Original Article

Quantitative PCR for Etiologic Diagnosis of Methicillin-Resistant *Staphylococcus aureus* Pneumonia in Intensive Care Unit

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Background: Ventilator-associated pneumonia (VAP) requires prompt and appropriate treatment. Since methicillin-resistant *Staphylococcus aureus* (MRSA) is a frequent pathogen in VAP, rapid identification of it, is pivotal. Our aim was to evaluate the utility of quantitative polymerase chain reaction (qPCR) as a useful method for etiologic diagnoses of MRSA pneumonia.

Methods: We performed qPCR for *mec*A, *S. aureus*-specific *fem*A-SA, and *S. epidermidis*-specific *fem*A-SE genes from bronchoalveolar lavage or bronchial washing samples obtained from clinically-suspected VAP. Molecular identification of MRSA was based on the presence of the *mec*A and *fem*A-SA gene, with the absence of the *fem*A-SE gene. To compensate for the experimental and clinical conditions, we spiked an internal control in the course of DNA extraction. We estimated number of colony-forming units per mL (CFU/mL) of MRSA samples through a standard curve of a serially-diluted reference MRSA strain. We compared the threshold cycle (Ct) value with the microbiologic results of MRSA.

Results: We obtained the *mec*A gene standard curve, which showed the detection limit of the *mec*A gene to be 100 fg, which corresponds to a copy number of 30. We chose cut-off Ct values of 27.94 (equivalent to 1×10^4 CFU/mL) and 21.78 (equivalent to 1×10^5 CFU/mL). The sensitivity and specificity of our assay were 88.9% and 88.9% respectively, when compared with quantitative cultures.

Conclusion: Our results were valuable for diagnosing and identifying pathogens involved in VAP. We believe our modified qPCR is an appropriate tool for the rapid diagnosis of clinical pathogens regarding patients in the intensive care unit.

Key Words: Methicillin-Resistant *Staphylococcus aureus*; Real-Time Polymerase Chain Reaction; Pneumonia, Ventilator-Associated

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Introduction

Hospital-acquired pneumonia (HAP) is the second most common nosocomial infection[1]. HAP is 6~20 times more common in mechanically-ventilated patient, and ventilator associated pneumonia (VAP) occurs in 9~27% of all intubated patients[2,3]. The mortality rate of VAP has been reported to range between 33% and 50%[4]. Because of increasing mortality, most patients with pneumonia are treated with broad-spectrum antibiotics, and this treatment increases hospital cost as well as the prevalence of resistant strains[5]. To minimize the

risk of mortality, clinicians are recommended to avoid delaying administration of appropriate antibiotic treatments[6].

The National Nosocomial Infection Surveillance from 2003 found that *Staphylococcus aureus* was the most common pathogen associated with VAP (27.8%) in US1. Methicillin resistance is caused by the penicillin-binding protein 2a (PBP2a), which is encoded by the methicillin-resistant *Staphylococcus aureus* (MRSA) *mecA* gene. Because genes for methicillin resistance in *Staphylococcus aureus* are well-known, *mecA* is used to differentiate between methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA[7].

Because the results of a microbiologic culture were not immediately available, empirical antibiotic therapy could lead to inadequate antibiotic treatment. Molecular-based diagnostic methods, such as quantitative polymerase chain reaction (qPCR), have been evaluated for their ability to identify MRSA by detecting the *mecA* gene[8-10]. This has the potential to deliver rapid, specific and sensitive detection. Previously, a study with the qPCR of blood indentified sepsis-relevant microorganisms[11]. Because qPCR for MRSA was limited to surveillance with nasal swabs, it was impossible to determine whether the specimens were true pathogens or colonization[12]. Multiplex PCR to detect Gram negative bacilli could not discriminate true pathogens, previously[13]. qPCR have not been completely evaluated for their ability to provide etiologic diagnostic criteria for clinical specimens[14,15].

