Original Article Rapid detection of *Pseudomonas aeruginosa* septicemia by real-time fluorescence loop-mediated isothermal amplification of free DNA in blood samples

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Abstract: Background: Pseudomonas aeruginosa septicemia poses significant healthcare-related problems including high mortality rates and substantial resource utilization. Successful antibiotic treatment relies on the accurate and rapid identification of infectious agents. However, current traditional methods of bacterial identification require extended processing steps including bacterial overnight culture, biochemical reactions, and antibiotic susceptibility testing. The sequential steps associated with infection diagnosis lack the celerity and sensitivity that is required to facilitate efficient result determination. We describe a rapid, sensitive, loop-mediated isothermal amplification assay for the direct detection of Pseudomonas aeruginosa DNA from clinical patient plasma. This technique is referred to as the "LAMP-Pa" (loop-mediated isothermal amplification of Pseudomonas aeruginosa) assay. Methods: Three primer pairs were designed to amplify the oprl gene, which encodes a Pseudomonas aeruginosa outer membrane protein. LAMP-Pa was performed using a 25 µl final volume at 65°C. For comparative purposes, we also used a traditional method of bacterial identification to evaluate assay efficacy. Results: The procedure enables direct detection from clinical patient plasma within 20 minutes without the requirement for DNA purification. The detection limit pertaining to Pseudomonas aeruginosa identification is 2.8 ng of total DNA/µl plasma. The diagnosis is specific to Pseudomonas aeruginosa, and the coincidence rate of LAMP-Pa and the traditional method was 100%. Conclusions: Our data indicate that the assay provides a sensitive, specific, and intuitive diagnostic tool for Pseudomonas aeruginosa septicemia detection.

Keywords: Detection, septicemia, Pseudomonas aeruginosa, loop-mediated isothermal amplification, blood

Introduction

Septicemia is a leading global cause of mortality, accounting for 6% of annual deaths in America. Additionally, this condition claims more lives than HIV, prostate cancer, and breast cancer combined [1]. Pseudomonas *aeruginosa* septicemia (PAS) is especially problematic because of high mortality rates, and antimicrobial resistance, which are characteristic of associated infections [2, 3]. PAS also requires substantial healthcare resource utilization. In 2010, the incidence of PAS was 6.5 individuals per 10,000 and the overall mortality rate was 16%, ranging from 10%-26% over the study period [4]. Surveillance studies have also reported mortality rates between 39% and 60% for *P. aeruginosa*-associated infections [5, 6]. However, early diagnosis of septicemia is more difficult than detection of other local infections. Detection of symptoms and signs such as fever (> 38.5°C) or hypothermia (< 36°C), chills, and increased leukocyte levels, do not address whether a patient has been infected or whether the infection is systemic or local. Furthermore, these determinations do not provide information regarding the infectious agent underpinning the infection. As a result, a persistent dif-

ficulty for practitioners involves decision making associated with determining whether to start patients on antibiotic treatment schedules or wait for bacterial test results. Delays incurred when attempting to initiate optimal therapeutic interventions for critically ill patients can result in increasing the patient's risk of morbidity and mortality [7, 8]. Indeed, a short turnaround time for test results is of the utmost importance for outpatients. Delays exceeding 30 minutes can result in patients requiring follow-up attention. Thus, treatment guidelines [9], currently advise prompt (1-3 h post-diagnosis) initiation of empiric combination broadspectrum antibiotic administration pending microbiology results. However, these empiric regimens are inappropriate in as many as 40% of cases [10]. Ultimately, successful antibiotic treatment of infections relies on accurate and rapid identification of the causative infectious agents. Unfortunately, current traditional methods of bacterial identification require lengthy steps including bacterial overnight culture, staining and microscopic examination, biochemical reactions, and antibiotic susceptibility tests. In addition to the obvious problems caused by extended processing times, traditional bacterial determination methods also lack the sensitivity and efficiency often required in important clinical diagnostic assays. Although molecular diagnostic techniques enable highly specific and sensitive pathogen detection, they do not represent a cost-efficient onsite screening option. Therefore, development of a sensitive, specific, simple, and cost-effective diagnostic tool for septicemia diagnosis is critical in the advancement and optimization of global clinical practice.

