Loop-mediated isothermal amplification polymerase chain reaction in place of a modified Knott test in screening dogs for heartworm (*Dirofilaria immitis*) infection combined with antigen detection test

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OBJECTIVE
To improve the current recommendations for the diagnosis of canine heartworm (*Dirofilaria immitis*) disease.

ANIMALS
Blood samples collected from 35 shelter dogs in the Republic of Korea.

METHODS
Samples were tested for the presence of microfilaria using the modified Knott (MK) test and *D. immitis* DNA using species-specific loop-mediated isothermal amplification (LAMP) PCR. The blood samples were additionally assessed for the presence of heartworm antigens using the Antigen Rapid Canine Heartworm AG Test Kit 2.0 (Bionote Co). The performance of the MK test and LAMP PCR was assessed through statistical analysis, with a paired McNemar test utilized for comparison.

RESULTS
The heartworm antigen was detected in 28.5% of the subjects. Of the 10 positive animals, the MK test detected microfilaria in 4 of 35 (11.4%) animals, and LAMP PCR detected *D. immitis* DNA in 6 of 35 (17.1%). The results of this study indicate that the LAMP PCR showed more positive results in samples compared to the conventional MK test.

CLINICAL RELEVANCE
The *D. immitis*-specific LAMP PCR assay has the potential to function as an alternative to current detection methods. It could complement the existing antigen detection tests in diagnosing canine heartworm infections.

Keywords: antigen detection test, *Dirofilaria immitis*, loop-mediated isothermal amplification (LAMP), modified Knott test, PCR

Canine heartworm disease is a mosquito-borne nematode infection caused by *Dirofilaria immitis*.1 It occurs worldwide in regions with suitable intermediate mosquito hosts and ambient temperature.2 A mosquito initially ingests microfilariae (first-stage larvae) that circulate in the blood of an infected host animal. The final molting into a stage-five larvae occurs between days 50 and 70 as the larvae migrate through the body. Under ideal conditions, the infection progresses to adult worms that produce microfilariae 7 to 9 months after infection.3

Methods for diagnosing canine heartworms generally include clinical symptoms, radiography, ultrasonography, echocardiography, detection of microfilariae, and immune response methods for detecting blood antigens and antibodies. The American Heartworm Society currently recommends using a combined diagnostic test that includes antigen and microfilaria detection tests for screening heartworm infections.4 Several methods may be used for microfilarial testing, including direct smear,
a modified Knott (MK) test, and buffy coat examination. Among these, the MK test, which is a technique for concentrating blood using formalin, is the most commonly used and highly sensitive. It allows for the assessment of microfilarial morphology using a microscope. D. immitis and other nonpathogenic nematodes may be additionally distinguished based on their shape or size using the MK test.

However, distinguishing D. immitis from other nonpathological nematodes by microscopy using the MK test may not be accurate considering observer subjectivity. In cases of occult infection, microfilariae are not detected in the blood despite their intermittent presence depending on the timing of blood sampling and season, whereas adult heartworms continue to parasitize the heart. Better diagnostic tests are, therefore, required to confirm and detect D. immitis microfilaraemia. These diagnostic tests require objectivity, in which the outcome does not depend on the observer, and are highly accurate for detecting D. immitis. Diagnostic methods using DNA amplification, such as quantitative PCR or loop-mediated isothermal amplification (LAMP) PCR, have, therefore, been developed.

LAMP PCR was developed as a new approach for amplifying DNA in an isothermal environment. The method does not require an expensive temperature control device because it is possible to amplify the target genetic material at a single temperature (room temperature or high temperature below 65 °C) without changing the reaction temperature. It has the advantage of being suitable for on-site diagnosis, which is generally challenging when using PCR. According to a recent study, LAMP PCR is widely used as a diagnostic method for parasites such as Trypanosoma and Babesia. It has also been demonstrated to have diagnostic value in the identification of genera- or species-specific parasites. Since few studies have validated the efficacy of LAMP PCR for the detection of canine heartworm, we evaluated the PCR method by comparing with other commonly used detection methods in the current study.

This study aimed to compare the performance of LAMP PCR for the detection of D. immitis DNA to that of the MK test technique in detecting heartworm microfilariae when used in conjunction with the canine heartworm antigen rapid test kit.

