*Available online at* www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.7160

**ISSN: 2320 – 7051**

*Int. J. Pure App. Biosci.* **6 (6):** 924-930 (2018)

International Journal of Pure & Applied Bioscience

*Research Article*

OPEN ACCESS

# Molecular Detection and Characterization of Canine *Babesia* Spp. from South India

**Mullur Prabhuswamy Roopesh, Eugene Placid D'souza and Gotakanapura Sanjeevamurthy Mamatha***

Department of Veterinary Parasitology, Karnataka Veterinary, Animal and Fisheries Sciences University (Regional Campus), Veterinary College, Hebbal, Bengaluru-560 024. India

*Corresponding Author E-mail: drmamathags@gmail.com

Received: 15.10.2018 | Revised: 23.11.2018 | Accepted: 1.12.2018

**ABSTRACT**

*The molecular detection and characterization of canine Babesia spp. was carried out by polymerase chain reaction (PCR) by targeting 18S rRNA and ITS1 gene from Bengaluru district, Karnataka state (South India). Out of 110 blood samples collected from both apparently healthy and clinically suspected dogs, 76 (69.09%) samples showed amplification of 410 bp DNA fragment specific for genus Babesia. In species specific PCR, out of 76 genus positive samples 22 (28.94%) and 39 (51.31%) samples showed amplification of 590 and 671 bp DNA fragments specific for Babesia canis vogeli and Babesia gibsoni, respectively. The mixed infections of B. c. vogeli and B. gibsoni were detected in 15 (19.73%) samples by species specific PCR. Hence, the present study indicated that PCR is highly specific and useful method for detection, characterization and clinical diagnosis of Babesia spp. upto the species level.*

***Key words:*** *Canine Babesia, Molecular detection, Phylogenetic analysis*

## INTRODUCTION

Babesiosis caused by intraerythrocytic apicomplexan parasites belonging to the genus *Babesia* is a common tick-borne haemoprotozoan disease of domesticated dogs and wild canids. It is one of the most important vector-borne disease of dogs caused by *Babesia canis*, a large form (3.0-5.0µm) and *Babesia gibsoni* a small pleomorphic organism (1.5-2.5µm) throughout the world. The disease is characterized by haemolytic anaemia, thrombocytopenia, fever and splenomegaly. It is found in almost all parts of Asia, Europe, Africa, America and Australia. Generally, *Babesia* spp. is identified by demonstrating the piroplasms in Giemsa stained blood smears under the light microscope and was presumed that *B. canis* is the only large species to infect dogs throughout the world. But recent molecular studies have shown the existence of three subspecies of *B. canis viz., B. canis canis, B. canis rossi* and *B. canis vogeli* which differ in their geographic location, antigenic variation, and in vector specificity.

**Cite this article:** Roopesh, M.P., D'souza, E.P. and Mamatha, G.S., Molecular Detection and Characterization of Canine *Babesia* Spp. from South India, *Int. J. Pure App. Biosci.* **6(6):** 924-930 (2018). doi: http://dx.doi.org/10.18782/2320-7051.7160

Further, the pathogenecity and treatment is also known to vary for each subspecies. Though, the direct microscopic examination of the stained blood smear is the most commonly used method as it is conclusive, feasible and cost effective diagnostic method[2]. But the molecular detection methods are more sensitive, specific, rapid for diagnosis and has allowed further genotypic characterization into several subspecies. Hence, the present study was carried out for detection and characterization of common *Babesia* spp. infecting dogs in south India.

## MATERIAL AND METHODS

### Study area and collection of samples

The blood samples (n=110) were collected from both apparently healthy dogs which were not showing any clinical symptoms and clinically suspected cases presented to the Veterinary College Hospital (Bengaluru), local Veterinary hospitals and clinics situated in Bengaluru urban district. The samples were transported to the laboratory and stored at -$20^0$C for further extraction of DNA.

### Genomic DNA extraction

The genomic DNA was extracted from 200 µl of venous blood and 20 µl of proteinase K using the "QIAamp DNA minikit" (Qiagen, Germany) as per the manufacturer's instruction. The extracted DNA was stored at -20°C until further use.

