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NOTE

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Molecular survey of tick-borne pathogens infecting backyard cattle and water buffaloes in Quezon province, Philippines

Eloiza May S. GALON[1)], Rochelle Haidee D. YBAÑEZ[1)], Paul Franck ADJOU MOUMOUNI[1)], Maria Agnes TUMWEBAZE[1)], Ralph Joselle A. FABON[2)], Mary Ruth R. CALLANTA[2)], Kim Joseph E. LABUTONG[2)], Gloria B. SALAZAR[2)], Mingming LIU[1)], Jixu LI[1)], Benedicto BYAMUKAMA[1)], Yongchang LI[1)], Shengwei JI[1)], Seung-Hun LEE[1,3)], Adrian P. YBAÑEZ[4)], Florencia G. CLAVERIA[5)] and Xuenan XUAN[1)]*

[1)]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[2)]Regional Animal Disease Diagnostic Laboratory, Department of Agriculture Regional Field Office IV-A, Marawoy, Lipa City 4217, Batangas, Philippines
[3)]College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea
[4)]Institute of Molecular Parasitology and Protozoan Diseases at Main Campus and College of Veterinary Medicine at Barili Campus, Cebu Technological University, Cebu City 6000, Cebu, Philippines
[5)]Biology Department, College of Science, De La Salle University, Taft Avenue, Manila 1004, Philippines

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ABSTRACT. Tick-borne diseases (TBD) cause enormous losses for farmers. Backyard raising comprises majority of the livestock population in the Philippines, but TBD information in backyard livestock is scarce. In this study, 48 cattle and 114 water buffalo samples from Quezon province, Philippines were molecularly screened for tick-borne pathogens. *Anaplasma marginale* (16.67%) and hemoplasma (20.99%) were detected in the samples. *A. marginale* infection (P=0.0001) was significantly higher in cattle, while hemoplasma infection (P=0.011) was significantly higher in water buffaloes. *A. marginale* isolates from this study were highly similar to previous isolates from the Philippines while *Mycoplasma wenyonii* and *Candidatus* Mycoplasma haemobos were the identified hemoplasma species. Our findings reveal additional information on the TBD situation of Philippine backyard livestock.

KEY WORDS: *Anaplasma*, hemoplasma, Philippines, Quezon, tick-borne

The livestock industry is a significant contributor to the agriculture sector of the Philippines, accounting for 17.65% of the total agricultural production value in 2018 [18]. Backyard raising by smallholder farmers has been the predominant practice in livestock farming, particularly for large ruminants. Cattle are commonly raised as source of meat and milk, while water buffaloes (carabaos) are indispensable as draft animals providing labor power for small-scale crop production [7]. Unlike animals raised in commercial farms, field cattle and water buffaloes are often neglected, thus receiving inadequate nutrient supplements and health care [10]. Costly veterinary expense is also seen as additional economic burden for subsistence backyard livestock farmers.

Tick infestation is commonly observed in cattle and water buffalo in the field causing hide damage and tick worry. These ticks transmit numerous pathogens that negatively impact large ruminant health. Among the most economically important tick-borne pathogens (TBPs) are protozoan parasites *Babesia* and *Theileria* and bacteria *Anaplasma* and hemotropic *Mycoplasma* (hemoplasma), all of which were previously reported in the Philippines [23]. Anemia is the typical clinical sign associated with the diseases caused by these TBPs leading to decreased meat and milk production, reduced draft power, and in more severe cases, mortality [3, 4, 14, 19].

Most of the previous tick-borne disease (TBDs) investigations in livestock in the country focused on animals raised in commercial and semi-commercial farms and on imported breeds. This study aimed to detect TBPs in backyard-raised cattle and water buffaloes and to evaluate animal profile and hematological parameters related to TBDs in Quezon province, Philippines.

The animal handling procedures performed in this study observed the Philippine Animal Welfare Act (R.A. 8485 as amended by R.A.