The object of this study was to develop and validate a qPCR assay from MRSA detection on bronchoalveolar lavage (BAL) or bronchial washing samples.

Materials and Methods

1. Specimen preparation and extract clinical data

This study was performed at a 52-bed intensive care unit (ICU) in Konyang University Hospital (Daejeon, Korea). Samples consisting of a BAL and bronchial washing samples were collected directly from patients admitted to the ICU. The study included 72 clinical samples that consisted of 72 VAP episodes in 64 patients, during a period of 11 months (March 2010 to February 2011). The study was approved by the Institutional Review Board of Konyang University Hospital, and we received informed consent from all patients.

2. BAL and bronchial washing samples

We obtained clinical samples from patients who had "clinically-suspected pneumonia" that was defined as follows: patients received mechanical ventilation for at least 48 hours in the ICU and had new or progressive infiltration on chest X-rays. In addition, patients had at least two of following symptoms indicating infection: 1) temperature$<36^{\circ}$C or $\geq38.3^{\circ}$C, 2) white blood cell $<5,000$ cells/mm^3 or $>10,000$ cells/mm^3, and 3) purulent sputum[4].

BAL samples were taken from pulmonary subsegments and bronchial washing samples were taken, using fiberoptic bronchoscopy from a segment or lobe in which new or progressive infiltration had developed. If lesions were not identified, samples were taken from basal segments of lower lobe. We performed the BAL procedure and bronchoscopy methods standard in the ICU[16].

3. Identification and antimicrobial susceptibility: breakpoint by Clinical and Laboratory Standards Institute guidelines

Quantitative cultures of BAL or bronchial washing specimens were performed in a microbiology laboratory. The specimens were inoculated in blood and MacConkey agar plates with a 0.001 mL-loop. After incubation at 37°C in a 5% CO$_2$ incubator, for 24 hours, a colony count was performed and expressed as the number of colony-forming units per mL (CFU/mL). In general, the number of CFU/mL is equal to the number of colonies on the agar plate multiplied by the inoculation factor. In our quantitative culture, the pathogen was evaluated by the diagnostic criteria for BAL specimens ($\geq1\times10^4$ CFU/mL) and bronchial washing specimens ($\geq1\times10^5$ CFU/mL). Organisms isolated from specimens were confirmed using Combo Panels of

the MicroScan WalkAway 96 system (Dade Behring, West Sacramento, CA, USA) for antimicrobial susceptibility and species identification.

4. DNA extraction

To extract DNA from BAL or bronchial washing samples, we used a NucleoSpin® Tissue extraction kit (MACHEREY-NAGEL, Neumann-Neander-Str., Germany), closely following the manufacture's protocols. The clinical sample volume was 1 mL, and an equal volume of N-acetyl/NaOH was added to the sample. After incubating the mixture for 25 minutes at room temperature, while shaking it, the mixture volume was adjusted to 25 mL with sterile water and the mixture was centrifuged at 4,000 ×g for 30 minutes. The supernatant was discarded, two hundred μL of buffer T1 was used to re-suspend the pellet, and 25 μL of proteinase K was added. For inhibitor control, 100 pg of spiked an internal control (SPUD) (Table 1) was also added. The samples were incubated at 56°C and were shaken until complete lysis was obtained. Two hundred μL of buffer B3 was added for lysis, and the sample was incubated at 70°C for 10 minutes. Then, 210 μL of ethanol (96~100%) was added, and the sample was placed on a NucleoSpin® tissue column, in a collection tube. After centrifugation for 1 minute at 11,000 ×g, the flow-through was discarded, and 500 μL of buffer BW were added for first wash. The sample was then centrifuged for 1 minute at 11,000 ×g. Next, 600 μL of buffer B5 were added to the column for the second wash and centrifuged for 1 minute at 11,000 ×g. The flow-through

was the discarded, and the column was centrifuged for 1 minute at 11,000 ×g in order to remove residual ethanol. Then, 50 μL of pre-warmed elution buffer BE (70°C) was added, and the sample was incubated at 25°C for 1 minute. After centrifugation for 1 minute at 11,000 ×g, the extracted DNA preparations were adjusted to a final volume of 50 μL.

