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Original Article

Evaluation of bronchoalveolar lavage fluid combined with the loop-mediated isothermal amplification assay in lower respiratory tract infections

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Abstract: The clinical application of the loop-mediated isothermal amplification (LAMP) assay has been problematic because of conflicting results obtained from the LAMP assay and bacterial culture. In order to eliminate the interference of oral microorganisms and more accurately evaluate the diagnostic performance of the LAMP assay, we utilized bronchoalveolar lavage fluid (BALF) as a sample to test whether the LAMP assay and bacteria culture yielded similar results. A total of 1092 BALF samples from patients with suspected lower respiratory tract infections were collected. For each sample, parallel studies using both bacterial culture and the LAMP assay were carried out. We were the first to utilize BALF as a sample to study the consistency between the LAMP assay and bacterial culture results. The present study demonstrated that the positive rate from the LAMP assay was higher than that from bacterial culture, and the two methods had a better consistency than previously reported.

Keywords: Loop-mediated isothermal amplification, lower respiratory infections, pathogens, bacterial culture, bronchoalveolar lavage fluid

Introduction

Lower respiratory tract infections (LRTIs) are the sixth leading cause of mortality for all ages and the leading cause of death among children younger than 5 years [1]. Timely and accurate identification of specific pathogens in LRTIs can reduce mortality and the misuse of antibiotics [2]. In the clinic, bacterial culture has been considered the gold standard for pathogen diagnosis, but it is time-consuming, easy to contaminate, and has a low positive rate [3-5]. Quantitative real-time polymerase chain reaction (qPCR) is much faster and can screen for multiple pathogens simultaneously [6, 7]. However, qPCR is too expensive and technologically intensive for general use in clinical practice. Unlike qPCR, the loop-mediated isothermal amplification (LAMP) assay is performed in a single step and increasingly used in pathogen detection because of its low cost, simplicity and rapidity [8]. However, the LAMP assay has not been clinically adopted because of conflicting results between the assay and bacterial culture [9]. Discrepancies between the two methods need to be understood before the LAMP assay can be applied routinely in LRTI diagnosis.

Sputum culture is usually used as a reference standard to evaluate the performance of the LAMP assay, but agreement between the LAMP assay and sputum culture is low. The testing principle of the LAMP assay is different from that of bacterial culture. The LAMP assay amplifies and detects pathogen target genes. If the number of pathogen genes reaches the detection limit, the result is positive whether the pathogen is alive or dead. In contrast, the culture method requires that the pathogen must be live bacteria, and only the dominant flora can be cultivated [5]. Hundreds of microbial species inhabit the pharynx, forming a changing and complex microflora [10]. Oral microorganisms easily contaminate sputum specimens during collection [11, 12], resulting in a nega-