Bacterial proliferation in the human body is often concomitant with a rapid reduction in cell numbers. Following immune system responses, bacterial cell death precedes cell lysis and the release of DNA. Upon initial infection, bacterial cells undergo a transitionary phase where adjustment to the new environment is required. This stage of the infection is called the lag phase. The duration of this phase, which is generally 1-4 hours, is dependent on a number of factors including the bacterial species involved, the number of bacteria causing the infection, and the status of the individual's immune system. The fittest bacteria adjust to their new environment by proliferation following active adapted metabolism, which is adapted for future division and growth. However, unfit bacterial cells undergo an alternative process that involves immune cell activation, bacterial cell lysis, and subsequent DNA release. Therefore, as an infection progresses, increased concentrations of free bacterial DNA or RNA occur in the blood. As a result, the pathogenic microorganisms involved can be identified using molecular identification methods that facilitate detection of free bacterial DNA or RNA directly from blood samples without the requirement for prior DNA or RNA extraction and purification.

Currently, PCR-based techniques are widely applied for clinical pathogenic microorganism detection. Although PCR-based detection is highly sensitive and specific, it is only suitable for centralized hospital facilities, requiring trained personnel and the use of expensive automated sample preparation machinery and real-time thermocyclers. Some recently developed amplification systems (such as loop-mediated isothermal amplification) are relatively simple to use and can be further modified for improved performance, thereby permitting the detection of DNA directly from patient urine samples [11-13].

The aim of this study was to verify that free bacterial DNA, present in infected blood samples, could be used to facilitate Pseudomonas aeruginosa septicemia diagnosis using a rapid LAMP (loop-mediated isothermal amplification) assay. LAMP is an amplification technique that offers highly sensitive and specific DNA detection without the requirement for thermocycling. The highly conserved oprl gene, encoding for a Pseudomonas aeruginosa outer membrane protein [14-16], was utilized as the target gene for real-time fluorescence loop-mediated isothermal amplification detection. Amplification of the oprl gene resulted in 100% specificity when compared with traditional bacterial culture methods utilized for the detection of Pseudomonas aeruginosa [17].

This study was performed at the Chinese PLA General Hospital from 2013 to 2014. Our results suggest that the LAMP assay employed as part of this study is a rapid, sensitive, and direct method for the detection of *Pseudomonas aeruginosa* from patients' plasma samples. The method takes less than 21 min and DNA purification is not required prior to the amplifi-

| Primer | Sequence | Amplification tem- perature (°C) | Product size of endo- nuclease digestion (bp) |
|--------|--|-------------------------------------|--|
| F3 | CGCGTCGAGCTGAAGAAGT | 65 | 135 |
| ВЗ | CCCACCTCCGGTAAAGGC | | |
| FIP | TCCGCCCTGGCCGCTAATGAATTCCCAAGCACCTGCGTGTC | | |
| BIP | TCCTGTGTATGACGGCGTTGCGAATTCACCTGCCGTGCCATACC | | |
| LF | AGCGCGAGGAACGTCAG | | |
| LB | CAACAATGGCGGCAACGT | | |

Table 1. The primer sets used for specific isothermal amplification of Pseudomonas aeruginosa

The bold italics are *E*coR I restriction endonuclease recognition sites.

cation reaction. To the best of our knowledge, this is the first study demonstrating the use of the LAMP assay for this purpose. From this point onwards, the *P. aeruginosa*-specific LAMP assay developed during this study will be referred to as "LAMP-Pa".

