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Original Article

Molecular epidemiology and its risk factors for efflux pump gene expression of multi-drug resistant pseudomonas aeruginosa isolated from ICU patients

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Abstract: *Objective:* The purpose of this study was to evaluate the molecular epidemiology and the risk factors for efflux pump gene expression of multi-drug resistant pseudomonas aeruginosa (MDRPA) isolated from ICU patients. *Methods:* Specimens were derived from sputum, blood, or wound secretions of 78 ward admitted patients diagnosed with MDRPA in the department of ICU of our hospital from January 2014 to June 2016. Polymerase chain reaction (PCR) and real-time PCR assays were used to detect the efflux pump gene (MexAB-OprM, MexCD-OprJ, MexEF-OprN) expression of MDRPA isolated from ICU patients. *Results:* The MexAB-OprM, MexCD-OprJ and MexEF-OprN positive expression rates of MDRPA were 100% (78/78), 35.9% (28/78) and 46.2% (36/78), respectively. The resistance rates to gentamicin, chloramphenicol and ampicillin/sulbactam were 100%. The resistance rates to levofloxacin and ciprofloxacin were >90%. The resistance rates to cefoperazone, cefotaxime, cefoperazone/sulbactam and aztreonam were >50%. Application of carbapenem antibiotics (OR=5.17, P<0.05), invasive medical procedure (OR=5.32, P<0.05), hypolekocytosis (OR=4.80, P<0.05), chronic diseases (OR=2.55, P<0.05) and cancer (OR=5.54, P<0.05) were associated with high MexAB-OprM gene expression. Application of carbapenem antibiotics (OR=11.61, P<0.05), application of macrolide antibiotics (OR=3.57, P<0.05), invasive medical procedure (OR=12.50, P<0.05) and hypolekocytosis (OR=3.13, P<0.05) were risk factors for positive MexCD-OprJ gene expression. Application of carbapenem antibiotics (OR=4.67, P<0.05), macrolide antibiotics (OR=9.92, P<0.05), invasive medical procedure (OR=8.17, P<0.05) and hypolekocytosis (OR=6.42, P<0.05) were independent factors for positive MexEF-OprN gene expression. *Conclusion:* MDRPA strains isolated from the ICU ward of our hospital were severely resistant to antibiotics. The normalized use of carbapenems and glycopeptides antibiotics, the reduction of unnecessary invasive procedures and the severely grasped standard of hospitalized in ICU ward played important roles in the prevention of the overexpression of the efflux pump genes of MDRPA.

Keywords: Pseudomonas aeruginosa, multi-drug resistant, efflux pump gene, molecular epidemiology

Introduction

Pseudomonas aeruginosa (Pa) is the main pathogen of refractory hospital infection, which mainly results from the continuous appearance of Pa multiple drug-resistance (MDR) strains [1, 2]. Drug-resistant nodular cell differentiation family is one of the bacterial efflux pump systems and plays an important role in Pa's multiple drug resistance [3-5]. Multidrug-resistant Pa is the main source of nosocomial infections, especially refractory pathogens in ICU and infection departments [6]. The high expression of multi-drug efflux pump genes, such as MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN is the important cause for the resistance to most commonly used antibiotics, which can make the organism excrete drugs, thereby resulting in the reduction of the thallus concentration and failure to kill the bacteria [7]. In this study, real-time PCR was used to predict the molecular epidemiological trend and analyze the risk factors of high expressions of MexAB-OprM, MexCD-OprJ and MexEF-OprN of MDRPA efflux pump genes among ICU patients.

Materials and methods

Specimens

Specimens were derived from sputum, blood, or wound secretions of 78 ward admitted

Table 1. Sequences of PCR primers for MexAB-OprM (mexA), MexCD-OprJ (mexC), and MexEF-OprN (mexE)

Gene	Sequence 5'-3'	Product (bp)
mexA-F	CCTGCTGGTCGCGATTTCGG	325
mexA-R	CCAGCAGCTTGTAGCGCTGG	
mexC-F	TTGGCTATGGCCATCGCGTT	851
mexC-R	ATCGAAGTCCTGCTGGCTGA	
mexE-F	ATCCCACTTCTCCTGGCGCT	265
mexE-R	GGTCGCCTTTCTTCACCAGT	

patients diagnosed with MDRPA in the Department of ICU of our hospital from January 2014 to June 2016. Samples from 78 patients infected by MDRPA were isolated and cultured. The same MDRPA strain could be cultured based on the above specimens for at least 2 times. The strains were identified by VITEK-Am s60 automatic bacterial identification system and gone for routine biochemical test. A total of 78 specimens consisted of 41 specimens of sputum, 19 specimens of blood, and 18 specimens of wound secretions.

