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Values of combined detection of bronchoalveolar lavage fluid endotoxin and serum procalcitonin levels for the rapid diagnosis of early methicillin-resistant *Staphylococcus aureus*-caused ventilator-associated pneumonia.

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Abstract

This study investigated the usefulness of the combined detection of Bronchoalveolar Lavage Fluid (BALF) endotoxin and serum Procalcitonin (PCT) levels for the rapid diagnosis of early Methicillin-Resistant *Staphylococcus aureus* (MRSA)-caused Ventilator-Associated Pneumonia (VAP) in the Intensive Care Unit (ICU). BALF and venous blood samples were obtained from 69 patients, admitted to the ICU, who required invasive mechanical ventilation. PCT levels were assessed within 72 to 96 h of ICU admission regardless of the VAP status. Based on the BALF-endotoxin and serum PCT levels, the patients were divided into four groups: group A, BALF endotoxin<6 endotoxin units per mL (EU/ml) and PCT>0.5 ng/ml; group B, BALF endotoxin<6 EU/ml and PCT<0.5 ng/ml; group C, BALF endotoxin>6 EU/ml and PCT>0.5 ng/ml; group D, BALF endotoxin>6 EU/ml and PCT<0.5 ng/ml. The proportion of MRSA infection in group A was significantly higher than that in the other groups (P<0.05); when the BALF-endotoxin was<6 EU/ml and the serum PCT was>0.5 ng/ml, the sensitivity of early MRSA-VAP was 63.64%, with specificity, positive predictive value, and negative predictive value of 91.89%, 70.00%, and 89.47%, respectively. Therefore, the evaluation of BALF-endotoxin and serum PCT levels may be a fast, economical, and effective diagnostic method for the early identification of MRSA-VAP in the ICU.

Keywords: Endotoxin, Bronchoalveolar lavage fluid, Ventilator-associated pneumonia, Procalcitonin, Methicillin-resistant *Staphylococcus aureus.*

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Introduction

Ventilator-Associated Pneumonia (VAP) is the most common hospital-acquired infection in the Intensive Care Unit (ICU), with mortality as high as 24-50% [1]; therefore, timely and accurate diagnosis and treatment are essential to improve the prognosis of patients with VAP. Among hospital-acquired infections, Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a common pathogen of hospital-acquired infections, including VAP; its infection rate has shown an increasing trend, reaching 20-40% [2,3]. ICUs have a high incidence of MRSA, which is also an important pathogen of VAP in the ICU. Compared with other pathogens, MRSA causes more adverse consequences, poorer prognosis, significantly prolonged hospital stay, and higher mortality [4,5]. Inadequate anti-infection protocols against VAP may lead to poor prognosis, but overtreatment may also lead to bacterial resistance and increase the economic burden to patients; therefore, providing timely etiological information and guiding the rational use of antibiotics have become urgent problems to be solved by medical workers. Thus, the early and rapid diagnosis of VAP due to MRSA is of the utmost importance.

Current VAP diagnostic methods mainly depend on clinical manifestations, imaging changes, and etiological diagnosis; however, clinical manifestations and imaging changes lack specificity and etiological diagnosis is time-dependent, requiring 48-72 h for the quantitative culture of airway secretions, making them less conducive to the early diagnosis of VAP. Although the inspection of Gram-stained smears of airway secretions is a quick inspection method, a meta-analysis found that the VAP-positive and negative predictive values of this method were 40% and 90%, respectively, suggesting the limited diagnostic value of this method for the detection of VAP, and that negative findings might be more meaningful for the exclusion of VAP [6].

The standard methods of cell counting, sorting, and calculation of intracellular bacteria percentages in Bronchoalveolar Lavage Fluid (BALF) for the diagnosis of pneumonia remain non-standardized; therefore, the findings of these methods may be affected by a variety of factors, which result in poor accuracy [7]. Although the sensitivity and specificity of microscopic

