研究报告

Rapid detection of *Shigella* and *Salmonella* in rhesus monkeys by loop-mediated isothermal amplification assay

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【**Abstract**】 **Objective** To establish a loop-mediated isothermal amplification (LAMP) method for detecting diarrhea pathogens (*Shigella* and *Salmonella*) in rhesus monkeys and evaluate the application of the LAMP method for detecting bacterial diseases in non-human primate laboratory animals. **Materials and Methods** A total of 205 fecal samples of rhesus monkeys were detected in this LAMP assay. The specificity and sensitivity of LAMP for *Shigella* and *Salmonella* were analyzed, and real-time polymerase chain reaction (REAL-TIME PCR) assay was employed as control. **Results** The LAMP method established here needed only 45 min to complete the reaction at 63℃. Its detection limit was 10 pg/μL and with a high specificity. The positive rate of *Shigella* and *Salmonella* was 1.5% and 6.3%, respectively. **Conclusions** Here we have established a fast and simple *Shigella* and *Salmonella* LAMP detection method that has strong specificity and high sensitivity and is suitable for rapid detection of bacterial disease in macaques. The development of this rapid detection kit is underway, and it will be helpful to the diarrhea detection.

【**Key words**】 *Shigella*; *Salmonella*; Loop-mediated isothermal amplification, LAMP; Rhesus monkey; Bacteria detection; Diarrhea
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【中图分类号】Q95-33 【文献标识码】A 【文章编号】1005-4847(2016) 01-0007-07
Doi:10.3969/j.issn.1005-4847.2016.01.002

There are hundreds of thousands of monkeys kept in cages in China as laboratory animals. According to the national rules, the monkeys should be healthy to be used in medical experiments. While many monkey farms were obsessed with diarrhea, which is common in captive Rhesus monkeys. The diarrhea is caused by bacterial infections, cage replacement, seasonal alternations, and so on. Bacterial infection is the most harmful cause, with *Shigella* and *Salmonella* as the most common diarrhea pathogens[1]. These bacteria adversely affect the animals' physical health and may infect animal keepers and other staff members. The current isolation and culturing method used for the routine diagnosis of these pathogens requires considerable time and effort. As a result, symptomatic medications cannot be promptly administered, which may result in illness delay and loss of optimal treatment timing. A monkey farm may shell out millions per year for the monkey death from diarrhea. Furthermore, blind medications can easily enhance the antimicrobial resistance of pathogens and increase difficulties in treatment. Therefore, a rapid and cheap detection technique of bacteria is urgently needed.

With the development of molecular techniques, a

[**基金项目**]十二五后勤科研项目(CWS11J092);国家自然科学基金(31272385);十二五重大专项(2011ZXJ09201-031);北京市自然科学基金(5152023)。
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variety of methods such as polymerase chain reaction (PCR) and immunological methods are available to identify causative pathogens. However, these approaches require expensive equipment (PCR instrument and Multiskan Ascent) and skilled technicians. Therefore, a rapid, simple, and economical detection technique is needed for diagnosing diseases in captive rhesus monkeys.

The loop-mediated isothermal amplification (LAMP) technique is based on the strand displacement reaction and stem-loop structure that amplifies the target gene fragment under isothermal conditions.[2] This method requires only a water bath and the result can be judged by naked eyes. The LAMP technique can be used in rapid diagnosis of different pathogens, and some researchers have already used this technique for bacterial detection.[3,4] However, they were only able to obtain the LAMP reaction results using an electrophoresis map. These results are lack of real-time reaction process monitoring. Furthermore, the risk of aerosol pollution exists in the laboratory. Therefore, no accurate judgment of the process could be made to date.

In the current study, we used a Loopamp® Real-time Turbidimeter LA-320C (Eiken Chemical Co. Ltd., Tokyo, Japan) to monitor the LAMP reactions in real time and established a method that can directly detect *Shigella* and *Salmonella* in rhesus monkey feces. This method may be helpful in monitoring the healthy status of non-human primate laboratory animals.

