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Diagnosis of subclinical hemoprotozoal and rickettsial infections in Bovine by blood smear and ELISA

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Abstract
Subclinical hemoparasitic infections are the major cause of morbidity and mortality in bovines as they easily evade being diagnosed during routine physical examination. Hence a study was carried out to know the incidence and prevalence of hemoprotozoan and ricketsial infections in cattle with no overt clinical manifestation by blood smear examination and ELISA. Examination of blood smears revealed *Babesia* organisms in 24 (40%) animals, *Anaplasma* in 28 (44%) animals and *Theileria* in 15 (23%) animals. In ELISA, 38 animals were positive for *Babesia bigemina* antibodies and 42 animals were positive for *Anaplasma marginale* antibodies.

Keywords: Bovine, haemoprotozoans, *Babesia* spp, *Anaplasma* spp, *Theileria* spp, blood smear, ELISA

Introduction
India has a cattle population of 199 million according to the quinquennial livestock census 2012 and its contribution to the production of milk made the country the largest producer of milk in the whole world. But the average milk production per animal in 2012-13 is 7.02 kg/day which is below the world average of 7.86 kg/day mainly because of absence of proper culling, poor nutrition and diseases (Birthal and Jha 2005) [1]. Haemoprotozoan diseases cause devastating losses to the livestock industry and pose major constraints to the dairy industry throughout the world (Perry *et al.*, 2002 and Minjaw and McLeod, 2003) [2, 3]. Rajput *et al.* (2005) [4] reported haemoparasitic diseases like Babesiosis, Trypanosomiasis, Theileriosis, Anaplasmosis and Microfilariasis as major impediments in the health and productive performance of cattle.

The various haemoparasites affecting the dairy cattle in India include Microfilaria, *Trypanosoma evansi*, Babesia, Anaplasma, and Theileria spp. They are diagnosed based on clinical signs exhibited by the animals, blood smear and lymph node aspiration smear examination, serological and molecular tests. The various clinical signs exhibited by cattle infected with blood parasites include pyrexia, inappetance, reduction in milk yield, lymph node enlargement, discoloured urine etc.

Magonigle and Newby (1984) [5] reported that the cattle which survive initial infection with *A. marginale* remain carriers of the parasites for several years and are immune to further clinical manifestations. Hence the present study was taken up to investigate the incidence of subclinical cases of hemoprotozoan and rickettsial infection by optical microscopy and Enzyme Linked Immunosorbant Assay (ELISA) method in the chosen cattle farm at Vithura in apparently healthy animals.

Materials and Methods
Study area
The present study was conducted in Jersey Farm, Vithura which is located at Thiruvananathapuram. As Vithura is a hilly area sharing boundaries with forest region, the cattle in the farm were maintained mostly in free range condition.

Study population
A total of 64 milch cows in the farm were randomly selected for the study. All animals which were apparently healthy and with no intestinal parasitic infection ruled out by examination of

dung sample for ova by concentration method were included for the study.

Staining of Blood Smears

Peripheral blood smears were prepared, air dried, fixed in methyl alcohol for 2-3 min, stained with Leishman and Giemsa stain for 30 min in a staining jar and rinsed in buffered distilled water (Kamani *et al*., 2010) [6]. Haemoparasites were then detected by direct microscopic examination of stained blood smears based on morphologic keys as described by Soulsby (1982) [7] using a compound microscope (Olympus, USA).

Detection of *Babesia* and *Anaplasma* antibodies by ELISA

ELISA (Enzyme-Linked Immunosorbant Assay) was performed with serum samples collected from cattle for detection of antibodies against *B. bigemina* and *A. marginale*. Antibodies in serum for *Anaplasma* were detected using VMRD cELISA kit. The positive and negative controls and serum samples were loaded into a transfer plate and incubated for 30 minutes at temperature of 23±2 ^{0}C. Then 50 µl of controls and samples were transferred to the corresponding well of the *Anaplasma* antigen –coated plate and the plate were incubated for 1 hour at a temperature of 23±2 ^{0}C. After the 1 hour incubation, the plates were washed 2 times with PBS- Tween solution. Then 50 µl of diluted 1X antibody-peroxidise conjugate was added and incubated for 20 minutes at a temperature of 23 ±2 ^{0}C. Again the plates were washed with PBS- Tween solution thrice and then 50 µl of substrate solution was added to each well. The plates were incubated for 20 min at 23 ±2 ^{0}C. After incubation, 50 µl of stop solution was added to each well and the OD of wells was recorded at 620 nm with ELISA plate reader. The Percentage Inhibition (PI) was calculated as; PI= 100[1-(OD_{sample} / $OD_{negative\ control}$)]. The samples with percentage inhibition ≥30 were interpreted as positive samples and samples with percentage inhibition <30 were interpreted as negative samples.

