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A longitudinal investigation of common haemoparasites in cattle calves in the Namakkal district of Tamil Nadu, India

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Abstract
A study was carried out to investigate the blood protozoan parasites in cross-bred cattle calves in the Namakkal district of Tamil Nadu. Whole blood samples from 20 numbers of cross-bred calves were collected at monthly intervals from the first month of birth to 6 months of age. Haematological studies were carried out by Auto analyzer and DNA was extracted from blood and screened for haemo-parasites using species-specific PCR. The blood smears were also examined microscopically for the presence of haemo-protozoan parasites. The haematological values like PCV, Hb, RBC, and WBC of normal calves were in the range of 23.9 to 35.7%, 6.5 to 13.5 g/dl, 7.8 to 10.6 x 10^6/µl, and 8.6 to 12.4 x 10^3/µl, respectively, whereas in the infected calves, they were 19.1 to 32.5%, 6.9 to 10.5 g/dl, 7.3 to 10.7 x 10^6/µl, and 7.9 to 11.5 x 10^3/µl, respectively. Out of 20 calves screened by PCR, 2 calves were positive for *Theileria annulata* and 3 calves were positive for *Anaplasma marginale*, but, none of these calves showed positive for *Babesia bigemina* infection. No swelling of lymph nodes and changes in mucous membrane was observed and there were no ticks observed on the body during the study period. This study suggests that infected young calves may act as a carrier for haemo-protozoan diseases and may lead to death under stressful conditions such as transportation and vaccination.

Keywords: Cross-bred cattle calves, *Theileria* spp., *Babesia* spp., *Anaplasma* spp., PCR

Introduction
Haemo-parasitic infections are widespread in cattle, cause substantial economic losses to the livestock industry, and pose a significant threat to the worldwide dairy industry. Tropical bovine theileriosis is a tick-borne disease caused by *Theileria annulata*. In India, the prevalence of theileriosis is very high among cross-bred cattle like Jersey and Holstein Frisian, causing huge economic losses to poor farmers. Although the native breeds and buffaloes are also affected, they are primarily asymptomatic carriers. Usually, they act as a source of infection to tick vectors, spreading the disease to susceptible cross-bred animals. In India, *Theileria annulata* is transmitted by *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum isaaci* (Ponnudurai *et al.* 2017) [16]. Several blood parasites (*Anaplasma* and *Theileria* spp. in cattle) cause transplacental infection (Costa *et al.* 2016) [4]. Determination of transplacental transmission of blood parasites in cattle calves would be a valuable study from an epidemiological point of view for devising efficient control strategies (Kolte *et al.* 2017) [10]. The conventional parasitological technique involving Giemsa-stained blood smears for detecting blood parasites is tedious and lacks specificity and sensitivity (Morzaria *et al.* 1992) [11]. Hence, the molecular method using PCR is a precise and sensitive technique for detecting haemoparasites in cattle (Norval *et al.* 1992) [12]. The main aim of our study is to investigate the blood protozoan parasites in cross-bred cattle calves in the Namakkal district of Tamil Nadu.

Materials and Methods
Ethical approval
Prior consent was taken from the owners of the cattle calves for blood collection. Complete care and measures were taken to avoid accidental injury to the cattle calves while collecting the blood.

Field Study area
The Namakkal District is situated in the North-western agro-climatic zone of Tamil Nadu at 11º2'N latitude and 78º2'E longitude at an altitude of 192 M above the mean sea level.

The study was carried out for a period of 6 months from April 2018 to September 2018 in Namakkal District, Tamil Nadu, India.

Collection of blood samples

Fresh whole blood samples were collected from 20 numbers of cross-bred cattle calves at monthly intervals from one month of age up to 6 months. A total of 2 mL of blood from the jugular vein of each calf was collected into an EDTA containing vacutainer tubes for haematological analysis according to the method followed by Emery *et al*. (2021) [6]. All the blood samples were stored under refrigeration in an isothermal box. Haematological studies were carried out by the Auto analyzer.

Blood smear examination

The blood smears were made from the ear tip of each cross-bred cattle calf. The blood smears were air dried, stained with Giemsa stain, examined with immersion oil under a light microscope, and evaluated for the presence of haemo-protozoan parasites (Agrawal *et al*. 2023) [1].

DNA isolation

Genomic DNA was extracted from the blood using a commercial QIAmp DNA extraction mini kit (Qiagen, Germany), and 5 µL of the template DNA was used per reaction. The extracted DNA was stored at –20 °C until further use (Ponnudurai *et al*. 2017) [16].

