NOTE *Parasitology*

A PCR Based Survey of *Babesia ovata* in Cattle from Various Asian, African and South American Countries

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ABSTRACT. *Babesia ovata* is a tick-transmitted hemoprotozoan parasite that infects cattle. In our study, bovine blood samples (n=2,034) were collected from 10 different countries (Brazil, China, Ghana, Japan, Mongolia, the Philippines, South Africa, Sri Lanka, Thailand and Vietnam) and DNA extracted. The DNA samples were screened using an established and specific polymerase chain reaction (PCR) assay targeting the *Apical membrane antigen 1* (*AMA-1*) gene. Parasite DNA was detected among samples collected from Japan, Mongolia and Thailand. Sequence analyses confirmed that the PCR assay detected only *B. ovata AMA-1*, and that amplicons from different geographical locations were conserved. Our findings highlight the importance of designing adequate strategies to control *B. ovata* infection in Japan, Mongolia, and Thailand.

KEY WORDS: AMA-1, *Babesia ovata,* cattle, Mongolia, Thailand.

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Babesia ovata is an intraerythrocytic protozoan parasite of cattle and is transmitted by *Haemaphysalis longicornis*, an ixodid tick vector [10]. *B. ovata* was first described in Japan [10], then China and Korea [4, 15]. It has been classified into a large group of *Babesia* parasites [10]. The pathogen is known to be a parasite of low pathogenicity in comparison with *B. bovis* and *B. bigemina*. However, previous reports described the clinical significance of *B. ovata* in immunocompromised animals such as splenectomized calves or in *Theileria orientalis* infected cattle [6, 14].

Our previous study suggested the possible presence of *B. ovata* in Mongolia, a country where the parasite has not been previously reported [11]. Therefore, it is important to accurately detect *B. ovata* in cattle populations and determine its prevalence in different regions across the world.

Until recently, the only available technique for the detection of *B. ovata* was microscopy. However, parasitemia of *B. ovata* in infected cattle tends to be very low making microscopy difficult [3]. Furthermore, as the morphology among many large *Babesia* parasites is similar, microscopy is not an effective diagnostic tool for species differentiation [5]. Therefore, we developed a PCR assay based on the *Apical membrane antigen 1* (*AMA-1*) gene for the specific detection of *B. ovata* in blood samples of grazing cattle in the Japanese field [14]. More blood samples collected from different geographical locations throughout the world should be tested to validate this assay as a universal diagnostic tool for the molecular detection of *B. ovata*.

In this study, we examined 2,034 bovine DNA samples originating from various countries across Asia, Africa and South America using the AMA-1 PCR assay. Blood samples were collected from cattle populations of Japan [16], Mongolia [1], China [12], Vietnam [9], Thailand [2], the Philippines, Sri Lanka [13], Ghana, South Africa and Brazil. In each country, samples were collected across several selected locations. All animals from which samples were collected appeared clinically normal and were over one year of age. Details regarding sample numbers from each location are summarized in Table 1.

The AMA-1 PCR assay was conducted according to our

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Table 1. Summary of the PCR detection of *B. ovata*

Country	Area	No. of samples	Sampling period	No. of positive (%)
Japan	Otofuke	57	July, 2009	4 (7)
	Shibecha	87	June, 2011	0
Mongolia	Dadal	45	May, 2010	0
	Undurkhan	18		1(5.6)
	Binder	46		4 (8.7)
	Jargaltkhan	62		2 (3.2)
	Ulaangom	49		0
	Bogd	38		0
	Tsenkher Mandal	44		0
China	Fujian	51	October, 2009	0
Vietnam	Hue	79	February, 2010	0
	Bavi	105	July, 2011	0
Thailand	Chiang Rai	96	September, 2009	3 (3.1)
	Lampang	50		2 (4)
	Chiang Mai	54		0
Philippines	Cebu	300	April, 2012	0
Sri Lanka	Nuwara-Eliya	83	May - June, 2011	0
	Polonnaruwa	84		0
	Ampara	88		0
	Jaffna	61		0
Ghana	Accra	255	July, 2008	0
South Africa	Limpopo	100	April, 2008	0
	Gauteng	18		0
Brazil	Bahia state	164	March, 2009	0

The highly specific AMA-1 PCR assay was conducted on DNA extracted from bovine blood samples.

previous report [15]. Briefly, 1 μl of DNA template (approximately 6 $\mu g/\mu l$) was added to 200 μM dNTPs (Applied Biosystems, Branchburg, NJ, U.S.A.), 1 μl of 10× PCR buffer, 0.5 μM forward (5’-GATACGAGGCTGTCGGTAGC-3’) and reverse (5’-AGTATAGGTGAGCATCAGTG-3’) primers [14], 0.5 U of Taq polymerase (Applied Biosystems) and 5.9 μl of double-distilled water, and then subjected to cycling conditions [14]. Following gel electrophoresis, detection of a 504 bp amplicon under ultraviolet light was considered positive for *B. ovata*.

