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Review Article

Drug resistance and genotyping of non-fermented gram-negative bacteria in hospital

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Abstract: The drug resistance of non-fermented gram-negative bacteria in hospital severely affects patients' health. The study of distribution and genotype pattern of non-fermented gram-negative bacteria in hospital thus will benefit the understanding of molecular mechanism underlying drug resistance and clinical management of nosocomial infection. Clinical samples from our hospital were isolated and cultured for non-fermented gram-negative bacteria. Automatic equipment was used to identify and classifying those bacteria. The drug sensitivity was examined by paper diffusion method. WHONET software was used to analyze the drug resistance, which was then compared by chi-square test. In a total of 208 isolates, most two common bacteria were determined as *Acinetobacter baumannii* (AB, 42%) and *Pseudomonas aeruginosa* (PA, 38%). Those gram-negative bacteria were mainly isolated from sputum (80%), urine (8%) and body exudates (12%). Types of bacteria were mostly distributed in intensive care unit (ICU) and respiratory department. AB and PA isolated had relatively lower sensitivity to antibiotic drugs tested, while PCR and western blot analysis revealed the existence of CTX-M-P, the drug resistance gene, in 30 out of 61 strains of AB and PA. Our data highlights the severe condition of drug resistance in nosocomial non-fermented gram-negative bacteria, which showed significant correlation with drug resistance gene, CTX-M-P.

Keywords: Non-fermented gram-negative bacteria, drug resistance, genotype analysis, *Acinetobacter baumannii, Pseudomonas aeruginosa*

Introduction

Non-fermented gram-negative bacteria are commonly categorized as conditional pathogenic bacteria [1]. As aerobic or facultative anaerobic bacteria [2], they obtain energy and metabolites without fermentation [3]. Non-fermented gram-negative bacteria family mainly consists of *Acinetobacter baumannii* [4, 5], and *Pseudomonas aeruginosa* [6-8]. As one major cause for clinical infection [9], this type of bacteria, especially those with drug resistance, severely affects patients' health. Therefore the systemic investigation of the distribution and genotypes of those bacterial strains requires for both the understanding of molecular mechanism underlying bacterial drug resistance, and the clinical management of nosocomial bacterial infection.

The prevention and treatment of non-fermented gram-negative bacteria are relatively difficult [10], mainly due to drug resistance [11]. Multiple pathways have been developed in these bacteria [12], including depressed membrane permeability [13], altering of molecular target for anti-bacterial drugs [14], and modulating enzyme productivity [15] or activity [16]. Recent study showed the aggravation of occurrence and progression of multi-drug resistance of these bacteria by the application of new-generation anti-bacterial agent, anti-tumor or anti-metabolic syndrome [17]. The knowledge of distribution and genotype of nosocomial non-fermented gram-negative bacteria is thus of critical importance [18]. This study thus aimed to provide evidences for guideline of clinical application of anti-bacterial agents.

Materials and methods

Sample collection

From July 2014 to October 2015, 196 patients consisted of 118 males and 78 females with a median age of 45 years old from in the First

Table 1. Identification of non-fermented gram-negative bacteria

Group	AB	PA	Others	Total
Numbers	87	79	42	208
Percentage	42%	38%	20%	100%

Note: AB, *Acinetobacter baumannii*; PA, *Pseudomonas aeruginosa*. Others including Pseudomonas diminuta (6%), Xanthomonas maltophilia (8%) and Acinetobacter calcoaceticus (12%).

Table 2. Sources of non-fermented gram-negative bacteria

Sources	Sputum	Urine	Body exudate	Total
Numbers	166	17	25	208
Percentage	80%	8%	12%	100%

Table 3. Distribution of non-fermented gram-negative bacteria

Clinical department	ICR	Respiratory	Other departments	Total
Numbers	162	33	13	208
Percentage	78%	16%	6%	100%

Other departments including Department of Infection (2%) and Department of Clinical Laboratory (4%).

