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Original Article

Application of loop-mediated isothermal amplification (LAMP) assay for the rapid diagnosis of pathogenic bacteria in clinical sputum specimens of acute exacerbation of COPD (AECOPD)

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Abstract: The present study explores the application of LAMP for rapid diagnosis of pathogenic bacteria in clinical sputum specimens of AECOPD as compared with conventional sputum culturing method. 120 sputum specimens of AECOPD patients, 46 sputum specimens of healthy controls, as well as 166 serum specimens as negative controls, were evaluated by LAMP assay using primers of eight typical respiratory pathogens. No cross-reactivity was observed in these negative control species using LAMP assay. The lower detection limit of LAMP assay was approximately 10^3 copies. 25 cases (20.8%) were detected at least one positive bacteria species by conventional sputum culturing method, while 73 cases (60.8%) were tested positive in LAMP assay. Moreover, compared with sputum culture, bacterial titers results of LAMP assay were more consistent with FEV_1/FVC value of AECOPD patients. These results indicated that the sensitivity of LAMP assay was significantly higher than that of sputum culturing method.

Keywords: Acute exacerbation of COPD, pathogenic bacteria, loop-mediated isothermal amplification (LAMP), conventional culturing method, sputum specimen

Introduction

Acute exacerbation (AE) is a frequent episode during the prolonged chronic course of chronic obstructive pulmonary disease (COPD), which entails significant morbidity and mortality [6]. The common aetiological factors of AECOPD are bacterial, viral infection and air pollutants [8]. It is evident that bacterial infections contribute to and are implicated in the majority of AECOPD episodes, the most commonly associated organism being *Haemophilus influenzae*, *Streptococcus pneumonae*, *and Moraxella catarrhalis* [13]. Recent studies have shown that *Haemophilus parinfluenzae* or *Pseudomonas aeruginosa* were also present in exacerbations of COPD, particularly in patients with more severe airflow limitation [2, 9]. In addition, data confirmed that *Chlamydia pneumoniae* present in 18% of AECOPD while in 5-10% of mild to moderate exacerbations [15], which means sputum atypical bacterial pathogen is associated with AECOPD. However, causal infective agents may not be isolated from up to 50% of purulent sputum samples based on conventional culturing methods from these patients.

Since conventional bacterial culturing methods for etiologic diagnoses of AECOPD take at least 48 to72 hrs to complete and frequently produce false-negative results [14], the treatment of AECOPD is often imprecise. Recently, several investigators have reported PCR-based detection systems such as real-time PCR and loop-mediated isothermal amplification (LAMP) for rapid diagnosis of bacterial infection in clinical specimens [1, 19], suggesting that nucleic acid-based assays hold greater promise for diagnosis of bacterial infection.

LAMP is highly specific for the target sequence because of the recognition of the target sequence is strictly manipulated by six independent sequences in the initial stage and by four

Table 1. Clinical characteristic of AECOPD patients and healthy controls

Group	AECOPD	healthy controls
No. of sputum specimens	120	46
Age, year*	59.4 (11.7)	51.4 (12.6)
Male/Female	74/46	31/15
Duration of acute symptoms, d*	10.3 (6.1)	
Smoking, n/N (%)		
Nonsmokers	57 (47.5)	12 (26.1)
Ex-smokers	46 (38.3)	5 (10.9)
Current smokers	17 (14.2)	29 (63)
Respiratory rate/min*	19.7 (3.3)	17.6 (3.8)
Pulse rate/min*	83.9 (12.4)	79.1 (13.7)
Temperature > 37.5°C, n/N	18/120	
Leukocytes > 10.5×10^9/L, n/N	13/120	
Lung function*		
FEV_1, L	1.6 (0.4)	3.1 (0.8)
FEV_1, % pred	43.1 (7.7)	83.5
FVC, L	2.3 (0.6)	3.7 (0.7)
FVC, % pred	59.4 (6.2)	95.6
FEV_1/FVC	68.3% (15.3)	83.8
Dyspnea, n/N (%)#		
Mild	9 (7.5)	
Moderate	64 (53.3)	
Severe	47 (39.2)	
Cough, n/N (%)#		
Mild	2/120 (1.7)	
Moderate	57/120 (47.5)	
Severe	61/120 (50.8)	
Increased sputum, n/N (%)#		
Mild	5/120 (4.2)	
Moderate	46/120 (38.3)	
Severe	69/120 (57.5)	
Wheeze, n/N (%)#		
Mild	4/120 (3.3)	
Moderate	75/120 (62.5)	
Severe	41/120 (34.2)	