PCR amplification

DNA was extracted and screened for the 18S rRNA gene sequences of *Theileria annulata, Babesia bigemina*, and 16s rRNA gene sequences of *Anaplasma marginale*, which were amplified by polymerase chain reaction (PCR) using species-specific primers (Table 1) as described by Kolte *et al*. (2017) [10]. The PCR was performed in a total reaction volume of 25 µL, containing 4 µL of DNA template, 4.5 µL of Nuclease-free water, 12.5 µL of 1.5 mm $Mgcl_2$ (Taq 2X Master mix, Red Ampliqon), and 2 µL of each primer at 10 pmol concentration. The PCR was carried out under the following condition, 35 cycles of three steps each, comprising denaturing at 95 °C for 30 S, annealing at 55 °C for 30 S, and product extension at 72 °C for 30 S. Amplified products were separated by electrophoresis on a 1% agarose gel and visualized under a gel documentation system (BioRad, USA).

Statistical analysis

Statistically, a one-way ANOVA test was used to determine the significant association between healthy and affected cattle calves. The sample data analyses were performed with SPSS version 18.0 (Hurley *et al*. 2014) [8].

Results

Clinical examination of cattle calves revealed average body temperature (38.7 °C), heart rate, and respiration rate. No swelling of lymph nodes and changes in the mucous membrane was observed, and no ticks were observed on the body during the study period. The haematological values of PCV, Hb, RBC, and WBC of normal calves were in the range of 23.9 to 35.7%, 6.5 to 13.5 g/dl, 7.8 to 10.6 x 10^6/µl, and 8.6 to 12.4 x 10^3/µl, respectively, whereas in the infected calves, they were 19.1 to 32.5%, 6.9 to 10.5 g/dl, 7.3 to 10.7 x 10^6/µl and 7.9 to 11.5 x 10^3/µl, respectively. Statistically, there is no significant difference in haematological values between healthy and affected calves was observed in this study. However, the haematological values of affected calves were slightly lower than healthy calves (Table 2).

None of the blood smears were found positive for any of the blood protozoan parasites. Infected cattle calves exhibited no clinical signs. However, out of 20 cattle calves screened by PCR, 2 were found positive for *Theileria annulata*, and 3 calves were positive for *Anaplasma marginale* infection. Interestingly, none of the cattle calves showed positive for *Babesia bigemina* infection. The polymerase chain reaction amplified *Theileria annulata* with a size of 193 bp (Fig.1) and *Anaplasma marginale* with 335 bp (Fig. 1).

Table 1: List of species-specific primers used for PCR amplification of 18s rRNA gene sequences of *Theileria sp. and Babesia sp*. and 16s rRNA gene sequences of *Anaplasma sp.*

Parasite Species	Primer Sequence	Amplicon size
Theileria annulata	F: 5' ACGGAGTTTCTTTGTCTGA 3' R: 5'CTAAGAATTTCACCTCTGACAGT 3'	193bp
Babesia bigemina	F: 5'-GACACAGGGAGGTAGTGACAAG 3' R: 5'CTAAGAATTTCACCTCTGACAGT 3'	276 bp
Anaplasma marginale	F:5'GGTTTAATTCGATGCAACGCGA 3 R: 5'GCTCAGCCTTGCGACGTT 3'	335 bp

Table 2: Range of haematological values of cross-bred cattle calves from the first month of birth to six months of age

Blood Parameters	Normal calves	Affected calves	Reference unit
PCV	23.9 - 35.7	19.1- 32.5	%
Hb	6.5- 13.5	6.9 - 10.5	g/dl
RBC	7.8 - 10.6	7.3 - 10.7	10^6/µl
WBC	8.6 - 12.4	7.9 - 11.5	10^3/µl

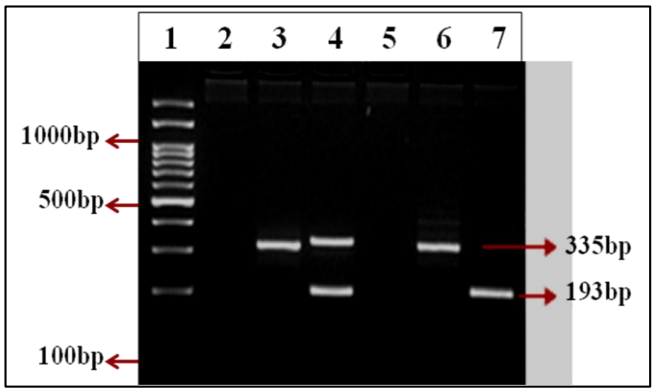

Fig 1. Agarose gel electrophoresis of amplified DNA using species-specific primers.
Lane L1-100 bp DNA marker; L2- Negative control; L3- *A. marginale*; L4- *T. annulata* + *A. marginale*; L5- Negative sample; L6- *A. marginale*; L7- *T. annulata*.

