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Pathogen diagnosis of children sepsis by LAMP technology

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ABSTRACT

Objective: To explore a rapid diagnostic method in neonatal sepsis and bacterial meningitis. **Methods:** The primers were designed and synthesized based on 16S rRNA gene of *Staphylococcus aureus*. Four specimens of *Staphylococcus aureus*, 16 specimens of coagulase-negative *Staphylococci*, 2 specimens of *Enterococci*, 3 specimens of *Streptococcus*, 1 specimen of *Micrococcus*, 3 specimens of *Escherichia coli*, 4 specimens of *Klebsiella pneumoniae*, 3 specimens of *Pseudomonas aeruginosa*, 2 specimens of *Enterobacter cloacae*, and 5 specimens of *Acinetobacter* were tested by loop-mediated isothermal amplification (LAMP) assay. A total of 118 clinical specimens of sepsis and non-sepsis were collected and detected with both LAMP assay and blood culture. **Results:** By designing primers specific for *Staphylococcus aureus*, specimens containing different kinds of pathogens were carried out by LAMP assay, and our data showed LAMP technology for the specific detection of *Staphylococcus aureus* in samples was successfully established. All clinical specimens of sepsis and non-sepsis were tested by both blood cultures and LAMP, and our data showed that compared wit blood culture method, the LAMP technology showed significantly high detection rate (P <0.01). **Conclusions:** As a quick and easy detection of *Staphylococcus aureus*, the LAMP technology was successfully established, laid the foundation for the diagnosis and treatment of children *Staphylococcus aureus* sepsis, and showed great promotion and application value.

1. Introduction

Children sepsis is one of the most common diseases in neonatal infection diseases. Although the infection rate of opportunistic pathogen such like *Staphylococcus epidermidis* is on the increase, *Staphylococcus aureus* infection is still the major cause for children sepsis in China[1]. Children sepsis caused by *Staphylococcus aureus* showed a mortality rate of 12.0%-20.5%[2], early diagnosis and treatment are the major factors influence prognosis. Blood culture is considered as the gold standard for the diagnosis with low positive rate and time-consuming. Loop-mediated isothermal amplification (LAMP), a novel gene amplification method, is an autocycling and strand displacement DNA synthesis method involving the use of the large fragment of Bst DNA polymerase and a set of four specially designed primers. In the LAMP reaction process, pyrophosphate ions generated from the dNTP may bind to Mg^{2+} in reaction solution, and became ivory precipitation magnesium pyrophosphate. The results also can be determined under naked eye by adding fluorescent dye. LAMP reaction can be processed at a constant temperature (63 ℃) by using simple devices with no need for temperature cycle, and its rapid and simple features give it an advantage over PCR[3,4].

In this study, we applied LAMP to *Staphylococcus aureus* DNA phagocytized by neutrophils. In order to pave the way for the diagnosis and treatment of children sepsis, we designed two pairs of specific annular primers according to 16S ribosomal RNA (rRNA), and accomplish rapid detection of *Staphylococcus aureus* by LAMP.

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2. Material and methods

2.1. Material

One hundred and eighteen children (boy: 58, girl: 60) participated in this study. According to the discharge diagnosis, these children were divided into 2 groups. Sepsis group (n=65): children sepsis was diagnosed in accordance the national children's sepsis diagnosis revised proposal[5]. There were 23 cases showing positive result on blood bacterial culture, and 53 cases showing negative result. Non sepsis group (n=53): there were 28 cases of hyperbilirubinemia, 11 cases of hypoxic ischemic encephalopathy, 2 cases of congenital heart disease, 8 cases of aspiration pneumonia, 1 case of peripheral neonatal cyanosis. Four strains of *Staphylococcus aureus*, 16 strains coagulase negative *Staphylococcus aureus*, 2 strains *Enterococcus*, 3 strains *Streptococcus*, 1 strain *Micrococcus*, 3 strains *Escherichia coli*, 4 strains *Klebsiella pneumoniae* bacteria, 3 strains *Pseudomonas aeruginosa*, 2 strains *Sewer enterobacter*, and 5 strains *Acinetobacter* were conserved in our lab.

dNTP, ultra-pure water, DNAiso reagent (TaKaRa, Dalian, China), Bst DNA polymerase (NEB), chemicals: such as Betaine, $MgSO_4$, $MnCl_2$ (Sigma), minriunG100 Gel electrophoresis apparatus and image analysis system (BIO-RAD), DB-20 metal cracking apparatus (TECHNE), WH-2 miniature vortex mixing apparatus (Shanghai Qingpu-Huxi Instruments Factory).

