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Title: Epidemiological Survey of *Babesia bovis* and *Babesia bigemina* Infections of Cattle in Philippines

Running title: Survey of bovine babesiosis in Philippines

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ABSTRACT. A total of 250 blood samples were collected from clinically healthy cattle in five provinces of Philippines. DNA was extracted from the samples and analyzed by nested PCR assays for an epidemiological survey of *Babesia bovis* and *Babesia bigemina* infections. Out of the 250 samples, 27 (10.8%) and 16 (6.4%) were positive for *B. bovis* infection and *B. bigemina* infection, respectively. Mixed infections were detected in a total of 4 samples (1.6%). Our data provide baseline information regarding the epidemiology of *B. bovis* and *B. bigemina* infections in cattle in Philippines, which can be utilized in developing proper strategies for disease control and management.

KEY WORDS: *Babesia bigemina*, *Babesia bovis*, nPCR, Philippines.

Bovine babesiosis is a tick-borne disease caused by the protozoan parasites of the genus *Babesia.* The disease is generally characterized by high morbidity and mortality worldwide [4]. Of the *Babesia* species affecting cattle, *B. bovis* and *B. bigemina* are the most prevalent species that cause babesiosis, making these two species economically important worldwide due to their impact on dairy and beef industries [7]. The economic losses from the infection are incurred not only from production losses and the cost of therapeutic intervention, but also through its impact on the international cattle trade [4]. The acute infections with *B. bovis* and *B. bigemina* are characterized by anemia, fever, hemoglobinuria and death. Additionally, cattle may remain persistently infected with no clinical symptoms and thus serve as reservoir animals for the disease transmission, even if they recover from the infections. Therefore, the effective control strategies must include the detection of reservoir animals [4]. Molecular method based on the detection of *Babesia* DNA in blood by PCR techniques provides reliable results with high sensitivity and specificity. Recently, nested PCR (nPCR) targeting *B. bovis* spherical body protein 2 (BboSBP2) and *B. bigemina* rhoptry-associated protein-1a (BbiRAP-1a) have proven to be a powerful tool for epidemiological investigations [1, 5, 10-11].

Philippines is a developing agricultural country located in South East Asia, and in 2003, the livestock sector contributed 13.53% to total agricultural production [6]. The beef and dairy industries significantly contribute to the socio-economic development of this country, providing nutritional sources for the population as well as supplementary cash income for rural agricultural households. The growing demand of national livestock products emphasizes the necessity to improve the productivity of cattle industry in the country. Occurrence of bovine babesiosis in the cattle may undermine the improvement in this sector and lead to huge economic losses [4]. Although local

62 veterinarians have often detected infections of domestic animals with these parasites
63 through classical microscopic examination of Giemsa-stained blood smears,
64 distinguishing between *B. bovis* and *B. bigemina* infections is not easy. Although the
65 occurrences of *B. bovis* and *B. bigemina* in cattle have been reported in Philippines,
66 these studies covered only a single province and thus are not enough for comprehensive
67 insight about the prevalence of the disease [2, 8]. Therefore, in the current study, we
68 have reported the detection of *B. bovis* and *B. bigemina* in cattle by nPCR assays in five
69 provinces of Philippines. Thereafter, the DNA samples were amplified by nPCR using
70 pairs of primers designed from the *B. bovis*-SBP2 and *B. bigemina*-RAP-1a genes
71 (Table 1).

