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CLINICAL MICROBIOLOGY - RESEARCH PAPER

CelB is a suitable marker for rapid and specific identification of *Klebsiella pneumoniae* by the loop-mediated isothermal amplification (LAMP) assay

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Abstract

Klebsiella pneumoniae belongs to *Enterobacteriaceae*, which is the commonest bacterium causing nosocomial respiratory tract infection. It ranks second in bacteremia and urinary tract infection in gram-negative bacteria. Therefore, the rapid and accurate identification of *K. pneumoniae* was of great significance for the guide of clinical medication, and timely treatment of patients. The purpose of this study was to establish a rapid and sensitive molecular detection method for *K. pneumoniae* based on loop-mediated isothermal amplification (LAMP) technology. Firstly, local BLAST and NCBI BLAST were used to analyze the genome of *K. pneumoniae*. According to the principle of interspecific and intraspecific specificity, *CelB* (GenBank ID 11847805) was selected as the specific gene. Then, the LAMP and PCR identification systems were established with this target gene. Thirty-six clinical isolates of *K. pneumoniae* and 50 non-*K. pneumoniae* were used for the specific evaluation, and both LAMP and PCR could specifically distinguish *K. pneumoniae* from non-*K. pneumoniae*. A 10-fold series diluted positive plasmids and simulated infected blood samples were used as the templates in the sensitivity assay, and the results showed that the sensitivity could reach 1 copy/reaction. In summary, a rapid, specific, and sensitive LAMP method was established to detect *K. pneumoniae* in clinics.

Keywords *Klebsiella pneumoniae* · Molecular detection · Loop-mediated isothermal amplification · Polymerase chain amplification

Introduction

K. pneumoniae was widely distributed in nature, which was one of the normal microorganisms in the intestine [1]. As a conditional pathogen, it belongs to gram-negative bacteria. When the body's immunity declines, it enters the lung through the respiratory tract and causes the fusion of large lobes or small lobes, and finally caused pneumonia [2, 3]. It also ranks second in bacteremia and urinary tract infection in gram-negative bacteria, which is a great threat to people's health [4–6].

At present, traditional methods were used for bacterial identification and drug sensitivity identification in many hospitals, including microscopy, disk diffusion method, biochemistry, serotype, and antibiotic dilution method (minimal inhibitory concentration method) [7]. Although it has the advantages of easy operation and low cost, it was time-consuming and has low accuracy. With the rapid development of bioinformatics and molecular biology, conserved nucleic acid sequences with interspecies-specific and intraspecies commonality in the pathogens have been discovered one after another [8].

Yichen Tian and Lefei Wang contributed equally to this work.

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Based on this, an army of detection techniques has been established, such as the polymerase chain amplification (PCR), real-time fluorescent PCR, loop-mediated isothermal amplification (LAMP) [9–11]. The specific DNA fragments were amplified in vitro by PCR technology was developed in 1985 by Mullis et al. [12]. In 2000, Notomi et al. developed a novel, constant-temperature, enzyme-based nucleic acid amplification technique, and named it LAMP, which was a viable and cost-effective alternative to molecular diagnostics [13]. This technique was based on a set of specific primers (at least four primers, two internal primers (BIP and FIP) and two external primers (B3 and F3)) to recognize six different regions of the DNA at constant temperature (60 °C to 65 °C) [13]. Complementary strand synthesis was initiated base on the target DNA region, produce the mixture of stem-loop DNA with various fragment sizes and shapes, and the by-product was white pyrophosphate ion precipitated [14, 15]. Thus, the LAMP could be used in the POCT (point-of-care testing) with the advantages of rapidity, specificity, sensitivity, simplicity, and low cost of operation [16–18] (Table 1).

In this study, the clinical strains of *K. pneumoniae* were used as the research object to establish a rapid LAMP detection method. It can make up the deficiency of traditional detection methods by accurately detect patients infected with pathogens in a timely and accurate manner during clinical practice, and guide the use of drugs promptly and accurately.