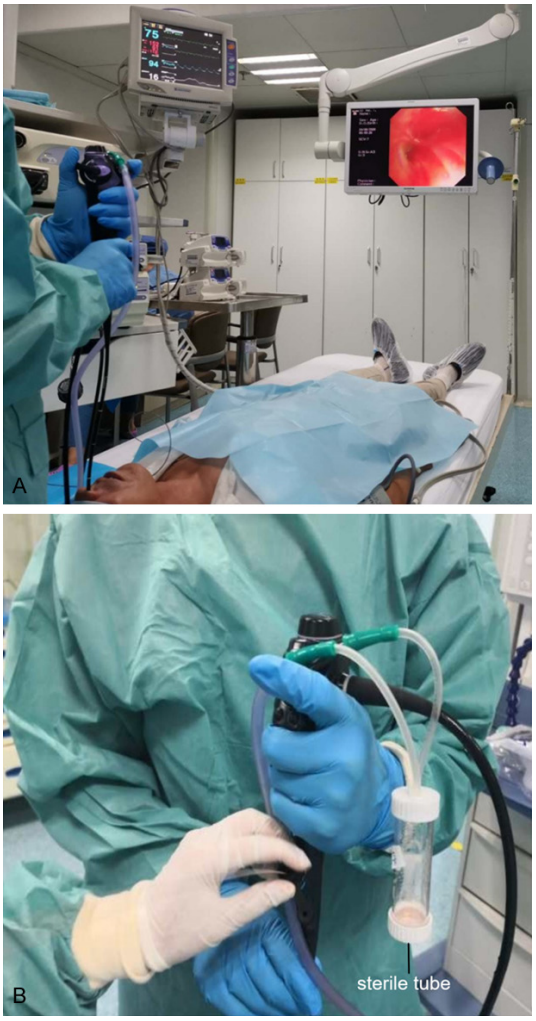

Figure 1. The lavage fluid was collected using a fiber-optic bronchoscope. A. The suspected infected site was rinsed using a bronchoscope. B. The bronchoalveolar lavage fluid (BALF) was sucked into a sterile tube by applying negative pressure.

tive sputum bacterial culture. However, the LAMP assay can still detect the pathogen genes. If sputum bacterial culture is regarded as the reference standard for diagnosing LRTIs, the LAMP assay results will be mistaken for a false-positive. However, the causative pathogens were not detected in the sputum bacterial culture. Therefore, the contamination of sputum specimens with oral microorganisms resulted in reduced consistency between the two methods [13]. Lower respiratory tract constitutes a more sterile environment than that of upper airways, the differences between upper respiratory tract microbiome and lower respiratory tract microbiome highlight the limitations of using upper respiratory tract samples as a substitute to study the lower respiratory tract microbiome [14]. Though bronchoscopy with BALF is an invasive diagnostic, it is a useful and safe tool in diagnosing pulmonary infection [15]. Bronchoalveolar lavage fluid (BALF) is collected through fiber-optic bronchoscopy and the probability of oral microorganism contamination is much lower than with sputum specimens.

To eliminate the interference from oral microorganisms and more accurately evaluate the diagnostic performance of the LAMP assay, we compared the performance of the LAMP assay with that of bacterial culture in detecting a panel of nine common respiratory bacterial pathogens in BALF samples.

Materials and methods

Patients

A retrospective observational study was conducted at the Tang Du Hospital, China. All participants were recruited from patients of Tang Du Hospital between January 2018 and September 2018. All patients were enrolled in this study based on an initial clinical diagnosis of LRTI if they met one of the following inclusion criteria: (1) symptoms of pneumonia or bronchitis, confirmed using chest X-rays or computed tomography scan or (2) one or more respiratory symptoms, including fever > 38.5°C, coarse breath sounds with wet or dry rale, exacerbated dyspnea, exacerbated sputum production, or purulence. Exclusion criteria from enrollment were: (1) diagnosed with a non-infectious disease or (2) infected with non-bacterial pathogens or tuberculosis. All procedures were conducted with the approval of the ethics committee of the Air Force Medical University.

Detection of pathogenic bacteria

The tip of the bronchoscopy was wedged into the area with the greatest radiologic abnormality under anesthesia. When no infiltrates were identified, BALF was performed in the middle lobe. The catheter was inserted into airway through the negative pressure suction hole. The sampling opening at the top of the catheter was pressed against the segmental or subsegmental bronchus opening, and 50 mL physiologic saline was injected into the bronchi. After standing for 4 minutes, manually sucked back saline into a sterile tube, as shown in **Figure 1**. For each sample, a parallel study

Table 1. Age and sex distribution of the patients

Characteristics	n	Total (%)
Gender		
Male	682	62.5
Female	410	37.5
Age group (years)		
< 20	26	2.4
≥ 20, < 39	104	9.5
≥ 39, < 60	372	34.1
≥ 60	590	54.0

was carried out using both the LAMP assay and bacterial culture. Experienced laboratory staff working in the respective laboratories performed the LAMP assay and bacterial culture. Nine bacterial pathogens were assessed: *Streptococcus pneumoniae* (Spn), *S. aureus* (Sau), methicillin-resistant *S. aureus* (MecA), *Escherichia coli* (Eco), *Klebsiella pneumoniae* (Kpn), *Pseudomonas aeruginosa* (Pae), *Acinetobacter baumannii* (Aba), *Stenotrophomonas maltophilia* (Sma), and *Haemophilus influenzae* (Hin).