5. Reference strains

Three reference strains of *Staphylococcus* spp., including methicillin-resistant and, methicillin-susceptible strains, were included in the study. The strains were MRSA (ATCC 33591), MSSA (ATCC 25923), and methicillin-sensitive *Staphylococcus epidermidis* (MSSE) (ATCC 12228). All strains were provided by Kyungbook National University Hospital and the Korean Federation of Culture Collections, Korean Culture Center of Microorganisms.

6. Quantitative PCR

Multiplex PCR was performed in a 20 μL volume that contained 10 μL of iQ™ Supermix (Bio-Rad, Hercules, CA, USA), 125 nM of each primer, 125 nM probes, and 4 μL of template. The amplification procedure for the Bio-Rad iQ5 took place at 95°C for 5 minutes, followed by 50 cycles at 95°C for 10 seconds and 57°C for 30 seconds with, fluorescence acquisition after every cycle. The sequence of the TaqMan primers and probes for MRSA qPCR was based on work from Francois et al.[17] and modified by the Beacon Designer (Premier Biosoft, San Diego, CA, USA) software, as described in Table 2.

Table 1. Sequence of primers and probes for spiked an internal control (SPUD)

Gene and primer or probe name	Sequence (5'→3')	Length	5' dye
SPUD			
F SPUD	ACGGCAAATAAAGACATAATACATA	25	
R SPUD	CATCCTTACATGGCACCA	18	
P SPUD	TCCTAACTTGGCTTTAATGGACCTCCA - BHQ1	27	FAM
A SPUD	ACGGCAAATAAAGACATAATACATAAGCATTATCCTAACTTGGCTTTAATGGACCTCCAATTTTGAGT GTGCACAAGCTATGGAACACCACGTAAGACATAAAACGGCCACATATGGTGCCATGTAAGGATG	132	

F: forward primer; R: reverse primer; P: probe; A: amplicon.

Table 2. Sequence of primers and probes for the *mecA*, *femA*-SA, and *femA*-SE genes

Gene and primer or probe name	Sequence (5'→3')	Length	5' dye
mecA			
F *mecA*	ACGAGTAGATGCTCAATA	18	
R *mecA*	GACGCTATGATCCCAATC	18	
P *mecA*	AACTACGGTAACATTGATCGCAACG - BHQ2	25	CalRed610
femA-SA			
F *femA*-SA	GGTGCCTTTACAGATAGC	18	
R *femA*-SA	TTCCCACTAAATGTGTTTCA	20	
P *femA*-SA	AGTCATTTCACGCAAACTGTTGGC - BHQ1	24	FAM
femA-SE			
F *femA*-SE	TCCGTTTGAAGTAGTTTAC	19	
R *femA*-SE	TACCGATTAATACCATGTTC	20	
P *femA*-SE	AATCATCTTCCATTGAACCGCATAGC - BHQ1	26	YAK

F: forward primer; R: reverse primer; P: probe.

Using this method, detection of only the *femA*-SA indicated the presence of MSSA, and detection of both the *femA*-SA and *mecA* genes indicated the presence of MRSA. If only the *femA*-SE gene was detected, the presence of MSSE was inferred. Furthermore, the detection of *femA*-SE and *mecA* genes, or the detection of the mecA gene alone, indicated the presence of methicillin-resistant coagulase negative Staphylococci (MRCNS).

7. Statistics

Data are shown as the mean±SD. Differences between the two groups were assessed using the student's t-test.