Materials and methods

Strain culturing and the extraction of genomic DNA

Thirty-six clinical isolates of *K. pneumoniae* and fifty (5 species) other non-*K. pneumoniae* clinical isolates including *Escherichia coli* (15), *Pseudomonas aeruginosa* (7), *Staphylococcus epidermidis* (10), *Staphylococcus aureus* (8), and *Acinetobacter baumannii* (10) were collected from March 2018 to July 2018 and kindly provided by the laboratory of the First People's Hospital of Yunnan Province. The above strains were identified by routine isolation culture, gram staining, and mass spectrometry (VITEK®MS) [19, 20]. All the strains were inoculated in LB liquid medium, in 37 °C, 180 rpm for 12 h, and preserved for later use. The bacterial genomic DNA kit (Zomanbio, China) was used to extract the genome DNA from bacteria. The extracted genome DNA was stored at − 20 °C for further use.

Specific gene screening

The specific gene of *K. pneumoniae* was screened similarity as the previous study [21, 22]. Briefly, the formatted non-redundant nucleic acid database was downloaded from the National Center for Biotechnology Information (NCBI) as the local database. The local BLAST was performed using *K. pneumoniae* subsp. *pneumoniae HS11286* (GenBank No. NC_016845.1) genome as a query sequence against the above-mentioned database. Potential specific genes were further analyzed by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn) with the database excluding or including the sequences of *K. pneumoniae*. The specific gene must be highly conserved among the *K. pneumoniae* strains and show no significant similarity to other species.

Primer designing

After the specific gene screening, *CelB* (ID: 11847805) as the target gene was finally obtained. Four oligonucleotide primers (outer and inner primers, F3/B3 and FIP/BIP, respectively) used for the LAMP reaction were designed by Primer Explorer version 5 software (http://primerexplorer.jp/lampv5/index.html). The outer primers (B3/F3) were also used in the PCR assays, all the primers were synthesized by TSINGKE Biological Technology Co., Ltd. (Kunming, China).

Establishment of LAMP and PCR reactions

The genomic DNA of *K. pneumoniae* as a template was subjected to the LAMP and PCR amplification. The LAMP reaction system consisting of 12.5 μL of 2 × isothermal Mastermix (pH 8.8, with SYBR green I as the fluorescence dye to combine with the minor groove of the DNA double helix), 8 μM FIP and BIP (4 μL), 1 μM B3 and F3 (0.5 μL), 100 ng of genomic DNA, and finally add sterilizing water up to 25 μL.

Table 1 LAMP primers for the *CelB* gene used in this study

Primer	Sequence(5′-3′)	Size (bp)
F3	TCATGAGCGGTATGATGC	18
B3	CGGAGAAAGCGACAATCA	18
FIP	CTGATATGGAGCAAACGGAAACACTTTTGCCAGTAACGATCATGAGT	47
BIP	TTGTTACGACACAACTTAACTCGCTTTTCCAAAAAATTATTCGT GAAGGT	50

The reaction was carried out in Genie®II with a process of incubation at 65 °C for 30 min and melting curve analysis at 98 °C to 80 °C. The PCR reaction was carried out in a 25 μL system, including 12.5 μL 2 × TSINGKE Master Mix (containing 1 U DNA polymerase, 1.5 mM $MgCl_2$, 200 μM dNTP), 10 μM B3 and F3, 50 ng of genomic DNA. The PCR reaction process was pre-denaturation at 95 °C for 5 min, with 35 cycles including denaturation at 95 °C for 30 s, annealing for 30 s, extension at 72 °C, and a final extension at 72 °C for 5 min. 5 μL of the PCR products were used in the 2% agarose gel electrophoresis at 120 V, 30 min, and the agarose gel was stained by Gel stain (Beijing TRANSGEN BIOTECH Co., Ltd.).