DNA extraction and the LAMP assay

Each BALF sample was liquefied with 4% NaOH solution for 30 minutes. Then, 1 mL was removed and centrifuged at 12000 rpm for 5 minutes. Then, the supernatant was discarded and DNA was extracted from the precipitate using the Universal Kit for Bacterial DNA Extraction (CapitalBio Technology, Beijing, China). The LAMP assay detection of pathogenic bacteria was done according to the instructions of the Pathogenic Bacteria Nucleic Acid Detection Kit (CapitalBio Technology, Beijing, China). Briefly, 34.5 μL DNA solution was mixed with 20 μL thermostatic amplification reagent (containing fluorescent dyes and enzymes). Next, 50 μL of the above mixture was removed and added to the microfluidic chip. The chip was centrifuged at 6000 rpm for 30 seconds. The amplification reaction was performed on a RTisochip™-A analyzer (CapitalBio Technology, Beijing, China) at 37°C for 3 minutes and 65°C for 47 minutes. After the reaction, if the time-to-positive value of the pathogen was less than or equal to that of the positive control value, the result was interpreted as positive.

Bacterial culture

The BLAF was seeded on bacteriological media such as blood agar plates, chocolate agar plates, or blue agar plates using sterile wire loops and incubated at 35°C for 48 hours in a 5% carbon dioxide environment in a thermostatic incubator. Subsequently, the dominant colonies were picked for bacterial detection using a VITEK2-Compact from BioMerieux (France) automatic bacterial analyzer. When the BALF contained ≥ 10^3 colony-forming units of bacteria/mL, the identified strains were considered the causative pathogens.

Statistical analysis

The agreement between the two tests was measured using the Kappa coefficient. The difference in positive rates between the LAMP assay and culture was evaluated using the McNemar χ^2 test. Statistical analyses were performed with SPSS statistics software (version 19.0) and GraphPad Prism 7.0 software. *P* values < 0.05 were considered statistically significant.

Results

Demographic characteristics

A total of 1160 BALF samples from presumptive LRTI patients were collected in the current study. Only nine species in the pathogen panel were assessed. Therefore, 68 patients were excluded and 1092 patients remained for analysis. Of those eligible participants, 62.5% were male and 37.5% female. The majority (54.0%) of the participants were over 60 years of age. Age and sex distribution of the patients are shown in **Table 1**.

Distribution of pathogens detected by using the LAMP assay and bacterial culture

The distribution of pathogens detected using the LAMP assay is shown in **Table 2**. In total, 414 out of the 1092 samples were positive using the LAMP assay, and the total positive rate was 37.91%. Among them, there were 201 cases of single infection and 213 cases of mixed infection, accounting for 48.55% and 51.45% of the total infection cases, respectively. MecA was the predominant bacterial pathogen (9.43%) and most of the cases were a single infection (66.99%). Other pathogens

Table 2. Distribution of pathogens detected by the LAMP assay

Pathogen	n	Positive rate (%)	Single infection		Mixed infection	
			n	Constituent ratio (%)	n	Constituent ratio (%)
Spn	59	5.40	32	54.24	27	45.76
Sau	17	1.56	5	29.41	12	70.59
MecA	103	9.43	69	66.99	34	33.01
Eco	10	0.92	1	10.00	9	90.00
Kpn	38	3.48	12	31.58	26	68.42
Pae	40	3.66	20	50.00	20	50.00
Aba	60	5.49	19	31.67	41	68.33
Sma	24	2.20	7	29.17	17	70.83
Hin	63	5.77	36	57.14	27	42.86
negative	678	-	-	-	-	-
Overall	1092	37.91	201	48.55	213	51.45

Note: LAMP, loop-mediated isothermal amplification; Spn, *Streptococcus pneumoniae*; Sau, *S. aureus*; MecA, Methicillin-resistant *S. aureus*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pae, *Pseudomonas aeruginosa*; Aba, *Acinetobacter baumannii*; Sma, *Stenotrophomonas maltophilia*; Hin, *Haemophilus influenzae*.

