

DETECTION OF *Anaplasma marginale* INFECTION IN A DAIRY CATTLE FARM BY STAINED BLOOD SMEAR EXAMINATION AND NESTED POLYMERASE CHAIN REACTION

Arrol Jan B. Aquino¹, Billy P. Divina², Ariel M. Bombio¹
and Flor Marie Immanuelle R. Pilapil³

ABSTRACT

Bovine anaplasmosis is caused by *Anaplasma marginale*. The study was conducted to detect the presence of *A. marginale* in a dairy cattle farm using stained blood smear examination (SBSE) and nested polymerase chain reaction (nPCR). Blood samples from 281 dairy cattle were collected and subjected to SBSE and nPCR. Based on the results, 73.7% and 67.3% *A. marginale*-infected cattle were detected using SBSE and nPCR, respectively. The higher detection rate of SBSE was attributed to false positives. Detection of infection rate using SBSE was significantly higher than nPCR. Detection of *A. marginale* infection using SBSE and nPCR showed a 77% agreement with a kappa coefficient of 0.44. The results of the study would help in assessing the infection status of the herd and help establish diagnostic protocols for the detection of *A. marginale* specific for acute and carrier state of infection.

Keywords: *Anaplasma marginale*, dairy cattle, microscopy, polymerase chain reaction

INTRODUCTION

Bovine anaplasmosis is caused by an intraerythrocytic pathogen *Anaplasma marginale* (Kocan *et al.*, 2010ab). It is a tick-borne disease of cattle that can also be transmitted mechanically by biting flies and through fomites (Kocan *et al.*, 2003; Ashuma *et al.*, 2013). Transplacental transmission from cow to calf has also been reported (Kocan *et al.*, 2003; Aiello *et al.*, 2012). The disease occurs worldwide (Ybañez *et al.*, 2012) being endemic in one-third to one-half of livestock production regions causing significant economic losses. It is considered of great importance in cattle production systems in tropical and subtropical regions of the world (Fosgate *et al.*, 2010). In the Philippines, only limited information about *A. marginale* infection in cattle can be accessed (Ybañez *et al.*, 2012). Published reports on *A. marginale* infection in cattle in the Philippines include those of Molina and Montenegro (1977), Ybañez *et al.* (2012, 2013, 2014) and Ochirkhuu *et al.* (2015). In this latest study by

¹Department of Basic Veterinary Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, Laguna 4031, Philippines, ²Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, Laguna 4031, Philippines, ³Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, Laguna 4031, Philippines (e-mail: abaquino@up.edu.ph).

Ochirkhuu *et al.* (2015) in Luzon, Philippines; the authors have reported a 95.5% prevalence of *A. marginale* and was the most prevalent vector-borne diseases (VBDs) of cattle in the area

Severe anemia and jaundice without hemoglobinemia and hemoglobinuria characterize the disease. Weight loss, decreased milk production, abortions, hyperexcitability (as a result of cerebral anoxia), and sudden death are other clinical manifestations (Fosgate *et al.*, 2010; Ashuma *et al.*, 2013). Recovery from acute stage of the disease results in persistent infection in cattle, which may serve as long-term reservoirs for transmission within herds and hence, detection of persistent infection is important to control the movement of infected cattle into and from disease-free regions to avoid outbreaks and significant economic losses (Noaman and Shayan, 2010).

Detection for the presence of *A. marginale* infection in cattle includes both direct and indirect methods. The commonly used technique is through evaluation of peripheral blood smear by light microscopy and is suitable for acutely infected cattle. A method that has higher degree of sensitivity and specificity in detecting persistently infected cattle is polymerase chain reaction (PCR), which detects DNA of the organism.

The dairy cattle farm of interest had a previous history of anaplasmosis and therefore has possible existence of carrier state of infection. The need to conduct regular monitoring for detection and determination of infection status of the herd will help the farm in formulating measures to prevent outbreaks of the disease. The study was conducted to detect the presence of *A. marginale* within the dairy cattle farm using microscopy method through stained blood smear examination (SBSE) and by molecular method using nested PCR (nPCR). In addition, comparison of the detection rate and percent agreement of the two methods were determined. The results of the study would help in assessing the infection status of the herd using the two different methods, which could help established diagnostic protocol for the detection of *A. marginale* in acute and carrier state of infection.

