



## NOTE

Bacteriology

## Molecular evidence of hemotropic mycoplasmas in goats from Cebu, Philippines

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**ABSTRACT.** Hemotropic mycoplasma (hemoplasma), a neglected vector-borne pathogen in goats, causes extensive economic damage to farmers due to production losses. In this study, 107/295 (36.27%) goats sampled from 4 farms (Barili, Danao City, Dumanjug and Minglanilla) in Cebu, Philippines tested positive for PCR targeting the *16S rRNA* gene of *Mycoplasma*. All hemoplasma-positive goats were from Barili and no clinical sign was observed. Sex ( $P=0.0005$ ) and age ( $P=0.03$ ) were found associated with hemoplasma infection. *Mycoplasma ovis*, *Candidatus Mycoplasma haemobos*, *Candidatus Mycoplasma haemominutum* and 3 Uncultured *Mycoplasma* sp. sequences were identified by sequencing analysis. This is the first report of molecular detection and genetic characterization of hemoplasmas in goats in the Philippines.

**KEY WORDS:** goat, hemoplasma, *Mycoplasma*, Philippines

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Hemotropic mycoplasma (hemoplasma) is a group of cell wall-less epierythrocytic bacteria infecting a wide variety of vertebrate hosts. Formerly known as *Haemobartonella* and *Eperythrozoon* classified under the Anaplasmataceae family, hemoplasmas have since been transferred to the genus *Mycoplasma* after phylogenetic confirmation based on the *16S rRNA* gene sequences [12–14]. Depending on host susceptibility and species pathogenicity, manifestation of hemoplasma infection ranges from asymptomatic to severe clinical signs [11].

*Mycoplasma ovis* and *Candidatus Mycoplasma haemovis* are the most common species in small ruminants. They often cause hemolytic anemia and decreased exercise tolerance in acutely infected sheep [1, 5, 11, 19] while goats develop markedly lower bacteremia and become persistent carriers [2, 10]. Natural transmission is effected by blood-sucking arthropods such as ticks (*Rhipicephalus bursa* and *Haemaphysalis plubeum*) and mosquitoes (*Aedes camptorhynchus* and *Culex annulirostris*) while mechanical transmission is by contaminated medical equipment [13]. The economic impact brought about by hemoplasmas to small ruminant farming has been substantial, mainly due to mortalities during outbreaks [5, 16] and productivity losses in chronically infected animals.

Goat farming is a vital sector of smallholder agriculture in the Philippines. Due to the minimal investment and resources required, raising the so-called “poor man’s cow” has been a common venture for subsistence farming families [3]. Cebu province, located in the Visayas region, raises 6% of the 3.71 million goats in the country [15]. Bovine hemoplasma *Mycoplasma wenyonii* was previously detected in Cebu, Philippines [22], but hemoplasma infection in small ruminants has not been reported in the country. The current study documented the first molecular detection and characterization of hemoplasmas in goats in the Philippines.

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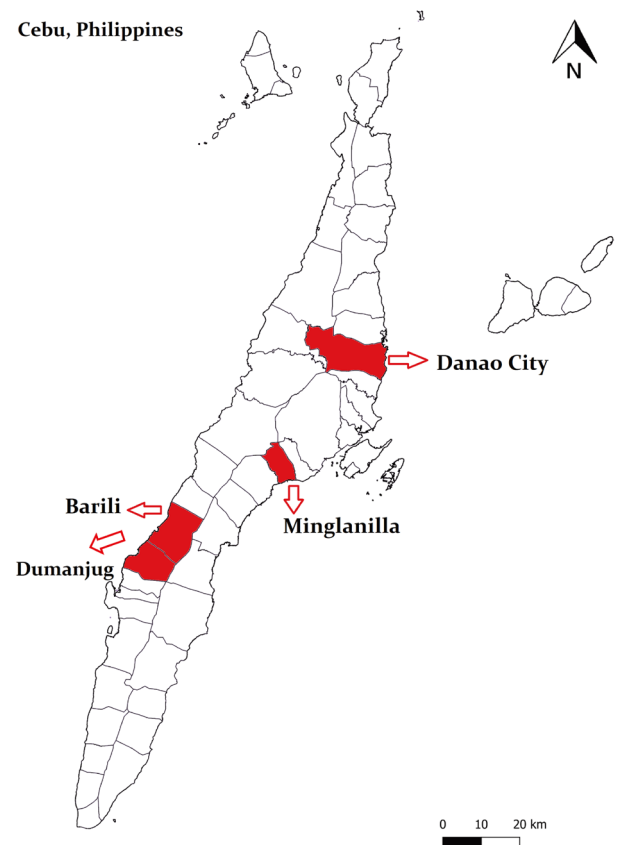
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Two hundred ninety-five goats were randomly selected from 4 farms in Cebu, Philippines (Barili, N=226; Danao City N=29; Dumajug, N=18; Minglanilla, N=22) (Fig. 1). Sex and age of the animals were recorded (Table 1) and animals were also examined for clinical signs. Thereafter, 2 ml of anti-coagulated blood was collected from the jugular vein of each animal and DNA was extracted using QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. All procedures conducted adhered to the principles of Animal Welfare Act of the Philippines (R.A. 8485 as amended by R.A. 10631). Permission for animal experiments (permit no. 29-69) and permission on the use of DNA (permit no. 1723-1724) were obtained from Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan. The primer pair F2 (5'-ACGAAAGTCTGATGGAGCAATA-3') and R2 (5'-ACGCCCAATAAATCCGRATAAT-3'), which targets the *16S rRNA* gene of most hemoplasma species, was used to amplify an approximately 200 base pair (bp) fragment by PCR [7]. Briefly, the PCR reaction mixture consisted of 7.8  $\mu$ l of double-distilled water, 200  $\mu$ M of dNTP solution mix (New England Biolabs, Ipswich, MA, U.S.A.), 2  $\mu$ l of 10x Ex Taq buffer (Takara, Kusatsu, Japan), 10 pmol of each primer, 0.2  $\mu$ l of Ex Taq polymerase (Takara) and 4  $\mu$ l of DNA sample. Double-distilled water and a *Mycoplasma* spp.-positive canine DNA sample [9] were used as negative and positive control, respectively. Thermocycling protocol by Jensen *et al.* [7] was used with slight modifications: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 30 sec, and final extension at 72°C for 7 min. PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide, then visualized under UV light. Association between animal parameters (sex and age groups) and status of hemoplasma infection was evaluated using Pearson's chi-squared test ( $P < 0.05$ ).