Instruments and equipment

High performance PCR instruments (Biometra), fluorescence quantitative PCR (ABI, model: ABI7900), electrophoresis apparatus (BIO-RAD), nucleic acid protein analyzer (Beckman, model: DU800), tabletop refrigerated centrifuge (Beckman, model: AllegraX-22R), oscillating incubator (Harbin Donglian Electronic Technology Development Co., Ltd.) and UV gel imaging system (US BIO-RAD) were used in this study.

Major reagents

Trizol reagent (Invitrogen), reverse transcriptase MMLV (TAKARA), ribozyme inhibitor RNAsin, dNTP, DEPC water (all from Tiangen), PCR primer (synthesis by The Beijing Genomics Institute BGI), Taq enzyme, EX TaqTMR-PCR mixture (both from TAKARA), isopropyl alcohol, trichloromethane and anhydrous ethano (all from Sinopharm Chemical Reagent Co., Ltd.) were used in this study.

Antimicrobial susceptibility experiment

Susceptibility experiment was done using drug sensitive method, and the drug sensitivity result was calculated based on the criteria of Clinical and Laboratory Standards Institute (NCCLS) 2008. Quality control strain for Pa strain was ATCC27853.

Bacterial treatment

Multidrug-resistant Pa strains cultured from clinical specimens were collected. These strains were enriched in LB medium and stored in 20% sterile glycerol in an -80°C refrigerator.

Gene expression detection

Total cDNA of the MDRPA strain was used as the PCR template, and the primers were listed in **Table 1**. rpsL was as the the internal reference for each strain. Reaction system composition: 25 μl of premix Taq, 1 μl each of forward (F) and reverse (R) primerd (20 μmol/L), 0.5 μg of the total bacteria cDNA and sterile double distilled water added to 50 μl. The procedure was as follows: 94°C for 7 min; 30-40 cycles of 94°C for 30 s, 57°C (mexA) or 55°C (mexC, mexE) for 30 s, 61°C for 45 s; 72°C for 10 min; 4°C for 10 min. Gel electrophoresis was performed in agarose (1.5%) at 100 V for 5 min. Image was obtained by gel electrophoresis imager [8, 9].

Real time-PCR assay

Reaction system composition: 10 μl of SYBR Premix Ex Taq™ II, 1 μl each of forward (F) and reverse (R) fluorescence quantitative PCR primer (10 μmol/L), 0.4 μl of ROX Reference Dye or DyeII (50 ×), 1.0 μl of cDNA solution and sterile double distilled water added to 20 μl. The procedure was as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s and 61°C for 34 s. The dissolution curve was analyzed in the end. The qualified specimens of dissolution curve were selected for CT analysis. The $2^{-\Delta\Delta CT}$ semi-quantitative method was used to analyze gene expression. The calculated values were as follows: ΔΔCT = experimental group (CT value of target gene-CT value of reference gene)-control group (CT value of target gene-CT value of reference gene). The calculated result showed the fold changes of target gene expressions in the experimental group normalized to those in the control group. Each gene was set with two holes. The average CT value was calculated, and results were obtained in triplicate [10].

