pISSN 1598-298X / eISSN 2384-0749
J Vet Clin 37(1) : 23-27 (2020)
http://dx.doi.org/10.17555/jvc.2020.02.37.1.23

Journal of Veterinary Clinics
www.ksvc.or.kr

Ultra Fast Real-Time PCR for Detection of *Babesia gibsoni* as Point of Care Test

Yong-Sung Yang*, Myung-Jun Mun and Young-Min Yun[1]**

College of Veterinary Medicine and Veterinary Medical Research Institute, Jeju National University, Jeju 63243, Korea
**Seogwipo Animal Medical Center, Seogwip 63590, Korea*
***CAREVET Co., Ltd., Ansan 15610, Korea*

(Received: January 23, 2020 / Accepted: February 12, 2020)

Abstract : Between May and November 2018, babesiosis was examined in 162 bloods samples obtained to an animal hospital in Jeju island for anemia and medical examination. Sixty-two of 162 (38.3%) were positive by PCR. The ultra fast real-time PCR test with blood directly analyzed without DNA extraction showed the same results. Accurate diagnosis, treatment and prognosis of babesiosis should be combined with clinical symptoms, blood tests, the babesia antibody test, and the PCR antigen test. Ultra fast real-time PCR, with these tests, is expected to be a point-of-care testing (POCT) for easy, fast and accurate diagnosis of babesiosis in the veterinary clinic.

***Key words* :** *babesia gibsoni*, ultra fast real-time PCR, POCT, dog.

Introduction

Babesiosis is a tick-borne protozoan disease whose hosts are rodents, humans, dogs, cats, horses and cattle. Canine babesiosis is caused by *Babesia gibsoni*, *B. canis* supsp., *B. microti*, *B. equi*, *B. conradae* and *B. vitalii* (2,9,13,14,18,19). Canine babesiosis is morphologically classified into small and large babesia. *B. gibsoni* which is small babesia and mediated by *Haemaphysalis longicornis* and *H. bispinosa*, occurs mainly in South Korea and throughout Asia (10,15,16). *B. gibsoni* is generally infected by the mites bite and are also infected by transfusions from infected dogs (7) and through placental infection (8). In general, the pathogenicity of *B. gibsoni* is more severe than *B. canis* (3,10). In recent years, the outbreak of babesiosis is increasing in South Korea as well as Jeju island. The main clinical symptoms are mucosal pale, hemoglobinuria, tachycardia, tachypnea, fever, decreased appetite, depression, jaundice, splenomegaly, lymphadenopathy and thrombocytopenia following hemolytic anemia (5,11,12,17). Rapid treatment is needed through differential diagnosis with various causes of anemia.

Diagnosis of babesiosis includes travel to epidemics, listening to mites such as blood sucking, transfusion of infected blood, Giemsa staining through blood, serological evaluation using indirect immunofluorescent antibodies (IFAT) (4), blood babesia antibody tests (6,20) and polymerase chain reaction (PCR) assays that directly detect antigens in the blood (1,13). Recently, the newly developed ultra fast real-time PCR method has been used as a point of care test (POCT) for diagnosing pathogenic microorganisms in the field.

This study diagnosed babesia through the general PCR test in patients who visited the veterinary clinic in Jeju island May and November 2018 and examined the effectiveness of ultra fast real-time PCR as POCT.

[1]Corresponding author.
E-mail : dvmyun@jejunu.ac.kr

Materials and Methods

Animals and grouping

From May through November 2018, blood samples of 162 dogs that visited Jeju and Seogwipo city animal hospital with anemia and non-anemia symptoms were included. In this study, 2 ml of blood was collected from the jugular or cephalic vein. The collected blood was dispensed into EDTA anticoagulant tubes for 0.5 ml for complete blood count (CBC) and 0.5 ml for babesia gene detection by PCR. CBC and blood smears were examined immediately after the blood collection. DNA was extracted from whole blood and stored in the deep freezer until conducting PCR test. Experimental animals were selected and approved by the Animal Experimental Ethics Committee of Jeju National University (Approval No. 2018-0041).

Complete blood count

Anticoagulant blood was tested for CBC using ADVIA 2120i (SIEMENS). Red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) and platelet (PLT) were examined.

