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PCR-based survey of vector-borne pathogens in dogs in Dhaka, Bangladesh

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Abstract

To identify tick-borne pathogens in blood samples from 68 dogs were examined for *Babesia*, *Anaplasma*/*Ehrichia*, *Mycoplasma*, and *Hepatozoon* spp. by using a molecular tool. The detection of DNA was performed using specific primers for partial 16S rRNA or 18S rRNA sequence, and amplicon sequences were analyzed. Our investigation detected *Babesia gibsoni* (26 dogs) and *Anaplasma* sp. AnHl446 (2 dogs), *Mycoplasma haemocanis* (27 dogs), and an unknown *Mycoplasma* spp were detected (2 dogs), which was most closely related to *Candidatus Mycoplasma turicensis*, on the basis of phylogenetic analysis. *Hepatozoon* DNA could not be detected in this study.

Keywords: Tick-borne-pathogen, *Babesia gibsoni*, *Mycoplasma haemocanis*, *Anaplasma* sp. AnHl446

Introduction

Canine tick-borne diseases are an important problem, especially in tropical and subtropical regions. In Asia, tick-borne bacterial or parasitic infections by *Babesia*, *Ehlichia, Anaplasma*, *Mycoplasma*, and *Hepatozoon* species have been predominantly recognized. These pathogens are transmitted mainly by the Ixodid tick species from the genera *Ixodes, Dermacentor*, *Rhipicephalus*, *Haemaphysalis*, and *Ambloymma*. In South Asia, tick-borne diseases have been recognized since an early time, for example, *Babesia gibsoni* was first recognized in India (Patton, 1910) and *Hepatozoon canis* was first recognized in India (James, 1905). Although, the incidence of *B. gibsoni* was reported from Iran (Niak *et al.,* 1973), there is little information about the current state of infections of dogs in the South Asia region. In Bangladesh, there have been no epidemiological studies regarding the prevalence of these infections among dogs. Using blood samples isolated from various sick dogs in the animal hospital in Dhaka, the capital of Bangladesh, we examined the state of infections of tick-borne diseases in dogs by using a molecular tool.

Materials and Methods

Peripheral blood samples from 68 dogs were randomly obtained from the animal hospital in Dhaka, Bangladesh, between 2009 and 2010. Each 200µl blood sample was fixed on an FTA Nucleic Acid Collection Card (Whatman, UK) and stored at room temperature in the dark. DNA was extracted from the blood on the FTA Cards by using the Whatman FTA Card DNA Isolation Kit (Whatman, UK). Of the 68 dogs, 31 were female and 37 were male. Age ranged from 1 to 6 years (median, 3 years). There were 31 Mongrel dogs and 33 dogs of other breeds (Bulldogs: n=8, German shepherds: n=6, Greyhounds: n=5, Labrador Retrievers: n=5, Spaniels: n=4, Shih Tzu: n=4, and Chinese crested dog: n=1). The breed was not determined for 4 dogs. On physical examination, 7 of these were found to be healthy; 2 were pregnant; 11 were anemic; 17 were lethargic; 13 had dermatitis; and 10 had fever.

The sequences of the primers used to amplify *Babesia* spp., *Anaplasma*/*Ehrichia* spp., *Mycoplasma* spp., and *Hepatozoon* spp. are shown in Table 1. Polymerase chain reaction (PCR) was performed on 25 µl of a mixture containing template DNA, 10 pmol of each primer, 200 µm deoxynucleoside triphosphate (dNTP), and 1.25 units of Taq DNA polymerase (TaKaRa, Shiga, JAPAN). PCR was repeated for 40 cycles with denaturation for 1min at 94°C; annealing at for 1 min at 55°C for *Babesia* spp. and *Anaplasma/Ehrichia* spp., 50°C for *Mycoplasma* spp., and57°C for *Hepatozoon* spp.; and extension for 1min at 72°C. The PCR product was separated by electrophoresis using a 1.5% agarose gel with TBE

buffer, stained with ethidium bromide, and photographed in ultraviolet light. The amplified DNA was purified using a commercial kit (QIAamp purification kit; Qiagen, USA) and sequenced directly (ABI PRISM 310 genetic Analyzer; Applied Biosystems, USA). The sequences were evaluated and edited using Genetyx Version. 10.0 (Genetyx, Co., Tokyo, Japan), and consensus sequences were submitted to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the sequence identity in order to find orthologous sequences available in the GenBank database.