MATERIALS AND METHODS

Two hundred eighty-one dairy cattle, composed of 278 Holstein-Friesian x Sahiwal crosses and 3 pure Holstein-Friesian, irrespective of age and sex, that are available for blood collection were chosen randomly and examined for *A. marginale* infection using SBSE and nPCR. This comprised approximately 70.6% of the total farm population.

Sample animals were restrained properly with the use of a nose lead and rope tied in a metal post (for bulls), individually in their feeding area using rope (for cows, heifers, and calves), or in groups using metal pipes fences that served like a large chute. Blood samples were collected once using a 19-gauge hypodermic needle attached to a 5 ml sterile disposable syringe and with a size-20 vacutainer needle via jugular venipuncture in bulls, heifers, and calves; whereas venipuncture of the median caudal vein was done in cases of cows. At least 5 ml of blood were collected and placed in a vial containing ethylenediaminetetraacetic acid (EDTA).

Blood smears were prepared, air dried, and stained using a rapid stain (Medic Diagnostic Reagents® Hema-Quick Stain Set, Medical Center Trading Corp., Pasig, Philippines). Slides were examined and evaluated using light microscopy under oil immersion objective (1000X). At least 20 oil immersion microscopic fields using Meander search system were examined for presence of *A. marginale* inclusion bodies.

DNA were extracted from 200 µl anticoagulated blood using a blood DNA extraction kit (Vivantis® GF-1 Blood DNA Extraction Kit, Vivantis, Malaysia) following the manufacturer's instruction. Extracted DNA were stored at - 20°C until testing.

Oligonucleotide primers for the nPCR assay were obtained from AITbiotech Pte Ltd (Singapore). The external primers used were patterned after Molad *et al.* (2006). Internal primers used were AM100 5'-CGAGAGCGTGGGACTACGTGC-3' and AM101 5'-TGGCCTTCCGCGAGCATGTG-3'. Nested PCR was performed according to the procedures of Molad *et al.* (2006) with some modifications of cycling (initial denaturation at 94°C for 30s, followed by 40 cycles of denaturation at 94°C for 30s) and nesting cycling protocol (annealing at 60°C for 30s). Reaction products were analyzed by agarose gel electrophoresis using 2% agarose gel and stained using GelRed™ Nucleic Acid Gel Stain - 10,000x in water (Biotium, Inc., California). Gel was viewed using UVP-DigiDoc-It® Imaging System (UVP, California). Selected 20 positive samples were sent for sequencing analysis (Macrogen Inc., Korea).

Number of positive and negative results using SBSE and nPCR were statistically analyzed using an epidemiological statistical software (OpenEpi ver. 3.01, CDC, France) utilizing the Chi square test and/or Fisher exact test at $P < 0.05$. Percent agreement and kappa statistic of the two methods were calculated based on the formula used by Bryington *et al.* (2002).

RESULTS AND DISCUSSION

The study confirms the presence of *A. marginale* infection in the farm. Based on the results (Table 1), stained blood smear examination (SBSE) detected 73.7% (207/281) *A. marginale*-infected cattle (Figure 1) and nested polymerase chain reaction (nPCR) detected 67.3% (189/281) *A. marginale*-infected cattle (Figure 2). Comparison of infection rate based on SBSE and nPCR showed that the infection rate obtained using SBSE was significantly higher than that obtained with nPCR. But in the detection of negatives, nPCR was better than SBSE (Table 1). This could be due to false positive results of SBSE with possible identification of non-*A. marginale* inclusion bodies as *A. marginale*. Moreover, nPCR was able to detect 24 positive samples (8.5%), which were negative in SBSE. These samples could be considered as true positives because nPCR is considered to be more

Table 1. Detected positives and negatives using SBSE and nPCR and the percent agreement of the two methods.