independent sequences during the later stages of the LAMP reaction [11, 16]. The amplification efficiency of LAMP is higher than PCR, because there is no time loss for thermal change based on its isothermal reaction [4, 10]. In the conventional LAMP assay, the production of LAMP reaction can be directly monitored real-time by turbidity of magnesium pyrophosphate. Moreover, using of two additional loop primers not only accelerates LAMP reaction, but also further improves the kinetics and sensitivity of the LAMP reaction [7]. Therefore, LAMP is performed under isothermal conditions as a promising approach with high specificity, selectivity, and cost-effective for nucleic acid amplification [12]. In our previous studies, we compared the performance of LAMP with standard bacterial culturing method in detecting a panel of eight common respiratory bacterial pathogens from sputum samples, and demonstrated that LAMP assay is a reliable method for the quantification of pathogens in sputum samples of lower respiratory tract [5].

The present study explores the application of LAMP assay for rapid diagnosis of eight kinds of pathogenic bacteria in clinical sputum specimens as compared with conventional culturing methods. Data on the sensitivity and specificity of the method compared with standard bacterial culturing method are reported, and applicability of the technology for clinical diagnosis of pathogenic bacteria in sputum specimens of AECOPD is discussed.

Materials and methods

Ethics statement

The study was approved by and carried out under the guidelines of the Ethical Committee of The First Affiliated Hospital of Nanchang University, Jiangxi Province, China. All patients or negative controls provided written informed consent for the collection of samples and subsequent analysis. The sputum specimens were collected between 1st January 2012 and 31st December 2013.

AECOPD patients, healthy and negative controls selection

A total of 120 AECOPD patients were diagnosed according to the criteria of the American Thoracic Society. Acute exacerbation was defined by the presence of an increase in at least two of the three following symptoms: dyspnea, cough and sputum purulence. Admission to the hospital was deemed necessary based on the clinical situation of the patient or the presence of complicating factors as respiratory failure. In all cases, the need for admission was decided by a senior chest physician, experienced in the management of COPD patients.

Table 2. Primer sequences used for amplification of pathogenic bacteria DNA by LAMP assay

