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# Rapid Diagnosis of Babesia Gibsoni by Point-of-Need Testing by Insulated Isothermal PCR in Dogs at High Risk of Infection

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## Standard Article

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Rapid Diagnosis of *Babesia gibsoni* by Point-of-Need Testing by Insulated Isothermal PCR in Dogs at High Risk of InfectionK.L. Cooke , P. Frenzer, S.J. Tucker, P.C. Crawford, S.K. Kirk, and J.K. Levy 

**Background:** Dogs seized by law enforcement agencies during dogfighting investigations are at increased risk of *Babesia gibsoni* infection. A rapid and cost-effective diagnostic test would increase the feasibility of mass screening of dogs for infection and monitoring treatment efficacy in *B. gibsoni*-infected dogs.

**Objective:** To determine the performance of a point-of-need insulated isothermal PCR (iiPCR) test for diagnosis of *B. gibsoni* in dogs rescued in dogfighting investigations.

**Animals:** Two hundred and thirty-three dogs seized in dogfighting investigations.

**Methods:** Cross-sectional study. Whole blood samples were tested for *B. gibsoni* and *Babesia* spp. by iiPCR. Results were compared to a reference standard comprised of concordant results from real-time PCR in a commercial diagnostic laboratory and antibody titers.

**Results:** The iiPCR system was quick to learn, portable, and had a short processing time of <2 hours. Sensitivity and specificity of the iiPCR assay for *B. gibsoni* were 90% (95% confidence interval [CI] 81–95%) and 99% (CI, 95–100%), respectively. Sensitivity and specificity of the iiPCR assay for *Babesia* spp. were 87% (CI, 78–93%) and 98% (CI, 0.94–99%), respectively.

**Conclusions and Clinical Importance:** The iiPCR system produced few false-positive results, indicating that positive results are likely to represent true infections when used in high-risk animals. The iiPCR system can fail to identify 10–15% of truly infected dogs. However, the portability, speed, and economy of the iiPCR system compared to testing through a reference laboratory can allow rescue groups to screen and identify infection in more dogs.

**Key words:** Babesiosis; Canine; iiPCR; Pit bull.

Although federal law defines organized dogfighting as an illegal activity across the United States, this cruel blood sport persists throughout the country. Dogs in fighting operations typically receive little preventive health care or veterinary oversight. More than half of dogs seized during one fighting investigation harbored vector-borne infections, the most common of which was *Babesia gibsoni*.<sup>1</sup> In the United States, *B. gibsoni* is most commonly diagnosed in American Pit Bull Terriers or “pit bull-type” dogs, in which prevalence can range as high as 55%.<sup>1–3</sup> Dogs with evidence of previous fighting (scarring) are 5.5 times more likely to be infected with *B. gibsoni* than dogs without scarring.<sup>4</sup>

Law enforcement agencies confiscate large numbers of dogs from dogfighting organizations every year. Animal welfare organizations use behavioral and health

## Abbreviations:

CI	confidence interval
iiPCR	insulated isothermal polymerase chain reaction

evaluations to select dogs suitable for adoption as opposed to the historic policy of blanket euthanasia for all seized dogs. Although it is ideal for rescue groups to screen and treat for infectious diseases before placing dogs for adoption, funding and time constraints frequently limit the degree to which this is feasible. This is especially true for infections such as *B. gibsoni* that require expensive testing in reference laboratories or prolonged treatment protocols. An inexpensive and rapid point-of-need test for *B. gibsoni* would facilitate mass screening of high-risk dogs and monitoring of response to treatment for infected dogs.