SBSE	nPCR		Total
	Positive	Negative	
Positive	165	42	207 ^a (73.7%)
Negative	24	50	74 ^d (26.3%)
Total	189 ^b (67.3%)	92 ^c (32.7%)	281

^{abcd}Values with a different letter superscript are significantly different ($\alpha=0.05$)

Percent of agreement = $165 + 50 / 281 = 0.77$

Chance agreement = $(207 \times 189 / 2812) + (74 \times 92 / 281^2) = 0.59$

Kappa = $(0.77 - 0.59) / (1 - 0.59) = 0.44$

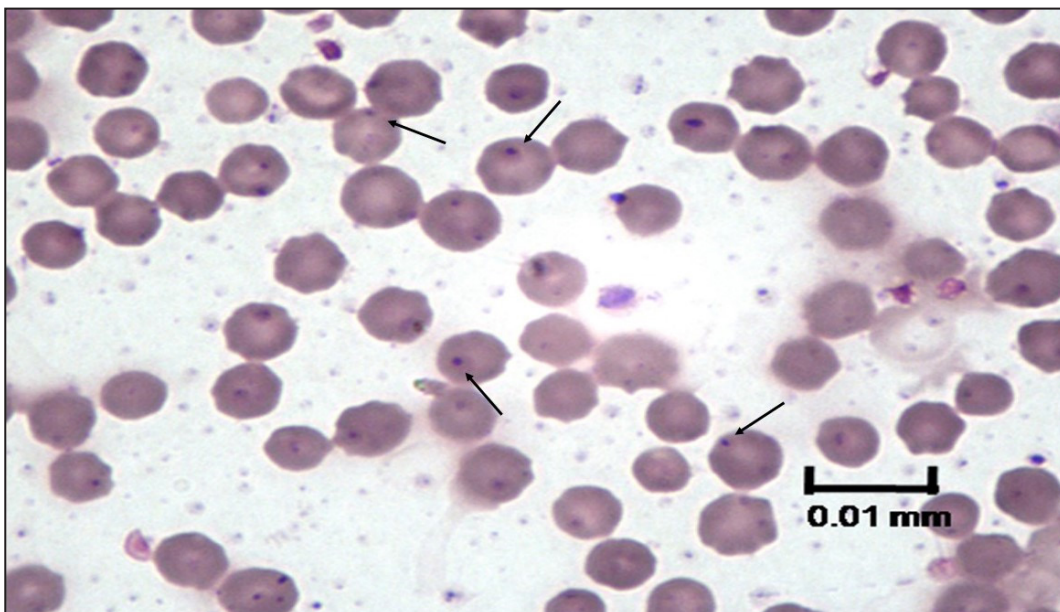


Figure 1. Representative image of stained cattle blood smear positive for *A. marginale* (arrows) under oil immersion objective (1000X) using Nikon E200 with DS-Fi1 camera and DS-L2 control unit attachments.

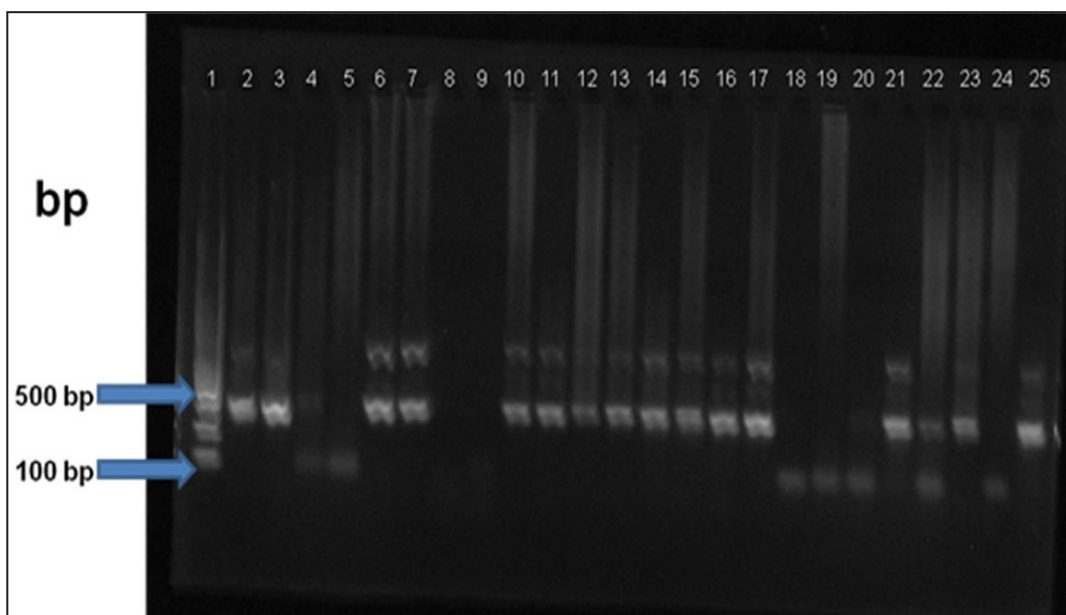


Figure 2. Representative picture of agarose-gel electrophoresis showing result of nPCR amplification product obtained from using *A. marginale* specific primers. Lane 1: 100 bp DNA Ladder, Lane 24: negative control, Lane 25: positive control, and Lanes 2-3, 6-7, 10-17, 21-23: positive samples.