Species	Gene	GenBank No.	Primer	Sequence
S. pneumoniae	*lyt*A	AJ243399	F3	CTGGAGGAAGCACACAGA
			B3	GTCTGGTTTGAGGTAGTACC
			FIP	CACCTTCTTCGTTGAAATAGTACCA-CTGGTTCGACAACTCAGG
			BIP	GACAGGCTGGGTCAAGTACAA-TGGATAAAGGCATTTGATACC
			LF	AGCGATTTTCTTCCAGCC
			LB	CTTAGACGCTAAAGAAGGCG
S. aureus	*fem*A	BA000033	F3	TGAATCATGATGGCGAGAT
			B3	CGTGTTTCTTTTTCTAAGTCCA
			FIP	ATGGAATCCAGTATGTTCAAATCCT-AGGTAATGCTGGTAATGATTGG
			BIP	AAGGATTTGATCCTGTGCTACAAA-TTTAATGATGTCATCTGCTGTT
			LF	AAGTTACTCATTTTATCAAAGA
			LB	TTCGTTATCACTCAGTGTTAGA
E. coli	*pho*A	U00096	F3	GCGCGTGGTTATCAGTTGG
			B3	CCAGGGTTGGTACACTGTC
			FIP	CGTCAGCAAACAGGCCAAGCA-TCACTGAATTCGGTGACGGA
			BIP	AGGACCGAAAGCAACGTACCAC-GCGGATTAGGCGTACAGG
			LF	GGGGTTTTTGCTGATTCGCT
			LB	TATCGATAAGCCCGCAGTCA
K. pneumoniae	KPN_04473	CP000647	F3	CGCCACTATCGACAGTCAG
			B3	TGCAGACCGGTAAAACTCAA
			FIP	ATCGCTGTGGCTATAGGTGCTG-AGCCTGGGCTGAATCTGG
			BIP	TCTTGCCCGCGATATTCACACC-CGAAAATGCCGGAAGAGGTA
			LF	GCAGGCGCCATGGTC
			LB	AGCCAGCTGGTGGTCG
P. aeruginosa	*opr*I	M25761	F3	CTGGATATTTTTTGAACAAACGA
			B3	GTTCATCGTGTTCCCCTTA
			FIP	TAAACTGACCAAGCGCAAGC-CCAACTACTGCTAAAGTCGG
			BIP	ACAAGTAATGGGTAGTATGTAGCCG-GGACATTTCCATAACAGCAATC
			LF	AGCAACTTTTTTTTTAGTCCCC
			LB	GCTAATTTCCCCGGCTG
A. baumannii	*ade*S	CP000521	F3	TGTGCCAATTAACTTCTTAGC
			B3	CTTGTAATATCGTTATAGGCGTT
			FIP	AGTGAATTCGGTTATCGTAAGCTC-GAAGCAGCAAAAAAAATTAGTCAC
			BIP	AATGATATGGCTCAAAAGCTAGAGG-CTTAACTCATGTGCGATGG
			LF	TAGCAGAGAGGTCGCC
			LB	CCGTTAAAAATGCGCAGG
S. maltophilia	*stm*Pr	AY253983	F3	ATGCCGACCTGTACGT
			B3	CCCATGGAGAGGGTCTTG
			FIP	GTCTCGCTGTTGCCGCTCA-CAGTGCCCCGACCGATA
			BIP	GTGGTATGTGCGGGTGAAGG-AGTACTGGGCGTTGAGG
			LF	TACGGACGGCAGGTGTA
			LB	TACAGCACCTTCTCGGG
H. influenzae	*omp*P6	CP000671	F3	GCAGATGCAGTTAAAGGTT
			B3	GCTAATTGGTTAAATTACAAACGA
			FIP	ACCTAATACTGCAGGTTTTTCTTCA-GGTAAAGGTGTTGATGCTGG
			BIP	GAAGCTGCATATTCTAAAAACCGTC-AAAAATGGATCCTGTTTTTCAAGT
			LF	CCGTAAGATACTGTGCCTAATT
			LB	GCAGTGTTAGCGTACTAATTCT

Chest X-rays were performed in each patient on admission and patients with lobar infiltrates or radiologic signs of pneumonia on chest X-ray were excluded from the study.

For all included patients the following data were assessed: (1) Lung function; (2) duration of acute symptoms; (3) dyspnea; (4) purulent sputum, as defined by a Gram stain showing; (6) sputum leukocytes; (7) cough; and (8) written informed consent.

Exclusion criteria were: (1) hospitalized status; (2) treatment with any antibiotic for 24 h or longer within 72 h before the baseline visit; (3) absence of an adequate sputum specimen as determined by Gram stain; (4) evidence of bronchiectasis and/or pneumonia; and (5) malignancy or severe immunosuppression.

46 healthy controls were defined as healthy individuals residing in Jiangxi Province, who were COPD negative, had no clinical symptoms related to respiratory infections.

After a thorough history and examination, the enrolled AECOPD patients and negative controls cleaned the mouth with 0.9% sodium chloride, and then processed in accordance with the standard procedures of induced sputum. Each patient collected two sputum specimens which used for routine culture and LAMP assays. While sputum sample of healthy individuals was defined as healthy control, the serum of each individual was defined as negative controls (**Table 1**).

Primer design

Oligonucleotide primers used for LAMP assay were targeted to highly conserved sequence in these eight kinds of bacteria as shown in **Tables 2** and **3**, which have been successfully applied in our previous study [5]. These primers include two outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and two loop primers (LF and LB) (**Table 2**).

Standard bacterial strains and standard concentration of bacteria

Standard Streptococcus pnuemoniae, Staphylococcus aureus, Escherichia coli, Klebsiella pnuemoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia, and Haemophilus influenzae were purchased from Biochip Beijing National Engineering Research Center. The standard bacteria strains were diluted by 10-fold gradient to establish a standard curve of bacterial titers, which including a total of four concentrations: 1×10^3, 1×10^4, 1×10^5, 1×10^6 copies/ml. After LAMP reaction, the standard curve was established based on bacterial titers as the abscissa, while Ct value as the ordinate. And then the titers in sputum specimens would be calculated based on the obtained Ct values.