2.2. Primer design

Gene sequences of *Staphylococcus aureus* 16S rRNA was searched from the Genbank EST database. Specific primers of species highly conserved sequence (F3, B3, FIP, BIP, LPF, LPB) which searched by online BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was designed by Primer Explorer V4 (http://primerexplorer.jp/e/). The primers were composed by Shanghai biological engineering company:

F3: 5′-AGTCCACAAGGAAAGTAAAGATG-3′:

B3: 5′-ATTCCACGCCAACCAAGAT-3′;

FIP: 5′-GCAAGGTGATTCCTTAATTCCTCTCTTTCACACTTATTGGATGGTCTC-3′;

BIP: 5′-CATCGAAACAAGGCCAGTTTTTTACCTTCCTCAGCTATAGGGTGCTT-3′;

LPF: 5′-CGTCATAGCCATGTTAGGCC-3′;

LPB: 5′-AGCTACCACATCTAAGGAAGGCAG-3′.

2.3. Methods

Three blood samples for culture were collected from different body regions in each child within 48 hours of hospitalization under sterile condition. The bacterial genomic DNA were extracted with DNAiso Reagent (TaKaRa) according the instruction. Twenty five microlitre of a reaction mixture containing 1.6 μL FIP, 1.6 μL BIP, 0.2 μL F3, 0.2 μL B3, 1.2 μL LPF, 1.2 μL LPB, 0.6 mM dNTP, 0.8 M betaine, 20 mM Tris-HCl (pH 8.18), 0 mM KCl, 10 mM NH_4SO_4, 4 mM $MgSO_4$, 0.1% Tritonx-100, 25 μM calcium and 0.5 mM $MnCl_2$ was incubated at 60 ℃ for 60 min and then heated at 80 ℃ for 2 min. The result was distinguished by two ways: distinguished by products with the naked eye: precipitation or turbidity was considered to be positive; or distinguished by 2% agarose gel electrophoresis: DNA ladder was considered to be positive. We consider the results as positive if both were positive.

Two positive results in 3 blood cultures or positive LAMP result was considered as Pathogen infection. One positive results in 3 blood cultures or negative LAMP result was considered as contaminated bacterial.

2.4. Statistical analysis

Sensitivities and specificities of the different diagnostic methods were calculated by two by two analysis and significant differences were calculated by the *chi*-square (χ^2) test using SPSS 14.0 software. $P < 0.05$ was considered significant.

3. Results

3.1. Developing specific detection method for Staphylococcus aureus by LAMP

LAMP reaction was used to detect 4 strains of *Staphylococcus aureus*, 16 strains coagulase negative *Staphylococcus aureus*, 2 strains *Enterococcus*, 3 strains *Streptococcus*, 1 strain *Micrococcus*, 3 strains *Escherichia coli*, 4 strains *Klebsiella pneumoniae* bacteria, 3 strains *Pseudomonas aeruginosa*, 2 strains *Sewer enterobacter*, and 5 strains *Acinetobacter* with special primers target to *Staphylococcus aureus*. After reaction, there was obvious turbidity or precipitation (Figure 1) and DNA lander (Figure 2) in the samples with *Staphylococcus aureus*. The results show that the primers were specificity to *Staphylococcus aureus*, and specific detection method for *Staphylococcus aureus* by LAMP was developed.

3.2. Comparison between blood culture and LAMP

Eight cases were positive for *Staphylococcus aureus* in sepsis group were detected by blood culture and 18 positive by LAMP. Only 2 cases positive for *Staphylococcus aureus* in non-sepsis group were detected by blood culture. The positive test rate was significant different between two methods (χ^2=7.69, P<0.01).

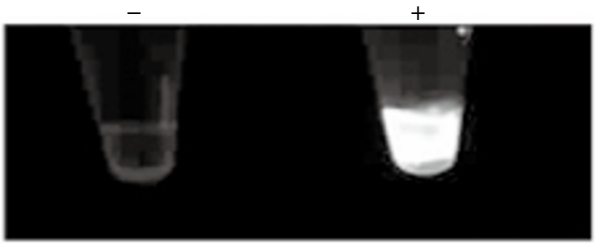

Figure 1. Precipitation after LAMP reaction.
+ sample with *Staphylococcus aureus*, −Negative sample.

