

Molecular detection of *Anaplasma* spp. in blood and milk of dairy cattle in the Philippines

Angelito Pangan DELA CRUZ¹, Remil Linggatong GALAY^{1*}, Kristina Andrea Claveria SANDALO^{1,2},
Flor Marie Immanuelle Rafallo PILAPIL-AMANTE³, Tetsuya TANAKA⁴

¹Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, the Philippines

²Veterinary Teaching Hospital Los Baños Station, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, the Philippines

³Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, the Philippines

⁴Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan

Received: 12.03.2019 • Accepted/Published Online: 05.07.2019 • Final Version: 07.08.2019

Abstract: Anaplasmosis has become a major concern in the cattle industry throughout the world due to its great economic impact. The causative agents, *Anaplasma* species, are primarily transmitted by ticks, occurring intracellularly within blood cells, with some species being zoonotic. In this study, the presence of *Anaplasma* spp. was investigated in the blood and milk of dairy cattle in the Philippines. Blood and milk samples were collected from 98 dairy cattle from selected farms in five provinces in the southern part of Luzon Island in the Philippines. After DNA extraction, a conventional PCR for the control gene *actin* was performed, followed by nested PCR for *Anaplasma* spp. Selected amplicons were purified and subjected to sequence analysis. It was found that 97 (98.97%) blood samples and 6 (6.12%) milk samples were positive for *Anaplasma*. Sequence analysis revealed that the positive amplicons from milk samples and their corresponding blood samples shared a high identity (98%–100%) with reported *Anaplasma marginale* isolates. To the authors' knowledge, this study provides the first molecular evidence of the presence of *A. marginale* in milk from dairy cattle under field conditions in the Philippines.

Key words: *Anaplasma*, anaplasmosis, cattle, milk

1. Introduction

The Philippine dairy industry has been continuously growing over the years. In the first half of 2018, the National Dairy Authority (NDA) reported increased dairy cattle population and milk production [1]. The Philippine government continues to implement programs that support and further develop the dairy sector. In fact, the increased number of dairy cattle stocks is a result of ongoing government herd build-up programs and the growing number of NDA dairy-multiplier farms. Thus, there is also a need to improve herd health management to secure dairy production. Among the diseases that threaten the dairy cattle industry is anaplasmosis.

Bovine anaplasmosis is one of the most prevalent tick-borne diseases worldwide, particularly in tropical and subtropical countries in Africa, Asia, Australia, Europe, North America, and South America [2]. The Office International des Epizooties Animal Health Code classifies anaplasmosis as a notifiable disease due to its

socioeconomic impact and international trade restrictions [3]. Bovine anaplasmosis is primarily caused by infection of erythrocytes with *Anaplasma marginale*, characterized by high pathogenicity with clinical manifestations such as progressive hemolytic anemia associated with fever, jaundice without hemoglobinemia and hemoglobinuria, decreased milk production, abortions, hyperexcitability, and sudden death [4]. Another intraerythrocytic species, *A. centrale*, is considered mildly pathogenic or nonpathogenic. Meanwhile, *A. phagocytophilum* (formerly *Ehrlichia phagocytophila*) infects neutrophils and can infect a wide range of hosts aside from cattle, including sheep, goats, dogs, and horses, as well as humans. Infection with *A. phagocytophilum* may cause anorexia, reduced milk production, respiratory distress, coughing, and reproductive problems in cattle but is usually subclinical in many cases. In humans, it causes granulocytic anaplasmosis characterized by leukopenia, thrombocytopenia, and/or anemia [5].

* Correspondence: rlgalay@up.edu.ph

Anaplasma spp. are primarily transmitted by hard ticks belonging to the genera *Rhipicephalus*, *Dermacentor*, *Ixodes*, and *Hyalomma* [4]. It can also be transmitted by biting flies such as horseflies and stable flies, and by blood-contaminated fomites such as contaminated hypodermic needles, dehorning instruments, and other tools [2]. Transplacental transmission from infected dams to fetuses has also been reported to occur particularly during the second or third trimester, which may subsequently lead to abortion [6].

