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Studies of Molecular Detection and Therapeutic Management of Tropical Theileriosis in Cattle

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Abstract
The present research was undertaken at in and around Tirupati district of Andhra Pradesh during the year 2022-23 to study the diagnosis and therapy of tropical theileriosis. A total of 170 cattle had shown typical characteristic signs of tropical theileriosis and from all the suspected cattle blood samples and lymph node aspirates was collected. Among them 24 were found to be positive for tropical theileriosis and all the 24 whole blood samples were subjected for PCR for detection of cytochrome b gene of *T.annulata* and all the 24 DNA samples were positive and yielded amplicon size of 312 bp. The positive cattle were randomly divided in to two groups and named as G-I and G-II. The G-I cattle were treated with inj buparvaquone, where as G-II cattle were treated with Inj buparvaquone along with oral anti-oxidant preparation. Hence the G-II cattle showed better recovery when compared to G-I.

Keywords: Andhra Pradesh,Tropical theileriosis, whole blood samples, lymph node aspirates and Polymerase chain reaction.

Introduction

Bovine tropical theileriosis is a lymphoproliferative, devastating, and dominant protozoan parasitic disease of dairy caused b*y Theileria annulata,* in tropical and sub-tropical countries (Brahmbhat *et al.,* 2019, Gaine *et al.,* 2019).The incidence of bovine tropical theileriosis shows a huge economic loss to an Indian farmers (Gupta *et al*., 2019). The illness of the diseases affects the farmers in different ways like sudden drop in milk production (Saravanan *et al.,*2017), reduced reproductive performance such as stunted growth, delayed puberty (Shivakumar *et al.,*2022), and increase in the death rates of the affected ones (Nayak *et al.,* 2017).

In tropical countries, after the rainy season there will be an increased vector population namely *Hyalomma anatolicum*, which carries an obligatory intracellular protozoan parasite (*T. annulata*), which intern leads to bovine tropical theileriosis (Parmar and Chandra 2019), that tends to strike exotic, and crossbred livestock (Singh *et al.,* 2017). In tropical theileriosis-affected cattle may show the clinical signs like pyrexia, weight loss, mild nasal and ocular discharge, anorexia, salivation, cough, petechial hemorrhages on conjunctival mucosa, enlargement of superficial lymph nodes, respiratory distress, tarry-coloured faeces, decreased milk yield, profound anemia, jaundice, and asphyxia-related death (Gupta *et al*., 2019). To identify and remove carrier animals from the flock in an effective way, screening the animals in a herd for the presence of illness is a prerequisite (Choudhary *et al*., 2022). The presence of piroplasms in red blood cells and schizonts in lymphocytes can be detected under a microscope during the examination of Giemsa-stained thin blood and lymph node smears, which is the

primary method of diagnosing theileriosis. (Parmar and Chandra 2019) Indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA) were two more serological tests that were employed (Radostits *et al*., 2007). With greater sensitivity and specificity than conventional testing, the polymerase chain reaction is now regarded as the method of choice for the diagnosis of theileriosis. (Shivakumar *et al*., 2022).

Hence, the present study was conducted to diagnose tropical theileriosis infected cattle and to treat the infected cattle with suitable therapeutic regimen.

Materials and Methods

The present study was conducted in cattle affected with tropical theileriosis, which were presented to the Department of Veterinary Clinical Complex, College of Veterinary Science, Tirupati and Veterinary Dispensaries located in and around Tirupati during the period December 2022 to September 2023. A total of 170 cattle showing the clinical signs of anorexia, pyrexia, pale conjunctival mucus membranes, enlargement of superficial lymph nodes, were suspected for tropical theileriosis are included in present study. Among them twenty four cattle were found suspected for *Theileria annulata* through blood smears and lymph node smears(Giemsa staining). later on it twenty four cattle were confined by polymerase chain reaction and they were randomly divided in to two groups (G-I and G-II). G-I cattle was treated with Inj. Buparvoquone @ 2.5 mg/kg body weight I/M as single injection along with symptomatic treatment (Inj. Meloxicam @ 0.25 mg/kg body weight I/M, Inj. Belamyl @ 15 ml per adult cattle and Bolus Feritas @ 3 boli per day) and G-II cattle was treated with Inj. Buparvoquone @ 2.5 mg/kg body weight I/M as single injection along with oral antioxidant preparation (containing Vitamin E 100 mg, Selenium 0.5 mg and Ocimum sanctum extract per ml) 25 ml daily for 10 days along with symptomatic treatment (Inj. Meloxicam @ 0.25 mg/kg body weight I/M, Inj. Belamyl @ 15 ml per adult cattle and Bolus Feritas @ 3 boli per day).

