Novel Loop-Mediated Isothermal Amplification Method for Detection of the JP2 Clone of *Aggregatibacter actinomycetemcomitans* in Subgingival Plaque[▽]

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We developed a loop-mediated isothermal amplification method that detects the JP2 clone of *Aggregatibacter actinomycetemcomitans*, which induces aggressive periodontitis in adolescents of North and West African descents. Being independent of special equipment, this specific and sensitive method offers significant advantages for screening of patients on a population basis and in clinical settings.

Aggregatibacter (Actinobacillus) actinomycetemcomitans (11) has long been suspected as an etiological factor in periodontitis in juveniles (17, 19). Members of a particular clonal lineage (JP2) of *A. actinomycetemcomitans* (18) show a unique 530-bp deletion (Δ 530) in the promoter region of the *ltx* operon resulting in enhanced production of leukotoxin, a key virulence factor (1). The JP2 clone shows marked racial tropism as it has been isolated almost exclusively from adolescent periodontitis patients of West and Northwest African descent, including both Africans and Arabs (2, 3, 4, 5, 6). Substantial evidence shows that the JP2 clone induces aggressive periodontitis in these populations and constitutes a particularly pathogenic subpopulation of *A. actinomycetemcomitans* (see reference 6a); reviewed in reference 8).

Because the presence of the JP2 clone has significant implications for prevention and treatment of periodontitis in juveniles, it is essential to be able to accurately determine its presence in subgingival plaque samples. Cultivation by itself does not distinguish between the JP2 clone and other genotypes of the species. PCR detection of the Δ 530 deletion has been used to identify the JP2 clone in plaque samples and among isolates and to distinguish it from other *A. actinomycetemcomitans* strains (13, 15, 20). However, there is a need for a simple and low-technology method for detection of the JP2 clone. For that purpose, the loop-mediated isothermal amplification (LAMP) method is an attractive alternative to conventional PCR (12).

The LAMP reaction runs at a constant temperature, usually around 65°C, and uses four primers that recognize six distinct regions of the target DNA, combined with the *Bst* DNA polymerase large fragment, which has strand displacement activity and is heat inactivated at 80°C (12). The reaction can be accelerated by inclusion of additional loop primers (10). The resulting amplification products are a complex mixture of

stem-loop DNAs with inverted repeats and cauliflower-like structures (12) and induce a white precipitate of magnesium pyrophosphate proportional to the amount of amplified DNA and visible to the naked eye (9). Usually, LAMP primers are designed to specifically recognize a target sequence that is unique to the microorganism in question. A LAMP method targeting the *dam* gene, which is common to all members of *A. actinomycetemcomitans*, was reported recently (14). We wanted to develop a method that specifically detects the JP2 clone and took advantage of the specific Δ 530 deletion. The low percentage of G+C in the sequence around Δ 530 made it impossible to design appropriate LAMP primers in this region, including a primer spanning the site of deletion. As an alternative, we developed a novel approach based on the differences in distance between primer regions ("spacer regions") in the JP2 clone and in other *A. actinomycetemcomitans* strains. The primers shown in Fig. 1 were designed on the basis of the genome sequence of the JP2 clone strain HK1651 (<http://www.genome.ou.edu/act.html>) by using the program PrimerExplorer V3 (<https://primerexplorer.jp/lamp3.0.0/index.html>). The recommended distance between the 5' ends of F2 and B2 is 120 to 180 bp for optimal amplification, and DNA of more than 500 bp amplifies very poorly (12). When the primers shown in Fig. 1 are used, this distance is 167 bp in members of the JP2 clone and 697 bp in non-JP2 clones of *A. actinomycetemcomitans*.