Methods

Animals and sampling
Whole-blood samples were collected from 35 shelter dogs from June through October 2022 in Gunsan Jeollabukdo, Republic of Korea. The study was approved by the IACUC of Jeonbuk National University.

Approximately 2 mL of blood was collected from the cephalic or external jugular veins. The blood was collected using a 3-mL syringe and stored in EDTA tubes. The blood samples were then stored at 4 °C until the test was performed. One microliter of blood was used for the MK test, and the remaining 1 mL was sent to Coscience Co Ltd for LAMP PCR analysis.

Modified Knott test
The MK test was used to confirm the absence or presence of microfilariae. The MK test remains the preferred test for observing morphology and measuring body size to differentiate D. immitis from nonpathogenic filarial species, such as Acanthocheilonema reconditum and Dirofilaria repens.

The MK test was conducted by mixing 1.0 mL of EDTA blood with 9 mL of 2% formalin in a centrifuge tube. The tube was rotated several times to mix the blood with formalin solution to break down the red blood cells. The tube was then placed in a centrifuge, where it spun at 1,500 X g for 5 minutes. The liquid was then poured off, leaving the sediment behind. Methylene blue was added to the sediment, and a cover slip was placed on top for protection. The entire coverslip was scanned visually; if there were any observations, the morphology was assessed at high power (400X) for species identification.

Antigen detection test
The Antigen Rapid Canine Heartworm AG Test Kit 2.0 (Bionote Co) is based on the principle of immunochromatographic assays and has 99.5% sensitivity and 94% specificity.

The sample used for the test is EDTA whole blood, and 2 drops were employed. The antigen testing and interpretation were performed as per the manufacturer’s instructions to assess the samples for changes in line numbers, indicating antigen detection. A positive result was indicated by 2 lines, whereas a negative result was indicated by a single line.

Deoxyribonucleic acid extraction and LAMP PCR

Deoxyribonucleic acid extraction—A phenol-based DNA extraction method was used to purify the total genomic DNA. The sample was incubated at 60 °C for 2 hours in a 1.5-mL microcentrifuge tube containing 200 μL of lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% Triton X-100, and 200 μg/mL of proteinase K). This was followed by extraction with equal volumes of Tris-EDTA saturated phenol (pH 8) and precipitation of the DNA with ethanol. This was resuspended in 50 μL of DNAse/RNase-free, double-distilled water. The extracted lyophilized DNA was kept at -20 °C until needed.

Test DNA extraction—The complete genomic DNA was obtained for the test using DNA lysis buffer created by Coscience Co Ltd. The DNA was then extracted 10 minutes after being combined with 200 μL of DNA lysis buffer and 10 μL of blood sample.

Loop-mediated isothermal amplification primer design
Sets of LAMP primers (external primers, F3 and B3; internal primers, FIP [F1c–F2] and BIP [B1c–B2]) were designed. The DNA sequences from different Dirofilaria species were used to build the sequences for the sets. The distances between the primers and required melting temperatures were taken into consideration when designing the primers. The set...
of primers targeting the conversed *D immitis* gene used in this study are presented (Table 1), and the results of the LAMP PCR electrophoresis are shown (Figure 1). Figure 1 presents the nucleotide sequences of the newly designed LAMP primers and their locations in the conserved *D immitis* gene.

**Table 1**—Primer sets used for loop-mediated isothermal amplification PCR in the current study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>F3</td>
<td>ATG CCT ATT TTG ATT GGT GG</td>
</tr>
<tr>
<td>LF</td>
<td>CAG GGG CAC CCA ATA TCA A</td>
</tr>
<tr>
<td>FIP</td>
<td>GCA TTA ACA CGA GGA AAA GCC ATT TTT</td>
</tr>
<tr>
<td>TTG GTA ATT GGA TGT TGC C</td>
<td></td>
</tr>
<tr>
<td>BIP</td>
<td>GTT GCG TTG TTG ATG GTT TAT CAA AAG</td>
</tr>
<tr>
<td>TTC AAC TAC TCC CAG G</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>TCT TTT TTT ATT GGG GGG GGT</td>
</tr>
<tr>
<td>B3</td>
<td>CTC TAC ACT CAA AGG AGG AT</td>
</tr>
</tbody>
</table>

BIP = Backward inner primer. FIP = Forward inner primer. LB = Loop backward. LF = Loop forward.

