



ELSEVIER

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

First molecular detection and characterization of tick-borne pathogens in water buffaloes in Bohol, Philippines

Eloiza May S. Galon^a, Paul Franck Adjou Moumouni^a, Rochelle Haidee D. Ybañez^a, Aaron E. Ringo^a, Artemis Efstratiou^a, Seung-Hun Lee^a, Mingming Liu^a, Huanping Guo^a, Yang Gao^a, Jixu Li^a, Caro B. Salces^b, Bon Christian A. Maurillo^c, Damdinsuren Boldbaatar^d, Adrian P. Ybañez^{e,f,*}, Xuenan Xuan^{a,*}

^a National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Hokkaido, Japan

^b Philippine Carabao Center at Ubay Stock Farm, Ubay, 6315, Bohol, Philippines

^c National Dairy Authority, Lomangog, Ubay, 6315, Bohol, Philippines

^d Institute of Veterinary Medicine, Zaisan, 17024, Ulaanbaatar, Mongolia

^e Institute of Molecular Parasitology and Vector-borne Diseases at Main Campus and College of Veterinary Medicine at Barili Campus, Cebu Technological University, Cor.

M.J. Cuenco and R. Palma St., Cebu City, 6000, Cebu, Philippines

^f Regional Center for Molecular Diagnostics and Research and College of Science, University of the Philippines Cebu, Lahug, Cebu City, 6000, Cebu, Philippines

ARTICLE INFO

Keywords:

Anaplasma
Babesia
Theileria
Water buffalo
Bohol province
Philippines

ABSTRACT

The water buffalo industry is a vital part of the Philippine livestock economy and is an essential contributor to the developing local dairy industry. Although relatively less susceptible to diseases, water buffaloes can still be infected and can act as reservoirs of tick-borne pathogens (TBPs). However, limited information is available regarding the prevalence of tick-borne infections in water buffaloes in the Philippines. This study was conducted to identify TBPs harbored by water buffaloes and to characterize these pathogens molecularly. One hundred water buffalo blood samples collected from three areas in Bohol, Visayas region, Philippines were screened for various TBPs using pathogen-specific PCR assays. TBPs were detected in 46% of the samples (39% singly infected, 7% coinfecting). The pathogens detected were *Anaplasma marginale* (29%), *Babesia bovis* (21%), and *B. bigemina* (3%). None of the blood samples were positive for *Theileria annulata*, *T. orientalis*, and *B. ovata*. *A. marginale* infection rates were significantly higher (37.5%) among water buffaloes aged ≤ 6 years ($P = 0.046$) than those > 6 years old (18.2%) and was detected only in Bulgarian Murrah (36.1%) and US Murrah (25.9%) breeds. Phylogenetic analyses revealed that *groEL* sequences of *A. marginale* were 100% identical with isolates from the Philippines (Batangas and Cebu) and China. Two *B. bigemina* RAP-1a gene sequences were identical to each other and were homologous with previous isolates from Thailand, Indonesia, Uruguay, and the Philippines. Moreover, four *B. bovis* *SBP-2* partial sequences obtained in this study had 92.4–99.7% identities. This study is the first molecular detection and characterization of *A. marginale*, *B. bigemina* and *B. bovis* in water buffaloes in the Visayas region, and the first molecular confirmation of *B. bovis* infection in water buffaloes in the country. The findings presented in this study may serve as baseline data for crafting effective tick-borne disease surveillance and prevention programs in Bohol and in the Philippines.

1. Introduction

The water buffalo (*Bubalus bubalis*) is an integral part of the livestock industry of the Philippines. It is mainly utilized as a source of draft power for farmers, and as a source of meat and milk. Water buffaloes outnumber cattle population in the Philippines with an inventory of

2.88 million heads (Philippine Statistics Authority, 2017). Regarded as “farmers’ best friend”, 99.6% of these animals are raised by backyard farmers. Often reared together with cattle, it is conspicuous that water buffaloes are more resistant and tolerant to diseases despite exposure to unsanitary environment and conditions favorable for disease development (National Research Council, 1981). More often, infected water

* Co-corresponding authors at: (X. Xuan) National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Hokkaido, Japan; (A.P. Ybañez) Institute of Molecular Parasitology and Vector-borne Diseases at Main Campus and College of Veterinary Medicine at Barili Campus, Cebu Technological University, Cor. M.J. Cuenco and R. Palma St., Cebu City, 6000, Cebu, Philippines.

E-mail addresses: dr.adrianpybanez@gmail.com (A.P. Ybañez), gen@obihiro.ac.jp (X. Xuan).

<https://doi.org/10.1016/j.ttbdis.2019.03.016>

Received 16 July 2018; Received in revised form 8 March 2019; Accepted 22 March 2019

Available online 23 March 2019

1877-959X/© 2019 Elsevier GmbH. All rights reserved.

buffaloes seldom show clinical signs. Thus, their potential as carrier animals and pathogen reservoirs is frequently overlooked.

Tick-borne diseases (TBDs) are known to cause adverse impacts on livestock health and impose considerable limitations in enhancing livestock productivity. In 1999, a model simulated by McLeod and Kristjanson estimated an annual cost of 0.6 million US\$ for the Philippine livestock industry due to losses and control measures for anaplasmosis and babesiosis (Bock et al., 2004). This highlights the significant risk and impact of TBDs to the livestock economy of the country.

Anaplasma marginale, an obligate intraerythrocytic rickettsia, is the primary cause of bovine anaplasmosis and the most prevalent bovine vector-borne pathogen worldwide (Suarez and Noh, 2011). It can be biologically transmitted by ticks, mechanically by biting flies and blood-contaminated fomites, and vertically through the movement of infected red blood cells across the placenta in the uterus (Aubry and Geale, 2011). Bovine anaplasmosis results in fever, weight loss, abortion, lethargy, icterus, and sometimes, death in adult cattle (Kocan et al., 2010). In water buffaloes, same clinical signs are observed but diseased animals also manifest tachycardia, labored respiration, and pale mucous membranes (da Silva et al., 2014; Vatsya et al., 2013). Although commonly infected, water buffaloes rarely develop the disease and thus have been implicated as reservoir hosts (de la Fuente et al., 2005; Kocan et al., 2010; Kuttler, 1984).