Giemsa staining

To identify babesia merozoite in erythrocytes, blood was smeared onto slides immediately after collection and fixed with methanol. Giemsa staining sol (Sigma-Aldrich) and distilled water (DW) were diluted 1 : 9 and dipped on the slide and left for 1 hour. Babesia was identified in red blood cells

under a high magnification (× 1,000) of an optical microscope (Olympus Optical Co. Tokyo, Japan).

DNA extraction

EDTA-treated blood was extracted with genomic DNA using G-DEXTM IIb Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea). The extracted DNA was adjusted to a final concentration of 100 ng/μl using a spectrophotometer (NanoVu, GE healthcare bioscience, USA), and stored frozen until the PCR test.

PCR

PCR was performed using primers reported by Birkenheuer (1) and Oh (15) to amplify the 18s rRNA gene of *Babesia gibsoni*. The PCR was performed using thermal cycler (C1000TM Thermal Cycler, BIO-RAD, USA) with 20 μl reaction solution containing canine babesia 18s rRNA forward primer 10 pmole, reverse primer 10 pmole, 2x AccuPower PCR master mix (Bioneer Inc, Korea), DNA (100 ng/μl). The initial denaturation was repeated 5 times at 94°C for 5 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec 35 times amplified at the last 72°C. The PCR products were identified by electrophoresis on 1.5% agarose gel (Red safe stain) with a 100 bp DNA ladder marker (Enzynomics, Korea). The amplification of *Babesia* spp. using a common primer was 1,676 bp.

Ultra fast real-time PCR

For the primer design, *hsp*70 (heat shock protein 70) gene sequences of *B. gibsoni*, were obtained from the NCBI Genbank Database (Genbank accession number AB083515) and aligned using Genious Software (Genious 11.1.5, Biomatters Ltd., Auckland, New Zealand). Highly conserved regions of this gene were identified, and the primers were designed against the conserved region (Fig 1). Information regarding the primer sequences is summarized in Table 1.

Ultra fast real-time PCR reaction was performed in a total volume of 10 μl containing 1 μl primer mix (10 pmole each primer), 2x master mix with SYBR green dye (Genesystem, Daejeon, Korea), 2 μl of template DNA, and DNase free water. A UF-150 real-time PCR (Genesystem, Dajeon, Korea) (Fig 2) was used with the following protocol: initial denatur-

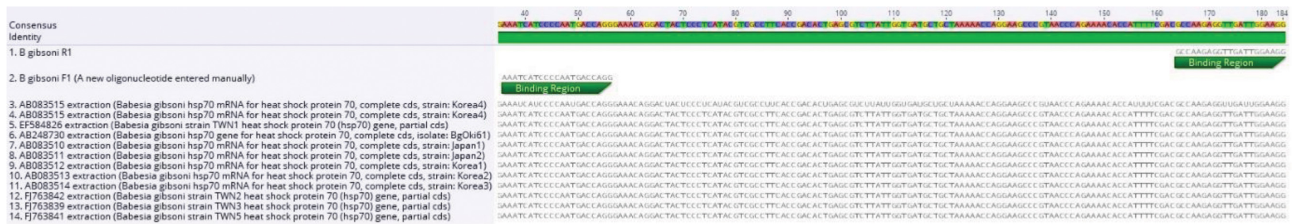

Fig 1. Alignment result of *Babesia gibsoni*. Nucleotide sequences collection and alignment have used Genious software.