Of the 10 countries surveyed in this study, positive results were obtained from Japanese (2.8%), Mongolian (2.3%) and Thai (2.5%) samples (Table 1). The prevalence of *B. ovata* in Japan was lower than previously reported (24.5% at Shin-Hidaka farm, Japan) [14]. This observed difference might be due to distribution of the possible vectors in surveyed areas, as our previous study suggested the *Haemaphysalis megaspinosa*, which was not detected in Otofuke, as the major tick species in Shin-Hidaka [16]. Additionally, *B. ovata* was not detected in Chinese samples in this study, despite the previous report of the parasite in this country [4]. This would suggest the importance of surveying adequate sample numbers or locations. In contrast to other bovine hemoprotozoan parasites, the prevalence of *B. ovata* was lower. *B. ovata* maintains a low parasitemia in infected cattle so that immune responses are kept at a minimum level, thereby ensuring survival of the parasite [3]. Therefore, there is the possibility that bovine DNA samples might contain a very low concentration of *B. ovata* DNA, which cannot be detected by PCR. Identification of ticks that can transmit *B. ovata* and determination of their densities at each location surveyed in this study might explain the differences among countries in the distribution of *B. ovata*. Although the DNA samples from several countries in this study were negative according to the AMA-1 PCR assay, the presence of *B. ovata* cannot be ruled out in the cattle populations of these countries, unless several other locations in each country are surveyed for the parasite.

All PCR amplicons were cloned and sequenced as described previously [1]. Briefly, amplicons were ligated into a plasmid vector (pCR 2.1-TOPO; Invitrogen, Carlsbad, CA, U.S.A.), and the nucleotide sequences of inserted fragments were determined by a genetic analyzer (ABI PRISM 3100; Applied Biosystems, Foster City, CA, U. S. A.). Sequence analyses confirmed that the AMA-1 PCR assay amplified only the *B. ovata AMA-1* gene. All sequences (GenBank accession numbers AB703297, AB703298, and AB733631) were identical to each other and to the previously reported Miyake strain of *B. ovata*(AB634843) [7, 11].

B. ovata AMA-1 sequences determined in the present study, together with already registered *AMA-1* sequences for *B. ovata, B. bovis*, *B. bigemina*, *B. divergens*, *B. gibsoni, T. annulata*, *T. parva*, *Plasmodium falciparum*, *P. vivax*, *P. chabaudi, P. berghei, Toxoplasma gondii* and *Neospora caninum,* were analyzed using GENTYX 7.0 software (GENTYX, Tokyo, Japan). A phylogenetic tree was constructed using the online MAFFT software program [8]. Estimation of bootstrap values was also performed using the same program. In the phylogenetic tree (Fig. 1), *B. ovata*