Bovine babesiosis is a disease caused by several species of *Babesia* parasites transmitted by ticks (Bock et al., 2004; Uilenberg, 1995). Significant *Babesia* species known to infect bovines in Asia include *B. bovis*, *B. bigemina*, *B. orientalis*, and *B. ovata* (Uilenberg, 2006). Babesiosis is regarded as an important arthropod-borne disease in bovines because of the massive economic losses it inflicts on the global livestock industry (Bock et al., 2004; de Castro, 1997; Minjauw and McLeod, 2003; Sackett and Holmes, 2006).

Members of the genus *Theileria* are tick-transmitted obligate protozoan parasites that infect a broad range of wild and domestic animals. They are characterized by the complex biology among various species (Mans et al., 2015). *Theileria* spp. are known for the millions of annual losses they bring to Asian and Sub-Saharan African agriculture sectors (Bishop et al., 2004). Notable species of importance in bovines are *T. annulata*, *T. parva*, and *T. orientalis* group (Sivakumar et al., 2014).

Epidemiological surveys on tick-borne pathogens (TBPs) using molecular detection techniques have been conducted in cattle in various parts of the Philippines (Belotindos et al., 2014; Foronda et al., 2010; Herrera et al., 2017; Ochirkuu et al., 2015; Ybañez et al., 2014, 2013, 2012; Yoshinari et al., 2012; Yu et al., 2013). To the best of the authors' knowledge, only *A. marginale* and *B. bigemina* have been molecularly detected in water buffaloes in the country (Mingala et al., 2009).

To date, information on the status of TBDs in Bohol, Philippines and the genetic characterization of TBPs in the area is limited. With the recent plan of the Philippine Department of Agriculture to raise in Bohol about 5000 heads of Girolando dairy cattle imported from Mexico, there is a need to survey the presence of TBPs in the area, and to assess the genetic diversity of the detected TBPs. Hence, this study was conducted.

2. Materials and methods

2.1. Ethical statements

All procedures performed in animals in this study were in accordance to the principles of Philippine Animal Welfare Act (R.A. 8485 as amended by R.A. 10631) and Administrative Order No. 45 of the Bureau of Animal Industry of the Philippines. Permission on the use of DNA (Permit no. 1723-1724) and animal experiments (Permit no. 29-69) was also obtained from Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan.

2.2. Sample population and area

A total of 100 female dairy water buffaloes were sampled from three areas in Bohol, Philippines (Ubay, N = 92; Mabini, N = 7; Alicia, N = 1) from December 2016 to May 2017 (Fig. 1). Selection of sampling locations was done based on convenience while animals were randomly chosen for sampling. Profile parameters such as breed (Bulgarian Murrah, US Murrah, crossbred, Philippine Carabao), age groups (≤ 6 years, > 6 years), body condition score (underweight, optimal, overweight) and presence or absence of ticks were recorded for analysis.

2.3. Blood sample collection and DNA extraction

Five milliliters of whole blood were collected from each of 100 water buffaloes in Bohol, Philippines. The blood samples were stored at -20°C until DNA extraction. DNA from blood samples was extracted using QIAamp® DNA Blood Mini Kit (QIAGEN, Germany) according to manufacturer's instructions.

2.4. PCR assays for the screening of TBPs

Oligonucleotide primers and PCR assays used in the screening of the samples are listed in Table 1. *T. orientalis* and *B. ovata* were screened utilizing single PCR targeting the major piroplasm surface protein (MPSP) (Ota et al., 2009) and apical membrane antigen (AMA)-1 (Sivakumar et al., 2012), respectively. Screening for *B. bigemina*, *B. bovis*, *T. annulata*, and *A. marginale* used nested PCR assays based on rhoptry-associated protein (RAP)-1a (Terkawi et al., 2011), spherical body protein (SBP)-2 (Aboulaila et al., 2010), merozoite surface antigen (TAMS)-1 (Martin-Sanchez et al., 1999), and *A. marginale* groEL (Ybañez et al., 2012) genes. The PCR mixture consisted of 4.9 μL of double-distilled water, 200 μM of dNTP solution mix (New England Biolabs, U.S.A.), 1 μL of 10x Ex Taq buffer (Takara, Japan), 0.5 mM of each primer, 0.1 μL of Ex Taq polymerase (Takara, Japan) and 1 μL of gDNA. Thermocycling conditions were performed for each pathogen as presented in Table 1. Double-distilled water was used as negative control and positive cattle DNA samples from a previous study (Ringo et al., 2018) were used as positive controls. After electrophoresis and staining with ethidium bromide, amplicons were visualized in 1.5% agarose gel under UV light.

2.5. Cloning and sequencing of detected pathogens

Amplicons of positive samples (*A. marginale*, N = 3; *B. bovis*, N = 4; *B. bigemina*, N = 2) were randomly selected, excised from the gel and purified using NucleoSpin® Gel and PCR Clean-up (Macherey Nagel, Germany). Thereafter, amplicons were inserted into pGEM®-T Easy Vector (Promega Corporation, USA) plasmids and cloned in DH5 α *E. coli* competent cells. Purification of plasmids was done by using NucleoSpin® Plasmid QuickPure Kit (Macherey Nagel, Germany) and sequencing analysis was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA).

2.6. Phylogenetic analysis

Nucleotide sequencing results were trimmed manually and identities were confirmed with EMBOSS Pairwise Sequence Alignment (https://www.ebi.ac.uk/Tools/psa/emboss_matcher/nucleotide.html) and BLASTn search. Sequences of *A. marginale* groEL (JQ839002.1), *B. bovis* SBP-2 (JX648555.1) and *B. bigemina* RAP-1a (JX648554.1) previously isolated in cattle from the Philippines were used as references for alignment. Multiple sequence alignment and construction of phylogenetic trees were done with the Molecular Evolutionary Genetics Analysis 7.0 software (Kumar et al., 2016) using maximum likelihood