1 Materials and methods

1.1 Sample collection

A total of 205 fecal samples were collected from 4 primate centers located in 4 cities (Haikou, Nanning, Yibin and Beijing) (Fig. 1). Bacterial DNA was extracted from 1 mL of cultural suspension using TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). DNA was extracted from 0.1 mg feces using CHLEX

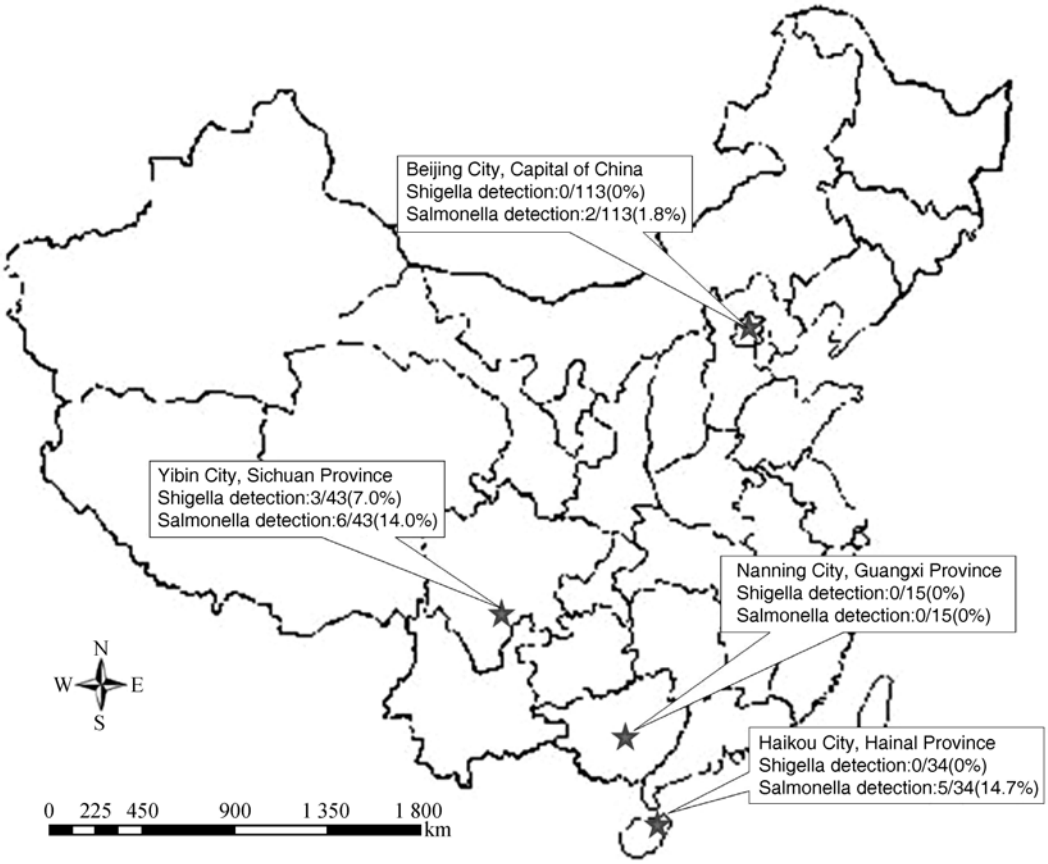

Fig. 1 Locations of the primate centers in China where macaques were investigated for *Shigella* and *Salmonella*.
A total of 205 rhesus macaques feces samples were collected from Haikou, Nanning, Yibin and Beijing Cities.
The results of positive rate of *Shigella* and *Salmonella* were shown in the picture.

reagent.

1.2 Bacterial strains and cultural conditions

Standard bacteria were purchased from the National Institutes for Food and Drug Control included *Salmonella enteritidis* (CMCC 50041), *Salmonella typhi* (CMCC 50071), *Salmonella Typhimurium* (CMCC 50115), *Shigella dysenteriae* (CMCC 51252), *Shigella flexneri* (CMCC 51571), *Shigella flexneri* (CMCC 1.1868), and *Escherichia coli* (CMCC 44102).

Our research complied with the protocols approved by the Animal Care and Use Committee of Laboratory Animal Center of the Academy of Military Medical Sciences. This research adhered to the legal requirements of China as well as the American Society of Primatologists principles for the ethical treatment of primates.

1.3 Design of *Shigella* and *Salmonella* specific LAMP primers

According to the nucleotide sequences of *Shigella ipaH* gene (M76444.1) and *Salmonella invA* gene (NC_003197.1) that we retrieved from the GenBank database, LAMP primers were designed using PrimerExplorer (http://primerexplorer.jp/elamp4.0.0/index.html). Five groups of primers for these two bacteria were obtained: F3 and B3 (outer primers), FIP and BIP (inner primers), and LF or LB (loop primer).

