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Original Article

Molecular mechanism of drug resistance of non-fermentative Gram-negative bacteria in hospitals

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Abstract: The development and persistence of antibiotic resistance in non-fermentative Gram-negative (NFGN) bacteria in hospitals has become one of the major causes for clinical infection. This study aimed to investigate the species, source, drug resistance and relevant molecular mechanism of NFGN bacteria in hospitals. NFGN bacteria isolates from samples of blood, sputum, urine, pus, secretion and throat swab in our hospital was cultured and identified using a VITEK 2 Compact automated microbiology system. Drug susceptibility was determined using the conventional KB disk diffusion method. Antibiotic resistance was analyzed with WHONET 8.6 software, and resistance rate was compared by χ^2 test using SPSS 16.0. The genotypes of these NFGN bacteria were analyzed by polymerase chain reaction (PCR). The expression of CTX-M type extended spectrum β-lactamase (ESBL) was measured by Western blot analysis. The correlation between CTX-M ESBL level and antibiotic resistance was analyzed by Spearman rank test using SPSS 16.0. A total of 608 isolates of NFGN bacteria were identified including 198 isolates of *Pseudomonas aeruginosa*, 152 *Acinetobacter baumannii*, 96 *Pseudomonas spp.*, 54 *Escherichia coli,* 58 *Staphylococcus aureus* and 50 *Klebsiella pneumoniae*. These NFGN bacteria were isolated from blood (30%), sputum (18%), urine (12%), pus (10%), throat (8%) and secretion (22%). The highest proportion of these bacteria was isolated in ICU (40%), followed by the respiratory department (26%). The remaining (34%) was isolated in other departments. High level of CTX-M ESBL was expressed in these NFGN bacteria and was positively correlated with the antibiotic resistance of these bacteria. The antibiotic resistance of hospital NFGN bacteria is positively correlated with the expression level of CTX-M ESBL. The finding may provide valuable information for infection control in hospital settings.

Keywords: Non-fermentative gram-negative bacteria, *pseudomonas aeruginosa*, *acinetobacter baumannii*, antibiotic resistance, CTX-M type β-lactamase gene

Introduction

Nonfermenters are a class of gram-negative aerobic bacilli or facultatively anaerobic bacteria that cannot ferment carbohydrates. They are generally conditional pathogenic bacteria and are abundantly in nature [1]. In recent years, non-fermented Gram-negative (NFGN) bacteria in hospitals have become important pathogens causing clinical infections and antibiotic resistance [2]. It has been found that hospital NFGN bacilli primarily include *Pseudomonas aeruginosa, Acinetobacter baumannii, Pseudomonas sp., Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae* [3-6]. The emergence of drug resistance in these NFGN bacteria is currently one of the important causes for clinical infections [7]. Therefore, the study of species, source, drug resistance and relevant molecular mechanism of NFGN bacteria in hospital settings may significantly reduce the incidence rate of clinical infections.

The issue of antibiotic resistance of NFGN bacteria has become increasing serious. Resistance to various antibiotics such as imipenem, amikacin, tazobactam, piperacillin, ceftazidime, aztreonam and cefepime has been observed [8-12]. Inappropriate use of antibiotics not only leads to treatment failure, but also worsens the patient's condition. The outer membrane of NFGN bacteria is composed of lipid A, the major toxic site, core polysaccharide and specific

polysaccharides. The long-term improper use of antibiotics has destroyed the outer membrane, exposing lipid A and releasing endotoxin, which might be associated with the antibiotic resistance in these bacteria. The latest researches suggest that antibiotics may directly or indirectly regulates the permeability of NFGN bacterial cell membranes, leading to a higher intracellular level of lactamase [13-15]. It has also been found that long-term, heavy use of antibiotics may cause covalent modification of lactamase in NFGN bacteria, which further changes the permeability of their cell membrane and thus induces antibiotic resistance in these bacteria [16-18]. Therefore, the investigation of the molecular mechanism of antibiotic resistance may provide a theoretic basis for a practical solution for the issue of antibiotic resistance in these bacteria.