*Correspondence to: Xuan, X.: gen@obihiro.ac.jp

10631). Permission for conducting animal experiments (permit no. 29-69) and DNA use (permit no. 1723-1724) were obtained from Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan. Sampling was done in 7 different municipalities in the east district of Quezon province, Luzon area, Philippines (Fig. 1). Blood samples were collected from a total of 162 apparently healthy cattle (N=48) and water buffaloes (N=114) in October 2018. Animal profile (sex, breed and presence or absence of ticks) was recorded (Table 1). About 2 m*l* of blood were collected from the jugular vein into EDTA-coated vacutainers. Approximately 1 m*l* of each blood sample was kept at −20°C for DNA extraction while the remaining blood samples were used for the complete blood count (CBC) test. Extraction of DNA was done using QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Hematological values, namely red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hgb) and packed cell volume (PCV), were obtained using KT-6180 3-part Hematology Analyzer (Shenzen Uniper Medical Technology, Shenzen, China).

Previously established polymerase chain reaction-based assays were used in evaluating the presence of *Babesia bovis, B. bigemina, Theileria* spp., *Anaplasma marginale* and hemoplasma in the blood samples. Genes targeted were spherical body protein *(SBP)-2* [1], rhoptry-associated protein *(RAP)-1a* [20], *18S rRNA* [5], *groEL* heat-shock operon [21] and *16S rRNA* [6], respectively. A final volume of 10 *µl* consisting of 4.85 *µl* of double-distilled water, 1 mM of dNTP solution mix (New England Biolabs, Ipswich, MA, USA), 1 *µl* of 10x ThermoPol® buffer (New England Biolabs), 10 *µ*M of each primer, 0.05 *µl* of Taq DNA polymerase (New England Biolabs) and 2 *µl* of gDNA was used. Thermocycling conditions were performed for each pathogen as before, except for the extension and final extension temperatures, which were set at 68°C. Double-distilled water was used as negative control and cloned positive DNA samples were used as positive controls. After electrophoresis and staining with ethidium bromide, amplicons were visualized in 1.5% agarose gel under UV light. Amplicons of hemoplasma (N=7) and *A. marginale* (N=8) were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and were directly sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequences were manually trimmed and submitted to the NCBI GenBank database. Species of obtained isolates were confirmed with BLASTn search while shared identity among isolates was determined by EMBOSS Matcher. Fisher's exact and Pearson's χ^2 tests were used to assess the association between animal profile parameters and PCR positivity while differences in hematological values were evaluated by Mann-Whitney *U* test. Statistically significant *P* value was <0.05.

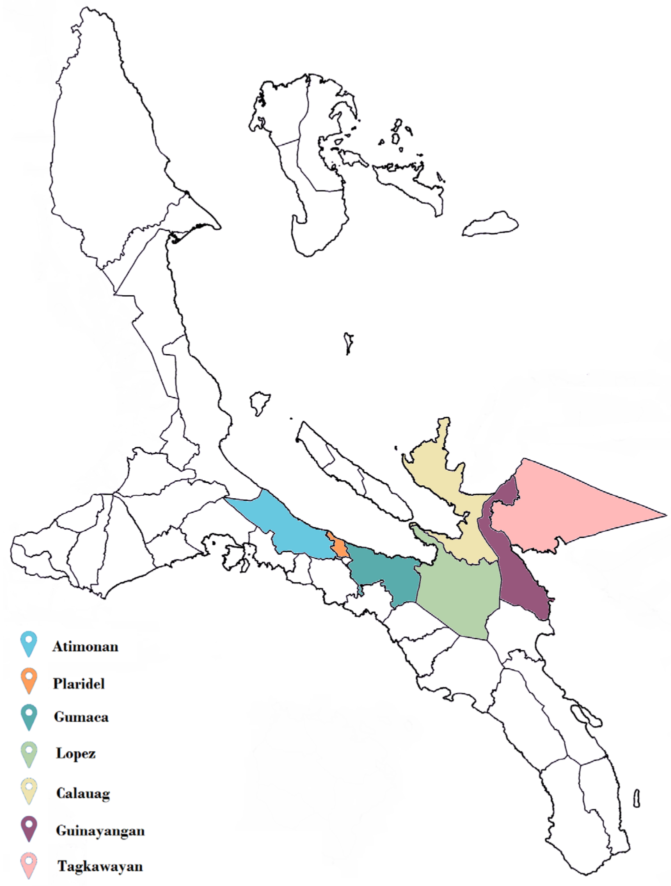

Fig. 1. Map of sampling sites in Quezon province, Philippines.