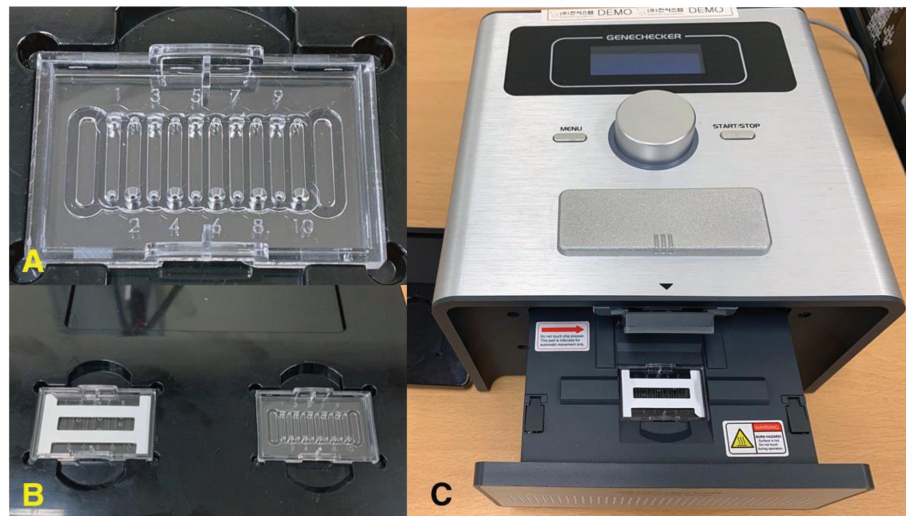

Fig 2. Ultra fast real-time PCR chip (A), frame for filling PCR reagents in chip (B) and PCR device (C).

Table 1. Oligonucleotide sequences of conventional and ultra fast real-time PCR primer sets used in this study

PCR	Primer set	Sequences (5′->3′)	Size
Conventional	Cbabe-18sF Cbabe-18sR	GTTGATCCTGCCAGTAGT AACCTTGTTACGACTTCTC	1,676
Ultra fast real-time	*B. gibsoni* F *B. gibsoni* R	AAATCATCCCCAATGACCAGG CCTTCCAATCAACCTCTTGGC	149

ation at 98°C for 30 sec, and 40 cycles of 98°C for 3 sec, 60°C 8 sec, 72°C 3 sec. After amplification, a melting curve with a ramp speed of 0.5°C/s between 60°C and 95°C was determined with using fluorescence of SYBR green. Melting peaks were automatically calculated by GeneRecorder software (Genesystem, Daejeon, Korea).

Statistical analysis

The blood test results of each group were analyzed by ANOVA using SPSS statistical program (*ver* 22, SPSS Inc. IBM Corp, Chicago, USA) to verify the significance between the groups ($p < 0.05$).

Results

CBC

In the babesia positive group, all RBC, Hct, and Hb levels significantly decreased compared with non-anemia patients. Platelet count was also significantly lower than that of non-anemia group. The leukocyte count increased slightly but not significantly (Table 2).

Blood smear test

In the blood smear, we could identify the suspected *B. gibsoni* merozoite with ovoid shape of 1.1-2 μm in erythrocytes under high magnification optical microscope after Giemsa staining (Fig 3).

PCR

Of the gDNA samples extracted from a total of 162 blood samples, 62 (38.3%) were amplified by 1,676 bp PCR products (Fig 4).

Ultra fast real-time PCR test

The total of 62 PCR-positive blood samples showed the same positive results by ultra fast real-time PCR and showed one peak at Tm value 82°C (± 2°C) (Fig 5). All the ultra fast real-time PCR tests showed the same negative for the general PCR negative samples.

Discussion

Babesiosis is frequently occurring from spring through

Table 2. The CBC of dogs in babesia infectious and normal group

	B (n = 62)	N (n = 100)	Reference range
RBC (10^6/μl)	4.61 ± 2.06^a	6.58 ± 1.39^A	5.7-8.8
Hct (%)	29.8 ± 12.58^a	42.3 ± 7.97^A	37.1-57.0
Hb (g/dl)	9.1 ± 3.99^a	13.0 ± 2.61^A	12.9-18.4
MCV (fL)	68.3 ± 7.86	65.3 ± 6.66	58.8-71.2
MCHC (g/dl)	30.1 ± 2.61	30.8 ± 2.25	31.0-36.2
PLT(/μl)	133.4 ± 75.23^a	286.9 ± 121.01^A	143-400
WBC (× 10^3/μl)	17.5 ± 7.96	14.3 ± 5.53	5.2-13.9

B; Babesia positive group, N; Normal group, A; a Tukey group ($P < 0.05$).

