



## First molecular detection of *Mycoplasma wenyonii* and the ectoparasite biodiversity in dairy water buffalo and cattle in Bohol, Philippines



Adrian P. Ybañez<sup>a,b,1</sup>, Rochelle Haidee D. Ybañez<sup>b,c,1</sup>, Reynald Klint M. Armonia<sup>b</sup>, James Knowell E. Chico<sup>b</sup>, Kevin James V. Ferraren<sup>b</sup>, Emerson P. Tapdasan<sup>d</sup>, Caro B. Salces<sup>d</sup>, Bon Christian A. Maurillo<sup>e</sup>, Eloiza May S. Galon<sup>c</sup>, Adrian Miki C. Macalanda<sup>c,f</sup>, Paul Franck A. Moumouni<sup>c</sup>, Xuenan Xuan<sup>c,\*</sup>

<sup>a</sup> College of Veterinary Medicine at Barili Campus and Center for Vector-borne and Protozoan Diseases at Main Campus, Cebu Technological University, Cebu, Philippines

<sup>b</sup> College of Science, University of the Philippines Cebu, Gorordo Avenue Lahug, Cebu City, Philippines

<sup>c</sup> National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro City, Japan

<sup>d</sup> Philippine Carabao Center at Ubay Stock Farm, Lomangog, Ubay, Bohol, Philippines

<sup>e</sup> National Dairy Authority, Lomangog, Ubay, Bohol, Philippines

<sup>f</sup> Department of Immunopathology and Microbiology, College of Veterinary Medicine and Biomedical Sciences, Cavite State University, Cavite 4122, Philippines

### ARTICLE INFO

#### Keywords:

Dairy water buffalo

Dairy cattle

*Mycoplasma wenyonii*

Ectoparasites

PCR

### ABSTRACT

Hemoplasmosis caused by *Mycoplasma* spp. have been associated with major economic losses in the global dairy production. Hemoplasma studies in the Philippines are limited despite its potential impact. This study mainly aimed to detect the presence of hemoplasma species in dairy water buffaloes and cattle and know their ectoparasite biodiversity in Bohol, Philippines. Detection of *Mycoplasma* spp. was performed using peripheral blood smear examination (PBSE) and standard PCR using whole blood samples collected from 100 dairy water buffaloes and 40 dairy cattle. Available records on the average annual, monthly and daily milk production were compared between PCR-positive and PCR-negative animals. Ectoparasites were manually collected and identified. While PBSE results were all negative, PCR testing showed that 80% (80 water buffaloes and 32 cattle) were positive for *Mycoplasma* spp. On the other hand, a total of 1436 ectoparasites were collected (609 *Haematopinus* and 827 *Rhipicephalus* spp.). DNA sequencing revealed that obtained sequences (193 bp) from 7 animals were 99.5 to 100% similar to registered *Mycoplasma wenyonii* sequences. The study reports the first molecular characterization of *M. wenyonii* in the Philippines and probably the first detection in dairy water buffaloes in Southeast Asia.

### 1. Introduction

The livestock industry has contributed to the Philippine economy, with the dairy sector reporting a gross income of almost three-fourths of a billion in 2016 [1]. The main milk-producing animals that accounted for 97% of the country's milk production are cattle and water buffaloes [2]. These animals under the family Bovidae are vulnerable to several economically important diseases, including those that are transmitted by ticks [3].

Tropical regions, including the Philippines, are suitable environments for ticks. Ticks can infest a variety of hosts and transmit tick-borne diseases (TBDs) to humans, livestock, and companion animals. In many countries, TBDs are major health impediments to efficient

livestock production and have caused major economic losses worldwide [4]. TBDs have affected bovine productivity in tropical and subtropical regions of the world, leading to a significant impact on the livelihood of resource-poor farming communities. In the Philippines, among the reported TBDs include hemoplasmosis in cattle [5]. It is caused by hemoplasmas or hemotropic *Mycoplasma* species, which are wall-less bacteria that cause infectious anemia in a variety of mammalian species [6]. The pathogen can be transmitted by blood-sucking arthropods such as ticks [7]. It has been known to cause infertility in dogs [8,9], delayed estrus in pigs [10] and decreased milk productivity in a variety of mammals including sows [11,12] and cattle [13]. The major effect of hemoplasmosis is on its economic impact as infected animals may not show obvious clinical signs (and thus underdiagnosed), but will have a

\* Corresponding author.