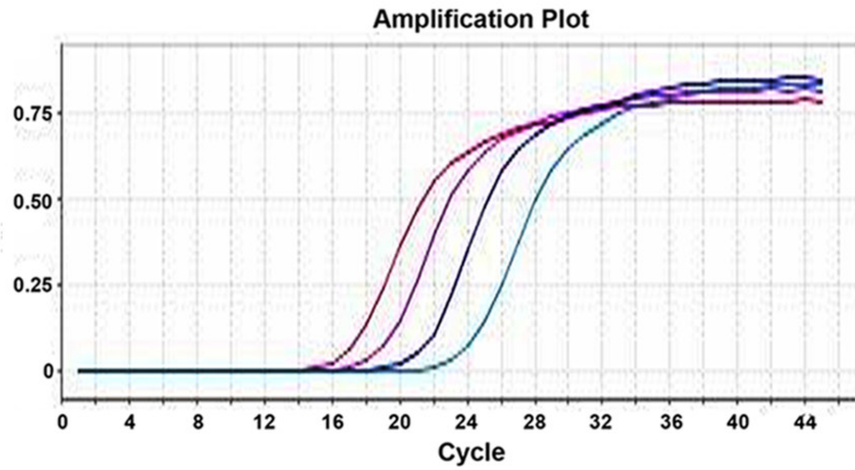
DNA extraction and LAMP assay

Each sputum sample or serum sample was liquefied in equal volume of 10% NaOH, and then the DNA in sputum samples was isolated using the universal kit for bacterial DNA extraction (Capitalbio Corporation, P. R. China). The concentration of DNA was quantified by Thermo Bio-Mate3 and diluted into 1 ng/μL. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan). A reaction mixture (25.0 μL) containing 12.5 μL Isothermal Master Mix, 1.6 μM of each inner primer (FIP and BIP), 0.2 μM of each outer primer (F3 and B3), 0.8 μM of each loop primer (LPF and BPF), 8 U of Bst DNA polymerase, 0.8 mM of dNTPs, 0.8 M betaine, 8 mM $MgSO_4$, 0.3 mM EvaGreen and 2 ng template DNA. The LAMP reaction was performed at 65°C for 45 min in a Loopamp real-time turbidmeter (LA-200; Teramecs) and then heated above 80°C for 2 min to terminate the reaction. Negative controls (double distilled water and genomic DNA in serum of AECOPD patients) were included in each run. The identity and purity of LAMP products were assessed by melting curve analysis. The reaction parameter is set to 95°C for 15 s, 60°C for 60 s, 95°C for 30 s, 60°C for 15 s, and then warmed to 95°C using a heating rate of 1°C/min.

Sputum cultures

Samples were collected in sterile sputum cups and sent to the laboratory within 1 h after expectoration. A Gram stain of the sputum in the area of maximal purulence was examined for polymorphonuclear leukocytes and epithelial cells. The number of leukocytes was semi-quantitatively described as: none, sporadic, few, moderate or many. A sputum sample was considered representative if many leukocytes were present in the absence of epithelial cells. Another portion of a documented purulent material was used for microbiological analysis. Sputa were processed according to standard microbiological methods. Sputum cultures were considered as positive (proving bacterial infection) if significant bacterial growth was

A



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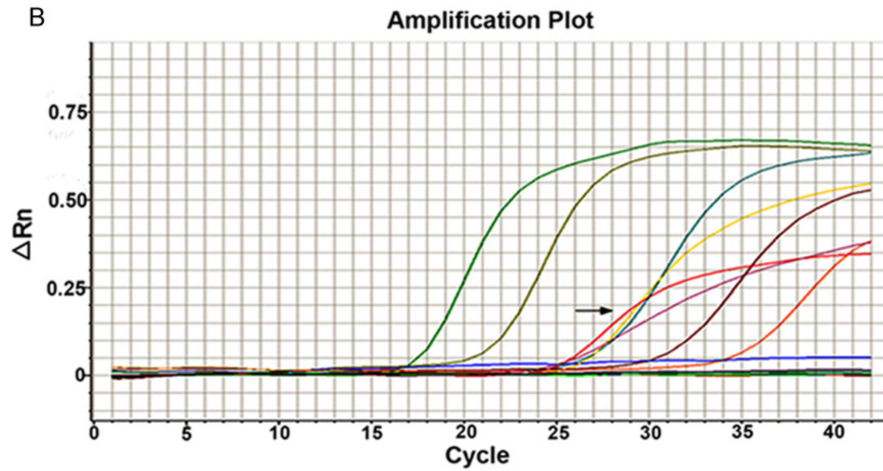

