FULL PAPER *Bacteriology*

High Genetic Diversity of *Anaplasma marginale* Detected from Philippine Cattle

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ABSTRACT. A total of 658 cattle in 6 provinces in the Philippines were screened for *Anaplasma marginale* infection by using a diagnostic heat-shock operon (*groEL*) gene-PCR assay. The screening-positive samples were further tested using the major surface antigen protein 1a (*Msp1a*) gene-PCR assay. Screening PCR results showed 130 cattle (19.8%) were positive for the *A. marginale* infection. Subsequent amplification using the *Msp1a* gene only showed 93 samples (14.1%) to be positive. In addition, 37 tandem-repeat structures, including 20 novel structures, and 41 distinct genotypes were identified. Interestingly, multiple infections of 4 different genotypes were also observed in *A. marginale*-infected cattle. The present study demonstrated the prevalence and characterization of diverse genotypes of *A. marginale* in the Philippine cattle.

KEY WORDS: *Anaplasma marginale*, cattle, *groEL*, *Msp1a*, Philippines.

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Anaplasma marginale, which is the most widely distributed agent causing bovine anaplasmosis, is a rickettsial Gram-negative, intra-erythrocyte pathogen [10]. It causes serious anemia and occasional death in the infected cattle. Cattle that recovered from the disease often maintain the infection and become the reservoirs for transmitting ticks [17]. *Rhipicephalus microplus* have been implicated as the tick vectors [5]. The pathogen has gained high interest worldwide, because it has caused great economic losses in several countries [30].

World strains or geographic isolates of *A. marginale,* which may differ in the biology, protein sequence and antigenicity, have been analyzed using the major surface protein 1a (*Msp1a*) gene of pathogen [1, 8, 27]. In the genome of *A. marginale,* the *Msp1a* is a single copy gene that encodes a 70–100 kDa protein (MSP1a) containing variable number of tandem-repeat sequences [1, 24]. Due to its diversity, the gene has been used as a stable marker to determine the genotypes of *A. marginale* distributed in the different geographic locations, utilizing the codes of established tandem-repeat forms [6, 8, 26]. The MSP1a is known to function as an adhesin against bovine erythrocytes and tick cells, in which it becomes important in the adhesion, infection and transmission of *A. marginale* between animals and ticks [6, 7, 20]. On the other hand, its potential use has also been suggested in the development of recombinant vaccines against bovine anaplasmosis [4]. Recently, immunization of recombinant MSP1a fused with tick antigen was shown to protect the cattle (>60% vaccine efficacy) from subsequent experimental infection by tick infestation [3].

While the geographic isolates of *A. marginale* in America, Europe and some parts of Asia have been characterized for genotyping, reports in Southeast Asia, including the Philippines, have been limited. Previously, only few cattle in a limited area were used to demonstrate the genotypes of *A. marginale* in the Philippines [32]. Thus, the present study was endeavored to determine the genetic diversity of *A. marginale* using more number of bovine blood samples collected from different geographic locations in the Philippines.

MATERIALS AND METHODS

DNA sample: A total of 658 DNA samples extracted from cattle blood from Cebu, Iloilo, Negros Oriental, Negros Occidental, Cavite and Batangas in the Philippines [33, 34] were used (Fig. 1). In brief, the DNA extraction was performed using a QIAamp DNA blood Mini Kit (QIAGEN, Hilden, Germany). The DNA samples were stored at −30°C until use. DNA concentrations were measured using a Thermo Scientific Nano Drop 2000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). A DNA sample prepared from blood of a Japanese black cattle infected with *A. marginale* [25] was used as the positive control for the subsequent PCR assays.