E-mail address: [gen@obihiro.ac.jp](mailto:gen@obihiro.ac.jp) (X. Xuan).

<sup>1</sup> Equal author.

decreased milk production.

In bovine hemoplasmosis, two species have been reported: *Mycoplasma wenyonii* [14] and *Candidatus Mycoplasma haemobos* [15]. Bovine hemoplasmosis has been mostly detected in cattle. However, *Ca. M. haemobos* has been detected in water buffaloes in China [16]. At present, hemoplasma studies in livestock in the Philippines have been very limited despite its potential economic impact to the industry. Hence, this study aimed to detect the presence of hemoplasma species (*Mycoplasma* spp.) in dairy water buffaloes and cattle, know the ectoparasite biodiversity and environmental profile of the selected animals, and assess its implications on milk productivity in Bohol, Philippines. The present study reports the first molecular detection of hemoplasma (*Mycoplasma* spp.) in dairy water buffaloes in the country.

## 2. Materials and methods

### 2.1. Animals and study site

A total of 100 dairy water buffaloes and 40 dairy cattle, regardless of age and breed, were selected from the Ubay dairy stock farm and selected local farms in National Dairy Authority farm in Bohol, Philippines. Body condition score was evaluated [17] and clinical signs were noted. Ectoparasite collection was performed in the field, while its identification was performed at the University of the Philippines Cebu. Peripheral Blood Smear Examination (PBSE), DNA extraction, and Polymerase Chain Reaction (PCR) testing was also performed in the same institution.

### 2.2. Blood sample collection and peripheral blood smear examination

Approximately 5 ml of blood sample were collected from the jugular vein of the water buffaloes using a BD K3 EDTA Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ, and the USA). Thin blood smears were prepared from the samples for PBSE. The slides were stained with Hemacolor color regent blue and red stains [18]. Blood samples were stored at  $-20^{\circ}\text{C}$  until DNA extraction was conducted [19].

### 2.3. Ectoparasite collection and identification

Ectoparasites were carefully collected from the animals using forceps and preserved in 70% ethyl alcohol. Identification was performed under a stereomicroscope using appropriate guides [20–22].

### 2.4. DNA extraction and PCR

DNA extraction was performed as previously described [23]. For PCR, the target fragments were amplified by using the forward primer (F2 – ACGAAAGTCTGATGGAGCAATA) and reverse primer (R2 – ACGCCCAATAAATCCGRATAAT [24]). The final volume used was 10  $\mu\text{l}$  which was composed of 4.3  $\mu\text{l}$  DDW, 2  $\mu\text{l}$   $5\times$  PCR buffer, 1  $\mu\text{l}$  of  $\text{MgCl}_2$  (25 mM), 0.15  $\mu\text{l}$  of dNTPs (10 mM each), 0.25  $\mu\text{l}$  each of the forward and reverse primers (0.22  $\mu\text{M}$ /reaction), 0.05  $\mu\text{l}$  of Taq DNA Polymerase (5 U/  $\mu\text{l}$ ) (Promega Corporation, USA) and 2  $\mu\text{l}$  of DNA template. Amplification cycles included initial denaturation at  $95^{\circ}\text{C}$  for 3 min, 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $52^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min followed by a final extension at  $72^{\circ}\text{C}$  for ten mins to amplify a 190-bp target. Agarose gel electrophoresis was performed as previously described [23].

### 2.5. Data processing, sequencing, and analyses

Data from the survey instrument (profile), daily milk yield, and PBSE and PCR results were manually tabulated in a tally sheet and then encoded to a Microsoft Excel using appropriate coding to facilitate statistical analyses. Association between daily milk yield and the PCR

**Table 1**

Profile of selected water buffaloes in Bohol ( $n = 100$ ).

