

## Original papers

# Prevalence of babesiosis (*Babesia bovis* and *Babesia bigemina*) in cattle and water buffalo in Nueva Ecija, Philippines using Nested Polymerase Chain Reaction

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**ABSTRACT.** The study was conducted to determine the prevalence of *Babesia bovis* and *Babesia bigemina* infection in blood samples of cattle and water buffaloes using nested polymerase chain reaction (nested-PCR). It also aimed to generate a spot map showing areas in Nueva Ecija, the Philippines where *B. bovis* and *B. bigemina* were detected. Whole blood samples of cattle (148) and water buffalo (65) were collected for DNA extraction and subsequent nested-PCR to detect *B. bovis* and *B. bigemina*. To further confirm and validate the nested-PCR results, three selected positive samples for each *B. bovis* and *B. bigemina* were sequenced and examined for homology analysis. The results showed that the prevalence of *B. bovis*, *B. bigemina* and mixed infection in cattle were 11.49% (17/148), 10.81% (16/148) and 5.41% (8/148), respectively. Homology analysis of nucleotide sequence of three selected DNA samples for each *B. bovis* showed two 99% and one 96% (partial sequence analysis) identities with *B. bovis* Thailand strain, while *B. bigemina* positive samples showed all 100% identities with *B. bigemina* Philippine strain. The result did not demonstrate in all water buffalo samples. These findings provide information about the prevalence of *B. bovis* and *B. bigemina* in cattle and water buffaloes in Nueva Ecija, which can be beneficial for strategic planning, disease management, and control and prevention.

**Key words:** water buffalo, *Babesia bovis*, *Babesia bigemina*, nested PCR

## Introduction

Bovine babesiosis is a tick-borne disease of cattle which is generally characterized by significant morbidity and mortality worldwide [1]. Bovine babesiosis threatens the livestock industry, particularly the productivity of cattle and buffaloes in many Asian countries [2]. *Babesia bovis* and *Babesia bigemina* are known as the most important species of *Babesia* spp. as these contribute to enormous economic losses in livestock industry in tropical and subtropical regions of the world [1,3–4]. *Rhipicephalus microplus* is the tick vector of both *B. bovis* and *B. bigemina* [3]. Once the eggs

laid by ticks hatch on the ground, the larvae actively seek a host [5].

The Philippines is a developing agricultural country where its livestock sector contributed 13.53% to total agricultural revenue in 2003 [6]. The prevalence of *B. bigemina* in buffalo in the Philippines has been described as 4.4% by Mingala et al. [7] using polymerase chain reaction (PCR) in blood samples collected from various provinces in the Philippines. In the related epidemiological study conducted in the Philippines that covering different provinces, *B. bovis* and *B. bigemina* infection in cattle had 10.8% and 6.4% prevalence rates, respectively [8]. According to Cresencio [personal

communication], there is limited data about bovine babesiosis in the Philippines due to its sporadic nature. This was reflected in the Philippines' report to the World Animal Health Organization (OIE) denoting the presence of the said disease, but cases were not confirmed in the laboratory or were not reported to authorities to warrant further investigation.

The asymptomatic nature of *B. bovis* and *B. bigemina* in water buffaloes [1], the inverse relationship between age and resistance to babesiosis, the persistence of the infections from several months up to several years of a very low parasitemia and the adaptation of *Babesia* spp. by means of vertebrate as reservoir host makes parasite viable for long periods. Moreover, transovarial and transtadial transmission in *Babesia* spp. are means of main adaptation which facilitate long-term persistence of *Babesia* spp. in the environment [9]. Water buffaloes may possibly serve as reservoirs for infections of cattle, since they are raised together in the pastures where tick vectors continue to infect and feed on them [3,10].

Giemsa-stained blood smear examination is the gold standard test for diagnosing babesiosis. However, this method has low sensitivity especially when the level of parasitemia is too low in the bloodstream. Similarly, serologic approach, such as enzyme-linked immunosorbent assay (ELISA) may not be sensitive enough in detection of infection in samples harbouring low parasitic burdens [11]. Besides, antibodies have the tendency to disappear in long-term carriers [12]. *Babesia* spp. are difficult to diagnose by smear and serology, but it can be accomplished by amplification of *Babesia* spp. DNA using PCR [13].