Out of the 162 screened samples, 42 were positive for at least one TBP (25.93%). *A. marginale* was only found in cattle (16.67%) while hemoplasma was detected in both water buffalo (26.32%) and cattle (8.33%) (Table 1). *A. marginale* is the most prevalent and extensively reported TBP in the Philippines [23]. The 16.67% (8/48) bovine *A. marginale* detection rate in this survey was significantly lower compared to a recent study which reported a 100% *Anaplasma* spp. detection rate in 21 dairy cattle in the same area [8]. In the Philippines, hemoplasmas have only been reported in cattle and goats in Cebu [13, 22] and dairy cattle and water buffaloes (80%) in Bohol [24] in the Visayas area. The current study is the first report of livestock hemoplasma infection in the Luzon area. The lower infection rates of *A. marginale* and hemoplasma in this study might be due to the difference in the breeds of the animals and raising system. Native breeds, normally used for draft power or meat production, dominate backyard farms while dairy animals are commonly imported breeds or crossbreeds. Native breeds of cattle and water buffaloes are usually more resistant against TBPs such as *A. marginale* [15, 17]. In the current study, 97% of water buffaloes were Philippine carabao breed and 77% of cattle were Philippine native breed. While the breed was not associated with any detected TBPs (*P*>0.05) in this study (Table 1), the unequal proportion of native to imported or crossbred animals sampled may have affected this result.

Based on statistical analysis, *A. marginale* infection (*P*=0.0001) was significantly higher in cattle, while hemoplasma infection (*P*=0.011) was significantly higher in water buffaloes (Table 1). Although both ruminants are natural hosts, clinical anaplasmosis affects cattle more frequently, while water buffaloes act as reservoir hosts [12, 14]. On the other hand, there is little information on the susceptibility of water buffaloes to hemoplasma. Previous studies detected comparable rates of hemoplasma infection between water buffaloes and cattle [9, 24]. This finding is particularly interesting; hence, the extent of hemoplasma susceptibility of water buffaloes should be assessed in future studies.

There were 19 animals found to be tick-infested despite acaricide use of some farmers a week prior to the sampling, which might suggest emerging resistance of ticks to acaricides. The resistance of cattle-infesting *Rhipicephalus microplus* ticks to chemical acaricide was recently reported in Luzon area [11]. In this study, tick infestation ($P>0.05$) was neither associated with *A. marginale* nor hemoplasma infection (Table 2). Backyard-raised livestock, which are often pastured and tethered, have more exposure to blood-sucking arthropods which could transmit TBPs [22]. As both TBPs are not exclusively transmitted by ticks, mechanical transmission by other ectoparasites is a likely possibility.

The lack of clinical signs in animals positive for *A. marginale* and hemoplasma denotes persistent subclinical infection, a

Table 1. Association of tick-borne pathogen positivity with profile parameters of backyard cattle and water buffaloes in Quezon province, Philippines

Parameter		N	*Mycoplasma* spp.		*Anaplasma marginale*	
			No. of positives (%)	*P* value[a)]	No. of positives (%)	*P* value[a)]
Location						
	Atimonan	12	1 (8.33)	n.a.	1 (8.33)	n.a.
	Calauag	21	6 (28.57)		1 (4.76)	
	Guinayangan	21	9 (42.86)		1 (4.76)	
	Gumaca	23	5 (21.74)		0	
	Lopez	4	0		0	
	Plaridel	37	4 (10.81)		2 (5.41)	
	Tagkawayan	44	9 (20.45)		2 (6.82)	
Host						
	Water buffalo	114	30 (26.32)	0.011[b)]	0	0.0001[c)]
	Cattle	48	4 (8.33)		8 (16.67)	
Sex						
	Female	133	26 (19.55)	0.33	6 (4.51)	0.63
	Male	29	8 (27.59)		2 (6.90)	
Breed						
	Native	148	32 (21.62)	0.74	6 (4.05)	0.15
	Crossbred or imported	14	2 (14.29)		2 (14.29)	
Tick infestation						
	Yes	19	1 (5.26)	0.13	3 (15.79)	0.053
	No	143	33 (23.08)		5 (3.50)	
Total		162	34 (20.99)		8 (4.94)	

a) Analyzed by Fisher's exact or χ^2 test; b) $P<0.05$; c) $P<0.001$. n.a.: not analyzed.

