PCR detection of *Babesia ovata* from questing ticks in Japan

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Abstract

Babesia ovata is a tick-transmitted hemoprotozoan parasite of cattle. In the present study, we analyzed tick DNA samples (n=1459) prepared from questing ticks collected from various cattle pastures in Hokkaido (Shibecha, Taiki, Otofuke, Memuro, and Shin-Hidaka districts) and Okinawa (Yonaguni Island) prefectures of Japan for *B. ovata*. When all the tick DNA samples were screened by a previously described *B. ovata*-specific apical membrane antigen-1 (AMA-1) gene-based polymerase chain reaction (PCR) assay, none of the DNA samples was positive. Therefore, we developed a PCR assay based on the protozoan *beta-tubulin* (β -*tubulin*) gene to detect *B. ovata* from ticks in Japan. In the specificity test, the PCR assay amplified the expected 444-bp target gene fragment from *B. ovata* DNA. No PCR products were amplified from DNA samples from other blood pathogens, bovine blood, or ticks. In addition, the PCR assay detected 100 fg of *B. ovata*-genomic DNA extracted from an in vitro culture of the parasites. Subsequently, when all the tick DNA samples were screened by this new PCR assay, 18 were positive for *B. ovata*. Positive samples were found only in the Yonaguni and Memuro areas. In Okinawa, where all the ticks were identified as *Haemaphysalis longicornis*, 9.7% of the samples were PCR-positive, while a single tick (*Ixodes ovatus*) from Memuro was infected with *B. ovata*. When the nucleotide sequences of the PCR amplicons were phylogenetically analyzed, they formed a separate clade containing a previously described β -*tubulin* gene sequence from *B. ovata* (Miyake strain), confirming that the PCR assay had detected only *B. ovata* from the tick DNA samples. This is the first report that describes the PCR detection of *B. ovata* in ticks. The findings warrant transmission experiments to evaluate *I. ovatus* as a potential vector of *B. ovata*.

Keywords: *Babesia ovata*; Beta-tubulin; Japan; PCR; Ticks

Introduction

Babesia ovata is a tick-borne hemoprotozoan parasite belonging to the genus *Babesia* (order Piroplasmida, phylum Apicomplexa) and is transmitted by the ixodid tick *Haemaphysalis longicornis* (Minami and Ishihara, 1980). *Babesia ovata*, a large-sized *Babesia* species, has a lower pathogenicity in cattle than the more virulent *B. bovis* and *B. bigemina* (Fujinaga, 1981). However, the infection can result in severe anemia in immunocompromised or *Theileria orientalis*-infected cattle (Fujinaga, 1981, 1982; Sivakumar et al., 2012c). Previous studies have shown the presence of *B. ovata* in cattle populations of Asia, including Japan, China, Korea, Mongolia, and Thailand (Minami and Ishihara, 1980; Bai et al., 1990; Suh,

1987; Yoshinari et al., 2013). Notably, *B. ovata* endemicity in Japan was reconfirmed in recent polymerase chain reaction (PCR)-based surveys (Sivakumar et al., 2012c; Yoshinari et al., 2013).

Several studies on *B. ovata* transmission have been conducted in the past. These have highlighted the several developmental stages of the *B. ovata* life cycle, such as macrogametes, microgametes, zygotes, kinetes, and sporozoites, in its tick host *H. longicornis* (Higuchi et al., 1987, 1989, 1994). Additionally, in an animal experiment, transovarially transmitted parasites in *H. longicornis* were able to infect cattle when the *B. ovata*-infected larval-, nymphal-, and adult-stage ticks fed on them (Bai et al., 1992). Although cattle populations infected with *B. ovata* were detected in eastern Hokkaido of Japan (Sivakumar et al., 2012c; Yoshinari et al., 2013), the absence of *H. longicornis* suggests that there might be other tick vectors that can transmit *B. ovata* in this region (Inokuma et al., 2007; Yoshimoto et al., 2010).