Detection of *Anaplasma* spp. has been traditionally done by microscopic examination of Giemsa-stained blood smears. Serological tests such as ELISA, card agglutination tests, and complement fixation tests and molecular techniques such as PCR have been developed and are increasingly used in recent years [2]. In the Philippines, studies employing PCR have shown the widespread occurrence of *A. marginale* in cattle in the country [7]. Since *Anaplasma* spp. are known to infect blood cells, most of the studies only focused on detection in the blood. The presence of antibodies against *A. marginale* in cow's milk has been demonstrated through ELISA [8]. Meanwhile,

the presence of *A. phagocytophilum* in leukocytes from the milk of experimentally infected cows was demonstrated by microscopic observation and nested PCR [9]. In other animals, *A. phagocytophilum* has been detected in the connective tissue of horses [10] and in skin lesions of sheep and dogs [11,12]. Recently, the presence of *Anaplasma* spp. in sheep's and goat's milk has also been demonstrated by PCR [13]. This study was conducted to determine whether *Anaplasma* spp. can be detected in the milk of dairy cattle from selected farms under natural field conditions in the Philippines.

2. Materials and methods

2.1. Study area and sample collection

The study area included the five provinces of Region IV-A, also known as CALABARZON, in the southern part of Luzon, the Philippines, namely Cavite, Laguna, Batangas, Rizal, and Quezon, located at 14.1008°N, 121.0794°E (Figure). Blood and milk samples were collected between September 2017 and June 2018 from a total of 98 lactating dairy cattle, regardless of breed, age, and health status,

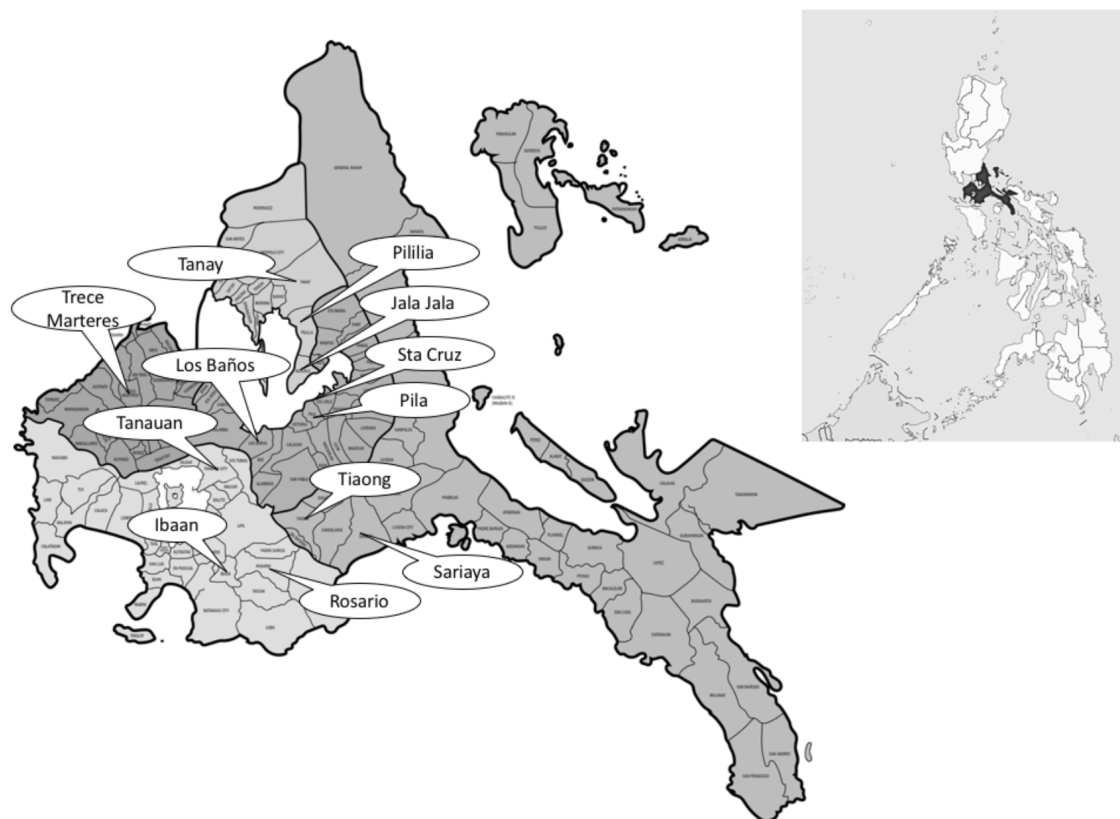


Figure. Map of the area of study, Region IV-A (CALABARZON), in the southern part of Luzon, the Philippines. The smaller map shows the relative location of CALABARZON in the Philippines. The municipalities from which the samples were collected are marked and labeled on the larger map. The smaller map was downloaded from Wikimedia Commons (<https://commons.wikimedia.org/wiki>).