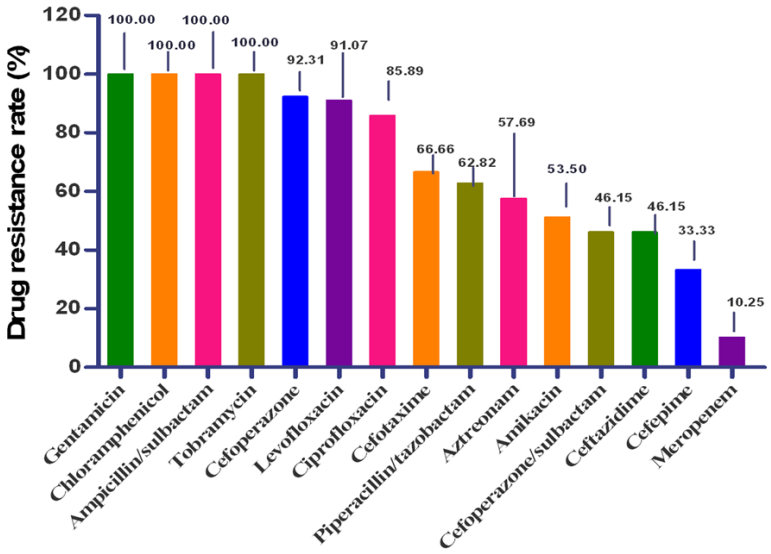

Figure 1. Drug resistance of specimens for the 78 strains of MDRPA.

Statistical analysis

Bacterial susceptibility data was analyzed by Whonet5.0. Expressions of Pa multi-resistant efflux pump gene was completed with Staga10.0. Univariate analysis was performed on risk factors of efflux pump gene expression. The comparison of intergroup was done by χ^2, with α=0.05 as the statistical standard and P<0.05 as of statistical differences.

Results

The expression of efflux pump genes

Among 78 strains of MDRPA, the positive expression rates of MexAB-OprM, MexCD-OprJ, and MexEF-OprN were 100% (78/78), 35.9% (28/78) and 46.2% (36/78), respectively. 37 strains highly expressed MexAB-OprM, and 41 strains lowly expressed MexAB-OprM.

Drug resistance of specimens

Among the isolated and cultured 78 specimens, MDRPA strains showed varying degrees of resistance to the commonly used antibiotics. The resistance rates to gentamicin, chloramphenicol, and ampicillin/sulbactam were all 100%. The resistance rates to levofloxacin and ciprofloxacin were >90%. The resistance rates to cefoperazone, cefotaxime, cefoperazone/sulbactam, and aztreonam were all >50%. The resistance rates to common antibiotics were shown in **Figure 1**.

Risk factors prediction for positive/high efflux pump gene expression

Application of carbapenem antibiotics (OR=5.17, P< 0.05), invasive medical procedure (OR=5.32, P<0.05), hypolekocytosis (OR=4.80, P<0.05), chronic foundation diseases (OR=2.55, P<0.05) and cancer (OR=5.54, P< 0.05) were associated with high MexAB-OprM expression. Application of carbapenem antibiotics (OR=11.61, P<0.05), macrolide antibiotics (OR=3.57, P<0.05), invasive medical procedure (OR=12.50, P<0.05) and hypolekocytosis (OR=3.13, P<0.05) were risk factors for positive MexCD-OprJ expression. Application of carbapenem antibiotics (OR=4.67, P<0.05), macrolide antibiotics (OR=9.92, P<0.05), invasive medical procedure (OR=8.17, P<0.05) and hypolekocytosis (OR=6.42, P<0.05) were independent factors for positive MexEF-OprN expression (**Table 2** and **Figure 2**).

Discussion

Pa is an aerobic gram-negative bacillus with polar flagella. It lively and widely presented in the hospital environment. It is an important conditional pathogenic bacterium that causes nosocomial infection [11, 12]. The excessive and un-standard use of antibiotics and the popularity of a variety of invasive operations both lead to drug resistance of Pa to commonly used antibiotics in clinical medicine. What's worse, extensive drug-resistant strains were generated, thereby bringing a great challenge to clinical treatment [1, 13]. The causes and mechanisms of Pa drug resistance are complex, including the alteration of thalli membrane permeability, antibiotic binding sites, and introduction of a variety of drug resistance genes and activation of efflux pump genes. Among these roles, the activated efflux pump system of resistant nodular cell differentiation family plays a leading role in Pa multiple drug resistance.

