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Molecular diagnosis and successful therapeutic management of theileriosis in a dairy cattle

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Abstract
A total of 85 suspected crossbred cows were screened for *Theileria annulata.* Out of which 45 were positive by molecular diagnosis in and around Namakkal district. Animals with the history of weakness, fever, drop in milk production and clinical signs of anorexia, dehydration, debility, staggering gait, anaemia, lethargy, recumbency, enlargement of lymph nodes, pale mucus membrane etc. were also included in this study. Haematology and biochemical analysis were done for assessment of infection status and also assessed therapeutic efficacy with respect to haemoprotozoan diseases. Whole blood, serum, peripheral blood smear and fine needle lymph node aspiration were collected and analyzed. Giemsa stained blood smear revealed numerous annular shaped Theileria piroplasm in the erythrocytes. The blood picture and serum biochemistry showed anaemia, hypoproteinemia, hypoalbuminemia and elevated liver enzyme (AST). In fine needle aspiration cytology (FNAC) numerous intracellular schizonts (Koch's blue bodies, KBB) and extracellular schizonts were observed. For further confirmation polymerase chain reaction was performed in whole blood using the primer specific for Tamulti gene of *Theileria annulata* and their amplification size was 751 bp was observed. The animal was treated with inj. Buparvaquone @ 2.5mg/Kg IM (Single dose) and Inj. Oxytetracycline@ 20mg/Kg IV with Dextrose normal saline and along with supportive for 3 days. Topical synthetic pyrethroids (1% Flumethrin) also recommended as tick control measures. The animal recovered uneventfully after 7 days of treatment.

Keywords: Anaemia, buparvaquone, Cow, *Theileria* spp

Introduction
Theileriosis is an important tick- borne protozoan disease of cattle (Constable *et al.*, 2007) [3], it has been associated with a range of losses include mortality, expenses related to vaccination and treatment, decreased live weight in subclinical cases, extended inter-calving period, reduced milk production and delayed maturation (Gharbi *et al*., 2006) [5]. The major Theileria species infecting the cattle are *Theileria annulata, Theileria parva* and *Theileria orientalis* (Constable *et al.*, 2007) [3], which is primarily transmitted through ticks, *Rhipicephalus* spp is main vector for *Theileria parva*, whereas *T. annulata* is predominantly transmitted by *Hyalomma* spp ticks. Ticks can retain their infection for as long as two years on pasture (Nampoothiri., 2021) [6]. *Theileria* parasites enter the bovine host during tick feeding as sporozoites, which rapidly invade mononuclear leukocytes and mature into macroschizonts and induce proliferation of the host cell. Macroschizonts further develop into microschizonts and ultimately into merozoites, which are released from the lymphocytes. The merozoites invade erythrocytes and develop into piroplasms. In initial stages, tropical theileriosis manifests as a lymphoproliferative disease, accompanied by lymph node enlargement. As the disease progresses and pyrexia develops, a lympho destructive phase begins, leading to a pronounced leucopenia and marked anaemia (Christine *et al*., 1995) [8]. In severe cases, the schizont can be seen in spleen, lymph nodes, liver and whole blood from *Theileria* infected animals (Nampoothiri.,2021) [6]. Cattle with subclinical infections in endemic regions serve as carriers of piroplasm and act as source of infection for ticks (Brown, 1990) [4]. The conventional method of diagnosis involves examination of blood smear and lymph node aspirates from live animals and impression smear from spleen in dead animals through staining (Aktas *et al*.,2001) [1].

In recent times, polymerase chain reaction (PCR) has become the preferred method for diagnosis of tropical theileriosis in epidemiological studies. This report describes the application of PCR for the sensitive and specific amplification of *T. Annulata* from clinically affected animals.

Materials and Methods

During clinical examination, the animal was dull and depressed with emaciated body condition, the conjunctival and vaginal mucous membranes were icteric with petechial haemorrhage (Fig.1-2), enlarged prescapular lymph node (Fig. 3) along with tick infestation in ear, but the rectal temperature was normal (38.3 °C). The vital clinical parameters like heart rate (102 bpm), respiratory rate (40breaths/min) and pulse rate (60/min) were observed. Based on general clinical examination the case was diagnosed as haemoprotozoal disease. For further confirmation whole blood, serum, peripheral blood smear and lymph node aspiration were collected. Both complete blood count and serum biochemical analysis was performed in before treatment, after 5th and 10th day post treatment. The peripheral blood smear and lymph node aspiration smear were stained with Giemsa for 30 minutes after methanol fixation (Souls by, 1982) [7], and screened for blood parasites under microscope (100X). For molecular confirmation, the DNA was extracted from whole blood using commercial blood and tissue DNA extraction kit as per the procedure described by manufacturer (Qiagen India, New Delhi). The polymerase chain reaction was performed using primer specific for tamulti gene (Kundave *et al*., 2018) [16] and Tams 1 gene (Ganguly *et al*., 2019) [11] of *T. annulata*.