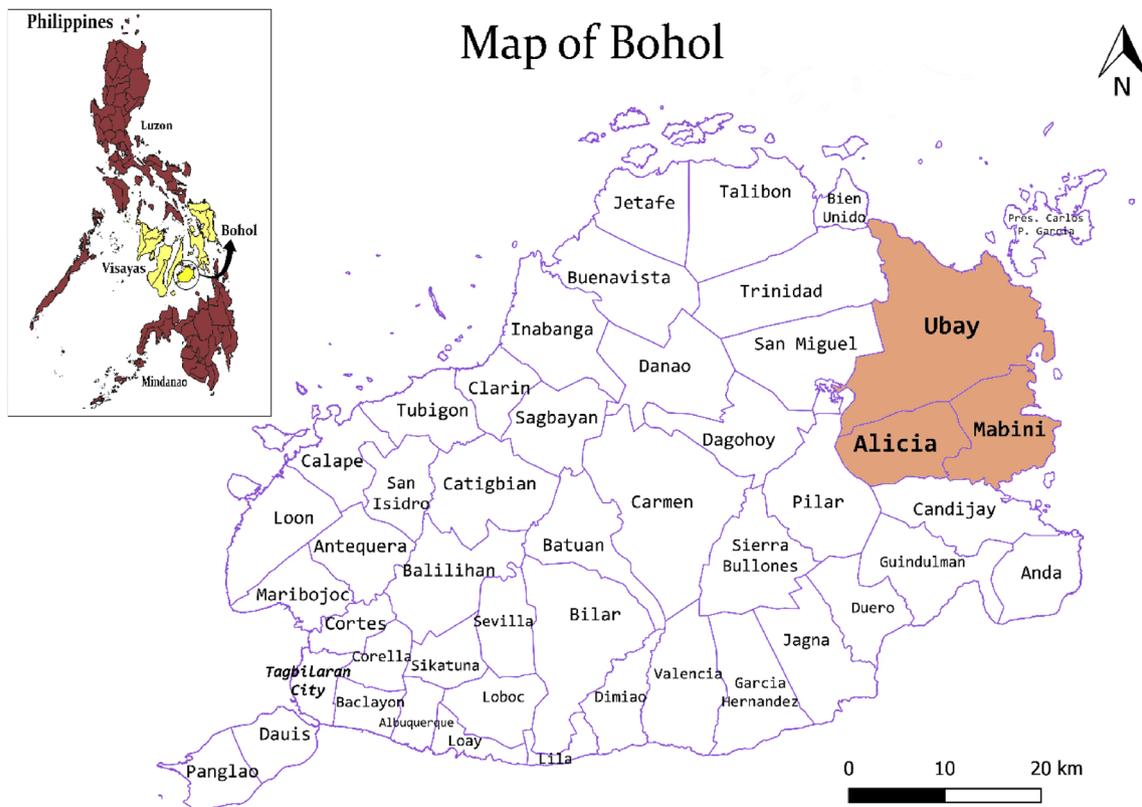


Fig. 1. Map of sampling area in Bohol, Philippines.

Table 1
Primers and PCR assays used in the detection of TBPs in water buffaloes from Bohol, Philippines.

Pathogen	Target gene	Oligonucleotide sequence (5' > 3')		Annealing temperature (°C)	Amplicon size (bp)	Detection limit	References
		Forward	Reverse				
<i>A. marginale</i>	<i>groEL</i>	GACTACCACATGCTCCATACTGACTG	GACGTCCACAACACTACTGCATTCAAG	74-65	866	10 ⁻³ ng ^a	Ybañez et al., 2012
		GTCTGAAGATGAGATTGCACAGGTTG	CCTTTGATGCCGTCCAGAGATGCA	74-68	618-768	10 ⁻⁸ ng ^a	
<i>B. bigemina</i>	<i>RAP-1a</i>	GAGTCTGCCAAATCCTTAC	TCCTCTACAGCTGCTTCG	55	879	10 ⁻⁸ % ^b	Terkawi et al., 2011; Cao et al., 2012
		AGCTTGCTTTCACAACFCGCC	TTGGTGCTTTGACCGACGACAT	55	412		
<i>B. bovis</i>	<i>SBP-2</i>	CTGGAAGTGGATCTCTGCAACC	TCACGAGCACTCTACGGCTTTGCAG	64	1236	10 ng ^a	Aboulaila et al., 2010
		GAATCTAGGCATATATAAGGCAT	ATCCCCCTCTAAGGTTGGCTAC	58	580	10 ⁻⁶ ng ^a	
<i>B. ovata</i>	<i>AMA-1</i>	GATACGAGGCTGTCGGTAGC	AGTATAGGTGAGCATCAGTG	56	504	10 ⁻⁴ ng ^a	Sivakumar et al., 2012
<i>T. annulata</i>	<i>Tams-1</i>	GTAACCTTTAAAAACGT	GTTACGAACATGGGTTT	55	721	3.8 × 10 ⁻⁴ % ^b	D'Oliveira et al., 1995; Martin-Sanchez et al., 1999
		CACCTCAAACATACCC	TGACCCACTTATCGTCC	60	453	n.a. ^c	
<i>T. orientalis</i>	<i>MPSP</i>	CTTTGCCTAGGATCTTCT	ACGGCAAGTGGTGAGAACT	58	777	n.a. ^c	Ota et al., 2009

RAP-1a: rhoptry associated protein-1a; *SBP-2*: spherical body protein-2; *AMA-1*: apical membrane antigen-1.

Tams-1: *T. annulata* merozoite surface-1; *MPSP*: major piroplasm surface protein.

^a Genomic DNA.

^b Parasitemia.

^c Not available.

method with Kimura 2-parameter model and 1000 bootstrap replications. Obtained sequences were deposited in the NCBI GenBank database [Accession numbers: MH265102-MH265104 (*A. marginale groEL*); MH265107-MH265110 (*B. bovis SBP-2*); MH265105-MH265106 (*B. bigemina RAP-1a*)].

2.7. Statistical analysis

Statistical significance of profile parameters of animals and PCR results were assessed using Pearson's chi-squared test and Fisher's exact test. The expected coinfection rates were computed using the conditional probability formula $P(A \text{ and } B) = P(A) \cdot P(B|A)$. A *P* value < 0.05 was considered statistically significant.

3. Results

In this study, only one water buffalo was found to be infested with ticks during blood collection. Fifty six percent of the animals were ≤ 6 years while 44% were > 6 years. Based on the body condition scores, most of the sampled animals were apparently healthy, with 51% in optimal health, 47% as overweight and 2% as underweight. Bulgarian Murrah, US Murrah, crossbred and Philippine carabao accounted for 67%, 27%, 11% and 1% of the sampled animals, respectively. No obvious clinical signs of tick-borne diseases were observed during sampling.