analysis for the detection of Polymorphonuclear-containing Intracellular Organisms (PIC) of the pathogens in BALF are high for the diagnosis of VAP, this method offers little guidance for targeted early antibiotic treatment [8]. Studies have shown that the detection of endotoxin levels in BALF may be an effective method, offering better sensitivity and specificity for the rapid diagnosis of Gram-negative bacterial VAP, with significantly higher levels in patients with VAP with Gram-negative bacteria than those in patients with VAP with Gram-positive cocci [9,10]. Endotoxins are an important component of the outer cell wall membrane of Gram-negative bacteria; however, Gram-positive cocci such as MRSA do not contain endotoxins. Therefore, low endotoxin levels in the BALF of patients with a pulmonary infection could indicate a high possibility of Gram-positive infections. However, the detection of endotoxins alone is not sufficient for the diagnosis of pulmonary infections. Therefore, this study assessed the levels of both Procalcitonin (PCT), an important indicator of bacterial infections and infection severity, and endotoxin in order to explore the usefulness of a single fast, simple, economical, and effective method for the early diagnosis of MRSA-caused VAP.

Materials and Methods

Subjects

The patients were admitted to the ICU and required invasive mechanical ventilation between August 2014 to February 2016. Bronchoalveolar Lavage (BAL) was performed and venous blood was collected to assess PCT levels within 72 to 96 hours of ICU admission no matter their VAP status. The diagnostic criteria of VAP were based on the clinical diagnostic criteria issued by the American Thoracic Society and the Infectious Diseases Society of America [11]. Patients with illness combined with pulmonary infection prior to the ICU admission, patients who denied treatment, who died during the experimental process, or from whom we were unable to obtain informed consent were excluded from the study.

A total of 69 patients were enrolled in this study, including 46 men (66.67%) and 23 women (33.33%) with a mean age of 49.91 ± 17.86 years. The enrolled patients included 26 cases of severe traumatic brain injury, 15 cases of cerebral hemorrhage, four cases of massive cerebral infarction, 19 cases of multiple injuries, two cases of postoperative cardiopulmonary resuscitation, one case of myasthenia gravis, and two cases of Guillain-Barre syndrome. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Fujian University. Written informed consent was obtained from all participants or their legally responsible relative.

BAL

BAL was performed by senior doctors in the ICU. Before the procedure, each patient stopped eating and nasal feeding for 2 h. Patients' heart rate, blood pressure, and transcutaneous oxygen saturation (SpO_2) were measured simultaneously and the ventilator was adjusted to the ventilation-controlling mode with Positive Expiratory End Pressure (PEEP) of 0 cmH_2O and inspired oxygen concentration (FiO_2) of 100%; each patient was administrated propofol or midazolam for sedation before performing BAL. One PENTAX FB-15BS bronchoscope was endotracheally intubated, connected to the three-way pipe, and then inserted into the main trachea *via* the air-sealing end of the flap. The intubation in patients with VAP was guided to the infected lung segment based on chest radiography or Computed Tomography (CT) scanning; 37°C saline was then infused *via* the biopsy hole for BAL. Two washes of 50 ml were performed and the second BALF was collected [12] and stored in a single pyrogen-free container for storage at -70°C. The right middle or lower lobes were lavaged in patients with diffuse lesion(s), non-exudative lesions in imaging, or those without VAP. If the SpO_2 dropped to 85% during BAL, the procedure was stopped immediately, restarting only after withdrawal of the bronchoscope and restoration of the SpO_2 to 90% or higher. The BALF specimen then underwent bacterial culture, sensitivity assay, and Gram staining.

Grouping

According to their BALF endotoxin and serum PCT levels, the patients were divided into four groups: group A, BALF endotoxin<6 endotoxin units per ml (EU/ml) and PCT>0.5 ng/ml; group B, BALF endotoxin<6 EU/ml and PCT<0.5 ng/ml; group C, BALF endotoxin>6 EU/ml and PCT>0.5 ng/ml; and group D, BALF endotoxin>6 EU/ml and PCT<0.5 ng/ml.

Detection of BALF endotoxin

The pre-melt BALF was centrifuged at 1,800 rpm for 10 min. Tachypleus Amebocyte Lysate (TAL) chromogenic substrate method and standard curves were used to measure the concentration of BALF endotoxin according to its absorbance (TAL rapid microbial detection system, Model: ELX808IULALXH, BIO-Tek Co., USA, provided by the Xiamen TAL factory).