Materials and methods

Primer design and Real Amp method

The primer pair (outer, loop and inner) used for the detection of Pseudomonas aeruginosa (shown in Table 1) was designed using LAMP Designer software (http://www.optigene.co.uk) (OptiGene Ltd, United Kingdom). This detection method permitted the amplification of the oprl gene, which encodes for an outer membrane protein. LAMP-Pa was performed using a 25 µl final volume. The reaction was conducted using a simple portable instrument, Genie® II (OptiGene Ltd, United Kingdom), and the commercially available Loopamp DNA Amplification Kit (Isothermal Master Mixes and DNA Polymerases, OptiGene Ltd, United Kingdom) following the manufacturer's instructions. DNA amplification was performed at 65°C. The Genie® II contains an amplification platform (heating block) and has a fluorescent detection unit for end-point use (acquiring real-time data). The platform allows isothermal amplification of DNA and RNA, and enables target detection following fluorescence measurement. Real-time temperature and fluorescence data are displayed graphically on a touch screen in realtime as the run progresses. Following amplification, a plot is generated where the X-axis denotes the time associated with the procedure in minutes, and the Y-axis shows the fluorescence. Amplification of the positive DNA yielded a sigmoidal amplification curve, while the negative control tube had no measurable fluorescence as indicated by a flat line in the plot. The reaction mixture was prepared by mixing the following components: 1 μ l each of 5 μ mol/L outer primers, F3 and B3, 1 μ l each of 40 μ mol/L inner primers, FIP and BIP, 15 μ l of mixed buffer, and 3.5 μ l of double-distilled H₂O. The loop primers LF and LB should not be used in the reaction, unless indicated otherwise. The reaction was initiated by adding 2.5 μ l of template and incubating at 65°C.

DNA extraction

Bacterial species isolated from hospitalized patient samples (including blood, sputum, urine, feces and gastric juice samples) included Candida, Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, Streptococcus viridans, Micrococcus, Enterococcus, coagulasenegative Staphylococcus, Acinetobacter baumannii and Stenotrophomonas maltophilia. Bacterial identification was performed by microscopic examination and biochemical reactions. Bacterial DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech (Beijing) Co. Ltd.) according to the manufacturer's instructions. Purified total DNA was quantified by determining absorbance at 260 nm using a micro-volume nucleic acid spectrophotometer (ACTGene NAS-99, ACTGene, USA). DNA samples were subsequently aliquoted for storage at -20°C. All DNA samples were tested using the LAMP-Pa assay.

LAMP-Pa assay clinical specificity

Bacterial isolates obtained from hospitalized patient samples included two *Candida albicans* isolates, a *Candida tropicalis* isolate, a *Candida parapsilosis* isolate, two *Klebsiella pneumonia* isolates, an *Enterobacter cloacae* isolate, an *Escherichia coli* isolate, two *Streptococcus viri*-

dans isolates, a Micrococcus isolate, an Enterococcus isolate, a coagulase-negative Staphylococcus isolate, an Acinetobacter baumannii isolate, a Stenotrophomonas maltophilia isolate and three Pseudomonas aeruginosa isolates (including a mucoid Pseudomonas aeruginosa isolate). Samples were identified by traditional microscopic examination and biochemical reactions. The bacterial isolates were cultured overnight on nutrient agar plates (0.5% Peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, pH 7) at 37°C. Single colonies were collected in 500 µl of sterile purified water and boiled for 30 min. The boiled bacterial suspensions were centrifuged at 10,000 ×g for 8 min and the supernatants were frozen at -80°C for further study. The analytical specificity of the LAMP-Pa assay was evaluated by testing the supernatant and comparing with the results of traditional methods.

Detection of bacterial cultures

To evaluate the detection capability of LAMP-Pa in bacterial cultures, processed patient blood cultures from automatic aerobic blood culture bottles (BacT/ALERT® FA, Biomerieux France) were used as amplification templates. *Pseudomonas aeruginosa*-positive blood cultures were verified using traditional methods.