which came from 12 selected commercial and backyard farms across the five provinces. From each cow, around 3 mL of blood was collected from the tail vein using a 5-mL syringe with a 21-G needle and stored in vacutainers containing ethylenediaminetetraacetic acid (EDTA). From the same animals, about 5 mL of milk was also manually collected and placed in conical tubes after the teats were disinfected with alcohol wipes. The samples were placed in a portable cooler with ice packs during transport and were stored at -20°C until DNA extraction. Animal handling and collection procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños, in accordance with applicable national guidelines.

2.2. DNA extraction and PCR

DNA from the blood and milk samples was extracted using a commercial spin column-based extraction kit (GF-1 Tissue Blood Combi Nucleic Acid Extraction Kit, Vivantis Technologies, Selangor Darul Ehsan, Malaysia) following the manufacturer's protocol. Milk samples underwent an initial process of butterfat removal, which involved a series of centrifugation and washing with phosphate-buffered saline [14], before proceeding to DNA extraction using the same extraction kit. DNA extraction was followed by a conventional PCR for the detection of the control gene *actin* to confirm successful extraction of DNA in all samples before proceeding to nested PCR, targeting the *groEL* gene of *Anaplasma* spp. The PCR mixtures were prepared using Tks Gflex DNA polymerase (Takara, Shiga, Kyoto, Japan) together with 2 μM primers and nuclease-free water. The primers and PCR conditions for detection of *Anaplasma* spp. were adapted from the study of Ybañez et al [15]. For the negative control, nuclease-free water was added to the PCR mixture instead of the DNA template. PCR products were run on 2% agarose gel in 1X Tris-acetate EDTA buffer, subsequently stained with ethidium bromide, and viewed in a gel documentation system (Bio-Print, Vilber Lourmat, France).

2.3. Sequence and data analysis

Positive amplicons from milk samples, as well as the amplicons from their corresponding blood samples, were purified using Nucleospin Gel and PCR Clean-Up Kit (Macherey-Nagel, Leicestershire, UK) following the manufacturer's protocol and were subjected to sequence reading using the forward primer for nested PCR [15]. The sequences were then compared to the sequences from reported *Anaplasma* spp. using the Basic Local Alignment Search Tool (BLAST) of the US National Center for Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignment of the nucleotide sequences of the positive amplicons was also done to compare the similarity using MAFFT version 7 ([\[mafft.cbrc.jp/alignment/server/index.html\]\(http://mafft.cbrc.jp/alignment/server/index.html\)\). Phylogenetic analysis was performed using online software \(<http://www.phylogeny.fr>\). Detection rate in the blood and milk samples was calculated by dividing the number of positive samples by the total number of samples, expressed as percentage.](https://</p>
</div>
<div data-bbox=)

3. Results

All DNAs extracted from blood and milk samples showed positive bands for the control gene *actin*, confirming successful DNA extraction, and were thus subjected to nested PCR, targeting the *groEL* gene of *Anaplasma* spp. A band with an expected size of 513 bp corresponding to *Anaplasma* spp. was observed in 97 of the 98 (98.97%) blood samples and 6 of 98 (6.125%) milk samples (Table). The positive milk samples came from the provinces of Cavite (one sample), Laguna (three samples), and Quezon (two samples). The sequences of the positive amplicons from milk and their corresponding blood samples were analyzed. BLAST analysis showed that the positive amplicons all shared 98% to 100% identity with reported *A. marginale* isolates (CP023730.1, CP006847.1, CP006846.1). Multiple sequence alignment showed that the amplicons were 99%–100% similar. Phylogenetic analysis was performed, and the constructed phylogenetic tree showed clustering of the isolates from this study with *A. marginale* isolates from other countries, suggesting a very close relationship (data not shown). The sequences of one amplicon from the blood and one amplicon from the milk were deposited in the DNA Data Bank of Japan and were assigned accession numbers LC461539 and LC461538, respectively.

