

## Original Article

# Research of the heteroresistance of *Pseudomonas aeruginosa* to imipenem

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**Abstract:** *Pseudomonas aeruginosa* (PA) plays an important role in nosocomial infection. To explore the heteroresistance of PA to imipenem (IMP), we detected the sensitivity of 140 strains of PA to IMP using the KB method and VITEK method. Combined with resistance mutation analysis, the heteroresistance of PA to IMP was determined. Whilst, the double disk synergy test and SYBGreen RT-PCR for efflux pump were performed in IMP-heteroresistant PA strains. In this study, we confirmed 20 IMP-heteroresistant strains. The double disk synergy tests suggested that none of 20 heteroresistant PA strains produced metalloenzyme. The SYBGreen quantitative RT-PCR revealed that the MexAB expression level of efflux pump in IMP-heteroresistant PA was significantly higher than that in the IMP-sensitive strains ( $P < 0.05$ ), while there was no significant difference between the MexCD expression between resistant strains and sensitive strains ( $P > 0.05$ ). We believe that the clinicians should pay more attention to the PA heteroresistance to IMP, and the heteroresistance of PA to IMP is related to high expression in the MexAB of PA efflux pump.

**Keywords:** *Pseudomonas aeruginosa*, imipenem, heteroresistance, antimicrobial susceptibility test

## Introduction

*Pseudomonas aeruginosa* (PA) plays an important role in nosocomial infection [1]. Imipenem (IMP) belongs to the carbene penicillin, which is commonly used in treating PA infection. At present, due to the widespread use of antibiotics, PA is becoming more serious resistance to multiple antimicrobials including carbapenems [2]. Therefore, the clinicians often relied on the antimicrobial susceptibility test (AST), however, even if the treatment is based on the AST, there still remains a considerable proportion of the failure cases. The reasons for clinical treatment failure may be either pharmacokinetics or pharmacodynamic, else reason may be linked with the heteroresistance produced by bacteria [3-6]. In clinical practice, we found that some PA isolates have the property of heteroresistance to IMP. In order to further understand its characteristics and elucidate the relevant mechanisms, we carried out the following research.

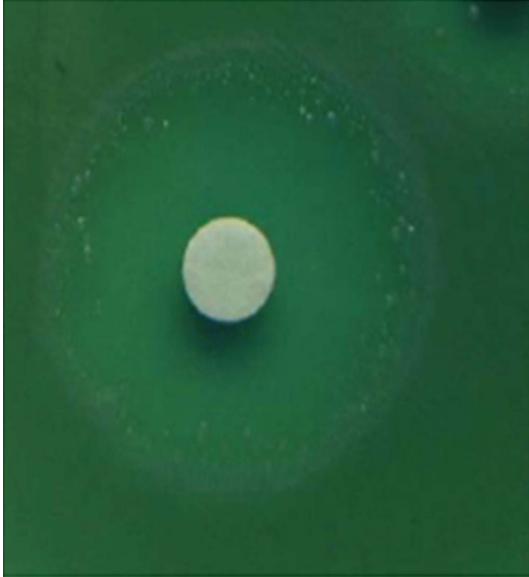
## Materials and methods

### Source of PA isolates

One hundred forty PA strains were isolated from hospitalized patients attending Kunshan Hospital Affiliated to Nanjing University of Traditional Chinese Medicine and Changzhou Tumor Hospital Soochow University between 2010 and 2013. All isolates were identified by Vitek-32 automatic microorganism identification instrument (BioMerieux Company, France) with non-fermentative bacteria identification card (BioMerieux Company, France).

### Antimicrobial susceptibility testing (AST) and heteroresistance identification

The PA susceptibility to IMP was tested by the KB method and VITEK method using GNS-119 card (BioMerieux Company, France), respectively. The AST was in accordance with 2012 CLSI. ATCC25922 and ATCC27853 were used as quality control strains.



**Figure 1.** The heteroresistance phenomenon of *Pseudomonas aeruginosa* to imipenem.

The screening for heteroresistant PA isolates was tested by the KB method. A number of colonies appeared in the bacteriostatic circle of IMP disk (visible to the naked eye observation in transmitted light) were considered as potential heteroresistance. And then the heteroresistant colonies to IMP was subcultured up to five generations and then repeated the AST, if the resistance to IMP still remains, we judge it as a heteroresistant PA to IMP. Finally, the colonies diluted with sterile saline were prepared in  $10^6$ - $10^{10}$  CFU/ml with different concentration of bacterial suspension. 10  $\mu$ l of bacterial suspension in above concentration were inoculated with M-H plate contained IMP at 35°C for 48 h, and counted the number of colonies to determine IMP heteroresistance frequency.

### *Metallo- $\beta$ -lactamase detection*

According to double-disk synergy test (DDST) in the previous literatures [7, 8], IPM-EDTA and sodium mercaptoacetic-acid (SMA) were used for the detection of metallo- $\beta$ -lactamase (MBL).

### *Quantitative RT-PCR detection for efflux pump protein*

Single colony of PA was cultured in 2 ml of LB at 37°C for 24 h, and bacteria suspension was centrifuged at 4°C with 0.1% DEPC. Bacterial precipitin was added with 1 ml of Trizol. Subsequently, cDNA was prepared by a com-

mercial kit. Quantitative detection for efflux pump protein MexAB and MexCD was used by RT SYBGreen quantitative PCR. Fluorescence quantitative PCR kit was purchased from ABI Company. The ABI7300 instrument was used for PCR amplification.