TBPs were detected in 46% of the samples (39% singly infected, 7% coinfecting). *A. marginale* was the predominant TBP infection with a

Table 2

Comparison between the expected and observed frequencies of TBP coinfections in water buffaloes from Bohol, Philippines.

	Coinfection rate					
	<i>A. marginale</i> and <i>B. bovis</i>		<i>A. marginale</i> and <i>B. bigemina</i>		<i>B. bovis</i> and <i>B. bigemina</i>	
	Expected (%)	Observed (%)	Expected (%)	Observed (%)	Expected (%)	Observed (%)
≤ 6 years old (N = 56)	8.03	5.36 (3/56)	1.31	1.79 (1/56)	0.75	0
> 6 years old (N = 44)	3.69	4.55 (2/44)	0.36	0	0.41	2.27 (1/44)
Total (N = 100)	6.09	5.00	0.80	1.00	0.60	1.00

detection rate of 29%, followed by *B. bovis* (21%), and *B. bigemina* (3%) infections. None of the blood samples were positive for *T. annulata*, *T. orientalis*, or *B. ovata*. Coinfection with *A. marginale* and *B. bovis* (5/7) occurred most frequently (Table 2). *A. marginale*, *B. bovis* and *B. bigemina* were detected from samples in Ubay. Four out of seven water buffaloes in Mabini were found to be singly infected with *B. bovis*.

A. marginale infection rates were significantly higher (37.5%) among animals aged ≤ 6 years ($P = 0.046$) than those > 6 years old (18.2%) (Table 3). *A. marginale* was detected in Bulgarian Murrah (36.1%) and US Murrah (25.9%) breeds, but not in crossbreds and Philippine carabao. However, detection rates among animal breeds were not statistically different ($P > 0.05$). Furthermore, the body condition score was not associated with *A. marginale* infection ($P > 0.05$). Meanwhile, none of the profile parameters were significantly associated with *B. bovis* or *B. bigemina* infection (Table 3).

Sequencing analysis revealed that three sequences (763 bp) of *A. marginale groEL* obtained in this study were identical to each other and were 100% identical to isolates from China, and sequences previously reported in cattle in Batangas and Cebu, Philippines (Fig. 2). The obtained sequences also showed 98.7–99.3% identity with those of isolates from South Africa, USA, Uruguay, Israel, Japan, Australia, and Uganda, and with previously reported cattle isolates in Batangas, Cebu, and Negros Oriental, Philippines (Fig. 2). On the other hand, this study generated four sequences of *B. bovis* based on SBP-2 gene, with homologies varying from 92.4 to 99.7%. Phylogenetic analysis indicated that three sequences belonged to the same subclade with Ghana and Thailand isolates while the other sequence grouped with Vietnam isolates and Texas strain (Fig. 3). Furthermore, two *B. bigemina RAP-1a* partial sequences (412 bp) obtained were identical to each other. BLASTn search and EMBOSS alignment showed that the genotype obtained in the present study had 100% homology with previous isolates from Thailand, Indonesia, Uruguay, and the Philippines. As shown in the phylogenetic tree, the RAP-1a gene is conserved across various geographical locations (Fig. 4).

Table 3

Detection rate and profile of water buffaloes screened for tick-borne pathogens in Bohol, Philippines.

Parameter	No. of tested	Detection rate (%)					
		<i>A. marginale</i>	<i>P</i> value	<i>B. bovis</i>	<i>P</i> value	<i>B. bigemina</i>	<i>P</i> value
Age							
≤ 6 years old	56	37.5 (21/56)	0.046*	21.4 (12/56)	1.0	3.6 (2/56)	1.0
> 6 years old	44	18.2 (8/44)		20.5 (9/44)		2.3 (1/44)	
Breed							
Bulgarian Murrah	61	36.1 (22/61)	0.051	16.4 (10/61)	0.44	3.3 (2/61)	1.0
US Murrah	27	25.9 (7/20)		29.6 (8/27)		3.7 (1/27)	
Crossbred	11	0		27.3 (3/11)		0	
Philippine Carabao	1	0		0		0	
Body Condition Score							
2 (underweight)	2	50.0 (1/2)	0.68	0	0.77	0	0.63
3 (optimal)	51	27.5 (14/51)		23.5 (12/51)		2.0 (1/51)	
4 (overweight)	47	29.8 (14/47)		19.1 (9/47)		4.3 (2/47)	
Total	100	29.0		21.0		3.0	

* P value < 0.05 is considered statistically significant.

4. Discussion

This study identified *A. marginale*, *B. bovis*, and *B. bigemina* as TBPs harbored by water buffaloes in Bohol, Philippines. This is the first report of detection of these pathogens in water buffaloes in the Visayas region of the country. *A. marginale* detection rate in the current survey (29%) was higher than that of a previous report in water buffaloes in the Philippines by Mingala et al. (2009) (10.3%). Meanwhile, *B. bigemina* infection rate (3%) in water buffaloes in Bohol was comparable to the results of a previous study in Luzon area which detected a 4.4% prevalence (Mingala et al., 2009).

Additionally, we report the first molecular confirmation of *B. bovis* infection in water buffaloes in the Philippines. The *B. bovis* infection rate (21%) in the current study is higher than that of Thailand (11.2%) (Terkawi et al., 2011), but lower than the documented *B. bovis* infection rates of 23.3% and 32.7% in Vietnam (Li et al., 2014; Weerasooriya et al., 2016). The relatively high infection rate may suggest endemicity of *B. bovis* in the area, although seroprevalence in animals and infection rate in tick vectors should be evaluated to prove this hypothesis. Although fatal to naïve cattle, natural infection with *B. bovis* is known to be asymptomatic in water buffaloes (Benitez et al., 2018), as observed in the infected animals in this study. This finding underpins the role of water buffalo as a reservoir of this economically important TBP of cattle.

