Original Article

Infection Route Analysis of Ventilator Associated Pneumonia

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Objective A prospective study was conducted in a tertiary care center to identify the risk factors of ventilator associated pneumonia (VAP) through phenotypic and molecular biological methods.

Methods The patients who were mechanically ventilated in the respiratory intensive care unit (RICU) and the neurological internal intensive care unit (NICU) were enrolled in our study, and samples were collected from the lower respiratory tract, oropharynx and stomach. Other samples, including the environmental air, swabs of nurses' hands, subglottic secretion and ventilator circuit, were also collected. Microorganisms in the collected samples were recovered and identified at species level by biochemical detection. Genetic relationship of dominant species was further characterized by pulsed field gel electrophoresis (PFGE).

Results Out of 48 enrolled patients, 22 cases developed VAP and bacterial cultures were recovered from the lower respiratory tract samples of 14 cases. The average hospitalization time with VAP was significantly longer than that of patients without VAP ($P < 0.05$). Among the recovered bacteria cultures, multidrug-resistant *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were dominant. It was more likely that subglottic secretion and gastric juice samples contained the same isolates as recovered in the lower respiratory tract by PFGE analysis.

Conclusions Mechanical ventilation in RICU and NICU was a high risk factor for VAP development. Special emphasis of VAP prophylaxis should be paid on subglottic secretion and gastric juice reflux.

Key words: Ventilator; Pneumonia; Pulsed field gel electrophoresis; Molecular epidemiology

Ventilator associated pneumonia (VAP) is a common pulmonary infection among patients using mechanical ventilation with trachea intubation,¹ with the incidence ranging from 9% to 27%, and the fatality fluctuates as 30% -70%.¹ The characterization of the microorganisms and their sources have been recognized as the prerequisite for the proper prophylaxis and treatment of VAP.² Currently, most studies on VAP pathogen investigations were carried out with the methods of species level with limited discriminatory power for intraspecific isolates.^{3,4} Several bacterial species, including *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, have been demonstrated as the dominant pathogens among VAP patients.^{5,6} Since these bacteria are common in hospital environment, the characterization and differentiation of isolates belonging to the same species mechanical ventilation are extremely important for nosocomial VAP prophylaxis and treatment. At present, different methods with high intraspecific discriminatory power are available, including pulsed

field gel electrophoresis (PFGE), multilocus sequence typing (MLST), multiple-locus variable-number tandem repeat analysis (MLVA), etc.⁷ After characterization of epidemiological related clinical isolates through these methods, the possible sources and infection routes of VAP might be recognized. And these data could help the physicians to establish or adopt helpful VAP prevention procedures.

In this study, the lower respiratory tract and other related specimens from the patients using mechanical ventilation in the intensive care unit (ICU) of our hospital were collected and suspect pathogens were cultured. The possible infection routes and vehicles were identified through genetic characterization of dominant species by DNA fingerprinting profiles resulting from digestion of genomic DNA by restriction enzymes *Xba* I.

PATIENTS AND METHODS

Patient recruitment and sampling

From February 2006 to June 2007, patients who were mechanically ventilated for > 48 hours in the respiratory intensive care unit (RICU) and the neurological internal intensive care unit (NICU)

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of Beijing Tiantan Hospital (Beijing, China) were considered for enrollment. The following patients were excluded: patients with significant preexisting chronic pulmonary diseases before admission (such as chronic bronchitis, bronchiectasis, etc.); patients who were extubated accidentally; patients died in three days after the intubation; patients exhibited alimentary tract bleeding, coagulation dysfunction, tracheoesophageal fistula or serious arrhythmia. Hospital ethical review board-approved consent forms were signed by the patient's relatives.

The development of the following clinical symptoms would be considered for lung infection: fever ($T \geq 38^\circ\text{C}$), leukocytosis, dry or wet rale, yellow or yellowish-white and viscous or purulent secretion in the airway. VAP was defined clinically by the infiltration focus of double or multiple lung lobes shown by chest radiograph or computed tomography scan.