1.4 LAMP reaction condition

The LAMP reactions were performed using a Loopamp DNA Amplification Kit (Eiken Chemical Co. Ltd. Tokyo). The reaction system (25 μL) included the following: 2 × reaction mix (12.5 μL), primer mix (4.6 μL), Bst DNA polymerase (1 μL), sample DNA (2 μL), distilled water (4.9 μL), and primer mix with F3, B3, FIP, BIP, and LF/LB in concentrations of 5, 5, 40, 40, and 20 pmol/μL, respectively. The reactions were performed in the Loopamp® Real-time Turbidimeter LA-320C (Eiken Chemical Co. Ltd.) for 60 min. The most optimal reaction temperature and primers were determined using a temperature gradient assay. Visual detection was performed using a fluorescent detection reagent (Eiken Chemical Co. Ltd.). The color change under normal conditions was recorded: a positive sample was green, while a negative sample was orange.

1.5 LAMP assay specificity

Shigella or *Salmonella* primers were used to detect standard bacteria (including CMCC 50041, 50071, 50115, 51252, 51571, 1.1868, and 44102). DNA was extracted as described earlier. All tests were performed in triplicate.

1.6 LAMP assay sensitivity

For the sensitivity tests, DNA mixes of *Shigella* (CMCC51252, 51571, and 1.1868) and *Salmonella* (CMCC50041, 50071, 50115) were serially diluted 10-fold with sterile water from 1.0×10^{-1} to 1.0×10^{-7}. All tests were performed in triplicate.

1.7 LAMP and real-time PCR detection

A total of 205 fecal samples from captive Rhesus monkey were examined using real-time PCR and the LAMP system which we developed for the detection of *Shigella* and *Salmonella*. Differences were found between these two methods.

2 Results

2.1 Specific LAMP primers of *Shigella* and *Salmonella*

The primer sets comprising the two outer primers (F3 and B3), two inner primers (FIP and BIP), and one loop primer (LF or LB) (Table 1) recognized six distinct regions on the ipaH of *Salmonella* and the invA of *Shigella* sequences. All of the primers were synthesized by Beijing AuGCT DNA-SYN Biotechnology (China).

2.2 LAMP reaction temperature

The reactions were performed in the real-time turbidimeter LA-320C at 60 – 64℃ for 90 min. The machine detects the turbidity values every 6 s and indicates positive amplification when the turbidity values reach 0.1.[5,6] The earliest reaction started at 63℃, and the optimal temperature of these two bacteria was 63℃ (Fig. 2).

2.3 LAMP result judgment

Fluorescent detection reagent (1 μL) was added to the *Shigella* and *Salmonella* reaction mixtures for visual detection by naked eyes. Positive amplification was green and negative amplification was orange (Fig.

3). The results also could be observed using a UV lamp (wavelength 240 – 260 nm or 350 – 370 nm), with a positive result of green and a negative result with no changes.

Tab. 1 The LAMP primer sequences of *Shigella* and *Salmonella*

Bacteria	Name of primer	Sequences of primers
Shigella	F3	ACATGAAGAGCATGCCAACA
	B3	TCCTCACAGCTCTCAGTGG
	FIP	AATCCGGAGGTATTGCGTGCA CCTTTTCCGCGTTCCTTGA
	BIP	GTCGCTGCATGGCTGGAAAAAC GCAGCAACAGCGAAAGACT
	LF	ACGGTATCGGAAAGGCGG
Salmonella	F3	GAACGTGTCGCGGAAGTC
	B3	CGGCAATAGCGTCACCTT
	FIP	CCGGCCTTCAAATCGGCATCAAG CCCGATTTTCTCTGGATGG
	BIP	GAACGGCGAAGCGTACTGGA CATCGCACCGTCAAAGGAA
	LB	AAGGGAAAGCCAGCTTTACGG

Note. LAMP: loop-mediated isothermal amplification.