4. Discussion

The occurrence of *Anaplasma* spp. in cattle in the Philippines has been well established already through PCR detection from the blood. Previous studies have shown the prevalence of *Anaplasma* spp. in several provinces of Luzon, including the current area of study [16–18]. CALABARZON in southern Luzon is reported to be among the regions with the highest cattle populations and contributes greatly to the country's milk production [19]. There are also previous studies from other Philippine islands such as Cebu [15,16,19], Negros, and Panay [17], which all together showed the endemicity of bovine anaplasmosis. Most of those previous studies only reported detection of *A. marginale*. However, in one study, some sequences of detected *Anaplasma* spp. were found to share high identity (99%–100%) with known *A. phagocytophilum* sequences [20]. Most of these previous studies in the Philippines only examined the presence of the pathogen in the blood of cattle. Recently, a study from China reported detection of *Anaplasma* in sheep's

Table. Number of *Anaplasma* spp.-positive samples and detection rate (%) in blood and milk from dairy cattle in southern Luzon (CALABARZON) provinces in the Philippines using nested PCR

| Sampling sites | | No. of blood samples tested | No. of blood positive (%) | No. of milk samples | No. of milk positive (%) |
|----------------|----------------|-----------------------------|---------------------------|---------------------|--------------------------|
| Province | Municipality | | | | |
| Cavite | Trece Martires | 10 | 10 (100) | 10 | 1 (10) |
| Laguna | Los Baños | 10 | 10 (100) | 10 | 2 (20) |
| | Pila | 3 | 3 (100) | 3 | 0 (0) |
| | Sta. Cruz | 9 | 9 (100) | 9 | 1 (11.11) |
| Batangas | Ibaan | 9 | 8 (88.88) | 9 | 0 (0) |
| | Rosario | 13 | 13 (100) | 13 | 0 (0) |
| | Tanauan | 2 | 2 (100) | 2 | 0 (0) |
| Rizal | Jala-jala | 2 | 2 (100) | 2 | 0 (0) |
| | Pililia | 10 | 10 (100) | 10 | 0 (0) |
| | Tanay | 9 | 9 (100) | 9 | 0 (0) |
| Quezon | Candelaria | 11 | 11 (100) | 11 | 1 (9.09) |
| | Tiaong | 10 | 10 (100) | 10 | 1 (10) |
| TOTAL | | 98 | 97 (98.97) | 98 | 6 (6.12) |

and goat's milk [13]. With the widespread occurrence of *Anaplasma* in dairy cattle not only in the Philippines but also in many other countries [21], it is interesting to know whether the pathogen can also be detected in the milk of naturally infected dairy cattle under field conditions, and hence this study was conducted.

The result of nested PCR detection of *Anaplasma* spp. in blood samples with an almost 100% detection rate implies widespread occurrence in dairy cattle from the five provinces and agrees with the results of previous studies [17,18]. Similarly, a high detection rate was reported in previous studies in the same provinces [16,18]. The high detection rate could be attributed to the prevalence of *Rhipicephalus (Boophilus) microplus* infestation in the study area. All farms included in this study had history of tick infestation, and on four farms, ticks have been actually observed in cattle from which samples were taken. During sample collection, none of the animals were observed to be exhibiting any clinical signs, implying that the animals may have been infected previously with *Anaplasma* and had become carriers, or may be infected with the nonpathogenic *A. centrale*. Additionally, since information on whether the sampled animals received vaccinations against anaplasmosis prior to their acquisition by the farms was not included in their records, the possibility that the positive result in the blood was due to the administered vaccine cannot be ignored.

Meanwhile, six of the milk samples were positive for *Anaplasma* spp. after nested PCR. Sequence analysis was done to determine the exact identity of the *Anaplasma* spp. amplicons from the milk. Interestingly, it was found that

those amplicons shared very high identity of 98% to 100% with reported *A. marginale* isolates, which also coincides with the sequence analysis result of the amplicons from the corresponding blood samples, suggesting that those cattle are indeed infected with *A. marginale*. Phylogenetic analysis showed clustering, most likely due to high homology shared with reported isolates from other countries. The targeted gene in this study was the heat shock *groEL*, which is known for high homology among closely related species. Thus, for phylogenetic analysis, we recommend targeting another gene, such as *Msp1a*, which is known to have greater variation [17].