sensitive and specific than SBSE. The result of the study is in contrast with the study of Ybañez *et al.* (2012) wherein examination using SBSE revealed 3 cattle (25%) showing *A. marginale* inclusion bodies while using a *16S rRNA* screening PCR and subsequent sequencing revealed 8 cattle (66.7%) to be positive for *A. marginale* infection.

In general, PCR-based methods such as nPCR are more sensitive and specific than other diagnostic methods such as SBSE (Molad *et al.*, 2006; Fosgate *et al.*, 2010; Noaman and Shayan, 2010; Ashuma *et al.*, 2013) particularly in cases of low parasitemia or mixed infections (Kieser *et al.*, 1990; Carelli *et al.*, 2007; Noaman and Shayan, 2010). PCR-based method uses a thermostable DNA polymerase to specifically amplify the target sequence up to 200,000-fold or more which helps in detecting even the lowest levels of parasitemia found in carrier cattle (Eriks *et al.*, 1989). DNA-based detection methods are most useful in cases of species and strain differentiation tests wherein serological tests have limitations. PCR detects DNA of the parasite and therefore recognizes active infection, and the relative amount of DNA detected correlates with the level of parasitemia the animal is having (Fosgate *et al.*, 2010). One example is nested-PCR (nPCR) which can reveal carrier cattle by amplification of *A. marginale* DNA. It is usually 10-10,000 times more sensitive than standard PCR assay (Torioni de Echaide *et al.*, 1998). However, some published studies about malaria have reported that PCR may occasionally yield false negative results (Barker *et al.*, 1994; Singh *et al.*, 1996). According to Coleman *et al.* (2006), there are limitations that can affect PCR performance. This includes selection of appropriate primers, collection method, storage of blood, and extraction method. In addition, Jelinek *et al.* (1996) reported in a study about *Plasmodium falciparum* that as SBSE is linked to parasite density, PCR-based methods could also be affected by parasite density. Barker *et al.* (1994) confirms that false negative PCR results could occur; however, major discrepancies in using SBSE and PCR come mainly from SBSE.

According to Noaman and Shayan (2010), SBSE is indeed accompanied with problems. SBSE is not suited for determination of pre-symptomatic or carrier animals and is difficult to interpret in those instances (Carelli *et al.*, 2007). According to Kieser *et al.* (1990), rickettsemia in carrier animals is usually below the limit detectable by SBSE (< 0.1% infected erythrocytes). Additionally, it is difficult to differentiate between *A. marginale* inclusions and structures like Heinz bodies, Howell-Jolly bodies, and staining artifacts especially in case of carrier state of infection. It needs special experiences (Noaman and Shayan, 2010). Moreover, differentiation of *A. marginale* and *A. centrale* in low level rickettsemia is difficult (OIE, 2012). In the study done by Ybañez *et al.* (2013) in Cebu, Philippines; the authors have reported that anaplasmosis is the most prevalent vector-borne disease (54.7%) and 24% of the reported prevalence was a multiple infection and 89.8% of it were associated with *Anaplasma species*. Hence, one could assume that multiple infection of *Anaplasma species* such as *A. centrale* could have also occurred in the study and probably could have affected the SBSE by listing *A. centrale* inclusion bodies as positive. Another limitation in the study is the number of microscopic fields to be examined. In the study, 20 microscopic fields were examined for the presence of *A. marginale* inclusion bodies. However, in the study by Noaman and Shayan (2010), SBSE was used even in carrier animals by examination of 50 and 100 microscopic fields. SBSE using 50 microscopic fields yield 25.8% sensitivity and 99% specificity and SBSE using 100 microscopic fields yield 91.4% sensitivity and 76.1% specificity in reference with PCR-restriction fragment length polymorphism (RFLP) which has 100% sensitivity and specificity. However, even with the increased

sensitivity of using 100 microscopic fields compared to using 50 microscopic fields, specificity was decreased. Hence, detection of *A. marginale* infection in carrier cattle by SBSE is very difficult (Noaman and Shayan, 2010).