Diagnosis of *B. gibsoni* is typically made by PCR testing of whole blood samples in reference laboratories, a process that delays results and adds costs. Insulated isothermal PCR (iiPCR) is an innovation that utilizes thermal gradients created by convection currents in the reaction mix to permit all phases of the PCR reaction (denaturation, annealing, extension) to occur simultaneously, resulting in more rapid assay completion compared to conventional PCR.<sup>5–7</sup> Recent development of portable closed tube iiPCR systems brings PCR technology to the point of need.<sup>5,7–10</sup> One such portable system is the POKKIT Nucleic Acid Analyzer,<sup>a</sup> which uses reaction tubes prefilled with specific lyophilized reagents to perform iiPCR with fluorescent probe hydrolysis detection. The POKKIT Xpress mobile laboratory is a fully contained system including a POKKIT Nucleic Acid Analyzer, a cubee mini-centrifuge,<sup>b</sup> 2 micropipettes, and reagent package packed into a hard-shell

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carry case. The entire system weighs 14 kg, can be operated on AC electricity or car battery, and has a short processing time of <2 hours for DNA extraction and iiPCR. The purpose of this study was to evaluate the POKKIT iiPCR system as a practical and accurate alternative to reference laboratory PCR testing for screening high-risk dogs for *B. gibsoni*.

## Materials and Methods

Archived blood samples from 233 dogs seized in a multistate dogfighting raid were used in the study. During the first week in custody, EDTA-anticoagulated whole blood and serum were collected to screen for a panel of infectious diseases, including for *B. gibsoni*, by real-time PCR and serological assays as previously described.<sup>1,c</sup> Residual EDTA whole blood samples were stored at  $-80^{\circ}\text{C}$  for 2 years before this study. For the purpose of this study, the reference standard for *B. gibsoni* infection status was defined as concordant real-time PCR and antibody titer test results from the original screening. None of the samples were PCR-positive for any *Babesia* species other than *B. gibsoni*.<sup>1</sup> A total of 79 samples met the criteria for infection with *B. gibsoni*, and 154 samples were defined as negative. Use of surplus samples was approved by the University of Florida Institutional Animal Care and Use Committee.

Blood samples were thawed at room temperature and mixed by inverting several times. DNA was extracted from a 200- $\mu\text{L}$  sample with a commercial kit according to the manufacturer's instructions.<sup>d</sup> Extracted DNA was processed with the POKKIT nucleic acid analyzer with two different kits according to the manufacturer's instructions: One kit was specific for *B. gibsoni* 18S rRNA gene<sup>e</sup> and the other detected both *B. gibsoni* and *Babesia canis* 18S rRNA genes<sup>f</sup> (babesiosis). Each PCR reaction run included a positive and negative control supplied in the kit and 6 test samples. The negative control was a no template control. Results were reported as positive, negative, or indeterminate based on the analyzer display. Samples with indeterminate results were retested.

The sensitivity of each iiPCR assay for *B. gibsoni* and babesiosis was calculated as the proportion of dogs infected with *B. gibsoni* that were identified as positive by the iiPCR assays. The specificity of each iiPCR assay was calculated as the proportion of dogs not infected with *B. gibsoni* that were identified as negative by the iiPCR assays. Sensitivity and specificity of the *B. gibsoni* and babesiosis assays were compared with the McNemar's test with  $P < 0.05$  considered to be significant. Agreement between each iiPCR test and the reference standard was assessed by Cohen's kappa coefficient ( $\kappa$ ). Positive and negative predictive values were calculated for each assay by Bayes' theorem for hypothetical 5%, 30%, and 60% prevalence rates. All statistical analyses were performed with an online statistical calculator.<sup>g</sup>

## Results

The results for the iiPCR assays relative to the reference standard are shown in Table 1. There were 2 samples with initial indeterminate results for *B. gibsoni* that yielded positive results on repeat testing. The sensitivity and specificity for the *B. gibsoni* kit were 90% (95% CI, 81–95%) and 99% (95% CI, 95–100%), respectively. The sensitivity and specificity for the babesiosis kit were 87% (95% CI, 78–93%) and 98% (95% CI, 94–99%), respectively. There were no significant differences in sensitivity ( $P = 0.51$ ) and specificity ( $P = 1.0$ ) between the *B. gibsoni*-specific test and the babesiosis test. The  $\kappa$  values for agreement between the iiPCR and reference standard results were 0.90 (95% CI, 0.84–0.96) for

**Table 1.** Test results for 233 blood samples with the POKKIT insulated isothermal PCR (iiPCR) assays for *Babesia gibsoni* and babesiosis compared to a commercial laboratory reference standard. (+), positive; (–), negative.