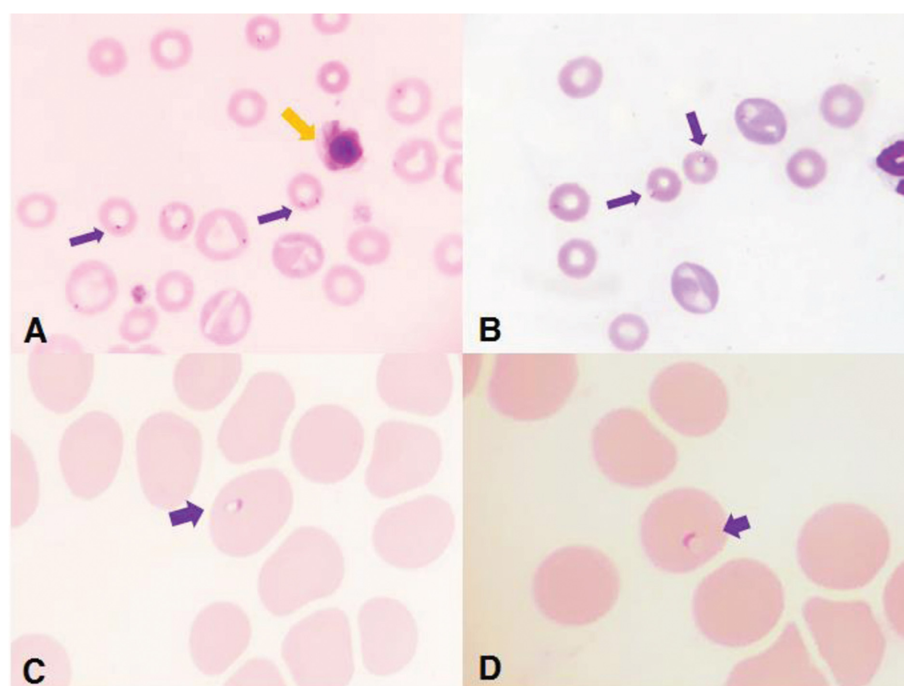

Fig 3. Photomicrograph (× 1,000) of Giemsa-stained thin-smear blood films showing erythrocytes parasitized by *B. gibsoni* merozoite. Purple arrow; *Babesia gibsoni*, yellow arrow; nucleated RBC.

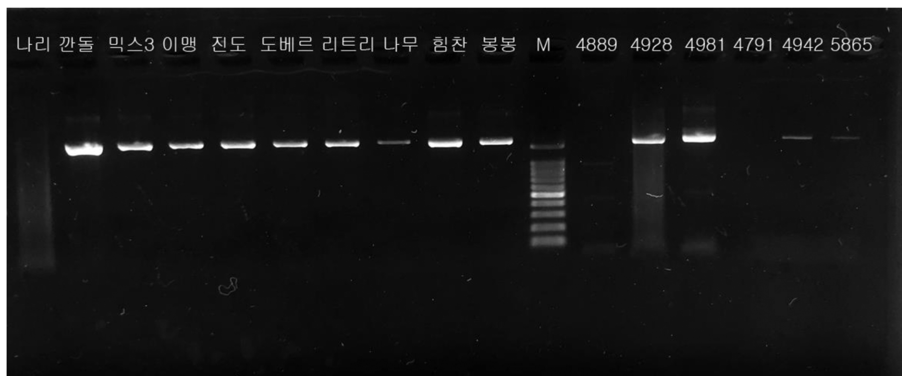

Fig 4. PCR products of *Babesia* spp. by 1.5% agarose gel electrophoresis. The sizes of PCR product of amplified 18s rRNA of *Babesia* spp. is 1,676 bp. M: 100 bp ladder marker (upper size; 1,500 bp).