Parameter	Category	Frequency	Percentage
Age	Below 5	19	19%
	Mean = 7.31 5 to 6	33	33%
	SD = 3.53 above 6	48	48%
Breed	Bulgarian Murrah	61	61%
	United States Murrah	27	27%
	Philippine Carabao	1	1%
	Cross breed	11	11%
Body condition score	< 3	2	2%
	Mean = 3.75 3	51	51%
	SD = 0.71 > 3	47	47%
Feeding method	Grazing only	80	80
	Cut and Carry	0	0
	Both	20	20
Animal location	PCC-Ubay Stock Farm	80	80
	Ubay Smallholder farms	12	12
	Alicia Smallholder farms	1	1
	Mabini farms	7	7
Clinical signs status	Present	4	4
	Absent	96	96

**Table 2**

PCR results of water buffaloes and cattle tested for *Mycoplasma* spp. in Bohol, Philippines ( $n = 100$ ).

Animal	PCR Results				Total
	Positive	Relative %	Negative	Relative %	
Water buffalo	80	80	20	20	100
Cattle	32	80	8	20	40
Total	112	80	28	20	140

results (positive or negative) was assessed using Chi-square method. Sequencing and phylogenetic analysis were similarly performed as previously described [23–25]. Phylogenetic analyses were performed using MEGA 5.0 software by Neighbor Joining Method and Maximum Likelihood (1000 replicates). Several sequences with low homology with the obtained sequences were removed in the final tree.

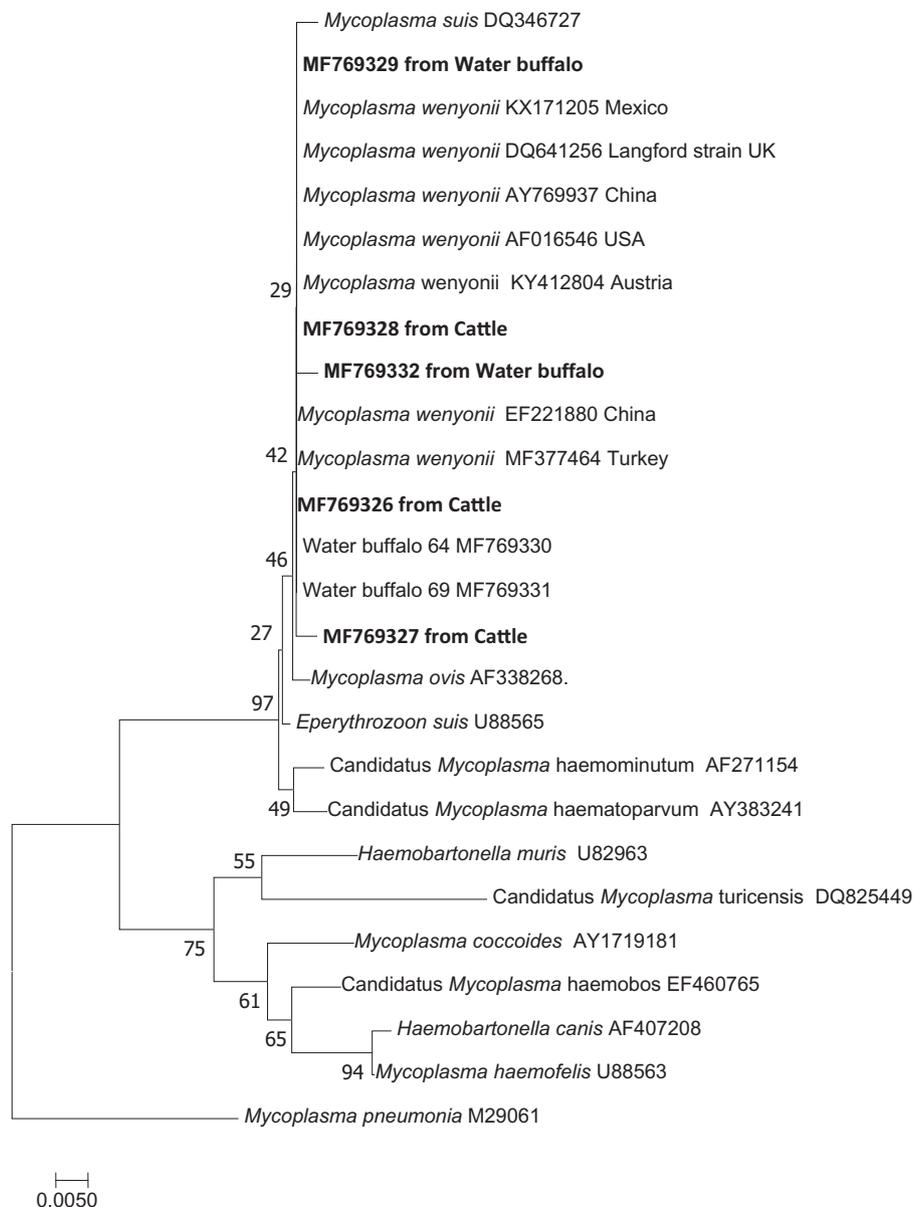
### 2.6. Ethical considerations

The study was conducted in accordance with the Animal Welfare Law of the Philippines (RA 8485) and the Department of Agriculture Administrative Order 40 of the Philippines.