	Reference standard (+)	Reference standard (–)	Total
iiPCR <i>B. gibsoni</i> (+)	71	2	73
iiPCR <i>B. gibsoni</i> (–)	8	152	160
Total	79	154	233
iiPCR babesiosis (+)	69	3	72
iiPCR babesiosis (–)	10	151	161
Total	79	154	233

*B. gibsoni* and 0.87 (95% CI, 0.80–0.94) for babesiosis. Calculated positive and negative predictive values for various hypothetical prevalence values are shown in Table 2.

## Discussion

The *B. gibsoni* and babesiosis iiPCR point-of-need tests had high sensitivity and specificity using the laboratory test results as the reference standard. The high specificity of the iiPCR tests, when compared to the reference laboratory, indicates that positive results likely represent true infections in high-risk dogs. However, the number of false-negative results suggests that approximately 10–15% of truly infected dogs would not be identified by the POKKIT iiPCR system. As there was no significant advantage gained by the broader test for *Babesia* spp. in this population of dogs, the use of the *B. gibsoni* test would be most practical for mass screenings of high-risk dogs and rapid identification of infected dogs requiring antimicrobial treatment.

When calculating predictive values of each test, we chose infection prevalences that would reflect different populations. The lowest reflects the prevalence commonly reported for dogs other than “pit bull-type” dogs, and the higher values reflect prevalence ranges reported for “pit bull-type” dogs.<sup>1–3,11</sup> The 34% proportion of positive samples in this study is typical for pit bull-type dogs rescued in dogfighting investigations. Both positive and negative predictive values were high

**Table 2.** Calculated positive and negative predictive values for 2 point-of-need iiPCR tests in hypothetical populations of dogs with low-risk (5% prevalence) to high-risk (60%) prevalence for *Babesia gibsoni* infection. PPV, positive predictive value; NPV, negative predictive value.

	5% Prevalence		30% Prevalence		60% Prevalence	
	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
<i>B. gibsoni</i>	82	99	97	96	99	89
Babesiosis	70	99	95	95	98	83

in this prevalence range. The positive predictive values decreased somewhat at a hypothetical prevalence of 5% or less expected in low-risk populations, and the negative predictive values decreased somewhat at a hypothetical prevalence of 60%, which has been reported in at least one dogfighting case.

In addition to screening for *B. gibsoni* infection, the iiPCR point-of-need system could be used for monitoring response to treatment, especially when large numbers of dogs are being treated. Although no treatment protocol has been shown to consistently resolve *B. gibsoni* infections in all dogs, current treatment recommendations include multidrug treatment followed by PCR retesting at monthly intervals for up to 120 days after treatment completion.<sup>12–16</sup> At the time of this study, drug and monitoring costs exceed several hundred dollars per dog; a cost that can put mass treatment of *B. gibsoni* out of reach in large cases.<sup>17</sup> Dogs that tested positive for *B. gibsoni* could be monitored during treatment with the iiPCR system. Once a negative result was achieved, a sample could be submitted to a reference laboratory for confirmation. Because there is no universally accepted gold standard test for *B. gibsoni*, and because false-negative results occasionally occur with PCR and serological assays, it has been recommended that both PCR and serology be used together to maximize the likelihood of a diagnosis.<sup>16</sup> Under circumstances of limited resources, that ideal may not be achievable. Further testing including serology, real-time PCR, and/or microscopy could be recommended in high-risk dogs with anemia or thrombocytopenia that had a negative iiPCR test. Users should also be cognizant of the fact that the *B. gibsoni* test is species-specific and so, is likely to miss *Babesia conradae*- or *Babesia microti*-like infections. Although somewhat less sensitive than real-time PCR, iiPCR is a practical, timely, and cost-effective method that could help bridge the accessibility gap to encourage investment in diagnosis, treatment, and post-treatment monitoring of more dogs.

