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Research Article

A Pilot Study of Quantitative Loop-Mediated Isothermal Amplification Guided targeting therapies for Hospital-Acquired Pneumonia

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Abstract

Background and Objectives: Quantitative loop-mediated isothermal amplification (qLAMP) has been considered an efficient approach for validating infectious pathogens. We assessed that the qLAMP could be served as a great implement for steering of the antibiotic decision making in hospital-acquired pneumonia.

Methods: Total 76 respiratory tract aspiration samples were prospectively collected from 60 patients with HAP, who were admitted to Peking University People's Hospital between August 2011 and March 2014. DNA was isolated from these samples. Specific DNA fragments for identifying 11 pneumonia-related bacteria were amplified by qLAMP assay. Culture results of these patients were compared with the qLAMP results. Clinical data and treatment strategies before and after reporting the qLAMP results were analyzed to evaluate the effects of qLAMP results on clinical data. Data were analyzed by McNemar test and Fisher's exact tests with SPSS 19.0.

Results: The detection of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Stenotrophomonas maltophilia, Streptococcus pneumonia, Acinetobacter baumannii* by qLAMP were consistent with sputum culture ($P>0.05$). The improvement of clinical condition was more significant ($P<0.001$) in patients with pathogen target-driven therapy based on qLAMP results than those with empirical therapy.

Conclusions: qLAMP is a more promising method for detection of pathogens in an early, rapid, sensitive and specific manner than culture, which is a good alternative for steering the targeted therapies in time.

INTRODUCTION

Hospital-acquired pneumonia (HAP) is defined as a low respiratory tract infection developed 48 h after hospital admission in a patient without infection at admission [1]. HAP currently ranks second among nosocomial infections, and accounts for one fourth of the infections in intensive care units (ICU) [1-4]. HAP has a significant impact on the financial burden of health care, and new cases drive the increasing emergence of pathogens with multi- or pan-antibiotic resistant traits. Therefore, identifying the infectious etiology in different settings is the key step for mitigating or obviating the severe infection in time. This is especially true for HAP, since early identification of specific pathogens could significantly impact the morbidity and mortality of HAP, and lower the cost of treatment as well. So far, the most

common method used to implement HAP etiological diagnosis is still tied to sputum culture. Sputum culture, however, shows significant disadvantages as a method for identifying pathogens. In addition to sputum culture's relatively low sensitivity and the difficulty with which it identifies atypical pathogens [1,5,6], the time required to obtain results from sputum culture always leads to empirical, broad-spectrum antibiotic therapies rather than targeted, narrow-spectrum therapies for patients with HAP. This, in turn, often exacerbates the rise of antibiotic resistance.

An innovative rapid method for etiology diagnosis of HAP, the quantitative loop-mediated isothermal amplification (qLAMP), has already been used in the diagnosis of virus, fungus, parasite and tuberculosis infections and is now commercially available [7-12]. It is a novel assay that focuses on the genetics of pathogens based on rapid nucleic acid amplification method. Therefore, this manner has two important advantages: rapid diagnosis and high sensitivity [13-15]. Furthermore, qLAMP is also a high specific assay, which could detect different bacteria with quantified copies [16]. As the excellent timeliness and accuracy of qLAMP for etiological diagnosis to the lower respiratory tract infection by our group[16], we initiated a pilot, prospective and interventional study to investigate the value of qLAMP to guide targeted, narrow-spectrum antibiotics therapies in a small group of patients with HAP. Based on the results, we found that qLAMP was a good alternative for steering the early-targeted narrow-spectrum antibiotics therapies in HAP patients.