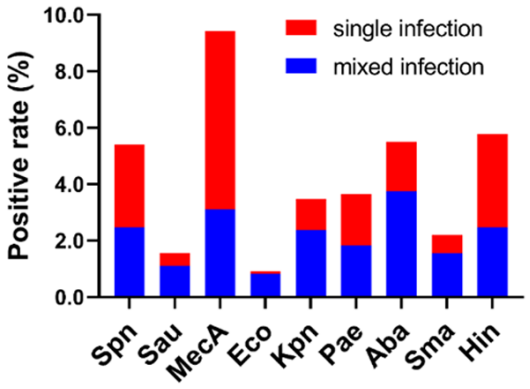

Figure 2. The positive rate of each pathogen in the loop-mediated isothermal amplification (LAMP) assay and the proportion of mixed infection and single infection for each pathogen infection. Note: Spn, *Streptococcus pneumoniae*; Sau, *S. aureus*; MecA, Methicillin-resistant *S. aureus*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pae, *Pseudomonas aeruginosa*; Aba, *Acinetobacter baumannii*; Sma, *Stenotrophomonas maltophilia*; Hin, *Haemophilus influenzae*.

with a high positive rate were Hin (5.77%), Aba (5.49%) and Spn (5.40%). In cases positive for Hin and Spn, the proportion of single infection and mixed infection was approximately equal, whereas Aba was predominantly present as a mixed infection (68.33%). Eco had the lowest positive rate (0.92%) and was detected primarily with other pathogens as a mixed infection (90.00%). In addition, Sau, Kpn, and Sma tended to coexist with other pathogens in mixed infections (**Figure 2**). However, only 129 cases were positive in bacterial culture (total positive rate 11.81%). The positive rate of each pathogen in bacterial culture was low and the pathogens with the top three positive rates were Aba (4.67%), Pae (2.75%) and Spn (1.10%), as shown in **Table 3**. The comparison of pathogen distribution between the LAMP assay and bacterial culture is shown in **Figure 3**.

Comparison of positive rate between LAMP and bacterial culture

The total positive rate from the LAMP assay was significantly higher than that from bacterial culture (37.91% vs 11.81%). The comparison of positive rates for each pathogen between the LAMP assay and bacterial culture is shown in **Figure 4**. The positive rates of Spn, Sau, MecA, Kpn, Pae, Sma, and Hin detected using the LAMP assay were significantly higher than those detected using bacterial culture ($P < 0.05$). The positive rates of Aba and Eco from the LAMP assay also were higher than those from bacterial culture, although the difference was not significant.

Congruence between the LAMP assay and bacterial culture

In this study, many samples that were negative in culture tested positive in the LAMP assay. Out of the 414 samples that were positive using the LAMP assay, 98 samples were positive and 316 samples were negative in bacterial culture. Meanwhile, out of all the negative samples detected using the LAMP assay, 31 samples were positive in bacterial culture. The two methods showed good agreement in pathogen detection, especially for Pae (Kappa = 0.735) and Aba (Kappa = 0.687). The positive cases identified using the LAMP assay and bacterial culture are summarized in **Table 4**.

Discussion

LRTIs are a leading cause of mortality and morbidity worldwide [16] and comprehensive

Table 3. Distribution of pathogens detected by bacterial culture

Pathogen	n	Positive rate (%)
Spn	12	1.10
Sau	3	0.27
MecA	3	0.27
Eco	5	0.46
Kpn	7	0.64
Pae	30	2.75
Aba	51	4.67
Sma	11	1.01
Hin	7	0.64
negative	963	-
Overall	1092	11.81

Note: LAMP, loop-mediated isothermal amplification; Spn, *Streptococcus pneumoniae*; Sau, *S. aureus*; MecA, Methicillin-resistant *S. aureus*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pae, *Pseudomonas aeruginosa*; Aba, *Acinetobacter baumannii*; Sma, *Stenotrophomonas maltophilia*; Hin, *Haemophilus influenzae*.