Table 2. Hematological analysis of cattle positive for *Anaplasma marginale* and hemoplasma in Quezon province, Philippines

Hematological parameter	Hemoplasma infection (N=4)	Hemoplasma negative (N=36)	*P* value[a)]	*A. marginale* infection (N=5)	*A. marginale* negative (N=35)	*P* value[a)]
Red blood cell ($10^{12}/l$)	5.80	6.24	0.25	6.06	6.22	0.92
White blood cell ($10^9/l$)	21.73	12.21	0.14	11.98	13.33	0.85
Hemoglobin (g/d*l*)	8.60	9.46	0.20	8.86	9.45	0.64
Packed cell volume (%)	35.75	40.80	0.12	37.94	40.63	0.42

a) Analyzed by Mann-Whitney *U* test.

Table 3. Hematological analysis of hemoplasma-positive water buffaloes positive in Quezon province, Philippines

Hematological parameter	Hemoplasma infection (N=26)	Hemoplasma negative (N=81)	*P* value[a)]
Red blood cell ($10^{12}/l$)	4.99	4.98	0.87
White blood cell ($10^9/l$)	9.93	9.17	0.29
Hemoglobin (g/d*l*)	9.57	9.84	0.61
Packed cell volume (%)	39.88	40.32	0.71

a) Analyzed by Mann-Whitney *U* test.

feature of *Anaplasma* and hemoplasma infections. Persistent *A. marginale* infection provides life-long protection against clinical anaplasmosis [14]. In the case of hemoplasma chronic infection, animals become pathogen carriers, and substantial losses in milk production are observed [19]. Only 147 samples (107 water buffaloes and 40 cattle) had hematological data, thus, samples with no information were excluded from the analysis (Tables 2 and 3). Overall, *A. marginale* and hemoplasma positivity did not cause significant changes in hematological values, indicating persistent subclinical infections which were also noted in past investigations [9, 19].

B. bovis, *B. bigemina,* and *Theileria* spp. were not detected in the current study. Previous studies in neighboring provinces like Batangas, Cavite and Laguna reported detection of *B. bovis*, *B. bigemina* and *Theileria* spp. in cattle [2, 16, 25]. The limited number of samples as well as the natural resistance of native animals might be attributed to the absence of these TBPs in the current survey. Despite non-detection, continuous surveillance of these economically TBPs should be conducted.

Eight *A. marginale* isolates (MN961201-MN961208) shared 99.1–100% identities with each other. *A. marginale* is considered an endemic TBP in the Philippines [23] and the high identities of current isolates with previous Philippine isolates corroborate past findings. Seven isolates of hemoplasma were confirmed as *Mycoplasma wenyonii* (MT241310–MT241312) and *Candidatus* Mycoplasma haemobos (MT241313–MT241316). *M. wenyonii* was earlier identified in cattle and water buffaloes in Bohol [22, 24] while *Candidatus M. haemobos* was detected in goats in Cebu [13]. Although both species can infect bovids, it was noted that *Candidatus M. haemobos*-infected cattle develop more severe clinical signs compared to *M. wenyonii*-infected cattle [19]. Nonetheless, prevention measures against hemoplasma infection in livestock should be implemented to minimize the economic impact caused by this tick-borne infectious disease.

In this study, *A. marginale* and hemoplasma were detected in backyard-raised cattle and water buffaloes in Quezon, Philippines. The current study is the first report of livestock hemoplasma infection in the Luzon area and the first confirmation of *Candidatus M. haemobos* in cattle and water buffaloes in the Philippines. Our findings reveal additional information on the diversity of TBPs in livestock in the country.

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