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# A loop-mediated isothermal amplification (LAMP) assay for rapid detection of *Anaplasma phagocytophilum* infection in dogs

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**Abstract:** A simple and rapid diagnostic method of *Anaplasma phagocytophilum* infection was established, employing the loop-mediated isothermal amplification (LAMP) reaction. With 1 set of 4 specific primers targeting the citrate synthase (*gltA*) gene of *A. phagocytophilum*, the LAMP assay rapidly amplified the target DNA sequence in a single tube in 1 h, requiring only a water bath for reaction. When visualized by gel electrophoresis, the LAMP products appeared as a ladder-like pattern, with many bands of different sizes from 180 base pairs up to the loading well. The results obtained from testing 32 clinical blood samples of dogs demonstrated that the LAMP method was more sensitive than nested PCR in the diagnosis reaction for the detection of *A. phagocytophilum* DNA.

**Key words:** *Anaplasma phagocytophilum*, *gltA* gene, LAMP assay, dogs

*Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), formerly known as the human granulocytic ehrlichiosis (HGE) agent, *Ehrlichia equi*, and *Ehrlichia phagocytophila*, is the causative agent of granulocytic anaplasmosis (1). In humans, the disease is characterized by fever, headache, myalgia, malaise, leukopenia, thrombocytopenia, and evidence of hepatic injury (2,3). Tick-borne fever is reported as a febrile disease of cows, deer, hares, and sheep, with clinical signs varying from undetectable illness to severe febrile disease associated with opportunistic infections (4). Equine and canine diseases are characterized by fever, depression, anorexia, leukopenia, and thrombocytopenia. Equine infection also frequently results in limb edema and ataxia and may lead to opportunistic infections (4,5).

Laboratory techniques used to diagnose *A. phagocytophilum* infection include primary isolation by culture, microscopic identification of morulae in stained blood smears, serology, and molecular methods. Although isolation is the "gold standard" for diagnosis, cultivation entails prolonged incubation and requires a level of technical expertise not likely to be found in most laboratories (6). The recognition of morulae in infected cells during the examination of stained blood smears can be diagnostic. However, morulae have not been observed in several cases subsequently confirmed by other criteria (7). Serological methods are commonly used, but these assays are often negative in the early acute phase of the illness, as antibody will typically be absent during the first week of infection (8). Furthermore,

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serological cross-reactions may occur between closely related organisms, leading to misinterpretation and misdiagnosis (9). With the recent development of molecular biology methods, specific and sensitive assays such as polymerase chain reaction (PCR) and nucleotide sequencing are now used for the detection of *A. phagocytophilum* in clinical and field-collected samples (10-14).

Notomi et al. (15) previously described a novel method, termed loop-mediated isothermal amplification (LAMP), which can amplify DNA with high specificity, efficiency, and rapidity. The aim of this study was to establish a LAMP-based *A. phagocytophilum* DNA amplification technique and examine its applicability and reliability for the diagnosis of granulocytic anaplasmosis caused by *A. phagocytophilum*.

Ethylenediaminetetraacetic acid (EDTA)-treated blood samples used in the present study were collected from 32 dogs, clinically suspected of being *A. phagocytophilum*-infected, that were referred to the Veterinary Teaching Hospital of National Chung Hsing University and 8 veterinary clinics throughout Taiwan between 2007 and 2010. For each sample, DNA was extracted from 200 μL of whole blood by using the QIAamp DNA Blood Mini kit (Qiagen), according to the instructions of the manufacturer. The extracted DNA was eluted in a total volume of 100 μL of elution buffer and was stored at –20 °C until further use.