Table 1. Primer sets used for detection of DNA of tick borne pathogens in dogs in Bangladesh

Pathogen	Gene	Primer	Sequence (5'-3')	fragment size	Reference
Babesia spp.	18S rRNA		GTGAAACTGCGAATGGCTCA	650bp	Inokuma *et al.*, 2003.
			CCATGCTGAAGTATTCAAGAC		
Anaplasma/ Ehrlichia spp.	16S rRNA	EHR16SD	GGTACC(C/T)ACAGAAGAAGTCC	345bp	Brown *et al.*, 2001.
		EHR16SR	TAGCACTCATCGTTTACAGC		
Mycoplasma spp.	16S rRNA	fHF5	AGCAGCAGTAGGGAATCTTCCAC	674bp	Messick *et al.*, 1998.
		rHF6	TGCACCAACCTGTCACCTCGATAAC		
Hepatozoon spp.	18S rRNA	HepF	ATACATGAGCAAAATCTCAAC	666bp	Inokuma *et al.*, 2002.
		HepR	CTTATTATTCCATGCTGCAG		

Results and Discussion

Of the 68 dogs, 26(38.2%) were positive for *Babesia* spp., from the results of PCR. The partial 18S rRNA gene sequences of these positive PCR products were determined using 602bp of the amplified product, excluding the primer region. BLAST analysis revealed that all the samples were 100% identical (602/602) to the sequence of *B. gibsoni* Asia-2 (GenBank accession no.AF175301). PCR results of 2dogs (2.9%) showed they were positive for *Ehlichia*/*Anaplasma* spp. The partial 16S rRNA gene sequences of these positive PCR products were determined using 304bpof the amplified region excluding the primer region. BLAST analysis revealed that both samples were 100% identical to the partial 16S rRNA gene of *Anaplasma* sp. AnHl446 (GenBank accession no.AF497579). Twenty-nine dogs (42.6%) were positive for *Mycoplasma* spp., from the PCR results. The partial 16S rRNA gene sequences of these positive PCR products were determined using a 627-bp sequence of the amplicon excluding the primer region. BLAST analysis revealed that the 627-bp samples were 99.5–100% (624–627/627) identical to the sequence of *Mycoplasma haemocanis* (GenBank accession no.EF416568, EF416567, EF416566), except in the case of 2 samples, which were both 89.6% (562/627) identical to *M. haemocanis*. The sequence of this strain was similar to that of *Candidatus Mycoplasma turicensis* (DQ464425, DQ464424) with 98.7% (619/627) identity.

A phylogenetic tree of partial 16S rRNA genes for this unknown *Mycoplasma* isolate and other *Mycoplasma* species was constructed using neighbor-jointing analysis in MEGA 5.0 (Tamura *et al.,* 2007) and is shown in Fig. 1. This *Mycoplasma* sp. was the most closely related to *Candidatus Mycoplasma turicensis*, but showed significant phylogenetic distance from *M. haemocanis* and *Candidatus Mycoplasma haematoparvum*, which are commonly recognized as canine pathogens. No dogs were positive for *Hepatozoon* spp. in this study.

Amongst the 68 dogs examined, 42 (61.8%) dogs were positive for one or more of the pathogens. One dog was positive for *B. gibsoni*, *M. haemocanis*, and *Anaplasma* sp. AnHl446. Twelve dogs were positive for *B. gibsoni* and *M. haemocanis*, and one for *B. gibsoni* and *Anaplasma* sp. AnHl446. Twelve dogs were singly positive for *B. gibsoni*, 14 for *M. haemocanis*, and 2 for an unrecognized *Mycoplasma* sp. (Table 2). In this study, vector-borne pathogens were investigated in blood samples of dogs from Bangladesh. The infection rates in dogs with these pathogens, especially with *B. gibsoni* and *M. haemocanis*, were extremely high. Canine babesiosis is caused by a protozoan parasite of the genus *Babesia,* which is distributed worldwide.