In order to determine an optimal (simple and efficient) template preparation method, the following different processing procedures were employed. Method 1: The positive blood cultures (0.5 ml) were boiled for 30 minutes and centrifuged at 10,000 ×g for 8 min and the supernatants were used as amplification templates (Template 1). Method 2: The positive blood cultures were processed using two centrifugation steps at 900 ×g for 10 min and 4000 ×g for 6 min, respectively. The samples were then boiled for 30 minutes, and the supernatant generated after a centrifugation step of 10,000 ×g for 8 min was used as an amplification template (Template 2). Method 3: The positive blood cultures were centrifuged at 900 ×g for 10 min and 4,000 ×g for 6 min, and the collected sediments were re-suspended in 0.45 ml of distilled water prior to boiling for 30 minutes. The supernatant generated following centrifugation for 8 min at 10,000 ×g was used as an amplification template (Template 3). Method 4: The positive blood cultures were centrifuged at 900 ×g for 10 min, and the sediments were re-suspended in 0.45 ml of distilled water. The re-suspensions were boiled for 30 minutes and centrifuged at 10,000 ×g for 8 min, and the supernatants were used as amplification templates (Template 4). Method 5: The positive blood cultures were centrifuged at 4,000 ×g for 6 min, and the supernatants were boiled for 30 minutes. They were subsequently centrifuged at 10,000 ×g for 8 min, and the supernatants were used as amplification templates (Template 5). Method 6: The positive blood cultures were centrifuged at 4,000 ×g for 6 minutes, the sediments were re-suspended in 0.45 ml of distilled water and boiled for 30 minutes. They were subsequently centrifuged at 10,000 ×g for 8 min, and the supernatants were used as amplification templates (Template 6). A non-Pseudomonas aeruginosa positive blood culture was used as a negative control and was processed in the same manner as Template 1.

In vitro experimental infection

This study was approved by the Ethics Committee of the Chinese PLA General Hospital.

To confirm that free bacterial DNA was present in infected blood and could be detected, in vitro experimental infections were performed. Furthermore, in vitro experimental infections were also used to test the sensitivity of the LAMP-Pa assay. Pseudomonas aeruginosa (isolated from patient samples) was cultured overnight on nutrient agar plates, and intact single bacterial colonies were inoculated into 4 ml of venous blood. The venous blood was aseptically and individually taken from four healthy volunteers and aliquoted into a BD Vacutainer® Lithium Heparin (BD Franklin Lakes NJ USA) tube. The blood samples were randomly divided into two groups. An experimental group containing three blood samples in three separate tubes was inoculated with three intact Pseudomonas aeruginosa colonies. The remnant blood from the three samples was used as the control group. All blood samples were incubated at 35°C. Following the initiation of blood sample incubation, 0.5 ml samples were taken at the following time points: 30 min, 1 hour, 1.5 hours, 2 hours, 4 hours, and 7 hours, respectively. Samples were taken from tubes pertaining to the experimental and control group, and plasma (Template 7) was prepared by centrifugation at 4,000 ×g for 8 min. The separated plasma (Template 7) was



Figure 1. The amplification curves of the extracted DNA of *Pseudomonas aeru*ginosa and other bacteria with the LAMP-Pa assay. 1, 2 and 3: *Pseudomonas aeruginosa*; 4, 5 and 6: *Candida albicans*, *Acinetobacter baumannii* and negative control (containing no bacterial DNA), respectively.

boiled for 30 minutes and centrifuged at 12,000 ×g for 10 min. The supernatant was collected and used as an amplification template (Template 8). Each template was tested for the presence of Pseudomonas aeruginosa using the LAMP-Pa assay. The assay was performed with and without the loop primers (1 µl each of 20 µmol/L loop primers, LF and LB, in a total volume of 25 µl). Total DNA from the plasma samples collected at each of the different time points was extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech (Beijing) Co. Ltd.), and the extracted DNA was quantified by determining the absorbance at 260 nm using ACTGene NAS-99 (ACTGene, USA). According to the method outlined by Katrin [18], the volume of the purified total DNA was adjusted to reflect the volume of the sample that was originally subject to purification. Following adjustment, 1 ul of purified DNA corresponded to the amount of total DNA present in 1 µl of the plasma sample from which it was derived.