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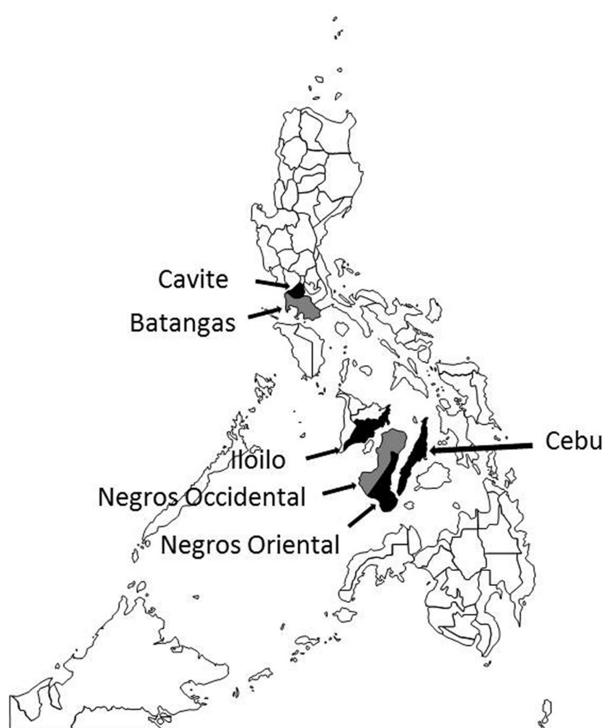

Fig. 1. The Philippine map indicating the sampling area (shaded).

PCR assays: The oligonucleotide sequences of PCR primers used in the present study are presented in Table 1. Briefly, for the *A. marginale*-specific *groEL* nested PCR assay, 2 primer pairs, AM265F1/AM1574R1 and AM424F2/AM1289R2, were respectively used for the first and second round PCRs to amplify a final 866-bp amplicon [32]. For the *Msp1a* gene, a hemi-nested PCR was performed using two primer pairs, MSPa733F1/MSPa3134R1 and MSPa733F1/MSPa2957R2, for the first and second round PCRs, respectively [19]. The amplification products were visualized in a 1.5% agarose gel after migration. The presence of single or multiple infections of different genotypes were assessed based on the presence of different sizes of visualized bands.

Cloning and sequencing of PCR products: Selected PCR amplicons were purified using either a QIAquick PCR Purification Kit or a QIAquick Gel Extraction Kit (Qiagen). DNA cloning and sequencing of the purified amplicons were performed as described previously [32]. Briefly, direct sequencing was initially performed using the 2nd round PCR primers. In some cases where the obtained sequence was of low quality, the PCR amplicons were cloned into a PCR 2.1-TOPO plasmid (Invitrogen, Carlsbad, CA, U.S.A.). The nucleotide sequences were then determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Sequence and phylogenetic analyses: Obtained sequences were manually trimmed to include only the sequence of interest. The sequence comparison and percent identity computation were performed as described previously [32]. Multiple sequence alignment (MSA) was performed using a MUSCLE program [11] employed in a MEGA5 program [31], as suggested by Hall [13]. Phylogenetic analyses using a Bayesian inference method were performed in a MrBayes 3.2 program [28] and guided by the best model testing results of the MSA in MEGA5.

RESULTS

A. marginale was detected in all the examined locations and was most and least prevalent in Cavite (62.5%) and Cebu (9.6%), respectively. Among the *groEL* PCR-positive samples (19.8%), 93 samples (14.1%) were amplified using the *Msp1a* PCR assay. Cattle infected with single (47 or 7.1%) and multiple (46 or 7.0%) genotypes of *A. marginale* were observed in the PCR assay (Table 2).

The partial *groEL* gene fragments of *A. marginale* detected from the Philippine cattle (GenBank Acc. KC113449-81) revealed 98.6–100% identities to each other and 99.2–100% to already registered sequences, including those from Japan (Ishigaki; FJ226455), Israel (Non-tailed; AF414861) and Australia (F12; AF414860), indicating a high conservation of the *groEL* gene among all known *A. marginale* strains. On the other hand, the lengths of partial *Msp1a* nucleotide (GenBank Acc. KC181866-915) obtained in the present study were variable, ranging from 272 to 983 bp. These sequences were 10.1–99.9% identical to each other.