Efflux pump primers were as follows: MexAB P1 5'-CTGGAGATCGACGACGAGAAG-3' P2 5'-GGT-CGATGAAATCGTTGACGT-3' MexCD P1 5'-GCG-ATACTCTTCTGGCGAGAT-3' P2 5'-TTCTCCCG-GTCGATCAACA-3' The reaction conditions were for 16S rRNA: 95°C for 10 min, 95°C 5 s, 7 s, 58°C, 72°C 10 s, 40 cycles; for MexAB and MexCD: 95°C 10 min, 5 s, 95°C, 58°C 8 s, 72°C 18 s, 40 cycles. The amplified PCR product was analyzed by melting curve analysis. Analysis parameters were set as following: 95°C 5 s, 65°C 20 s, heating to 97°C 5 s at 0.1°C/s, 40°C 20 s.

### *Statistic analysis*

The statistical analysis is performed using SPSS (statistical program for social sciences software) 13.0 version. The matched-pair t-test is used for the analysis of the difference of the expression levels between MexAB and MexCD.

## Results

According to the results of VITEK, there were 106 IMP-sensitive strains and 34 non-sensitive strains. According to the results of KB, among 106 IMP-sensitive isolates, 33 colonies were observed to exist heteroresistance phenomena (the heteroresistance judgement: the minor PA colonies can be observed in the IMP-disk inhibitory cycle, seen in **Figure 1**). The 33 colonies were subcultured after five generations, and 13 colonies recovered sensitivity, while the remaining 20 colonies have still kept resistance according to the AST of KB method. Therefore, we confirmed that 20 PA strains (18.87%) had heteroresistance to IMP. The results also showed that the PA mutation frequencies with IMP-heteroresistance were from the  $6 \times 10^{-7}$  to  $4.5 \times 10^{-9}$ . DDST revealed that none of 20 IMP-heteroresistant PA strains were found to be produce metallo- $\beta$ -lactamase.

Melting curve analysis revealed there had no nonspecific amplification and the detection results were reliable. According to the results of quantitative PCR in 20 IMP-heteroresistant PA, the means and standard deviations of Ct in

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MexAB and MexCD were  $14.14 \pm 0.88$ , and  $13.54 \pm 3.30$ , respectively; while in 20 IMP-sensitive PA, that of Ct in MexAB and MexCD were  $16.98 \pm 1.06$  and  $13.90 \pm 0.99$ , respectively. It suggested that the MexAB expression level of efflux pump in IMP-heteroresistant PA was significantly higher than that in IMP-sensitive PA ( $P < 0.05$ ), while there had no significant difference in MexCD expression level between them ( $P > 0.05$ ).

### Discussion

Heteroresistance is a special type of bacterial resistance. The heteroresistance can lead to clinical detection error and the clinical anti-infection failure, thus raised the concern of researchers. The earlier report about the heteroresistance appeared in *Staphylococcus* [9]. In 1997, Japanese scholar [10] firstly found one methicillin-heteroresistant *Staphylococcus aureus* isolate from sputum specimens of one patient with infectious disease. Up to now, many countries have been reported the vancomycin-heteroresistant *Staphylococcus aureus* [6, 11, 12], and teicoplanin-heteroresistant or vancomycin-heteroresistant *Enterococcus* [13-15]. In recent years, some studies showed that *Acinetobacter Baumanni* to polymyxin and carbapenem [13, 16, 17], and PA to meropenem and *Enterobacter aerogenes* to carbapenems was likely to occur heteroresistance [18, 19].

At present, automatically AST microbial instruments have been widely used in hospital setting. However, we found that, when the bacteria appear heterogeneous resistance, the microbial VITEK instrument can't be correctly detected. In this study, we use the KB method and 33 colonies were observed to have heteroresistance. Furthermore, the 33 colonies subcultured after five generations, 13 colonies recovered sensitive, while the remaining 20 colonies have still kept resistance, suggesting that the heteroresistance in some PA strains is unstable. Besides, the results also showed that the PA mutation frequencies of IMP-heteroresistance were from the  $6 \times 10^{-7}$  to  $4.5 \times 10^{-9}$ , which showed that the IMP-heteroresistance incidence was higher. Thus, PA heteroresistance should be paid more attention in clinic practice.

The Metallo- $\beta$ -lactamase and the abnormal expression of efflux pump were the two main

reasons conferring to PA resistance to carbapenem including IMP. DDST revealed that none of 20 IMP-heteroresistant PA strains were found to be produce metallo- $\beta$ -lactamase. According to the results of quantitative PCR, the MexAB expression level of efflux pump in IMP-heteroresistant PA was significantly higher than that in IMP-sensitive PA, while there had no significant difference in MexCD expression level between them. Thus, we demonstrated that the IMP-resistance mechanism of PA is associated with the high MexAB expression of efflux pump. Due to the high expression of efflux pump, PA can pump out of the bacterial membrane and occur resistance [20, 21]. However, the mechanism of overexpression of MexAB remains unknown and need be further study in the future.

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### Disclosure of conflict of interest

None.

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