MATERIALS AND METHODS

Study design

Patients with suspected HAP from August 2011 to March 2014 at Peking University People's Hospital were recruited to the study, which were approved by the ethical committee of Peking University People's Hospital (2011-83). These patients were initially diagnosed as suspected cases of HAP occurred more than 48 h after admission and were not incubating at the time of admission, having typical characteristics of pneumonia, which were firmly inferred from chest X-rays and the following criteria[17]: (1) at least one of the following: ① fever (>38.5 ^{0}C), ② leukopenia (peripheral white blood cell count(WBC) <4.0×10^9/L) or leukocytosis (WBC >10.0 ×10^9/L), ③ for adults 70 years old or older, mental status changes with no other recognized cause, and (2) at least two of the following: ① new onset of purulent sputum, or change in character of sputum, or increased respiratory secretion, or increased suctioning requirements, ② new-onset or worsening cough, or dyspnea, or tachycardia, ③ rales or bronchial breath sounds, ④ worsening gas exchange (PaO_2/FiO_2 ≤240), increased oxygen requirements, or increased ventilation demand. Patients with non-infectious diseases, viral infection, or TB were subsequently excluded from the study.

Once patients were enrolled, lower respiratory secretion samples were collected on the 1st day for both routine culture and qLAMP assays, of which the results were reported to the clinicians. Data of each patient was also collected from the medical chart, with particular attention to clinical manifestations and treatment strategies before and after the qLAMP results reporting.

To determine the final diagnosis and assess the treatment response for each patient, 2 independent pulmonologists blinded to qLAMP results reviewed all available medical records (including patient history, physical examination, results of laboratory tests, including blood routine examination, biochemical indicators, plasma electrolytes, blood gas analysis and chest radiograph) pertaining to the patient from the time of HAP presentation to discharge/death. Cases were reviewed and adjudicated by a third pulmonologist when confronting a disagreement.

Methods

After liquefied in equal volume of 10% NaOH, DNA specimen of each sample was isolated by using the Universal Kit for Bacterial DNA Extraction (Capitalbio Corporation, P. R. China). The specimens were then prepared for qLAMP by using a set of specific primers for *Streptococcus pneumonia (SP), Staphylococcus aureus (SA), Escherichia coli (EC), Klebsiella pneumonia (KP), Pseudomonas aeruginosa (PA), Acinetobacter baumannii (AB), Stenotrophomonas maltophilia (SM), Haemophilus influenzae (HI), Legionella pneumophila (LP), Mycoplasma pneumonia (MP), Chlamydophila pneumonia (CP)*. qLAMP primer system of each species of pathogen is composed of six primers recognizing eight distinct regions on the target DNA, termed a forward outer primer (F3), a backward internal primer (B3), a forward internal primer (FIP), a backward internal primer (BIP), and loop primers (LF and LB). Eight-pathogen primer sequences are used same as we did before, including SP, SA, EC, KP, PA, AB, SM and HI [16]. Those for atypical pathogens were redesigned as in table 1, and both their sensitivity and specificity were ensured by quantified DNA isolated in 27 bacterial species as we did before [16].

The reaction was performed at 65^{0}C for 45 min in a 25μl reaction mixture consisting of 1.6μM each of FIP and BIP, 0.2μM each of F3 and B3, 0.4μM each of LF and LB, 8U of the Bst DNA polymerase large fragment (New England Biolabs Inc., Beverly, Mass., USA), 0.4mM dNTP, 0.1mM dUTP, 0.8M betaine, 6mM $MgSO_4$, 0.5mg/ml BSA, 0.6 × EvaGreen (Biotium, Inc., CA, USA), 0.1U/ml Uracil-DNA Glycosylase (Fermentas Inc., MD, USA), 20mM Tris-HCl (pH 8.8 at 25^{0}C), 10mM KCI, 10 mM $(NH_4)_2SO_4$, 0.1%Triton X-100, and 2μl template DNA or PCR grade H_2O as negative control, and then heated at 80^{0}C for 5 min to terminate the reaction. All amplifications were performed with a RT-Cycler Real-time Fluorescence Quantitative PCR Instrument (Capitalbio Corporation, Beijing, China). The titer was quantified according to standard curves obtained from pre-quantified DNA templates, as described previously [16]. Biochip technology was introduced in January 2013 at Peking University People's Hospital and the reaction was performed on a microfluidic device after then.

The qLAMP tests and routine cultures were conducted by two experienced technicians awareless of the sample identities in two separated laboratories of Peking University People's Hospital.

Statistical analysis

We constructed a contingency table and used McNemar test to evaluate the congruence of qLAMP and culture results. The differences between patients with or without treatment strategies adjustment based on qLAMP results were tested with Fisher's exact tests.

