study, 100 *Pseudomonas aeruginosa* were surveyed. Gentamicin and imipenem-resistant strains were confirmed by disc diffusion method and MIC. Active proton motivation pumps were screened by pumps inhibitor. Semi-quantitative Real-Time PCR assay was used to confirm gene overexpression.
Results: Seventy-six and 79 out of 100 strains were resistant to gentamicin and imipenem, respectively. Seventy-five strains were resistant to both gentamicin and imipenem. The results of proton pump inhibitor test showed the involvement of active proton motivation pump in 22 of 75 imipenem- and gentamicin-resistant strains. According to Real − Time PCR assay, *mexX* efflux gene was overexpressed in the majority of isolates tested.
Discussion: The collateral sensitivity effect cannot explain the involvement of active proton motivation pumps in both imipenem and gentamicin-resistant strains simultaneously. Active and/or inactive proton pump in gentamicin-sensitive and/or resistant strains cannot be a suitable example for explanation of collateral sensitivity between aminoglycosides and beta-lactam antibiotics.

1. Introduction

Antibiotic resistance is a serious problem within the health community worldwide. The appearance of multi-drug resistant (MDR) strains of bacteria in clinical settings enforce more expense for both the healthcare system and the patients [1–6]. Infection with MDR strains can cause limitation in antibiotic therapy and can lead to more morbidity and mortality, especially in vulnerable patients, including those with immune suppression [1,5,6]. There are many studies on the detection of antibiotic resistant strains and different mechanisms precipitating in antibiotic resistance [1,6–11]. However, MDR strains are unfortunately increasing worldwide along with many victims [6–10]. Researchers have been trying to develop a new way, such as selection inversion to confront the MDR strains of bacteria [6,11–13]. They have proposed the use of a method in antibiotic therapy to select antibiotic-susceptible strains instead of utilizing more broad-spectrum antibiotics and to confront more resistant bacteria [6,11–13]. Collateral sensitivity between aminoglycosides and other antibiotics, such as beta-lactam, is an example for selection inversion by collateral sensitivity effect [6,11–13]. According to this hypothesis, aminoglycoside resistant strains, developed by mutational mechanisms resulting in inactivation of the proton motivation pumps that involves the entrance of antibiotic to bacterial cell, can become more sensitive to other antibiotics (e.g., beta-lactam). This is because one of the main mechanisms for resistance to beta-lactam is the presence of proton motivation pumps, such as RND family efflux pumps [6,11–13]. The aim of this study was to prove that collateral sensitivity associated with the proton motivation pump between aminoglycosides and

* Corresponding author. Clinical Microbiology of Iran University of Medical Sciences, P.O. Box 14515-717, Tehran, Iran.
E-mail address: azizlari@gmail.com (A. Rastegar Lari).

https://doi.org/10.1016/j.micpath.2017.09.049
0882-4010/

beta-lactam antibiotics may not be true in all cases, using semi quantitative Real-Time PCR assay.

2. Materials and methods

2.1. Bacterial strains

In this cross-sectional study, 100 *Pseudomonas aeruginosa* were collected from wounds of burn patients during 6 months in one of the teaching hospitals in Tehran, Iran in 2013. Conventional biochemical and microbiological tests, including TSI, oxidase, gelatinase, growth on 42° C were used for phenotypic identification. *oprI* and *oprL* genes were used for genus and species confirmation by PCR assay. The sequences of primers used are shown in Table 1.

2.2. Antibiotic susceptibility testing

The antibiotic susceptibility testing to gentamicin (10 μg), and imipenem (10 μg) was performed by the disc diffusion method on Mueller-Hinton agar. MIC value of these antibiotics was also determined by the agar dilution assay in triplicate replication. Clinical and laboratory standards institute (CLSI) 2013 break points [15] were used for detection of gentamicin and imipenem resistant strains. Antibiotic discs used in this study were obtained from MAST Company (Mast Diagnostics, UK) and antibiotic reference standard 1097636 (for gentamicin) and 1337809 (for imipenem) (Sigma-Aldrich, France) was used for MIC determination. *P. aeruginosa* ATCC 27853 was used as control strain in the both antibiotic susceptibility testing.