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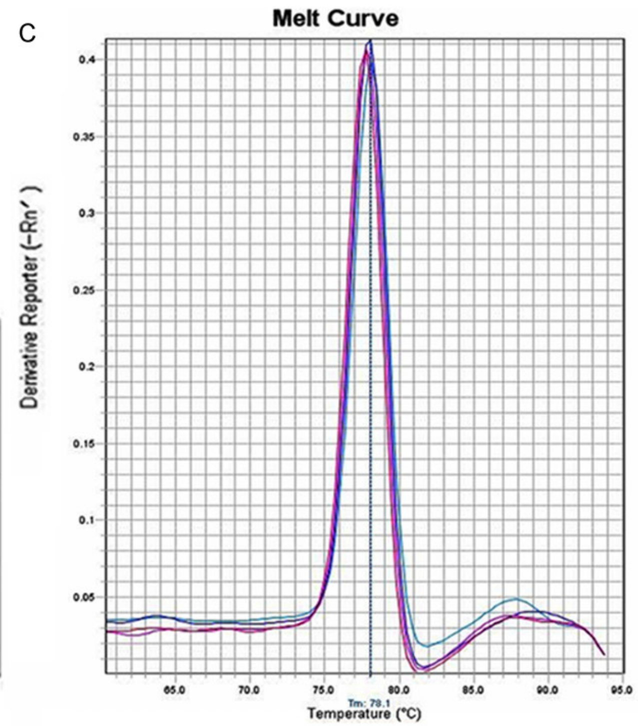

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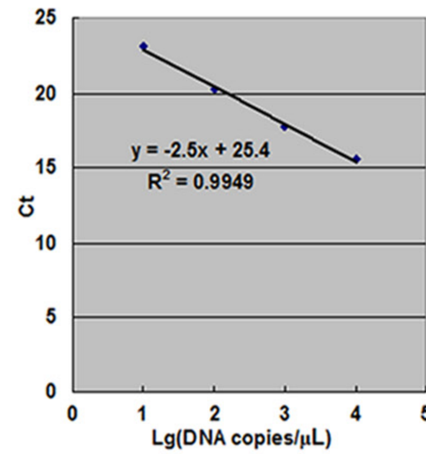

Figure 1. Sensitivity and specificity of LAMP assay. A. The Ct value of four titers of *S. aureus* detected by LAMP assay; B. Real-time monitoring of LAMP products using serial dilutions of 10^6 to 10^3 copies per reaction, the sputum samples reaction, as well as the negative controls reaction; C. The melting curve of LAMP products detected for *S. aureus*; D. The standard curve of *S. aureus* was established based on standard strain titer as the abscissa, while Ct value as the ordinate.

Table 3. Data from LAMP assays and sputum cultures

Groups	Brands		Sputum culture (+)	Sputum culture (-)	Total	P
Healthy controls	LAMP	(+)	*2*	3	5	> 0.1
		(-)	*5*	36	41	
	Total		7	39	46	
AECOPD patients	LAMP	(+)	*20*	53	73	0.027
		(-)	*5*	42	47	
	Total		25	95	120	

present as defined by the number of bacteria (higher than 10^5 colony forming units¼cfu) in a representative sample.

Statistical methods

Data was analyzed using SPSS16.0 statistical package (©IBM Corporation 2010). To evaluate the congruence of LAMP and culture results, we constructed a contingency table and used Fisher's exact and McNemar's tests. The FEV_1/FVC value less than 60% is considered positive, while the relatedness with results of bacterial titers was analyzed by Fisher's exact and McNemar's tests.