Discussion

Tick-borne diseases are a significant impediment to the productivity of livestock in India (Velusamy *et al*. 2017) [16]. Detailed epidemiological data would assist control of these diseases. In our study, there is no significant association of haematological values between healthy and affected calves was observed. However, the haematological values of affected calves were slightly lower than healthy calves. The lower reference ranges of haematological values in affected calves compared to healthy calves may be due to poor nutritional status among calves. Further, none of the blood smears were positive for any haemo-protozoan diseases. This might be due to insensitivity and non-specificity of the test when animals maintain a carrier status of infection.

Out of 20 calves screened by PCR, two were found positive for *Theileria annulata*, and three calves were positive for *Anaplasma marginale*. This might be due to the contraction of infection from the mother through the transplacental route. Transplacental transmission of *Theileria* spp. has been reported with no known natural exposure to the tick vector in areas where tick vectors may be present (Issi & Gul 2008) [9].

Our study found that cross-bred cattle calves below six months of age (15%) were infected with *Theileria annulata* without exhibiting any clinical signs. Similarly, documentation on the transplacental transmission of *T. annulata* in 1-3 days old cross-breed calves was registered in India (Sudan *et al.* 2012) [18].

Moreover, Onoe *et al*. (1992) [13] added that carrier dams could transmit *T. annulata* to their offspring, and calves born at a term can have massive parasitemia. Reports of vertical transmission of various *Theileria* species are well-documented in literature worldwide, *Theileria equi* in mare (Phipps & Otter 2004) [14], *T. lestoquardi* in ewes (Zakian *et al*. 2014) [20] and *T. sergenti* in cows (Baek *et al*. 2003) [3], Vertical transmission of *T. annulata* had also been reported in India (Godara *et al*. 2009) [7].

Bovine anaplasmosis caused by *A. marginale* is a significant constraint to livestock production and cattle health due to its devastating economic impact on the production potential of cattle. *A. marginale* can be transmitted through biological, mechanical, and transplacental routes. In transplacental transmission, infected erythrocytes move across the placenta in the uterus from infected cows to their offspring without amplifying *A. marginale* (da Silva *et al*. 2014) [5].

Incidence cases of *A. marginale* in a diverse range of cattle breeds and evidence of transplacental dissemination from India have been rarely reported. *A. marginale* can also be transplacentally transmitted from a persistently infected cow to the calf during pregnancy (Zaugg 1985) [21]. We found that 20% of calves (below six months of age) were infected with *Anaplasma marginale*. However, in most cases, we were unsure of the exact day and time of infection. Other reports indicated a comparatively lower incidence (12.5%) in cattle calves.

However, they did not mention the status of the diseases in the mother and the time of the infection when the mother was infected. The feature of uterine transfer implies the epidemiology of anaplasmosis in infection-free areas. Interestingly, we observed a comparatively moderate prevalence of transplacental transmission of *A. marginale* in cattle calves compared to reports of 13% in Neonatal Dairy Calves from Jhang district, Punjab, Pakistan, by Atif *et al*. (2021) [2].

Conversely, Costa *et al*. (2016) [4] mentioned higher transplacental positivity (26.47%) in the cross-bred neonatal calves using the nested PCR. Da Silva *et al*. (2014) [5] from Brazil reported a higher incidence of transplacental transmission of 41% in newborn cattle calves by PCR. Similarly, Salabarria and Pino (1988) [17] from Cuba mentioned a higher 86.4% (32/37) frequency of vertical transmission under clinical anaplasmosis in the last month of gestation. The variation in the results may be due to different diagnostic techniques, genetic diversity, and different agro-climatic conditions of the area (Costa *et al*. 2016) [4].

Vertical transmission in cattle is mainly due to persistent infection in a population (Pohl *et al*. 2013) [15]. The rate of in-utero transmission depends upon the timing of fetal infection during gestation, as the occurrence of transmission is higher at the end of gestation. The mechanism of transplacental transmission needs further investigation to understand its dissemination. The higher transplacental transmission rate may be due to anaplasmosis clinical or acute infection.

Conclusion

The present investigation suggests that haemo-parasitic infections pose a major threat to adult animals and may cause mortality in young cattle calves under stressful conditions. The infected young calves may act as a carrier or source of infection for susceptible animals but are at higher risk for death under stressful conditions such as transportation and vaccination. Further studies are needed to explore the transplacental transmission potential of the disease in other domestic animals.

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Competing Interest

The authors declare that they have no competing interests.

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