RESULTS

qLAMP assaying outcome of the specimen from HAP patients

Seventy-six samples were recruited out of 103 eligible samples overall in our study (Figure 1). The 76 samples were collected from 60 patients with HAP several times. None of the samples were collected from the same onset of HAP. As seen in Table 2, there were 70 samples with qLAMP results greater than 10^5 copies/ml, 23 ones with qLAMP results between 10^3 and 10^5copies/ml, and 16 ones with qLAMP results below 10^3copies/ml.

Congruence of qLAMP and Culture Results

The concordance rates of the two assays for detecting *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Stenotrophomonas maltophilia, Streptococcus pneumonia, Acinetobacter baumannii* are 90.79%, 98.68%, 89.47%, 93.42%, 93.42%, 100.00%, 77.63%, respectively (Table 3). We also evaluated the differences between qLAMP and culture results by McNemar test, of which no significant difference was found ($p > 0.05$) (Table 3, Table S1-S7 in the online supporting information). The qLAMP results of 4 samples for *Haemophilus influenzae*, *Legionella pneumophila*, or *Mycoplasma pneumonia* were positive, while the culture results for these specimens were negative probably because of their low detectable rates in culture. We then studied the clinical data of these 4 samples and found that the qLAMP results were all reliable except 1 *Mycoplasma pneumonia* positive sample due