In this study, NFGN bacteria isolates from samples of blood, sputum, urine, pus, secretion and throat swab in our hospital was cultured and identified using a VITEK 2 Compact automated microbiology system. Drug susceptibility was determined using the conventional KB disk diffusion method. Antibiotic resistance was analyzed with WHONET 8.6 software, and resistance rate was compared by χ^2 tests. The genotypes of these NFGN bacteria were analyzed by polymerase chain reaction (PCR). The expression of CTX-M type extended spectrum β-lactamase (ESBL) was measured by Western blot analysis. The correlation between CTX-M ESBL level and antibiotic resistance was analyzed by Spearman rank tests. The study shall provide insight into the molecular mechanism of antibiotic resistance in NFGN bacteria which might be a theoretic basis for appropriate use of antibiotics and reduction in the occurrence of hospital infections.

Material and methods

Reagents, instruments and software

The VITEK 2 Compact automated microbiology system was purchased from (bioMerieux, France). KB disk diffusion susceptibility testing kit and polymerase chain reaction (PCR) kit were purchased from Sangon Biotech. (Shanghai, China). Anti-β-actin antibody and anti-CTX-M ESBL antibody were purchased from Sigma (St. Louis, MO, USA). Both WHONET 8.6 software and SPSS 13.0 were purchased from SPSS Inc. (Chicago, IL, USA).

Isolation and culture of bacteria

NFGN bacteria were isolated from samples of blood, sputum, urine, pus, secretion and throat swab in our hospital as described previously [19]. Briefly, samples of blood, sputum, urine, pus, secretion and throat swab were mixed with sterile purified water, and centrifuged at 500 rpm for 6 min. The supernatant was inoculated in BP medium and cultured at 37°C for 72 h.

Identification and classification of bacteria

The bacterial isolates were identified based on the conventional identification method [20] using a VITEK 2 Compact automated microbiology system. Briefly, bacterial suspension (approximately 8000 cells/ml) was loaded onto the test card of the system through the connection pipe and incubated for 48 h. The information on bacterial growth was transmitted to the computer in order to analyze the species and number of NFGN bacteria.

Antibiotic susceptibility test

The Antibiotic susceptibility test was performed using KB disk diffusion susceptibility test kit according to the manufacture's instruction [21]: each bacterial isolate was inoculated on agar plate. Disk with the antimicrobial agent is placed on the surface of the plate. The susceptibility was recorded after 24-h incubation at 37°C.

Analysis of bacterial genotype

Bacterial genomic DNA was extracted using the conventional methods, and analyzed using PCR kit according to the manufacture's instruction [22] to determine the genotype. Briefly, bacterial cultures were collected by centrifugation at 600 rpm for 8 min. Bacterial genomic DNA was extracted and quantified using NanoDrop™ 3000 Spectrophotometer. The 50-μl reaction system was prepared: 26 μl of DreamTaq PCR Master Mix, 1 μl of forward primer, 1 μl of reverse primer, 80 ng of template DNA, and nuclease-free water. And PCR analysis was performed using the following program: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, a final extension at 72°C for 5 min, and an indefinite hold at 4°C. The sequences of primers for the internal control β-actin

Table 1. Identification of NFGN bacteria isolates

	Acinetobacter baumannii	*Pseudomonas aeruginosa*	*Escherichia coli*	*Pseudomonas spp.*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	Total
Number	152	198	54	96	58	50	608
Percentage	25.0%	32.6%	8.9%	15.8%	9.5%	8.2%	100%

Table 2. Summary of the source of NFGN bacterial isolates

Source	Blood	Sputum	Urine	Pus	Throat swab	Secretion
Number	182	109	73	61	49	134
Percentage	30%	18%	12%	10%	8%	22%

Table 3. The distribution of NFGN bacterial isolates in different clinical departments in the hospital

Clinical departments	ICU	Respiration department	Other departments
Number	243	158	207
Percentage	40%	26%	34%

were as follows: 5'-TTTGATCTGGCTCTTTGA-TAGAGAGAAGAG-3' and 5'-GCTCTTGGATCATG-GAAAGTTTGATAGTAG-3'. The sequences of primers for CTX-M ESBL were: 5'-CATGGCTATT-GATGAGTTTGATCAGAGAGT-3' and 5'-GGATCAG-CTCA AAGAGTTTGATGTTTTGGA-3'. PCR products were analyzed by 0.8% agarose gel electrophoresis. The gray value of each band was quantified using BandScan 860 software. Each sample was measured three times and the mean value was calculated. The relative amount of CTX-M ESBL gene was determined as the ratio of grey value of CTX-M ESBL to that of β-actin.