In the initial study, a species-specific nested polymerase chain reaction (PCR) was used to identify samples infected with *A. phagocytophilum*. Oligonucleotide primers used for nested PCR were designed, based on the citrate synthase (*gltA*) gene sequence of *A. phagocytophilum* (accession numbers AF304136 to AF304138 and AY464132 to AY464138, DQ160228, and AY339602 in GenBank) after comparing the alignment of *gltA* gene sequences from other genetically related rickettsia (*Anaplasma platys*, AY077620, AF058782, AF478130, and AY530807; *Anaplasma marginale*, AF304140; *Anaplasma centrale*, AF304141; *Ehrlichia ewingii*, DQ365879; *Ehrlichia canis*, AF304143; *Ehrlichia chaffeensis*, AF304142; *Ehrlichia muris*, AF304144; *Ehrlichia ruminantium*, AF304146; and *Neorickettsia risticii*, AF304147) (16). The name, location, and sequence of each primer are given in Figure 1. The specificities of the designed primers were initially checked using the Basic Local Alignment Search Tool (BLAST) program on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) before being confirmed by the assays. The first round of PCR amplification was carried out in a 25-μL reaction mixture containing 1 μM each of the primers Aph-F4 and Aph-B4, 0.2 mM of each dNTPs, 1 × Taq buffer (Invitrogen), 2.5 mM $MgCl_2$, 2 μL of template DNA, and 1.25 U of *Taq* DNA polymerase (Invitrogen). The amplification was performed in capillary tubes using the Gene Amp PCR system 9700 thermal cycler (Applied Biosystems) with the following program: an initial 5 min denaturation at 94 °C; 35 repeated cycles of denaturation (94 °C for 1 min), annealing (57 °C for 30 s), and extension (72 °C for 30 s); followed by 5 min extension at 72 °C. Distilled water (no template) and DNA extracted from the blood sample of a healthy dog were included as negative controls. After electrophoresis on 1% agarose gel, the amplification products were visualized by ethidium bromide staining under UV fluorescence. As several non-specific bands were observed in the first PCR (data not shown), it was difficult to evaluate the results. Then the first-round PCR product from each sample was diluted 1 to 5 with distilled water and 2 μL of the diluted solution was used as the template DNA for the second round of PCR with primers Aph-F3 and Aph-B3 (see Figure 1) to amplify a 221-bp fragment. The annealing step was performed at 55 °C for 30 s. The other PCR conditions were the same as in the first-round PCR. The amplification products were visualized on a 1% agarose gel after electrophoretic migration. As shown in Figure 2, in all of the infected samples (lanes 2-5), the expected 221-bp band is obvious.

To further confirm the specificity of the PCR method, an amplification product chosen randomly from 21 PCR-positive samples was purified with an MinElute Gel Extraction kit (Qiagen) and then subjected to direct sequence analysis using the Big Dye Terminator kit (Applied Biosystems) with an automated DNA sequencer (ABI PRISM 310 genetic analyzer). The sequence data of the PCR product


| Method | Primer | Sequence |
|---|---|---|
| Nested PCR | Aph-F4 | 5′-CACTGCTGCTTGCAGATCAG-3′ (150-169, forward) |
| | Aph-B4 | 5′-GCATCATCCATGTCGCCAAA-3′ (598-617, reverse) |
| | Aph-F3 | 5′-AAGAGTTTGCGCGGATTT-3′ (311-328, forward) |
| | Aph-B3 | 5′-AGTGTGCCTATAAATACTTGCA-3′ (510-531, reverse) |
| LAMP | Aph-FIP (F1c-F2) | 5′-CATAGGATGCGAATCTCGAGGCGCTGAATACGATGTGCC-3′ |
| | Aph-BIP (B1c-B2) | 5′-TTACCACGCAAGTCGCATTCAATGCCGGGTACTTTCG-3′ |

Figure 1. Oligonucleotide primers of LAMP for the detection of *A. phagocytophilum*. Nucleotide sequence of the *gltA* gene of *A. phagocytophilum* (GenBank accession number AF304138) was used as target. The nucleotide sequences and the positions used to design the primers are shadowed and labeled. Numbers indicate the nucleotide positions. The outer primers for LAMP (F3 and B3) were the same as the inner primers for nested PCR. F1c, sequence complementary to F1; B1c, sequence complementary to B1.

were analyzed by BLAST for the homology search. The result showed that the 181-bp fragment of the positive PCR excluding the primer regions was 100% homologous to the registered *gltA* gene sequence of *A. phagocytophilum* (GenBank accession numbers AF304137 and AF304138) (16). Thus, the genomic DNA extracted from this blood sample was used as a positive control to determine the appropriate conditions for the *A. phagocytophilum*-specific LAMP assay.

During the development of a LAMP assay, we evaluated the specificity of the *A. phagocytophilum*-specific LAMP primers first. The primers used for LAMP amplifications were designed according to the nucleotide sequence of the *gltA* gene of *A. phagocytophilum* (16) (see Figure 1 for detailed sequences). The LAMP method requires a set of 4 specially designed primers, 2 outer and 2 inner, which recognize 6 distinct regions on the target sequence (15). The outer primers for LAMP (F3 and B3) were the same as the inner primers for nested PCR. The inner primers for LAMP were denoted as forward inner primer (FIP) and backward inner primer (BIP). The FIP consisted of a complementary