### PCR for detection of *Babesia* spp.

The PCR amplification was performed using a gradient thermal cycler (Eppendorf, Germany) in a final reaction volume of 25 µl containing 12.5 µl of PCR master mix (Merck's), 2 µl of each forward and reverse primer (10 picomoles per µl), 5 µl of template DNA and 3.5 µl of nuclease free water. The DNA extracted from blood samples were subjected for *Babesia* genus specific PCR by using primers PIRO-A Forward: 5'AATACCCAATCCTGACACAGGG 3' and PIRO-B Reverse: 5'TTAAATACG AATGCCCCCAAC3'[6]. The cycling conditions was as follows: Initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min with final extension at 72°C for 7 min. The samples which were found positive for *Babesia* genus were subjected to species specific PCR with the primers BAB1 F: 5' GTGAACCTTATCACTTAAAGG 3' and BAB4 R: 5' CAACTCCTCCACGCAATCG 3' for *B. c. vogeli*3 and Gib599 F: 5' CTCGGCTACTTGCCTTGTC 3' Gib1270 R: 5' GCCGAAACTGAAATAACGGC 3' for *B. gibsoni* [7] by targeting 28S rRNA and 18S rRNA, respectively. The PCR cycling conditions were initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min, final extension at 72°C for 7 min for *B. c. vogeli.* For *B.gibsoni,* the initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1.30 min for 40 cycles with final extension at 72 °C for 5 min was followed. The known positive control was obtained from Department of Medicine, Veterinary College, Bengaluru. Nuclease free water was used as a negative control and no template control (NTC) was used in all PCR amplifications. The PCR products were analysed on 1.5 per cent agarose and the images were captured in a gel documentation unit.

### Sequencing and Blast Analysis

Out of 70 confirmed positive samples which yielded 410 bp DNA fragment specific for *Babesia,* four samples were randomly selected and were sequenced by using Sanger dideoxy sequencing in an ABi nucleotide sequencer at M/s Bioserve biotechnologies private limited, Hyderabad. The BLAST search was performed on GenBank database at National centre for Biotechnology information (NCBI) gov.website http://www.ncbi.nlm.nih.govt/ BLAST by using the complete 18S rRNA gene sequence of genus *Babesia.*

### Phylogenetic analysis of *B. canis vogeli* and *B. gibsoni isolates*

The obtained sequences were aligned with the published sequences deposited in the GenBank database (Accession numbers KY608902, AY371196, KU662366, LC008284,

KF171474, KC811802 and KC811801 and the phylogenetic tree was constructed by maximum likelihood method using MEGA 6.0 software. The 18S rRNA gene sequence of *Plasmodium* spp., (M19172) was included in the tree as an outgroup.

## RESULTS AND DISCUSSION

### Detection of *Babesia* spp. by PCR and sequencing

Out of 110 DNA samples analysed by *Babesia* genus specific PCR, 69.09 (76/110) per cent of the samples confirmed positive by specific amplification of 410 bp (Fig. 1). In BLAST analysis, out of four samples two samples showed 97.0 per cent homology with the sequences of *B. c. vogeli* isolates deposited in GenBank and other two showed 99.0 per cent homology with *B. gibsoni* isolates. Based on these results, all the samples which confirmed positive for genus *Babesia* were subjected for *B. c. vogeli* and *B. gibsoni* species specific PCR.

In *Babesia* spp. specific PCR, a total of 28.94 (22/76) per cent of the samples were confirmed positive for *B. c. vogeli* with a specific amplicon of 590 bp (Fig 2). In PCR assay which was optimized to amplify *B. gibsoni* by targeting a portion of the 18S rRNA gene, a total of 51.31 (39/76) per cent of the samples confirmed positive with a specific amplicon of 671 bp (Fig 3). The mixed infections of *B. c. vogeli* and *B. gibsoni* were recorded in 19.73 (15/76) per cent of the cases.