In nPCR as possible source of discrepancy in the study, false negative results could have obtained due to different *A. marginale* strain present in the farm where the primers used could have not detected. In the study done by Ybañez *et al.* (2012) in Cebu, Philippines; the authors have used *Msp1a* as basis of PCR method and only few have been detected positive in contrast to positives detected by using *16S rRNA*, *Msp5*, and *groEL*. According to Lew *et al.* (2002), *Msp1a* is proven highly sensitive and specific for detecting *A. marginale* in Australia. However, based on the results, *Msp1a* PCR method could have differed in sensitivity if used with the Philippine isolates. Ybañez *et al.* (2012) added that *Msp1a* is probably more suitable only for Australian isolates having only 1 genotype compared to Philippine isolates wherein at least 3 genotypes have been identified. In the study, *Msp1b* was used as the basis of the PCR method. According to Barbet and Allred (1991), *Msp1b* is a multigene family, which varies within the strain. One could assume that the primers used were not able to completely detect the strain present in the farm resulting to the decreased detection of positives compared to SBSE assuming that the detected positives using SBSE were true positives. In the study, true disease status of sample animals was not confirmed. Hence, one could assume that both SBSE and nPCR probably could have false positive and false negative results. This could be true in the study where there were positive samples in nPCR but were negative in SBSE and vice versa.

The results of the study showed that there is a statistically significant difference in using SBSE and nPCR in the detection of *A. marginale* infection. The result (Table 1) shows a 77% agreement in the detection of *A. marginale* using SBSE and nPCR with a kappa of 0.44 showing that there is fair clinical significance between the two diagnostic methods.

Based on the results of the study, it is recommended to consider regular monitoring of the herd using both microscopy and molecular methods with possible infection of *A. marginale* to identify those that are persistently infected and can serve as carrier of infection. Likewise, an established criterion (detection of *A. marginale* in at least 20 microscopic fields) using stained blood smear examination can prove useful for the immediate treatment of acutely infected animal.

In conclusion, the results of the study highly recommend that the farm attend to the problem of high infection rate with *A. marginale* and look into the production impact of the infection. A control program that considers the presence of vectors (ticks and biting flies), management practices and physiological state of the animals should be planned to decrease transmission among the animals in the farm and finally prevent outbreaks of the disease.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Therese Marie A. Collantes, Dr. Alisha Wehdnesday B. Reyes and Dr. Claro N. Mingala for their guidance and advice for the molecular experimentation of the study.