Genomic DNA isolation was done as per the protocol given on DNA blood mini Kit (Qiagen).

Oligonucleotide primer sequences used for the identification of *T. annulata* by PCR

Organism	**Primer sequence pair**	**Product size (bp)**	**Reference**
Theileria Annulata	**Tams 1 F**: ACT TTG GCC GTA ATG TTA AAC **Tspm 1 R**: CTC TGG ACC AAC TGT TTG G	312	Gaurav *et al.* (2020)

The reaction mixture composition for PCR reactions is as follows

Components	**Volume** (µl)
Nuclease free water	8.5
External forward primer	1
External reverse	1
Master mix	12.5
DNA template	2
Final volume	25

PCR conditions

S.no		Step	Temperature	Time	
1		Initial Denaturation	95 ºC	5 min	
2	A	Denaturation	95 ºC	30 sec	35 cycles
	B	Annealing	55 ºC	30 sec	
	C	Extension	72 ºC	1 min	
3		Final extension	72 ºC	10 min	
4		Hold	Till retrieval		

The PCR products were checked for amplification by Gel electrophoresis.

Results

Examination of Giemsa-stained blood smears and lymph node aspiration smears from 170 cattle showing the clinical signs suggestive of theileriosis revealed presence of theileria piroplasms in 24 cattle blood smears (14.11 %). The theilerial piroplasms appeared as, comma, dot, annular and irregular shape, but higher proportion of theilerial piroplasms were in comma shaped and the same number of cattle had the presence of schizonts in the lymphocytes (Koch's blue bodies) as depicted in plate 02.

The DNA was extracted from whole blood samples which were collected from 24 blood smear positive cattle was subjected to PCR for detection of cytochrome b gene of *T. annulata.* All the 24 DNA samples from smear positive cases yielded amplicon size of 312 bp specific for *T. annulata* as shown in plate *03.*

The clinical signs recorded in tropical theileriosis infected cattle were anorexia (62.5 %), lymph node enlargement (58.33 %), reduced milk yield (45.83 %), pyrexia (37.5 %), pale mucus membrane (29.16 %), tick infestation (25 %), tarry colored faeces (12.5 %), weight loss (8.33 %), diarrhea (12.5 %) and icteric mucus membrane (4.16 %). The clinical signs of present study were depicted in Table 01 and Plate 01.

Table 01. Clinical signs in cattle affected with tropical theileriosis

Clinical signs	No.of cases (24)	Percentage (%)
Anorexia	15	62.5
Lymph node enlargement	14	58.33
Reduced milk yield	11	45.83
Pyrexia	09	37.5
Pale mucous membranes	7	29.16
Tick infestation	6	25
Tarry coloured faeces	3	12.5
Weight loss	2	8.33
Diarrhea	3	12.5
Icteric mucous membrane	1	4.16