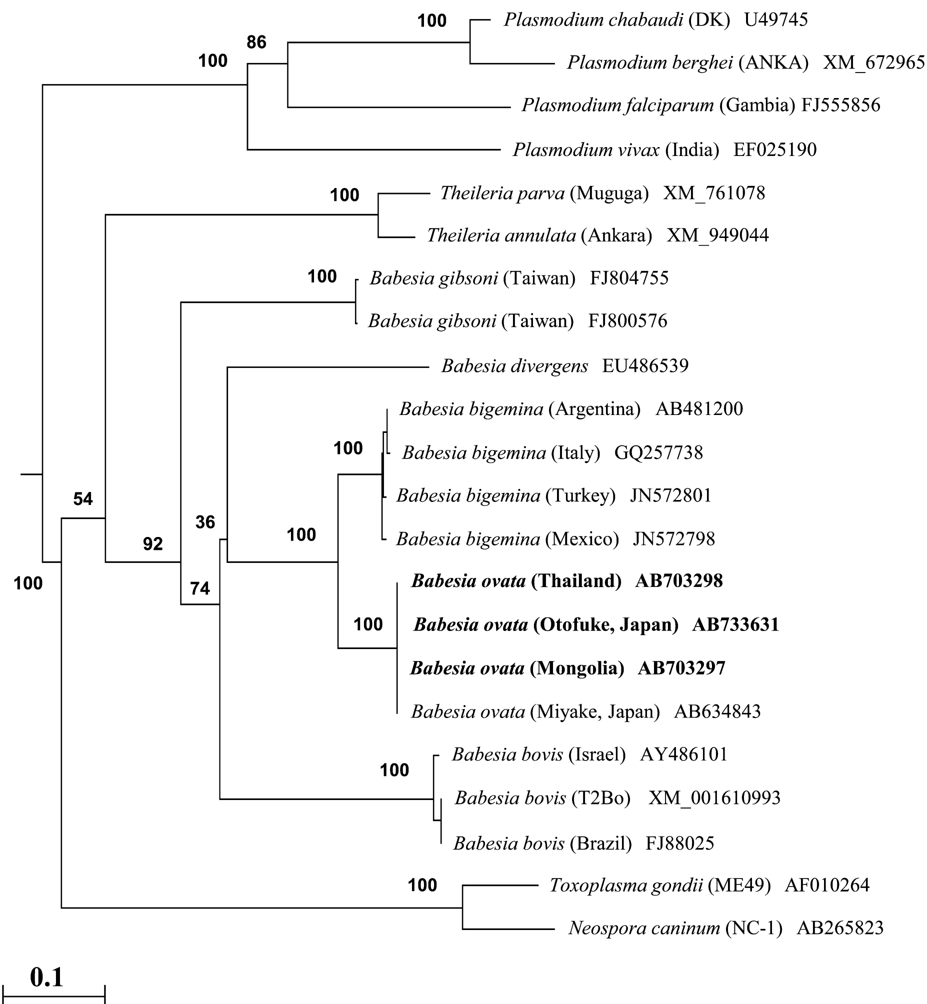

Fig. 1. Phylogenetic analysis of *AMA-1* sequences of Apicomplexan parasites. Boldface letters indicate sequences determined in the present study. Bootstrap values are provided at the beginning of each branch. Note that *B. ovata AMA-1* sequences form a separate clade close to those in *B. bigemina*.

AMA-1 sequences formed a separate cluster close to *AMA-1* of *B. bigemina,* a large *Babesia* parasite that morphologically resembles *B. ovata*. The conserved nature of *B. ovata AMA-1* combined with the high specificity of the AMA-1 PCR assay [14], makes it an excellent molecular diagnostic tool for the detection of *B. ovata*.

Although *B. ovata* was first described a number of decades ago [10], limited research has been conducted to date. As the pathogen is a relatively benign parasite circulating within limited geographical boundaries, extensive research on *B. ovata* has not been carried out. Additionally, unavailability of species-specific diagnostic methods with high sensitivity was another stumbling block affecting *B. ovata* studies. In this study, we determined that our PCR assay did not detect any other blood pathogen from more than 2,000 bovine DNA samples taken from geographically distinct areas. Therefore, we conclude that our standardized AMA-1 PCR assay is an appropriate and specific diagnostic tool that can be applied to identify *B. ovata* in cattle. In addition, the PCR method may also be useful to differentiate the *B. ovata* from other virulent piroplasms that might morphologically resemble the parasite.

We also report the presence of *B. ovata* in Mongolia and Thailand for the first time. The next step is to isolate and characterize Mongolian and Thai *B. ovata* to determine its pathology. Furthermore, identification of the tick vectors capable of transmitting *B. ovata* to cattle is vital, as infection control methods should also include tick control strategies.