The LAMP reaction was performed with 25 μ l containing 1.6 μ M each of the primers FIP and BIP, 0.2 μ M of primers F3 and B3, 0.4 μ M of primers LF and LB, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs), 1.4 mM each of the four deoxynucleoside triphosphates, 0.8 M betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 0.1% Tween 20, and template DNA in a volume of up to 5 μ l. The high-performance liquid chromatography-purified primers purchased from DNA Technology were dissolved in Tris-EDTA buffer. The mixture was incubated at 63°C for 60 min and then heated at 80°C for 2 min to terminate the reaction. In agreement with our hypothesis, an amplification product was obtained when DNA from *A. actinomycetemcomitans* strain HK921 (JP2) was the template, whereas DNA from

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FIG. 1. LAMP primers used to detect $\Delta 530$ in the *ltx* promoter region of JP2 strains of *A. actinomycetemcomitans*. (a) Locations of the primer sequences in the promoter region of the *ltx* gene operon. The ATG start codon of the *lxC* gene (the first gene in the *ltx* operon) is shown in bold and underlined. The site of $\Delta 530$ and the BsmI restriction site are indicated by arrows. The numbers to the left refer to positions in the *A. actinomycetemcomitans* strain HK1651 genome sequence (<http://www.genome.ou.edu/act.html>). (b) Structure and sequence of the six primers used in the LAMP reaction.

non-JP2 strain HK975 (Y4) did not result in a product, as revealed both by visual inspection and by agarose gel electrophoresis. Throughout this study, evaluation of the LAMP reactions showed complete agreement between a white precipitate recorded by visual inspection and an amplification product detectable by agarose gel electrophoresis.

Specificity of the LAMP reaction. To evaluate the specificity of the LAMP reaction, we tested 27 *A. actinomycetemcomitans* strains, including 11 JP2 strains and 16 non-JP2 strains, selected to represent the different subpopulations in the species (16), and members of the three closely related species *Aggregatibacter* (*Haemophilus*) *aphrophilus*, *Aggregatibacter* (*Haemophilus*) *segnis*, and *Mannheimia haemolytica*, the last of which produces a repeat-in-toxin leukotoxin homologous to that of *A. actinomycetemcomitans*. When approximately 1 ng whole-cell DNA was used in the LAMP reaction, amplification products from all JP2 strains were observed, whereas none of the non-JP2 strains of *A. actinomycetemcomitans* and representatives of other species resulted in amplification products. Addition of excess DNA (25 ng) from non-JP2 strain HK975 did not result in a product (Fig. 2). To ensure that the amplification products corresponded to the selected sequence target, we took advantage of the fact that strain JP2 has a BsmI recognition site between sequences F1c and B1c (Fig. 1). In full agreement, the products demonstrated after digestion with BsmI had predicted sizes of 106 and 116 bp (Fig. 2). Their identities were further confirmed by sequencing using primers F2 and B2. Thus, the LAMP reaction is highly specific for the JP2 clone.

Sensitivity of the LAMP reaction. DNA from *A. actinomycetemcomitans* strain HK921 (JP2) was purified using a DNeasy kit (Qiagen), and the absorbencies A_{260} and A_{280} were used to measure the quantity of DNA. The number of genome

FIG. 2. Analysis of DNA fragments amplified by LAMP reactions on isolates and directly on plaque samples. (a) Visual detection of a white precipitate in positive samples. The figure shows a positive (strain JP2) and a negative (non-JP2 strain Y4) sample, together with a negative control (neg.). (b) LAMP products were separated by 3% agarose gel electrophoresis and stained with ethidium bromide. Lane 1, molecular size marker in base pairs (GeneRuler 50-bp DNA ladder; Fermentas); lane 2, strain JP2; lane 3, strain HK1651 (JP2 clone strain); lane 4, strain HK975 (Y4, non-JP2 strain); lane 5, negative control; lane 6, clinical sample 362 M (positive for the JP2 clone and negative for non-JP2 types in PCR); lane 7, clinical sample 436 I (positive for the JP2 clone and non-JP2 types in PCR); lane 8, clinical sample 507 I (negative for *A. actinomycetemcomitans* in PCR); lane 9, clinical sample 510 I (negative for the JP2 clone and positive for non-JP2 types in PCR); lanes 10 to 13, same as lanes 2, 3, 6, and 7, respectively, except with BsmI digestion; lane 14, molecular mass marker. The arrows to the right indicate the positions of fragments in bp in the molecular mass marker. In lane 4, an excess of DNA was used as described in the text, and the genomic DNA is seen as a faint band at the top of the gel. The low molecular smear in lanes 5, 8, and 9 is presumably due to primer-dimers, and it did not result in a visible white precipitate. In lanes 10 to 13, the two fragments of 106 and 116 bp migrate together in the gel. The band in lane 10 is weak because only a fraction of the sample was loaded onto the gel.