The present findings were in agreement with the epidemiological studies conducted by Porchet *et al*.[12] who had reported widespread distribution of *B. canis* in the Lake Geneva region, with 62.0 per cent prevalence of *B. canis* through blood smear and PCR. Similar findings were reported by Laha *et al*. [9] who had reported 56.75 per cent prevalence of *Babesia* infection by PCR in Assam comprising 8.10 per cent of *B. canis* and 2.70 per cent of *B. gibsoni* with 45.94 per cent of mixed infections. Similarly, Foldvari *et al.* [5] had reported the highest prevalence of *Babesia* infection (88.63%) in dogs in Hungary by piroplasm-specific PCR amplifying the partial 18S rRNA gene which yielded an 450 bp PCR product in 39 (88.63%) samples and Paulauskas *et al.*[11] also reported higher prevalence of babesiosis in dogs (87.8%) in Lithuania where *Babesia* isolated from dogs were detected and characterized by nested-PCR and sequence analysis of a fragment of the 18S rRNA gene. In the present study, the samples that were positive by microscopy were also positive by PCR. The higher prevalence of *Babesia* by PCR during this study could be probably due to ability of PCR in detection of *Babesia* parasites in carrier animals possessing low parasitemia that could not be detected by microscopy[14]. However, Jain *et al*.,[8] reported the validation of multiplex PCR for simultaneous detection of *B.c.vogeli, B.gibsoni* and *E.canis* in dogs from Kerala, South India and suggested the suitability of the assay to assist in the selection of pathogen specific treatment protocols.

During this study, the prevalence of *B. gibsoni* (51.31%) was higher when compared to *B. c. vogeli* (28.94%) this is may be due to re-occurrence of disease in *B. gibsoni* infected cases as observed in the study were more when compared to *B. c. vogeli* infected cases and also may be due to lack of effective drug treatment for *B. gibsoni*. Further, PCR has showed higher sensitivity for detection of *Babesia* infection by detecting organisms in samples where no organisms were found by microscopic examination.

### Phylogenetic analysis

The phylogenetic analysis of 410 bp sequences showed that *B. c. vogeli* Karnataka isolate (MG252701) shared 99.99 per cent nucleotide homology with *B. c. vogeli* isolates from Brazil (KU662366) and China (KY608902) deposited in GenBank and 99.98 per cent nucleotide homology with *B. c. vogeli* isolates from Brazil (AY371196) (Fig 4.). Whereas, *B. gibsoni* Karnataka isolates (MG252702) shared 100.0 per cent nucleotide homology with *B. gibsoni* isolates from Assam (KC811802), Bareilly (KC811801), Kolkata (KF171474) and Bangladesh (LC008282) and were in same clade. The isolates that were identified as *B. c. vogeli* and *B. gibsoni* during

this study were completely different from *Plasmodium* spp. (M19172) which was used as an outer group.

Similarly, Tiskina *et al.*[13] conducted a phylogenetic study in a splenectomized Estonian dog and showed that the sequences were identical to other European *B. c. canis* isolates that display AG genotype at positions 610/611 of the whole-length gene. Phylogenetic characterization of *B. c. vogeli* in dogs in Brazil by Duarte *et al.*[4] had aligned partial sequences of 18S rRNA of the autochthonous *B. c. vogeli* GO 1 and *B. c.* vogeli GO4 isolates and found that the sequences were clustered into two distinct clades *viz.*, one clade (bootstrap = 99%) was composed of *B. gibsoni*, *B. c. vogeli*, *B. c. canis* and *B. c. rossi*. The other clade (bootstrap = 74%) included the babesid species *B. microti* and *B. conradae* and the theilerids *T. annae*, *T. equi* and *T. annulata*; 2) *B. canis vogeli* GO 1 and GO 4 were clustered together to form a well-defined group with other *B. c. vogeli* strains from different geographical regions. Buddhachat *et al*.[1] reported that *B. canis* from Chiang Mai was determined as *B. c. vogeli* and was more closely related to those subspecies from China (HM590440), Japan (AY077719), Venezuela (DQ297390), and Brazil (AY371196). Caccio *et al*.[2] concluded that three *B. canis* subspecies form a monophyletic, well-supported group that is the sister group to the *B. odocoilei/B. gibsoni* group. Mandal *et al.*[10] determined genetic characterization and phylogenetic relationships based on 18S rRNA and ITS1 region of small form of canine *Babesia* spp. from India, by using maximum likelihood method and found that all the isolates of *B. gibsoni* Asian genotype formed a separate major clade named as *Babesia* spp. sensu stricto clade with high bootstrap support. The two unnamed *Babesia* sp. (Malbazar and Ludhiana isolates) clustered close together with *B. orientalis*, *Babesia* sp. (Kashi 1 isolate) and *B. occultans* of bovine.