A study that investigated the prevalence of babesiosis in cattle and water buffalo was initiated with the application of molecular approach. The method provides greater sensitivity and specificity over the methods routinely used in most laboratories [14]. Recently, nested-PCR targeting *B. bovis* spherical body protein 2 (BboSBP2) and *B. bigemina* rhoptry-associated protein-1a (BbiRAP-1a) was reported to be a significant and powerful method especially in epidemiological investigations [1,15–17]. Detection of carrier animals and differential diagnosis between the *B. bovis* and the *B. bigemina* infections and other diseases with similar clinical signs are important in the development of disease management programs and control strategies [1].

## Materials and Methods

**Study area.** Backyard or commercial farms within the province of Nueva Ecija, the Philippines were enrolled in the study. Sampling inclusion criteria were the presence of ticks or previous tick infestation in the animal. The area was investigated first for the occurrence of ticks in cattle and water buffaloes before collection. Prior visitation to different areas was done before the sample collection. The epidemiological design of the study was random sampling. The animals to be collected with blood were randomly selected from different locations.

**Restraining of animal.** The animals were properly restrained using chute or restrained by means of casting down depending on the field condition. The head was secured if the site of collection was on the facial or ear vein. Safety of both animal and researcher were considered.

**Sample collection.** During clinical examination, data on animals where tick infestation or history of previous tick infestation were indicated, and obtained from the owners. The animals included in the study were one-year old and above. Field samples of blood from cattle and water buffaloes that were currently or previously infested with *R. microplus* or together with animal/s that was/were tick infested or previously infested were collected from selected farms.

The study required 148 samples for cattle and calculated using 10.8% prevalence, 95% confidence level, and 5% degree of error. While the study required 65 samples for water buffalo and was calculated using 4.4% prevalence, 95% confidence level, and 5% degree of error. Approximately five ml of whole blood was collected from either the jugular/tail/facial/ear veins of individual cattle and water buffalo, both with inclusion of heparin or EDTA. Blood samples were placed in cooler with ice during transportation and were refrigerated at 4°C until processed

**DNA extraction.** Before extraction of genomic DNA, blood samples were centrifuged at 3000 rpm for 15 minutes. After centrifugation, 500 µl of whole blood and 600 µl of cell lysis buffer were transferred into a sterile 1.5 ml microcentrifuge tube. The mixture was centrifuged at 14000 rpm for one minute. Supernatant was discarded using pipette. 700 µl of Cell lysis buffer was added and was mixed thoroughly by using vortex. The mixture was centrifuged at 14000 rpm for one minute.

Supernatant was discarded using pipette, careful not to pipette the pellet. 300 µl of Nuclei lysis buffer and 100 µl of Protein precipitation buffer were added to the pellet and were mixed thoroughly by using vortex. Then, it was centrifuged at 14000 rpm for 10 minutes. Supernatant was transferred into a new, sterile microcentrifuge tube. 50 µl of isopropanol was added and was mixed by inverting the tube. Mixture was centrifuged at 14000 rpm for one minute then the supernatant was discarded. 70% ethanol was added and was mixed again by inverting the tube. The tube was centrifuged at 14000 rpm for one minute then the supernatant was discarded. Tubes were placed in a clean bench until the pellet or tube was dry. 50–100 µl of DNA rehydration solution or sterile Tris-EDTA buffer was added into the tubes. It was stored at 4°C or at –20°C until used.

**PCR amplification for beta actin.** Beta actin was used commonly as internal control gene [18]. The internal control gene of host origin must be amplified in pre-diagnostic PCR reaction to determine the overall DNA quality of the sample. This is significant in epidemiological pathogen surveys for veterinary medical applications [19].

Fragments of expected size (200 bp) were generated from template DNA representing beta actin (Fig. 1. lanes 1–22) isolates using specific primer set.

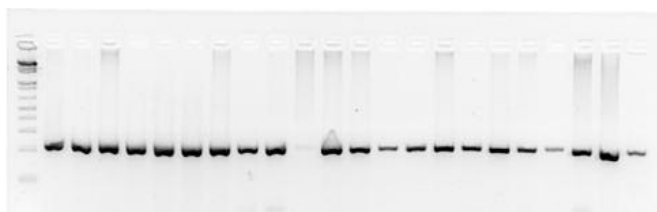


Fig. 1. Agarose gel electrophoresis of PCR using DNA isolated from bovine and bubaline field samples. M, 1 KB+ ladder; Lanes 1–15 (bovine) and lanes 16–22 (bubaline) template DNA isolated for beta-actin. Arrow indicates 200 base pair amplicons generated using beta actin primer set.