Specificity evaluation of LAMP and PCR reactions

Thirty-six clinical isolates of *K. pneumoniae* and 5 other non-*K. pneumoniae* clinical isolates (*E. coli*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*, and *A. baumannii*) were used for the specific evaluation of LAMP and PCR reactions. The PCR test was used as the gold standard in preliminary experiments for the specific test prior to the LAMP test. All experiments were repeated twice.

Sensitivity evaluation of LAMP and PCR reactions

The sensitivity of LAMP and PCR reactions were evaluated using two different templates, the serially diluted 10-fold positive plasmids (10^8–10^0 copies) (constructed as the previous study [21]) and blood sample mimicking infection. The counted *K. pneumoniae* strain was serially diluted by 10-fold in PBS and then mixed with blood from the healthy specific-pathogen-free female Kunming mice (Laboratory Animal Center of Kunming Medical University, Kunming, Yunnan; weight, 22–25 g; age, 5 weeks) at 1:1 proportion to mimic infection. The mice were housed in the animal experiment center of Kunming University of Science and Technology at 25 °C with a 12 h/12 h light/dark cycle and access to food and water ad libitum. The blood sample was lysed by a solution containing 125 mM NaOH, 1 mM EDTA, and 0.1% Tween 20, and then a solution containing 125 mM HCl and 10 mM Tris-HCl, and the suspension was used as the template for LAMP and PCR assays. All experiments were repeated 2 times.

Results

Screening the specific gene and primer designing

Through local BLAST, 700 potential specific genes were obtained. Conclusively, 4 potential specific genes were obtained after NCBI BLAST, which met the criterion about the interspecific specificity and intraspecies universality. Then, the primers for LAMP reaction were designed on the Primer Explorer V5 software (http://primerexplorer.jp/lampv5/index.html). In this step, four primers were designed for the six regions of the target gene in the LAMP, and the primers should be confirmed by the primer BLAST and PCR assay. Considering the above harsh selection conditions, the *CelB* gene (GenBank ID 11847805) was finally identified as the only one that can be used in the detection of *K. pneumoniae*. The *CelB* gene was involved in the encoding of cellobiose-specific PTS family enzyme IIC component (YP_005227087.1), which participated in the metabolism of *K. pneumoniae*, and could be as a new target for the identification of *K. pneumoniae*.

Specificity of LAMP and PCR reactions

Thirty strains of *K. pneumoniae* and 50 other non-*K. pneumoniae* clinical isolates were used for the specificity evaluation. Before the LAMP reaction, PCR test was used for the specific test. All the *K. pneumoniae* were positive, while all the non-*K. pneumoniae* were negative, the partial results were shown in Fig. 1A. The LAMP results including the amplification curve, melting curve, and the fluorescence visualization were consistent with the PCR, which was shown in Fig. 2 A–C.

Sensitivity of LAMP and PCR reactions

After calculation, the initial concentration of the positive plasmid was 2.13×10^8 copies/μL, which was first diluted to 1×10^8 copies/μL, then subjected to 10-fold serial dilution, diluted from 10^8 to 10^0. One microliter of each gradient concentration of the recombinant plasmid was used as the template for the sensitivity detection of the PCR reaction. Take a 10-fold gradient of *K. pneumoniae* with an initial concentration of 1.05×10^{10} CFU/mL, dilute from 10^8 to 10^1, 2 μL of each gradient of the bacterial solution to make a blood sample, lysed by heat. Two microliters each gradient concentration (10^7 to 10^0 for PCR, 10^5 to 10^0 for LAMP, positive plasmid (1×10^6 copies/μL) as the positive control for LAMP) of the blood sample lysate was used as a template for sensitivity detection of the LAMP and PCR reaction. As can be seen from Figs. 1 B and C and 2 D–F, both the positive plasmid and the blood sample were used as templates, the detectability of LAMP and PCR reaction can achieve 1 copy/reaction.

