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Establishment of the molecular beacon-loop-mediated isothermal amplification method for the rapid detection of *Porphyromonas gingivalis*

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ABSTRACT

Porphyromonas gingivalis, a clinically important oral pathogen causing periodontal disease, is difficult to culture in routine conditions. Hence, it is necessary to establish a reliable technique to detect this pathogen. Previously, our laboratory developed a new isothermal detection method, called MB-LAMP (molecular beacon-Loop-mediated isothermal amplification), which combines the advantages of LAMP and qPCR through the accurate and quantitative detection of LAMP products. This approach offers significant potential for the point-of-care detection of *P. gingivalis*. Here, MB-LAMP was used to detect *P. gingivalis* targeting a specific fragment, and the sensitivity was as high as 1.4×10^{-1} pg μL^{-1}. The method showed no cross-reaction with 14 other bacterial pathogens. For clinical samples, this assay showed a high diagnostic sensitivity (100%) and specificity (100%), equivalent to that of real-time quantitative polymerase chain reaction (real-time qPCR). Moreover, detection with MB-LAMP was significantly faster than that with real-time qPCR, reducing the time required for clinical diagnosis. Finally, we established an absolute quantification method with MB-LAMP for *P. gingivalis* using pilot samples. Thus, the highly specific, sensitive, and rapid assay developed in this study makes it feasible to diagnose *P. gingivalis*.

1. Introduction

Periodontitis is a chronic infectious disease in periodontal support tissues that can induce or aggravate multi-system diseases such as diabetes (Taylor et al., 2013), cardiovascular disease (Kalburgi et al., 2014), and chronic obstructive pulmonary disease (Tan et al., 2014). Periodontitis has become one of the most common human diseases causing damage to both oral and general health. In humans, *Porphyromonas gingivalis* has been implicated in periodontitis risk factors, suggesting a relevant role in disease establishment and progression (van Winkelhoff et al., 2002; Ciandrini et al., 2017). Culturing has been used to detect *P. gingivalis* in root canals during endodontic treatment (Gomes et al., 2004; Tomazinho and Avila-Campos, 2007); however, this method is poorly suited for clinical diagnosis because it is time-consuming and has a low detection rate (Rolph et al., 2001).

Molecular biological techniques, including quantitative real-time polymerase chain reaction (qPCR) and loop-mediated isothermal amplification (LAMP) have been well developed, and possess great potential to facilitate the diagnosis of *P. gingivalis* (Maeda et al., 2005; Saito et al., 2009; Kitano et al., 2016). Recently, our group developed a new detection method, called molecular beacon-LAMP (MB-LAMP) which combined the advantages of LAMP and qPCR, enabling the detection of LAMP products directly at constant temperature (Liu et al., 2017). With a molecular beacon probe, LFP (Loop Forward Probe) or LBP (Loop Backward Probe) rectified the issue of non-specific amplification to some extent and this method displayed higher specificity than traditional LAMP (Liu et al., 2017). In addition, the reaction conditions were simpler than those of qPCR. Here, considering the need for *P. gingivalis* diagnosis, we developed an MB-LAMP detection method for *P. gingivalis* and compared its sensitivity, specificity, and practicality with the qPCR method.

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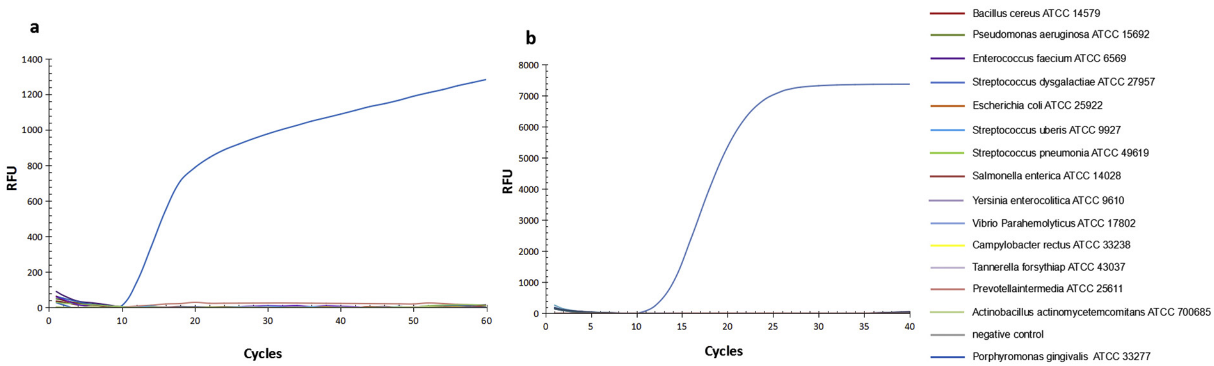

Fig. 1. Specificity of MB-LAMP and qPCR for *P. gingivalis*. (a) Specificity of MB-LAMP. (b) Specificity of qPCR. The specificities of MB-LAMP and qPCR assays were determined using nucleic acid extracted from 15 bacterial strains as indicated in the material and methods. Water served as a negative control in all three experiments.