Western blot analysis

The expression of CTX-M ESBL was quantified by Western blot analysis as described previously [23]. Briefly, bacterial cultures were collected by centrifugation at 600 rpm for 8 min. The bacterial pellet was treated in cell lysis buffer on ice for 15 min. Bacterial lysates were analyzed by Western blot analysis using mouse anti-β-actin or anti-CTX-M ESBL antibody (1:1000 dilution) as primary antibody and goat anti-mouse antibody as secondary antibody (1:3000 dilution). The gray value of each band was quantified using BandScan 860 software. Each sample was measured three times and the mean value was calculated. The relative expression level of CTX-M ESBL protein was determined as the ratio of grey value of CTX-M ESBL protein to that of β-actin.

Statistical analysis

Numeric data were expressed as mean ± standard deviation and analyzed using SPSS 13.0. Difference in bacterial resistance rates was analyzed by χ^2 tests. The correlation between CTX-M ESBL expression and antibiotic resistance of bacterial isolates was analyzed by Spearman rank tests. $P<0.05$ was considered statistically significant.

Results

Identification of NFGN bacterial isolates

As shown in **Table 1**, a total of 608 NFGN bacterial isolates were obtained in this study including 198 *P. aeruginosa*, 152 *A. baumannii*, 96 *Pseudomonas*, 54 *E. coli*, 58 *S. aureus* and 50 *K. pneumoniae*.

Source of NFGN bacterial isolates

As shown in **Table 2**, the NFGN bacterial isolates were obtained from samples of blood (30%), sputum (18%), urine (12%), pus (10%), throat swab (8%) and secretion (22%).

Distribution of NFGN bacterial isolates

The distribution/location of NFGN bacterial isolates in the hospital was summarized in **Table 3**.

Drug susceptibility of NFGN bacterial isolates

The sensitivity rate of NFGN bacterial isolates to antibiotics including mezlocillin, imipenem, amikacin, tazobactam, piperacillin, ceftazidime, aztreonam and cefepime was shown in **Table 4**.

PCR analysis

Antibiotics may directly or indirectly regulate the permeability of NFGN bacterial cell mem-

Table 4. Drug susceptibility of NFGN bacterial isolates

	Acinetobacter baumannii	*Pseudomonas aeruginosa*	*Escherichia coli*	*Pseudomonas spp.*	*Staphylococcus aureus*	*Klebsiella pneumoniae*
Sensitivity rate	18%	26%	37%	29%	37%	25%

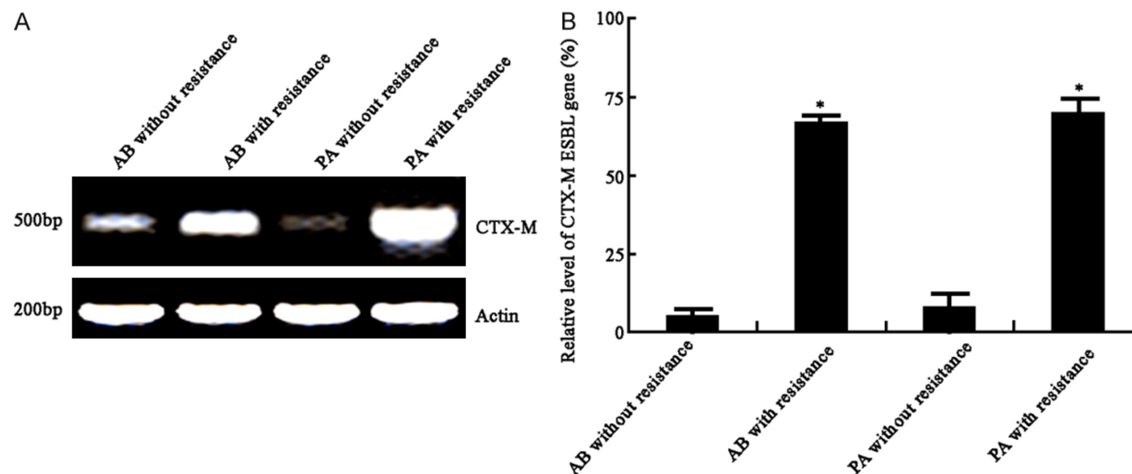

Figure 1. PCR analysis of genotypes of *Acinetobacter baumannii* (AB) and *Pseudomonas aeruginosa* (PA) isolates. *, P<0.05 compared with isolates without antibiotic resistance.