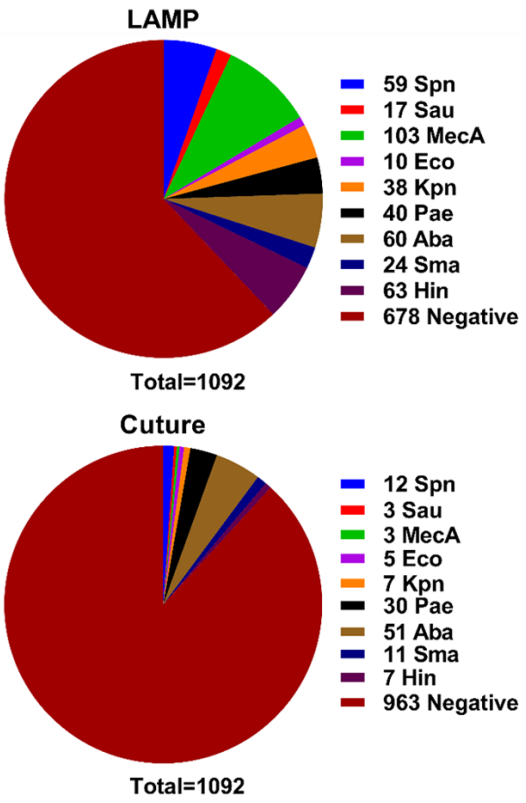

Figure 3. Comparison of pathogen distribution between the loop-mediated isothermal amplification (LAMP) assay and bacterial culture. Note: Spn, *Streptococcus pneumoniae*; Sau, *S. aureus*; MecA, Methicillin-resistant *S. aureus*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pae, *Pseudomonas aeruginosa*; Aba, *Acinetobacter baumannii*; Sma, *Stenotrophomonas maltophilia*; Hin, *Haemophilus influenzae*.

microbiological evaluation is very important for them treatment [17]. The LAMP assay is a rapid, simple and cost-effective method for determining pathogens. However, inconsistent results between the LAMP assay and bacterial culture hinder its popularization and adoption. Previous studies used sputum specimens as the research object and compared the LAMP assay with sputum culture to reflect the detection efficiency of the LAMP assay [9, 18]. The consistency between the two methods tends to decrease when oral microorganisms contaminate sputum, which leads to undervaluing of the diagnostic performance of the LAMP assay. Although some studies suggest filtering out the influence of contamination from the normal pharyngeal flora using statistical methods to identify disease-causing pathogens [5, 9], the application of these models are relatively difficult. Compared with sputum specimens, BALF is less likely to be contaminated with oral microorganisms during collection. Hence, BALF is a more suitable sample to accurately reflect the detection performance of the LAMP assay.

In this study, 1092 BALF samples were assessed using the LAMP assay and bacterial culture simultaneously. The results demonstrated that the consistency between the two tests was good, especially for Pae (Kappa = 0.735) and Aba (Kappa = 0.687). In another study, the LAMP assay and bacterial culture were performed on 1855 samples (1767 sputum and 88 lavage fluid) and the consistency between the two approaches was low. Of all the pathogens tested, Pae had the highest Kappa coefficient, which was only 0.311 [18]. The explanation for the discrepancy between these studies may be that the proportion of BALF in the total samples was much lower in the previous study (88/1855, 4.7%) compared to our study. This might indirectly reflect that oral flora have a significant impact on sputum samples. The consistency between the LAMP assay and bacterial culture was better with BALF than with sputum samples.