Meanwhile, a total of 38 kinds of tandem-repeat structures of *A. marginale* MSP1a, including 20 novel structures that were unique to the Philippine samples, were identified in the present study (Table 2). These novel structures were 90.0–96.6% identical to those found in Mexico, Brazil, Argentina, South Africa, Venezuela, Japan, Israel, China, U.S.A., Italy and South Africa. As shown in Table 3, a total of 44 new genotypes were identified, of which 4 were not area-specific. Out of 46 samples with multiple infections, 3 samples were found co-infected with 4 different genotypes and another 4 samples with 3 different genotypes. The rest of the samples (39) only had dual infections. Additionally, *Msp1a* phylogenetic trees showed very low bootstrap values on monophyletic clades that contained the obtained partial sequences (data not shown).

DISCUSSION

The present study is the first molecular-epidemiological report of *A. marginale* in cattle covering several geographical areas in the Philippines. Past studies dealt with only either water buffaloes or a few cattle in limited geographic areas [21, 23, 32]. The diversity of livestock vector-borne diseases is interesting to correlate with the unique geography of the Philippines, which is composed of several islands.

The prevalence of *A. marginale* in the present study (19.8%) was higher than those of previous reports (10.3–16.7%) in water buffaloes [21]. Water buffaloes living in close contact with backyard cattle is not uncommon in the Philippines. Therefore, cattle are in constant risk of the infection, because water buffaloes are known to serve as a reservoir for

Table 1. PCR Primers used in the present study

Primer	Oligonucleotide sequence	Final target amplicon (bp)	Reference
*groEL*gene			
AM265F1	GACTACCACATGCTCCATACTGACTG	866	[32]
AMA424F2	GTCTGAAGATGAGATTGCACAGGTTG		
AM1574R1	GACGTCCACAACTACTGCATTCAAG		
AM1289R2	CCTTTGATGCCGTCCAGAGATGCA		
Msp1a gene			
MSPa733F1	TGTGCTTATGGCAGACATTTCC	272–983	[19]
MSPa2957R2	AAACCTTGTAGCCCCAACTTATCC		
MSPa3134R1	TCACGGTCAAAACCTTTGCTTACC		

Table 2. List of MSP1a tandem-repeat forms of *A. marginale* detected from the Philippine cattle

Repeat Form	Encoded Sequence	Reference
Ph1	ADSSSASGVLSKSDQASTSSQLG	This study
Ph2	ADSSSAGDRQQESGVSSQSGQASTSSQLG	This study
Ph3	TDSSSASGQKQESSVLSQSDQASTSSQLG	This study
Ph4	ADSSSASGQQQDSSVLSQGDQASTSSQLG	This study
Ph5	TDSSSASGQQQESGVLPQSGQASTSSQLG	This study
Ph6	TDSSSASGQQQESSVLPQGDQASTSSQLG	This study
Ph7	TDSSSASGQQQESSVLSQGDQASTSSQLG	This study
Ph8	AGSSSASGQQQDSSVLSQGDQASTSSQLG	This study
Ph9	ADSSSAGDQQQESGVSSQSGQASTSSQLG	This study
Ph10	TDSSSTGDQQQESGVSSQSGQASTSSQLG	This study
Ph11	ADSSSASGQQQESSVSSQLG	This study
Ph12	ADSSSASDQQQESGVPSQSEASTSSQLG	[32]; This study
Ph13	ADSSSASDQQQESSVLSQSGQASTSSQLG	This study
Ph14	ADSSSASGQQQESGVPSQSEASTSSQLG	This study
Ph15	ADSSSAGDQQQESSVSSQSDASTSSQLG	This study
Ph16	TDSSSASGQRQESSVLSQSDQASTLSQLG	This study
Ph17	ADSSSASGQQQESSVLSQSDQASTLSQLG	This study
Ph18	TDSSSASGQQQESSVLSQSDQASTLSQLG	This study
Ph19	AYSSSAGDQQQESSVSSQSGQASTSSQLG	This study
Ph20*	TDSSSASGQKQESSVLPQSGQASTSSQLG	[32]
Ph21	ADSSSAGDQQQESSVSSQSGASTSSQLG	This study
62	TDSSSAGDQQQESSVSSQSDASTSSQLG	[2]
61	TDSSSAGDQQQESSVSSQSGASTSSQLG	[2]
β	TDSSSAGDQQQGSGVSSQSGQASTSSQLG	[8]
r	TDSSSASGQQQESSVSSQSDASTSSQ	[8]
3	ADSSSASGQQQESSVLSQSGQASTSSQLG	[8]
4	TDSSSASGQQQESSVLSQSGQASTSSQLG	[8]
13	TDSSSASGQQQESSVLSQSDQASTSSQLG	[8]
14	TDSSSASGQQQESSVLSQSGASTSSQLG	[8]
17	TDSSSASGQQQESGVSSQSGQASTSSQLG	[8]
21	ADSSSAGDQQQESSVLSQSGQASTSSQLG	[8]
27	ADSSSASGQQQESSVLSQSDQASTSSQLG	[8]
46	TDSSSASGQQQESSVLPQSGQASTSSQLG	[8]
F	TDSSSASGQQQESSVSSQSGQASTSSQLG	[8]
M	ADSSSASGQQQESSVSSQSGQASTSSQLG	[8]
MGl10	ADSSSASGQQQESSVLSQSGASTSSQLG	[29]
Is1	TDSSSAGDQQQESGVSSQSGQASTSSQLG	[22]
Me1 (provisional)	ADSSSASGQQQGSSVLSQSGQASTSSQLG	AEV59754 (Mexico; unpublished)