Results

1. Result of microbiologic cultures of clinical samples

The assay was applied to 72 clinical samples of 64 patients from medical (56.3%), surgical non-traumatic (15.6%) and surgical traumatic (28.1%) categories. Reasons for mechanical ventilation were neurologic diseases (31.3%), pulmonary diseases (29.7%), postoperative respiratory failure (12.5%) and the others (26.5%). The clinical characteristics and the identified species from patients are described and summarized in Table 3.

2. Measure reproducibility and obtained standard curve about *mecA*, *femA*-SA, and *femA*-SE genes

We measured the reproducibility of detecting the *femA*-SA, *femA*-SE and *mecA* genes in the reference strains (Table 4).

We obtained the *mecA* gene standard curves by serial dilution of genomic DNA under the assumption that 1 copy number of the *mecA* gene corresponds to 1 CFU[18]. The results indicate that the detection limit of the *mecA* gene is 100 fg:30 copy number (Figure 1).

3. Comparison between qPCR results and quantitative culture results

We detected MRSA, MSSA, and MRCNS using the qPCR. As a standard curve (Figure 1), we determined the cut-off Ct value (28.94) that corresponded to diagnostic criteria (1×10^3 CFU/mL) and compared the results of qPCR with results from the quantitative culture. We concluded that the sensitivity and specificity were 94.11% and 74.54%, respectively (data not shown). Additionally, we chose a quantitative microbiological method based on the inoculation in plates with a 0.001 mL-loop. This method did not allow for a culture of less than 10^3 CFU/mL.

After discriminating between MRSA cultures and other specimens for determining the presence of pathogens,

Table 3. Clinical characteristics of study patients

Characteristic	No.
Age, mean±SD, yr	64±15
Gender (male/female)	46/18
APACHE-2 score, mean±SD at admission in the ICU	22±8
No. of episodes receiving glycopeptides prior to sampling, %	25 (34.7)
Median (IQR) length of glycopeptides, days	6 (3~7)
Median (IQR) duration of ICU*, days	10 (7~18)
Median (IQR) duration of ICU receiving mechanical-ventilation prior to sampling, days	8 (6~12)
No. of sampling (total)	72
Bronchoalveolar lavage samples	21
Bronchial washing samples	51
No. of detected species (total)*,†	73
Methicillin-resistant *Staphylocccus aureus*	16
Methicillin-sensitive *Staphylococcus aureus*	1
Methicillin-resistant coagulase negative Staphylococi	1
Acinetobacter baumannii	35
Serratia marcescens	4
Stenotrophomonas maltophilia	3
Pseudomonas aeruginosa	6
Achromobacter spp.	2
Burkholderia cepacia	2
Candida spp.	1
Escherichia coli	1
Penicillin-susceptible *Streptococcus pneumoniae*	1

*Intensive care unit. †In 1 of 72 samples, 2 species were cultured.

Table 4. Average Ct values for triplicate determinations, with 100 pg of genomic DNA

Strain	Ct values for		
	femA-SA	*femA*-SE	*mecA*
MRSA (ATCC33591)	21.99 (±0.16)*	NA	20.48 (±0.05)
MSSA (ATCC25923)	26.54 (±0.13)	NA	NA
MSSE (ATCC12228)	NA	20.21 (±0.11)	NA

*Average Ct value (±standard deviation).
MRSA: methicillin-resistant *Staphylococcus aureus*; NA: not assessed; MSSA: methicillin-sensitive *Staphylococcus aureus*; MSSE, methicillin-sensitive *Staphylococcus epidermidis*.