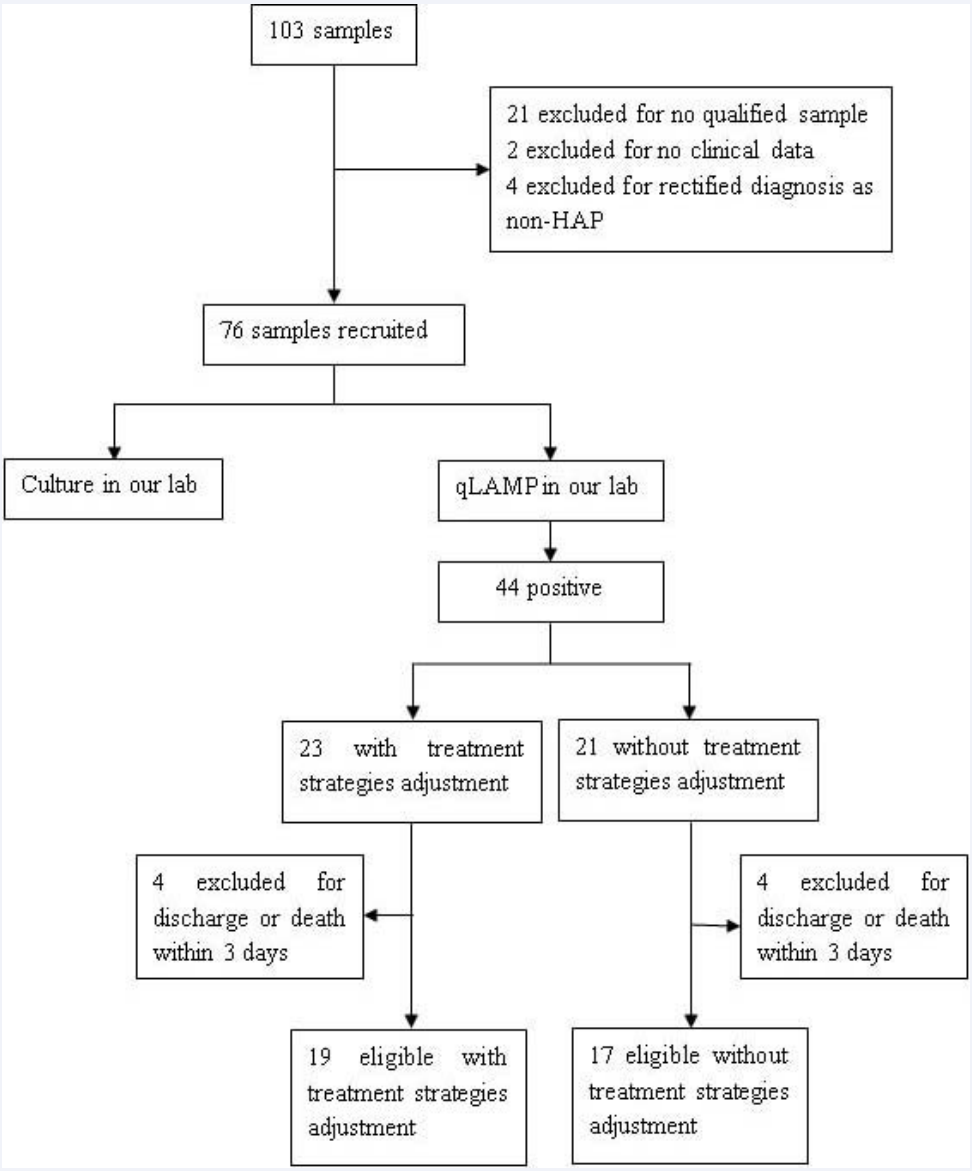

Figure 1 Study profile. For each eligible patient, we collected lower respiratory secretion samples on the 1st day for routine culture and qLAMP tests and reported the results to the clinicians. We also collected the clinical data and treatment strategies before and after reporting the qLAMP results.

Table 1: Primers for atypical pathogens used in this study.

Target species	Primers	Nucleotide sequence
Legionella pneumophila	F3	GCAAGACGCTATGAGTGG
	B3	TGATTACTTTGTATTGCAAACCA
	FIP	GCCATCAAATCTTTCTGAAACTTGT-CTCAATTGGCTTTAACCGAAC
	BIP	GCGGATGAAAATAAAGTAAAAGGGG-CTTGGCAATACAACAACGC
	LF	TAAGAACGTCTTTCATTTGCT
	LB	CTGAAAACAAAAACAAGCCAG
Mycoplasma pneumoniae	F3	GTTAAACCCGCAAACGCC
	B3	TGCTCATAGTACACCACGCT
	FIP	TGCAGCCCCACTCAAACCAA-GACCAAACCGGGCAGATC
	BIP	TCAAAAACAAGGTCCCCGTCGA-GGCACGAGTAAAACGGCAA
	LF	CGCCAAAGGGGTTAAAGGT
	LB	CAAGACCCCTCCAATCCCT
Chlamydophila pneumoniae	F3	AATTATAAGACTGAAGTTGAGCA
	B3	AGAGAGATATGGCATATCCG
	FIP	TTCTCTTAGAGGCAACGTAGACTTT-GGGAGATGCAGATTTAGATCA
	BIP	TCAAGTTGGAGATAAAATGGCTGG-CGGGAACGATTTTGGAAAC
	LF	ACCTTGGCGAATGACACCA
	LB	ACGACACGGAAATAAAGGTGTT

Table 2: qLAMP results*.

	qLAMP results				
	$>10^5$ copies/ml	10^3~10^5 copies/ml	$<10^3$ copies/ml	-	total
Streptococcus pneumonia	1	0	0	75	76
Staphylococcus aureus	11	5	6	54	76
Escherichia coli	1	0	1	74	76
Klebsiella pneumonia	8	4	3	61	76
Pseudomonas aeruginosa	17	3	0	56	76
Acinetobacter baumannii	20	5	1	50	76
Stenotrophomonas maltophilia	8	2	0	66	76
Haemophilus influenzae	1	1	3	71	76
Legionella pneumophila	1	3	0	72	76
Mycoplasma pneumonia	2	0	2	72	76
Chlamydophila pneumonia	0	0	0	76	76
total	70	23	16	727	836

*Data are presented as No. unless otherwise indicated.

Table 3: Congruence of qLAMP and culture results.

	Concordance rate	p value
Staphylococcus aureus	90.79%	0.453
Escherichia coli	98.68%	1.000
Pseudomonas aeruginosa	89.47%	0.070
Klebsiella pneumonia	93.42%	0.375
Stenotrophomonas maltophilia	93.42%	0.063
Streptococcus pneumonia	100.00%	1.000
Acinetobacter baumannii	77.63%	0.332

to the lack of specific species identified in final diagnosis. No *Chlamydophila pneumonia* positive results were reported in the 76 samples either by qLAMP or culture assay.