Seven out of the 46 infected animals were coinfecting with two pathogens. The observed rates for concurrent infections of *A. marginale* and *B. bovis* (5%), *A. marginale* and *B. bigemina* (1%), and *B. bovis* and *B. bigemina* (1%) correspond to their respective expected coinfection rates of 6%, 0.8%, and 0.6% (Table 2). Coinfection with *Anaplasma* and *Babesia* are common in the field and can result in exacerbation of ill health in bovines (Figueroa et al., 1993; Hofmann-Lehmann et al., 2004). Likewise, the effects of concurrent *Anaplasma* and *Babesia* infections lead to the display of aggravated clinical and pathological indicators (Todorovic et al., 1975). Interestingly, the majority of the

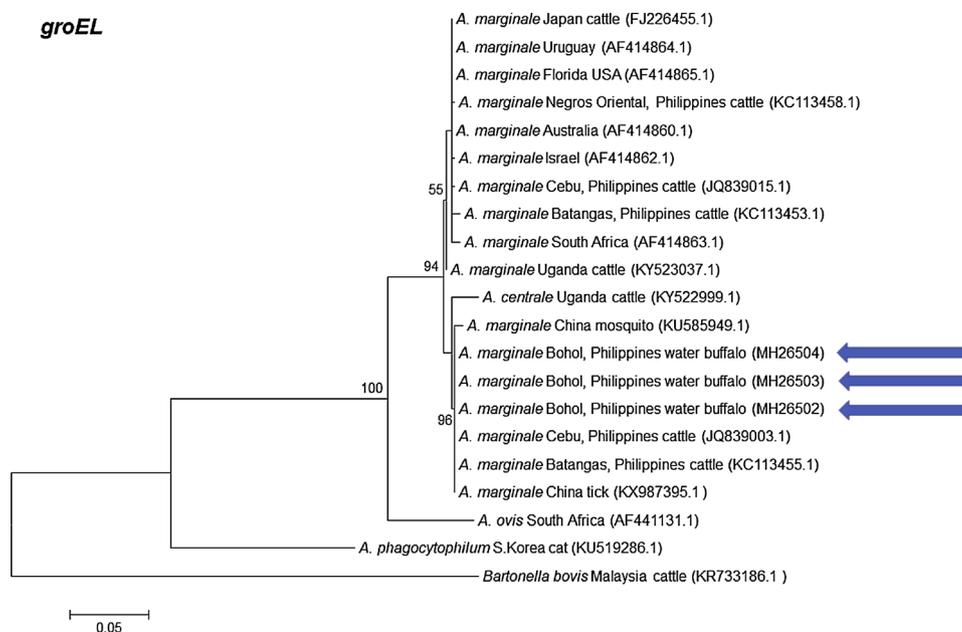


Fig. 2. Phylogenetic tree of *A. marginale* based on *groEL* gene. The tree was constructed using maximum likelihood method with Kimura-2 parameter model and 1000 bootstrap replications in MEGA 7.0. The three arrows indicate the sequences generated in this study and *Bartonella bovis* *groEL* sequence was used as the outgroup.

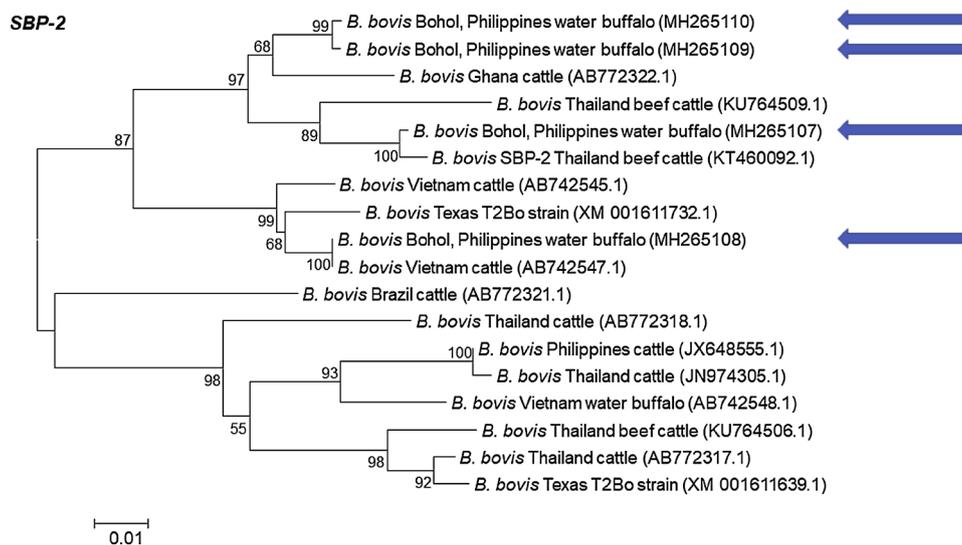


Fig. 3. Phylogenetic tree of *B. bovis* based on *SBP-2* gene. The unrooted tree was constructed using maximum likelihood method with Kimura-2 parameter model and 1000 bootstrap replications in MEGA 7.0. The four arrows indicate the sequences generated in this study.

animals tested in this study were apparently healthy despite being infected, indicating persistent infection with *Anaplasma* and *Babesia*. Pathogen persistence in the host is an important strategy for successful pathogen transmission to ticks and for developing resistance against reinfection of hosts (Brown, 2012; Chauvin et al., 2009). The evident minimal tick infestation observed in water buffaloes during sampling might have been a result of wallowing in mud puddles, which makes them less susceptible to ticks and other ectoparasites (Michelizzi et al., 2010). However, based on the high infection rates, it is likely that the animals have been previously exposed to ticks. In addition, the sampling period (December to May) may have influenced the observed presence of ticks as sampling was done prior to the monsoon season (Singh and Rath, 2018).

The current study also evaluated infection of *T. annulata*, *T. orientalis*, and *B. ovata* in water buffaloes, but none were detected. Studies conducted in cattle from the nearby Cebu island also yielded similar results (Yoshinari et al., 2012; Ybañez et al., 2013). However, *Theileria*

spp. was identified in cattle in other parts of the country, including Bohol (Belotindos et al., 2014; Ochirkhuu et al., 2015). It is highly likely that the aforementioned TBPs were not detected due to very low prevalence levels and the limited number of samples tested. Similarly, non-detection of *T. annulata* and *B. ovata* in water buffaloes may suggest the absence of *Hyalomma* spp. and *Haemaphysalis longicornis* ticks in Bohol and may be a worthwhile topic for future studies as the tick fauna in the area has not been explored yet.