Table 2. Risk factors for positive/high efflux pump gene expression of multi-drug resistant pa

Risk factors	MexAB-OprM		P	MexCD-OprJ		P	MexEF-OprN		P
	High (n=37)	Low (n=41)		+ (n=28)	- (n=50)		+ (n=36)	- (n=42)	
Cephalosporins			>0.05			>0.05			>0.05
Yes	34 (91.9%)	36 (87.8%)		24 (85.7%)	42 (84.0%)		22 (61.1%)	25 (59.5%)	
No	3 (8.1%)	5 (12.2%)		4 (14.3%)	8 (16.0%)		14 (38.9%)	17 (40.5%)	
Enzyme compound drug			>0.05			>0.05			>0.05
Yes	28 (75.7%)	29 (70.7%)		20 (71.4%)	31 (62.0%)		14 (38.9%)	13 (31.0%)	
No	9 (24.3%)	12 (29.3%)		8 (28.6%)	19 (38.0%)		22 (61.1%)	29 (69.0%)	
Carbapenem			<0.05			<0.05			<0.05
Yes	28 (75.7%)	6 (14.6%)		26 (92.9%)	4 (8.0%)		8 (22.2%)	2 (4.8%)	
No	9 (24.3%)	35 (85.4%)		2 (7.1%)	46 (92.0%)		28 (77.8%)	40 (95.2%)	
Aminoglycosides			>0.05			>0.05			>0.05
Yes	11 (29.7%)	12 (29.3%)		5 (17.9%)	7 (14.0%)		5 (13.9%)	5 (11.9%)	
No	26 (70.3%)	29 (70.7%)		23 (82.1%)	43 (86.0%)		31 (86.1%)	37 (88.1%)	
Macrolides			>0.05			<0.05			<0.05
Yes	23 (62.2%)	22 (53.7%)		26 (92.9%)	13 (26.0%)		17 (47.2%)	2 (4.8%)	
No	14 (37.8%)	19 (46.3%)		2 (7.1%)	37 (74.0%)		19 (52.8%)	40 (95.2%)	
Glycopeptides			>0.05			>0.05			>0.05
Yes	7 (18.9%)	8 (19.5%)		4 (14.3%)	7 (14.0%)		6 (16.7%)	4 (9.5%)	
No	30 (81.1%)	33 (80.5%)		24 (85.7%)	43 (86.0%)		30 (83.3%)	38 (90.5%)	
Invasive medical procedure			<0.05			<0.05			<0.05
Yes	24 (64.9%)	5 (12.2%)		14 (50.0%)	2 (4.0%)		7 (19.4%)	1 (2.4%)	
No	13 (35.1%)	36 (87.8%)		14 (50.0%)	48 (96.0%)		29 (80.6%)	41 (97.6%)	
Application of adrenal cortical hormone			>0.05			>0.05			>0.05
Yes	4 (10.8%)	5 (12.2%)		6 (21.4%)	9 (18.0%)		4 (11.1%)	4 (9.5%)	
No	33 (89.2%)	36 (87.8%)		22 (78.6%)	41 (82.0%)		32 (88.9%)	38 (90.5%)	
Hypolekocytosis			<0.05			<0.05			<0.05
Yes	13 (35.1%)	3 (7.3%)		14 (50.0%)	8 (16.0%)		11 (30.6%)	2 (4.8%)	
No	24 (64.9%)	38 (92.7%)		14 (50.0%)	42 (84.0%)		25 (69.4%)	40 (95.2%)	
Chronic disease			<0.05			>0.05			>0.05
Yes	23 (62.2%)	10 (24.4%)		12 (42.9%)	13 (26.0%)		10 (27.8%)	12 (28.6%)	
No	14 (37.8%)	31 (75.6%)		16 (57.1%)	37 (74.0%)		26 (72.2%)	30 (71.4%)	
Cancer			<0.05			>0.05			>0.05
Yes	10 (27.0%)	2 (4.9%)		6 (21.4%)	5 (10.0%)		6 (16.7%)	3 (7.1%)	
No	27 (73.0%)	39 (95.1%)		22 (78.6%)	45 (90.0%)		30 (83.3%)	39 (92.9%)	
Combined use of antibiotics			>0.05			>0.05			>0.05
Yes	33 (89.2%)	34 (82.9%)		13 (46.4%)	23 (46.0%)		24 (66.7%)	21 (50.0%)	
No	4 (10.8%)	7 (17.1%)		16 (57.1%)	27 (54.0%)		12 (33.3%)	21 (50.0%)	