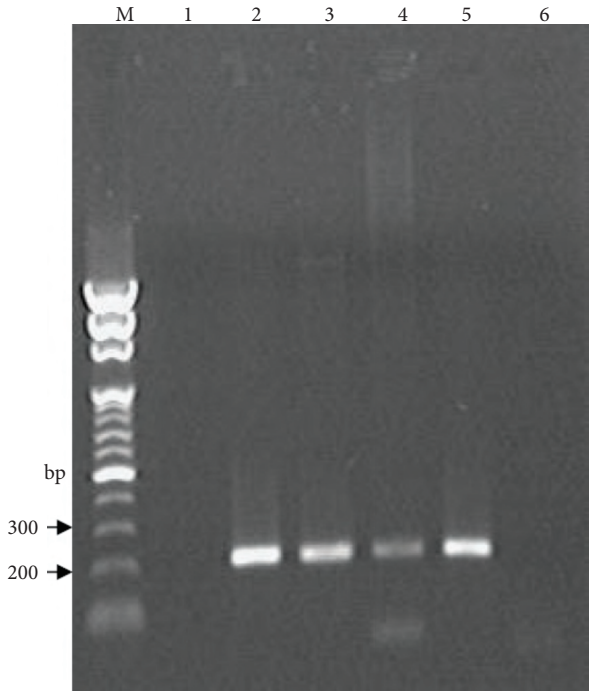


Figure 2. An ethidium bromide-stained agarose gel showing the nested PCR products amplified from blood samples of clinically *A. phagocytophilum*-suspected dogs. Lanes 2-5, the samples from naturally infected dogs; lane 1, no template DNA; lane 6, the sample from a healthy dog (negative control); and lane M, 100-bp DNA ladder marker.

sequence of F1 (F1c) and a sense sequence of F2. The BIP consisted of a complementary sequence of B1 (B1c) and a sense sequence of B2.

The LAMP reaction was carried out in 25 µL of a mixture containing 5 µL of the extracted DNA, 0.2 µM each of outer primers Aph-F3 and Aph-B3, 1.6 µM each of inner primers Aph-FIP and Aph-BIP, 1.4 mM each dNTP, 0.8 M betaine (Sigma-Aldrich), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, and 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs). The mixture was incubated at 65 °C for 60 min using a conventional water bath and was heated at 80 °C for 5 min to terminate the reaction. From each of the amplified products, 5 µL was analyzed by electrophoresis on 1.5% agarose gel, followed by ethidium bromide staining, visualization on a UV transilluminator, and photography. Distilled water and DNAs extracted from the *A. platys*-, *E. canis*-, and *Babesia gibsoni*-positive blood samples were included as negative controls. Due to the formation of stem-loop DNAs of varying stem length and cauliflower-like structures with multiple loops formed by sequentially inverted repeats of the target sequence, the LAMP product of the positive sample demonstrated many bands of different sizes upon agarose gel electrophoresis (15,17). As shown in Figure 3, the amplified *A. phagocytophilum* DNA exhibited a typical ladder-like pattern on the gel (lane 2), whereas no LAMP product was detected in reactions performed with any of other DNAs (lane 3-6). These results indicated that the LAMP assay had high specificity among DNA samples we tested.

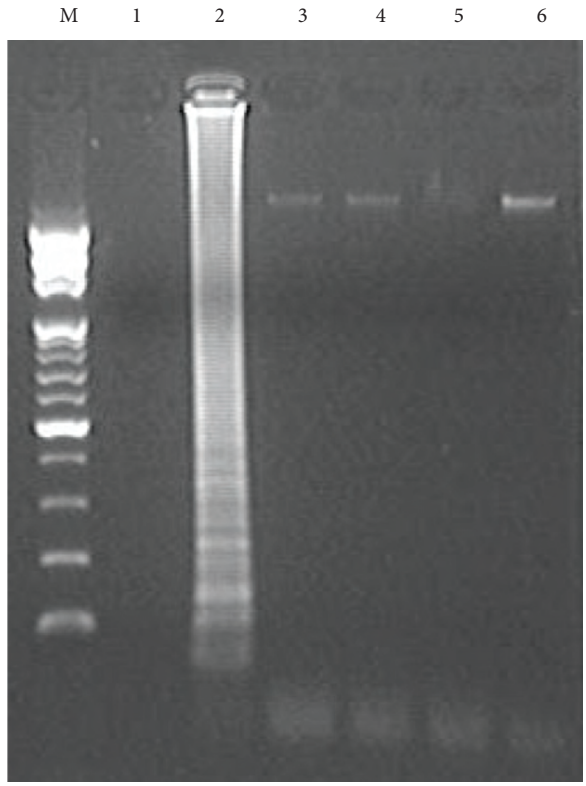


Figure 3. Specificity of LAMP primers for detection of *A. phagocytophilum*. DNAs were extracted from 200-µL blood samples of dogs infected by different rickettsia (lane 2, *A. phagocytophilum*; lane 3, *A. platys*; lane 4, *E. canis*; and lane 5, *B. gibsoni*) and were resuspended in 100 µL of buffer. An aliquot of 2 µL was subjected to LAMP. The LAMP products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. Lane 1, no template DNA; lane 6, the sample from a healthy dog (negative control); and lane M, 100-bp DNA ladder marker.