A. marginale infection rate was significantly higher in young (≤ 6 years) compared to older animals, respectively (Table 3). Inverse age immunity is a well-known, distinct feature of *A. marginale*, *B. bovis*, and *B. bigemina* infections in < 1 -year-old bovines (Jonsson et al., 2012). However, since the ages of animals in the current study ranged from 2 to 18 years, the recorded infection rates cannot be explained based on this immunity feature. Regarding breeds, it is generally known that crossbred water buffaloes and Philippine carabao develop stronger disease resistance compared to purebred imported buffaloes (Nanda

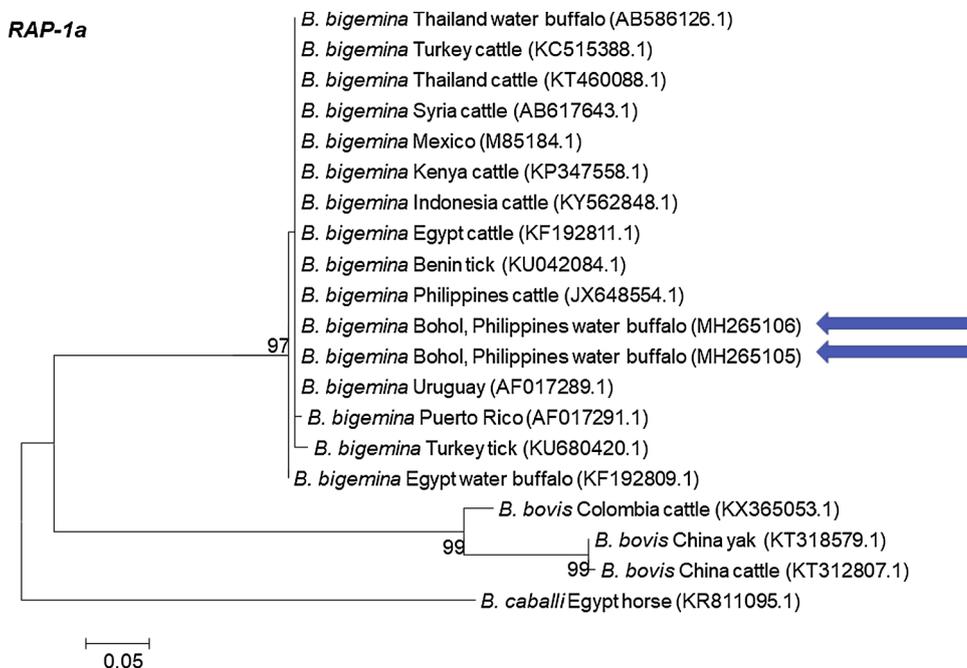


Fig. 4. Phylogenetic tree of *B. bigemina* based on RAP-1a gene. The tree was constructed using maximum likelihood method with Kimura-2 parameter model and 1000 bootstrap replications in MEGA 7.0. The two arrows indicate the sequences generated in this study and *Babesia caballi* RAP-1a sequence was used as the outgroup.

et al., 2003; Mingala et al., 2009). Nevertheless, the limited number of crossbred and Philippine carabao samples prevented this study from confirming the association of breed with TBP infections.

The genotype of *A. marginale* based on groEL gene identified in this study shared 98.7–100% identity with documented isolates from Negros Oriental, Batangas, and Cebu, Philippines. It has been suggested that the *Anaplasma* groEL gene is conserved across various locations in the Philippines and this might be an indication of endemicity of the pathogen in the country (Ybañez et al., 2014, 2012). Similarly, the phylogenetic tree of *B. bigemina* RAP1-a partial sequences revealed that the water buffalo-derived *B. bigemina* variant is highly identical to isolates from different regions of the world, suggesting its high conservation (Niu et al., 2016). *B. bigemina* RAP1 is known to be a family of several genes with five tandemly arranged polymorphic genes and is characterized with conserved protein features (Suarez et al., 1998; Niu et al., 2016). Conversely, the four sequences obtained for *B. bovis* based on the SBP-2 gene clustered in one clade different from a previously reported Philippine cattle isolate. The finding that *B. bovis* SBP-2 sequences showed some degree of diversity is compelling, considering that SBP-2 is recognized as a conserved gene among various geographical isolates (Jasmer et al., 1992; Gallego-Lopez et al., 2018). Further studies in different areas and other animal species utilizing different genes are suggested to verify the extent of genetic diversity of this pathogen.

The present study is the first molecular detection and characterization of *A. marginale*, *B. bigemina*, and *B. bovis* in water buffaloes in Bohol, Philippines. It also documents the first molecular confirmation of *B. bovis* infection in water buffaloes in the country. As interprovince trading of livestock animals is active between and among different areas in the Philippines, the information obtained in this study is beneficial, especially with the huge economic damages caused by TBDs to livestock farmers. Should the plan of importing 5000 heads of dairy cattle push through, the findings gathered in this study will be useful as baseline data for crafting effective TBD surveillance and prevention programs in the Philippines.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and

Technology of Japan, a grant from the Japanese Society for Promotion of Science Core-to-Core Program, Japan and a grant from the Commission on Higher Education of the Philippines.