## 3. Results and discussion

Most of the sampled water buffaloes were Bulgarian Murrah (61%), five years old and above (81%), and with an optimal body condition score (51%). Most were fed by grazing (80%), and no observed clinical signs (96%) (Table 1). This indicates that the dairy water buffaloes were mostly healthy and properly nourished. The predominant breed was Murrah, which is usually preferred due to its high average milk yield and efficient milk and fat production [26]. It is the top milk breeder in Southeast Asia and is regarded as the best breed improver. From those with observed clinical signs, three had red eyes and nasal discharges. One showed weight loss. For the cattle, the profile was unfortunately not provided.

A total of 1436 ectoparasites (609 lice and 827 ticks) were collected from the selected water buffaloes and cattle in the area (Table not shown). A total of 2 genera were identified. The collected lice were morphologically identified as *Haematopinus* spp., which was consistent to the description of [27]. On the other hand, the collected ticks were identified as *Rhipicephalus* spp. [21,22]. These species have been identified as potential vectors of *Mycoplasma* spp. [28,29]. Not all of the



**Fig. 1.** Phylogenetic analysis of *Mycoplasma wenyonii* from cattle and water buffalo in the Philippines based on the 16S rRNA gene using Neighbor-Joining method (1000 replicates). Obtained sequences are highlighted in bold.

**Table 3**  
Comparison of daily milk average (in liters) of *Mycoplasma* spp.-infected and *Mycoplasma* spp.-uninfected dairy water buffaloes in Bohol, Philippines (n = 100).

Category	Daily milk average per year (L)					
	2015		2016		2017*	
	Yield	SD	Yield	SD	Yield	SD
<i>Mycoplasma</i> spp. infected (n = 80)	5.2	1.48	4.69	1.3	5.33	1.42
<i>Mycoplasma</i> spp. uninfected (n = 20)	5.35	1.53	4.27	1.38	4.47	1.27

\*p value = .048 df = 1

sampled water buffaloes and cattle had ectoparasites (Table not shown). Only 115 out of 140 animals were observed with ectoparasites. All cattle (n = 40) had ticks, while only one water buffalo was found with tick. Conversely, no lice was found on cattle but was observed in

74 water buffaloes. Only one water buffalo had both the tick and lice. This observed difference where lice was only found in water buffaloes may be due to the wallowing practice of the animal [30]. Compared to ticks, lice can survive under water for several hours and can close their breathing holes. Although cattle can also wallow, those in this study were not allowed to do so.

All samples were negative for PBSE. The diagnosis of hemoplasma infection has been traditionally performed via microscopic examination of the bacterial pathogen on the surface of the erythrocyte or in the plasma, but the sensitivity and specificity of this method are low [7,31]. PBSE requires proficiency in identifying pathogens and is error-prone because artifacts can be interpreted as inclusion bodies or pathogens [23]. PBSE's diagnostic sensitivity is generally < 20% in animals that are chronically infected, and its specificity is hampered by artifacts such as Howell-Jolly bodies [29,32]. Previous studies have shown the low reliability of PBSE in the detection of *Mycoplasma* spp. in cattle [5].

PCR revealed that 80% of the tested animals were found to be positive for *Mycoplasma* spp. (Table 2). The results show that PCR is more sensitive than PBSE in detecting the pathogen [5]. Several other studies

have favored the use of PCR in diagnosing bovine hemoplasmosis [7,15,33,40]. On the other hand, DNA sequencing revealed that obtained sequences (193 bp) from 7 animals were 99.5 to 100% identical to registered *M. wenyonii* sequences from several countries around the world. Phylogenetic analyses revealed similar clade patterns regardless of the method used which indicated that the obtained sequences were not divergent from *M. wenyonii* (Fig. 1).