72 A total of 250 blood samples were collected randomly from apparently healthy
73 Brahman cattle in 5 provinces of Batangas, Cavite, Iloilo, Negros Occidental and
74 Negros Oriental in Philippines in April 2011. The cattle genomic DNA was extracted
75 and purified using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), and
76 then stored at -30ºC until use [12]. Briefly, primary PCR reaction was performed in a 20
77 μl reaction mixture containing 2 μl of a 10×PCR buffer (Takara, Kyoto, Japan), 200 μM
78 of each dNTP (Takara, Kyoto, Japan), 0.5 μM of the outer forward and reverse primers,
79 1 unit of *Taq* DNA polymerase (Takara, Kyoto, Japan) and 2 μl of the template DNA
80 sample. After the first PCR, 1 μl of the PCR products was transferred to new PCR tubes,
81 each containing a reaction mixture with the same composition as that of the first PCR
82 mixture except for the outer primers, which were replaced with the inner forward and
83 reverse primers. The cycling conditions were: an initial denaturation of 5 min at 95ºC,
84 followed by 35 cycles of 30 sec at 95ºC, 1 min at appropriate annealing temperature and
85 1 min at 72ºC, and a final extension of 7 min at 72ºC. The appropriate annealing

86 temperatures for all PCR amplifications are shown in Table 1. To determine positive
87 PCR reactions, an agarose gel electrophoresis was performed, and the DNA samples
88 were detected after staining with ethidium bromide.

89 To further validate the nPCR results, one positive DNA sample from each province
90 was sequenced as described previously [12]. Briefly, the nPCR products were cloned
91 into a pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced using the
92 ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The
93 obtained nucleotide sequences were analyzed using the GENETYX version 7.0 software
94 program (GENETYX Corporation, Tokyo, Japan). The nucleotide sequences were
95 analyzed for similarities and identities using GenBank BLASTN program and
96 EMBOSS Needle program. Any statistically significant differences ($P < 0.05$) in the
97 prevalences of cattle babesiosis between the different locations, genders and age groups
98 were determined by using chi-square test.

99 Out of the 250 blood samples, the overall prevalences of *B. bovis* and *B. bigmina*
100 were 10.8% and 6.4%, respectively (Table 2). Out of these positive samples, mixed
101 infections with both *Babesia* species were detected in 4 samples. These results
102 suggested that *B. bovis* infection is more prevalent than *B. bigemina* in cattle in
103 Philippines. The present data were comparable to the earlier epidemiological
104 investigations of *B. bovis* and *B. bigemina* infections. For instance, an earlier study
105 using nPCR documented 4.4% prevalence of *B. bigemina* in water buffalo in Philippines
106 [8]. In neighboring country such as Thailand, the prevalences of *B. bovis* and *B.*
107 *bigemina* were reported to be 12% and 21% in cattle and 11.2% and 3.6% in water
108 buffalos, respectively [5, 11].

Moreover, *B. bovis* was detected in the samples from all the regions, while *B. bigemina* was not detected in the samples from Batangas, Iloilo and Negros Oriental (Table 2). There was a significant difference in the prevalence of *B. bovis* among the provinces ($P < 0.05$). Notably, the Negros Oriental province showed highest prevalence (25.0%), and the lowest was in Batangas province (6.4%). The differences in the prevalence can be explained by the geographic distribution of the tick vectors in the regions under study. This lack of information on the epidemiology of tick-vector in Philippines necessitates a further study about the geographical distribution of tick and the molecular detection of *Babesia* in the vectors. Furthermore, the prevalence of *B. bovis* infection and *B. bigemina* infection was analyzed based on genders and ages. There were no statistically significant differences among different genders and ages groups (Tables 3 and 4). In the present study, genders seemed to be irrelevant with regard to the prevalence of the two *Babesia* infections, which was consistent with previous reports [3]. Previous studies have shown that cattle infected with *B. bovis* remain carriers for long periods, while those infected with *B. bigemina* remain carriers for only a few months [4]. The lack of significant differences in the young and adults indicated that cattle in the surveyed regions were early and persistently infected with the *Babesia*.