Plate 01. Clinical signs in cattle affected with tropical theileriosis

B. Enlarged pre scapular lymph node

A. Pale mucus membrane

A. Dysentery

B. Icteric Mucus membrane

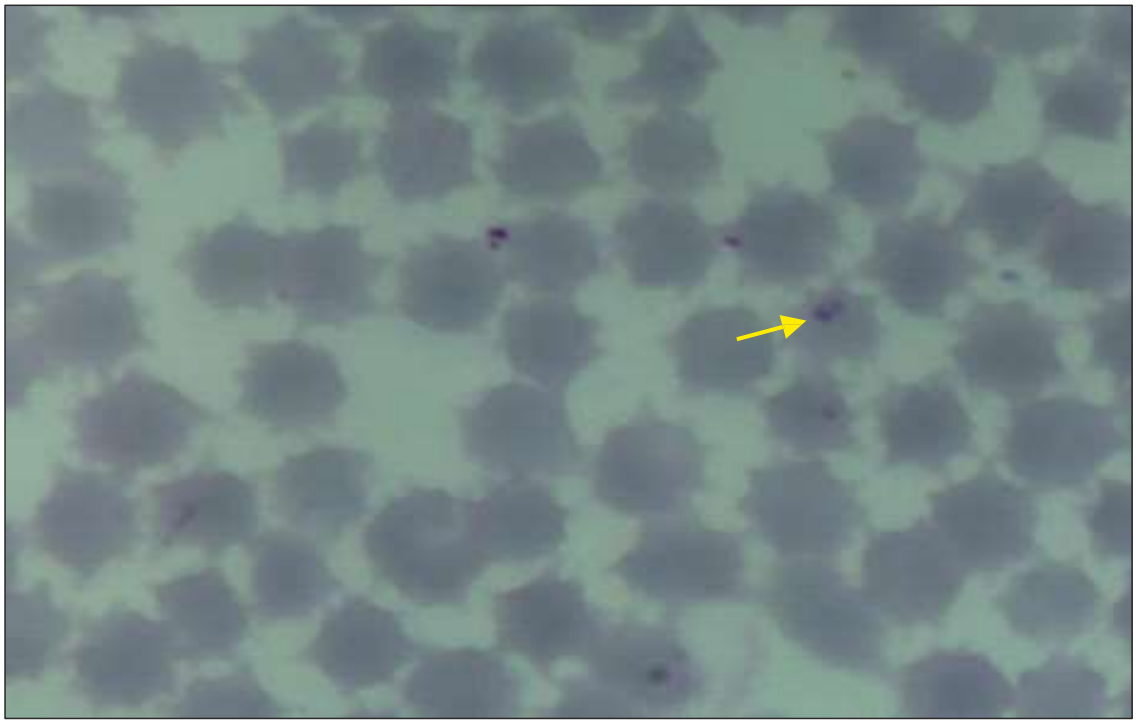

Plate 2 Blood film stained with Giemsa stain showing intracellular trophozoit "signet ring" of Theileria species (X100).