Animal was treated with single dose of Inj. Buparvaquone @ 2.5 mg/kg I/M, Inj. Oxytetracycline @ 20 mg/kg I/V with Dextrose normal saline 500 ml IV, Chlorpheniramine maleate @ 0.5 mg/kg and Inj. Vitamin B complex 10 ml IM for 3 days. The supplements like hematinicssharkoferrol @ 50 g/day and ethnovetoral medication of moringa, curry and keezhanelli leaves with jaggery for 1 month. For tick control measures topical synthetic pyrethroids (Flumethrin 1%) was advised.

Results and Discussion

Peripheral blood smear revealed more number of annular and signet ring shaped piroplasmin erythrocytes and Koch's blue bodies in lymphocytes (Fig. 3).

Giemsa staining technique is the traditional method that involves microscopic examination of piroplasm in blood smear (Aktas *et al.*, 2006) [2]. This method is frequently used for detection of parasites as it is comparatively inexpensive. However, this method is insensitive and not suitable for carrier animals because the pathogen level is usually low in the blood stream.Fine needle aspiration cytology revealed that numerous koch's blue bodies in lymphocytes (Fig. 4), extra cellular schizonts and irregular shaped lymphocytes due to invasion of organism. Lymph node aspirates showed schizonts of *T. annulata* (Koch's Blue Body) where ruptured Koch's blue bodies with large extracellular schizonts from clinical and subclinical cases. Clinical cases revealed lymphoid hyperplasia in the current study. Similar findings observed by Sudan *et al.* (2012) [15], who observed KBB in lymph node cytology and the findings unisons with Singh *et al.* (2017) [14].

Hematology revealed anaemia, leucopenia and neutrophilia. Serum biochemistry showed hypoproteinemia, hypoalbuminemia and hyperbilirubinemia. Animals were treated with buparvaquone @ 2.5 mg/kg body weight once in intramuscular route, oxytetracycline @10 mg/kg body weight intravenously for 5 days and supportive treatment for 5 days. Then affected animals recovered uneventfully. After 5th and 10th day post treatment, results showed negative for causative organism and remarkable improvement in haematology and serum biochemistry values after treatment (Table 1). The haematological profile of infected animals revealed significant decrease in Hb, PCV and RBC, whereas increased parameter of WBC, MCV and MCH. In serum biochemistry decrease in total protein, ALB, Ca, P and Glu levels and increase in the values of liver enzyme ALT and AST were recorded. There were significant positive changes noticed on 5th day and 10th day post treatment. Changes include Hb, RBC, PCV, total protein, ALB, Ca, P and Glu significant (p<0.01) increased in 5th day, when compared with 0 day, significantly (p<0.01) increased in 10th day after treatment. The values of WBC, MCV, MCH, ALT and AST were significantly (p<0.01) decreased in 5th day and when compared with 0 day, significantly (p<0.01) decreased in 10th day after treatment in both clinical and subclinical infection.

Haematological parameters like Hb, PCV and RBC were decreased in clinically affected dairy cattle, whereas increased values of WBC, MCV and MCH were observed (Yadi *et al.*, 2017) [13].

Serum biochemistry of total protein, albumin, calcium, phosphorus and glucosevalues were significantly reduced, whereas ALT and AST increased in clinically infected animals. Similar findings were observed by Saber *et al.* (2008) [12].

Polymerase chain reaction confirmed Tams 1(tamulti) gene with amplicon size of 751 bp was amplified and the Tams 1 gene with amplicon size of 156 bp was amplified presence of both genes of *Theileria annulata*. Polymerasechain reaction has largely superseded other methods and is widely used species-specific molecular diagnostic assay to determine piroplasm-carrier animals. These are highly sensitive tools employed for diagnosis of pathogens in carrier animals as compared to conventional techniques (Ganguly *et al*., 2019) [11].

Marked clinical improvement as well as haematological and serum biochemical parameters were noticed after 5th and 10th day of post therapeutics. Clinical and subclinical *T. annulata* affected animals were treated with buparvaquone @ 2.5mg/kg body weight once in intramuscularly, oxytetracycline @ 10 mg/kg body weight intravenously for 5 days with supportive treatment for 5 days. Therapeutic recommendations were in agreement with Nasir (2000) [10] stated that remedy for theileriosis is best treated by buparvaquone was more effective and reliable drug which is active against both schizonts and piroplasms than any other theilericidal drugs in field condition, additional recommendation that the use of long acting oxytetracycline @ 10 mg/kg body weight intravenously for 5 days, since it has certain activity against Theileria and also prevent secondary infections, mainly respiratory signs. Similar therapeutic recommendations suggested by Azhahianambi *et al.* (2021) [9].

