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First Report on Molecular Identification of *Babesia bigemina* in a Nervous Signs Evident Naturally Infected Holstein-Friesian Cattle from Central India

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ABSTRACT

Background: Bovine babesiosis caused by an intraerythrocytic apicomplexan protozoon responsible for the most prevalent and costly tick borne diseases (TBD's) of cattle throughout the globe. Cerebral babesiosis of bovine is fatal and mainly caused by *Babesia bovis*. To the knowledge of author, there is no confirm molecular report of *Babeisa bigemina* caused cerebral babesiosis in cattle. Therefore, authors want to report *Babesia bigemina* caused cerebral babesiosis on record.

Methods: In the year 2015, a Holstein-Friesian cow aged 3 years and weighing approximately 300 kg, was attended at Jabalpur, (M.P.) with the clinical signs of high rise in temperature (104°F), recumbency, severe dysponea, peculiar sound during open mouth breathing, pale color of eye conjunctiva and mucous membrane of vagina, convulsions, sever anaemia, paddling of legs at frequent interval. After preparation of peripheral thin blood smear from animal at the site of collection and fixation with methanol, blood sample brought to Department of Veterinary Parasitology, College of Veterinary Science and A.H, Jabalpur and stained by standard protocol for Giemsa staining. Genomic DNA was isolated from the collected blood sample using QIAamp® DNA blood mini kit following the manufacturer's recommendations and PCR was performed.

Conclusion: The thin blood smear examination revealed the presence of *Babesia* parasite. The species of *Babesia* was confirmed by molecular amplification of genomic DNA as *B. bigemina*. This might be the first confirmed report of cerebral babesiosis caused by *B. bigemina* from Central India.

Key words: *Babesia bigemina,* Cattle, Cerebral babesiosis, PCR.

INTRODUCTION

Bovine babesiosis is worldwide distributed and responsible for serious burden on livestock production and human livelihood and is attaining interest due its emerging zoonotic nature (Bock *et al.,* 2004). Bovine babesiosis is inadequately controlled diseases in various part of the globe. Ixodid ticks are responsible for transmission of babesial parasite to vertebrate host. (Agrawal *et al.,* 2017 and Sharma *et al.,* 2013) reported only 2.17% and 2.43% prevalence of babesiosis in cattle in central part and Punjab state of India, respectively. Blood protozoans have been found to produce nervous disorders in a variety of host *viz.* cerebral malaria in human caused by *Plasmodium falciparum* (Cecil and Loeb, 1959), turning sickness in cattle of East Africa, caused by *Theileria parva* (Mettam and Carmichael, 1936) and cerebral babesiosis in cattle caused by *B. bovis* (Bock *et al.,* 2004). Economic losses caused by babesiosis in livestock of India are 57.2 million USD annually (Mcleod and Kristjanson, 1999). Depending on the size of merozoites, *Babesia* species have been grouped in to large (3-5 µm) and small form (0.5-2.5 µm). There are different species of *Babesia* examined from bovine namely *B. bigemina*, *B. bovis*, *B. divergens* and *B. major*. In India, *B. bigemina* is the major species reported from cattle although there are very few reports of *B. bovis* from India (Shastri *et al.,* 1991; Gautam and Chhabra, 1983; Muraleedharan *et al.,*1984; Maharana *et al*., 2016). Intravascular haemolysis and haemoglobinuria is the main pathogenesis caused by *B.bigemina* while excessive production of cytokinin particularly IL-6, tumor necrosis factor -6 resulting in *B.bovis* caused cerebral babesiosis (CB). Cerebral babesiosis has been reported mainly by *B. bovis* and few cases of CB have been reported due to *B. argentina*. Although, *B.argentina* is considered as synonym of *B. bovis* (Mahoney and Mirre, 1977). Due to the

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evidence of broader diversity of *Babesia* species by molecular studies, it is imperative to identify the *Babesia* species by molecular methods. The thin blood smear is a tradition method but gold standard for diagnosis of babesiosis and other blood protozoans but have limited sensitivity (Kaur *et al*., 2016). The molecular method using specific primer for PCR based amplification of genomic DNA overcome not only sensitivity issue but also gives confirmatory diagnosis (Singh *et al*., 2013). This paper highlights the first case of molecular based evidence of cerebral babesiosis caused by *Babesia bigemina* in naturally infected Holstein Friesian cattle.

MATERIALS AND METHODS

In the year 2015, a Holstein Friesian cattle aged 3 years and weighing 300 kg, owned by a dairy owner was showing high rise of temperature (104°F), lateral recumbency, paddling of legs at frequent interval, convulsion, severe dyspnea, peculiar sound during open mouth breathing, pale color of eye conjunctiva and mucous membrane of vagina, sever anemic (Fig 1). The previous case history revealed that animal was showing the said clinical signs from last four days along with passing of coffee color urine. But at the time of examination the urine was transparent.