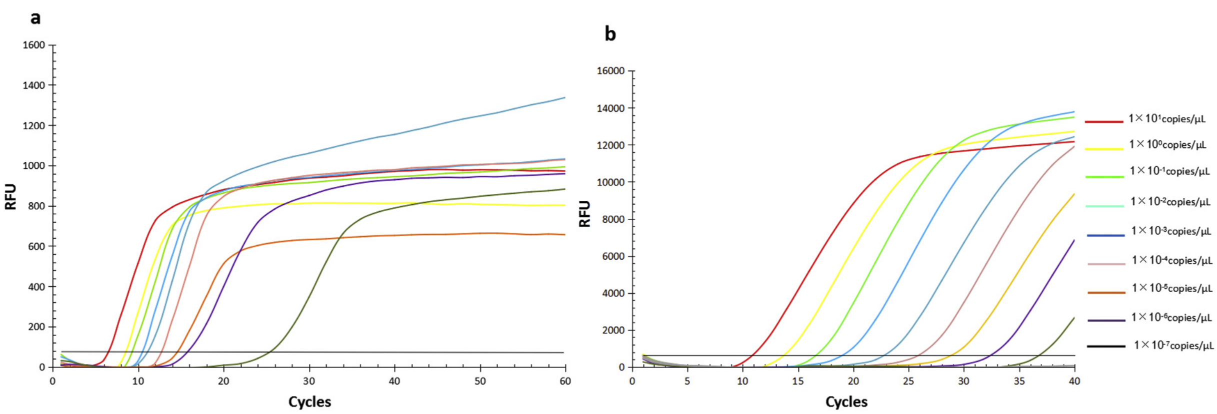

Fig. 2. Sensitivity of MB-LAMP and qPCR for *P. gingivalis*. (a) Sensitivity of LAMP. (b) Sensitivity of qPCR. Plasmids were serially diluted to 10 ng μL^{-1}, 1 ng μL^{-1}, 1×10^{-1} ng μL^{-1}, 1×10^{-2} ng μL^{-1}, 1×10^{-3} ng μL^{-1}, 1×10^{-4} ng μL^{-1}, 1×10^{-5} ng μL^{-1}, 1×10^{-6} ng μL^{-1}, 1×10^{-7} ng μL^{-1}, 1×10^{-8} ng μL^{-1}, 1×10^{-9} ng μL^{-1}, and 1×10^{-10} ng μL^{-1}. The lowest detection limit of all two methods was 1×10^{-7} ng μL^{-1}. Dilutions are indicated in the figures.

2. Materials and methods

2.1. Subjects and sample collection

Ethical approval for the study was granted by the Ethics Committee of the Academy of Military Medical Sciences (China). Forty patients with periodontitis (20 males and 20 females), ranging from 35 to 55 years of age, were selected from those referred for scaling and root planning at the No. 307 Hospital of People's Liberation Army. All the subjects provided written informed consent. Patient inclusion and exclusion criteria are detailed in the Supplementary File S1. Dental plaque samples were collected from the roots of teeth, beyond the gingival margin, using a Gentra Puregene Buccal Cell Kit (QIAGEN Sciences, MD, USA) brush and delivered to the laboratory on ice.

2.2. DNA preparation

The following bacterial strains were used as negative controls: *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 15692, *Enterococcus faecium* ATCC 6569, *Streptococcus dysgalactiae* ATCC 27957, *Escherichia coli* ATCC 25922, *Streptococcus uberis* ATCC 9927, *Streptococcus pneumoniae* ATCC 49619, *Salmonella enterica* ATCC 14028, *Yersinia enterocolitica* ATCC 9610, *Vibrio parahemolyticus* ATCC 17802, *Campylobacter rectus* ATCC 33238, *Tannerella forsythiap* ATCC 43037, *Prevotella intermedia* ATCC 25611 and *Actinobacillus actinomycetemcomitans* ATCC 700685. *Porphyromonas gingivalis* ATCC 33277 was used as the positive control. All strains were grown in their respective medium, and genomic DNA was extracted from these strains using a TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China), following the manufacturer's protocol. DNA concentrations were > 20 ng μL^{-1}, as measured using Qubit 3.0 (Life Technologies, Carlsbad, CA, USA). Dental plaque DNA was extracted using a Gentra Puregene buccal cell kit (Qiagen, Hilden, Germany) and all sample were diluted to a concentration of approximately 20 ng μL^{-1}. All samples were stored at −20 °C until further processing. In addition, a 248-bp target region in the *P. gingivalis* genome (File S2) was generated from the specific sequence used for the design of primers, and the acquired DNA fragment was cloned into the plasmid vector pBR322 by Sangon Biotech Co., Ltd. (Shanghai, China). The plasmid was quantified using Qubit 3.0 according to the manufacturer's protocol and serially diluted from 10 ng μL^{-1} to 1×10^{-12} ng μL^{-1} in ddH2O to be used as templates. The copy number (copies mL^{-1}) calculation formula was $Y = 6.02 \times 10^{23} \times (g/mL)/DNA\ length \times 660$.