The POKKIT iiPCR is user-friendly. The closed tube system minimizes risk of cross-contamination and user error, and specific training in PCR procedures is not necessary to perform the assay. The processing time for a batch of 6 samples from nucleic acid extraction to end result was approximately 90 minutes. Cost per test was approximately \$12, which was roughly one-third the cost for the commercial reference laboratory real-time PCR assay at the time of this study. In addition to ease of use, prompt results, and lower costs, the POKKIT iiPCR system is portable and self-contained. The equipment occupies about 2 square feet of counter space, all reagents are provided, and only a small box of reagents requires refrigeration. Currently, the reagents for the POKKIT iiPCR system can be used for research purposes with a United States Department of Agriculture permit for importation into the United States.

Limitations of our study include the lack of a universally accepted gold standard for diagnosis of *B. gibsoni*, potential sample selection bias, and use of stored samples.<sup>11</sup> The comparative reference standard for this

study was defined by a combination of real-time PCR and serum antibody titer testing. Only samples with concordant results (positive PCR/positive antibody titer or negative PCR/negative antibody titer) were included in this study. The exclusion of samples with discordant results could have introduced selection bias.

Previous studies have demonstrated the challenges faced in attempting to diagnose *B. gibsoni* infection in dogs.<sup>1,11</sup> Dogs can have discordant results on PCR, serology, and microscopy, for a variety of patient factors including level of parasitemia, duration of infection and immune competence, as well as antigenic and sequence variation between different *Babesia* species, strains, and isolates. While we tried to ensure that our reference standard was likely to reflect true positive or true negative status using only dogs that had concordant results for *B. gibsoni* on real-time PCR and serology, it is still possible that these results did not reflect the true infection status of any given dog. However, this is true for any individual test for *B. gibsoni* and is the reason that PCR, serology, and microscopy are recommended as the ideal diagnostic approach in dogs suspected of being infected with *Babesia*.<sup>11</sup> This testing can run in the hundreds of dollars for each dog for screening and monitoring, and can be cost-prohibitive for rescue groups working with large numbers of high-risk dogs.

Although there was strong agreement between the iiPCR and the reference standard results, there were some discordant results. False-negative results could have been caused by a low level of parasitemia in the sample, poor DNA extraction, the presence of PCR inhibitors, and primer/probe mismatches. Additionally, this study used archived samples rather than freshly collected whole blood. Initial testing by the reference laboratory was performed on fresh samples. The effect of sample storage and the freeze/thaw process on test performance in this study is unknown. False-positive results were uncommon, only 2 or 3 of 154 samples, but could have been caused by DNA cross-contamination during sample preparation. The babesiosis kit could also have detected other small *Babesias* such as *B. microti*-like sp. not detected by the reference laboratory. Additionally, as mentioned above, given the challenges associated with diagnosing babesiosis, it is possible that the reference standard was incorrect for some samples.

The POKKIT iiPCR system provides an efficient and cost-effective alternative for mass testing of high-risk dogs for *B. gibsoni*. Although this study focused on *B. gibsoni*, the field-deployable iiPCR platform has potential to aid in rapid detection of a variety of animal and human infections under field conditions.<sup>8–10,18–27</sup>

## Footnotes

<sup>a</sup> POKKIT Nucleic Acid Analyzer, GeneReach USA, Lexington, MA

<sup>b</sup> Cubee mini-centrifuge, GeneReach USA, Lexington, MA

<sup>c</sup> IDEXX Laboratories Inc, Westbrook, ME



<sup>d</sup> PetNAD Nucleic Acid Co-prep kit, GeneReach USA, Lexington, MA

<sup>e</sup> POCKIT *Babesia gibsoni* Reagent Set, GeneReach USA, Lexington, MA

<sup>f</sup> POCKIT Canine Babesiosis Reagent Set, GeneReach USA, Lexington, MA

<sup>g</sup> VassarStats, <http://vassarstats.net/> (accessed March 30, 2017)

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**Conflict of Interest Declaration:** Authors declare no conflict of interest.

**Off-label Antimicrobial Declaration:** Authors declare no off-label use of antimicrobials.

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