**Nested-PCR amplification.** The genomic DNA samples of cattle and water buffaloes were screened for the presence of *B. bovis* and *B. bigemina* using nested-PCR. Specific primers for *B. bovis* and *B. bigemina* targeting the BboSBP-2 and BbigRAP-1, respectively were used based on the study of Figueroa et al. [20] and Terkawi et al. [1].

A total volume of 10 µl PCR reaction contained 1 µl of the extracted DNA temple, 0.8 µl magnesium

chloride (MgCl<sub>2</sub>), 0.5 µl of each primers (10 pmol), 2 µl of 5×PCR Buffer, 1 µl of dNTP (200 µM), 0.05 µl of Flexi Taq polymerase (Promega, USA) and 4.2 µl of distilled water. The thermal cycling conditions for PCR amplifications were as follows: initial denaturation for five minutes at 95°C, followed by 35 cycles of one minute denaturation at 94°C, one minute annealing at 55°C, and one minute extension at 72°C and final extension at 72°C for 10 minutes. The nested-PCR products were subjected to agarose gel electrophoresis and were visualized under an ultraviolet light.

**DNA sequencing.** To further confirm and validate the nested-PCR results, three selected positive samples with most clear fragments of each *B. bovis* and *B. bigemina* were sequenced at First Base, Malaysia. Approximately 45 µl of nested-PCR products and purified DNA using agarose gel were sent for sequencing. The homology of *B. bovis* and *B. bigemina* partial gene sequence were analyzed using NCBI BLAST.

## Results and Discussion

A total of 148 blood samples from cattle and 65 blood samples from water buffaloes were randomly collected from different areas in Nueva Ecija for DNA extraction and subsequent nested-PCR to detect *B. bovis* and *B. bigemina*. To further confirm and validate the nested-PCR results, three selected positive samples for each *B. bovis* and *B. bigemina* were sequenced and examined for its homology to sequences registered in GenBank.

### Nested PCR amplification of *B. bovis* DNA

Fragments of expected size were generated from template DNA representing *B. bovis* (Fig. 2. lanes 1,

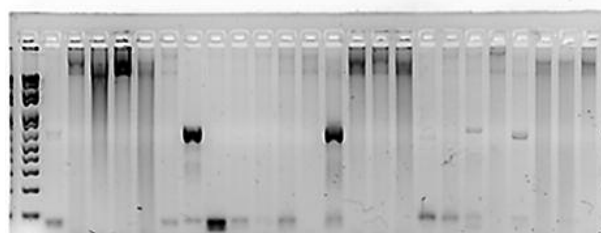


Fig. 2. Agarose gel electrophoresis of nPCR using DNA isolated from bovine field samples. M, 1 KB+ ladder; Lanes 1–22, template DNA isolated from bovine blood samples for *B. bovis*; lanes 23–24, negative PCR control (water). Arrow indicates 580 base pair amplicons generated using BboSBP2 primer set. Lanes 1, 7, 13, 17, 19 and 21, positive for *B. bovis*.

7, 13, 17, 19 and 21) isolates using species-specific primer set (*B. bovis* spherical body protein 2 [BboSBP2]) [8], while non-target species DNA displayed no evidence of fragment amplification. This indicated that the primer set was capable of specifically amplifying the target locus.

#### Nested-PCR amplification of *B. bigemina* DNA

Fragments of expected size (412 bp) were generated from template DNA representing *B. bigemina* (Fig. 3. Lanes 3, 4, 6, 7, 9, 15, 16, 19, 20 and 22) isolates using species-specific primer set (*B. bigemina* rhoptry-associated protein-1a [BbigRAP-1a]) [8], while non-target species DNA displayed no evidence of fragment amplification. This indicated that the primer set was capable of specifically amplifying the target locus.

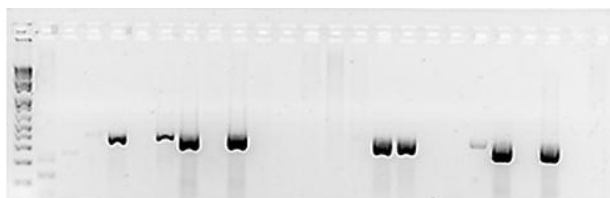


Fig. 3. Agarose gel electrophoresis of nested-PCR using DNA isolated from bovine field samples. M, 1 KB+ ladder; Lanes 1-22, template DNA isolated from bovine blood samples for *B. bigemina*; lanes 23-24, negative PCR control (water). Arrow indicates 412 base pair amplicons generated using BbigRAP-1a primer set. Lanes 3, 4, 6, 7, 9, 15, 16, 19, 20 and 22, positive for *B. bigemina*.