we divided the samples into four groups, based on the CFU results from quantitative cultures: group 1 contained samples with culture negatives, group 2 contained samples with cultures of $\geq 10^3$ to $< 10^4$ CFU/mL, group 3 contained samples with cultures of $\geq 10^4$ to $< 10^5$ CFU/mL, and group 4 contained samples with a culture of $\geq 10^5$ CFU/mL. The Ct values were 23.97± 3.97 for group 3 and 18.72±3.06 in group 4 (Figure 2). The difference between group 3 and group 4 was statistically significant ($p < 0.05$). In the standard curve of the *mecA* gene, the corresponding Ct to CFU values were 2.08×10^3 to 6.05×10^5 CFU/mL for group 3 and 1.87×10^5 to 1.65×10^7 CFU/mL for group 4. To minimize the differences between reference MRSA strains and extracted specimens, and to reduce false-positives, we determined that a cut-off value of 27.94 (mean+SD in group 4) corresponded to 1×10^4 CFU/mL, and a cut-off value of 21.78 (mean+SD in group 2) corre-
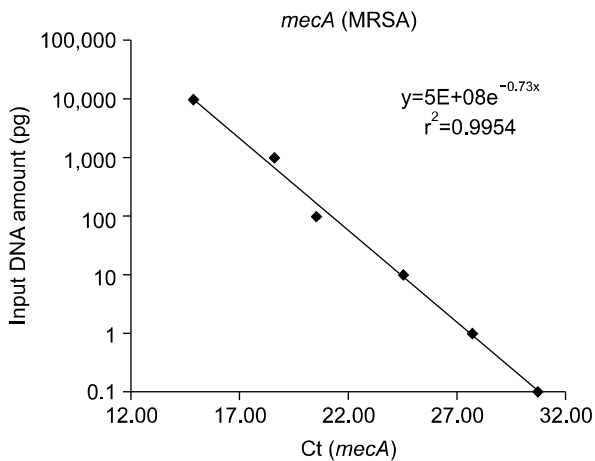
Figure 1. Correlation between input DNA amount and Ct values. MRSA: methicillin-resistant *Staphyloccous aureus*.
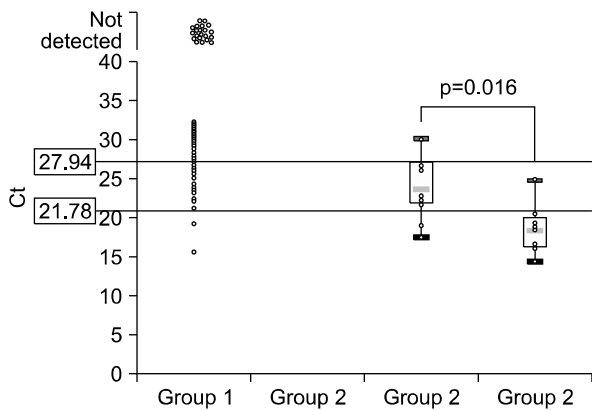

Figure 2. Box-plot of Ct values from methicillin-resistant *Staphyloccous aureus* (MRSA) cultured specimens. Of the total samples, we created 4 groups based on the colony-forming units (CFU) results from the quantitative culture. Group 1 (n=56) contained samples with negative cultures, group 2 (n=0) contained samples with cultures of $\geq 10^3$ to $<10^4$ CFU/mL, group 3 (n=8) contained samples with cultures of $\geq 10^4$ to $<10^5$ CFU/mL, and group 4 (n=8) had samples with a culture of $\geq 10^5$ CFU/mL. Boxes represent the 25th to 75th percentile interquartile range, with the line in the middle representing the median. The upper horizontal line represents the cut-off value of 1×10^4 CFU/mL, and the lower horizontal line represents the cut-off value of 1×10^5 CFU/mL.

sponded to 1×10^5 CFU/mL. To define whether the specimens were pathogens or colonization, we used a cut-off value such that sample Ct values of ≤ 27.94 from BAL specimens and ≤ 21.78 from bronchial wash-

Table 5. Comparison of results by qPCR with quantitative culture (n=72)

Quantitative culture result	qPCR results	
	MRSA pathogen	MRSA not pathogen
MRSA pathogen (n=9)	8	1
MRSA not pathogen (n=7)	2*	5
MRSA not cultured (n=56)[†]	5[†]	51[§]
Total specimens (n=72)[‖]		
Sensitivity	8/9 (88.9%)	
Specificity	56/63 (88.9%)	