Targeting Therapies Guided by qLAMP for HAP

A total of 44 qLAMP-positive samples were identified in the study. Treatment strategies were adjusted in 23 of them based on qLAMP results. 8 samples were subsequently excluded from analysis because of discharge or death within 3 days after admission. The final analysis group of 36 patients consisted of 19 with treatment adjusted to narrow-spectrum targeted antibiotics therapies according to qLAMP results (pathogen target-driven

therapy group) and 17 without treatment strategies adjustment (empirical therapy group).

Demographic and clinical characteristics of the two groups: Demographic and clinical characteristics of the two groups are shown in Table 4. There is no significant difference ($p > 0.05$) for the characteristics of the patients between the two groups, including gender, age, complications, clinical manifestation (temperature, cough, sputum, rales), blood routine examination, biochemical indicators, blood gas analysis, blood coagulation index and chest radiograph infiltration.

Patients taking an advantage for clinical condition with pathogen target-driven therapy: The clinical condition of three days after qLAMP assay is ameliorated in group with pathogen target-driven therapy based on qLAMP results, which is much better than the group with empirical therapy (Table 5). The differences of the remission rates between these two groups evaluated by Fisher's exact tests are statistically significant ($p=2\times10^{-6}$).

Daily mean temperature of group with pathogen target-driven therapy shows a more obvious tendency of improvement than the group with empirical therapy (Figure 2). On the 1st day when samples were collected, the mean temperature of target-driven therapy group and empirical therapy group were 37.95^{0}C and 37.68^{0}C respectively, while the mean temperature of those two groups changed to 37.42^{0}C and 38.02^{0}C three days later. That is, the decrease of daily mean temperature was 0.53^{0}C in the

Table 4: Patient's demography and clinical characteristics*.

Characteristics (normal value)	Patients with pathogen target-driven therapy (n=19)	Patients with empirical therapy (n=17)	p value
Male, n (%)	15(78.95%)	9(52.94%)	0.16
Age, yr	74.26±10.99	78.00±8.48	0.27
Complications			
Hypoproteinemia, n (%)	15(78.95%)	12(70.59%)	0.71
Coronary Heart Disease, n (%)	9(47.37%)	4(23.53%)	0.18
Acute cerebrovascular disease, n (%)	4(21.05%)	5(29.41%)	0.71
Clinical Manifestation			
Temperature, ^{o}C	37.95±1.06	37.68±0.81	0.40
Cough, n (%)	19(100.00%)	17(100.00%)	-
Sputum, n (%)	19(100.00%)	17(100.00%)	-
Rales, n (%)	19(100.00%)	17(100.00%)	-
Blood Routine Examination			
WBC, $\times10^9$/L (4.0-10.0)	11.63±5.26	10.45±4.55	0.48
NE, % (50-70)	81.48±10.74	85.37±9.80	0.27
NE, $\times10^9$/L(2.0-7.0)	9.60±5.01	9.08±4.50	0.74
Hb, g/L(110-170)	103.32±19.83	96.84±19.51	0.33
PLT, $\times10^9$/L(100-300)	211.21±104.93	197.20±88.14	0.67
Biochemical Indicators			
ALT, U/L(0-40)	41.11±68.58	28.35±17.39	0.46
AST, U/L(0-40)	36.95±29.20	35.06±20.13	0.83
ALB, G/L(35-55)	31.59±3.57	30.38±5.46	0.43
CRE, umol/L(20-106)	76.21±73.42	70.00±39.52	0.76
BUN, mmol/L(2.9-8.3)	10.28±7.80	10.28±5.64	1.00
Blood Gas Analysis			
pH(7.35-7.45)	7.52±0.05	7.51±0.06	0.37
PaO_2, mmHg(80.0-100.0)	115.17±43.97	97.47±32.29	0.18
$PaCO_2$, mmHg(35-45)	38.79±8.03	40.12±9.56	0.65
HCO_3^-, mmol/L(21.4-27.3)	31.97±6.47	31.57±6.37	0.85
Oxygenation Index, mmHg(400-500)	230.02±113.91	229.10±96.36	0.98
Blood Coagulation Index			
PT, s(9.8-13.1)	12.81±2.13	14.05±5.90	0.42
APTT, s(25.4-38.4)	33.22±6.85	32.88±6.32	0.88
Chest Radiograph Infiltration, n (%)	19(100%)	17(100%)	-

*Data are presented as mean ± standard deviation unless otherwise indicated.