In this study, the detection rate in milk was much lower than the detection rate in blood, which was similar to the previous report on detection of *Ehrlichia phagocytophila* (now *Anaplasma phagocytophilum*) in experimentally infected cows [9] and of *Anaplasma* spp. in goats [13]. *Anaplasma marginale* is known to occur mainly in erythrocytes and only the detection of antibodies against *A. marginale* in the milk was previously reported [8]. The presence of blood in the milk is uncommon, which may explain the low detection rate in this study. Occurrence of blood in the milk may happen due to trauma in the udder and teats from improper milking, which damages the epithelial lining of the teat cistern. The farms included in this study utilize milking machines to collect the milk in their daily operation, but it cannot be guaranteed that the farms' milking practices do not cause trauma to the udder. Blood cells can also appear in milk in the presence of mastitis, intravascular hemolysis, and capillary

damage in the udder due to systemic microbial infections and diseases, which may cause thrombocytopenia [22]. Additionally, there are pathogens that can infect the udder or be excreted through the udder from the milk [23]. During sample collection, none of the animals had visibly inflamed mammary quarters and none of the milk samples collected had visible discolorations or coagulation, which may suggest mastitis. However, mastitis cannot be ruled out since no test was performed. Furthermore, the animals might be affected with subclinical mastitis, wherein no visible signs can be observed. The leakage of blood cells during the early acute stage of mastitis occurs due to increased permeability and alteration in the blood/milk barrier [24] caused by some microbes or their endotoxins, which are able to disrupt the mammary alveolar tight junctions [25]. Additionally, erythrocytes infected with *A. marginale* undergo hemolysis, which can release the organism and may be brought to the milk through blood circulation.

In conclusion, this study reports the detection of *A. marginale* in the milk of dairy cows based on nested PCR and sequence analysis. To our knowledge, this study

provides the first molecular evidence of *A. marginale* being excreted in the milk. Further studies are needed to determine whether the pathogen is viable in the milk, since detection of its DNA does not indicate its viability. Moreover, whether *A. marginale* can be transmitted through the infected milk remains to be elucidated. The risk factors associated with the occurrence of *A. marginale* in milk, including the presence of mastitis, should also be examined.

Acknowledgments

This work was funded by the University of the Philippines Enhance Creative Work and Research Grant (ECWRG 2017-1-008) and Balik PhD (Foreign-trained PhD) grant, and the Japan Society for Promotion of Science (15H05264). The authors are also grateful to the National Dairy Authority's South Luzon Regional Office for assistance in sample collection; the participating farms and cattle owners; Dr. Billy P. Divina, Dr. Jesalyn Lapitan-Constante, and Dr. Emmanuel P. Hernandez for technical assistance; and Mr. Fernando P. Micoso for the map of study area.