The main reason for the low consistency between the two methods for detecting the pathogens in our study may be that bacterial culture generates false-negative results. Notably, 316 LAMP assay results were positive but culture results were negative, suggesting that many pathogens may not be detected in bacterial culture. Culture results can yield a false negative, if inoculated bacteria die during

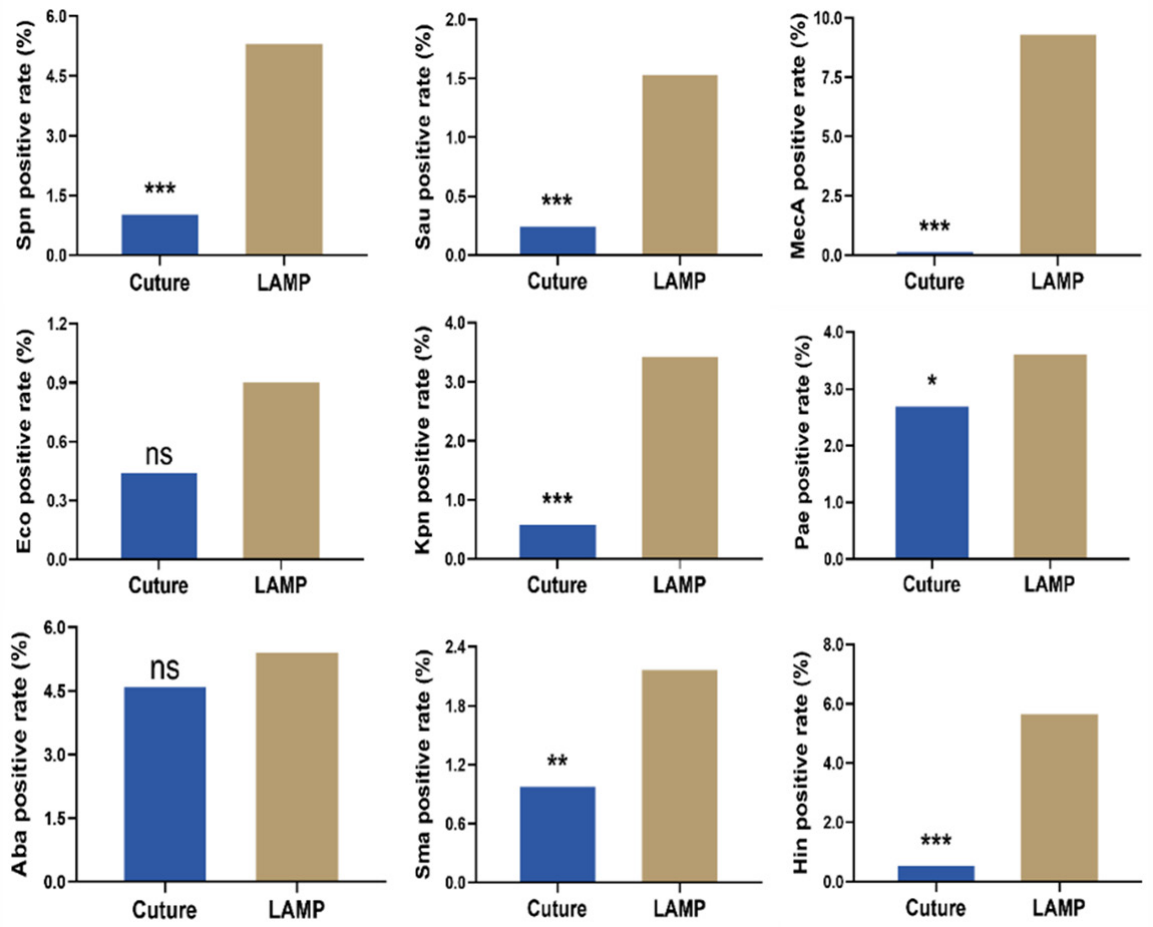

Figure 4. Comparison of positive rate for each pathogen between the loop-mediated isothermal amplification (LAMP) assay and bacterial culture. Notes: Spn, *Streptococcus pneumoniae*; Sau, *S. aureus*; MecA, Methicillin-resistant *S. aureus*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pae, *Pseudomonas aeruginosa*; Aba, *Acinetobacter baumannii*; Sma, *Stenotrophomonas maltophilia*; Hin, *Haemophilus influenzae*; NS, no significance; The statistical analysis was performed using the McNemar χ^2 test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 4. Agreement of the results between the LAMP assay and bacterial culture