The high detection rate in this study may be caused by several factors, including the presence of ectoparasites in the majority of the animals. Although little is known in the means of transmission of *Mycoplasma* spp., some evidences have suggested that flies, lice, and fleas may facilitate mechanical transmission [34,35] while blood-sucking arthropods such as ticks may serve as biological vectors [36–38]. Another factor can be the feeding method where animals were mainly fed via grazing, which allows increased exposure to ectoparasites in open grass area. Pastured animals are believed to be more susceptible to TBDs than those confined [7]. Despite being PCR-positive, those infected in the present study were apparently healthy. These findings were similar to another study wherein majority of the hemoplasma-infected cattle appeared to be healthy [39].

Although the hematological analysis was not performed in this study, PCR detection is sufficient in the diagnosis of hemoplasma infection. Studies have reported that cattle infected with *M. wenyonii* did not show any significant difference in their hematological parameters [40] and hematocrit values [33] with those hemoplasma-negative cattle. Moreover, cattle infected with bovine hemoplasmas showed significantly lower red blood cells (RBC), hemoglobin (HB) and packed cell volume (PCV) levels and a higher mean corpuscular volume (MCV) [7]. These conflicting findings indicate that the sole use of hematological results as a basis for diagnosing bovine hemoplasmosis can be unreliable.

Milk yield data from the cattle were not complete as only 27 had the information. Thus, only the water buffalo information was used (Table 3). *Mycoplasma* sp.-infected animals showed lower milk yield in 2015 than uninfected animals. However, milk yield was observed to be higher in PCR-positive animals than those negative in 2016 and 2017. Statistical analysis showed that there is a significant difference in the milk productivity among *Mycoplasma* spp.-infected and uninfected water buffaloes in 2017. No significant differences were observed in the previous years.

Records showed that the daily milk average of *Mycoplasma* spp.-negative animals was sub-optimal. Optimal milk values of Murrah buffaloes, which comprised majority of the water buffaloes, ranges from 6 to 8 l per day [41]. This implies that milk production of the animals is indeed lower than normal, but the degree of the drop is higher for PCR-negative animals than *Mycoplasma* sp.-infected groups.

The results of the present study did not coincide with the study of [13] where hemoplasma-infected groups showed consistent lower milk yields than hemoplasma-negative animals. In the present study, opposite results were observed as hemoplasma-infected groups showed higher daily milk average than non-infected animals. Several factors may have contributed to such results. Among which is the possible occurrence of co-infection with other pathogens in *Mycoplasma* sp.-infected animals, which can lead to interference phenomenon where the effect of one pathogen can hinder the effect of a prior pathogen. The effect of *Mycoplasma* sp. in milk productivity was possibly resisted or hampered by the presence of another pathogen. This interaction was observed between *Theileria orientalis* and hemotropic *Mycoplasma* spp. in grazing cattle [13]. Another factor is the possible presence of other pathogens in PCR-negative animals. Previous studies have shown that besides *Mycoplasma* spp., other pathogens such as *Anaplasma* species and *Babesia* species can decrease milk production in animals [42,43].

Statistical analysis revealed no significant association between *Mycoplasma* sp. positivity and ectoparasites presence. It implies that an animal can be positive for *Mycoplasma* sp. even if no ectoparasites are observed. This observation has been reported in similar studies [44,45].

Profile parameters in water buffalo also revealed no significant association with *Mycoplasma* sp. positivity.

The improvement of the dairy industry is among the targets of the Philippine government. Several research and development endeavours have been conducted to improve dairy productivity. However, only a few studies have dealt with pathogens that may have a direct impact on the dairy animals. Hemoplasmosis is known to affect milk productivity. Its greatest effect may not be on the direct observable clinical signs, but on the economic impact it may cause on the dairy production which may go unnoticed. The present study calls for more studies especially on the potential effects of this pathogen in the different areas of the country. As other tick-borne pathogens have been detected, including Anaplasmosis and Babesiosis, its interaction with the *Mycoplasma* sp. can be further studied.