References

1. National Dairy Authority Central Office. Reports of Accomplishments First Semester 2018. Quezon City, the Philippines: National Dairy Authority; 2018.
2. Aubry P, Geale DW. A review of bovine anaplasmosis. *Transboundary and Emerging Diseases* 2011; 58: 1-30.
3. Reinbold JB, Coetzee JF, Sirigireddy KR, Ganta RR. Detection of *Anaplasma marginale* and *A. phagocytophilum* in bovine peripheral blood samples by duplex real-time reverse transcriptase PCR assay. *Journal of Clinical Microbiology* 2010; 48: 2424-2432.
4. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA. The natural history of *Anaplasma marginale*. *Veterinary Parasitology* 2010; 167: 95-107.
5. Atif FA. *Anaplasma marginale* and *Anaplasma phagocytophilum*: Rickettsiales pathogens of veterinary and public health significance. *Parasitology Research* 2015; 114: 3941-3957.
6. Correa WM, Correa CN, Gottschalk AF. Bovine abortion associated with *Anaplasma marginale*. *Canadian Journal of Comparative Medicine* 1978; 42: 227-228.
7. Ybañez AP, Mingala CN, Ybañez RHD. Historical review and insights on the livestock tick-borne disease research of a developing country: the Philippine scenario. *Parasitology International* 2018; 67: 262-266.
8. de Echaide ST, Bono MF, Lugaresi C, Aguirre N, Mangold A et al. Detection of antibodies against *Anaplasma marginale* in milk using a recombinant MSP5 indirect ELISA. *Veterinary Microbiology* 2005; 106: 287-292.
9. Pusterla N, Huder J, Wolfensberger C, Braun U, Lutz H. Laboratory findings in cows after experimental infection with *Ehrlichia phagocytophila*. *Clinical and Diagnostic Laboratory Immunology* 1997; 4: 643-647.
10. Chang YE, Novosel V, Dubovi E, Wong SJ, Chu FK et al. Experimental infection of the human granulocytic ehrlichiosis agent in horses. *Veterinary Parasitology* 1998; 78: 137-145.
11. Granquist EG, Aleksandersen M, Bergström K, Dumler SJ, Torsteinbø WO et al. A morphological and molecular study of *Anaplasma phagocytophilum* transmission events at the time of *Ixodes ricinus* tick bite. *Acta Veterinaria Scandinavica* 2010; 52: 43.
12. Berzina I, Krudewig C, Silaghi C, Matise I, Ranka R et al. *Anaplasma phagocytophilum* DNA amplified from lesional skin of seropositive dogs. *Ticks and Tick-borne Diseases* 2014; 5: 329-335.
13. Zhang Y, Lu Y, Cui Y, Wang J, Cao S et al. First molecular evidence for the presence of *Anaplasma* DNA in milk from sheep and goats in China. *Parasitology Research* 2016; 115: 2789-2795.
14. Haguigan JB, Gordoncillo MN, Bautista JN, Hikiba M, Sarmago IG. Detection and identification of bacterial carriage of subclinical mastitis cases in backyard dairy cows. *Philippine Journal of Veterinary Medicine* 2010; 47: 21-25.
15. Ybañez AP, Sivakumar T, Ybañez RHD, Ratilla JC, Perez ZO et al. First molecular characterization of *Anaplasma marginale* in cattle and *Rhipicephalus (Boophilus) microplus* ticks in Cebu, Philippines. *Journal of Veterinary Medical Science* 2013; 75: 27-36.

16. Ochirkhuu N, Konnai S, Mingala CN, Okagawa T, Villanueva M et al. Molecular epidemiological survey and genetic analysis of vector-borne infections of cattle in Luzon Island, the Philippines. *Veterinary Parasitology* 2015; 212: 161-167.
17. Ybañez AP, Ybañez RHD, Claveria FG, Cruz-Flores MJ, Xuenan X et al. High genetic diversity of *Anaplasma marginale* detected from Philippine cattle. *Journal of Veterinary Medical Science* 2014; 76: 1009-1014.
18. Aquino AJB, Divina BP, Bombio AM, Pilapil FMIR. Detection of *Anaplasma marginale* infection in a dairy cattle farm by stained blood smear examination and nested polymerase chain reaction. *Philippine Journal of Veterinary and Animal Science* 2018; 44: 68-75.
19. Philippine Statistics Authority. Performance of Philippine Agriculture October-December 2018. Quezon City, the Philippines: Philippine Statistics Authority; 2018.
20. Ybañez AP, Sivakumar T, Ybañez RHD, Vincoy MRB, Tingson JA et al. Molecular survey of bovine vector-borne pathogens in Cebu, Philippines. *Veterinary Parasitology* 2013; 196: 13-20.
21. Paramanandham K, Mohankumar A, Puttahnappa Suresh K, Susan Jacob S, Roy P. Prevalence of *Anaplasma* species in India and the World in dairy animals: A systematic review and meta-analysis. *Research in Veterinary Science* 2019; 123: 159-170.
22. George LW, Divers TJ, Ducharme N, Welcome FL. Diseases of the teats and udder. In: Divers TJ, Peek SF (editors). *Rebhun's Diseases of Dairy Cattle*. 2nd ed. St. Louis, MO, USA: Saunders-Elsevier; 2008. p. 335.
23. Griffiths MW. The microbiological safety of raw milk. In: Griffiths MW (editor). *Improving the Safety and Quality of Milk*. Cambridge, UK: Woodhead Publishing Limited; 2010. p. 28.
24. Burton JL, Erskine RJ. Immunity and mastitis some new ideas for an old disease. *Veterinary Clinics of North America Food Animal Practice* 2003; 19: 1-45.
25. Kobayashi K, Oyama S, Numata A, Rahman MM, Kumura H. Lipopolysaccharide disrupts the milk-blood barrier by modulating claudins in mammary alveolar tight junctions. *PLoS One* 2013; 8: e62187.