Aseptically 3 ml blood sample were collected from ear vein of suffering cow in blood collection vial containing EDTA anticoagulants. The reason to collect the blood sample from ear vein was that as capillary blood may contain up to 20 times higher *B. bovis* than blood of general circulation. While there is no distribution discrimination of *B. bigemina* parasitized cell in the blood circulation of host (Callow *et al.,* 1993). Peripheral thin blood smear was prepared from animal at the site of collection and immediately fixed with methanol. Blood sample brought to Department of Veterinary Parasitology, College of Veterinary Science and A.H., Jabalpur and blood smear stained by standard protocol for Giemsa staining and examined under oil immersion of microscope.



Fig 1: Holstein Friesian cattle suffering from cerebral form of Babesiosis.

Genomic DNA was isolated from the collected blood sample using QIAamp® DNA blood mini kit following the manufacturer's recommendations. In brief, approximately 200 µl of the blood sample was mixed with 20 µl of proteinase-K and 200 µl of lysis buffer in a 2.0 ml microcentrifuge tube. The homogenous suspension was thoroughly vortexed and incubated at 56°C for 10 min. Subsequently, 200 µl of ethanol was added to the lysate and again vortexed. The mixture was then applied to QIAamp spin column and centrifuged at 8000 rpm for 1 min. Thereafter, 2 washings were given with wash buffers and DNA was eluted in 200 µl of elution buffer and stored at -20°C till further use. Genomic DNA of *B. bigemina* was isolated from infected blood showing parasitaemia in Giemsa stained blood smear examination and utilized as positive control. Genomic DNA was also isolated from the whole blood of infection-free, 3-day-old bovine calf and used as a negative control.

PCR assay

B.bigemina specific primers as per the protocol of (Figueroa *et al.,* 1992) were used during the PCR assay.
The sequences of the primers were as follows:

Bbig278 Forward: 5′-CAT CTA ATT TCT CTC CAT ACC CCT CC-3′

Bbig278 Reverse: 5′-CCT CGG CTT CAA CTC TGA TGC CAA AG-3′

For the PCR following components were used; master mix consisted of 2.5 µl of 10X PCR buffer, 0.5 µl of 10 mM dNTP mix, 2.0 µl of 25 mM $MgCl_2$, 1.0 U of recombinant TaqDNA polymerase, 1 µl each (15 pmol) of Bbig278 Forward and Bbig278 Reverse primers and 5 µl of template DNA isolated from field and the volume was made up to 25 µl with nuclease-free water. The cycling conditions were: initial denaturation at 94°C for 5 min, 37 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min and the final extension was done at 72°C for 10 min. The PCR product was checked for amplification by electrophoresis on a 1.2% agarose gel and visualized using gel documentation system (Syngene, UK).

RESULTS AND DISCUSSION

Haematological values namely haemoglobin, PCV, TLC, Neutrophils, Lymphocytes and Eosinophils of suffered animal were found 3.0 g/dl, 11%, 4000/µl, 36%, 62% and 2%, respectively which indicated that cow was suffering from severe anemia, sever leucopenia, neutrophilia and eosinophilia at the last stage of disease. The clinical picture of microscopic examination of blood smears showed anisocytosis with *Babesia* organism approximately in 30% RBCs. Morphologically these were visualized as pear shaped lied in pair forming an acute angle with size more than 3 µm average length (Fig 2). The same sample in PCR based amplification gave the confirmed the presence of *B.bigemina* (Fig 3).

The high rise of temperature as response to effect of unspecific toxic substances produced during the metabolism

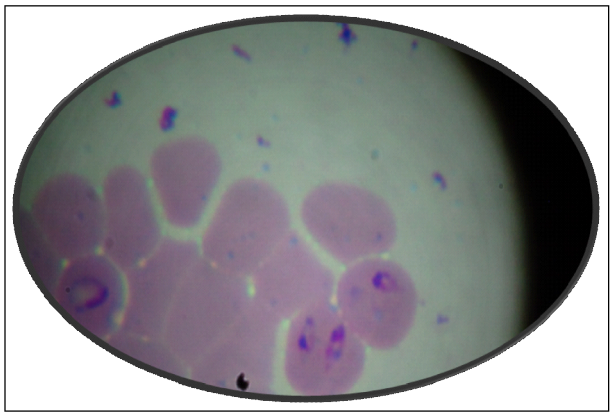

Fig 2: Intra erythrocytic *Babesia bigemina* organism (in pair) under oil immersion lens (x1000).