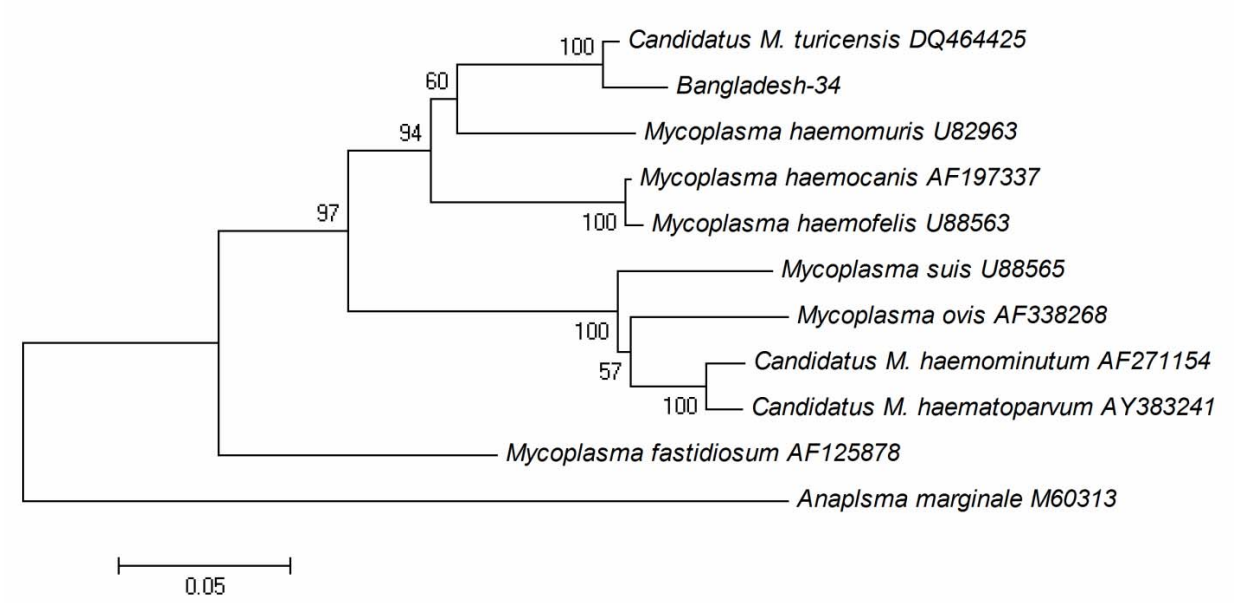

Fig. 1. Phylogenetic relationship between *Mycoplasma* detected in 2 Bangladesh dogs (Bangladesh-34) and other *Mycoplasma* species based on the partial sequences of the 16S rRNA gene. The neighbor-jointing method was used to construct the phylogenetic tree using the MEGA 5 program. The scale bar represents 0.05% divergence. The numbers at the nodes are the proportions of 100 bootstrap re-samplings that support the topology shown.

Table 2. Infection rates of agents in 68 dogs in Bangladesh examined by PCR

PCR and sequencing	Number of dogs (%)
B. gibsoni+ M. haemocanis+ Anaplasma sp. AnHl446	1 (1.5)
B. gibsoni+ Anaplasma spp. AnHl1	1 (1.5)
B. gibsoni+ M. haemocanis	12 (17.7)
B. gibsoni	12 (17.7)
M. haemocanis	14 (20.6)
Unknown *Mycoplasma* sp.	2(3.2)
None detected	26 (38.2)
Total	68 (100)

Two species of *Babesia* have been traditionally identified as the principal cause of canine babesiosis, *Babesia canis*, which is large in size, and the small-sized *Babesia gibsoni* (Boozer and Macintire, 2003). However, with the development of new molecular methods, more piroplasmid species have been found as canine pathogens. These include the small piroplasms *B. gibsoni* (Kjemtrup *et al.,* 2000); *B. conradae* (Kjemtrup *et al.,* 2006); *B. microti*-like piroplasm (also referred to as *Theileira annae* (Camacho, 2006 and Zahker *et al.,* 2000); *Theileria* spp. (Matjila *et al.,* 2008); and the large piroplasms *B. canis*, *B. vogeli*, *B. rossi*, and an unidentified large-sized *Babesia* spp. (Solano-Gallego *et al.,* 2012). In Asia, *B. gibsoni* and *B. vogeli* have been reported. In this study, *B. gibsoni* identical to *B. gibsoni* Asia-2 genotype was found to be dominantly distributed in Bangladesh.

M. haemocanis, formerly classified as a *Haemobartonella* species, has recently been positioned within the genus *Mycoplasma* by 16S rRNA analysis (Messick *et al.,* 2002). This is a small, uncultivable, cell wall-lacking group of bacteria that can infect the red blood cells of mammalian species. In dogs, *M. haemocanis* and *Candidatus Mycoplasma haematoparvum* have been characterized as pathogens (Messick *et al.,* 2002 and Sykeks *et al.,* 2004). Infection with *M. haemocanis*, which is found worldwide, generally induces clinically significant anaemia, particularly in splenectomized or immunocompromised dogs (Inokuma *et al.,* 2006; Novacco *et al.,* 2010 and Ramos *et al.,* 2010). However, in Asia, detailed