2.3. Treatment of efflux pump inhibitor

Carbonyl Cyanide 3-cholorophenylhydrazone (CCCP) as proton motivation pump inhibitors (efflux pump inhibitor) was used for detection of efflux pump phenotype. CCCP (C2759 Sigma-Alderich, France) was added to individual Mueller-Hinton agar (MHA) plates, one containing 0.5–1024 μg/ml gentamicin and another containing 0.5–256 μg/ml imipenem. The final concentration of CCCP in the each MHA plates was 25 μg/ml CCCP [5,8,9]. The positive criterion for presence of active efflux pump in isolates was that MIC of tested antibiotics decreases at least 4-fold when CCCP was added [5,8,9]. Seven of these strains were selected for Real-Time PCR assay to determinate the level of expression of efflux genes *MexA, MexC and MexX*.

2.4. RNA extraction

The strains were cultured in LB medium (5 mL) and were grown to mid-exponential phase (OD600 = 1.5–2.0). Then, the bacteria (5×10^8) were added to 0.5 mL of RNeasy bacteria protect solution (Qiagen, 74104, Germany) to extract RNA according to the manufacturer's instruction. Furthermore, contaminating DNA was eliminated using 20 U of RQ1 RNAse-free DNAse (Promega, Madison, WI) and finally, DNA-free RNA solution was resuspended in 50 μL of DEPC-treated water (0.1% v/v).

2.5. cDNA synthesis

The RNA sample (1 μg) was incubated with 250 ng random hexamer primers (Sigma) and was added to the pre-mix cDNA synthesis kit (BioNEER, Cat. No. K- 2041. Korea). The reaction was performed for 60 s at 15 °C, 60 min at 55 °C and then at 95 °C for 5 min.

2.6. Real-Time PCR reaction

The semi quantitative Real-Time PCR using the primers recommended by other authors was done to evaluate the expression level of efflux pumps (Table 1) in seven selected isolates with results opposed to collateral sensitivity theory. Duplicated PCR reactions using the QuantiT ect SYBR Green RT-PCR Kit (Qiagen, Cat. No. 204243) were carried out in a Rotor Gene RT-PCR instrument (Corbett Research, Sydney, Australia; Model RG3000, software version 6). After 12 min activation of the modified Taq polymerase at 95° C, 40 cycles of amplification consisted of 15 s at 95 °C, 30 s at each gene annealing temperature (Table 1) and 30 s at 72 °C were performed. Then, the ΔΔCT values were used for data analysis [17].

3. Results

In this study, 100 *P. aeruginosa* were confirmed by phenotypic and molecular assays. Seventy-five (75%) of 100 strains were resistant to both of gentamicin and imipenem and 21 (21%) of strains were susceptible to both antibiotics. One (1%) and four (4%) strains were resistant to gentamicin and imipenem alone according to the results of disc diffusion and MIC, respectively. The results of MIC with and without CCCP, as a proton pump inhibitor, showed the presence of active proton motivation pump in 22 of 75 imipenem- and gentamicin-resistant isolates. According to the Real –

Table 2
Percentage of gene over expression in proton motivation pump.

	Percentage of strains							
	1024	512	256	128	32	8	4	2
Increase of *mexX* gene expression	8	8	17	25	8	_	32	_
Increase of *mexC* gene expression	_	_	_	_	_	40	20	20
Increase of *mexA* gene expression	_	_	_	_	17	17	66	_

Table 1
Primers sequences of *oprI, oprL*.

Primers	Primer sequence	RCR product size	References
oprI-F	5′ATGAACAACGTTCTGAAATTCTCTGCT-3′	249bp	[14]
oprI-R	5′-CTTGCGGCTGGCTTTTTCCAG-3′		
oprL-F	5′-ATGGAAATGCTGAAATTCGGC-3′	504bp	[14]
oprL-R	5′-CTTCTTCAGCTCGACGCGACG-3′		
MexA-F	5′-CGACCAGGCCGTGAGCAAGCAGC-3′	316 bp	[16]
MexA-R	5′-GGAGACCTTCGCCGCGTTGTCGC-3′		
MexC-F	5′-GTACCGGCGTCATGCAGGGTTC–3′ 5′-TTACTGTTGCGGCGCAGGTGACT-3′	164 bp	[16]
MexC-R			
MexX-F	5′-TGAAGGCGGCCCTGGACATCAGC-3′	326 bp	[16]
MexX-R	5′-GATCTGCTCGACGCGGGTCAGCG-3′		