References

- Aboulaila, M., Yokoyama, N., Igarashi, I., 2010. Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. *Vet. Parasitol.* 169, 45–50.
- Aubry, P., Geale, D.W., 2011. A review of bovine anaplasmosis. *Transbound. Emerg. Dis.* 58, 1–30.
- Belotindos, L.P., Lazaro, J.V., Villanueva, M.A., Mingala, C.N., 2014. Molecular detection and characterization of *Theileria* species in the Philippines. *Acta Parasitol.* 59, 448–453.
- Benitez, D., Mesplet, M., Echaide, I., de Echaide, S.T., Schnitger, L., Florin-Christensen, M., 2018. Mitigated clinical disease in water buffaloes experimentally infected with *Babesia bovis*. *Ticks Tick Borne Dis.* 9, 1358–1363.
- Bishop, R., Musoke, A., Morzaria, S., Gardner, M., Nene, V., 2004. *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. *Parasitology* 129, S271–S283.
- Bock, R., Jackson, L., De Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. *Parasitology* 129, S247–S269.
- Brown, W.C., 2012. Adaptive immunity to *Anaplasma* pathogens and immune dysregulation: implications for bacterial persistence. *Comp. Immunol. Microbiol. Infect. Dis.* 35, 241–252.
- Cao, S., Aboge, G.O., Terkawi, M.A., Yu, L., Kamyngkird, K., Luo, Y., Li, Y., Goo, Y.K., Yamagishi, J., Nishikawa, Y., Yokoyama, N., 2012. Molecular detection and identification of *Babesia bovis* and *Babesia bigemina* in cattle in northern Thailand. *Parasitol. Res.* 111, 1259–1266.
- Chauvin, A., Moreau, E., Bonnet, S., Plantard, O., Malandrin, L., 2009. *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet. Res.* 40, 37.
- D'Oliveira, C., Weide, M., Habela, M.A., Jacquet, P., Jongejan, F., 1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *J. Clin. Microbiol.* 33, 2665–2669.
- da Silva, J.B., dos Santos, P.N., de Santana Castro, G.N., da Fonseca, A.H., Barbosa, J.D., 2014. Prevalence survey of selected bovine pathogens in water buffaloes in the north region of Brazil. *J. Parasitol. Res.* 2014, 603484.
- de Castro, J., 1997. Sustainable tick and tickborne disease control in livestock improvement in developing countries. *Vet. Parasitol.* 71, 77–97.
- de la Fuente, J., Naranjo, V., Ruiz-Fons, F., Höfle, U., Fernández De Mera, I.G., Villanúa, D., Almazán, C., Torina, A., Caracappa, S., Kocan, K.M., Gortázar, C., 2005. Potential vertebrate reservoir hosts and invertebrate vectors of *Anaplasma marginale* and *A. phagocytophilum* in central Spain. *Vector Borne Zoonotic Dis.* 5, 390–401.
- Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. *Vet. Parasitol.* 50, 69–81.
- Foronda, J., Baticados, W., Baticados, A., 2010. Molecular evidence of *Babesia* spp. in cattle in the Philippines. *Online J. Vet. Res.* 14, 188–193.
- Gallego-Lopez, G.M., Lau, A.O.T., Brown, W.C., Johnson, W.C., Ueti, M.W., Suarez, C.E., 2018. Spherical body protein 2 truncated copy 11 as a specific *Babesia bovis*