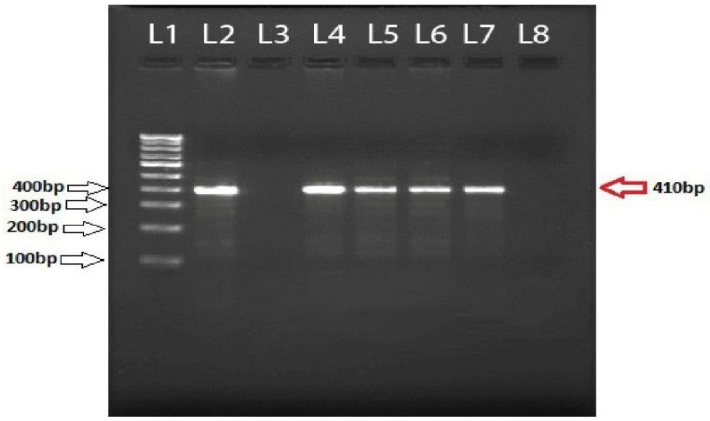


**Fig 1: Gel showing amplification of *Babesia* genus specific amplicon at 410 bp from dog blood sample**

Lane 1 -100bp molecular weight DNA Ladder

Lane 2 - Positive Control

Lane 3 - Negative Control

Lane 4, Lane 5, Lane 6 and Lane 7 - clinical samples

Lane 8- No template control (NTC)

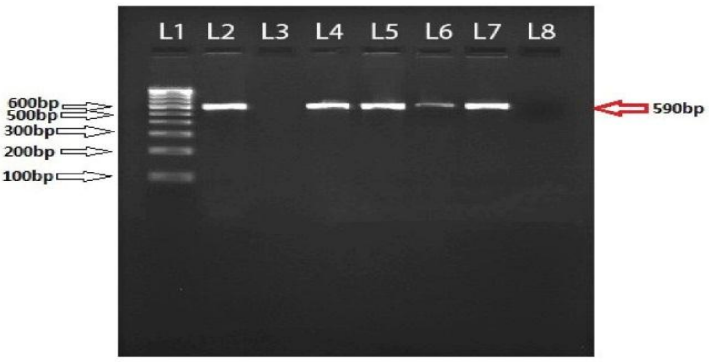


**Fig 2: Amplification of 590 bp *Babesia canis vogeli* specific fragment from dog blood by PCR**

Lane 1 -100bp molecular weight DNA Ladder

Lane 2 - Positive Control

Lane 3 - Negative Control

Lane 4, Lane 5, Lane 6 and Lane 7 - clinical samples

Lane 8- No template control (NTC)

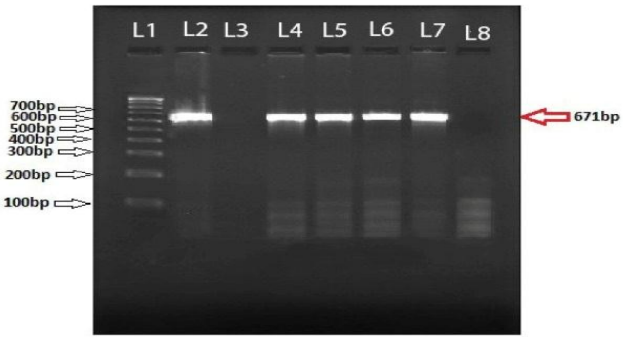


**Fig. 3 Amplification of 671 bp *Babesia gibsoni* specific fragment from dog blood by PCR**

Lane 1 -100bp molecular weight DNA Ladder

Lane 2 - Positive Control

Lane 3 - Negative Control

Lane 4, Lane 5, Lane 6 and Lane 7 - clinical samples

Lane 8- No template control (NTC)