Recently, a diagnostic PCR assay based on the *apical membrane antigen-1* (*AMA-1*) gene (*AMA-1* PCR assay) was developed to detect *B. ovata* in cattle (Sivakumar et al., 2012c) because microscopy lacks the specificity and sensitivity needed for detecting this parasite in cattle (Böse et al., 1995). The *AMA-1* PCR assay was subsequently used to identify *B. ovata* in several countries where the parasite had not been reported previously (Yoshinari et al., 2013). However, PCR detection of *B. ovata* in ticks has not yet been described. In the present study, *AMA-1* PCR was initially employed to screen tick populations of different geographical locations in Japan for the presence of *B. ovata*. However, none of the DNA samples prepared from these ticks was positive for *B. ovata*, which suggested the importance of developing an alternative PCR assay that can detect this parasite in tick vectors.

Beta-tubulin (β -tubulin) is a component of microtubules, which are major components of the cytoskeleton in eukaryotic cells. This component plays important roles in maintaining the shape of the parasite and in host cell penetration (Morrisette, 2002). Although the β -tubulin gene sequences of apicomplexan parasites are highly conserved, several differences have been observed in their sequence lengths and nucleotide compositions among different hemoprotozoan parasite species (Caccio et al., 2000). In the present study, we developed a *B. ovata*-specific PCR assay based on the *β -tubulin* gene (β -tubulin PCR assay) and used it to survey tick populations in Japan for *B. ovata*, and the utility of this β -tubulin PCR assay is described herein.

Materials and methods

Tick collection and DNA extraction

Questing ticks were collected from ground level grasses in and around cattle farms after the end of the winter season, mainly before the commencement of grazing. Ticks collected from Hokkaido [Taiki, Otofuke, Shin-Hidaka (Yokoyama et al., 2012), Memuro, and Shibecha districts] and Okinawa [Yonaguni Island (Yokoyama et al., 2012)] prefectures were subjected to DNA extraction, following species identification of each tick, as described previously (Yokoyama et al., 2012). Briefly, individual ticks were digested with the extraction buffer provided with Isohair kit (Nippon Gene Co., Tokyo, Japan), and the DNA samples were extracted using phenol:chloroform method. After ethanol precipitation, DNA samples were dissolved in 100 μ L of double distilled water (DDW) and stored at -30°C until further use. The species and numbers of ticks collected from each sampling location are summarized in Table 1.

DNA extraction from cultured *B. ovata*

Genomic DNA from *B. ovata* was extracted as described previously (Sivakumar et al., 2012a). Briefly, a commercial DNA extraction kit (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany) was employed to extract DNA from in vitro culture-derived *B. ovata*-infected erythrocytes according to the manufacturer's instructions. After DNA concentration measurement (Nanodrop, Wilmington, DE, USA), the extracted samples were stored at -20°C until use.

AMA-1 PCR assay

All the tick DNA samples were initially screened using the previously described AMA-1 PCR assay (Sivakumar et al., 2012c). Briefly, 1 μ L of DNA sample was added to a 24- μ L reaction mixture that contained 2.5 μ L of $10 \times$ PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 2.5 μ L of 200 mM dNTPs (Applied Biosystems), 2 μ L of a 10- μ M solution of each forward (5'-GATACGAGGCTGTCGGTAGC-3') and reverse (5'-AGTATAGGTGAGCATCAGTG-3') primer, 1 unit (0.2 μ L) of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), and 14.8 μ L of DDW. After an initial enzyme activation step at 95°C for 5 min, reaction mixtures were subjected to 45 cycles each of which included a denaturing step 95°C for 30 s, an annealing step at 56°C for 1 min, and an extension step at 72°C for 1 min. The final elongation step at 72°C for 10 min was followed by gel electrophoresis and ethidium bromide staining. Detection of a band close to 504 bp under ultraviolet illumination was considered positive.