- attenuation marker. *Parasit. Vectors* 11, 169.
- Herrera, P.C., Vilorio, V.V., Balbin, M.M., Mingala, C.N., 2017. Prevalence of babesiosis (*Babesia bovis* and *Babesia bigemina* in cattle and water buffalo in Nueva Ecija, Philippines using nested polymerase chain reaction. *Ann. Parasitol.* 63, 309–316.
- Hofmann-Lehmann, R., Meli, M.L., Dreher, U.M., Gónczi, E., Deplazes, P., Braun, U., Engels, M., Schüpbach, J., Jörgler, K., Thoma, R., Griot, C., Stärk, D.C.K., Willi, B., Schmidt, J., Kocan, K.M., Lutz, H., 2004. Concurrent infections with vector-borne pathogens associated with fatal hemolytic anemia in a cattle herd in Switzerland. *J. Clin. Microbiol.* 42, 3775–3780.
- Jasmer, D.P., Reduker, D.W., Perryman, L.E., McGuire, T.C., 1992. A *Babesia bovis* 225-kilodalton protein located on the cytoplasmic side of the erythrocyte membrane has sequence similarity with a region of glycogen phosphorylase. *Mol. Biochem. Parasitol.* 52, 263–269.
- Jonsson, N.N., Bock, R.E., Jorgensen, W.K., Morton, J.M., Stear, M.J., 2012. Is endemic stability of tick-borne disease in cattle a useful concept? *Trends Parasitol.* 28, 85–89.
- Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The natural history of *Anaplasma marginale*. *Vet. Parasitol.* 167, 95–107.
- Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874.
- Kuttler, K.L., 1984. *Anaplasma* infections in wild and domestic ruminants: a review. *J. Wildl. Dis.* 20, 12–20.
- Li, Y., Luo, Y., Cao, S., Terkawi, M.A., Lan, D.T., Long, P.T., Yu, L., Zhou, M., Gong, H., Zhang, H., Zhou, J., 2014. Molecular and seroepidemiological survey of *Babesia bovis* and *Babesia bigemina* infections in cattle and water buffaloes in the central region of Vietnam. *Trop. Biomed.* 31, 406–413.
- Mans, B.J., Pienaar, R., Latif, A.A., 2015. A review of *Theileria* diagnostics and epidemiology. *Int. J. Parasitol. Parasites Wildl.* 4, 104–118.
- Martin-Sanchez, J., Viseras, J., Adroher, F.J., Garcia-Fernandez, P., 1999. Nested polymerase chain reaction for detection of *Theileria annulata* and comparison with conventional diagnostic techniques: its use in epidemiology studies. *Parasitol. Res.* 85, 243–245.
- Michelizzi, V.N., Dodson, M.V., Pan, Z., Amaral, M.E.J., Michal, J.J., McLean, D.J., Womack, J.E., Jiang, Z., 2010. Water buffalo genome science comes of age. *Int. J. Biol. Sci.* 6, 333.
- Mingala, C.N., Konnai, S., Cruz, L.C., Onuma, M., Ohashi, K., 2009. Comparative molecular-immunological analysis of swamp- and riverine-type water buffaloes responses. *Cytokine* 46, 273–282.
- Minjauw, B., McLeod, A., 2003. Tick-borne Diseases and Poverty, the Impact of Ticks and Tick-borne Diseases on the Livelihood of Small Scale and Marginal Livestock Owners in India and Eastern and Southern Africa. DFID Animal Health Programme. Centre for Tropical Veterinary Medicine, University of Edinburgh, UK.
- Nanda, A.S., Brar, P.S., Prabhakar, S., 2003. Enhancing reproductive performance in dairy buffalo: major constraints and achievements. *Reprod. Suppl.* 61, 27–36.
- National Research Council, 1981. *The Water Buffalo: New Prospects for an Underutilized Animal*: Report. National Academy Press, Washington, D.C., USA.
- Niu, Q., Bonsergent, C., Rogniaux, H., Guan, G., Malandrini, L., Moreau, E., 2016. RAP-1a is the main rhoptry-associated-protein-1 (RAP-1) recognized during infection with *Babesia* sp. BQ1 (Lintan)(*B. motasi*-like phylogenetic group), a pathogen of sheep in China. *Vet. Parasitol.* 232, 48–57.
- Ochirkhuu, N., Konnai, S., Mingala, C.N., Okagawa, T., Villanueva, M.A., Pilapil, F.M.I., Murata, S., Ohashi, K., 2015. Molecular epidemiological survey and genetic analysis of vector-borne infections of cattle in Luzon island, the Philippines. *Vet. Parasitol.* 212, 161–167.
- Ota, N., Mizuno, D., Kuboki, N., Igarashi, I., Nakamura, Y., Yamashina, H., Hanzaike, T., Fujii, K., Onoe, S., Hata, H., Kondo, S., 2009. Epidemiological survey of *Theileria orientalis* infection in grazing cattle in the eastern part of Hokkaido, Japan. *J. Vet. Med. Sci.* 71, 937–944.
- Philippine Statistics Authority, 2017. *Livestock and Poultry: Inventory by Animal Type, Farm Type, by Region, by Province, by Quarter and by semester/year*. (Accessed 21 August 2017). <http://countrystat.psa.gov.ph/?cont=10&pageid=1&ma=C00PNLPI>.
- Ringo, A.E., Mounouni, P.F.A., Lee, S.H., Liu, M., Khamis, Y.H., Gao, Y., Guo, H., Zheng, W., Efstratiou, A., Galon, E.M., Li, J., Tiwananthagorn, S., Inoue, N., Suzuki, H., Thekisoe, O., Xuan, X., 2018. Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens isolated from cattle on Pemba Island, Tanzania. *Ticks Tick-Borne Dis.* 9, 1437–1445.
- Sackett, D., Holmes, P., 2006. Assessing the Economic Cost of Endemic Disease on the Profitability of Australian Beef Cattle and Sheep Producers. Final Report on Project AHW.087. Meat and Livestock Australia Limited, North Sydney, Australia, pp. 21–29.
- Singh, N.K., Rath, S.S., 2018. Epidemiology of ixodid ticks in buffaloes (*Bubalus bubalis*) in Punjab, India. *Buffalo Bull.* 35, 347–353.
- Sivakumar, T., Tagawa, M., Yoshinari, T., Ybañez, A.P., Igarashi, I., Ikehara, Y., Hata, H., Kondo, S., Matsumoto, K., Inokuma, H., 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.
- Sivakumar, T., Hayashida, K., Sugimoto, C., Yokoyama, N., 2014. Evolution and genetic diversity of *Theileria*. *Infect. Genet. Evol.* 27, 250–263.
- Suarez, C.E., Noh, S., 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. *Vet. Parasitol.* 180, 109–125.
- Suarez, C.E., Palmer, G.H., Hötzel, I., Hines, S.A., McElwain, T.F., 1998. Sequence and functional analysis of the intergenic regions separating babesial rhoptry-associated protein-1 (rap-1) genes. *Exp. Parasitol.* 90, 189–194.
- Terkawi, M.A., Huyen, N.X., Shinuo, C., Inpankaew, T., Maklon, K., Aboulaila, M., Ueno, A., Goo, Y.K., Yokoyama, N., Jittapalpong, S., Xuan, X., 2011. Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand. *Vet. Parasitol.* 178, 201–207.
- Todorovic, R.A., Gonzalez, E.F., Adams, L.G., 1975. *Babesia bigemina*, *Babesia argentina*, and *Anaplasma marginale*: coinfectious immunity in bovines. *Exp. Parasitol.* 37, 179–192.
- Uilenberg, G., 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol.* 57, 19–41.
- Uilenberg, G., 2006. *Babesia*—a historical overview. *Vet. Parasitol.* 138, 3–10.
- Vatsya, S., Kumar, R.R., Singh, V.S., Arunraj, M.R., 2013. *Anaplasma marginale* infection in a buffalo: a case report. *Vet. Res. Int.* 1, 51–53.
- Weerasooriya, G., Sivakumar, T., Lan, D.T.B., Long, P.T., Takemae, H., Igarashi, I., Inoue, N., Yokoyama, N., 2016. Epidemiology of bovine hemoprotozoa parasites in cattle and water buffalo in Vietnam. *J. Vet. Med. Sci.* 78, 1361–1367.
- Ybañez, A.P., Sivakumar, T., Ybañez, R.H.D., Ratilla, J.C., Perez, Z.O., Gabotero, S.R., Hakimi, H., Kawazu, S., Matsumoto, K., Yokoyama, N., Inokuma, H., 2012. First molecular characterization of *Anaplasma marginale* in cattle and *Rhipicephalus (Boophilus) microplus* ticks in Cebu, Philippines. *J. Vet. Med. Sci.* 75, 27–36.
- Ybañez, A.P., Sivakumar, T., Ybañez, R.H.D., Vincoy, M.R.B., Tingson, J.A., Perez, Z.O., Gabotero, S.R., Buchorno, L.P., Inoue, N., Matsumoto, K., Inokuma, H., Yokoyama, N., 2013. Molecular survey of bovine vector-borne pathogens in Cebu, Philippines. *Vet. Parasitol.* 196, 13–20.
- Ybañez, A.P., Ybañez, R.H.D., Claveria, F.G., Cruz-Flores, M.J., Xuan, X., Yokoyama, N., Inokuma, H., 2014. High genetic diversity of *Anaplasma marginale* detected from Philippine cattle. *J. Vet. Med. Sci.* 76, 1009–1014.
- Yoshinari, T., Sivakumar, T., Asada, M., Battsetseg, B., Huang, X., Lan, D.T.B., Inpakaew, T., Ybañez, A.P., Alhassan, A., Thekisoe, O., De Macedo, A.C.C., Inokuma, H., Igarashi, I., Yokoyama, N., 2012. A PCR based survey of *Babesia ovata* in cattle from various Asian, African and South American countries. *J. Vet. Med. Sci.* 75, 211–214.
- Yu, L., Terkawi, M.A., Cruz-Flores, M.J., Claveria, F.G., Aboge, G.O., Yamagishi, J., Goo, Y.K., Cao, S., Masatani, T., Nishikawa, Y., Xuan, X., 2013. Epidemiological survey of *Babesia bovis* and *Babesia bigemina* infections of cattle in Philippines. *J. Vet. Med. Sci.* 75, 995–998.