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Molecular detection of *Babesia* sp. infection in bovines from slaughter house, Mizoram

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Abstract
Bovine babesiosis is a one of the serious threat to cattle industry due to treatment and tick control costs, production loses and animal mortality. It is mainly caused by *Babesia bovis* and *Babesia bigemina* in the tropics and subtropics. Slaughterhouse was regularly visited and blood samples were randomly collected in EDTA vials from bovines being slaughtered, for the study of different hemoparasitic infections. Total 100 number of bovine blood samples were collected properly in EDTA vials from Govt. slaughterhouse Mizoram. Thin blood smears were prepared and stained with 10% Giemsa stain and observed under oil immersion lens (100 X magnifications) for presence of *Babesia* sp. Further blood samples were undergone Molecular detection through PCR analysis. Total 22 number of samples were found positive out of 100 no. of blood samples in morphological diagnosis whereas 27 samples were found positive for *Babesia bigemina* in molecular diagnosis using a portion of the mitochondrial DNA of *Babesia bigemina*. The incidence of *Babesia* sp. infections was more common in summer followed by winter and monsoon.

Keywords: *Babesia* sp., bovine, molecular, morphological, Mizoram, slaughter house

Introduction
Although cattle play an important role in the economy in North Eastern Hill region, several limiting factors come to play in the way of ensuring sustainable productivity and profitability from these animals. Parasitic diseases are one of the major problems which adversely affect the health and productivity of cattle (Bhatnagar *et al*., 2015) [1]. The exotic and cross breeds of animals reared in India are of more susceptible to tick infestation and thereby tick borne diseases compared to indigenous animals (Kumar *et al*., 2015) [2]. The diverse climatic zones of India are highly conducive for survival and propagation of vectors and vector-borne pathogens (Bhattacharjee and Sarmah, 2013; Laha *et al*., 2015) [3, 4]. The two most important haemoprotozoan diseases encountered in cattle are babesiosis and theileriosis (Rajput *et al*., 2005) [5]. Anaplasmosis, one of the most important rickettsial disease occurred in cattle, has also great impacts on animal productivities (Radostitis *et al*., 2000) [6].
Bovine babesiosis is a one of the serious threat to cattle industry due to treatment and tick control costs, production loses and animal mortality (McLeod and Kristjanson, 1999) [7]. It is mainly caused by *Babesia bovis* and *Babesia bigemina* in the tropics and subtropics (Bock *et al*., 2004) [8]. Disease development in cattle is affected by many factors like age, management practices, immunity and breed (Bock *et al*., 2004) [8]. Usually animals with less than 9 months old use to show resistant to clinical babesiosis while adults are more prone to clinical infections (Zintl *et al*., 2005; Goff *et al*., 2001) [9, 10]. Immunity acquired by young animals protects them from developing clinical babesiosis when they become infected as adults. Also *Bos indicus* is relatively more resistant as compared to *Bos taurus* to clinical infections (Bock *et al*., 1999) [21].
Abattoirs have an important role mainly in the surveillance of various diseases of animals and human beings (Alton *et al*., 2010) [12]. Abattoir study also plays a significant role to know about the extent of exposure of the public to certain zoonotic diseases and helps in estimation of the financial losses due to condemnation of affected organs (Cadmus and Adesokan, 2009; Raji *et al*., 2010; Singla and Juyal, 2014) [13, 14, 15]. In India cattle, bulls and cow slaughters are allowed by the Government in states like Arunachala Pradesh, Assam, Goa, Kerala, Mizoram, Meghalaya, Nagaland, Tripura and West Bengal.

The present study was conducted as there is no proper data regarding incidence of haemoparasitic infections in bovines which are slaughtered in Mizoram or even in India.

Materials and Methods

Govt. Slaughter house was regularly visited and blood samples were randomly collected in EDTA vials from bovines being slaughtered, for the study of different hemoparasitic infections. Total 100 number of blood samples were collected properly in EDTA vials and brought to the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Central Agricultural University, Mizoram, India for further examination.

Blood Smear Examination

Thin smears were prepared on a clean grease free slides from the anticoagulated blood samples and were fixed with absolute methanol for 30 seconds. Fixed smears were stained with 10% Giemsa stain for 45 mins. The stained smears were washed under gentle running tap water. Then the smears were air dried and observed under oil immersion lens (100X magnifications) for presence of *Babesia* sp. The *Babesia* sp. were identified on the basis of characteristics morphology (Schalm *et al*., 1975) [16].