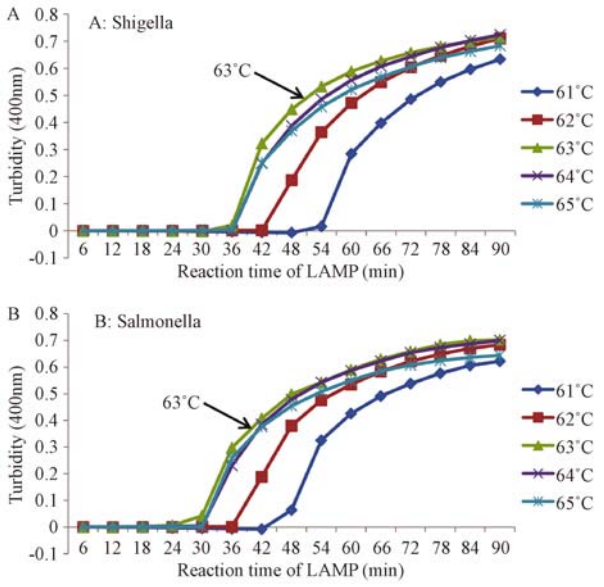

Fig. 2 The optimal temperature in detection of *Shigella* (A) and Salmonella (B), with LAMP reaction at 63℃.

2.4 Specificity and sensitivity *of the Shigella* and *Salmonella* specific LAMP assay

With the *Shigella* primer, the *Shigella* samples were positively amplified, while the *Salmonella* and *E. coli* samples were not (Fig. 4). The *Salmonella* detection results were the same. These results showed that the LAMP primers of *Shigella* and *Salmonella* had strong specificity.

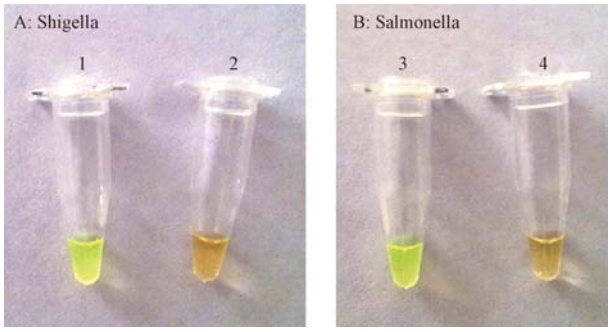

Fig. 3 Fluorescence detection of *Shigella* (left) and Salmonella (right) by the LAMP assay.
Visual detection of *Shigella* (At) and *Salmonella* (B) in the LAMP products using fluorescence detection.
1 and 3: Positive LAMP reaction (green);
2 and 4: Negative LAMP reaction (orange).

Shigella and *Salmonella* mixture DNA samples (212.3 ng/μL and 185.7 ng/μL, respectively) were prepared for the sensitivity assay. The loopamp® real-time turbidimeter LA-320C curve analysis showed that all positive amplifications reached a velocity curve peak >0.1 within 60 min (Fig. 5 and 6). When the DNA template concentration decreased, the reaction rate constant and response slope was decreased and the peak time delayed. The minimum detectable limits for *Salmonella* and *Shigella* were both 10^{-5} (DNA concentration was approximately 10 pg/μL).

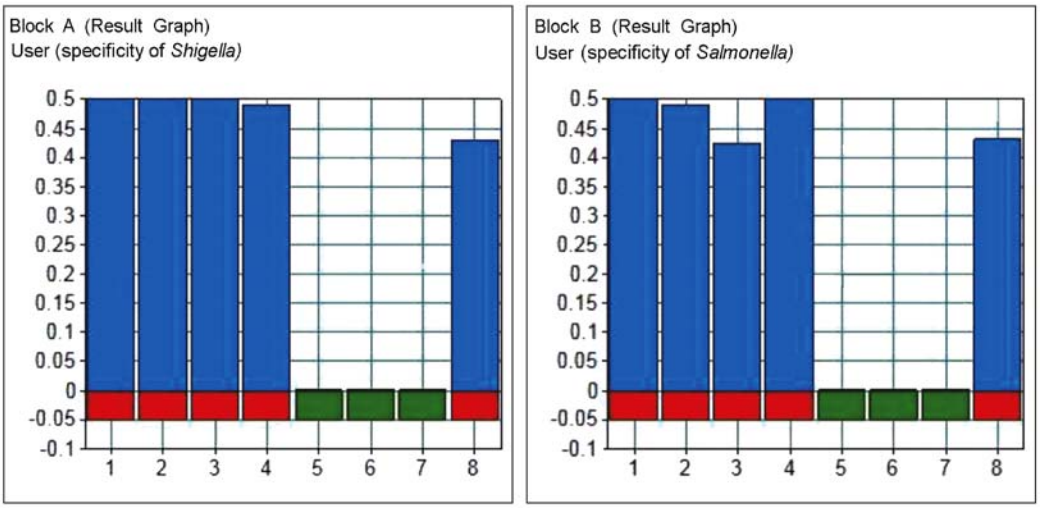

Fig. 4　Specificity of *Shigella* and *Salmonella*.