For the VAP patients, specimens were collected from the lower respiratory tract, oropharynx and stomach. Specimens of the lower respiratory tract were taken through the protected specimen brush (PSB) directed by the fiberoptic bronchoscopy (FOB). Other related samples, including the environmental air, swabs of nurses' hands, subglottic secretion and ventilator circuit were also collected. Specimens were sent immediately to the clinical microbiology laboratory in the hospital for bacteria culture.

Isolation, identification and antimicrobial susceptibility detection of bacterial isolates

After PSB samples were collected, the brushes were immediately immersed into 1 ml saline physiological saline and shaken vigorously for 60 seconds. Diluted samples were inoculated onto blood agar, chocolate agar, sabouraud agar and MacConkey agar, respectively and incubated at 35°C for 24 - 48 hours. If fewer than 10^3 cfu/ml microbes were recovered, then sample contamination was considered. Specimens from other sources were directly inoculated onto those four agar plates. PSB samples with isolates showing the same phenotype on agar plates were kept and further tested with Phoenix-100 ID/AST System (BD Diagnostics, China). *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were taken as control microorganisms.

PFGE genotyping

Pseudomonas aeruginosa and *Stenotrophomonas maltophilia* were further characterized by PFGE to determine their genetic relationship.⁸ PFGE analysis was performed according to reference method with minor modifications. Briefly, bacteria were grown on blood agar plates and suspended in cell suspension buffer (100 mmol/L Tris HCl, 100 mmol/L EDTA; pH

8.0) and adjusted to an optical density of 0.52 to 0.56. The cell suspension (200 μl) was mixed with 10 μl of proteinase K (20 mg/ml) and an equal volume of melted 1% agarose. After solidification, the agarose plugs were treated with proteinase K for 2 hours in a water bath at 54°C with agitation at 180 rpm/min. The plugs were washed and treated with 50 U *Xba* I (Roche, Indianapolis, IN) for two hours in a water bath at 37°C . The resulting DNA fragments were resolved by electrophoresis in a 1% agarose gel using a Chef Mapper (Bio-Rad, Beijing, China) with pulse times of 2.16 - 63.8 s. The gel was stained with ethidium bromide, and DNA bands were visualized with a UV transilluminator. The interpretation of the PFGE patterns was aided by BioNumerics program (Applied-Maths, Kortrijk, Belgium).

Data Analysis and Statistical Methods

The data were analyzed by Chi-square test and Matel-Haenszel test using Epi Info 6 software (www.cdc.gov/epiinfo/).

RESULTS

Clinical data of patients and samples

In total, 48 patients were enrolled, including 26 male and 22 female cases. Out of these 48 patients, 35 cases (72.9%) had received antimicrobial treatment before sampling, including cefoperazone/sulbactam, levofloxacin and clindamycin/clindamycin. The average time of antimicrobial treatment was 4.00 ± 0.36 days. All the patients had detained gastric tubes. Out of the 48 enrolled cases, 22 cases (45.8%) with mechanical ventilation suffered from VAP, with the ages ranging from 29 to 87 years old. After administered by appropriate antimicrobials according to the antimicrobial susceptibility test results, 20 patients recovered or improved and two patients died. The VAP patient's ventilation time, length of time in ICU, and the length of hospitalization time were significantly longer than those of patients without VAP ($P < 0.01$) (Table 1).

Isolation and identification of bacterial strains

Out of the 22 VAP patients, microorganisms were isolated from the lower respiratory tract samples of 14 (63.6%) patients, including *Pseudomonas aeruginosa* (n = 6), *Stenotrophomonas maltophilia* (n = 4), *Acinetobacter baumannii* (n = 1), *Pasteurella multocida* (n = 1), *Sphingomonas paucimobilis* (n = 1) and *Chryseobacterium meningosepticum* (n = 1). Microorganisms that showed the same phenotype on agar plates as those from the lower respiratory tract samples were also isolated from the oropharynx

Table 1. Characteristics of the patients in ICU with and without ventilator-associated pneumonia