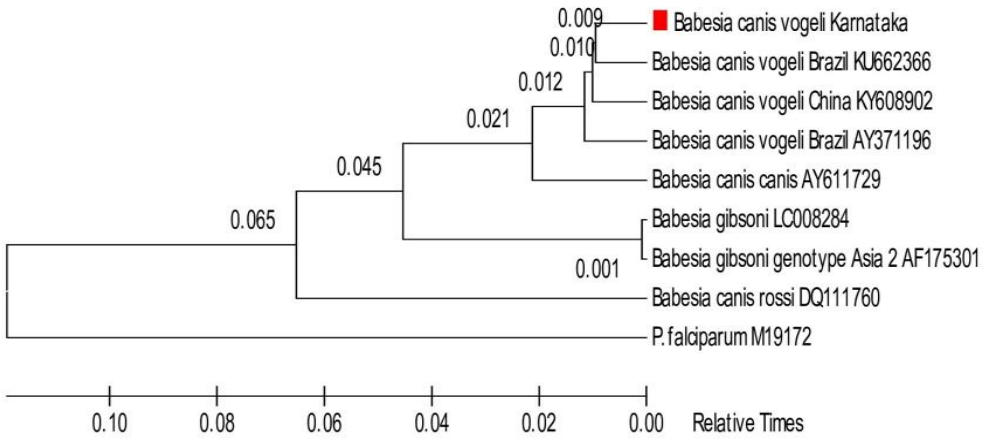


**Fig 4. Phylogenetic tree of the *Babesia canis vogeli* Karnataka isolates**

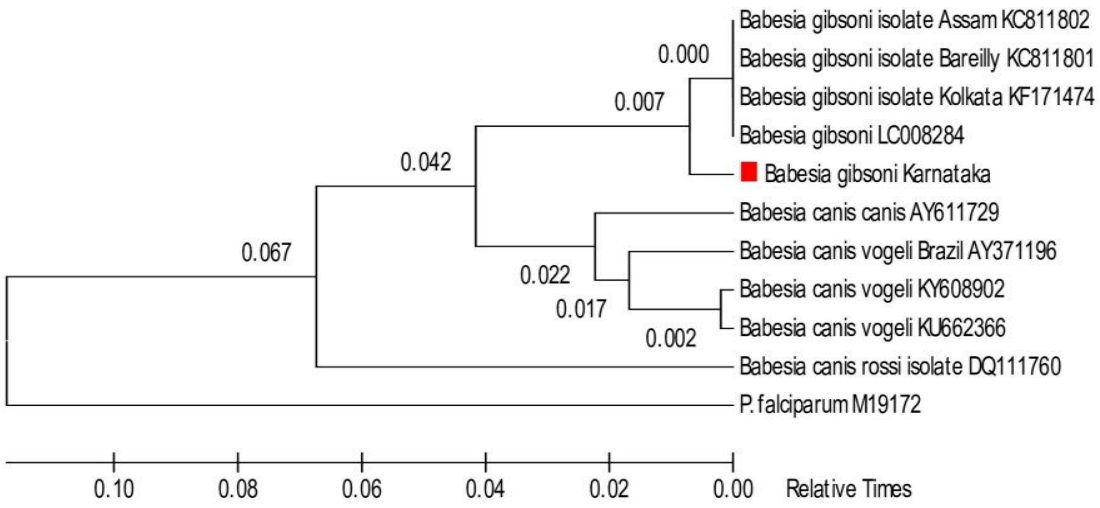


**Fig 5. Phylogenetic tree of the *Babesia gibsoni* Karnataka isolates**

## CONCLUSION

Based on the present findings, babesiosis has been considered as one of the emerging/re-emerging haemoprotozoan disease of dogs in Bengaluru, Karnataka state. Since, the animals which recover from acute infection become carriers, creating a potential source of infection to healthy susceptible population and concurrent occurrence of disease along with other diseases like Ehrlichiosis, Hepatozoonosis etc. Hence, the early accurate diagnosis of carriers and subclinically infected animals is essential to overcome the disease. It also has a great epidemiological significance, as they are the source of infection for the vectors [10]. Therefore, PCR was found to be very sensitive, rapid and reliable method for detection of *Babesia* infection in both carriers and clinically suspected animals.

## Acknowledgements

The financial support extended through Centre of Advanced Faculty Training, ICAR, New Delhi is gratefully acknowledged.