Discussion

In China, the infection rate of *K. pneumoniae* has become second only to that of *Escherichia coli* [5]. The World Health Organization reported in 2013 that

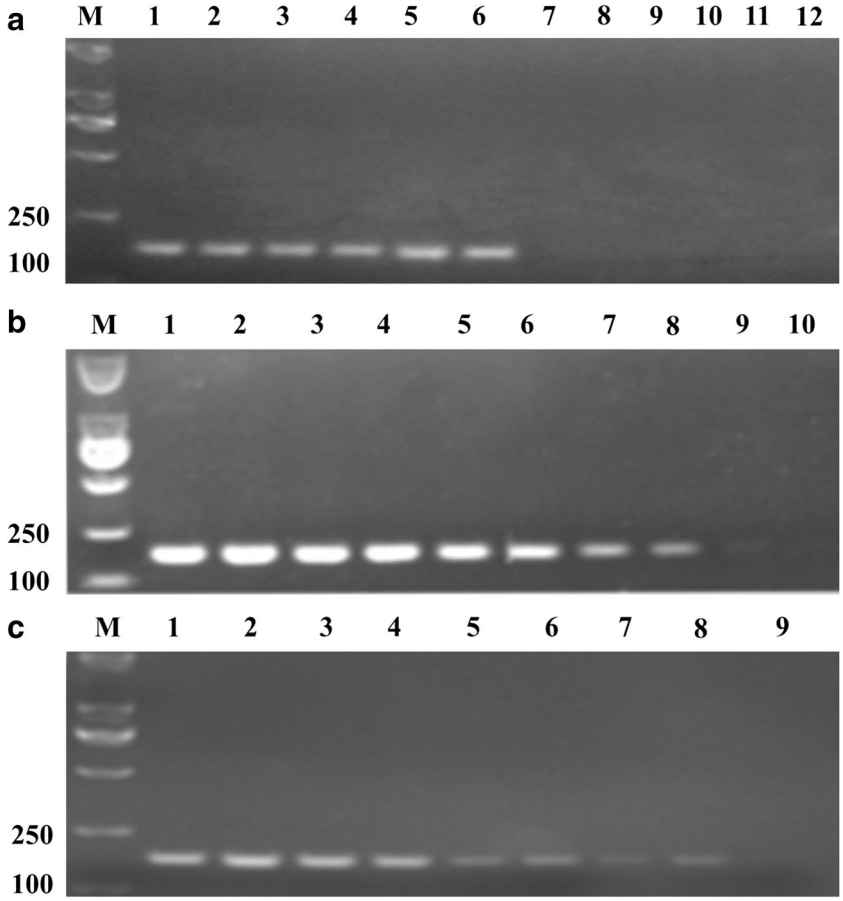

Fig. 1 Specificity and sensitivity of the PCR assay for detecting the target gene *CelB* using the primers B3/F3 (**a**–**c**). **a** Specificity of the PCR assay for detecting the target gene of *CelB*. Genomic DNA of *K. pneumoniae* (14A0882, 14A0794, 13A14310, 13A14525, 13A13753, 13A13943) was used as the template for PCR in lane 1 to lane 6, the template in lane 7 to lane 11 were *E. coli* (1611NY0004), *P. aeruginosa* (0007280366), *S. epidermidis* (14B13012), *S. aureus* (15A14046). Negative control (NC), with the sterile distilled water as the template in lane 12. **b** Sensitivity of the PCR assay for detecting the target gene of *CelB* in the positive plasmids. The positive plasmids of *K. pneumoniae* and they were serially diluted 10-fold as templates for PCR assay. The lane 1 to 9 were 10^8–10^0, lane 10 was the negative control. **c** Sensitivity of the PCR assay for detecting the target gene of *CelB* in the bacterial solutions. The bacterial solutions were serially diluted 10-fold with the mice blood as a volume ratio of 1:1. These mixtures were lysed and then the suspension was used for PCR assay. The lane 1 to 8 were 10^7–10^0, lane 9 was the negative control. M: 2000 marker (Takara Biomedical Technology (Beijing) Co., Ltd). All the experiments were repeated three times