Affiliated Hospital of Xinxiang Medical University were enrolled in this study. These enrolled patients met one or two of the following criteria: the same non-fermented gram-negative bacterial isolated after screening twice and/or more than 10^6 CFU/mL non-fermented gram-negative bacterial bacteria after culturing. Using previously documented methods [19], non-fermented gram-negative bacterial samples were isolated and cultured. In brief, patients' sputum, urine and body exudates were diluted in sterilized water. Under room temperature, the sample was centrifuged at 1000 g for 10 min. The supernatants were inoculated onto BP basic culture medium at 37°C for 48-hour. The study has been approved by the ethnic committee of the First Affiliated Hospital of Xinxiang Medical University. Informed consents have been obtained from all patients before this study.

Identification and typing of bacteria

Automatic bacterial identifying equipment (MicroScan Walk Away 96 Plus) was used to identify isolated bacteria as previously reported [20]. In brief, bacteria were prepared for suspensions (10000 per mL) and were loaded onto the test plate provided. Incubated in a closed chamber, the proliferation and biochemical fingerprint were examined to determine the type and quantity of sampled bacteria.

Drug sensitivity assay

Using previously reported method [21], paper diffusion method was used to test the drug sensitivity of bacteria according to CLSI2015 standard. The data was analyzed by WHONET 6.8 software. In brief, bacterial culture was inoculated onto solid culture medium using sterilized inoculation ring. Different drug sensitivity pieces were placed on the surface of culture medium for recording the growth of bacteria at each region. The whole plate was then cultured at 37°C, for analysis after 24 hours.

PCR

PCR was used to be genotyped different bacterial strains as previously documented [22]. In brief, bacterial culture was collected and centrifuged at 1000 g for 10 min to collect precipitation, which was re-suspended in 50 μL sterilized water. After heating at 100°C for 5 min. the supernatant was used as the template for detecting the presence of CTX-M-9 gene (Forward primer: 5'-AGAGT TTGAT CATGG CTCAG AGAGT TTGAT-3'; Reverse primer: 5'-TTGGA TCATG GGCTC AAAGA GTAGT TTGAT-3'). β-actin was used as the internal reference (Forward primer: 5'-AGAGT TTGAT CATGG CTCAG AGAGT TTGAT-3'; Reverse primer: 5'-GCTCA AAGAG TTTGG ATCAT GGAGT TTGAT-3'). BanScan 680 system (Bio-Rad, USA) was used to analyze the intensity of each PCR bands, which were performed in triplicates. The relative expression of CTX-M-9 was determined by probing against β-actin.

Western blotting

Protein level of drug resistance factor CTX-M-9 was semi-quantified using Western blotting method from clinical samples as previously established [23]. In brief, bacterial culture medium was firstly centrifuged at 1000 g for 10 min to obtain bacterial precipitation, which was then re-suspended with cell lysis buffer. After incubated on ice for 30 min, protein solutions were separated in SDS-PAGE and were transferred to PVDF membrane. Using specific antibodies against CTX-M-9 or β-actin, the membrane was developed and exposed. Band-

Table 4. Drug sensitivity of non-fermented gram-negative bacteria

Drugs	AB (87) (numbers, %)		PA (79) (numbers, %)	
	Sensitivity	Resistance	Sensitivity	Resistance
Imipenem	39, 45%	48, 55%	44, 56%	35, 44%
Cefoperazone	45, 52%	42, 48%	51, 65%	28, 35%
Amikacin	43, 49%	44, 51%	48, 61%	31, 39%
Piperacillin	40, 46%	47, 54%	40, 51%	39, 49%
Ciprofloxacin	35, 40%	52, 60%	55, 70%	24, 30%

Note: AB, *Acinetobacter baumannii*; PA, *Pseudomonas aeruginosa*.