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REFERENCES

1. Altangerel, K., Sivakumar, T., Battsetseg, B., Battur, B., Ueno, A., Igarashi, I. and Yokoyama, N. 2012. Phylogenetic relationships of Mongolian isolates of *Babesia bovis* based on the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c genes. *Vet. Parasitol.* **184**: 309–316. [Medline] [CrossRef]
2. Altangerel, K., Sivakumar, T., Inpankaew, T., Jittapalapong, S., Terkawi, M. A., Ueno, A., Xuan, X., Igarashi, I. and Yokoyama, N. 2011. Molecular prevalence of different genotypes of *Theileria orientalis* detected from cattle and water buffaloes in Thailand. *J. Parasitol.* **97**: 1075–1079. [Medline] [CrossRef]
3. Arai, S., Tsuji, M., Kim, S. J., Nakada, K., Kirisawa, R., Ohta, M. and Ishihara, C. 1998. Antigenic and genetic diversities of *Babesia ovata* in persistently infected cattle. *J. Vet. Med. Sci.* **60**: 1321–1327. [Medline] [CrossRef]
4. Bai, Q., Liu, G., Zhang, L. and Zhou, J. 1990. Studies on the isolation and preservation of a single species of bovine haematocytozoon: the finding and isolation of *Babesia ovata* in China. *Chin. J. Vet. Med.* **16**: 2–4.
5. Böse, R., Jorgensen, W. K., Dalgliesh, R. J., Friedhoff, K. T. and De Vos, A. J. 1995. Current state and future trends in the diagnosis of babesiosis. *Vet. Parasitol.* **57**: 61–74. [Medline] [CrossRef]
6. Fujinaga, T. 1981. Bovine babesiosis in Japan: clinical and clinico-pathological studies on cattle experimentally infected with *Babesia ovata*. *Nippon Juigaku Zasshi* **43**: 803–813. [Medline] [CrossRef]
7. Igarashi, I., Avarzed, A., Tanaka, T., Inoue, N., Ito, M., Omata, Y., Saito, A. and Suzuki, N. 1994. Continuous *in vitro* cultivation of *Babesia ovata*. *J. Protozool. Res.* **4**: 111–118.
8. Katoh, K., Misawa, K., Kuma, K. and Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **30**: 3059–3066. [Medline] [CrossRef]
9. Khukhuu, A., Lan, D. T., Long, P. T., Ueno, A., Li, Y., Luo, Y., Macedo, A. C., Matsumoto, K., Inokuma, H., Kawazu, S., Igarashi, I., Xuan, X. and Yokoyama, N. 2011. Molecular epidemiological survey of *Theileria orientalis* in Thua Thien Hue. *J. Vet. Med. Sci.* **73**: 701–705. [Medline] [CrossRef]
10. Minami, T. and Ishihara, T. 1980. *Babesia ovata* sp. n. isolated from cattle in Japan. *Nat. Inst. Anim. Health Q. (Tokyo)* **20**: 101–113. [Medline]
11. Sivakumar, T., Altangerel, K., Battsetseg, B., Battur, B., Aboulaila, M., Munkhjargal, T., Yoshinari, T., Yokoyama, N. and Igarashi, I. 2012. Genetic detection of *Babesia bigemina* from Mongolian cattle using *apical membrane antigen-1* gene based PCR assay. *Vet. Parasitol.* **187**: 17–22. [Medline] [CrossRef]
12. Sivakumar, T., Altangerel, K., Igarashi, I., Xuan, X., Guo, G., Huang, X. and Yokoyama, N. 2011. Phylogenetic analysis of *Theileria orientalis* in cattle bred in Fujian province, China. *J. Protozool. Res.* **21**: 14–19.
13. Sivakumar, T., Kothalawala, H., Abeyratne, S. A., Vimalakumar, S. C., Meewewa, A. S., Hadirampela, D. T., Puvirajan, T., Sukumar, S., Kuleswarakumar, K., Chandrasiri, A. D., Igarashi, I. and Yokoyama, N. A PCR-based survey of selected *Babesia* and *Theileria* parasites in cattle in Sri Lanka. *Vet. Parasitol.* (in press). [Medline]
14. Sivakumar, T., Tagawa, M., Yoshinari, T., Ybanez, A. P., Igarashi, I., Ikehara, Y., Hata, H., Kondo, S., Matsumoto, K., Inokuma, H. and Yokoyama, N. 2012. PCR detection of *Babesia ovata* from cattle reared in Japan and clinical significance of coinfection with *Theileria orientalis*. *J. Clin. Microbiol.* **50**: 2111–2113. [Medline] [CrossRef]
15. Suh, M. D. 1987. Pure isolation and identification of Babesia ovata by morphological characteristics and complement fixation test in Korea. Korean. *J. Vet. Res.* **27**: 307–316.
16. Yokoyama, N., Sivakumar, T., Ota, N., Igarashi, I., Nakamura, Y., Yamashina, H., Matsui, S., Fukumoto, N., Hata, H., Kondo, S., Oshiro, M., Zakimi, S., Kuroda, Y., Kojima, N., Matsumoto, M. and Inokuma, H. 2012. Genetic diversity of *Theileria orientalis* in tick vectors detected in Hokkaido and Okinawa, Japan. *Infect. Genet. Evol.* **12**:1669–1675. [Medline]