2.3. Primer design

The MB-LAMP primers for *P. gingivalis* detection were designed using the Primer Explorer V4 software (Fujitsu, Tokyo, Japan), together with the *P. gingivalis*-specific target sequence (GenBank: CP01195.1; Region: 29273 to 132,746).

In addition to the basic LAMP primers, the molecular beacon LFP or LBP labeled with fluorophore was needed. The two molecular beacons were adapted from the LF and LB primers designed in LAMP as previously described (Liu et al., 2017) and replaced the original ones to form a complete set of MB-LAMP primers.

The qPCR primer sequences used were as follows: forward primer, 5′ CATGGAGAGGAAATCCCTGA 3′; reverse primer, 5′ AAAACCTTCAG CCCGTTTTT 3′; FAM probe, 5′ FAM-CATTGTCTCTACTCAGGCGCTCC

Table 1
Comparison of MB-LAMP and qPCR for *P. gingivalis* detection in clinical samples.

No.	Results		Reaction time(minutes)	
	MB-LAMP	qPCR	MB-LAMP	qPCR
1	+	+	13	24
2	+	+	13	24
3	−	> 35 cycles	−	−
4	+	+	14	23
5	+	+	17	30
6	−	−	−	−
7	−	> 35 cycles	−	−
8	+	+	13	24
9	+	+	13	25
10	+	+	13	25
11	−	> 35 cycles	−	−
12	−	> 35 cycles	−	−
13	+	+	13	26
14	+	+	13	25
15	−	−	−	−
16	+	+	14	28
17	+	+	14	28
18	+	+	13	26
19	−	> 35 cycles	−	−
20	+	+	14	27
21	+	+	13	26
22	+	+	13	25
23	−	> 35 cycles	−	−
24	−	> 35 cycles	−	−
25	+	+	14	27
26	−	> 35 cycles	−	−
27	+	+	15	26
28	−	> 35 cycles	−	−
29	+	+	13	25
30	+	+	15	26
31	−	> 35 cycles	−	−
32	+	+	15	30
33	+	+	14	26
34	+	+	18	32
35	+	+	17	31
36	−	−	−	−
37	−	> 35 cycles	−	−
38	+	+	14	28
39	+	+	15	29
40	+	+	14	28

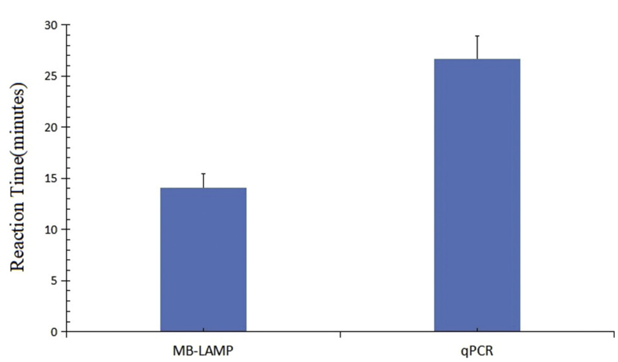

Fig. 3. Comparison of reaction times of MB-LAMP and qPCR for *P. gingivalis* in clinical samples. For the clinical samples with reaction cycles < 35, we found that the average reaction time of qPCR was significantly longer than that of MB-LAMP. There were significant differences in the reaction time between these two groups. *$p < .01$.

TCCT-Dabcyl 3′. The qPCR primer set for *P. gingivalis* detection was designed and synthesized by Sangon Biotech Co., Ltd. using the same target sequence as in the synthetic plasmid.