REFERENCES

- Aiello SE, Moses MA, Allen DG, Constable PD, Davies PR, Queensberry KE, Reeves PT, Sharma JM, Smith RKW and Treadwell T. 2012. The Merck Veterinary Manual. New Jersey: Merck & Co., Inc.
- Ashuma AS, Singla LD, Kaur P, Bal MS, Bath BK and Juyal PD. 2013. Prevalence and haemato-biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India). *Asian Pac J Trop Med* 6(2): 139-144.
- Barbet AF and Allred DR. 1991. The *msp1 β* multigene family of *Anaplasma marginale*: nucleotide sequence analysis of an expressed copy. *Infect Immun* 59(3): 971-976.
- Barker RH Jr., Banchongaksorn T, Courval JM, Suwonkerd W, Rimwungtragoon K, and Wirth DF. 1994. *Plasmodium falciparum* and *P. vivax*: factors affecting sensitivity and specificity of PCR-based diagnosis of malaria. *Exp Parasitol* 79: 41-49.
- Bryington AA, Palmer DJ and Watkins MW. 2002. The estimation of interobserver agreement in behavioral assessment. *Behav Anal Today* 3(3): 323-328.
- Carelli G, Decaro N, Lorusso A, Elia G, Lorusso E, Mari V, Ceci L and Buonavoglia C. 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet Microbiol* 124: 104-107.
- Coleman RE, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, Kengluetcha A, Rachapaew N, Zollner G, Miller RS, Vaughan JA, Thimasarn K and Khuntirat B. 2006. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum*/*vivax* endemic area in Thailand. *Malar J* 5: 121-127.
- Eriks IS, Palmer GH, McGuire TC, Allred DR and Barbet AF. 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle using a nucleic acid probe. *J Clin Microbiol* 27(2): 279-284.
- Fosgate GT, Urdaz-Rodriguez JH, Dunbar MD, Owen Rae D, Arthur Donovan G, Melendez P, Dobek GL and Alleman AR. 2010. Diagnostic accuracy methods for detecting *A. marginale* infection in lactating dairy cattle of Puerto Rico. *J Vet Diagn Invest* 22: 192-199.
- Jelinek T, Proll S, Hess F, Kabagambe G, von Sonnenburg F, Loscher T and Kilian AH. 1996. Geographic differences in the sensitivity of a polymerase chain reaction for the detection of *Plasmodium falciparum* infection. *Am J Trop Med Hyg* 55: 647-651.
- Kieser ST, Eriks IS and Palmer GH. 1990. Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. *Infect Immun* 58(4): 1117-1119.
- Kocan KM, de la Fuente J, Guglielmone AA and Melendez RD. 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 16(4): 698-712.
- Kocan KM, de la Fuente J, Step DL, Blouin EF, Coetzee JF, Simpson KM, Genova SG and Boileau MJ. 2010a. Current challenges of the management and epidemiology of bovine anaplasmosis. *Bov Pract* 44(2): 93-102.
- Kocan KM, de la Fuente J, Blouin EF, Coetzee JF and Ewing SA. 2010b. The natural history of *Anaplasma marginale*. *Vet Parasitol* 167: 95-107.

- Lew AE, Bock RE, Minchin CM and Masaka S. 2002. A *msp1a* polymerase chain reaction assay for specific detection and differentiation of *Anaplasma marginale* isolates. *Vet Microbiol* 86: 325-335.
- Molad T, Mazuz ML, Fleiderovitz L, Fish L, Savitsky I, Krigel Y, Leibovitz B, Molloy J, Jonjegan F and Shkap V. 2006. Molecular and serological detection of *A. centrale*- and *A. marginale*-infected cattle grazing within an endemic area. *Vet Microbiol* 113: 55-62.
- Molina JQ and Montenegro MM. 1977. Anaplasmosis in two Philippine cows. *Philipp J Vet Med* 16: 181-187.
- Noaman V and Shayan P. 2010. Comparison of microscopy and PCR-RFLP for detection of *Anaplasma marginale* in carrier cattle. *Iran J Microbiol* 2(2): 89-94.
- Ochirkhuu N, Konnai S, Mingala CN, Okagawa T, Villanueva M, Pilapil FMI, Murata S and Ohashi K. 2015. Molecular epidemiological survey and genetic analysis of vector-borne infections of cattle in Luzon island, the Philippines. *Vet Parasitol* 212(3-4): 161-167.
- OIE. 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 7th ed. Vol. 1, Section 2.4 Bovidae, Chapter 2.4.1 Bovine Anaplasmosis. Accessed 25 September 2012 http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.01_BOVINE_ANAPLASMOSIS.pdf
- Singh B, Cox-Singh K, Miller AO, Abdullah MS, Snounou G and Rahman HA. 1996. Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. *Trans R Soc Trop Med Hyg* 90: 519-521.
- Torioni de Echaide S, Knowles DP, McGuire TC, Palmer GH, Suarez CE and McElwain TF. 1998. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J Clin Microbiol* 36(3): 777-782.
- Ybañez AP, Sivakumar T, Ybañez RHD, Ratilla JC, Perez ZO, Gabotero SR, 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(1): 27-36.
- Ybañez AP, Sivakumar T, Ybañez RHD, Vincoy MRB, Tingson JA, Perez ZO, Gabotero SR, Buchorno LP, Inoue N, Matsumoto K, Inokuma H and Yokoyama N. 2013. Molecular survey of bovine vector-borne pathogens in Cebu, Philippines. *Vet Parasitol* 196(1-2): 13-20.
- Ybañez AP, Ybañez RHD, Claveria FG, Cruz-Flores MJ, Xuenan X, Yokoyama N and Inokuma H. 2014. High genetic diversity of *Anaplasma marginale* detected from Philippine cattle. *J Vet Med Sci* 76(7): 1009-1014.