We used a commercial TAL assay kit to quantitate the concentration of endotoxin within the BALF. All BAL specimens were collected in sterile non-pyrogenic containers and freshly prepared. Thirty microliters of BALF was diluted in 270 μL of pyrogen-free water and incubated at 37°C for 5 min, after which it was placed in an ice bath. Additional dilutions were made using pyrogen-free water to achieve three final concentrations of 1:50, 1:100, and 1:200. In the presence of endotoxin, factors in the Limulus Amebocyte Lysate (LAL) are activated in a proteolytic cascade resulting in the cleavage of a colorless artificial peptide substrate present in the pyrochrome LAL. Proteolytic cleavage of this substrate liberates p-nitroaniline (pNA), resulting in a yellow color that absorbs light at 405 nm. A standard curve consisting of measured optical density plotted against known standard endotoxin concentrations was used to determine the endotoxin concentrations in the BAL specimens. The limit of detection of the assay was 0.001 EU/ml.

Detection of PCT (PCT detector model)

Two milliliters of venous blood were sampled and centrifuged within 20 min; the serum was then placed at -70°C for the assay. A fluorescence immunological sandwich assay was used with a detection accuracy of 0.01 ng/ml. PCT levels>0.5 ng/mL was considered to be positive [13].

Determination of MRSA

All the specimens were isolated and cultured according to conventional methods and the isolated strains identified using a Vitek GPI card and *S. aureus* Slidex Stap-Plus kit (VITEK-32 automatic microbial identifier, bioMerieux, France). The susceptibility assay used the disk diffusion method (K-B method) and the results were categorized as Resistant (R), Intermediate (I), or Sensitive (S) using the thresholds established by the National Committee for Clinical Laboratory Standards (NCCLS) in 2010. The MRSA surveillance used the 30 Lg/piece Cefoxitin-coated test paper was provided by Beijing Tiantan Biological Products Co. Ltd. The results were interpreted in accordance with the 2010 CLSI/NCCLS standards [14,15]. The strain used for quality control and susceptibility was ATCC 25923 *S. aureus*, while the reference MRSA strain was ATCC 43330.

Statistical analysis

SPSS version 18.0 was used for the statistical analysis. The measurement data were expressed as $\bar{x} \pm s$, and the count data were compared using chi-square tests. Comparisons of multiple measurement data were made by Analysis of Variance (ANOVA), with $P<0.05$ considered statistically significant. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated based on the formulas as follows: Sensitivity=true positive/(true positive +false negative) × 100%, specificity=true negative/(true negative+false positive) × 100%, positive predictive value=true positive/(true positive+false positive) × 100%, negative predictive value=true negative/(true negative+false negative) × 100%.

Results

General patient information

A total of 56 patients with early VAP were finally enrolled, including 40 patients with Gram-negative bacteria and 16 patients with Gram-positive cocci; among the patients, 10 were infected with MRSA, two cases with methicillin-resistant *Staphylococcus epidermidi*s (MRSE), and four patients with *Streptococcus pneumoniae*.

Classification of diseases and pathogens

The proportions of diabetes, severe brain injury, and multiple injuries in group A were significantly higher than those in the other groups ($P<0.05$), while the acute physiology and chronic health evaluation-II (APACHE II) score in group B was significantly lower than those of other groups ($P<0.05$). The proportions of cases with Gram-positive cocci and MRSA in group A were significantly higher than those in the other groups ($P<0.05$), but the proportions of MRSE and *Streptococcus pneumoniae* did not differ significantly between the groups ($P>0.05$); the proportion of cases with Gram-negative bacilli in group C was significantly higher than that in the other groups ($P<0.05$); the proportion of infection-free patients in group B was significantly higher than that in the other groups ($P<0.05$).

Detection sensitivity and specificity

For BALF endotoxin level<6 EU/ml and the plasma PCT level>0.5 ng/ml, the diagnostic sensitivity, specificity, positive predictive value, and negative predictive value against early VAP with Gram-positive cocci were 69.23%, 91.89%, 75.00%, and 84.47%, respectively; when MRSE was classified as MRSA, the values against early MRSA-VAP were 63.64%, 91.89%, 70.00%, and 89.47%, respectively (Tables 1 and 2).