The nested-PCR had its advantage in a way that it reduced the presence of undesirable bands due to contamination and it increased the specificity of

amplifying the target gene. Usually, the first PCR product would not show bands after electrophoresis like in cases of low DNA yield of the target gene, thus it only produces invisible bands. The second PCR product with expected amplicon size is usually viewed as confirmation of the presence of the target gene [21]. The second amplification will definitely form a band because it is specific to the sequence found within the DNA of initial amplification during nested-PCR [22].

Table 2 shows the overall summary of the prevalence of *B. bovis* and *B. bigemina* in cattle. The results presented that in cattle, out of 148 samples, 17 (11.49%) and 16 (10.81%) were positive for *B. bovis* and *B. bigemina*, respectively. Furthermore, mixed infections were detected in a total of 8 (5.41%) samples.

The prevalence of *B. bovis*, *B. bigemina* and mixed infection in cattle was 11.49% (CI=6.83–17.75), 10.81% (CI=6.30–16.96) and 5.41% (CI=2.36–10.37), respectively. Previous studies in the Philippines stated that prevalence of *B. bovis* and *B. bigemina* in cattle was 10.8% and 6.4%, respectively, in five different provinces [8], 10% and 15.4%, respectively in province of Cebu [23] and 45.4% and 61.6%, respectively, in Luzon island [24]. In other country, studies suggest that the prevalence of *B. bovis* and *B. bigemina* in cattle was 17.1% and 9.7%, respectively, in Myanmar [25], 21.8% and 10.9%, respectively, in Vietnam [14] and 12% and 21%, respectively, in Northern Thailand [16]. The results were statistically comparable to the studies of Yu et al. [8], Ybañez et al. [23] and Bawm et al. [25] for the prevalence of *B. bovis* and *B. bigemina*. Moreover, the results were statistically comparable to the study of Li et al. [14] for the

Table 1. Primer sets specific for *B. bovis* BbSBP-2 and *B. bigemina* BbigRAP-1a genes for nested-PCR assays [8]

Genes	Assays	Oligonucleotide primers	Product sizes
BboSBP-2	PCR	5'-CTGGAAGTGGATCTCATGCAACC-3'	1236 bp
		5'-TCACGAGCACTCTACGGCTTTGCAG-3'	
	Nested-PCR	5'-GAATCTAGGCATATAAGGCAT-3'	580 bp
		5'-ATCCCCCTCCTAAGGTTGGCTAC-3'	
BbigRAP-1a	PCR	5'-GAGTCTGCCAAATCCTTAC-3'	879 bp
		5'-TCCTCTACAGCTGCTTCG-3'	
	Nested-PCR	5'-AGCTTGCTTTCACAACCTCGCC-3'	412 bp
		5'-TTGGTGCTTTGACCGACGACAT-3'	

Table 2. Summary of results of nested-PCR for *Babesia bovis* and *Babesia bigemina* infection in cattle

	P (+) nested-PCR	Prevalence	95 % confidence interval	
			lower level	upper level
<i>B. bovis</i>	17/148	11.49%	6.83	17.75
<i>B. bigemina</i>	16/148	10.81%	6.30	16.96
Mixed infection	8/148	5.41%	2.36	10.37

prevalence of *B. bovis* and Cao et al. [16] for the prevalence of *B. bigemina*. Nevertheless, the results were non-comparable to the study of Ochirkhuu et al. [24] because of the higher prevalence of both *B. bovis* and *B. bigemina* compared to the current study.