Results

Sensitivity and specificity of LAMP assay

To determine the sensitivity of the LAMP assay, the DNA from standard bacterial strains with known titer was serially diluted by 10-fold gradient up to 1×10^3 copies/ml. As shown in **Figure 1A**, the four titers of any standard bacterial strain were detectable in this LAMP assay, and the titer of sputum bacterial more than 1×10^3 copies/ml was defined as positive result. Both LAMP products and melting curves of DNA in sputum samples and serum samples were used to analyze the specificity of the LAMP assay. As shown in **Figure 1B**, real-time monitoring of LAMP products using serial dilutions of 10^6 to 10^3 copies per reaction, the sputum samples reaction, as well as the negative controls reaction. The results demonstrated that partial sputum DNA samples from AECOPD patients can be detected positive, but all DNA samples from the negative controls showed no positive products. As shown in **Figure 1C**, all of eight LAMP products appeared only a dissolution peak after temperature gradient warming. In addition, the standard curve of eight standard bacterial strains had good linear relationships, such as $r^2 = 0.9970$ for *Staphylococcus aureus* (**Figure 1D**).

Data from LAMP assays and sputum cultures

In the total of 120 sputum specimens isolated from AECOPD patients, a total of 25 sputum specimens (20.8%) were detected positive bacteria by sputum culture, while 73 sputum specimens (60.8%) were detected positive bacteria by LAMP assay (**Table 3**). However, 7 sputum samples (15.2%) in 46 healthy controls were detected positive bacteria by sputum culture, while 5 sputum samples (10.9%) were detected positive bacteria by LAMP assay (**Table 3**). During the Fisher's exact test, we dealt with the results as both positive if more than one same positive bacterium was detected both by LAMP and sputum culture in the same sputum specimen. The results showed that the positive rate of LAMP assay for AECOPD was proved to be significantly higher than that detected by sputum culture ($P = 0.027$) (**Table 3**).

Relatedness between LAMP and culture assays

As shown in **Table 5**, data from LAMP assay showed that the predominant bacteria were *S. pnuemoniae* (22.5%), *K. pnuemoniae* (21.7%), *S. maltophilia* (20.0%), *A. baumannii* (19.2%), followed by *H. influenzae* (17.5%), *P. aeruginosa* (15.8%), *S. aureus* (10.8%), and *E. coli* (7.5%). The positive rate of each bacterial pathogen detected by LAMP assay was higher than that detected by sputum culture. Except for three sputum specimens, all of positive samples detected by sputum culture were also proved to be positive by LAMP assay. The Fisher's exact test results proved that LAMP assay was more sensitive than sputum culture in detecting sputum pathogenic bacteria (**Table 4**).

Table 4. Positive rate of eight brands of pathogenic bacteria detected by LAMP and sputum culturing methods

strains	LAMP			Sputum Culture			χ^2	P
	Positive Number		Positive Rate	Positive Number		Positive Rate		
S. pnuemoniae	Culture (+)	9	22.5%	LAMP (+)	9	8.3%	24.438	< 0.001
	Culture (-)	18		LAMP (-)	1			
S. aureus	Culture (+)	4	10.8%	LAMP (+)	4	3.3%	19.026	< 0.001
	Culture (-)	9		LAMP (-)	0			
E. coli	Culture (+)	3	7.5%	LAMP (+)	3	2.5%	15.167	< 0.001
	Culture (-)	8		LAMP (-)	0			
K. pnuemoniae	Culture (+)	11	21.7%	LAMP (+)	11	10.0%	34.048	< 0.001
	Culture (-)	15		LAMP (-)	1			
P. aeruginosa	Culture (+)	7	15.8%	LAMP (+)	7	5.8%	33.093	< 0.001
	Culture (-)	12		LAMP (-)	0			
A. baumannii	Culture (+)	9	19.2%	LAMP (+)	9	7.5%	17.678	< 0.001
	Culture (-)	14		LAMP (-)	0			
S. maltophilia	Culture (+)	9	20.0%	LAMP (+)	9	8.3%	28.807	< 0.001
	Culture (-)	15		LAMP (-)	1			
H. influenzae	Culture (+)	9	17.5%	LAMP (+)	9	7.5%	35.250	< 0.001
	Culture (-)	12		LAMP (-)	0			

Table 5. Relatedness between FEV_1/FVC values and results from LAMP assay or sputum culture

Brands		FEV_1/FVC (< 60%)		Total	χ^2	*P*
		(+)	(-)			
LAMP	(+)	62	11	73	2.333	> 0.1
	(-)	*4*	43	47		
Total		66	54	120		
Sputum culture	(+)	*21*	4	25	34.3	< 0.01
	(-)	*45*	51	96		
Total		66	54	120		

Relatedness between FEV_1/FVC values and bacterial titers results

As shown in **Table 5**, in these 120 sputum specimens isolated from AECOPD patients, a total of 11 sputum specimens (9.2%) were detected positive bacteria by LAMP assay, but with negative results in the FEV_1/FVC value. Meanwhile, only 4 sputum specimens (3.3%) with FEV_1/FVC value lower than 60%, but could not detected positive bacteria by LAMP assay. The result from Fisher's exact and McNemar's tests showed that bacterial titers results of LAMP assay were consistent with FEV_1/FVC value of these patients ($P > 0.1$).