Next, five *B. bovis* positive samples and two *B. bigemina* positive samples (one from each province) were selected for sequencing using BboSBP2 and BbiRAP-1a as target genes, respectively. The nucleotides sequences of BboSPB2 (accession number JX648555) and BbiRAP-1a (accession number JX648554) genes derived from all the provinces surveyed in the present study were identical to each other. In addition, the sequence of BboSBP2 of Philippines isolates showed 93.5% nucleotide sequence

identities with the *B. bovis* Texas T2Bo strain (accession number XM_001611639) and 99.7% with the Thailand isolate (accession number JN974305). Likewise, BbiRAP-1a gene derived DNA extracted from Philippines samples showed nucleotide sequence identities of 99.5% with the Puerto Rico isolate (accession number AF017291), 99.5% with the Argentina isolate (accession number AF017296), 99.5% with the Thailand isolate (accession number JN974300), 99.8% with the Mexico isolate (accession number M85184), 99.8% with the Brazil isolate (accession number AF017295), 99.8% with the Syrian isolate (accession number AB617643) and 100% with the Uruguay isolate (accession number AF017297).

In conclusion, our data indicate the presence of *B. bovis* and *B. bigemina* infections in cattle in different provinces of Philippines, indicating an urgent need for a national strategy for the control of bovine babesiosis. Our data have also provided important information about the prevalence of *B. bovis* and *B. bigemina* in cattle in Philippines.

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191 Table 1. Oligonucleotide sequences and primer sets for the amplification of *B. bovis*
192 SBP2 gene and *B. bigemina* RAP-1a gene
193

Target	Assay	Sequences (5'→3')	Fragment (bp)	Annealing temperature	Reference
B. bovis SBP2 gene	PCR	CTGGAAGTGGATCTCATGCAACC TCACGAGCACTCTACGGCTTTGCAG	1236	64	[1]
	nPCR	GAATCTAGGCATATAAGGCAT ATCCCCTCCTAAGGTTGGCTAC	580	55	
B. bigemina RAP-1a gene	PCR	GAGTCTGCCAAATCCTTAC TCCTCTACAGCTGCTTCG	879	58	[5]
	nPCR	AGCTTGCTTTCACAACTCGCC TTGGTGCTTTGACCGACGACAT	412	50	[9]

194
195

196 Table 2. Prevalence of *B. bovis* and *B. bigemina* in cattle from five regions in
197 Philippines
198

Regions	No. examined	*B. bovis*		*B. bigemina*	
		Frequency	Prevalence (95% CI[†])	Frequency	Prevalence (95% CI[†])
Batangas	47	3	6.4%[a] (1.4-17.54)	0	0
Cavite	24	2	8.3% (1.03-27)	1	4.1% (0.11-21.12)
Iloilo	44	5	11.3% (3.79-24.56)	0	0
Negros Occidental	115	12	10.4% (5.56-17.67)	15	13.0% (7.56-20.77)
Negros Oriental	20	5	25.0%[a] (8.22-47.17)	0	0
Total	250	27	10.8% (7.24-15.32)	16	16% (3.7-10.19)

199 [a] The chi-square test was applied to evaluate significant differences ($P < 0.05$).
200 [†]Prevalence at 95% confident intervals
201
202

203 Table 3. Prevalence of *B. bovis* and *B. bigemina* in cattle of different genders in
204 Philippines
205

Gender	No. examined	*B. bovis*		*B. bigemina*	
		Frequency	Prevalence (95% CI†)	Frequency	Prevalence (95% CI†)
Male	173	18	10.4% (6.25-15.86)	10	5.8% (2.79-10.32)
Female	77	9	11.7% (5.56-21.29)	6	7.8% (2.95-16.4)

206 †Prevalence at 95% confident intervals
207
208 Table 4. Prevalences of *B. bovis* and *B. bigemina* in cattle of different age groups in
209 Philippines
210

Age (years)	No. examined	*B. bovis*		*B. bigemina*	
		Frequency	Prevalence (95% CI†)	Frequency	Prevalence (95% CI†)
1-2	18	1	5.6% (0.14-27.29)	2	11.1% (1.38-34.71)
3-4	175	17	9.7% (5.76-15.10)	10	5.7% (2.77-10.26)
> 5	57	9	15.8% (7.48-27.87)	4	7.0% (1.95-17.00)

211 †Prevalence at 95% confident intervals