Blind tests of clinical specimens

A total of 0.5 ml of unidentified positive blood (from an automatic aerobic blood culture bottle where the infectious agent involved was unidentified) was boiled for 30 minutes and centrifuged at 10,000 ×g for 8 min. The supernatant was tested using the LAMP-Pa assay with *Pseudomonas aeruginosa*-specific primers. Assay results were compared with that of the traditional identification method.

Venous blood samples collected from a febrile patient (38.9°C) with a high white blood cell count $(16,000/\mu I)$ were centrifuged at 4,000 ×g for 8 min to isolate plasma for the detection of Pseudomonas aeruginosa using LAMP-Pa. The amplification was performed using Pseudomonas aeruginosa-specific primers. The same process was carried out for the other three patients exhibiting fever and in-

creased leucocyte counts. The blood culture assay was also performed for these patients. The results of LAMP-Pa were compared with that of the blood culture assay.

Results

Specificity of the real Amp method

We developed a specific LAMP-Pa assay to detect Pseudomonas aeruginosa. This assay facilitated the amplification of DNA extracted from Pseudomonas aeruginosa within 10 min of initiation (Figure 1). The fluorescence peak typically persisted over time. Using this method, approximately 37 ng/µl of specific nucleic acids were detected after 7 min. No amplification curve was observed upon processing of 14 non-Pseudomonas aeruginosa bacterial DNA samples. An analysis of the specificity associated with the LAMP-Pa assay demonstrated that only Pseudomonas aeruginosa DNA (including mucoid Pseudomonas aeruginosa) resulted in the generation of a positive result. DNA extracted from Candida albicans, Candida tropicalis, Candida parapsilosis, Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, Streptococcus viridans, Micrococcus, Enterococcus, coagulase-negative Staphylococcus, Acinetobacter baumannii, Staphylococcus hominis, Cryptococcus and Stenotrophomonas maltophilia resulted in a negative result when

| No. | Strains | LAMP-Pa | Traditional methods | No. | Strains | LAMP-Pa | Traditional methods |
|-----|---------------------------------|---------|---------------------|-----|-----------------------------------|---------|---------------------|
| 1 | Candida albicans | - | - | 10 | Candida tropicalis | - | - |
| 2 | Candida albicans | - | - | 11 | Micrococcus | - | - |
| 3 | Klebsiella pneumoniae | - | - | 12 | Candida parapsilosis | - | - |
| 4 | Enterobacter cloacae | - | - | 13 | Enterococcus | - | - |
| 5 | Pseudomonas aeruginosa | + | + | 14 | Coagulase negative staphylococcus | - | - |
| 6 | Pseudomonas aeruginosa (mucoid) | + | + | 15 | Streptococcus viridans | - | - |
| 7 | Escherichia coli | - | - | 16 | Stenotrophomonas maltophilia | - | - |
| 8 | Streptococcus viridans | - | - | 17 | Acinetobacter baumannii | - | - |
| 9 | Pseudomonas aeruginosa | + | + | 18 | Klebsiella pneumoniae | - | - |

 Table 2. The results for extracted DNA tested with the LAMP-Pa assay and traditional identification

 methods following analysis of 18 bacterial samples



Figure 2. The amplification curves of differently treated positive cultures of automatic aerobic blood culture bottles of *Pseudomonas aeruginosa*. 1, 2, 3 and 5: the positive amplification curves of Templates 1, 2, 3 and 5, respectively; 4 and 6: the negative curves of Templates 4 and 6; N: the negative control.

| Table 3. The peak time of amplification curves w | vith |
|--|------|
| different templates | |

| Template | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------------|-------|-------|-------|---|-------|---|
| Peak time (mm: ss) | 23:44 | 30:14 | 25:14 | / | 30:14 | / |
| /: unreacted. | | | | | | |

used as template during the LAMP-Pa assay (**Table 2**). This demonstrated that the specificity for *Pseudomonas aeruginosa* was 100%.