β-tubulin PCR assay

β-tubulin gene sequences of bovine hemoprotozoan parasites (*B. ovata*, AB634844; *B. bigemina*, AB634846, DQ104522, and EF060267; *B. bovis*, L00978; *Theileria annulata*, XM_947161; *T. parva*, XM_759937), *Haemaphysalis* spp. ticks (AB642157), and *Bos taurus* (BC118413) were retrieved from GenBank. After multiple alignments of these gene sequences, a set of forward (5'-ACACTGTGCATCCTCACCGTCATAT-3') and reverse (5'-CTCGCGGATCTTGCTGATCAGCAGA-3') primers specific to *B. ovata* was designed for a new PCR assay. One microliter of each DNA sample was added to 19 μL of PCR reaction mixture containing 2 μL of 200 mM dNTPs (Applied Biosystems, Foster City, CA, USA), 2 μL of 10× buffer (Applied Biosystems), 2 μL of a 10-μM solution of each forward and reverse primer, 1 unit (0.2 μL) of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), and 10.8 μL of distilled water. The reaction mixtures were then subjected to an initial enzyme activation step at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. After final extension at 72°C for 10 min, the PCR products were detected by ultraviolet illumination after agarose gel electrophoresis and ethidium bromide staining. Detection of a band close to 444 bp was considered positive.

Specificity and sensitivity tests

The specificity of the β-tubulin PCR assay was assessed using a panel of DNA samples from *B. ovata* (Miyake strain), *B. bigemina* (Argentine strain), *B. bovis* (Texas strain), *Theileria annulata* (DNA sample was extracted from naturally infected cattle in Sri Lanka) (Sivakumar et al., 2012b), *T. orientalis* (Ikeda type), *Trypanosoma evansi* (Tansui strain), *Trypanosoma brucei gambiense* (Strain IL1922), *Trypanosoma theileri* (isolated from naturally infected cattle in Japan) (Sivakumar et al., 2012c), *Anaplasma marginale* (isolated from naturally infected cattle in the Philippines) (Ybañez et al., 2013), *A. bovis* (DNA sample was extracted from naturally infected cattle in Japan) (Jilintai et al., 2009), *A. centrale* (Aomori strain), bovine blood (*Bos taurus*), and ticks (*H. longicornis*). For the sensitivity analysis, 10 ng/μL of *B. ovata*-genomic DNA was 10-fold serially diluted and then subjected to the PCR assay. In addition, the sensitivities of the AMA-1 and β-tubulin PCR assays were determined using normal (*B. ovata*-uninfected) tick DNA samples (82 ng/μL), which were spiked with known concentrations of *B. ovata*-genomic DNA extracted as described above.

DNA cloning and sequencing

Cloning and sequencing of the PCR amplicons were carried out as described previously (Sivakumar et al., 2012a). Briefly, after gel electrophoresis, PCR products with high band intensities were extracted using a QIAquick Gel Extraction Kit (Qiagen). The extracted DNA was ligated into a PCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA, USA), and the nucleotide sequences of the inserted DNA fragments were determined using an ABI PRISM3100 genetic analyzer (Applied Biosystems). For each positive DNA sample, both positive and negative strands of 2 clones were sequenced.

Phylogenetic analysis

The nucleotide sequences obtained were initially analyzed using a basic local alignment search tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Subsequently, the sequences generated herein, together with those of other hemoprotozoan parasites available in GenBank, were used to construct a phylogenetic tree using the MAFFT program (Katoh et al., 2002) based on a neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor substitution model (Jukes and Cantor, 1969).

Results

The species of ticks collected from Taiki, Otofuke, Shin-Hidaka, and Yonaguni areas have been described previously (Yokoyama et al., 2012). Ticks collected from the Shibeche district included *Ixodes persulcatus*, *I. ovatus*, and *H. douglasi*, while those from the Memuro district were identified as *I. persulcatus* and *I. ovatus* (Table 1).