As shown in **Table 5**, in these 120 sputum specimens, 4 sputum specimens (9.2%) were detected positive bacteria by sputum culture, but with negative FEV_1/FVC value. Meanwhile, 45 sputum specimens (3.3%) with positive FEV_1/FVC value which lower than 60%, but could not detected positive bacteria by sputum culture. The result from Fisher's exact and McNemar's tests showed that positive bacterial results of sputum culture were inconsistent with FEV_1/FVC value of these patients ($P < 0.01$).

Discussion

Respiratory tract infections induce local and systemic inflammation, and a decline in FEV_1value, which are often considered as the initiating event contributing to the acute exacerbation of COPD [3]. Previous studies revealed that pathogenic bacterium were partially detected in sputum samples of both COPD and AECOPD patients. However, AECOPD patients had a higher incidence of infections with *Pseudomonas aeruginosa* and other Gram-negative bacteria especially in those patients with more compromised lung function [9]. Conventional sputum culture is still useful in researching the pathogenesis of exacerbations of COPD. However, previous studies have shown that sputum culture, when used alone as a detection method to delineate the pathogenic

bacterium of AECOPD, has major limitations: (1) long test period (2-3 days); (2) low positive rate; (3) atypical bacterium are difficult to be detected; (4) specimens susceptible to be contaminated [18].

In the present study, we demonstrated that LAMP assay is a potential diagnostic tool for pathogenic bacterium detection in low airway sputum specimens of AECOPD. The positive results of LAMP reaction were higher than standard sputum culture. However, as all DNA-based methods do, LAMP also has its potential drawbacks. The biggest drawback is that the sensitivity and accuracy of LAMP results are susceptible to be affected by the effect of sequence mutations on pathogenic bacterium. In order to minimize this effect, we have deliberately selected the most characteristic and conservative genes as target sequences. However, it is still possible that clinical strains harbor mutations on the sequences where primers are designed. Despite this, LAMP is less affected by mutations than other DNA-based methods, such as real-time PCR, for its long primers that cover over 160 bp in length [5]. One or two mutations often do not make distinguishable effects on amplification. Another drawback is that the sputum specimen is susceptible to be contaminated by the upper airway liquid. In order to minimize this effect, the sputum specimens were collected in lower trachea by a modified cytology brush technique through the channel of a flexible fiberoptic bronchoscope.

The clinical application of LAMP assay in detecting bacterium has a confessed handicap by the discrepancies between their results and those from sputum culture method. The targets for LAMP are representative sequences from both alive and dead pathogens, while sputum culture only detects all living bacterial cells grown in the medium [17]. Although the DNA from dead bacteria may cause some interference to LAMP result, the condition changes during sputum culture would have a greater deviation. In order to verify which one of these two methods is more consistent with the clinical symptoms of AECOPD, we chose FEV_1/FVC value as an indicator of AECOPD, and defined the FEV_1/FVC value less than 60% is a positive specimen of AECOPD. Our results demonstrated that bacterial titers results of LAMP assay, but not sputum culture results, were consistent with FEV_1/FVC value of these patients. It indicates that compared with conventional sputum culturing method, LAMP assay is a better clinical diagnostic tool for rapid detection of pathogenic bacteria in clinical sputum specimens of AECOPD.

Conclusions

LAMP assay is faster and more sensitive than sputum culturing method in detecting pathogenic bacteria in sputum specimen of AECOPD patients. Compared with sputum culture, bacterial titers results by LAMP assay were more consistent with FEV_1/FVC value of AECOPD patients. LAMP assay is a potential diagnostic tool for pathogenic bacterium detection in sputum specimens of AECOPD.

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Disclosure of conflict of interest

None.

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