Treated cultures from automatic aerobic blood culture bottles

The positive blood cultures from automatic blood culture bottles were prepared in an identical manner to the templates that were used for the initial amplification procedures. Each of the templates, apart from Templates 4 and 6, were successfully amplified (**Figure 2**). This indicated that positive amplification could be achieved using crudely processed positive

blood culture samples from automatic blood culture bottles. This crude processing procedure was conducted by boiling the sample and was followed by a centrifugation step. This method did not appear to affect amplification of the target gene. However, the peak time for amplification was delayed and occurred at 23-30 minutes following amplification initiation (Table 3). The negative reactions associated with Templates 4 and 6 sug-

gest that the introduction of inhibitors into the amplification reaction. These results indicate that boiling of the sample for 30 minutes followed by centrifugation at 10,000 ×g for 8 minutes, is optimal for blood cultures from automatic blood culture bottles.

In vitro experimental infection

In order to determine the sensitivity of the LAMP-Pa assay, an experimental in vitro infection experiment was performed. Two hours after culturing, *Pseudomonas aeruginosa* was directly detected in the untreated plasma with a 20-minute peak time. The amplification was performed using the loop primers, LF and LB, and the DNA detection limit of the assay was 2.8 ng of total DNA/ μ I plasma. Negative results were generated without any amplification curve upon amplification of the controls (**Figure 3**). The amplification reaction with loop primers was more efficient and resulted in a shorter



Figure 3. The amplification curves of plasma samples inoculated with experimental *Pseudomonas aeruginosa*. The figure contains the following: a. 1.5 μ l of Template 8 with loop primers; b. 2.5 μ l of Template 7 with loop primers; c. 2.5 μ l of Template 7 without loop primers.



Table 4. The peak time of amplification

Figure 4. The agarose gel electrophoresis of the amplified *Pseudomonas aeruginosa*-specific products. Lane M: DNA molecular weight marker; Lane 1: 1.5 μ l of Template 8 with loop primers; Lane 2: 2.5 μ l of Template 7 with loop primers; Lane 3: 2.5 μ l of Template 8 without loop primers; Lane 4: 2.5 μ l of Template 7 without loop primers.

peak time than reactions performed without loop primers (**Table 4**). This was further demonstrated using less concentrated templates, indicating that the loop primers greatly improved the efficiency of amplification (**Figure 3 a** and **b**). Indeed, the peak time of the amplification curve for Template 8 was shorter than that observed for Template 7, which suggested that simple treatment of the plasma by boiling and centrifugation resulted in fewer interfering substances than untreated plasma (**Figure 3 a-d**). A positive *Pseudomonas aeruginosa* LAMP reaction typically produced a ladder pattern when electrophoresed on an agarose gel (**Figure 4**).

Blind tests of clinical specimens

The unidentified positive blood culture was boiled and centrifuged to test for *Pseudomonas aeruginosa* presence using the LAMP-Pa assay. The test

result was negative. Two days later, the traditional identification method proved that the unidentified sample contained *Staphylococcus hominis* (**Figure 5A**). The results of four clinical blood specimens blindly tested for *Pseudomonas aeruginosa* by LAMP-Pa were all negative. Furthermore, the blood culture and traditional identification method confirmed that of four blood specimens, two had no bacteria. while one contained *Cryptococcus* and the other contained *Klebsiella pneumoniae* (**Figure 5B**).

The blind tests of clinical specimens showed that the primers used for the detection of *Pseudomonas aeruginosa* were highly specific. No false-positive results were obtained with blood samples from healthy volunteers and patients with other bacterial infections.