Table 5: Clinical condition improvement between two groups.

group	Clinical condition		
	Improved	**Unimproved**	**Improvement Rate, %**
Patients with pathogen target-driven therapy	16	3	84.2
Patients with empirical therapy	1	16	5.9
Total	17	19	47.2

*Data are presented as No. unless otherwise indicated.

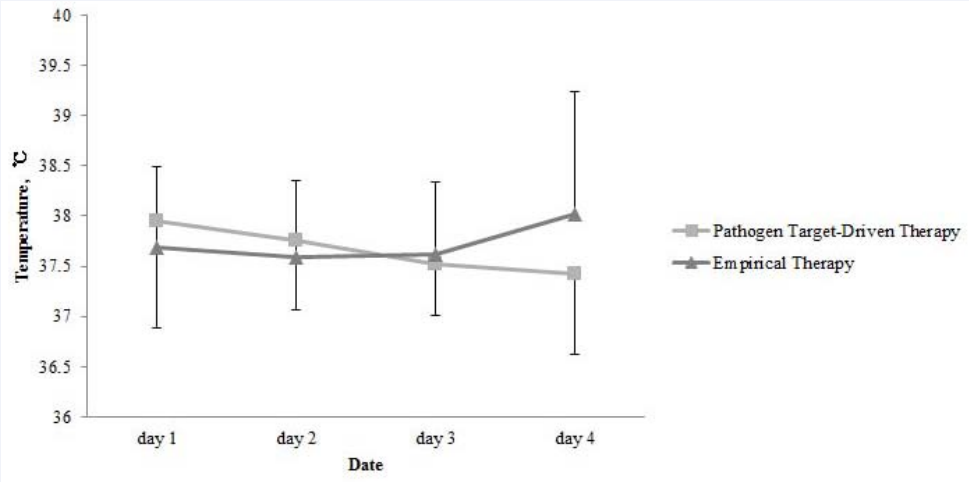

Figure 2 Temperature alteration between two groups. The body temperature of the group with pathogen target-driven therapy decreased, while the group with empirical therapy had no significant improvement in body temperature.

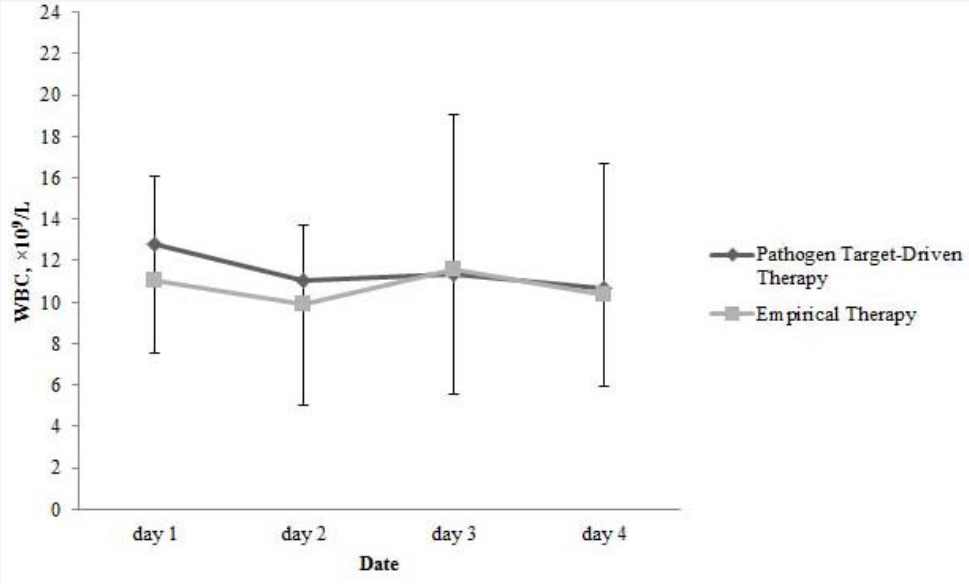

Figure 3 WBC alteration between two groups. The WBC of the group with pathogen target-driven therapy decreased, while the group with empirical therapy fluctuated in WBC.