* Ybanez *et al.,* in press; not detected in the present study.

Table 3. *A. marginale* MSP1a genotypes detected from the Philippine cattle

Area	MSP1a tandem repeat	Number of Repeats
Batangas	Ph1/β/β/г/β/β/г	7
	Me1/4/M/M/4/4/4	7
	Ph11/Ph11/Ph11/Ph11/M	5
	Ph1/27/27	3
	13/13	2
	13/27*	2
	46/F*	2
Cavite	Me1/4/4/4*	4
	Ph11/Ph14/3	3
	Ph13/4/4	3
	Ph16/Ph17/MGl10*	3
	Ph15/62	2
Cebu	13/13/14/14/13 /14/14	7
	Ph4/17/Ph5/Ph6/Ph5/Ph7	6
	13/13/13/14/14	5
	Ph12/M/Ph12/M/M**	5
	Ph21/62/62/61/61	5
	13/13/13/MGl10	4
	Ph9/Is1/Is1/Ph10	4
	13/14/14	3
	13/27/14	3
	13/27/27	3
	21/M/M	3
	46/Ph20/46**	3
	13/27*	2
	13/MGl10	2
	46/46**	2
	46/F*	2
	14	1
	17	1
	Me1	1
	Ph8	1
	13	1
Iloilo	Ph4/17/Ph5/Ph7/ Ph5/Ph7	6
	Ph12/M/3/3/M	5
	Ph4/17/Ph5/Ph5/Ph7	5
	Me1/4/4/4*	4
	Ph16/Ph17/MGl10*	3
	Ph19/M/F	3
	13/27/13/14	4
Negros Occidental	Me1/4/M/M/4/4	6
	Ph21/62/61/ 62/61/62	6
	Me1/4/M/M/4	5
	Ph2/Is1/Is1/Is1	4
	Ph18/MGl10	2
	Ph3	1
	13/14	2
Negros Oriental	Ph4/17/Ph5/ Ph7/Ph5/Ph7	6
	13/27*	2

*Not area specific; ** [32]; Not detected in the present study.

A. marginale [18]. On the other hand, the Australian Centre for International Agricultural Research (ACIAR) and the Bureau of Animal Industry of the Philippines had a previous collaborative project (ID:AS2/2000/098) partly dealing with the detection of bovine anaplasmosis. However, information on the national prevalence of *A. marginale* infection was still not made to be readily available. The project had relied on serological and peripheral blood smear examination methods, which might have sensitivity and specificity issues.