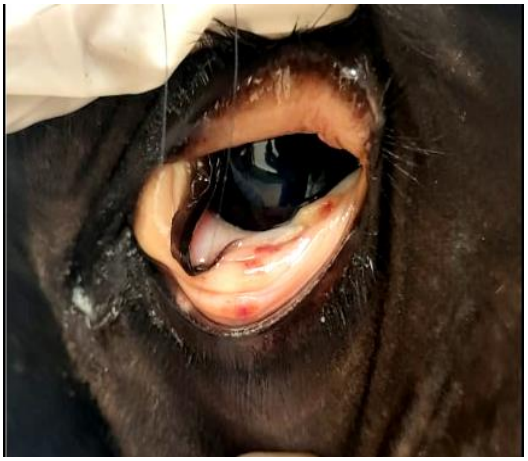

Fig 1: Conjunctival mucous membrane – icteric with petechiae haemorrhage

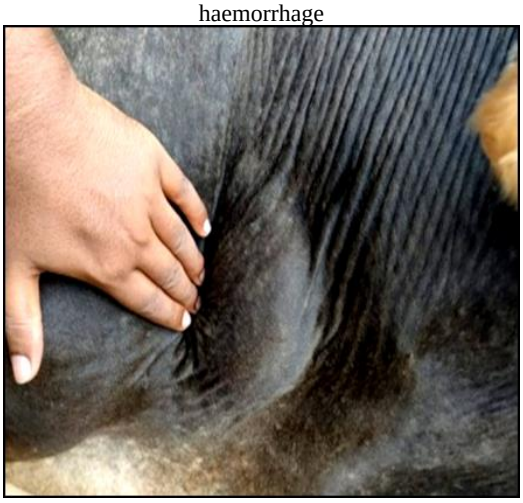

Fig. 2: Enlarged prescapular lymph node

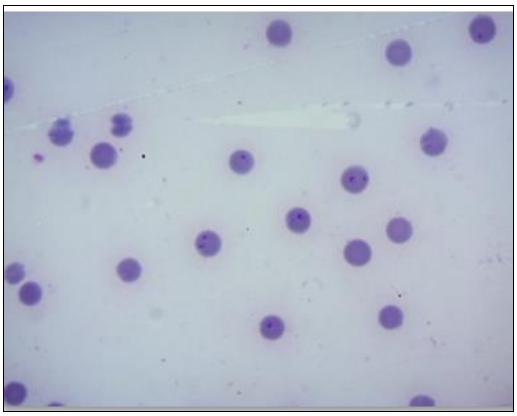

Fig 3: Peripheral blood smear- Annular shaped Theileria Piroplasm (100x)

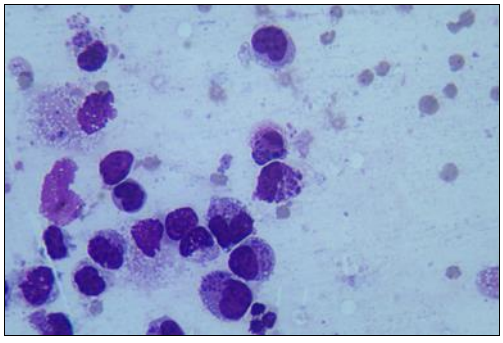

Fig 4: Lymph node aspiration smear- Theileria Schizonts (Koch blue bodies) in lymphoblast (100x)

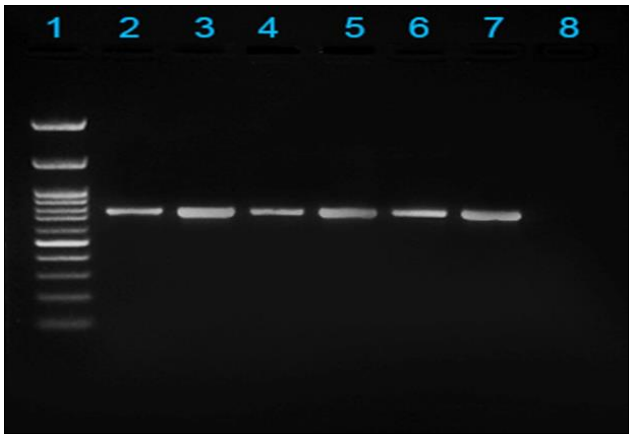

Fig 6: Gel picture showing amplified PCR product *T. annulata*-Tams 1(tamulti) gene (751bp) in sub clinically infected cases.
Lane 1 Ladder
Lane 7 Positive control
Lane 2-6 Positive samples (751bp)
Lane 8 Negative control