Furthermore, a LAMP thermocycler (Alltdtec; Coscience Inc) was used to acquire fluorescence data from the final amplification product.

**Detection of LAMPs**

Deoxyribonucleic acid amplification was performed by using 2 methods. The SYTO-9 Green Fluorescent Nucleic Acid Stains are cell-permeant nucleic acid stains that demonstrate significant fluorescence enhancement when they attach to DNA or RNA. SYTO-9 (2 μM; excitation/emission, 488/518 nM) was used to detect and measure the DNA accumulating in the reaction tubes. The second method for end-point quantification of DNA involves the addition of SYBR Green-I (> 1 μM; Thermo-Fisher Scientific). The color of the reaction changed from orange to fluorescent green immediately upon detection of the LAMP DNA. The results were verified through electrophoresis of the LAMP DNA and by observing it on agarose gels stained with ethidium bromide.

**Statistical analysis**

Each experimental group was categorized as positive or negative as per the results of MK, LAMP PCR, and antigen detection tests. The data were analyzed using a paired McNemar test. The MK test and LAMP PCR results were compared with each other based on *D immitis* infection, categorizing dogs as either positive or negative.

Statistical analyses were performed using SPSS Statistics software (version 29; IBM Corp).

**Results**

Of the 35 dogs screened, 10 of 35 (28.5%) tested positive with the antigen detection kit, whereas 25 of 35 (71.5%) turned out negative.

Among the 10 positive animals, 4 were found to have microfilaria, accounting for 4 of 35 (11.4%), morphologically identified as *D immitis*. In comparison, LAMP PCR demonstrated 2 additional heartworm-positive samples, including the DNA of *D immitis* in 6 of 35 (17.1%) of the animals. Among the 25 samples that tested negative in the antigen detection test, *D immitis* was not detected by either the MK test or LAMP PCR. (Tables 2 and 3)

**Table 2**—Diagnostic test results for *Dirofilaria immitis*-positive dogs (n = 35) from one shelter in Gunsan, Korea.

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Ag test</th>
<th>MK test</th>
<th>LAMP PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>4</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>4</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>2</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
</tbody>
</table>

Ag = Antigen detection (Antigen Rapid Canine Heartworm AG Test Kit 2.0; Bionote Co). LAMP = Loop-mediated isothermal amplification. MK = Modified Knott.
Modified Knott. 

MK test Positive increased through additional heat treatments. Females of assay methods have demonstrated high sensitivity in recent years. This study used LAMP PCR to diagnose infections using molecular biology have increased in microfilaria detection tests has increased. Various microfilaria detection tests have been developed as the significance of a Microfilaricide. Various microfilaria detection can trigger severe reactions after the administration of heartworm as a reservoir of infection and warned of the high microfilaremia burden that veterinarians of the high microfilaremia burden that heartworm found in this study was determined (100.0%) across Mississippi state. These differences may be influenced by the density of the local mosquito population and the choice of the antigen diagnostic test.

Although the sensitivity and specificity of commercial heartworm antigen kits vary widely, antigen testing is considered to be the most sensitive diagnostic method. Enzyme-linked immunosorbent assay methods have demonstrated high sensitivity and specificity depending on the number of mature females of D. immitis. The sensitivity may be further increased through additional heat treatments. A microfilaria detection test in addition to an antigen detection test should be performed if heartworm infection is suspected. The positive results of the microfilaria detection test identified canine heartworm as a reservoir of infection and warned veterinarians of the high microfilaremia burden that can trigger severe reactions after the administration of a microfilaricide. Various microfilaria detection tests have been developed as the significance of microfilaria detection tests has increased. Approaches for diagnosing canine heartworm infections using molecular biology have increased in recent years. This study used LAMP PCR to diagnose D. immitis. There was no statistically significant difference (P = .500) observed in this study. Despite this, the LAMP PCR assay detected the DNA of D. immitis in 6 of 35 (17%) of the samples, which was more than the 4 of 35 (11%) detected by the MK test. The LAMP PCR method was determined to be more effective than the MK test from the results of the positive antigen diagnostic test.