The number of the positive cattle in the *Msp1a* gene based-PCR assay was lower than that in the *groEL* gene based-PCR assay. This might be attributed to the varying sensitivities of PCR protocols despite testing the same sample [12]. The *groEL* PCR assay was previously shown to be highly sensitive and specific in detecting the *A. marginale* in Philippine cattle [32]. Furthermore, the high identities and the monophyletic clade formed by the obtained partial *A. marginale groEL* gene fragments suggest the high conservation of the *groEL* gene among Philippine isolates regardless of the geographic locations and also provide further evidence of its usefulness in the molecular detection of the pathogen in the country.

For the *Msp1a* gene, the lower nucleotide identities and presence of many genotypes demonstrated that there is a high genetic diversity of *A. marginale* distributed in the Philippines. In a previous study done in Cebu [32], the registered *Msp1a* gene sequences revealed 4 tandem-repeat structures: 2 already established structures (46 and M in Table 3) and 2 novel structures containing the sequences of ADSSSASDQQQESGVPSQSEASTSSQLG and TDSSSASGQKQESSVLPQS-GQASTSSQLG (designated as Ph12 and Ph20 in the present study, respectively). Although the Ph20 structure was not detected in the present study, Ph12 was identified together with 19 other novel structures. Moreover, the 3 previously identified genotypes from Cebu (with tandem repeats Ph12/M/Ph12/M/M, 46/Ph20/46 and 46/46) could not be detected in the present study.

Infection with multiple genotypes of *A. marginale* was reported in the present study. The presence of multiple infections of different genotypes indicates the superinfection of *A. marginale* in the Philippine cattle [26]. Meanwhile, as some genotypes of *A. marginale* were unexclusive in each study area, there might be a common exposure or source of the infection despite geographical boundaries, or it might be due to cattle trade or movement among different islands in the Philippines [9]. On the other hand, the possible co-infection of *A. marginale* with other pathogens could not be discounted [14, 16]. In a related study, co-infection of *Anaplasma* spp. with other vector-borne disease (VBD) pathogens was found to be prevalent with those considered ill animals harboring concurrent infections of up to 5 VBD pathogens [15]. Therefore, studies to determine the occurrence of other VBD pathogens in the studied areas can be useful in investigating their interaction with the different genotypes of *A. marginale* in the susceptible host.

In conclusion, *A. marginale* was molecularly detected from cattle populations in 6 different locations in the Philippines. Furthermore, the present study determined the prevalence of *A. marginale* and identified its different genotypes in cattle from geographically distant areas in the Philippines. The information on *A. marginale* genotypes in the Philip-

pines is apparently the first in Southeast Asia. Because genotypes may also vary in their pathogenicity, further studies are necessary to associate these genotypes with the clinical signs in cattle. In addition, farmers, local veterinarians, veterinary epidemiologists and the local government units in the Philippines should cooperate in preventing and controlling bovine anaplasmosis, as it can cause considerable economic losses.