Discussion

As part of this study, a traditional bacterial culture and identification technique was used as a comparative method for bacterial identification. Eighteen bacterial specimens isolated from clinical samples were used to evaluate the sensitivity and specificity of the LAMP-Pa assay. The results of the study showed that the sensitivity limit of the LAMP-Pa assay was 2.8 ng of total DNA/µl plasma. The assay exhibited no cross-reactivity with the genes of unrelated bacteria including *Candida*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli*, *Streptococcus viridans*, *Micrococcus*, *Enterococcus*, coagulase-negative *Staphylococcus*, *Acinetobacter baumannii* and *Steno-*



Figure 5. The results of blind tests of clinical specimens. A: The amplified templates were the boiled and centrifuged positive blood cultures from automatic blood culture bottles; B: The amplified templates were the untreated plasma from anticoagulant venous blood (with Lithium heparin) from clinical patients; p.c.: unidentified positive blood cultures; N: the negative control.

trophomonas maltophilia. The coincidence rate of the two methods was 100%.

Nested PCR and conventional PCR reactions require amplification times of 4-5 h and 2-3 h, respectively. Furthermore, these techniques require the utilization of multiple steps. However, the LAMP-Pa assay requires only two steps: blood or culture centrifugation and addition of the specimen into the reaction micro tube. As a result, the latter method is comparatively simpler, more convenient, and results in a substantial timesaving when compared with more established PCR-based *Pseudomonas aeruginosa* assays. Our method requires approximately seven minutes to generate a detectable amount of *Pseudomonas aerugino-*

sa-specific product when using as little as 92.5 ng of template DNA in a reaction (Figure 1). The in vitro infection experiment indicates that Pseudomonas aeruginosa can be detected in untreated plasma as early as two hours after infection, with an amplification peak time of 20 minutes using loop primers and 27 minutes when loop primers were not used (Figure 3). The sensitivity limit of the LAMP-Pa assay is 2.8 ng of total DNA/µl plasma, which is less than other published sensitivities [19]; however, purified and concentrated total DNA samples were utilized in the latter studies. These results indicate that LAMP-Pa is a sufficiently rapid isothermal amplification method.

Positive blood cultures from automatic blood culture bottles were used as templates for amplification to diagnose the presence of *Pseudomonas aeruginosa*. Of the four experimental infection specimens, the sample infect-

ed with Pseudomonas aeruginosa tested positive when the LAMP-Pa assay was used directly to test 2.5 µl of plasma. The LAMP-Pa assay described in this report also has the significant advantage of increased inhibition of detection interference when compared with nested PCR and conventional PCR. DNA extraction is not required for LAMP-Pa. The target DNA was detected directly from minimally processed blood samples, such as serum and plasma separated from whole blood using centrifugation, without interference to specificity or significant loss in sensitivity. In contrast, unprocessed blood normally requires DNA extraction prior to PCR amplification, since there are many inhibitors in blood, such as urea [20-22], heparin [23-28], hemoglobin [29, 30], lactoferrin [31],

immunoglobulin G [31, 32], proteinase [33], and myoglobin [34]. Hemoglobin, immunoglobulin G and lactoferrin are the three major PCR inhibitors found in blood [29, 31]. In adult blood, the normal concentration of immunoglobulin G and myoglobin is 7-16 mg/ml and 10-92 ng/ml, respectively. It has been reported that addition of \geq 0.1 to 0.0016 IU of heparin per reaction mixture (50 µl) suppressed DNA amplification in a dose-dependent fashion [26]. In a BD Vacutainer[®] Lithium Heparin tube, there is 68 IU of heparin, while the concentration of heparin in blood samples is generally > 17 IU/ml. Currently, phenol/chloroform extraction methods are used to remove the majority of the PCR inhibitors in blood, such as hemoglobin, lactoferrin, immunoglobulin G, and proteinase. In addition, the negative effects of ions (including Ca^{2+} and K^{+}) in the blood can be avoided by using optimal PCR buffers. However, due to a stronger adsorption capacity, heparin is difficult to remove completely. In this study, although plasma harbors many inhibitors, it was successfully used as an analyte for the direct detection of pathogenic bacterial DNA. Moreover, consistent results were achieved for each of the culture-positive and negative samples. These data demonstrate the effectiveness and specificity of the LAMP-Pa assay.