Genomic DNA isolation from Blood samples

Blood samples were further used for DNA isolation which was used in PCR assay latter. DNA extraction was carried out by use of DNeasy Blood and Tissue Kit (Qiagen®, Germany, Catalogue No. 69504) as per manufacturer's protocol. 20 µl of Proteinase K Solution was added to 200 µl of whole blood and mixed by vortexing. Then 400 µl lysis solution was added to that mixture thoroughly by vortexing for obtaining a uniform suspension. After that the mixture was incubated at 56 °C for 10 minutes in water bath for complete lysis. Then 200 µl of ethanol (96-100%) was mixed by pipetting and transferred to spin column and centrifuged for 1 minutes at 8,000 rpm. The collection tube with flow-through solution was discarded. Then after washing with 500 µl of Wash Buffer (WB I) and Wash Buffer II (WB II), final elution was done with 200 µl of Elution Buffer (EB). Recovery and purity of each DNA sample was estimated by Spectrophotometer (NanoDrop®ND-1000, PeqLab, Erlangen, Germany) according to manufacturer's instructions (NanoDrop® User Manual, 2004) and stored in -20 °C for further use.

PCR Assay

Species specific primers and PCR thermal profile for *Babesia bigemina* is shown in Table 1 and 2 respectively. The PCR reaction was carried out in 12.5 µl of 10 X PCR green buffers (Thermo Scientific, USA) containing 0.1 µl of Taq DNA polymerase, 0.5 µl of each primer (10 pmol/µl) and 0.3 µl of 10mM dNTPs and 1 µl of template DNA. Amplification was done in C1000 thermal cycler (BioRad, USA) and the amplicons were checked in agarose gel electrophoresis using 1.5% agarose gel containing ethidium bromide. The gel was then visualized on a UV transilluminator.

Table 1: Sequence of Oligonucleotide primer used for study

Species	Primer sequence pair	Amplification Target	Product size	Reference
Babesia bigemina	Bbi-F: 5'-TGGCGGCGTTTATTAGTTCG-3' Bbi-R: 5'-CCACGCTTGAAGCACAGGA-3'	A portion of the *Babesia bigemina* mitochondrial DNA	1124 bp	Laha *et al*., 2015 [4]

Table 2: Thermal Profile for *Babesia bigemina* (40 Cycles)

Step I	95 °C	5 mins	Initial denaturation
Step II	95 °C	30 sec	Denaturation
Step III	55 °C	30 sec	Annealing
Step IV	72 °C	1 mins	Extension
Step V	72 °C	10 mins	Final extension

Results

Morphological diagnosis

Thin blood smears were prepared from blood samples, stained with 10% Giemsa and observed under light microscope. *Babesia* sp. were intraerythrocytic and appeared as a single round (Fig. 1), ovoid, elongate or amoeboid trophozoites. The large shaped forms appeared as pair with their narrow ends at an acute angle while small forms formed obtuse angle. Total 22 number of samples were found positive out of 100 no. of blood samples in morphological diagnosis.

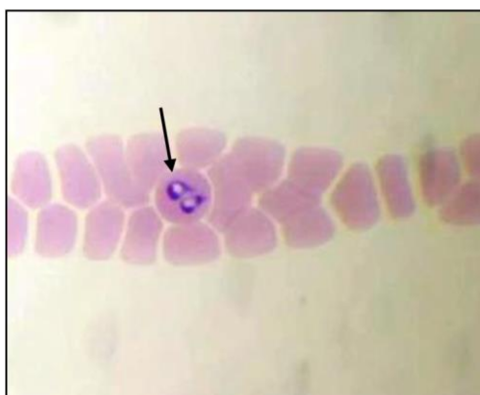

Fig 1: *Babesia* sp. inside RBC (Giemsa, 1000x).

Molecular Diagnosis

Blood samples examined microscopically for Babesia sp. infections were further tested by molecular method. These samples were tested by PCR for detection of a portion of the mitochondrial DNA of *Babesia bigemina*. 27 samples were found positive for *Babesia bigemina*. A 1124 bp size of fragment of a portion of mitochondrial DNA of *Babesia bigemina* was amplified (Fig. 2). More number of blood samples were found positive in molecular detection as compared to morphological diagnosis may be due to subclinical infections in those samples.