In the quantitative culture, the pathogen was evaluated by the diagnostic criteria for BAL specimen ($\geq 1 \times 10^4$ CFU/mL) or bronchial washing specimen ($\geq 1 \times 10^5$ CFU/mL), according to the materials and methods. The other 2 categories (MRSA, not pathogen and MRSA not cultured) were negative.
*,[†]Explained in the Discussion. [†]Other strains (except MRSA) were cultured. [§]This results contained the samples of MRSA not detected. [‖]Seventy-two were equal to VAP episode.
qPCR: quantitative polymerase chain reaction; MRSA: methicillin-resistant *Staphyloccous aureus*; BAL: bronchoalveolar lavage; CFU/mL, number of colony-forming units per mL; VAP: ventilator-associated pneumonia.

ing specimens were diagnosed as pathogens. These cut-off values resulted in a sensitivity of 88.9% and a specificity of 88.9% (Table 5). With the exception of the 25 VAP episodes that received antibiotics for MRSA, we analyzed a total of 47 VAP episodes. qPCR assay had a sensitivity of 85.7% and specificity of 90.0% for the pre-antibiotic samples. There were 4 false positives and 1 false negative in the qPCR assay.

Discussion

According to data from the Korean Nosocomial Infection Surveillance System, MRSA is the pathogen most frequently isolated in VAP[19]. Mortality caused by MRSA pneumonia ranges between 14% and 47% but is significantly lower among patients receiving early appropriate antibiotic treatment than in those requiring a change in treatment[20]. Recent studies have correlated qPCR with quantitative culture counts and have provided therapeutic information for applying diagnostic criteria (1×10^4 CFU/mL) to BAL specimens[14]. To in-

dicate appropriate therapeutic information, we correlated firstly qPCR results with clinical pathogens. Therefore, we not only reduced differences between qPCR and quantitative cultures but also suggested a clinical setting for bronchial washing specimens.

We estimated the number of MRSA CFUs isolated from BALs or bronchial washing specimens and compared the results of qPCR with the results of quantitative cultures. In our study, 1 of 72 clinical samples (1.4%) was classified as a false-negative (Table 5) and this patient may have received inappropriate treatment leading to fatality. The patient's clinical pulmonary infection score (CPIS) was greater than 7 and MRSA grew more than 10^5 CFU/mL. Combining the CPIS score with qPCR results would likely have yielded a more accurate diagnosis for this patient.

Seven patients' (qPCR, MRSA pathogen and quantitative culture, MRSA not pathogen [2 patients]; qPCR, MRSA pathogen and quantitative culture, MRSA not cultured [5 patients]) (Table 5) results classified as false-positives. Of the 7 false-positives, five patients' specimens (qPCR, MRSA pathogen and quantitative culture, MRSA not cultured) (Table 5) had no MRSA in a quantitative culture. According to their clinical records, all patients had "clinically-suspected VAP" and CPIS was exceeded 6. Two of the five patients received glycopeptides within the 48 hours prior to obtaining samples. One patient who maintained vancomycin was survived, but the remaining patients with acute respiratory distress syndrome did not respond to teicoplanin. The qPCR method might not distinguish nonviable organisms from viable ones, because the DNA of some nonviable organism could still be extracted[21,22]. We should consider recent antibiotic therapy before extracting the specimens for qPCR, because patients who showed a favorable clinical response to therapy had a rapid fall in colony counts within 24 to 72 hours[23]. One patient with high risk for MRSA pneumonia, whose medication was changed to teicoplanin, showed improvement. Two patients who had not received glycopeptides progressed. Five patients might have had MRSA, so our qPCR method might be more sensitive than a microbiologic

culture.