Table 1. *General conditions and main disease classifications of each group.*

Classification	A (n=12)	B (n=10)	C (n=40)	D (n=7)	χ^2 (F)	P
Age (years)	47.67 ± 15.53	48.50 ± 14.75	48.75 ± 16.74	53.71 ± 20.02	0.22	0.883
Gender (male)	8	7	27	4	0.348	0.951
APACHE II score	27.67 ± 8.37	19.60 ± 6.43	25.28 ± 6.35	28.14 ± 8.51	3.075	0.034
Smoking	7	5	23	4	0.206	0.977
COPD	3	3	8	2	0.625	0.891
Immunosuppressive drugs before admission	3	2	7	1	0.449	0.93
Acute renal insufficiency	4	1	3	1	5.53	0.317
Heart disease	2	4	12	2	0.961	0.811
Diabetes	8	2	11	1	8.687	0.034

Severe brain injury	9	4	10	3	9.959	0.019
Stroke	3	3	11	2	0.073	0.995
Multiple injuries	8	2	8	1	11.248	0.01
After cardiopulmonary resuscitation	0	0	1	1	3.904	0.272
Myasthenia gravis	0	0	1	0	0.736	0.805
Guillain-Barre	0	0	1	0	0.736	0.805

Table 2. *Comparison of different etiologies among the groups.*

Etiological classification	**A (n=12)**	**B (n=10)**	**C (n=40)**	**D (n=7)**	**χ^2**	**t**
Gram-negative bacilli	3	0	34	3	31.797	<0.001
Gram-positive cocci	9	2	4	1	22.36	<0.001
MRSA	6	0	4	0	15.741	0.001
MRSE	1	1	0	0	4.454	0.216
Streptococcus pneumoniae	2	1	0	1	6.305	0.098
Non- pathogens	0	8	2	3	34.899	<0.001

Note: Group A: BALF-endotoxin<6 EU/ml and PCT>0.5 ng/ml, group B: BALF-endotoxin<6 EU/ml and PCT<0.5 ng/ml, group C: BALF-endotoxin>6 EU/mL and PCT>0.5 ng/ml, and group D: BALF-endotoxin>6 EU/ml and PCT<0.5 ng/ml.

Discussion

Since its first clinical isolation in 1961, the drug resistance of MRSA has continued to increase along with its annual incidence; this strain has become one of the main pathogens of nosocomial infections, and the ICU has a high incidence of MRSA. After the 1980s, with the growing categories of antibiotics and their wide clinical use, the incidence of MRSA-induced serious infections has been rapidly increasing worldwide owing to its heterogeneity and multi-drug resistance, with a reported fatality rate of patients with systemic infection as high as 50% [16]. The National Nosocomial Infection Surveillance (NNIS) system reported that MRSA accounted for 35.9% of cases with *S. aureus* infections in the ICU in 1992 [17] and 64.4% in 2003, corresponding to an annual growth rate of 3.1%. Because of its multidrug resistance, easy outbreaks, difficulty in treatment, and high mortality, MRSA has become a problem for clinical timely diagnosis and treatment.

The current clinical MRSA detection methods mainly include oxacillin-MIC, K-B susceptibility assay, salt-containing oxacillin agar screening, molecular biological techniques such as Polymerase Chain Reaction (PCR), nucleic acid probe hybridization technique (detecting *mecA* gene), Cefoxitin-paper disk diffusion susceptibility assay, etc. In recent years, plasmid spectral analysis, immunoblotting, chromosomal restriction endonuclease analysis, etc., have also been applied to detect MRSA and analyse its drug resistance [18]. In addition, detection technologies such as multiple real-time quantitative PCR, loop-mediated isothermal amplification, and gene chips have also been used in clinical and laboratory studies of MRSA [18]. However, some of the above techniques and methods require significant time, some require complicated and cumbersome procedures or special equipment, and some are too expensive to facilitate their standard clinical use. Although the molecular biology methods are sensitive, specific, and rapid, most *S. aureus* contain the *mecA* gene, and only a small number of bacteria lack this drug resistance gene; thus, together with the bacterial heterogeneity, the detection of drug resistance genes may not be equivalent to the actual bacterial resistance.