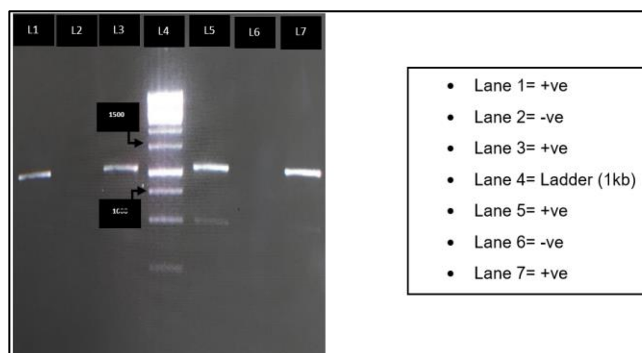

Fig 2: 1.2% Agarose gel electrophoresis stained with Ethidium bromide showing 1124 bp of a portion of the *Babesia bigemina* mitochondrial DNA in blood sample.

Season-wise Prevalence

Total 100 no. of blood samples were collected i.e. 32 no. in summer (March- June), 35 no. in monsoon (July-October) and 33 no. in winter (November-February). Overall incidence of *Babesia* sp. infections was 22% (i.e. 22/100) in morphological diagnosis. But the overall incidence was more i.e. 27% (27/100). The season wise incidence was given in the table no. 3. In summer 9 samples showed positive in morphological diagnosis out of 32 samples. In winter 7 samples showed positive out of 33 samples whereas in monsoon 6 samples showed positive out of 35 samples in morphological diagnosis. The incidence of *Babesia* sp. infections was more common in summer (281.15%) followed by winter (21.21%) and monsoon (17.14%). But in molecular detection 11 samples in summer, 7 samples in monsoon and 9 samples in winter was found positive from 32, 35 and 33 respectively. The incidence trend was similar with morphological diagnosis. The incidence of *Babesia* sp. infections in molecular detection was more common in summer (34.37%) followed by winter 27.27%) and monsoon (20.00%).

Table 3: Season wise incidence of *Babesia* sp. infection

Season	Total number of samples collected	Incidence % in Morphological Diagnosis	Incidence % in Molecular Diagnosis
Summer	32	28.15% (9/32)	34.37% (11/32)
Monsoon	35	17.14% (6/35)	20.00% (7/35)
Winter	33	21.21% (7/33)	27.27% (9/33)

Discussion

During this study, *Babesia* sp. infection was confirmed by blood smear examination and by the molecular detection using PCR. Bovine babesiosis have serious impact on the livestock production by causing severe economic loss to the livestock owner in our country (Bhattacharjee and Sarmah, 2013) [3]. During my study period, Overall incidence of *Babesia* sp. infections was 22% (i.e. 22/100) in morphological diagnosis whereas overall incidence was more i.e. 27% (27/100) in molecular diagnosis. But Velusamy *et al*. (2014) [17] recorded overall prevalence of 16.64% of hemoparasitic infections in Tamilnadu, India and in another study by Bhatnagar *et al*. (2015) [1] in Rajasthan, India, the overall prevalence was 9%. The difference in the results may be due to variations in the climatic and geographical conditions of the study areas and managemental practices of animals. Laha *et al*. (2015) [4] reported about the prevalence of Babesiosis in north eastern India i.e. 3.6%. The season wise prevalence showed that the hemoparasitic infections was more common in monsoon (52.78%) followed by summer (30.55%) and winter (16.67). In case of *Babesia* sp., infections were more common in summer (36.36%) followed by winter (33.33%) and monsoon (31.58%). During particular season, the high prevalence of tick vectors might be responsible for propagation and multiplication of hemoparasites in both hosts and vectors. Similar results were reported in previous studies (Mohanta *et al*., 2011; Kohli *et al*., 2014; Bhatnagar *et al*., 2015 and Ghosh *et al*., 2018) [18, 19, 1, 20]. A 1124 bp size of fragment of a portion of mitochondrial DNA of *Babesia bigemina* was amplified as per Laha *et al*. (2015) [4].

Conclusions

The present studies for the presence of *Babesia* sp. infections. Total 22 number of samples were found positive out of 100 no. of blood samples in morphological diagnosis whereas 27 samples were found positive for *Babesia bigemina* in molecular diagnosis. The incidence of *Babesia* sp. infections was more common in summer followed by winter and monsoon. As the haemoprotozoan infections are having great threat to the bovine industry, so proper managemental care should be taken along with treatment aspects.

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