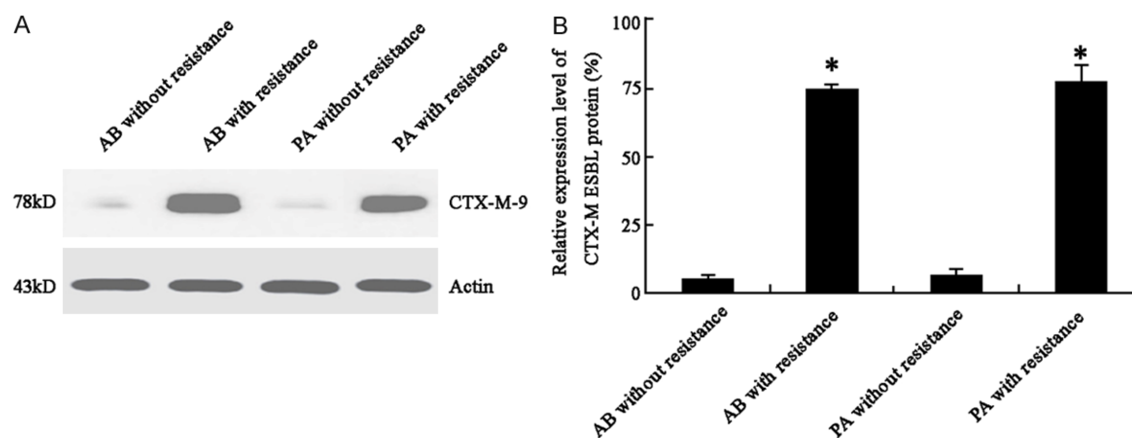

Figure 2. Western blot analysis of CTX-M ESBL protein in *Acinetobacter baumannii* (AB) and *Pseudomonas aeruginosa* (PA) isolates. *, P<0.05 compared with isolates without antibiotic resistance.

brane, leading to increased CTX-M ESBL level. In this study, we examined the genotypes of NFGN bacterial isolates in order to investigate the molecular mechanism of antibiotic resistance. *A. baumannii* and *P. aeruginosa* were analyzed and it was found that CTX-M ESBL gene was detected in 82 *A. baumannii* and 61 *P. aeruginosa* isolates (**Figure 1**). It was also shown that the relative CTX-M gene level in antibiotic-resistant *A. baumannii* and *P. aeruginosa* was significantly higher compared with *A. baumannii* and *P. aeruginosa* isolates without resistance (P = 0.021).

Western blot analysis

The molecular mechanism of antibiotic resistance of NFGN bacterial isolates was further analyzed on the protein level. *A. baumannii* and *P. aeruginosa* were analyzed and it was found that CTX-M ESBL protein was expressed in 59

Table 5. Correlation between positive rate of CTX-M ESBL and antibiotic resistance

	CTX-M				
	Positive (n)	Negative (n)	Positive rate	r	P
Acinetobacter baumannii	129	23	84. 9%	0.927	0.0083
Pseudomonas aeruginosa	168	30	84.8%	0.899	0.012
Total	297	53	84.8%		

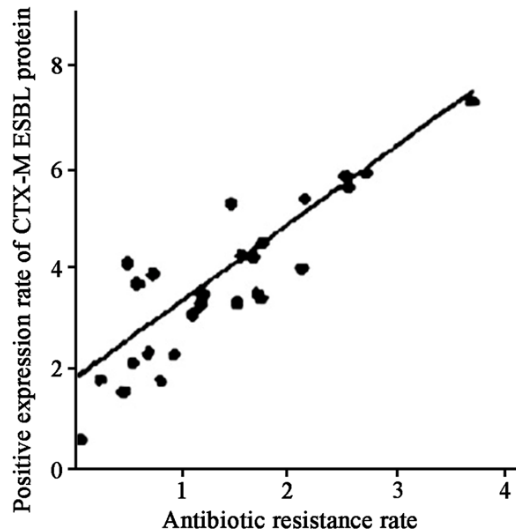

Figure 3. Correlation between positive expression rate of CTX-M ESBL and antibiotic resistance rate.