Characteristics	Patients with VAP (n = 22)	Patients without VAP ^a (n = 26)	P values ^b
Age (years)	69.05 ± 12.53	72.31 ± 5.39	0.235
Male (%)	13 (59%)	13 (50%)	0.573
Artificial airway (NT ^a /PT ^a /tracheotomy)	15/5/2	18/7/1	- ^c
Prior antimicrobials treatment	2.88 ± 1.45	2.74 ± 1.37	0.774
Ventilation time (days)	15.09 ± 2.88	10.50 ± 2.23	<0.001
Time of staying in ICU (days)	17.09 ± 2.38	14.25 ± 2.26	<0.001
Time of staying in hospital (days)	22.23 ± 2.43	18.42 ± 1.39	<0.001

Notes: ^a VAP denotes ventilator-associated pneumonia; NT nasal trachea; PT peroral trachea; ^b The difference of sex was compared by χ^2 test and other factors were compared by *t* test. *P* < 0.01 means the difference is extremely significant and *P* > 0.05 means no significant difference; ^c The statistic difference of this factor was not calculated due to limited cases.

Figure 1. Dendrogram of patterns generated by pulsed-field gel electrophoresis (PFGE) of VAP isolates.

secretion, subglottic secretion, gastric juice or ventilator circuit samples (including the seeper bottle) of the same patient. After biochemical examination, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* isolates were identified from six, three and one patient related specimens that were matched to the lower respiratory culture, respectively. No similar microorganism was found from the environmental air or swabs of nurses' hand samples. Antimicrobial susceptibility data and other related information were shown in Table 2 and Figure 1.

PFGE genotyping

Pseudomonas aeruginosa (n = 19) and *Stenotrophomonas maltophilia* (n = 9) isolates isolated from the related specimens of six and three patients were furtherly analyzed for genetic relationship by PFGE. Eleven PFGE patterns were identified among 19 *Pseudomonas aeruginosa* isolates (eight patterns) and nine *Stenotrophomonas maltophilia* isolates (three patterns) isolated from nine patients' related samples. Compared to the individual PFGE pattern of the isolates from the lower respiratory tract samples, isolates of

identical PFGE patterns were identified from subglottic secretion (n = 6), gastric juice (n = 4), ventilator circuit (n = 2) and oropharynx secretion specimens (n = 2) (Figure 1). Isolates of identical PFGE patterns were also identified from two pairs of patient's lower respiratory tract samples (patient 1 and 2, patient 12 and 17) (Figure 1).

DISCUSSION

VAP had a high morbidity and mortality in patients undergoing mechanical ventilation.⁹ In this study, 22 out of the 48 patients who were mechanically ventilated for > 48 hours suffered from VAP and two patients died. Bacteria were cultured from 14 patients, among which multidrug resistant isolates were dominant. After PFGE analysis, clonal isolates of the culture from the lower respiratory tract were found from oropharynx secretion, subglottic secretion, gastric juice and ventilator circuit samples. These data demonstrated that the sources were important pathogen reservoirs of VAP and should be paid more attention during proper VAP prevention procedure development.

Table 2. Isolates of different sources related to VAP patients that were not analyzed by PFGE

Cases number	Numbers of strains	Positions of specimens	Results of strains identification	Resistant profile
6	6-1	Lower respiratory tract	<i>Acinetobacter baumannii</i>	AMK-CAZ-CRO-CTX-FEP-GAT-GEN-LEV-PIP-PTZ -TM-T/S
	6-2	Subglottic secretion	<i>Acinetobacter baumannii</i>	AMK-CAZ-GAT-GEN-LEV-PIP-TM-T/S
	6-3	Gastric juice	<i>Acinetobacter baumannii</i>	PIP
13	13-1	Lower respiratory tract	<i>Chryseobacterium meningosepticum</i>	AMK-ATM-CAZ-CTX-GEN-IMP-PIP-TM
14	14-3	Gastric juice	<i>Chryseobacterium meningosepticum</i>	AMK-ATM -CAZ-CTX-GEN-IMP-PIP-TM
16	16-1	Lower respiratory tract	<i>Stenotrophomona maltophilia</i>	CAZ-LEV
	16-3	Gastric juice	<i>Pseudomonas aeruginosa</i>	CRO-CTX
18	18-1	Lower respiratory tract	<i>Sphingomonas paucimobilis</i>	AMK-ATM-CAZ-GEN-CRO-CTX-FEP-IMP-PIP-TM
	18-2	Subglottic secretion	<i>Stenotrophomona maltophilia</i>	T/S
	18-5	Ventilator circuit	<i>Pseudomonas aeruginosa</i>	ATM-CRO-CTX-FEP-GEN-IMP-LEV-PIP-PTZ-TM
19	19-1	Lower respiratory tract	<i>Pseudomonas multocida</i>	-
	19-2	Subglottic secretion	<i>Acinetobacter baumannii</i>	AMK-CAZ-CIP-CRO-FEP-GAT-GEN-IMP-LEV-PIP-PTZ-TM
	19-3	Gastric juice	<i>Pasteurella multocida</i>	-