Selected amplicons were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), inserted into pGEM®-T Easy Vector (Promega Corp., Madison, WI, U.S.A.) and cloned in DH5 $\alpha$  *Escherichia coli* competent cells. Two colonies were picked for each band observed. The plasmids were purified using NucleoSpin® Plasmid QuickPure Kit (Macherey-Nagel, Düren, Germany). BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems) were used for sequencing analysis. Obtained nucleotide sequences were manually trimmed and percentages of identity with other isolates were checked using BLASTn and EMBOSS program. The sequences were deposited in the GenBank database (accession nos. MK328955-MK328960). Phylogenetic tree was modeled using maximum likelihood method and Kimura 2-parameter model with 1000 bootstrap replications in the Molecular Evolutionary Genetics Analysis 7.0 software.

Overall, 107 of 295 goat blood samples (36.27%) were *Mycoplasma* spp.-positive. Three types of positive samples were observed: one band of the size 190–200 bp (type A), one band of the size 170–180 bp (type B), and an upper band the size of A and a lower band the size of B (type C). Among the 107 positive samples, 101 were type A, 1 was type B and 5 were type C. PCR-based investigation of hemoplasma infection in goats are relatively infrequent worldwide. In the current study, the obtained infection rate (36.27%) is slightly lower compared to reports in China (41–45.24%) [16–17, 20] and Brazil (39.30%) [10], but is higher than those of in Turkey (6.2%) [1], U.S.A. (18%) [8] and Hungary (20%) [6]. The disparity in infection rates between the current and previous investigations may be attributed to various factors such as geographic location, sample size, animal age, environmental conditions, presence of vectors and animal production systems [1]. All positive animals in the present study were from Barili. The farm in Barili is a commercial transitory farm that raises goats originating from various places. After a few weeks, goats are transported to different areas in Cebu and nearby provinces. On the other hand, the samples from Danao City, Dumanjug, and Minglanilla tested negative, but this finding may not fully describe the hemoplasma situation in these areas due to the limited number of samples tested.

Our survey revealed that the infection rate in female goats (41.38%) was significantly higher compared to the 17.46% in male goats (Table 1). Sex has been associated with hemoplasma-positivity in small ruminants in previous investigations [10, 20]. However, it is possible that our findings may have been influenced by the larger number of female goats. Age group was found to be related to hemoplasma infection ( $P=0.03$ ), with a considerably higher infection rate in adult goats (Table 1). This result corroborates similar findings in earlier reports [6, 10] but contrasted with results of Johnson *et al.* [8]. Longer exposure time



**Fig. 1.** Map of sampling area in Cebu, Philippines. Sampling sites are shaded in red.

**Table 1.** Hemoplasma infection in goats based on sex and age groups

Parameter	Farm location				Total no. of tested	Total PCR-positive	Infection rate (%)	P value
	Barili	Danao City	Dumanjug	Minglanilla				
Sex								
Female	171	25	17	19	232	96	41.38	0.0005 <sup>a)</sup>
Male	55	4	1	3	63	11	17.46	
Age group								
Young (<1 year)	144	2	8	15	107	27	25.23	0.03 <sup>b)</sup>
Adult (≥1 year)	82	27	10	7	188	80	42.55	
Total	226	29	18	22	295	107	36.27	

a)  $P < 0.001$ ; b)  $P < 0.05$ .