2.4. Reaction mixture and conditions

The MB-LAMP reaction mixtures (DNA Amplification Kit; Eiken Chemical Co., Ltd., Tochigi, Japan) comprised the following reagents (final concentration): 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 8 mM $MgSO_4$, 1.4 mM dNTPs, and 8 U Bst DNA polymerase in a total volume of 25 µL per reaction tube (Eiken Chemical Co.). Primer concentrations for LAMP were 40 pmol of FIP and BIP, 20 pmol of LF and LB, and 5 pmol of F3 and B3; the concentrations for MB-LAMP were 40 pmol of FIP and BIP, 20 pmol of LF, 8 pmol of LBP, and 5 pmol of F3 and B3. In addition, 2 µL of genomic DNA template was added. In order to prevent aerosol pollution in the MB-LAMP reaction, we also added impacting medium to each reaction tube. The reactions were performed at 65 °C. The qPCR mixture (20 µL per reaction tube) contained 10 pmol of forward primer, 10 pmol of reverse primer, 8 pmol of fluorescent probe, and 10 µL of Probe qPCR Mix (Takara Bio, Inc., Kusatsu, Japan). The amplification conditions were as follows: an initial activation step at 95 °C for 30 s; 40 amplification cycles at 95 °C for 30 s and 58 °C for 30 s (one cycle for 1 min); and a hold at 4 °C. An LA-320c turbidimeter (Eiken Chemical Co.) was used for MB-LAMP detection, and a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for qPCR and MB-LAMP monitoring.

3. Results

3.1. Determination of optimal MB-LAMP primers

Five sets of primers for LAMP were produced using Primer Explorer V4 (Table S1), and real-time turbidity curves indicated that the B1 set amplified the target sequences in the shortest time and was thus chosen for MB-LAMP detection (Fig. S1). The molecular beacons, LFP and LBP, were considered from LF and LB of B1 sets separately; the sequences are detailed as follows: LFP, 5′ FAM–TGTCACGGAGCAGAAGCCTGTGACA-Dabcyl 3′; LBP, 5′ FAM-GCGCCTGCTGATCATTGTCTCTACTCAGGCGC-Dabcyl 3′. In comparison, the LBP sets reacted faster than the LFP sets; hence, LBP was selected as the molecular beacon for MB-LAMP (Fig. S2).

3.2. MB-LAMP specificity and sensitivity determination

To avoid non-specific amplification, we tested the specificity of MB-LAMP using various strains. In this experiment, we verified the specificity of MB-LAMP using 14 standard strains from our laboratory collection. As shown in Fig. 1a. *P. gingivalis* was significantly amplified at 12 min, while the other standard strains and the blank control remained below the detection threshold (horizontal line) for the entire MB-LAMP methods. In comparison to qPCR shown in Fig. 1b, MB-LAMP exhibited the same high specificity.

As shown in Fig. 2a, MB-LAMP could detect serially diluted plasmid at 1×10^{-7} ng µL^{-1}, which is equivalent to the detection limit of qPCR (Fig. 2a and b). In addition, as demonstrated in Fig. 2, the reaction of MB-LAMP could be completed within 26 min, in contrast to 38 min of amplification during qPCR in the minimum concentration. Thus, the rate of qPCR reaction was slower than that of MB-LAMP.

3.3. Validation of MB-LAMP with clinical samples

The efficacy of MB-LAMP assay for clinical dental plaque samples was compared with that of qPCR using the clinical samples from 40 patients with periodontal disease. All detection experiments using 40 clinical samples were repeated thrice. The initial results indicated that the positive rate was 26/40 for MB-LAMP and 37/40 for qPCR (Table 1). However, the 11 positive qPCR results can be thought as false positive because it is generally considered to be false positive if the reaction cycle of some sample is > 35. Thus, we found that the results