## REFERENCES

1. Buddhachat, K., Meesong, O., Nganvongpanit, K., Osathanunkul, M., Chomdej, S., Molecular characterization and detection of *Babesia canis vogeli* in asymptomatic roaming dogs in Chiang Mai, Thailand, Thai, *J. Vet. Med.* **42(2):** 173-178 (2012).
2. Caccio, S. M., Antunovic, B., Moretti, A., Mangili, V. A., Marinculic, R. R., Baric, S. B., Slemenda, Pieniazek, N. J., Molecular characterisation of *Babesia canis canis* and *Babesia canis vogeli* from naturally infected European dogs, *Vet. Parasitol.* **106:** 285-292 (2002).
3. Duarte, S.C., Linhares, G.F.C., Romanowsky, T.N., Neto, O.J.S., Borges, L.M.F., Assessment of primers designed for the subspecies-specific discrimination among *Babesia canis canis, Babesia canis vogeli* and *Babesia canis rossi* by PCR assay, *Vet. Parasitol*. **152:** 16-20 (2008).
4. Duarte, S.C., Parente J.A., Pereira M., Soares C.M.A., Linhares G.F., Phylogenetic characterization of *Babesia canis vogeli* in dogs in the state of Goias, Brazil, *Rev. Bras. Parasitol. Vet.* **4:** 274-280 (2011).
5. Foldvari, G., Hell E., Farkas, R., *Babesia canis canis* in dogs from Hungary: detection by PCR and sequencing, *Vet. Parasitol.* **127:** 221-226 (2005).
6. Gallego, S.L., Trotta, M., Carli, E., Carcy, B., Caldin, M., Furlanello, T., *Babesia canis canis* and *Babesia canis vogeli* clinicopathological findings and DNA detection by means of PCR-RFLP in blood

from Italian dogs suspected of tick-borne disease, *Vet. Parasitol*. **157:** 211–221 (2008).

7. 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*. **121:** 341–346 (2004).
8. Jose, Jain, Bindu, Lakshmanan, Hitaishi, V. Nagaraj, Jose, E., Syamala, P.K. and Aravindakshan, T., Detection of *Babesia canis vogeli*, *Babesia gibsoni* and *Ehrlichia canis* by multiplex PCR in naturally infected dogs in South India, *Veterinarski Arhiv*. **88 (2):** 215-224 (2018).
9. Laha, R., Bhattacharjee, K., Sarmah, P. C., *Babesia* infection in naturally exposed pet dogs from a north-eastern state (Assam) of India: detection by microscopy and polymerase chain reaction, *J. Parasit. Dis.* **38(4):** 389-393 (2013).
10. Mandal, M., Banerjee, P.S., Garg, R., Ram, H., Kundu, K., Kumar, S., Kumar, G.R., Genetic characterization and phylogenetic relationships based on 18S rRNA and ITS1 region of small form of canine *Babesia* spp. from India, *Infection, Genetics and Evolution*. **27:** 325-331 (2014).
11. Paulauskas, A., Radzijevskaja, J., Karveliene, B., Grigonis, A., Aleksandraviciene, A., Zamokas, G., Babickaite, L., Sabunas, V., Petkevicius, S., Detection and molecular characterization of canine babesiosis causative agent *Babesia canis* in the naturally infected dog in Lithuania. *Vet. Parasitol*. **205:** 702-706 (2014).
12. Porchet, M. J., Sager, H., Muggll, L., Oppliger, A., Muller, N., Frey, C., Gottstein, B., A descriptive epidemiological study of canine babesiosis in the Lake Geneva region, *Schweiz Arch Tierheilkd*. **149:** 457-465 (2007).
13. Tiskina, V., Capligina, V., Must, K., Berzina, I., Ranka, R., Jokelainen, P., Fatal ,*Babesia canis canis* infection in a splenectomized Estonian dog. *Acta. Vet. Scand*. **58(7):** 13-19 (2016).
14. Yamane, I., I. A., Gardner, C. P. Ryan, M. Levy, J. Urrico and P. A,. Conrad Serosurvey of *Babesia canis*, *Babesia gibsoni*, and *Ehrlichia canis* in pound dogs in California, USA. *Prev. Vet. Med.* **18:** 293-304 (1994).