Initially, all the tick DNA samples were screened using the AMA-1 PCR assay that had been previously established (Sivakumar et al., 2012c). However, none of the tick samples was positive for *B. ovata* (Table 1). Therefore, a new PCR assay based on the *B. ovata* β -tubulin gene was developed to detect this parasite from ticks in Japan. The previously described *B. ovata* β -tubulin gene sequence (AB634844) shared 90.6% identity with the corresponding sequence from *B. bigemina* (AB634846). Therefore, special care was taken to validate the sequence differences in the primer-binding regions for *B. ovata* and *B. bigemina*, especially those at the 3' end (data not shown). The β -tubulin PCR assay was shown to be highly specific for detection of *B. ovata*: No amplicon was observed when individual DNA samples extracted from 10 other bovine blood pathogens, bovine blood, and ticks were included as PCR templates (Fig. 1A). In the sensitivity test, the PCR assay was able to detect 100 fg of *B. ovata* DNA (Fig. 1B). In addition, when the tick DNA samples were spiked with known amounts of *B. ovata* DNA and then subjected to the β -tubulin and AMA-1 PCR assays, the

sensitivities of the individual assays were both 10 pg (Fig. 2). However, the intensities of the gene-specific bands were higher for the β -tubulin PCR assay (Fig. 2A) than those of the AMA-1 PCR assay (Fig. 2B).

Among the tick populations that were collected from the 6 sampling locations in Japan, only those from Yonaguni Island and the Memuro district were positive for *B. ovata* infection in the β -tubulin PCR assay (Table 1). In Yonaguni Island, where all the collected ticks were identified as *H. longicornis*, 17 (9.7%) of the 175 ticks were positive for *B. ovata*. In contrast, for the Memuro district, only one DNA sample (*I. ovatus*) was *B. ovata*-positive (0.2%, Table 1). Out of 18 PCR amplicons, 15 were successfully sequenced (GenBank accession numbers: AB845492–AB845506). Although 2 clones were sequenced for each positive sample, the sequences of both clones were identical for all the samples analyzed. The length of the gene sequences were in the range of 441–448 bp, while the sequence identity values among these gene sequences were 94.8–100%. A previously described β -tubulin gene sequence from *B. ovata* (Miyake strain; AB634844) shared 97.1–100% identity with the β -tubulin gene sequences obtained in the present study. In addition, the β -tubulin gene sequences from *B. bigemina* (AB634846), *B. odocoilei* (AY144706), and *B. divergens* (AY144703) shared 87.0–90.6%, 83.7–86.3%, and 82.7–85.4% sequence identity, respectively, with the *B. ovata* β -tubulin gene sequences determined in the present study. In the phylogenetic analysis of the β -tubulin genes, the gene sequences obtained herein, including that from *I. ovatus* (AB845506), comprised a single clade containing a previously described *B. ovata* β -tubulin gene sequence (AB634844), while the *B. bigemina* sequences clustered as a separate clade close to that of *B. ovata* (Fig. 3). The clades were supported by high bootstrap values.

Discussion

Babesia ovata, a benign hemoprotozoan parasite of cattle, is still endemic in Japan. Past studies have suggested that this pathogen might affect the health of infected cattle under certain circumstances (Fujinaga, 1981; Sivakumar et al., 2012c). Previously, a PCR assay based on the AMA-1 gene was developed to detect *B. ovata* infection in cattle (Sivakumar et al., 2012c). Initially, we used the AMA-1 PCR assay to screen all the tick DNA samples used in the present study, but none of them was positive for *B. ovata* infection. Therefore, we developed a new PCR assay based on the β -tubulin gene, and resurveyed the questing ticks for the presence of *B. ovata* infection.