With the *Shigella* and *Salmonella* primers, the samples were positively amplified, while the control samples were not. (Block A) 1, *Salmonella* mix (including 50041, 50071, 50115); 2, 50041; 3, 50071; 4, 50115; 5, *Shigella* mix (including 51252, 51571, 1.1868); 6, 44102; 7, DEPC H_2O; 8, positive control. (Block B) 1, *Shigella* mix (including 51252, 51571, 1.1868); 2, 51252; 3, 51571; 4, 1.1868; 5, *Salmonella* mix (including 50041, 50071, 50115); 6, 44102; 7, DEPC H2O; 8, positive control.

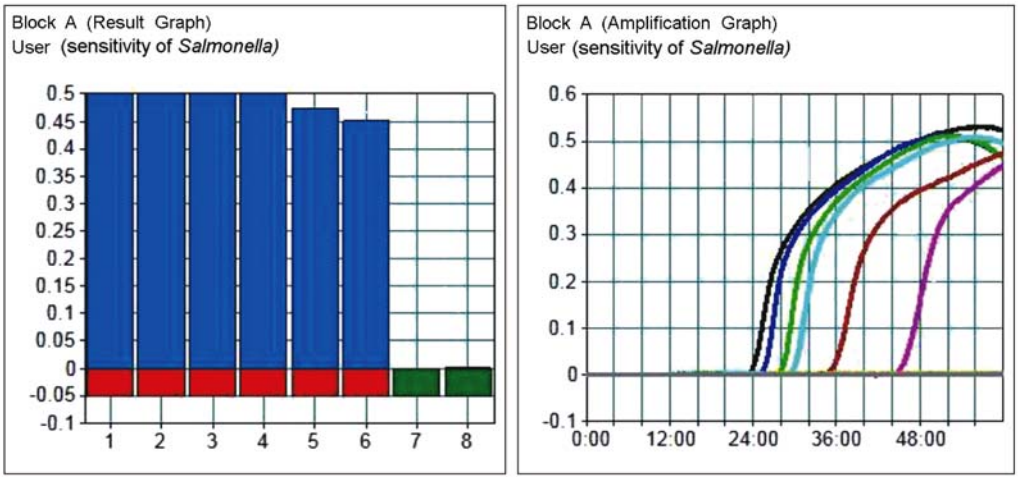

Fig. 5　Sensitivity of *Salmonella* in LAMP.

The minimum detectable limits for *Salmonella* was 10^{-5} (DNA concentration was approximately 10 pg/μL).

CH1, 1.0; CH2 – CH8, $1.0 \times 10^{-1} - 1.0 \times 10^{-7}$.

2.5　Comparison of the LAMP and real-time PCR assay

In the 205 clinical fecal samples we collected from captive rhesus monkeys from 4 provinces in China, 3 were tested as positive for *Shigella* and 13 tested as positive for *Salmonella* by LAMP and real-time PCR assay (Table 2, Fig. 1). No nonspecific amplification was observed. The LAMP and real-time PCR assays had the same positive detection rates of *Shigella* and *Salmonella*.