#### 4. Conclusion

*M. wenyonii* was detected in water buffaloes and dairy cattle in Bohol, Philippines. Two genera of ectoparasites (*Haematopinus* spp. and *Rhipicephalus* spp.) were observed. The present study reports the first molecular confirmation of *M. wenyonii* in dairy cattle and water buffaloes in the Philippines.

#### Acknowledgment

This research is supported by grants from the Commission on Higher Education (CHED) of the Philippines, and from the Japanese Society for Promotion of Science Core-to-Core Program Grant.

#### References

- [1] Philippine Statistic Authority, Performance of Philippine Agriculture January–December '16, (2016).
- [2] Philippine Statistic Authority, Foreign trade statistics 2016, NDA-PMSD Philippine Dairy Update (2016) 2016.
- [3] S. Ghosh, P. Azhahianambi, M.P. Yadav, Upcoming and future strategies of tick control: a review, *J. Vector Borne Dis.* 44 (2) (2007) 79.
- [4] J.J. de Castro, Sustainable tick and tickborne disease control in livestock improvement in developing countries, *Vet. Parasitol.* 71 (2–3) (1997) 77–97.
- [5] A.P. Ybañez, R.H.D. Ybañez, M. Tagawa, Molecular detection of hemoplasma species (*Mycoplasma* spp.) in Cattle in Cebu, Philippines, *J. Adv. Vet. Res.* 5 (1) (2015) 43–46.
- [6] E. Spada, D. Proverbio, P. Galuzzo, A. Pepa, G. Giorgi, R. Perego, E. Ferro, Prevalence of haemoplasma infections in stray cats in Northern Italy, *ISRN Microbiol.* (2013) 298352.
- [7] M. Tagawa, A.P. Ybañez, K. Matsumoto, N. Yokoyama, Prevalence and risk factor analysis of bovine hemoplasma infection by direct PCR in eastern Hokkaido, Japan, *J. Vet. Med. Sci.* 74 (9) (2012) 1171–1176.
- [8] J.B. Messick, Hemotropic *Mycoplasma* (Hemoplasmas): a review and new insights into pathogenic potential, *Vet. Clin. Pathol.* 33 (1) (2004) 2–13.
- [9] V. Chalker, Canine mycoplasma, *Res. Vet. Sci.* 79 (1) (2005) 1–8.
- [10] J. Wu, J. Yu, C. Song, S. Sun, Z. Wang, Porcine eperythrozoonosis in China, *Ann. N. Y. Acad. Sci.* 1081 (1) (2006) 280–285.
- [11] S.C. Henry, Clinical observations on eperythrozoonosis, *J. Am. Vet. Med. Assoc.* 174 (1979) 601–603.
- [12] G.M. Zinn, G.W. Jesse, A.W. Dobson, Effect of eperythrozoonosis on sow productivity, *J. Am. Vet. Med. Assoc.* 182 (1983) 369–371.
- [13] M. Tagawa, K. Yamakawa, T. Aoki, K. Matsuyama, M. Ishi, H. Inokuma, Effect of chronic hemoplasma infection on cattle productivity, *J. Vet. Med. Sci.* 75 (10) (2013) 1271–1275.
- [14] I. Nishizawa, M. Sato, M. Fujihara, S. Sato, R. Harasawa, Differential detection of hemotropic *Mycoplasma* species in cattle by melting curve analysis of PCR products, *J. Vet. Med. Sci.* 72 (1) (2010) 77–79.
- [15] M. Tagawa, K. Matsumoto, H. Inokuma, Molecular detection of *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* in cattle in Hokkaido, Japan, *Vet. Microbiol.* 132 (2008) 177–180.
- [16] Q.L. Su, H.Q. Song, R.Q. Lin, Z.G. Yuan, J.F. Yang, G.H. Zhao, W.Y. Huang, X.Q. Zhu, The detection of "Candidatus *Mycoplasma haemobos*" in cattle and buffalo in China, *Trop. Anim. Health Prod.* 42 (8) (2010) 1805–1808.
- [17] A. Alapati, S.R. Kapa, S. Jeepalyam, S.M. Rangappa, K.R. Yemireddy, Development of the body condition score system in Murrah buffaloes: validation through ultrasonic assessment of body fat reserves, *J. Vet. Sci.* 11 (1) (2010) 1–8.
- [18] J. Jaso, A. Nguyen, A.N. Nguyen, A synoptic reporting system for peripheral blood smear interpretation, *Am. J. Clin. Pathol.* 135 (3) (2011) 358–364.
- [19] C. Hartmann, K. Lennartz, H. Ibrahim, A. Coz, Y. Kasper, C. Lenz, M. Polidori, Stable 16-year storage of DNA purified with the QIAamp® DNA, Blood Mini Kit. QIAGEN