diarrhea caused by *K. pneumoniae* was the main reason for the 29% death (Two million children each year) of global children [23]. *K. pneumoniae* was reported to account for 9.03% of total bacterial infection in hospitals [24, 25]. Faced with such a severe situation, the rapid and accurate diagnostic methods are of great significance for drug use.

The main methods for pathogen detection were including the culture method, routine smear microscopic examination, and immunological assay in clinical testing. With the rapid development of molecular biology technology, PCR technology was considered to be a good choice in addition to the above techniques, and has been more commonly applied to pathogenic microorganism detection. Compared with traditional methods, PCR technology has the advantages of high specificity, sensitivity, and rapidity [26, 27]. However, PCR requires expensive thermal cycling equipment, and finally requires agarose gel electrophoresis to further verify the detection results, which was time-consuming and inapplicable. Since 2000, LAMP technology has been widely used for the detection of pathogenic bacteria, including bacteria, fungi, viruses, and parasites, due to its rapidity, simplicity, and high sensitivity [16, 18, 28]. In this study, LAMP combining PCR technology was established for the fast identification.

After local BLAST and NCBI BLAST, the *CelB* gene was finally obtained as the target gene for the detection of the *K. pneumoniae*. After the extensive literature review, we found that this gene had been annotated a few years ago, but it has never been used for the identification of *K. pneumoniae*. In this study, this gene was used in the identification for the first time. In the results, it has been found that this gene can successfully identify *K. pneumoniae*.