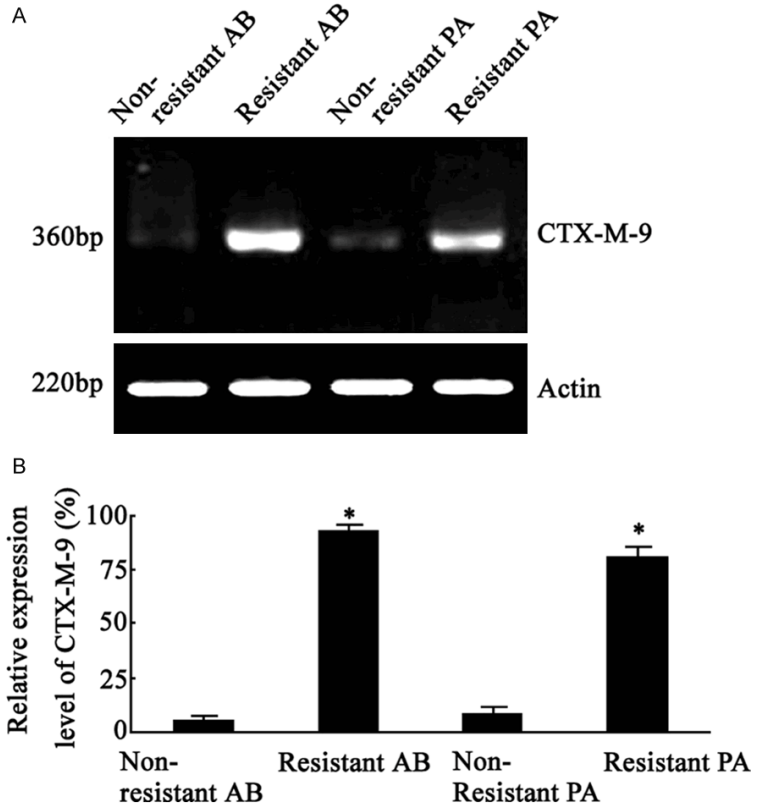

Figure 1. The detection of drug-resistance gene in bacteria. A. PCR detected of CTX-M-9 gene across AB and PA with/without drug resistance; B. Quantitative results of relative expression level of CTX-M-9 gene. *, P<0.05 compared to non-resistant genes. AB. *Acinetobacter baumannii*; PA. *Pseudomonas aeruginosa*.

Scan 680 system was used to analyze the intensity of each protein bands, which were performed in triplicates. The relative expression of CTX-M-9 was determined by probing against β-actin.

Statistical analysis

SPSS 13.0 software was used to analyze all collected data, which were presented as mean ± standard deviation (SD). The incidence of drug resistance was analyzed by chi-square test. A statistical analysis was defined when P<0.05.

Results

Identification of strains of non-fermented gram-negative bacteria

As shown in **Table 1**, in a total of 208 bacterial strains that were successfully isolated and identified, there were 42% of *Acinetobacter baumannii* (AB) and 38% of *Pseudomonas aeruginosa* (PA), while other types of non-fermented gram-negative bacteria occupied 20%.

Sources of bacteria

As shown in **Table 2**, those non-fermented gram-negative bacteria mainly came from sputum (80%), urine (8%) and body exudates (20%).

Bacterial distribution across clinical departments

As shown in **Table 3**, most of non-fermented gram-negative bacteria come from intensive care unit (ICU, 78%) and department of respiratory (16%).

Drug sensitivity

As shown in **Table 4**, AB had less than 48% sensitivity to all tested anti-bacterial drugs ranging from 40% to 52%, while PA presented drug sensitivity ranging from 51% to 70%.

Drug resistant gene

We further analyzed the genotype of AB and PA with different drug sensitivity using PCR approach targeting CTX-M-9 gene. Results showed that 30 out of 61 strains of those bacteria positively had CTX-M-9 genes (**Figure 1**). Further analysis also demonstrated the drug