copies was calculated on the basis of a genome size of 2.1 Mb. Serial 10-fold dilutions of the genomic DNA were tested in the LAMP reaction, and the results were compared with those of a conventional PCR test targeting the *ltx* promoter and performed as described previously (15). The detection limits for the LAMP assay and PCR were 10 and 100 genome copies, respectively, in a 5- μ l sample. When incubation was reduced to 30 min, the detection limit for the LAMP was 100 genome copies, indicating that the reaction was very rapid.

LAMP applied to analysis of clinical specimens. Subgingival plaque samples on paper points were collected from adolescents in Morocco and sent to Denmark in 1 ml 0.9% NaCl. After vortexing, the bacteria were collected by centrifugation, resuspended in 100 μ l water, and boiled for 5 min as described previously (15). The samples were stored at -20°C . A total of

TABLE 1. Detection of *A. actinomycetemcomitans* with and without $\Delta 530$ in 72 plaque samples by PCR with primers ltx3 and ltx4 (15) compared to that by the LAMP method described in the text

LAMP result	No. of samples with indicated result for PCR				Total
	No product	Non-JP2 type ^a	JP2 clone	JP2 clone and non-JP2 type ^a	
Negative ^b	12	12	0	0	24
Positive ^c	5	1 ^d	36	6	48
Total	17	13	36	6	72

^a Non-JP2 type of *A. actinomycetemcomitans*.

^b No product.

^c Amplification of a product.

^d The LAMP reaction is unable to detect non-JP2 types of *A. actinomycetemcomitans*, and a positive reaction is due to the simultaneous presence of both the JP2 clone (missed by the PCR) and non-JP2 types of the bacterium.

72 subgingival plaque samples were tested for the presence of the JP2 clone by both the LAMP test and the PCR (Table 1). In the PCR, 23 μ l of the subgingival plaque suspension was used in a 25- μ l reaction mixture. In the LAMP test, 5 μ l was used as described above. The two tests agreed on the presence of the JP2 clone in 42 samples and on the absence of the clone from 24 samples. For the remaining six samples (8.3%), the LAMP test demonstrated the presence of the JP2 clone while the PCR gave a negative result. Digestion of the LAMP products from the latter six samples with BsmI confirmed that these samples represented the specific target. A higher sensitivity of the LAMP test conceivably contributed to the higher detection rate. In addition, previous studies demonstrated that the LAMP reaction is more tolerant to potentially perturbing biological substances than the PCR methodology (7). In contrast to the LAMP test, the PCR test concurrently detects non-JP2 members of *A. actinomycetemcomitans*. The presence of non-JP2 *A. actinomycetemcomitans* seems not to influence detection of the JP2 clone by LAMP (Table 1).

In conclusion, we have developed a LAMP test using a novel principle based on differences in "spacer regions" for detection of the JP2 clone of *A. actinomycetemcomitans* and having a specificity equivalent to and a sensitivity exceeding those of previously described PCR methods. Because the LAMP reaction is easy to set up and does not require special equipment, it has obvious advantages in clinical settings and in population-based studies with limited access to laboratory technology.

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