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

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Detection of Anaplasma marginale in a dairy cattle farm

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’-TGGCCTTCCCGAGCATGTG-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>
<thead>
<tr>
<th>SBSE</th>
<th>nPCR</th>
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<tr>
<td>Positive</td>
<td>165</td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
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<tr>
<td>Total</td>
<td>189&lt;sup&gt;b&lt;/sup&gt; (67.3%)</td>
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<sup>abcd</sup>Values with a different letter superscript are significantly different ($\alpha=0.05$)

Percent of agreement = 165 + 50 / 281 = 0.77

Chance agreement = (207 x 189 / 281<sup>2</sup>) + (74 x 92 / 281<sup>2</sup>) = 0.59

Kappa = (0.77-0.59) / (1-0.59) = 0.44
Detection of *Anaplasma marginale* in a dairy cattle farm

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
Detection of *Anaplasma marginale* in a dairy cattle farm

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.

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