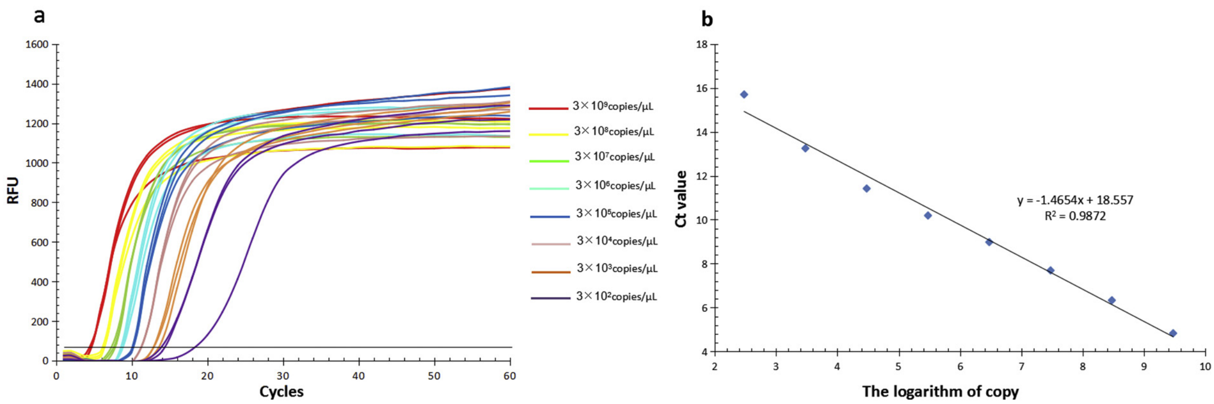

Fig. 4. Absolute quantification using MB-LAMP for *P. gingivalis*. (a) Amplification curves. Serially diluted plasmids (3×10^9, 3×10^8, 3×10^7, 3×10^6, 3×10^5, 3×10^4, 3×10^3, and 3×10^2 copies μL^{-1}) were repeatedly detected. (b) Regression curves analysis of absolute quantitative results. Y-axis: reaction time. X-axis: logarithm of the copy number of samples at different concentrations.

of MB-LAMP were consistent with that of qPCR in all the 40 clinical samples (26 positives in 40 samples).

Furthermore, we compared the reaction time of these two methods for positive samples with reaction time < 35 min. The average reaction time of MB-LAMP and qPCR was 14.16 and 26.69 min, respectively. Thus, for clinical samples, the reaction rate of qPCR was significantly slower than that of MB-LAMP (Fig. 3).

3.4. Absolute quantification using MB-LAMP

As mentioned previously, MB-LAMP had the potential to enable absolute quantification of the target fragments in samples because it has characteristics of the qPCR method that could be used for real-time detection by fluorescence probe (Liu et al., 2017). Thus, we performed the experiment with serially diluted plasmid (3×10^9 to 3×10^2 copies μL^{-1}) to explore the possibility of performing absolute quantification. As shown in Fig. 4a, the reaction times of the first six standards were stable, and the spacing between the samples was nearly the same. We analyzed the correlation between the logarithm of copy number and reaction time of eight standards and obtained the following correlation equation: $Y = -1.4654X + 17.374$ ($R^2 = 0.9872$) (Fig. 4b).

4. Discussion

Periodontal disease is the most common infectious disease affecting tooth-supporting structures. Although the concentration of pathogenic bacteria is low during the early stages of the disease, bacteria can undergo extensive replication, and have more serious consequences. Compared with the traditional culturing method for detection, nucleic acid detection methods can detect microorganisms that are more difficult to culture with high sensitivity. Therefore, this study established and evaluated an MB-LAMP method with relatively high sensitivity and simple operational requirements to detect *P. gingivalis*, a representative periodontal pathogen.

In this experiment, MB-LAMP could detect pure nucleic acids within 20 min, with the detection limit as low as 10^{-4} pM or 10^{-7} ng μL^{-1}. Increasing the reaction time did not allow the detection of lower concentrations of pure nucleic acids. Similarly, although the positive results from the lowest concentrations of pure plasmid were yielded at 38 cycles, as observed by qPCR, conventionally, the positive results were generally thought to be false positive when the reaction time was > 35 cycles (Johnson et al., 2012). Thus, we considered samples with reaction time < 35 min to be positive samples in clinical samples. Although these two methods showed similar sensitivity and specificity for detection in clinical samples, MB-LAMP displayed a better performance in amplification speed for clinical samples. As shown in Fig. 3b, the reaction time was evidently shorter for MB-LAMP when compared to that for qPCR. In addition, MB-LAMP requires simpler instruments and equipment, such as Genie II (GEN2–2201, OptiGene, Co., Horsham, UK), a kind of thermostatic fluorometer. This reduces the cost of MB-LAMP in comparison to that of CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The devices used for MB-LAMP are simple and suitable for on-the-spot rapid diagnosis.

Overall, the main advantages of MB-LAMP are that it solves the issue of non-specific amplification to a certain extent and improves the visualization degree of LAMP. The development of MB-LAMP in this study are not only suitable for *P. gingivalis*, but can also be extended to the detection of various other pathogenic microorganisms, which could inform future choices of detection methods.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.01.013.

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Conflict of interest

The authors have declared that no competing interests exist.

Author contributions

YS, SH, YK and LH wrote the main manuscript text. DZ helped to design the experiments. YS and SH executed the experiments. YT helped collect the strains. SC repeated and confirmed the experiments. XH helped prepare the reaction solution. YX and XL helped organize the funds. LL and LF helped organize the figs. WL helped to conceive project and LH reviewed the manuscript.

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