Two patients (qPCR, MRSA pathogen and quantitative culture, MRSA not pathogen) (Table 5) who had clinically-suspected VAP had MRSA that was less than the cut-off value for microbiologically-diagnosed VAP. One patient receiving glycopeptides improved, which suggests that the result of the qPCR were accurate. The other patients' results were false positives. One of seven patients, according to positive results of qPCR (1/72 VAP episodes, 1.4%), might have taken unnecessary glycopeptides during our study. In contrast, six of the seven patients with negative or low levels of CFU/mL of microbiologic cultures might not have received glycopeptides for MRSA pneumonia, which might have contributed to an unfavorable outcome.

In our study, 51 specimens were bronchial washing samples that were easier to obtain, and the less invasive to critically ill patients, than BAL or protected specimen brush (PSB). The quickest, easiest and least expensive sample to obtain was the endotracheal aspirate. We used, rather than blind sampling, bronchoscopic sampling; blind aspiration sampling could lead to errors and diagnosis could be missed in some patients[16]. Although washing samples were obtained from the distal portion of lobar bronchus and involved VAP, we used a cut-off value established by the endotracheal aspirate diagnostic criteria ($\geq 1 \times 10^5$ CFU/mL) instead of BAL ($\geq 1 \times 10^4$ CFU/mL), owing to misleading colonization to pathogen by using a low-cut off. In previous studies, when a bacterial count $\geq 1 \times 10^5$ CFU/mL showed positive culture results of endotracheal aspirate, the sensitivity and specificity were 80% (range, 60~97%) and 62% (range, 41~74%) respectively. There was no difference between the several modalities of sampling in obtaining clinically significant pathogens[24,25]. In addition, the Ct values of qPCR were well-correlated with the quantitative culture of specimens. When using the diagnostic criteria of ≤ 27.94 (equivalent to 1×10^4 CFU/mL) in BAL specimens and ≤ 21.78 (equivalent to 1×10^5 CFU/mL) in bronchial washing specimens, the sensitivity was 88.9% and the specificity 88.9%.

The major finding of our study was that the qPCR

method can be used to confirm whether colonized MRSA is a pathogen involved in VAP. A limitation is that it is difficult to differentiate patients infected only with MRSA from those who are co-infected (MSSA and methicillin-resistant *Staphylococcus epidermidis*). However, the co-infected patients are clinically uncommon cases.

We used a modified SPUD assay to evaluate experimental and clinical conditions[26]. We spiked the SPUD in the specimen and used a high-throughput column method of DNA extraction. This methodology allows the qPCR method to be utilized routinely. The recovery rate of this method was not constant, as in sample conditions: agglutination of sputum. For this reason, we needed to consider the recovery rate for absolute quantification. With this modification, we were able to consider not only inhibitors but also technical errors. Thus, the process more accurately reflected clinical conditions.

Diagnosis of VAP was very difficult in the ICU patient. Instead of developing a clinical suspected scoring system, diagnostic criteria and microbiology diagnosis were used[4]. Our study shows that the qPCR could be good for diagnosing pneumonia and might be an appropriate substitute for microbiologic cultures. In a study of PSB in suspected VAP, quantitative cultures led to antimicrobial changes in 38% of the patients with inadequate initial therapeutic plans[27]. In another study, patients with clinically-suspected VAP had a high mortality rate regardless of whether BAL cultures confirmed a clinical diagnosis of VAP[28]. When adequate antibiotic therapies were initiated very early, the mortality rate was reduced when compared to adequate therapies that were delayed until bronchoscopies were performed or BAL results were known[29]. Rapid turnaround times are therefore highly important in diagnosing and treating VAP. In our study, we not only demonstrated a rapid qPCR method to diagnose whether there were pathogens, but also reduced differences between qPCR and quantitative culture results. In addition, we suggested clinical settings for bronchial washing specimens, which had not yet been established. In conclusion, our results

were valuable for diagnosing and identifying pathogens for VAP. We suggest that our modified qPCR is an appropriate and rapid tool for diagnosing clinical pathogens in ICU patients.

Acknowledgements

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