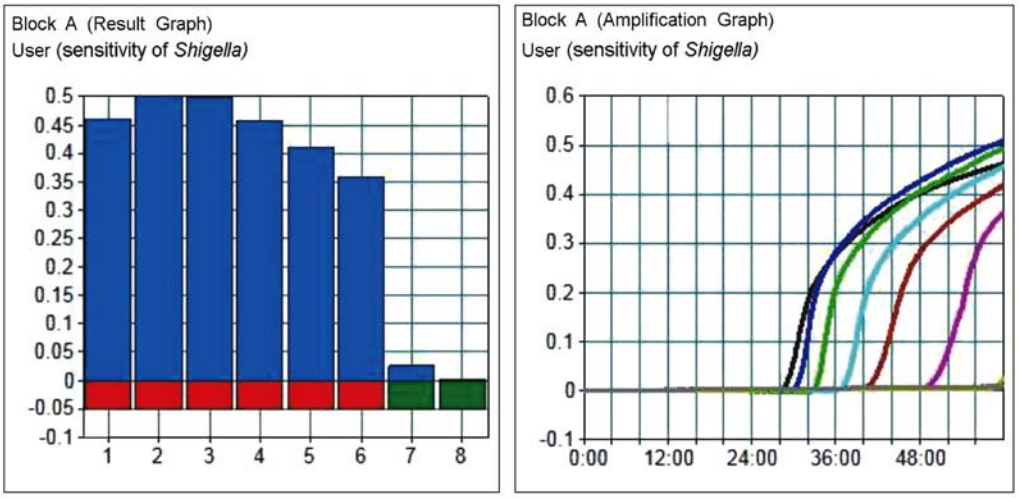

Fig. 6 Sensitivity of the detection of *Shigella* in LAMP.

The minimum detectable limit for *Shigella* was 10^{-5} (DNA concentration was approximately 10 pg/μL). CH1: 1.0; CH2 – CH8: $1.0 \times 10^{-1} - 1.0 \times 10^{-7}$.

Tab. 2 Positivity rates of the rhesus monkey fecal samples detected by LAMP and real-time PCR

Results	LAMP		Real-time PCR	
	Shigella	*Salmonella*	*Shigella*	*Salmonella*
Positive	3	13	3	13
Total	205	205	205	205
Positivity rate (%)	1.5	6.3	1.5	6.3

Note. LAMP: loop-mediated isothermal amplification. PCR: polymerase chain reaction

3 Discussion

Shigella and *Salmonella* are the major pathogens leading to diarrhea in rhesus monkeys. These two bacteria are commonly detected using culturing, PCR,[7] and enzyme-linked immunosorbent assay[8] techniques, among others. However, culture requires 2 – 3 days, and PCR requires specific equipment and >2 h for detection. As such, these methods cannot rapidly detect bacterial diseases in laboratory animals. In contrast, LAMP, a new nucleic acid amplification reaction technique, is advantageous due to its fast and simple reaction conditions used for pathogen detection. In our study, results could be determined after the bacterial nucleic acids were allowed to react in this system at 63℃ for 1 h. The LAMP approach is much faster than the traditional methods.

Most of the currently established LAMP reaction methods determine results using images from the UV analysis of agarose gel electrophoresis.[9] Furthermore, only the final LAMP reaction result is analyzed, and the methods carry a risk of aerosol contamination in the laboratory. Given the lack of real-time reaction monitoring, eliminating these interfering factors is quite difficult. In this study, we established a real-time LAMP method for detection of *Shigella* and *Salmonella*. The reaction progress was analyzed using a loopamp® real-time turbidimeter LA-320C (Eiken Chemical Co. Ltd., Tokyo, Japan). The instrument be used to automatically observe the reaction in real-time and exclude false-positive and non-specific reaction interference factors according to set analytical standards. In a sample analysis, when the reaction progressed to 24 min, generation of a precipitate was detected (Fig. 5). Analysis of the curves showed that the reaction peaked at 0.5, more than the default of 0.1 positive determination standards. During the efficient LAMP reaction, the amplified target sequence amount was enriched to a critical value and entered a higher reaction rate to achieve the reaction peak. Dilution of the template affected only the reaction starting time and not the reaction efficiency. Therefore, the sample reaction time was associated with the initial amount of DNA template used.

The lowest detection limit of PCR is 200 pg/μL commonly. However, in our study, the detection limits of *Shigella* and *Salmonella* were both 10 pg/μL, which indicated that the LAMP method had a higher sensitivity than PCR. In examination of the 205 fecal samples, the positive LAMP and PCR rates for *Shigella* and *Salmonella* were the same. These findings suggest that the LAMP method has the same sensitivity as real-time PCR for detecting *Shigella* and *Salmonella*, especially the former could be judged with eyes.

The two bacteria real-time LAMP detection methods established in this study are fast (completed within 1 h), sensitive (detection limit of approximately 10 pg/μL), with simple equipment requirements, and have simple experimental operation. In particular, this method has no side effect on animals and required only fecal sample collection. Our findings suggest that the LAMP method is properly applied for the diagnosis of the diarrhea pathogens in the rhesus monkeys. The LAMP primers designed in this assay are applying patents, and the development of a quick kit for macaque diarrhea detection is underway. This investment is scientific and economic for the macaque health maintaining and for the monkey farms.

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[收稿日期] 2015-09-09