Specificity and sensitivity of the β -tubulin PCR assay were comparable to those of the previously developed AMA-1 PCR assay. The β -tubulin PCR assay was also shown to be highly specific, in that it only amplified *B. ovata* DNA and not the 10 other blood pathogens of cattle that were tested. The sensitivities of the AMA-1 and β -tubulin PCR assays were identical (100 fg) (Sivakumar et al., 2012c), when pure *B. ovata* DNA was used. In contrast, both PCR assays experienced 100-fold lower sensitivities with the tick DNA samples that were spiked with *B. ovata* DNA. However, the sensitivities of the PCR assays remained the same (100 fg) when the bovine DNA samples that had been spiked with *B. ovata* DNA were tested (data not shown). This suggests that PCR inhibitors were possibly present in the tick DNA samples, which is in agreement with a previous study (Kim et al., 2013). Furthermore, agarose gel analysis revealed that the intensities of the *B. ovata*-positive bands were higher for the β -tubulin PCR assay than for the AMA-1 PCR assay. This suggests that the DNA amplification efficiency of the AMA-1 PCR assay with the tick DNA samples was lower than that of the β -tubulin PCR assay. Higher levels of non-specific binding of the PCR primers to the tick DNA could be one explanation for the lower amplification efficiency of the AMA-1 PCR assay, compared with that of the β -tubulin PCR assay. This might explain why the AMA-1 PCR assay was unable to detect *B. ovata* in the tick DNA samples sourced from the Japanese pastures.

Hence, the tick DNA samples were rescreened using the β -tubulin PCR assay. Detection of *B. ovata* from *H. longicornis* in Okinawa is not surprising, as this tick species is a well-known vector of *B. ovata* (Minami and Ishihara, 1980). In addition, although there are no published data, *B. ovata* is common among cattle in Okinawa according to the local veterinarians. However, in the Memuro district, we found a single *I. ovatus* DNA sample that was positive for *B. ovata*. This is the first report of the possibility that a non-*H. longicornis* tick species could be a vector for this parasite. However, detection of *B. ovata* in tick-DNA does not necessarily mean that the tick can be a vector of this parasite. Therefore, further studies to demonstrate the infective stages of *B. ovata* in the salivary glands of *I. ovatus* are essential. Furthermore, transmission experiments using *B. ovata*-infected *I. ovatus* ticks are also equally important to confirm this tick species as a potential vector of *B. ovata*.

Phylogenetic analysis using the β -tubulin gene sequences determined in the present study confirmed the specificity of the β -tubulin PCR assay, because the *B. ovata* β -tubulin gene sequences were found in a single clade containing a known *B. ovata* β -tubulin gene sequence. In addition, the clade formed by the *B. ovata* sequences was clearly separated from that of the *B. bigemina* sequences. This finding is particularly important because *B. ovata* resembles *B.*

bigemina both morphologically and genetically (Kawai et al., 1986; Luo et al., 2005).

Therefore, the PCR assay we developed has practical utility for epidemiological surveys of *B. ovata* in ticks. However, the β -tubulin PCR assay would benefit from further evaluation using tick DNA samples sourced from other countries where other *Babesia* parasites such as *B. ovata* and *B. bigemina* are endemic, as *B. ovata* is the only *Babesia* parasite of cattle in Japan. *Babesia ovata* was previously detected in the cattle populations of Otofuke and Shin-Hidaka districts of the Hokkaido prefecture (Sivakumar et al., 2012c; Yoshinari et al., 2013).

However, none of the tick DNA samples collected in these areas was positive for this parasite in the present study. *Babesia ovata* is thought to be transmitted by each of the 3 tick stages comprising the larva, nymph, and adult, which when infected with transovarially transmitted *B. ovata* can transmit the parasite to cattle (Bai et al., 1992). However, there are no reports detailing the parasite load for each of the tick stages. The present study examined only the nymphal and adult stages of the ticks for the presence of *B. ovata*. Therefore, in addition to surveying the larval stage, it is very important to determine the parasite loads in the *B. ovata*-infected larva and the resultant nymphal and adult stages to clarify whether the parasite load is higher in the larva than in the nymph and adult. Of interest, the ticks tested in the present study were collected in April–June (spring) from the sampling locations. However, unlike the tick vectors for *T. orientalis* (Yokoyama et al., 2012), the tick species that transmit *B. ovata* in the Hokkaido region might be active during different seasons of the year. Collection of questing ticks during different seasons of the year and PCR screening of their DNAs could shed additional light on the vectors of *B. ovata* in Hokkaido prefecture. Nevertheless, detection of the vectors of *B. ovata* is important for implementing a systematic tick control strategy based on the seasonal activities of the vectors.