The results were negative for both *B. bovis* and *B. bigemina* in water buffalo. Bovine babesiosis in water buffalo was not prevalent in Nueva Ecija. This could be explained by the rationale that water buffalo spend much of the time submerged in muddy waters, hence tick infestation was reduced

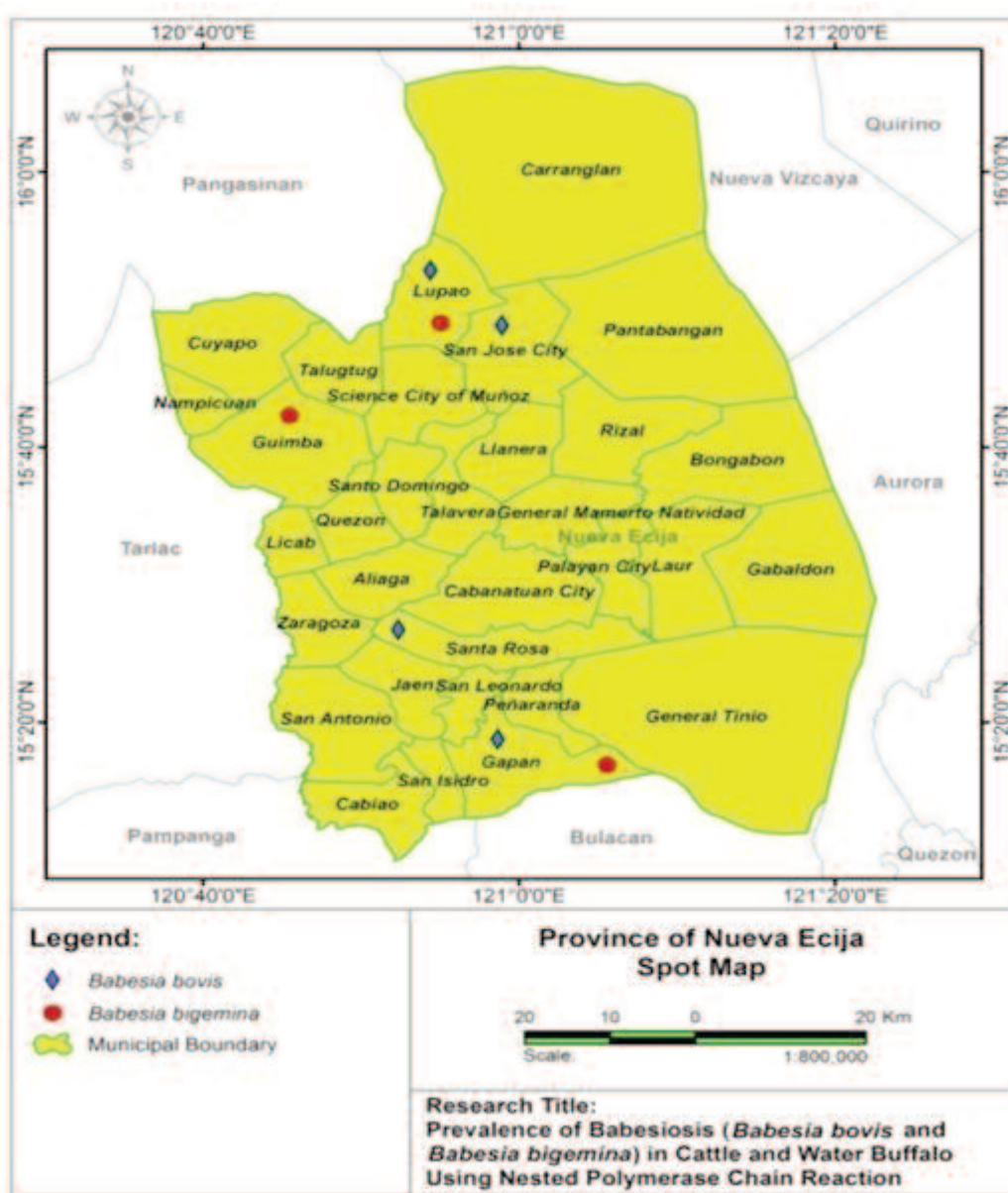


Fig. 4. Spot map of the province of Nueva Ecija for babesiosis

[26]. This was supported by [27] and [28], and as a result, decreasing the possibility of attachment of ticks, consequently the transmission of pathogen. Earlier study of [7] revealed that there was 4.4% prevalence of *B. bigemina* in the Philippines, mainly from Luzon area. In other Asian countries, the prevalence of *B. bovis* and *B. bigemina* infection in water buffalo were 11.2% and 3.6%, respectively in Thailand [1] and 23.3% and 0%, respectively in Vietnam [14].