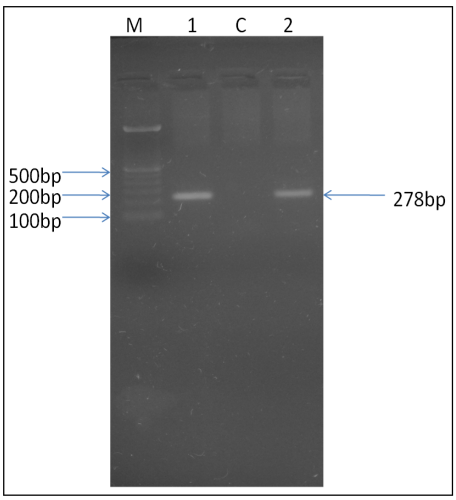

Fig 3: Agarose gel electrophoresis (1.5%) showing the band of 278 bp fragment from genomic DNA of *Babesia bigemina*. Lane M: 100 bp DNA ladder, Lane 1: Amplification of *B. bigemina* genomic DNA from the blood of animal positive for infection (positive control) Lane C: Negative control (No temple), Lane 2 Positive processed field samples.

of *Babesia*. Subsequently, the heart rate was increased, marked dyspnea was developed. Similar Plus minus findings were also reported by several workers in cattle suffering from cerebral babesiosis. In case of *B.bigemina*, mild form of anemia has been reported by various workers (Vikrant *et al.*, 2013) but in present study sever form of anemia has been reported might be due to high level of parasitaemia which leads to oxidative damage to the erythrocytes (Saleh, 2009) or suppression of erythropoiesis and phagocytosis of non infected erythrocyte due to changing in its antigenic surface (Esmaeilnejad *et al.,* 2020). Haemoglobinuria was present in early stage of infection but urine was transparent in latter stage of infection which is in corroborated with findings of (Bock, 2004).

The morphology of parasite in blood smear examination revealed that the present case is of *B. bigemina* and this was again confirmed by molecular method. In the previous studies it is stated that the cerebral form of babesiosis is caused by *B. bovis*. This might be the first report of cerebral babesiosis by *B. bigemina*. In the present study *B. bigemina* was confirmed by traditional as well as molecular methods, although further investigations are required to prove *B. bigemina* as cause of cerebral babesiosis. The relation of cerebral babesiosis by *B. bigemina* with other factors like breed of bovine, age, sex, prevalence of vectors should also be investigated.

Many species of *Babesia* have different characteristic with relation to severity of disease and drug susceptibility hence, accurate detection of *Babesia* species is required. Although there are number of reports of *B. bovis* caused cerebral babesiosis in cattle. *B. bovis* has the ability to markedly alter erythrocyte structure and function, causing parasitized RBCs to cytoadhesion or accumulate in the microvasculature and giving rise to fatal cerebral babesiosis, respiratory distress and multiorgan failure, contrary to *B. bovis*, in case of *B. bigemina* the pathogenesis of cerebral babesiosis is not studied (Wright and Goodger., 1988). There is lot of difference between biology of *B. bovis* and *B. bigemina* as sporozoites of *B. bigemina* are mainly transmitted by nymph while sporozoites of *B. bovis* are mainly transmitted by larvae of ticks. Intravascular sequestrations of infected erythrocytes are very common in *B. bovis* and rarely occur with *B. bigemina* infections. *B. bovis* is more pathogenic than *B. bigemina* and only 1% level of parasitaemia is sufficient to produce acute form of disease while in case of *B. bigemina* parasitaemia level exceeds 10%. Hence, exact pathogenesis behind cerebral babesiosis in case of *B. bigemina* is required. Cerebral babesiosis caused by *B. argentina* has been reported (Callow and Mcgavin, 1963) but latter on it was confirmed that *B. argentina* is not a separate species but it is *B. bovis*. In a study conducted by (Ajayi, 1978) of cerebral pathology in cattle, only 8% of the animals had babesiosis, of which only 10% exhibited heavily parasitized capillaries However, because of this low incidence and the fact that few studies have analysed CB, one should be careful in concluding that other *Babesia* species do not cause CB.

CONCLUSION

Haemoglobinuria is not a consistent feature of babesiosis in cattle hence, it is imperative if cattle is showing nervous symptoms along with anemia then same should be diagnosed to rule out the possibility of *B.bigemina* infection. Presently there is no report of occurrence of molecular evidence of *B.bigemina* caused cerebral babesiosis in Holstein Friesian cattle. This is a first report of its kind and hence placed on record.

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