A. baumannii and 48 *P. aeruginosa* isolates as indicated by Western blot analysis (**Figure 2**). It was also shown that the CTX-M ESBL protein level in antibiotic-resistant *A. baumannii* and *P. aeruginosa* was significantly higher compared with *A. baumannii* and *P. aeruginosa* isolates without resistance (P = 0.017).

Correlation between CTX-M ESBL and antibiotic resistance

The positive rate of CTX-M ESBL was positively correlated with antibiotic resistance in both *A. baumannii* (r = -0.927, P = 0.0083) and *P. aeruginosa* (r = -0.899, P = 0.012) (**Table 5** and **Figure 3**).

Discussion

Hospital infections have seriously threatened people's health and life. NFGN bacteria in hospitals have become important pathogens causing clinical infections [24, 25]. In recent years, the antibiotic resistance of these NFGN bacteria has become an issue [26]. In this study, the genotype, distribution and antibiotic resistance of NFGN bacterial isolates in our hospital was analyzed and the relevant molecular mechanism was investigated in order to provide a theoretical basis for clinical management of drug resistance in NFGN bacteria.

In this study, a total of 608 isolates of NFGN bacteria were identified including 198 isolates of *P. aeruginosa*, 152 *A. baumannii*, 96 *Pseudomonas spp.*, 54 *E. coli,* 58 *S. aureus* and 50 *K. pneumoniae*. These NFGN bacteria were isolated from blood (30%), sputum (18%), urine (12%), pus (10%), throat (8%) and secretion (22%). The highest proportion of these bacteria was isolated in ICU (40%), followed by the respiratory department (26%). The remaining (34%) was isolated in other departments. High level of CTX-M ESBL was expressed in these NFGN bacteria and was positively correlated with the antibiotic resistance of these bacteria. These results were generally consistent with previous studies [27, 28] despite of slight discrepancies. For instance, in both previous studies, the number of *P. aeruginosa* is far less than that of *A. baumannii*, which is opposite to our result. Such discrepancy might be associated with different drug resistance of bacteria in conditions with different temperatures and humidity.

Our result showed that the largest proportion of NFGN bacteria was isolated from blood samples (30%), which is substantially higher than the world standard (15%). The proportion of NFGN bacteria isolated in sputum, urine, pus, throat swab and secretion was 18%, 12%, 10%, 8% and 22%, respective, which was similar to the result in literature [26].

Our study also demonstrated that NFGN bacteria were mostly distributed in ICU, followed by respiratory department, indicating that both departments are important locations for the prevention of clinical infections, and should receive close attention.

Further, the molecular mechanism of antibiotic resistance of these NFGN bacterial isolates

was investigated. It was suspected that antibiotic resistance in NFGN bacteria might be associated with the intracellular CTX-M ESBL expression. Our results demonstrated that CTX-M ESBL protein was expressed in 59 *A. baumannii* and 48 *P. aeruginosa* isolates. PCR and Western blot analyses also demonstrated that CTX-M ESBL expression level in antibiotic-resistant *A. baumannii* and *P. aeruginosa* was significantly higher compared with *A. baumannii* and *P. aeruginosa* isolates without resistance ($P<0.05$). The positive rate of CTX-M ESBL was positively correlated with antibiotic resistance in both *A. baumannii* ($r = -0.927$, $P = 0.0083$) and *P. aeruginosa* ($r = -0.899$, $P = 0.012$).

There are three limitations in this study. Firstly, this study has included a relatively small number of samples. Secondly, the number of samples collected from some departments is too small. Finally, the expression of CTX-M ESBL has not been regulated through overexpression or RNAi technology to further confirm the correlation between antibiotic resistance and CTX-M ESBL expression in NFGN bacteria in hospitals.

In summary, the antibiotic resistance of hospital NFGN bacteria is positively correlated with the expression level of CTX-M ESBL. The finding may provide valuable information for infection control in hospital settings.

Disclosure of conflict of interest

None.

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