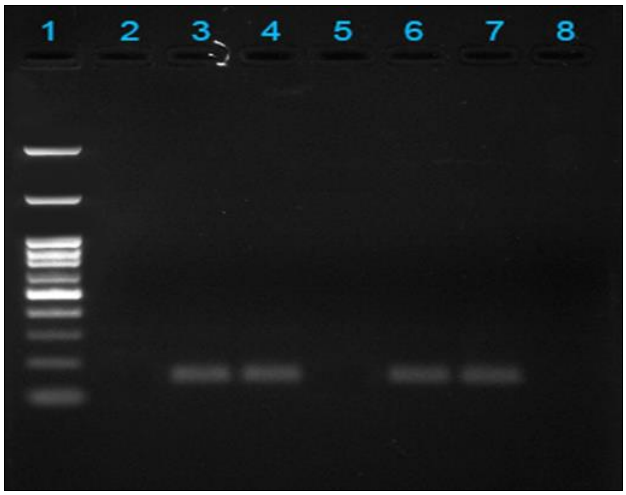

Fig 6: Gel picture showing amplified PCR product *T. annulata*-Tams 1 gene (156 bp) sub clinically infected cases
Lane 1 - Ladder
Lane 2 & 5 - negative
Lane 3,4,6 & 7 - Positive samples
Lane 8 - Negative control

Table 1: Mean (±S.E) of Haematology and Biochemistry parameters in *T. annulata* infected cattle before and after treatment

Parameters	***T. annulata***			
	0 day	5th day	10th day	F value
Hb (g/dl)	5.02^{a}±0.097	6.25^{b}±0.096	7.26^{c}±0.115	198.332**
PCV (%)	18.57^{a}±0.621	23.36^{b}±0.402	26.61^{c}±0.283	128.178**
RBC ($x10^{6}$/µl)	3.21^{a}±0.066	4.34^{b}±0.132	5.25^{c}±0.083	201.677**
WBC ($x10^{3}$/µl)	12.45^{c}±0.211	10.47^{b}±0.143	8.58^{a}±0.089	304.171**
MCV (pg)	61.77^{c}±0.509	56.22^{b}±0.559	51.06^{a}±0.816	151.260**
MCH (pg)	24.64^{c}±0.294	22.32^{b}±0.289	20.0^{a}±0.310	183.963**
TP (g/dl)	4.97^{a}±0.101	5.66^{b}±0.050	6.16^{c}±0.027	105.827**
ALB (g/dl)	2.49^{a}±0.039	2.8^{b}±0.041	3.11^{c}±0.031	179.358**
ALT (U/L)	59.67^{c}±0.172	53.67^{b}±0.651	46.29^{a}±1.014	158.525**
AST (U/L)	154.38^{c}±1.301	136.54^{b}±1.301	114.35^{a}±1.968	368.300**
CA (mg/dl)	7.68^{a}±0.134	8.19^{b}±0.112	8.60^{c}±0.100	71.354**
P (mg/dl)	6.32^{a}±0.089	6.63^{b}±0.099	6.98^{c}±0.099	77.466**
GLU (mg/dl)	51.16^{a}±0.734	58.03^{b}±0.930	66.09^{c}±0.959	189.314**

** P<0.01 – Statistically significant at 1 per cent level
Mean bearing similar superscript within classes do not differ significantly.

Conclusion

Identification of schizont stage of Theileria organism in peripheral blood smear is rare diagnosis, which indicate poor prognosis. The PCR assay is characterized by its speed, robustness, high sensitivity and specificity, making it an invaluable diagnostic tool. This method provides a practical and efficient means to identify early stage and subclinical infection. Haematology and biochemistry values were significantly affected. Infected animals successfully managed with combination of Inj. Buparvaquone and oxytetracycline along with supportive therapy.

Therapeutic efficacy was assessed with suitable drug combination with respect to different stage of infection, during 5th and 10th day of post treatment assessment of haematology and biochemical parameters showed significant positive effect.

PCR assays exhibited a significantly higher level of sensitivity compared to blood smear examination. Furthermore, it was noteworthy that in all animals suspected of clinical and subclinical infections. Early diagnosis with best treatment protocol got rid of disease consequences. Theileriosis in endemic area is controlled by implementation of vaccination and tick control measures to reduce economic losses.

Declaration of competing interest

The authors declare no conflicts of interest.

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