Endotoxin is an important component of the outer cell wall of Gram-negative bacteria. Its detection is simple, economical, and fast and also offers high sensitivity and specificity. Previous studies have suggested that BALF endotoxin detection is an effective method for the rapid diagnosis of Gram-negative bacterial pneumonia, with the sensitivity and specificity of 100% and 75%, respectively [9,10]. Flanagan et al. [12] found that BALF endotoxin levels>6 EU/ml, the diagnostic sensitivity for Gram-negative bacteria-caused VAP was up to 81%, with a specificity of 87%, indicating that BALF endotoxin level could not only rapidly diagnose Gram-negative bacteria-caused VAP but also have good effects for the identification of pathogens. Therefore, we could infer that BALF endotoxin levels in patients with VAP<6 EU/ml have an increased possibility of Gram-positive cocci infection. However, there are shortcomings with the use of endotoxin level alone as a diagnostic indicator of VAP; for endotoxin levels<6 EU/ml, the possibility of excluding Gram-negative bacterial infections, stopping antibiotic treatment, and the potential for cocci infections still needed to be answered.

As the precursor of calcitonin, PCT is a 116 amino acid glycoprotein existing in normal human serum. In healthy

humans, serum PCT concentration is very low; when bacterial infections cause inflammation, the levels increase significantly and consistent with the severity of the infection, suggesting its usefulness as an indicator of bacterial infections as well infection severity [19,20]. Recent studies have shown that PCT is not only the useful adjunct indicator for the diagnosis of VAP, which increases two days before the clinical diagnosis of VAP but also helpful to determine patient prognosis [21,22].

In this study, BALF endotoxin and serum PCT levels were combined for the diagnosis of early MRSA-caused VAP in the ICU. The results showed that the proportions of patients with pneumococcus and MRSA infection with BALF endotoxin concentrations<6 EU/ml and PCT>0.5 ng/ml were significantly higher than those in the other groups. In addition, the proportion of Gram-negative bacilli in patients with BALF endotoxin levels0>6 EU/ml and PCT>0.5 ng/ml was significantly higher than those in the other groups. This combined method had good sensitivity and specificity for the early diagnosis of MRSA-caused VAP. Patients with BALF endotoxin levels<6 EU/ml and PCT>0.5 ng/ml had a high possibility of pulmonary cocci infection, which had a better discrimination of Gram-negative bacilli and Gram-positive cocci infection. MRSA is the most common pathogenic bacteria in the ICU, so the probability of MRSA infection is significantly increased in the ICU. Detection of this condition suggests treatment for MRSA infection, which is more conducive to the recovery of the patient condition.

Among the 69 patients, only two cases were positive for Gram-positive bacteria, accounting for only 3.00% of the patient population; thus, they were not likely to have lung infections. Among the patients with BALF endotoxin concentrations>6 EU/ml and PCT<0.5 ng/ml, three had Gram-negative bacterial infections, accounting for 4.35% of the population, while one case (1.45%) was positive for a cocci infection. These results indicated that VAP could not be confirmed completely on PCT values alone and that certain corresponding clinical manifestations and laboratory parameters should also be considered; however, these findings also showed that the combination of two indicators had a good effect in excluding early VAP.

This study classified both MRSA and MRSE as MRSA, and the calculation revealed that the diagnostic sensitivity, specificity, positive predictive value, and negative predictive value for BALF endotoxin concentrations<6 EU/ml and PCT>0.5 ng/ml toward the detection of early MRSA-VAP were 63.64%, 91.89%, 70.00%, and 89.47%, respectively and 69.23%, 91.89%, 75.00%, and 84.47% for the diagnosis of early VAP caused by Gram-positive cocci, respectively. These findings showed the increased possibility of pneumococcus under these conditions. Since MRSA is the most common pathogen of cocci infections in the ICU, its infection rate would correspondingly be significantly increased. Thus, the identification of the above conditions would suggest the prompt active administration of anti-MRSA treatments.

This study also has several limitations. First, this study targeted only patients with early VAP while covered those with late-onset or receiving long-term mechanical ventilation. Second, the findings of this study require further assessment be under the conditions of mixed or fungal infections. Third, this study did not dynamically assess how the application of antibiotics would impact the findings, a limitation that warrants further studies and investigation.

Conclusion

In summary, the results of this study revealed that BALF-endotoxin concentrations<6 EU/ml and PCT levels>0.5 ng/ml had clinical significance and application for the rapid, economical, and effective diagnosis of Gram-positive cocci and early MRSA-VAP in the ICU.

Ethics Committee Approval

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Fujian University. Written informed consent was obtained from all participants.

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Conflict of Interest

All authors have no conflict of interest regarding this paper.

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