Currently, there are no Pseudomonas aeruginosa POC (Point-of-care) tests that offer sensitivity comparable to laboratory nucleic acid amplification-based techniques. Unlike traditional PCR-based approaches, the associated simplicity and short turnaround time makes the LAMP-Pa assay suitable for point-of-care use in outpatient settings, and even remote areas. The assay does not require the purification of total DNA from blood samples. Simple sample preparation, such as centrifugal separation of plasma, is enough to facilitate diagnosis of the pathogen in blood after the patient develops symptoms such as fever or chills. Using LAMP-Pa, positive blood cultures can also be directly tested for pathogen presence, eliminating lengthy identification procedures associated with bacterial detection, thereby speeding up the diagnosis. Moreover, elimination of the DNA purification step prior to amplification diminishes the chance of contamination. The lack of a purification step also makes the process less laborious and time-consuming, and therefore less expensive. The LAMP-Pa assay is a twostep procedure that takes < 20 minutes, and includes sample centrifugation and isothermal target amplification steps. This simple assay enables the highly specific detection of *Pseudomonas aeruginosa* with greatly improved sensitivity levels when compared with traditional *Pseudomonas aeruginosa* detection methods. Other isothermal amplification assays (such as the helicase-dependent amplification assay) regularly require 30 to 60 minutes for detectable amplification and product formation [12, 35, 36]. Therefore, the LAMP-Pa assay offers several significant advantages for on-site application when compared with other isothermal amplification methods.

In addition, LAMP-Pa is robust enough to facilitate the detection of septicemia even at the very early stages of infection in the human body. These advantages mean that the assay is especially suitable for postoperative detection and detection in elderly and debilitated patients and children. Indeed, this assay has the potential to be used in many applications apart from the detection of *Pseudomonas aeruginosa* septicemia. For example, pathogen-specific primers can be designed to amplify target regions according to clinical need, facilitating the generation of antimicrobial susceptibility information.

One limitation associated with this study is that the efficacy of the assay was only tested in two biological fluid types i.e. blood and blood cultures. Optimally the assay should facilitate the detection of lower copy numbers associated with partially treated infections (after empirical antibiotic administration), where the pathogens are reduced in number or not cultivable, but the DNA is still detectable.

This is the first report to demonstrate that it is possible to diagnose early septicemia by directly detecting the DNA of infectious bacteria from patient blood samples without prior DNA extraction and purification procedures. Our data indicate that the LAMP-Pa assay can greatly reduce handling times associated with bacterial culture, DNA extraction, purification, and amplification, thereby dramatically improving the diagnostic turn-around-time and medical treatment time associated with septicemia. The assay also provides a sensitive, specific, and two-step diagnostic tool that allows for the identification of suspected septicemia in point-of-care, outpatient settings and remote areas. Further efforts are required to determine the sensitivity, specificity, and anti-jamming ability of this assay using animal models infected with the pathogen.

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Disclosure of conflict of interest

None.

Authors' contribution

Jie Bai, designed the study, wrote the manuscript. Rungong Yang, designed the study and reviewed the manuscript. Shuhong Fu, collected patients' clinical data and provided blood samples, carried out in vitro experimental infection. Ling Ye, analyzed the results and edited the manuscript. Meiliang Gong, in charge of bacterial culture and identification. Jinghui Yang, carried out the isothermal amplification reaction. Yanfeng Fan, carried out the isothermal amplification reaction and analyzed the results. Xiaoxia Li, bacterial DNA extraction. Jihong Yu, carried out the isothermal amplification reaction. Hongyan Chen, DNA agarose gel electrophoresis. All authors have read and approve this version of the article. No conflict of interest exits in the submission of this manuscript.

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