- (2016) 1–4.
- [20] C.T. Chung, R.H. Miller, A rapid and convenient method for the preparation and storage of competent bacterial cells, *Nucleic Acids Res.* 16 (8) (1988) 3580.
- [21] A.R. Walker, Ticks of domestic animals in Africa: a guide to identification of species, Edinburgh: Bioscience Reports, 2003, pp. 3–210.
- [22] H.P. Portugaliza, M.A. Bagot, Different species of lice (Phthiraptera), fleas (Siphonaptera) and ticks (Ixodida) collected from livestock, poultry, reptile and companion animal in Leyte Island, Philippines, *Livestock Res. Rural Dev (LRRD)* 27 (2015) 151.
- [23] A.P. Ybañez, T. Sivakumar, R.H.D. Ybañez, M.R.B. Vincoy, J.A. Tingson, Z.O. Perez, S.R. Gabotero, L.P. Buchorno, N. Inoue, K. Matsumoto, H. Inokuma, Molecular survey of bovine vector-borne pathogens in Cebu, Philippines, *Vet. Parasitol.* 196 (1) (2013) 13–20.
- [24] W.A. Jensen, M.R. Lappin, S. Kamkar, W.J. Reagen, Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats, *Am. J. Vet. Res.* 62 (2001) 604–608.
- [25] A.P. Ybañez, M. Sashika, H. Inokuma, The phylogenetic position of *Anaplasma bovis* and inferences on the phylogeny of the genus *Anaplasma*, *J. Vet. Med. Sci.* 76 (2) (2014) 307–312.
- [26] National Research Council, The water buffalo: new prospects for an underutilized animal: report, *Natl. Acad.* 32 (1981).
- [27] R.L. Wall, D. Shearer, *Veterinary Ectoparasites: Biology, Pathology and Control*, John Wiley & Sons, 2008.
- [28] Q. Song, L. Wang, R. Fang, M.K. Khan, Y. Zhou, J. Zhao, Detection of *Mycoplasma wenyonii* in cattle and transmission vectors by the loop-mediated isothermal amplification (LAMP) assay, *Trop. Anim. Health Prod.* 45 (1) (2012) 247–250.
- [29] B. Willi, C. Filoni, J.L. Catao-Dias, V. Cattori, M.L. Meli, A. Vargas, F. Martinez, M.E. Roelke, M.P. Ryser-Degiorgis, C.M. Leutenegger, H. Lutz, R. Hofmann-Lehmann, Worldwide occurrence of feline Hemoplasma infections in wild feline species, *J. Clin. Microbiol.* 45 (2007) 1159–1166.
- [30] B.R. McMILLAN, M.R. Cottam, D.W. Kaufman, Wallowing behavior of American bison (*Bos bison*) in tallgrass prairie: an examination of alternate explanations, *Am. Midl. Nat.* 144 (1) (2000) 159–167.
- [31] L. McAuliffe, J. Lawes, S. Bell, A. Barlow, R. Ayling, R. Nicholas, The detection of *Mycoplasma* (formerly *Eperythrozoon*) *wenyonii* by 16S rDNA PCR and denaturing gradient gel electrophoresis, *Vet. Microbiol.* 117 (2–4) (2006) 292–296.
- [32] S. Tasker, C.R. Helps, M.J. Day, D.A. Harbour, S.E. Shaw, S. Harrus, G. Baneth, R.G. Lobetti, R. Malik, J.P. Beaufils, C.R. Belford, T.J. Gruffydd-Jones, Phylogenetic analysis of Hemoplasma species: an international study, *J. Clin. Microbiol.* 41 (2003) 3877–3880.
- [33] A. Giroto, A.F. Zangirólamo, A.L.G. Bogado, G.C.F.D. Silva, J.L. Garcia, L.A. Vilas Boas, O. Vidotto, Molecular detection and occurrence of 'Candidatus Mycoplasma haemobos' in dairy cattle of southern Brazil, *Rev. Bras. Parasitol. Vet.* 21 (3) (2012) 342–344.