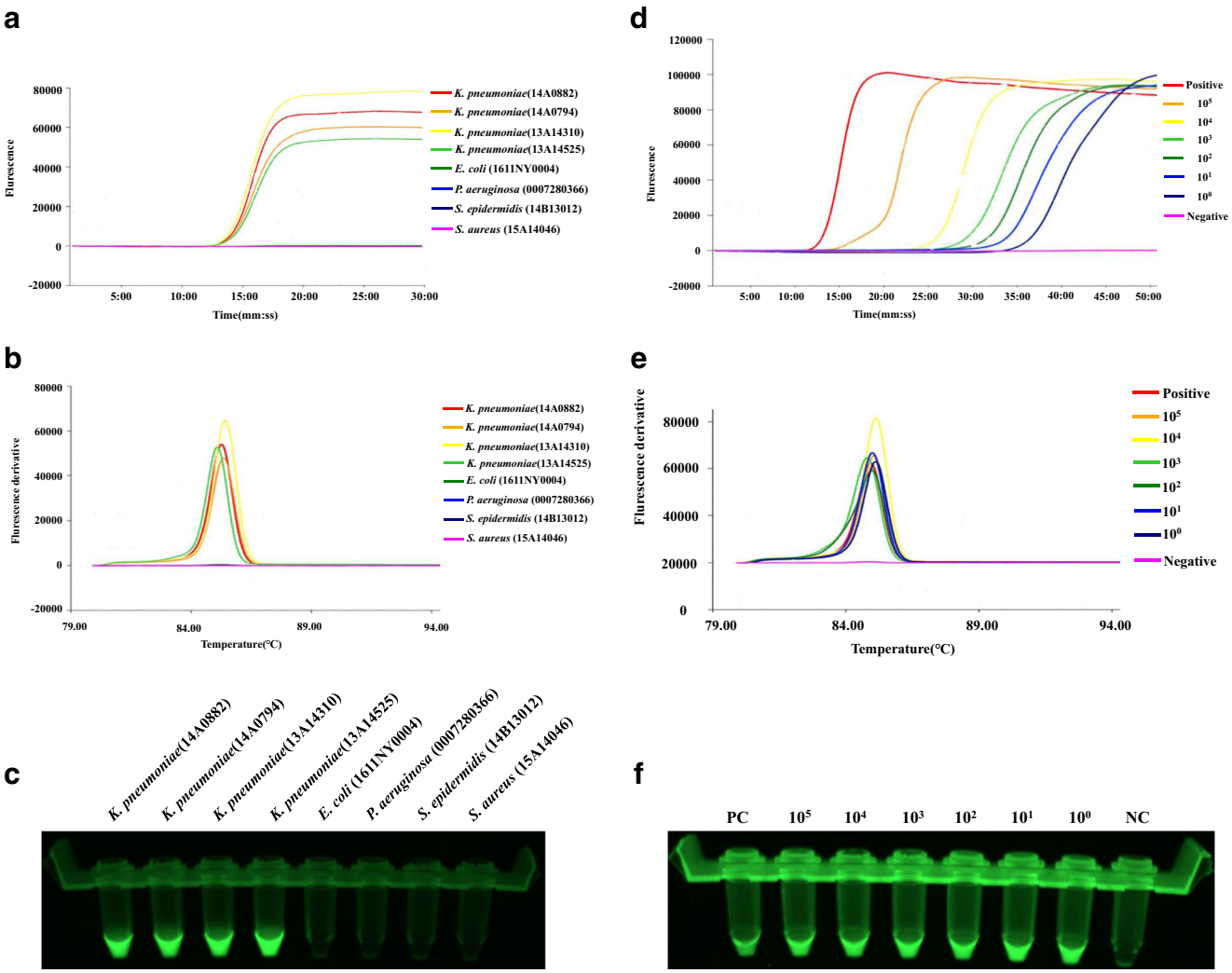

Fig. 2 Specificity and sensitivity of the LAMP assay for detecting the target gene *CelB* using the primers B3/F3, FIP/BIP (**a**–**f**). (**a**–**c**) Specificity of the LAMP assay for detecting the target gene of *CelB* by Genie®II. Genomic DNA of *K. pneumoniae* (14A0882, 14A0794, 13A14310, 13A14525) and four non-*K. pneumoniae* (*E. coli* (1611NY0004), *P. aeruginosa* (0007280366), *S. epidermidis* (14B13012), *S. aureus* (15A14046)) were used as the template for LAMP test. **a** the amplification curve, **b** melting curve, and the fluorescence visualization **c**. (**d**–**f**) sensitivity of the LAMP assay for detecting the target gene of *CelB* by Genie®II. Gradient diluted bacterial solution of *K. pneumoniae* (10^5–10^0) was mixed with the mice blood as a volume ratio of 1:1. These mixtures were lysed and then the suspension was used for LAMP assay, positive plasmid (PC) as the positive control, negative control (NC), with the sterile distilled water as the template. **d** The amplification curve, **e** melting curve, and the fluorescence visualization **f**. All the experiments were repeated three times

LAMP provides an alternative to PCR-based assays; due to its isothermal nature, it may be more suitable for the detection of pathogenic bacteria in the front-line or mobile laboratories in developing countries. LAMP technology was at least 10 times more sensitive than PCR technology [29–31]. But in this study, the sensitivity of PCR was same to the LAMP by optimizing the PCR reaction system, which suggested that there was still room for improvement in the sensitivity of PCR.

This study established a rapid LAMP detection system for *K. pneumoniae*. PCR and LAMP technology was used to detect 86 strains of pathogens (the 50 non-*K. pneumoniae* including the commonest multi-drug-resistant bacteria and the most likely to cause contamination bacteria); the positive rate was 100% and no false-positive results were found, indicating that the detection method with better specificity. The detectability of LAMP can reach 1 copy/reaction, which proved to be highly sensitive. The LAMP detection system established in this study laid the foundation for subsequent research.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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