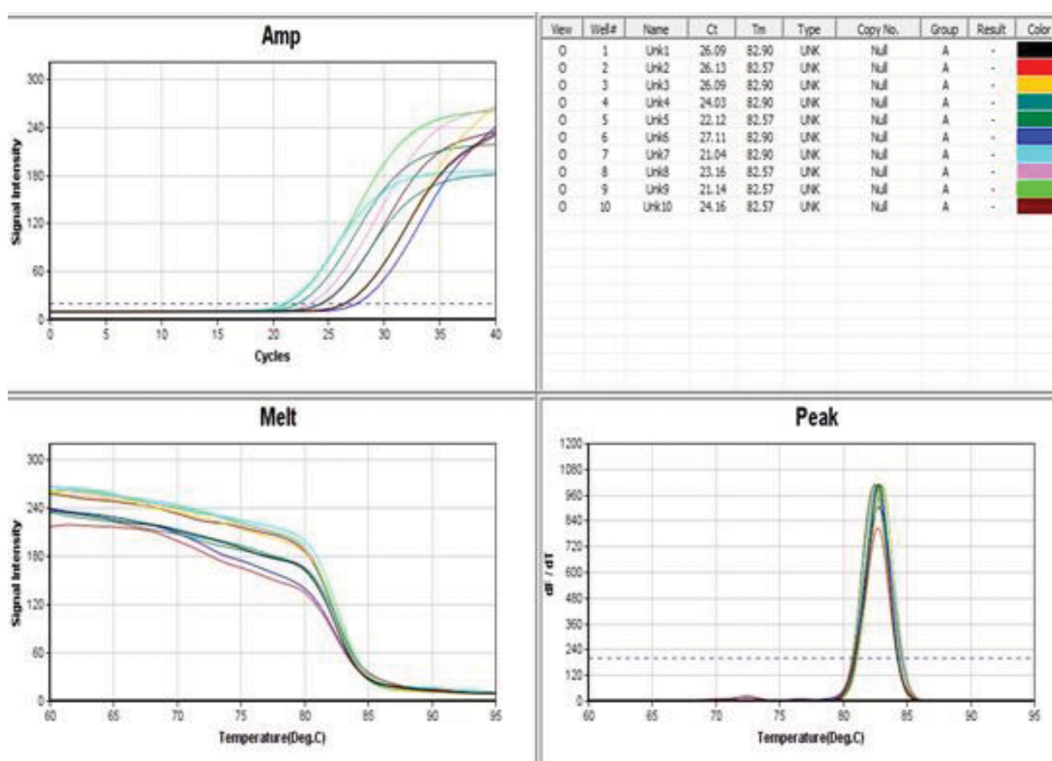

Fig 5. Results of ultra fast real-time PCR in the babesia positive samples.

autumn (14). At this time, 62 (38.3%) of the 162 patients were positive for PCR. The high positive result is deduced to be due to the period of the most active tick activity and the increasing number of patients manifesting anemia (14,15). Some of the PCR-positive individuals were found to be asymptomatic.

In the blood test, the babesia group showed significant decrease in RBC index and platelet count compared to the control group. WBC count tended to increase and decrease slightly at the beginning of babesia infection, and there was no significant difference from the control group. Many outdoor dogs bred on Jeju island are often found to be infected with babesia but do not exhibit clinical symptoms (latent infection). Babesia infection is endemic in dogs in Jeju island. Further studies are needed to detect antigens and antibodies following babesia infection and to determine when and how long antigens are lost.

The ultra fast real-time PCR is easier and faster to test than the general PCR: 15 minutes of whole blood incubation and initial denaturation at 98ºC for 30 sec, and 40 cycles of 98ºC for 3 sec, 60ºC 8 sec, 72ºC 3 sec in the quick PCR. The result of qPCR showed the consistency with the general PCR. To diagnose babesiosis in patients with anemia in the veterinary clinic, it is difficult to confirm the diagnosis only with decrease in the RBC index, regeneration anemia and thrombocytopenia. In addition to difficulty in distinguishing between erythrocytes and babesia merozoites in blood smear and staining results, it is often difficult to identify them in the early stages of infection or in case of mild infection. Currently, it is necessary to simultaneously diagnose clinical symptoms, general blood test, blood smear staining, babesia antibody diagnosis (20), and PCR antigen test (14). Highly specificity PCR anti-

gen tests require a multiple equipment and time from DNA extraction to gene amplification and identification. In this study, the ultra fast real-time PCR with gDNA from blood showed the same results as conventional PCR. And also ultra fast real-time PCR with blood directly without DNA extraction showed the same results. Ultra fast real-time PCR, with various diagnostic tests for the babesiosis, is expected to be a point-of-care testing (POCT) for easy, fast and accurate diagnosis of babesiosis in the veterinary clinic.