Notes: *As there was no executive standard of antibacterial susceptibility test for *Sphingomonas paucimobilis* and *Chryseobacterium meningosepticum*, the criteria of *Pseudomonas aeruginosa* were applied.

Abbreviation: AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; GAT, gatifloxacin; GEN, gentamicin; IMP, imipenem; LEV, levofloxacin; PIP, piperacillin; PTZ, piperacillin-tazobactam; TM, tobramycin; T/S, trimethoprim/sulfamethoxazole.

Previous studies showed that the VAP patient's condition was more serious and with a longer duration of mechanical ventilation.^{10,11} In this study, the VAP patients' ventilation time, length of time in ICU, and the length of hospitalization time were significantly longer than those of patients without VAP ($P < 0.01$). Importantly, our data showed the infection could be cured or improved if proper antimicrobials were applied following the antimicrobial susceptibility test results, which furtherly underlined the necessity to carry out bacteria isolation and antimicrobial susceptibility measurement. Especially, most isolates from VAP patients were multidrug resistant and proper selection of antimicrobials based on experience was difficult to be administered.

Our data showed that gram negative bacteria were the dominant isolates which were widely distributed in the hospital environment.⁶ Clonal isolates were isolated from different patients and related specimens after PFGE analysis, which indicated the nosocomial infections happened in the ICU. After PFGE analysis, we identified several reservoirs of VAP pathogens. These data demonstrated pathogen characterization by high discrimination power method was necessary to identify nosocomial infection routes and the data could help to develop proper prevention procedures.

Among molecular epidemiological methods, PFGE genotyping has been widely applied in the homologous analysis of bacterial infections due to its strong discriminatory power and reproducibility.¹² In this

study, the PFGE profiles of microorganisms from lower respiratory tract of VAP patients were in consistent with the isolates from subglottic secretion, gastric juice, ventilator circuit or oropharynx secretion specimens. These data demonstrated the possible reservoirs of bacteria in lower respiratory tract. For oropharynx secretion samples, the possible contamination routes might be that a lot of mechanical ventilation secretion accumulated on the catheter gasbag below the glottis forming a mucus lake, which made the secretion more or less flow into the lower respiratory tract, even though the catheter gasbag could prevent some oropharynx secretion inflowing.² To prevent VAP from oropharynx secretion, the following instructions should be adopted. Firstly, the pressure of the gasbag should be checked every 4 hours and the pressure should be kept between 25 - 30 cm H₂O.^{13,14} Secondly, the trachea cannula with subglottic secretion suction tube should be applied. The analysis on recent five large-scale investigations by Dezfulian et al showed that the trachea cannula technology could reduce 50% incidence of VAP.¹⁵ Thirdly, negative pressure with 20 - 30 cm H₂O should be used to continuously suck the subglottic secretion.¹⁴

Especially, the PFGE data showed that the gastric juice were another important pathogen reservoir of VAP. Since H₂-receptor antagonist and proton pump inhibitors were excessively used among patients in ICU to prevent stress ulcers, this might increase the stomach pH and delay the gastric evacuation. As a result, the pathogen could have a chance to multiply in

stomach and the reflux of gastric juice could become an independent risk factor for VAP.¹⁶ In our study, proton pump inhibitors were administered to three VAP patients and H₂-receptor antagonists were given for stress ulcer among 11 VAP cases, which may have contributed to the VAP occurrence.

In conclusion, our data demonstrated the necessity of bacteria culture, antimicrobial susceptibility analysis and isolates genotyping for proper VAP treatment and prevention. Possible infection routes and reservoirs could be identified and appropriate prevent procedures should be developed based on these data.

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