epidemiological evidence has not been reported. It is found that *M. haemocanis* infection is dominant and has a high infection rate among dogs in Bangladesh. In addition, an unknown *Mycoplasma* sp. was detected in 2 dogs; this species is most closely related to *Candidatus Mycoplasma turicensis*, a bacterium that has been detected in cats worldwide (Willi *et al.,* 2005 and Willi et al., 2006). In this study, we analyzed only the partial sequences of the 16S rRNA gene of the agent from these 2 dogs. To confirm the results of this study, pathogens from more dogs should be analyzed and compared to longer sequences of the 16S rRNA gene of this agent. Two dogs infected with *Anaplasma* spp. The sequences demonstrated a nucleotide identity of 100% with the 16S rDNA gene of *Anaplasma* sp. AnH1446, which was obtained from *Haemaphysalis lagrangei* ticks collected from a bear in Thailand (Parola *et al.,* 2003). This *Anaplasma* sp. AnHl446 is genetically similar to *A. bovis* (99.6%) and *A. phagocytophilum* (96.5%), and in the phylogenetic tree, this strain was thought to be contained in an *A. bovis* group (Parola *et al.,* 2003). Although Sakamoto *et al*., (2010) has demonstrated *A. bovis* DNA in the peripheral blood of dogs, there are no reports demonstrating that the *Anaplasma* sp. AnHl1446 exhibits pathogenicity against animals or humans. Of the two infected dogs, one was infected with both *B. gibsoni* and *M. haemocanis*. This dog was in poor health. The other dog was infected with *B. gibsoni*, and its only clinical signs was dermatitis. It was not possible to determine whether this *Anaplasma* infection is associated with clinical pathogenicity.

In Bangladesh, more than a dozen of tick species have been recorded in domestic animals. The common species infesting dogs in Bangladesh have been reported as *Boophilus microplus, Amblyomma variegatum*, *Rhipicephalus sanguineus,* and *Haemaphysalis bispinosa* (Ghosh *et al.,* 2007). These ticks can act as a vector for *Babesia, Mycoplasma,* and *Anaplasma* spp. It is possible that this is a cause of concurrent infections.

Conclusion

In conclusion, our molecular study revealed high infection rates of tick-borne diseases in dogs living in Dhaka, Bangladesh. Detailed studies to correctly identify the exact vector tick are required.

References

Boozer A.L. and Macintire, D.K. 2003. Canine babesiosis. *Veterinary Clinics of North America*: *Small Animal Practice,* 33:885–904.

Brown, G.K., Martin, A.R., Roberts, T.K. and Aitken, R.J. 2001. Detection of *Ehrlichia platys* in dogs in Australia. *Australian Veterinary Journal,* 79: 552–553.

Camacho-Garcia, A.T. 2006. Piroplasma infection in dogs in northern Spain. *Veterinary Parasitology,* 138:97–102.

Gal A., Loeb, E., Yisaschar-Mekuzas, Y. and Baneth, G. 2008. Detection of *Ehrilichia canis* by PCR in different tissues obtained from dogs surveyed for naturally occurring canine monocyticehrlichiosis. *Veterinary Journal,* 175:212–217.

Ghosh, S., Bansal, G.C., Gupta, S.C., Ray, D., Khan, M.Q., Irshad H., Shahiduzzaman, M., Seitzer, U. and Ahmed, J.S. 2007. Status of tick distribution in Bangladesh, India and Pakistan. *Parasitology Research,* 101: S207–216.

Inokuma, H., Okuda, M., Ohno, K., Shimoda, K. and Onishi, T. 2002. Analysis of the 18S rRNA gene sequence of a *Hepatozoon* detected in two Japanese dogs. *Veterinary Parasitology,* 106:265–271.

Inokuma, H., Yoshizaki, T., Shimada, Y., Sakata, Y., Okuda, M. andOnishi, T. 2003. Epidemiological survey of *Babesia* species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesiaodocoilei* and *Babesia divergens* DNA. *Journal of Clinical Microbiology,* 41:3494–3498.

Inokuma, H., Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Watanabe, M., Itamoto, K., Okuda, M. and Brouqui, P. 2006. Epidermiological survey of *Ehrlichiacanis* and related species infection in dogs in easetern Sudan. *Annals of the* New York *Academy of Sciences,* 1078:461–463.

James, S.P. 1905. On a parasite found in the white corpuscles of the blood of dogs. *Scientific Mem. Officers Med. & Sanitary Depts. Gov. India,* 14:1–12.

Kjemtrup,A.M., Kocan, A.A., Whitworth,L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K.,Stockham, S.L., Irizarry-Rovira, A. and Conrad, P.A. 2000. There are at least three genetically distinct small piroplasms from dogs. *International Journal for Parasitology,* 30:1501–1505.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L. and Carreno, R.A. 2006. *Babesiaconradae*, sp. Nov. a small canine *Babesia* identified in California. *Veterinary Parasitology,* 31:103–111.