Acknowlegement

"This study was supported by the research grant of Jeju National University in 2016".

References

1. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J Clin Microbiol 2003; 41: 4172-4177.
2. Boozer L, Macintire D. Canine babesiosis. Vet Clin North Am Small Anim Pract 2003; 33: 885-904.
3. Boozer L, Macintire D. *Babesia gibsoni*: an emerging pathogen in dogs. Compend Contin Educ Vet 2005; 27: 33-42.
4. Chisholm ES, Ruebush II TK, Sulzer AJ, Healy GR. *Babesia microti* infection in man: evaluation of an indirect immunofluorescent antibody test. Am J Trop Med Hyg 1978; 27: 14-19.
5. Conrad P, Thomford J, Tamane I, Whiting J, Bosma L, Uno T, Holshuh HJ, Shelly S. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. J Am Vet Med Assoc 1991; 199: 601-605.
6. Eichenberger RM, Štefanić S, Naucke TJ, Šarkūnas M, Zamokas G, Grimm F, Deplazes P. An ELISA for the early diagnosis of acute canine babesiosis detecting circulating antigen of large *Babesia* spp. Vet Parasitol 2017; 243: 162-168.
7. Farwell GE, LeGrand EK, Cobb CC. Clinical observations on *Babesia gibsoni* and *Babesia canis* infection in dogs. J Am Vet Med Assoc 1982; 180: 507-511.
8. Fukumoto S, Suzuki H, Igarashi I, Xuan X. Fetal experimental transplacental *Babesia gibsoni* infections in dogs. Int J Parasitol 2005; 35: 1031-1035.
9. Garcia AT. Piroplasma infection in dogs in northern Spain. Vet Parasitol 2006; 138: 97-102.
10. Inokuma H, Yoshizaki Y, Matsumoto K, Okuda M, Onishi T, Nakagome K, Kosugi R, Hirakawa M. Molecular survey of Babesia infection in dogs in Okinawa, Japan. Vet Parasitol 2004; 121: 341-346.
11. Irwin PJ, Hutchinson GW. Clinical and pathological findings of Babesia infection in dogs. Aust Vet J 1991; 68: 204-209.
12. Jacobson LS, Clark IA. The pathophysiology of canine babesiosis: new approaches to an old puzzle. J S Afr Vet Assoc 1994; 65: 134-145.
13. Kjemtrup AM, Kocan AA, Whitworth L, Meinkoth J, Birkenheuer AJ, Cummings J, Boudreaux MK, Stockham SL, Irizarry-Rovira A, Conrad PA. There are at least three genetically distinct small piroplasms from dogs. Int J Parasitol 2000; 30: 1501-1505.
14. Lee MJ, Yu DH, Yoon JS, Li YH, Lee JH, Chae JS, Park J. Epidemiologic and clinical surveys in dogs infected with *Babesia gibsoni* in South Korea. Vector Borne Zoonotic Dis 2009; 9: 681-686.
15. Oh ST, Woo HC. Prevalence of *Babesia* spp. in dogs of Seogwipo-si, Jeju-do, South Korea. Korean J Vet Serv 2009; 67: 467-471.
16. Shimada Y, Beppu T, Inokuma H, Okuda M, Onishi T. Ixodid tick species recovered from domestic dogs in Japan. Med Vet Entomol 2003; 17: 38-45.
17. Taboada J, Merchant SR. Babesiosis of companion animals and man. Vet Clin North Am Small Anim Pract 1991; 21: 103-123.
18. Uilenberg G, Franssen FFJ, Perrie NM. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet Q 1989; 11: 33-40.
19. Yonamine H, Ichiki H, Hamakawa M, Shimabukuro T, Sugiyama M, Isoda M. Studies on canine babesiosis in Okinawa Island. Jpn J Vet Sci 1984; 46: 511-518.
20. Zhou J, Zhang G, Nishikawa Y, Fujisaki K, Xuan X. A 38-kDa protein from *Babesia gibsoni* and its antibody response in an experimentally infected dog. Vet Parasitol 2006; 141: 345-348.