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REFERENCES

1. Allred, D. R., McGuire, T. C., Palmer, G. H., Leib, S. R., Harkins, T. M., McElwain, T. F. and Barbet, A. F. 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. *Proc. Natl. Acad. Sci. U.S.A.* **87**: 3220–3224. [Medline] [CrossRef]
2. Almazán, C., Medrano, C., Ortiz, M. and de la Fuente, J. 2008. Genetic diversity of *Anaplasma marginale* strains from an outbreak of bovine anaplasmosis in an endemic area. *Vet. Parasitol.* **158**: 103–109. [Medline] [CrossRef]
3. Almazán, C., Moreno-Cantú, O., Moreno-Cid, J. A., Galindo, R. C., Canales, M., Villar, M. and de la Fuente, J. 2012. Control of tick infestations in cattle vaccinated with bacterial membranes containing surface-exposed tick protective antigens. *Vaccine* **30**: 265–272. [Medline] [CrossRef]
4. Bowie, M. V., de la Fuente, J., Kocan, K. M., Blouin, E. F. and Barbet, A. F. 2002. Conservation of major surface protein 1 genes of *Anaplasma marginale* during cyclic transmission between ticks and cattle. *Gene* **282**: 95–102. [Medline] [CrossRef]
5. Connell, M. and Hall, W. T. K. 1972. Transmission of *Anaplasma marginale* by the cattle tick *Boophilus microplus*. *Aust. Vet. J.* **48**: 477. [Medline] [CrossRef]
6. de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., McEwen, B. R., Clawson, D. and Kocan, K. M. 2001. Major surface protein 1a effects tick infection and transmission of *Anaplasma marginale*. *Int. J. Parasitol.* **31**: 1705–1714. [Medline] [CrossRef]
7. de La Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Rodríguez, S. D., García, M. A. and Kocan, K. M. 2001. Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale*. *Anim. Health Res. Rev.* **2**: 163–173. [Medline]
8. de la Fuente, J., Ruybal, P., Mtshali, M. S., Naranjo, V., Shuqing, L., Mangold, A. J., Rodríguez, S. D., Jiménez, R., Vicente, J., Moretta, R., Torina, A., Almazán, C., Mbati, P. M., de Echaide, S. T., Farber, M., Rosario-Cruz, R., Gortazar, C. and Kocan, K. M. 2007. Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences. *Vet. Microbiol.* **119**: 382–390. [Medline] [CrossRef]
9. de la Fuente, J., Van Den Bussche, R. A., Prado, T. M. and Kocan, K. M. 2003. *Anaplasma marginale msp1alpha* genotypes evolved under positive selection pressure but are not markers for geographic isolates. *J. Clin. Microbiol.* **41**: 1609–1616. [Medline] [CrossRef]
10. Dumler, J. S., Barbet, A. F., Bekker, C. P., Dasch, G. A., Palmer, G. H., Ray, S. C., Rikihisa, Y. and Rurangirwa, F. R. 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma, Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int. J. Syst. Evol. Microbiol.* **51**: 2145–2165. [Medline] [CrossRef]
11. Edgar, R. C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**: 113. [Medline] [CrossRef]
12. Fernández, D., González-Baradat, B., Eleizalde, M., González-Marcano, E., Perrone, T. and Mendoza, M. 2009. *Trypanosoma evansi*: A comparison of PCR and parasitological diagnostic tests in experimentally infected mice. *Exp. Parasitol.* **121**: 1–7. [Medline] [CrossRef]
13. Hall, B.G. 2011. Phylogenetic Trees Made Easy: A How-To Manual, 4th ed., Sinauer Associates, Massachusetts.
14. Hoar, B. R., Nieto, N. C., Rhodes, D. M. and Foley, J. E. 2008. Evaluation of sequential coinfection with *Anaplasma phagocytophilum* and *Anaplasma marginale* in cattle. *Am. J. Vet. Res.* **69**: 1171–1178. [Medline] [CrossRef]
15. Hofmann-Lehmann, R., Meli, M. L., Dreher, U. M., Gönczi, E., Deplazes, P., Braun, U., Engels, M., Schüpbach, J., Jörger, K., Thoma, R., Griot, C., Stärk, K. D., Willi, B., Schmidt, J., Kocan, K. M. and 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. [Medline] [CrossRef]
16. Hornok, S., Micsutka, A., Fernández de Mera, I. G., Meli, M. L., Gönczi, E., Tánczos, B., Mangold, A. J., Farkas, R., Lutz, H., Hofmann-Lehmann, R. and de la Fuente, J. 2012. Fatal bovine Anaplasmosis in a herd with new genotypes of *Anaplasma marginale, Anaplasma ovis* and concurrent haemoplasmosis. *Res. Vet. Sci.* **92**: 30–35. [Medline] [CrossRef]
17. Kocan, K. M., de la Fuente, J., Blouin, E. F., Coetzee, J. F. and Ewing, S. A. 2010. The natural history of *Anaplasma marginale*. *Vet. Parasitol.* **167**: 95–107. [Medline] [CrossRef]
18. Kuttler, K. L. 1984. *Anaplasma* infections in wild and domestic ruminants: a review. *J. Wildl. Dis.* **20**: 12–20. [Medline] [CrossRef]
19. Lew, A. E., Bock, R. E., Minchin, C. M. and Masaka, S. 2002. A *msp1alpha* polymerase chain reaction assay for specific detection and differentiation of *Anaplasma marginale* isolates. *Vet. Microbiol.* **86**: 325–335. [Medline] [CrossRef]
20. McGarey, D. J., Barbet, A. F., Palmer, G. H., McGuire, T. C. and Allred, D. R. 1994. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. *Infect. Immun.* **62**:

4594–4601. [Medline]
21. Mingala, C. N., Konnai, S., Cruz, L. C., Onuma, M. and Ohashi, K. 2009. Comparative moleculo-immunological analysis of swamp- and riverine-type water buffaloes responses. *Cytokine* **46**: 273–282. [Medline] [CrossRef]
22. Molad, T., Fleidrovich, L., Mazuz, M., Fish, L., Leibovitz, B., Krigel, Y. and Shkap, V. 2009. Genetic diversity of major surface protein 1a of Anaplasma marginale in beef cattle. *Vet. Microbiol.* **136**: 54–60. [Medline] [CrossRef]
23. Molina, J. Q. and Montenegro, M. M. 1977. Anaplasmosis in two Philippine cows. *Phil. J. Vet. Med.* **16**: 181–187.
24. Oberle, S. M., Palmer, G. H., Barbet, A. F. and McGuire, T. C. 1988. Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. *Infect. Immun.* **56**: 1567–1573. [Medline]
25. Ooshiro, M., Zakimi, S., Matsukawa, Y., Yafuso, M., Katagiri, Y. and Inokuma, H. 2009. *Anaplasma marginale* infection in a Japanese Black cow 13 years after eradication of *Rhipicephalus (Boophilus) microplus* in Okinawa, Japan. *Vet. Parasitol.* **160**: 351–355. [Medline] [CrossRef]
26. Palmer, G. H., Knowles, D. P. Jr., Rodriguez, J. L., Gnad, D. P., Hollis, L. C., Marston, T. and Brayton, K. A. 2004. Stochastic transmission of multiple genotypically distinct *Anaplasma marginale* strains in a herd with high prevalence of Anaplasma infection. *J. Clin. Microbiol.* **42**: 5381–5384. [Medline] [CrossRef]
27. Palmer, G. H., Rurangirwa, F. R. and McElwain, T. F. 2001. Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. *J. Clin. Microbiol.* **39**: 631–635. [Medline] [CrossRef]
28. Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **61**: 539–542. [Medline] [CrossRef]
29. Silveira, J. A., Rabelo, E. M. and Ribeiro, M. F. 2012. Molecular detection of tick-borne pathogens of the family Anaplasmataceae in Brazilian brown brocket deer (Mazama gouazoubira, Fischer, 1814) and marsh deer (Blastocerus dichotomus, Illiger,1815). *Transbound. Emerg. Dis.* **59**: 353–360. [Medline] [CrossRef]
30. Suarez, C. E. and Noh, S. 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. *Vet. Parasitol.* **180**: 109–125. [Medline] [CrossRef]
31. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–2739. [Medline] [CrossRef]
32. 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. and Inokuma, H. 2013. First molecular characterization of *Anaplasma marginale* in cattle and *Rhipicephalus (Boophilus) microplus* ticks in Cebu, Philippines. *J. Vet. Med. Sci.* **75**: 27–36. [Medline] [CrossRef]
33. Ybañez, A. P., Sivakumar, T., Ybañez, R. H., Vincoy, M. R., Tingson, J. A., Perez, Z. O., Gabotero, S. R., Buchorno, L. P., Inoue, N., Matsumoto, K., Inokuma, H. and Yokoyama, N. 2013. Molecular survey of bovine vector-borne pathogens in Cebu, Philippines. *Vet. Parasitol.* **196**: 13–20. [Medline] [CrossRef]
34. 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. and Xuan, X. 2013. Epidemiological survey of *Babesia bovis* and *Babesia bigemina* infections of cattle in Philippines. *J. Vet. Med. Sci.* **75**: 995–998. [Medline] [CrossRef]