The sensitivity of the LAMP method was also determined and compared with that of the *gltA* nested PCR method as described above and that of the PCR method using the *epank1* primers designed in a previous study (14). An *A. phagocytophilum*-positive dog blood sample with approximately 6% parasitemia was subjected to 10-fold dilutions using normal dog blood, and DNA extracted from each diluted sample was used to determine the detection limit of each method. The results shown in Figure 4 indicated that the sensitivities of the LAMP and *gltA* nested PCR methods were equivalent, with detection limits of 0.000006% parasitemia each (lane 7). At this level of dilution, however, the positive LAMP result could be easily determined by visual examination of the DNA smear and the pattern of many DNA bands of different sizes. In contrast, the positive *gltA* PCR result only showed a very faint single band. These 2 methods had detection limits 10-fold lower than that for the *epank1* PCR method.

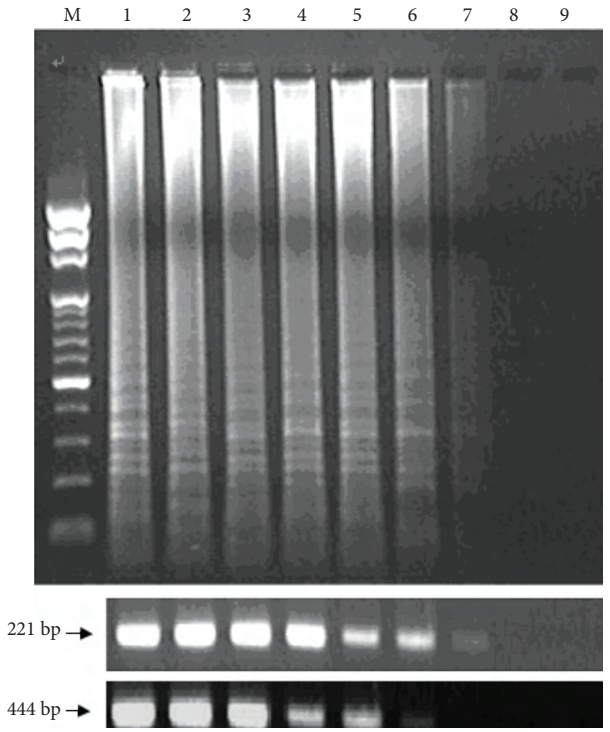


Figure 4. Relative sensitivities of LAMP and PCR for detection of *A. phagocytophilum*. The DNA extracted from an *A. phagocytophilum*-positive dog blood sample with 6% parasitemia was subjected to serial 10-fold dilutions from $10^0$ to $10^{-8}$ (lanes 1-9, respectively). Those diluted DNA samples were used to carry out the amplification reaction, followed by agarose gel electrophoresis, to determine the lowest detection limit of the method. The amplification by LAMP (top) showed a ladder-like pattern, whereas the *gltA* nested PCR (middle) and the *epank1* PCR (bottom) showed a 221-bp amplicon and a 444-bp amplicon, respectively. Lane M, 100-bp DNA ladder marker.

After these initial validation studies, 32 blood samples of clinically *A. phagocytophilum*-suspected dogs were tested for *A. phagocytophilum* by LAMP and nested PCR simultaneously. With LAMP, 23 of the 32 blood samples (71.9%) tested positive, whereas 21 of the 32 blood samples (65.6%) tested positive by nested PCR. When tested by both LAMP and nested PCR, 21 blood samples (65.6%) were positive, while 2 samples (6.3%) were positive by LAMP and negative by nested PCR. No sample that tested positive by nested PCR tested negative by LAMP; and 9 samples (28.1%) tested negative by both tests. The agreement between the 2 tests was 91.3%. None of the blood samples obtained from 8 adult dogs exhibiting no clinical signs of tick-borne diseases were positive by either one of tests, thereby indicating the specificity of the 2 assay systems.

In summary, a novel *A. phagocytophilum* DNA detection system has been developed employing the nucleic acid amplification method, LAMP. The LAMP assay described here is quite simple; it is performed in a single tube, incubating the mixture at a constant 65 °C for 1 h in an ordinary water bath. The advantages of the method are due to its simple operation, rapid reaction, and potential for visual interpretation without instrumentation. Although limited numbers of clinical samples were used in the present study, it has been shown that LAMP could be useful as an on-site diagnostic assay for detecting *A. phagocytophilum* infection. As a result, the LAMP assay is expected to be a valuable tool for epidemiologic studies that require screening of large numbers of potential vector and reservoir populations for *A. phagocytophilum*.

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