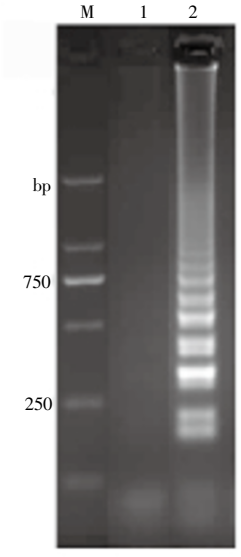

Figure 2. Agarose gel electrophoresis results after LAMP reaction.
1: Negative sample, 2: sample with *Staphylococcus aureus*, M: DNA marker.

4. Discussion

In recent years, molecular biology, especially LAMP, has become the most valuable technology for clinical microbiology diagnosis. LAMP is an autocycling and strand displacement DNA synthesis method involving the use of the large fragment of Bst DNA polymerase and two pairs of specific inner and outer annular primers designed based on the gene sequence of different purposes. In the LAMP reaction, the design of the inner primer is capable of hybridizing to the DNA region in the target sequence, synthetizing the complementary strand, and producing a type of dumbbell-shaped DNA. Then this special structure use itself as a template for DNA synthesis to convert stem-loop DNA, which is the starting configuration of the LAMP cycling reaction. Because the inner primer hybridization in the ring of the stem-loop structure, so after the replacement the primer can produce a gaped stem-loop-like DNA which may be attaching target sequence[3-7]. In the LAMP reaction process, pyrophosphate ions generated from the dNTP may bind to Mg^{2+} in reaction solution, and became ivory precipitation magnesium pyrophosphate. The results also can be determined under naked eye by adding fluorescent dye. LAMP reaction can be processed at a constant temperature (63 ℃) by using simple devices with no need for temperature cycle, and its rapid and simple features give it an advantage over PCR. Because 16S rRNA gene is highly conservative, so it plays an important role in bacteria identification as a specific detection of *Staphylococcus aureus*. The LAMP technology in our study which does not depend on the isolation and culture was successfully established, laid the foundation for the future use.

In 65 patients with children sepsis, it was discovered that the positive ratio detected by using LAMP was higher than using blood culture. Eleven positive cases which detected by LAMP were showed negative result in blood culture. Because 7 cases were treated with antibiotics before hospitalization and 4 cases unable to draw the adequate blood. Antibiotics can restrain bacterial growth, so leading to a negative result in blood culture[8]. One case which shows positive result by blood culture but negative result in LAMP has been confirmed to be pathogenic bacteria by repeated culture and LAMP. In 53 patients without children sepsis, 2 positive result detected by blood culture were demonstrated as pollution bacteria by Infection judgment standard. All *Staphylococcus aureus* detected by LAMP were pathogenic bacteria, but 5 of 8 *Staphylococcus aureus* detected by blood culture were pollution bacteria. There were numerous ways to distinguish pollution bacteria in blood culture, such as repeated culture[9], and pulsed-field gel electrophoresis[10]. It is not suitable for children, because repeated culture need lots of blood and PEGF is hard to operate.

LAMP technology is a quick and easy detection for *Staphylococcus aureus*, because *Staphylococcus aureus* detected by blood cultures alarms accounted for 54% within 24 h, 40% within 48 h[11], then after alarm it need 24-48 h for identify. Early symptoms of children sepsis is not obvious and can progress rapidly, so it often get worse before positive result appear in laboratory testing, and loss the best opportunity for treatment. LAMP provide an early diagnosis for sepsis, it reduced mortality, improve survival, and reduce

the use of unnecessary antibiotics[12].

This study compared the effect of blood culture and LAMP in sample detection. The results show that compare to blood culture, the LAMP technology is more sensitivity. In addition, LAMP is easy to operate and no need of complex instruments. In conclusion, as a quick and easy detection of *Staphylococcus aureus*, the LAMP technology was successfully established, laid the foundation for the diagnosis and treatment of children *Staphylococcus aureus* sepsis.

Conflict of interest statement

We declare that we have no conflict of interest.

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