The ability of LAMP PCR to identify single microfilaria suggests that this method may be applied to clinical cases with extremely low microfilaremia. The sequencing of LAMP PCR products additionally confirmed the specific amplification of the D. immitis DNA, indicating the high specificity of this method.

The American Heartworm Society recommends annual testing using both a heartworm antigen test to detect adult worms and a microfilaria detection test to detect circulating D. immitis microfilaria. Based on the result of the current study, the application of this LAMP assay in combination with the D. immitis antigen test may be an efficient substitute for primary diagnostic heartworm screening. A reconditum and D. repens should directly be compared under a microscope in the MK test, subject to the observer’s subjectivity. However, this is not easy. In the case of LAMP PCR, the probability of potential error is low considering it detects D. immitis microfilaria DNA species specifically. In clinical practice, the LAMP PCR is more convenient and less biased compared to the MK test.

The limitations of this study include its relatively small sample size. It is necessary to increase the sample size through a larger sample collection. Considering the subjects were dogs in shelters, it was not possible to determine the exact clinical symptoms of each patient and to know how much heartworm preventative medication was administered or how that affected the results. The infection status of the dog population was not confirmed through necropsy. Further studies on necropsy diagnosis are, therefore, required to establish the sensitivity, specificity, and accuracy of the LAMP PCR assay. A greater number of D. immitis was detected even though the LAMP PCR results were not statistically significant compared to those of the MK test.

In clinical use, compared to the MK test, LAMP PCR does not require a microscope and may be used simply as long as blood is available, such as in an advanced antigen test kit, regardless of the subjectivity of the observer. Together with antigen testing, this method could, therefore, be a valuable screening test for D. immitis infections in canine populations. Further research, such as antigen kit testing, is needed for its frequent use in actual clinical practice.

Table 3—Frequency of positive cases of Dirofilaria immitis using individual diagnostic methods (n = 35).

<table>
<thead>
<tr>
<th>Diagnostic method(s)</th>
<th>D. immitis-positive dogs (n)</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag test</td>
<td>10</td>
<td>28.5</td>
</tr>
<tr>
<td>MK test</td>
<td>4</td>
<td>11.4</td>
</tr>
<tr>
<td>LAMP PCR</td>
<td>6</td>
<td>17.1</td>
</tr>
<tr>
<td>MK test plus Ag test</td>
<td>4</td>
<td>11.4</td>
</tr>
<tr>
<td>LAMP PCR plus Ag test</td>
<td>6</td>
<td>17.1</td>
</tr>
</tbody>
</table>

Ag = Antigen detection (Antigen Rapid Canine Heartworm AG Test Kit 2.0; Bionote Co). LAMP = Loop-mediated isothermal amplification. MK = Modified Knott.

Table 4—Comparison between the results of the MK test and LAMP PCR (P = .500).

<table>
<thead>
<tr>
<th>Results</th>
<th>Positive</th>
<th>Negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK test</td>
<td>Positive</td>
<td>4</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>2</td>
<td>33.3</td>
</tr>
<tr>
<td>LAMP PCR</td>
<td>Positive</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>n</td>
<td>%</td>
</tr>
</tbody>
</table>

LAMP = Loop-mediated isothermal amplification. MK = Modified Knott.

(100.0%) for LAMP PCR-negative. The difference was, therefore, not statistically significant (P = .500; McNemar test).

Discussion

D. immitis infection was detected in 28% of shelter dogs in this study. The overall prevalence of heartworm found in this study was determined to be lower than that reported in previous studies (34.4%) across Mississippi state. These differences may be influenced by the density of the local mosquito population and the choice of the antigen diagnostic test.

All the dogs suspected of heartworm infection were tested for microfilaria using an antigen detection test. Although the sensitivity and specificity of commercial heartworm antigen kits vary widely, antigen testing is considered to be the most sensitive diagnostic method. Enzyme-linked immunosorbent assay methods have demonstrated high sensitivity and specificity depending on the number of mature females of D. immitis. The sensitivity may be further increased through additional heat treatments.

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Acknowledgments

None reported.

Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.
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