There are nine types of multi-drug efflux pump genes. Among these, MexAB-OprM, MexCD-OprJ and MexEF-OprN have high positive rates [14]. MexG and MexJ efflux pump systems are theoretically existed, but have not yet been found clinically. Our present study had examined the abovementioned three efflux pump genes in the sputum, blood, and wound secretions of ICU patients. Among 78 strains of MDRPA, the positive rates of MexAB-OprM, MexCD-OprJ, and MexEF-OprN were 100%, 28 35.9% and 46.2%, respectively. The positive rate of highly expressed MexAB-OprM was 47.4%, and that of lowly expressed MexAB-OprM was 52.6%. The positive rate of MexAB-OprM reached the maximum. The certain molecular epidemiological characteristics for the expression of efflux pump gene of MDRPA strain were demonstrated in our present study.

We conducted a univariate analysis to explore the possible risk factors associated with the positive expression of the abovementioned three efflux pump genes. Factors analyzed included the following: application of cephalosporins, carbapenems, aminoglycosides, macrolides, and glycopeptides; application of enzyme preparation; invasive manipulation;

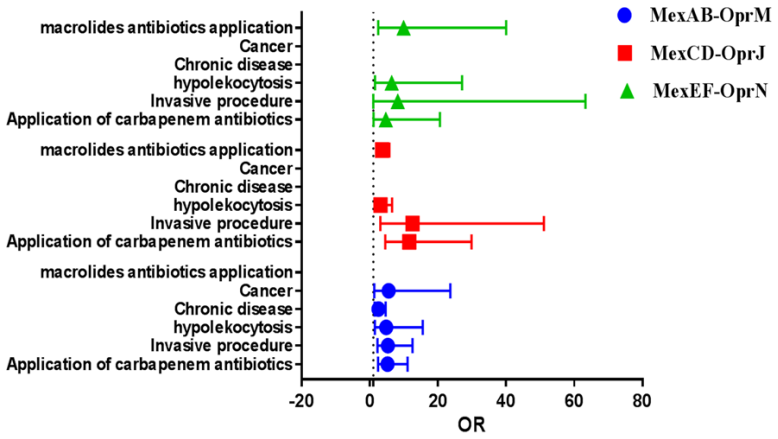

Figure 2. Bar graph of risk factors associated with positive/high efflux pump gene expressions of multi-drug resistant Pa.

application of adrenal cortical hormone; leucopenia; chronic basal disease; malignant tumors; and combined use of antibiotics. The result came out that the applications of carbapenase antibiotics, invasive manipulation, low WBC count, chronic foundation disease and cancer were associated with high MexAB-OprM expression. The applications of carbapenem antibiotics, macrolides antibiotics, invasive manipulation and low WBC count were related to high expression of MexCD-OprJ. The applications of carbapenase antibiotics, macrolides antibiotic, invasive operation, and low WBC count were related to the high expression of MexEF-OprN. The applications of carbapenem antibiotics, invasive operation, and low WBC count were common risk factors for the positive expression of multi-drug efflux pump genes, i.e., MexAB-OprM, MexCD-OprJ, and MexEF-OprN.

Most studies have confirmed that overexpression of multi-drug efflux pump gene is one of the important factors underlying the bacterial resistance. Herein, we prove that applications of carbapenase antibiotic, invasive operation, and low WBC count are the risk factors of overexpression of multi-drug efflux pump genes (MexAB-OprM, MexCD-OprJ and MexEF-OprN). Theoretically, ICU patients with the above three groups of risk factors are of high risk to MDRPA bacterial infection [15-17].

In conclusion, carbapenem antibiotics should be strictly applied to ICU patients to avoid leukopenia caused by a variety of factors. An invasive operation, such as deep vein catheterization, should be done carefully. The above mentioned targeted operations are expected to reduce the expression of MDRPA multi-drug efflux pump genes and reduce the drug resistance of Pa.

Disclosure of conflict of interest

None.

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