Matjila, P. T., Leisewitz, A. L., Oosthuizen, M. C., Jongejan, F. andPenzhorn, B. L. 2008. Detection of a *Theileria* species in dogs in South Africa. *Veterinary Parasitology,* 157:34–40.

Messick, J.B., Berent, L.M. and Cooper, S.K. 1998. Development and evaluation of a PCR- based assay for detection of *Haemobartonellafelis* in cats and differentiation of *H. felis* from related bacteria by restriction fragment length polymorphsm analysis. *Journal of Clinical Microbiology,* 36:462–466.

Messick J.B., Walker, P.G., Raphael, W., Berent, L. and Shi, X. 2002. '*Candidatus*mycoplasma haemodidelphidis' sp. nov., '*Candidatus* mycoplasma haemolamae' sp. nov. and *Mycoplasma haemocanis*comb. nov., haemotrophic parasites from a naturally infected opossum (*Didelphisvirginiana*), alpaca (*Lama pacos*) and dog (*Canisfamiliaris*): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas. *International Journal of Systematic and Evolutionary Microbiology,* 52:693–698.

Niak, A., Anwar, M. and Khatibi, S. 1973. Canine babesiosis in Iran. *Tropical Animal Health and Production,* 5:200–201.

Novacco, M., Meli, M.L., Gentilini, F., Marsilio, F., Ceci, C., Pennisi, M.G., Lombardo, G., Lloret, A., Santos, L., Carrapiço, T., Willi, B., Wolf, G., Lutz, H. and Hofmann-Lehmann, R. 2010. Prevalence and geographical distribution of canine hemotropic mycoplasma infections in Mediterranean countries and analysis of risk factors for infection. *Veterinary Microbiology,* 19: 276–284.

Patton, W. S. 1910. Preliminary report on a new piroplasm (*Piroplasma gibsoni* sp. nov.) found in the blood of the hounds of the Madras Hunt and subsequently discovered in the blood of the jackal *Canis aureus Bulletin de la Societe de Pathologie Exotique,* 3:274–280.

Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Thien, H.V., Gonzalez, J.P., Raoult, D., Telford, S.R. and Wongsrichanalai, C. 2003. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Mynmar border and Vietm. *Journal of Clinical Microbiology,* 41: 1600–1608.

Ramos, R., Ramos, C., Araujo, F., Oliveira, R., Souza, I., Pimentel, D., Galindo, M., Santana, M., Rosas, E., Faustino, M. and Alves, L. 2010. Molecular survey and genetic characterization of tick-borne pathogens in dogs in metropolitan Recife (north-eastern Brazil). *Parasitology Research,* 107:1115–1120.

Sakamoto, L., Ichikawa, Y., Sakata, Y., Matsumoto, K. and Inokuma, H. 2010. Detection of *Anaplasmabovis* DNA in the peripheral blood of domestic dogs in Japan. *Japanese Journal of Infectious Diseases,* 63:394–352.

Solano-Gallego, L., Baneth, G. Babesiosis in dogs and cats-expanding parasitological and clinical spectra. *Veterinary Parasitology* (In press)

Sykeks, J.E., Bailiff, N.L., Ball, L.M., Foreman, O., George, J.W. and Fry, M.M. 2004. Identification of a novel hemitropic mycoplasma in a splenectmized dog with hemic neoplasia. *Journal of the American Veterinary Medical Association,* 224:1946–1951.

Tamura, K., Dudley, J., Nei, M.& Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution,* 24: 1596–1599.

Willi, B., Boretti, F.S., Cattori, V., Tasker, S., Meli, M.L., Reusch, C., Lutz, H. and Hofmann-Lehmann, R. 2005.Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with haemolytic anemia in Switzerland. *Journal of Clinical Microbiology,* 43:2581–2585.

Willi, B., Tasker, S., Boretti, F.S., Doherr, M.G., Cattori, V., Meli, M.L., Lobetti, R.G., Malik, R., Reusch, C.E., Lutz, H.andHofmann-Lehmann, R. 2006. Phylogenetic analysis of "*Candidatus Mycoplasma turicensis*" isolates from pet cats in the United Kingdom, Australia, and South Africa, with analysis of risk factors for infection. *Journal of Clinical Microbiology,* 44:4430–4435.

Zahker, M., Rinder, H., Schein, E. and Gothe, R. 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. *Veterinary Parasitology,* 89:241–248.