Pathogen	LAMP (+) Culture (+)	LAMP (+) Culture (-)	LAMP (-) Culture (+)	LAMP (-) Culture (-)	Kappa	*P*-value
Spn	10	49	2	1031	0.268	< 0.001
Sau	3	14	0	1075	0.297	< 0.001
MecA	0	103	3	986	-	-
Eco	2	8	3	1079	0.262	< 0.001
Kpn	6	32	1	1053	0.259	< 0.001
Pae	26	14	4	1048	0.735	< 0.001
Aba	39	21	12	1020	0.687	< 0.001
Sma	7	17	4	1064	0.392	< 0.001
Hin	5	58	2	1027	0.133	< 0.001
Overall	98	316	31	-	-	-

Note: LAMP, loop-mediated isothermal amplification; Spn, *Streptococcus pneumoniae*; Sau, *S. aureus*; MecA, Methicillin-resistant *S. aureus*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Pae, *Pseudomonas aeruginosa*; Aba, *Acinetobacter baumannii*; Sma, *Stenotrophomonas maltophilia*; Hin, *Haemophilus influenzae*, the agreement between two tests was measured using the Kappa coefficient, $P < 0.05$ indicated that the consistency between the test results was statistically significant.

the lengthy culturing process [9, 19]. Rapid diagnostic testing use on BLAF for MecA significantly reduces use of anti-MecA antibiotic in patients with suspected pneumonia [20]. The LAMP assay and PCR show high consistency in identifying S. aureus and MecA [21, 22]. However, there was a significant difference in the detection of S. aureus and MecA between the LAMP assay and bacterial culture in our study. The most likely explanation is that the pathogens were not detected in bacterial culture, as only about one-third of patients with LRTIs was positive in BALF bacterial culture [23]. On the other hand, the false positives in the LAMP assay caused by nonspecific amplification may also reduce the consistency between the two methods.

Compared with single infection, mixed infections have a worse effect on the progression of LRTIs. It is not a single pathogen but a combination of multiple pathogens that leads to LRTIs has become the dominant view [24]. In addition to a significantly higher positive rate than that of bacterial culture, the LAMP assay detected more than half of the infections as mixed infections. Although bacterial culture is the gold standard for clinical pathogen diagnosis, it was not regarded as a reference standard for determining the sensitivity and specificity of the LAMP assay in our study. In addition, many studies show a high consistency between the LAMP assay and sequencing analysis [22, 25-27]. As new technologies emerge, the status of bacterial culture as the gold standard for pathogen diagnosis will be challenged.

There are a few limitations to our study. First, some patients were treated with antibiotics before arriving at the hospital. Recent antibiotic therapy would significantly reduce the positive rate of BALF bacterial culture [23, 28]. Due to the existence of dead bacteria, the LAMP assay would be positive but the culture negative, which would decrease the consistency between the two test results. Second, because our study was a retrospective, single-center observational study, it was difficult to control for potential confounding factors. Third, because simultaneous qPCR detection of the samples was not performed, we could not evaluate the performance of the LAMP assay using qPCR. Consequently, we could not determine if the positive cases identified by the LAMP assay were true pathogen infections or nonspecific gene amplification.

In conclusion, to our knowledge, this is the first report utilizing BALF as a sample to assess the consistency between the LAMP assay and bacterial culture. The present study demonstrated that the two methods might show better consistency in pathogen diagnosis than previously believed. Our study provides an evidence-based foundation for the clinical application of the LAMP assay. The LAMP assay is a suitable diagnostic tool for detecting pathogens from patients with LRTIs.

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Disclosure of conflict of interest

None.

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