- [34] R. Hofmann-Lehmann, M.L. Meli, U.M. Dreher, E. Gönczi, P. Deplazes, U. Braun, M. Engels, J. Schüpbach, K. Jörgler, R. Thoma, C. Griot, Concurrent infections with vector-borne pathogens associated with fatal hemolytic anemia in a cattle herd in Switzerland, *J. Clin. Microbiol.* 42 (8) (2004) 3775–3780.
- [35] J.B. Prullage, R.E. Williams, S.M. Gaafar, On the transmissibility of *Eperythrozoon suis* by *Stomoxys calcitrans* and *Aedes aegypti*, *Vet. Parasitol.* 50 (1–2) (1993) 125–135.
- [36] M.R. Lappin, B. Griffin, J. Brunt, A. Riley, D. Burney, J. Hawley, W.A. Jensen, Prevalence of *Bartonella* species, haemoplasma species, *Ehrlichia* species, *Anaplasma phagocytophilum*, and *Neorickettsia risticii* DNA in the blood of cats and their fleas in the United States, *J. Feline Med. Surg.* 8 (2) (2006) 85–90.
- [37] S. Taroura, Y. Shimada, Y. Sakata, T. Miyama, H. Hiraoka, M. Watanabe, H. Inokuma, Detection of DNA of 'Candidatus Mycoplasma haemominutum' and *Spiroplasma* sp. in unfed ticks collected from vegetation in Japan, *J. Vet. Med. Sci.* 67 (12) (2005) 1277–1279.
- [38] J.E. Woods, M.M. Brewer, J.R. Hawley, N. Wisniewski, M.R. Lappin, Evaluation of experimental transmission of *Candidatus Mycoplasma haemominutum* and *Mycoplasma haemofelis* by *Ctenocephalides felis* to cats, *Am. J. Vet. Res.* 66 (6) (2005) 1008–1012.
- [39] M.L. Meli, B. Willi, U.M. Dreher, V. Cattori, G. Knubben-Schweizer, K. Nuss, R. Hofmann-Lehmann, Identification, molecular characterization, and occurrence of two bovine hemoplasma species in Swiss cattle and development of real-time TaqMan quantitative PCR assays for diagnosis of bovine hemoplasma infections, *J. Clin. Microbiol.* 48 (10) (2010) 3563–3568.
- [40] M. Tagawa, K. Matsumoto, N. Yokoyama, H. Inokuma, Comparison of two hemoplasma species on hematological parameters in cattle, *J. Vet. Med. Sci.* 72 (2010) 113–115.
- [41] D.H.A. Subasinghe, N.U. Horadogoda, H. Abeygunawardena, J.D.S. Siriwardene, Water buffalo—improved utilisation through new technologies, *Natl. Sci. Fdn.* (1998) Sri Lanka.
- [42] E. Camus, G. Uilenberg, Anaplasmosis, in: P.-C. Lefevre, J. Blancou, R. Chermette, G. Uilenberg (Eds.), *Infectious and Parasitic Diseases of Livestock: Bacterial Diseases, Fungal Diseases, Parasitic Diseases*, Lavoisier, Paris, France, 2010, pp. 1247–1263.
- [43] N. Decaro, V. Larocca, A. Parisi, M. Losurdo, R.P. Lia, M.F. Greco, A. Miccolis, G. Ventrella, D. Otranto, C. Buonavoglia, Clinical bovine piroplasmosis caused by *Babesia occultans* in Italy, *J. Clin. Microbiol.* 51 (7) (2013) 2432–2434.
- [44] A.P. Ybañez, R.H.D. Ybañez, R.R. Villavelez, H.P.F. Malingin, D.N.M. Barrameda, S.V. Naquila, S.M.B. Olimpos, Retrospective analyses of dogs found serologically positive for *Ehrlichia canis* in Cebu, Philippines from 2003 to 2014, *Vet. World* 9 (1) (2016) 43.
- [45] A.P. Ybañez, R.H.D. Ybañez, M.G. Talle, M. Liu, P.F.A. Moumouni, X. Xuan, First report on *Babesia vogeli* infection in dogs in the Philippines, *Parasitol. Int.* 66 (1) (2017) 813–815.