Antibodies in serum for *B. bigemina* were detected using SVANOVA kit (Sweden Inc.). In duplicates, 100 µl of pre-diluted positive control and negative control were added and serum samples were added to respective selected wells and incubated at 37 ^{0}C for 30 minutes. The plates were rinsed with PBS- tween buffer and then 100 µl of HRP conjugate was added to each well. The plates were again incubated at 37 ^{0}C for 30 minutes. The plates were then washed with PBS- tween buffer and 100 µl substrate solution was added to each well. The plates were further incubated for 30 minutes at room temperature and then 100 µl of stop solution was added to each well. The optical density (OD) of the controls and samples were measured at 405 nm in ELISA plate reader. The percentage positivity was calculated as PP= OD_{sample} /$OD_{positive\ control}$ x100. Based on percentage positivity, samples with PP ≥ 40 were interpreted as positive samples and samples with PP ≤ 25 were interpreted as negative samples and samples with PP 26-39 were interpreted as borderline or doubtful cases.

Results and Discussion

Microscopical examination of blood smears

On microscopical examination of blood smears, *Babesia* organisms were detected in 24 (40%) animals, *Anaplasma* in 28 (44%) animals and *Theileria* in 15 (23%) animals. Mixed infection with *Babesia* and *Anaplasma* were detected in 2(3%) animals, *Anaplasma* and *Theileria* in 4(6%) animals and *Babesia* and *Theileria* in 2(3%) animals. (Table 1). Except one case, none of the smears had more than one haemoprotozoan organisms per 10 oil immersion fields.

Table 1: Incidence of haemoprotozoans in cows

Sl. No.	Organism	No. of animals (out of 64)
1	*Babesia*	24 (40%)
2	*Anaplasma*	28 (44%)
3	*Theileria*	15(23%)
4	*Babesia* and *Anaplasma*	2 (3%)
5	*Anaplasma* and *Theileria*	4(6%)
6	*Babesia* and *Theileria*	2(3%)
7	Negative for any haemoprotozoans	15(23%)

Detection of antibodies against *B. bigemina* and *A. marginale* by ELISA

Of the 64 samples tested, 38 were positive for *B. bigemina* Ab, 42 were positive for *A. marginale* Ab and 15 were negative for both by ELISA. 31 were positive for antibodies against both *B. bigemina* and *A.marginale.* The data on analysis of result of ELISA for detection of antibodies is shown in Table 2. The result showed a very high percentage of seropositivity among animals for *B. bigemina* and *A. marginale.* Seropositivity could be due to recent infection, premunity or due to antibodies produced during a previous infection. Salih *et al.* (2015) [8] opined that serodiagnosis is not different between current and past infection as the animal may already have cleared the pathogen but remain seropositive. De Waal (2012) [9] stated the difficulty in demonstrating parasites in carrier animals as the number of parasites in such animals fall below the detectable levels soon after the acute stages of the disease. Only a small percentage of the animals had concurrent presence/absence of organism in the blood and antibody titre.

Correlating the findings of blood smear examination and ELISA revealed that a higher percentage of animals had Ab titre but no organisms in the blood (84% for *Babesia* and 67% for *Anaplasma*). *Babesia* organisms were not demonstrable in blood smear in 85% of *Babesia* seronegative animals and *Anaplasma* organisms were not demonstrable in blood smear in 86% of Anaplasma seronegative animals.

Only 16% of animals with Ab titre against *B. bigemina* revealed *Babesia* organisms in blood smear and only 26% of animals with Ab titre against *A. marginale* revealed *Anaplasma* organisms in the blood smear. 15% of animals with *Babesia* in the blood smear were seronegative for *Babesia* and 14% of animals with *Anaplasma* in the blood smear were seronegative for *Anaplasma.* The correlation between the results of blood smear examination and ELISA for detection of antibodies is shown in Table 3.

Terkawi *et al.* (2011) [10] and Carelli *et al.* (2007) [11] reported that the direct method of diagnosis of haemoprotozoans by examination of stained blood smears shows a low sensitivity in subclinical and chronic phases of the infection. Hence ELISA method of antibody estimation will be a complementary screening tool in diagnosis of hemoprotozoan infections. Kachani *et al*. (1992) [12] stated the employment of ELISA in increasing rate for the detection of parasite-specific antibodies, antigens and immune complexes.

Table 2: Detection of *B. bigemina* and *A. marginale* antibodies by ELISA

Sl No.	Antibody detected	Positive	Positive for both	Negative	Negative for both
1	*B. bigemina* Ab	38 (59%)	31 (48%)	26 (41%)	15(23%)
2	*A.marginale* Ab	42 (66%)		22 (34%)	

Table 3: Detection of organism in blood smear vis. antibody titre

Sl. No.	Organism	Seropositivity (no. of animals)	Test	No. of animals	Percentage
1	*Babesia*	Ab titre + (38)	Smear +	6	16%
			Smear -	32	84%
		Ab titre – (26)	Smear +	4	15%
			Smear -	22	85%
2	*Anaplasma*	Ab titre + (42)	Smear +	11	26%
			Smear -	28	67%
		Ab titre – (22)	Smear +	3	14%
			Smear -	19	86%

Conclusion

The present study revealed that a significant percentage of animals in the cattle farm chosen for the study had Babesia, Theileria and Anaplasma organisms in the blood despite appearing apparently normal. Hence it gives the importance of screening blood smears even in apparently healthy animals especially in areas with heavy tick infestation by blood smear examination as well as by ELISA method to identify the cattle suffering with subclinical form of hemoprotozoan and rickettsial infections. Identifying such animals will help to start an early therapeutic intervention before the infections becoming severe and causing untoward morbidity and mortality losses in cattle.

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