In summary, the present study described the development of a new *B. ovata*-specific PCR assay and the first PCR screening of ticks for *B. ovata*. Our findings warrant further studies to confirm the usefulness of this PCR assay for detecting *B. ovata* in ticks. Additionally, transmission experiments should be conducted to confirm whether the *B. ovata*-infected *I. ovatus* ticks can transmit the parasite to cattle.

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Legends of figures

Fig. 1. Specificity and sensitivity of the β -tubulin PCR assay. The specificity of the PCR assay was tested using DNA samples from 11 bovine hemoparasites (panel A). Lanes 1–13 represent DNA samples from *Babesia ovata*, *B. bigemina*, *B. bovis*, *Theileria annulata*, *T. orientalis*, *Trypanosoma evansi*, *Trypanosoma brucei gambiense*, *Trypanosoma theileri*, *Anaplasma marginale*, *A. bovis*, *A. centrale*, bovine blood, and ticks, respectively. Lane 14: No-template control. PCR assay sensitivity was evaluated using *B. ovata* genomic DNA (panel B). Lanes 1–8 represent 10-fold serial dilutions (10 ng/ μ L to 1 fg/ μ L, respectively) of in vitro culture-derived *B. ovata* genomic DNA. Lane 9: No-template control. M (both panels): 100-bp DNA ladder marker.

Fig. 2. Sensitivities of *B. ovata*-specific PCR assays in the presence of tick DNA. Tick DNA samples were spiked with *B. ovata* DNA sourced from in vitro cultures and then subjected to β -tubulin (panel A) and AMA-1 (panel B) PCR assays to evaluate their sensitivities. The final concentrations of *B. ovata* DNA were adjusted to 10 ng/ μ L (lane 1), 1 ng/ μ L (lane 2), 100 pg/ μ L (lane 3), 10 pg/ μ L (lane 4), 1 pg/ μ L (lane 5), 100 fg/ μ L (lane 6), and 10 fg/ μ L (lane 7). Lane 8: Uninfected tick DNA. Lane 9: No-template control. M: 100-bp ladder marker.

Fig. 3. Phylogenetic analysis of *B. ovata* β -tubulin gene sequences. The phylogram was constructed from *B. ovata* β -tubulin gene sequences and those derived from other *Babesia* and *Theileria* parasite species based on a neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor substitution model (Jukes and Cantor, 1969). The gene sequences determined in the present study are shown in bold. Bootstrap values are provided at the beginning of each branch. Note that the *B. ovata* β -tubulin gene sequences form a separate clade.

Fig. 1

Fig. 2

Fig. 3

Table 1. Collection, identification, and PCR screening of questing ticks in Hokkaido and Okinawa prefectures (Japan).

Prefecture	District	Date (year/month)	Tick species					Total	Number of PCR-positive samples (%)	
			<i>Ixodes persulcatus</i>	<i>Ixodes ovatus</i>	<i>Haemaphysalis megaspinosa</i>	<i>Haemaphysalis douglasi</i>	<i>Haemaphysalis longicornis</i>		AMA- 1	β- tubulin
Hokkaido	Shibecha	2012/6	111	195	0	183	0	489	0 (0.0)	0 (0.0)
	Taiki ^a	2008/4	161	5	0	22	0	188	0 (0.0)	0 (0.0)
	Otofuke ^a	2008/4	41	0	0	1	0	42	0 (0.0)	0 (0.0)
	Memuro	2012/5	76	425	0	0	0	501	0 (0.0)	¹ (0.19) ^b
	Shin- Hidaka ^a	2008/6	6	5	49	4	0	64	0 (0.0)	0 (0.0)
Okinawa	Yonaguni ^a	2008/4	0	0	0	0	175	175	0 (0.0)	¹⁷ (9.71)
	Total		395	630	49	210	175	1,459	0 (0.0)	¹⁸ (1.23)

^a Tick species in these locations were described by Yokoyama et al. (2012).

^b *I. ovatus*.

PCR, polymerase chain reaction.