**Fig. 2.** Phylogenetic analysis based on *16S rRNA* gene. The tree was constructed using maximum likelihood method with Kimura-2 parameter model and bootstrap-replicated 1,000 times in MEGA 7.0. Sequences generated from this study are displayed in bold font. *Candidatus Mycoplasma haemolamae* was used as outgroup.

predisposes the adult goats to become more infected with hemoplasma compared to young ones [10]. In addition, Hornok *et al.* [6] asserted that older goats tend to be more infected as once-infected goats become long-term carriers of hemoplasma. All hemoplasma-infected goats in the current study did not show any clinical signs, further supporting the idea that these goats had persistent hemoplasma infection.

Eight randomly selected amplicons generated six *16S rRNA* sequence types. One sequence type (MK328958, 195 bp) was identified in 3 amplicons. MK328958 was confirmed as *Mycoplasma ovis* and had 99.5–100% identity with isolates from Japan, China, Hungary, India, Iran and Turkey. The obtained *M. ovis* sequence is likewise identical and most closely related to the U.S.A.-isolated *M. ovis* derived from a human clinical case [18] (Fig. 2). This interesting finding suggests that the *M. ovis* genotype infecting goats in Cebu might be zoonotic, hence, farmers and veterinarians should carry out appropriate preventive measures against *M. ovis* infection in both animals and humans. One nucleotide sequence (MK328955, 172 bp) was identified as *Candidatus Mycoplasma haemobos* and showed 99.4–100% identity with isolates from Cuba, Brazil, Taiwan, and China. Although primarily

a bovine hemoplasma, *Candidatus M. haemobos* was recently identified in sheep, goats and *Rhipicephalus microplus* tick [16]. Constant contact between animals in the same grazing areas may have facilitated transmission of *Candidatus M. haemobos* from cattle and water buffaloes to goats through common vectors like *R. microplus* tick and other ectoparasites. It is worth noting that a fatal outbreak in goats and sheep was attributed to the transplacental transmission of *Candidatus M. haemobos* in China [16], indicating a transmission risk from the *Candidatus M. haemobos*-infected goats to their offspring. Similarly, another sequence (MK328956, 193 bp) from the present study was identified as feline hemoplasma *Candidatus Mycoplasma haemominutum*. Although the *Candidatus M. haemominutum* sequence showed 99% identity with isolates from Thailand and China, the former did not appear in the same branch with the two isolates (Fig. 2), which suggests that the isolate in this study might be a new genotype of *Candidatus M. haemominutum*. In the Philippines, goats are usually kept in the backyard after grazing, often in close contact with domestic pets like cats. This may explain the detection of *Candidatus M. haemominutum* in goats in the present study. Nevertheless, the transmission route is unclear as this specific pathogen can be transmitted both by arthropod vectors [21] and directly through cat saliva [4]. Three sequences (MK328957, MK328959, and MK328960) generated in this study had relatively low identity with registered isolates in the database, thus, species were not confirmed. These sequences were designated as Uncultured *Mycoplasma* sp. BLASTn search revealed that MK328957 (174 bp) shared 95% identity with *Candidatus M. haemovis* isolates from China, but after phylogenetic analysis, the isolate appeared in the subclade of bovine *M. wenyonii* isolates from China, Mexico and Philippines (Fig. 2). MK328959 (170 bp) and MK328960 (195 bp) were each found in different subclades (Fig. 2). Among the five samples which yielded fragments of 190–200 bp and 170–180 bp (type C), sequencing analysis was done for 1 sample and it was confirmed to be infected with *M. ovis* (MK328958, 195 bp) and Uncultured *Mycoplasma* sp. (MK328959, 170 bp). Coinfection with *M. ovis* and another hemoplasma species (*Candidatus M. haemovis*) in sheep have been reported as the cause of severe clinical cases in Japan and fatal outbreak in Hungary [5, 19]. However, the exact species of MK328959 was not confirmed and no clinical sign was observed in the infected animal, hence, the actual effect of this coinfection type cannot be determined. In addition, considering that there are only 1 to 4 nucleotide differences among the sequences obtained (type A: 193 and 195 bp; type B: 170, 172, and 174 bp), the possibility that other types A and B-positive samples were infected with two or more hemoplasma species cannot be entirely ruled out. As only limited number of positive samples were sequenced in the present investigation, it is recommended that further studies focus on identifying all hemoplasma species in infected samples by using techniques that can differentiate pathogens in the species level.

This study documented the first confirmation of hemoplasma infection in goats in the Philippines using a PCR-based assay. Information generated about this neglected pathogen would be useful to create awareness among farmers and veterinarians, leading to better health practices and improved overall herd condition.

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