group with pathogen target-driven therapy while the decrease in the group with empirical therapy was -0.34^{0}C. Similarly, decrease of total WBC number in group with pathogen target-driven therapy is more significant than the group with empirical therapy (2.15×10^9/L with pathogen target-driven therapy vs 0.70×10^9/L with empirical therapy, Figure 3).

DISCUSSION

To our knowledge, this is the first study to report the value of qLAMP to guide early targeted narrow-spectrum antibiotic therapies of HAP. This study is an exploration for the targeted therapies of HAP, which may have big effects on the mortality of HAP and reduce the cost of these patients. Although bacterial

pneumonia is a kind of curable diseases due to the advent of the antibiotics, the mortality of bacterial HAP is still high, which contributes to the delay of targeted antibiotics therapies according to the results of sputum culture.

As a new manner of detecting the etiology of different kinds of infections, qLAMP is now commercially available. With the availability of this rapid (results are available within 1-2 hours), sensitive and specific test, early targeted antibiotic therapy of infectious is now possibly feasible. Therefore, we apply qLAMP for the decision-making regarding whether we selected broad-spectrum empirical antibiotic therapies or the narrow-spectrum targeted antibiotic therapies for HAP patients.

Since we would investigate the value of qLAMP steering therapies, the first important issue was whether qLAMP can etiologically diagnose HAP in time. As qLAMP assay was much more rapid than sputum culture, the most common assay in recent clinical practice, we first focus on the congruency of the results of qLAMP and sputum culture. Fortunately, there was no significance between qLAMP and culture results of HAP patients with infections of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Stenotrophomonas maltophilia, Streptococcus pneumonia, Acinetobacter baumannii.* Also qLAMP can detect *Haemophilus influenzae, Legionella pneumophila,* and *Mycoplasma pneumonia,* which were not detectable by culture. Besides, qLAMP was a candidate method which could differentiate the pathogens between colonized and infectious status. After that, we prospectively enrolled 36 patients of HAP with the same baseline data to evaluate the value of qLAMP steering early targeted therapies. Among these patients, the qLAMP results were all positive based on the cut-off value (1.0×10^5copies/ml) which was established in our former work (data not shown). We randomly adjusted the regimen of these patients with empirical therapies according to the 2005 ATS/IDSA HAP GUIDELINE [1] or narrow-spectrum targeted therapies based on the results of qLAMP. Interestingly, we found that the clinical condition was significantly improved in the group with pathogen target-driven therapy compared to the group with empirical therapies.

There are a few limitations in our studies. Firstly, it was performed with a small sample size, the stochastic effects was too big to drive a definite conclusion. A second limitation was that we did not test the infection of fungus and virus of HAP, which may contribute a small number of HAP infections. A third limitation was that the drug sensitivity cannot be tested by qLAMP. Perhaps we could combine qLAMP and sputum drug sensitivity test to individualize the HAP regimens. However, the definition conclusion can only be driven after multi-centered, randomized and large sample sized research. Since qLAMP cannot test the drug sensitivity, the combination of qLAMP and sputum culture is a good choice to guiding early targeted therapies in HAP patients.

In conclusion, the qLAMP assay was a good alternative for steering early targeted therapies of HAP.

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Authors contributions

Fang Wang and Ran Li had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: ZG, YK.

Acquisition of data: FW, RL, DZ, DY.

Analysis and interpretation of data: FW, ZG, YK.

Drafting of the manuscript: ZG, YK, FW.

Critical revision of the manuscript for important intellectual content: ZG, YK, PT

Statistical analysis: FW.

Obtaining funding: ZG.

Administrative, technical, or material support: FW, RL, YS, DZ, DY, WC, WG.

Supervision: ZG.

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