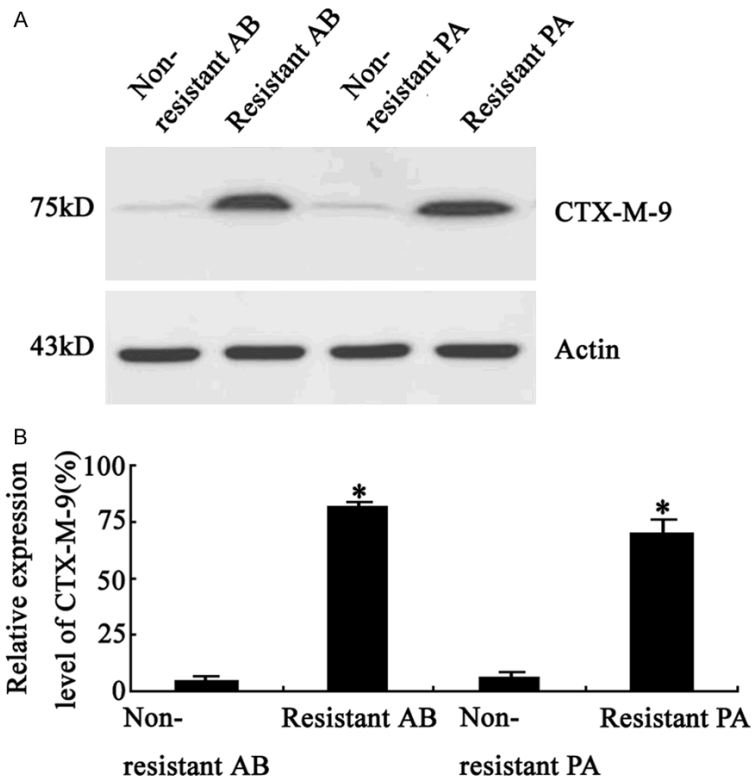

Figure 2. The expression of CTX-M-9 protein in bacteria. A. Western blotting bands showed the expression of CTX-M-9 gene across AB and PA with/without drug resistance; B. Quantitative results of relative protein level of CTX-M-9 gene. *, P<0.05 compared to non-resistant genes. AB. *Acinetobacter baumannii*; PA. *Pseudomonas aeruginosa*.

resistance of these bacteria had significant correlation with elevated expression level of CTX-M-9 gene (P<0.05). As consistent with those from PCR study, Western blotting revealed significantly enhanced protein expression level of CTX-M-9 in PA and AB with drug resistance (P<0.05, **Figure 2**).

Discussion

Multiple studies have confirmed the major role of non-fermented gram-negative bacteria in nosocomial infection in China [24]. With the high potency of drug resistance [25], those bacteria severely affect people's healthy. This study therefore investigated the distribution and genotype pattern of non-fermented gram-negative bacteria in hospital, in order to unravel the molecular mechanism underlying drug resistance of current nosocomial infection.

Our study showed the major sources of non-fermented gram-negative bacteria from sputum (80%), urine (8%) and body exudates (12%). Most common distribution of bacteria were located in intensive care unit (ICU, 78%) and respiratory department (16%), suggesting importance of counter measures in those departments for anti-bacterial infections. These results were consistent with previous surveillance of nosocomial bacterial drug resistance in China [25], though deviation in previous study mainly indicated more PA existed than AB did. This may be due to the differential phenotypes of drug resistance under unique environments.

80% of all those bacteria were isolated from patients' sputum, significantly higher than the 55% of averaged level. Other sources of bacteria included urine (8%) and body exudates (12%), as was consistent with previous reports [26]. Owing to the open nature of human respiratory tract, the opportunistic pathogen including surface yeast may invade the respiratory tract especially under the circumstance of immune suppression [27], thus causing infection [28].

We further explored the molecular mechanism underlying the drug resistance of those non-fermented gram-negative bacteria. The up-regulation of CTX-M-9, a critical bacterial drug resistant gene, was observed in those bacteria with drug-resistance. Those results indicated the potential relationship between the drug resistance and CTX-M-9 level.

Certain limitation still existed in this study. Firstly, the size of the samples relative affects the reliability of the conclusion. Secondly, although sample collection covered all clinical departments in our hospital, the uneven distribution of bacterial strains may be a factor to impact test results. Thirdly, the proposed model for drug resistance requires further substantiation and validation using gene over-expression or RNA interference.

Taken together, this study revealed the severity of drug resistance in nosocomial non-fermented gram-negative bacteria, probably due to the expression of drug resistant gene CTX-M-9, which should draw attention from clinicians.

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Disclosure of conflict of interest

None.

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