All of the samples from cattle were tick infested or had a history of tick infestation (*R. microplus*). Only few water buffaloes were infested with ticks, most were not infested and some were together with cattle or water buffalo that were tick infested or previously infested. The presence of ticks or history of tick infestation to the animal was associated with the presence of babesiosis in the study areas. *R. microplus* is the most significant vector of *Babesia* parasites of domestic cattle and buffalos in Southeast Asia [29].

#### Sequence analysis

Three nested-PCR positive samples with most clear fragments for *B. bovis* and *B. bigemina* were selected and sequenced for homology analysis of *B. bovis* spherical body protein 2 (BboSBP2) and *B. bigemina* rhoptry-associated protein-1a (BbigRAP-1a). The sequences of BboSBP2 (JX648555) of samples from three selected positive samples showed two 99% and one 96% (partial sequence analysis) nucleotide sequence identities with the *B. bovis* Thailand strain (KT460092). The sequences of BbigRAP-1a (JX648554) gene derived DNA extracted from three selected positive samples showed all nucleotide sequences identities of 100% with the *B. bigemina* Philippine isolate (JX648554).

The nested-PCR targeting *B. bovis* spherical body protein 2 (BboSBP2) and *B. bigemina* rhoptry-associated protein-1a (BbiRAP-1a) have confirmed to be powerful means for epidemiological investigations of bovine babesiosis [1,15–17].

#### Spot map

Areas in Nueva Ecija where *B. bovis* and *B. bigemina* infection in cattle and water buffaloes were presented using a spot map (Fig. 4).

In cattle, positive samples for *B. bovis* were gathered in the municipalities of Lupao, Gapan, Sta. Rosa and San Jose City whereas for *B. bigemina* were collected in the municipalities of Lupao, Gapan and Guimba. The spot map was presented

through geographic information system (GIS). There was presence of ticks or history of tick infestation in the animals in areas where *B. bovis* and *B. bigemina* were present.

In Gapan, cattle were highly infested with ticks and the animals were raised in ranch-type farming having a large population. In Lupao, animals that were collected with blood samples were all in backyard type of farm, which they were tethered in the field. All of the cattle collected with blood in Lupao were infested with ticks. In Sta. Rosa, the type of farming of cattle was commercial, and all of the cattle were tick infested. In San Jose City, areas where blood collection was conducted were both backyard and commercial farm. Tick infestations were common to the animals. In Guimba, history of tick infestations were common than currently infested. The animals collected were in backyard farms.

Geographic distribution of tick-vectors explained the differences in the occurrence of babesiosis [8]. Furthermore, factors that could affect the fluctuation of prevalence of babesiosis in different areas were explained by tick distribution, micro-climate pattern, breeds, farm management and the sampling condition [26].

This is the first molecular-epidemiological surveillance of *B. bovis* and *B. bigemina* in cattle and water buffalo in Nueva Ecija using nested-PCR.

Results showed that *B. bovis* and *B. bigemina* were present in cattle in Nueva Ecija, although not detected in water buffalo. The study also revealed that *B. bovis* was more prevalent than *B. bigemina* in cattle. Absence of information about the epidemiology of tick-vector in the Philippines accentuates the significance of further study to investigate the geographic distribution of tick in the province, likewise in whole country. Identification of *Babesia* spp. on its tick-vectors using molecular technique may also contribute to additional information on the possible occurrence of these diseases in a certain place. The negative result for both *B. bovis* and *B. bigemina* in water buffalo could be explained by the nature of the living of host. They tend to wallow their body to the muddy water, which might decrease the attachment of tick-vector and transmission of infection. Moreover, farmers in Nueva Ecija use their water buffalo in dairy and draft purposes, thus make sure to thoroughly bathe and clean their livestock for good production and condition. Hence external parasitic infestation was prevented; as a result the transfer of the pathogen

was diminished. Increasing the number of animals for blood sampling and inclusion all municipalities in Nueva Ecija as area of study may also be necessary to further validate the prevalence of *B. bovis* and *B. bigemina*.

The current data provide information about the incidence of *B. bovis* and *B. bigemina* infections in cattle and water buffalo that could serve as guide in the strategic plan for the control and prevention of those diseases. The presence of *B. bovis* and *B. bigemina* in Nueva Ecija necessitates the national surveillance of these diseases to observe the extent of the occurrence and to generate future strategy for the control of bovine babesiosis if present in the whole country and to prevent possible outbreak. Thus, rigid monitoring and testing of animals for babesiosis should be done prior to importation of cattle and water buffalo.

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