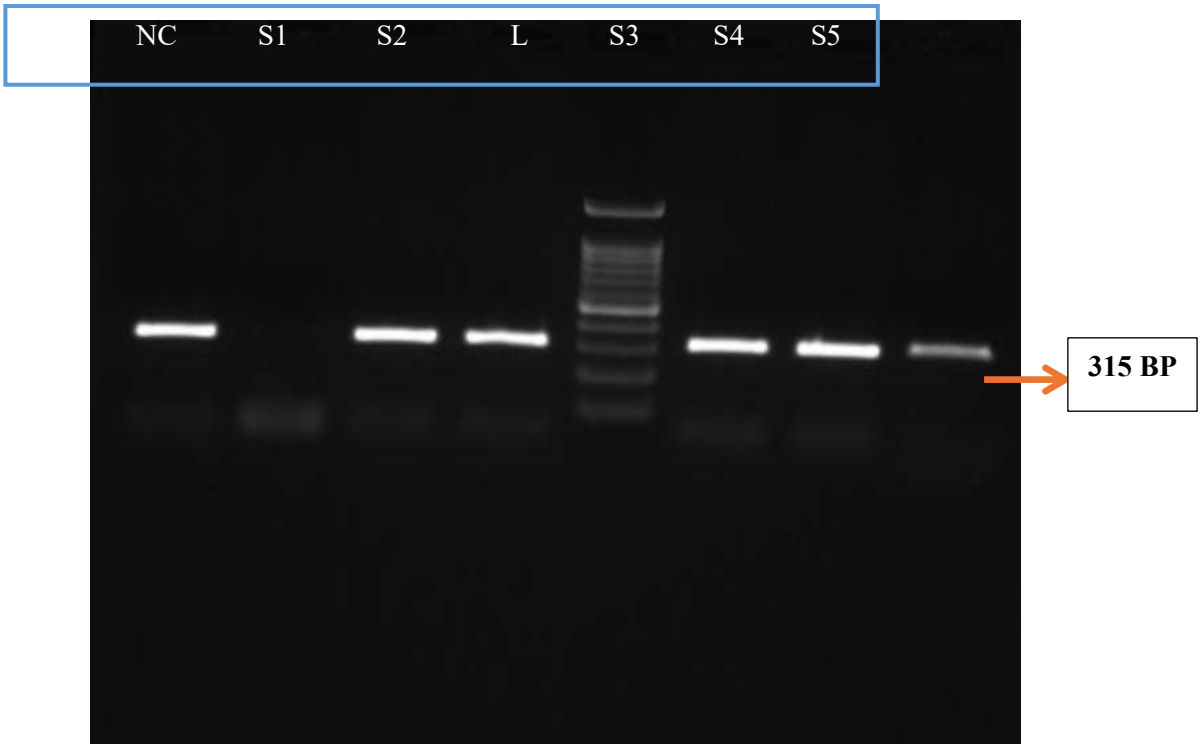

Plate 3 Agarose gel showing amplified PCR product of size 315 bp (Theileria)
(L-100 bp DNA ladder, S1-S5 Positive sample, PC- Positive control, and NC- Negative control)

Out of the 12 animals selected for treatment with buparvaquone @ 2.5 mg/kg body weight in G-I, three animals remained anorexic, lethargic and showed no improvement after 48 hrs of treatment. So these three animals were administered with a second dose of buparvaquone. All the three animals showed

marked clinical improvement after two days of second dose of buparvaquone with resolution of clinical signs. The remaining nine animals showed remarkable clinical improvement with resolution of most of the clinical signs and improvement in appetite was noticed after 48 hrs of treatment. Examination of peripheral blood smears after 48 hrs of treatment revealed presence of theilerial piroplasms in three out of 12 animals.

Where as the cattle infected with tropical theileriosis in G-II were treated with single dose of buparvaquone @ 2.5 mg/kg body weight along with oral anti-oxidant preparation (Vitamin E 100 mg, Selenium 0.5 mg and Ocimum sanctum extract per ml) 25 ml daily for 10 days. All animals in this group showed marked clinical improvement with remission of clinical signs after 48 hours of treatment. Examination of peripheral blood smears after 48hours of treatment revealed absence of theilerial piroplasm's in all 12 animals.

Discussion

In the present study, out of the 170 crossbred cattle suspected for tropical theileriosis 24 crossbred cattle were positive for tropical theileriosis by blood smear and lymph node aspirate examination thus representing an overall occurrence of 14.1 per cent. The results of the present study were almost in parallel with the findings of Selim *et al.* (2022) and Nair *et al.* (2011). Who reported over all occurrence of 16.49 per cent and 16 per cent in Egypt and Kerala respectively. On the contrary Singh *et al.* (2017) reported higher prevalence of 89 per cent Odisha. The variations in the occurrences in different studies might be due to differences in geographic location or epidemiological factors, animal breed, managemental practices and tick control practices study population (Selim *et al*. 2022).

In the current study, examination of Giemsa stained peripheral blood smears revealed presence of theileria piroplasms inside the RBC. The theileria piroplasms appeared in different shape and size viz., comma, dot, annular and irregular shaped but majority of the piroplasms appeared as comma shaped only. This finding was in agreement with Al-Emarah *et al* (2012). Conventional method of blood smear examination is useful technique for identification of theilerial piroplasms in the RBC. But it could not detect the carrier animals with low level of parasitaemia (Agina *et al*. 2020). Further, in the present study examination of lymph node biopsy smear stained by Giemsa stain revealed the presence of Koch blue bodies (KBB) which are characteristic of tropical theileriosis. The sporozites after entry into the blood of the host infect the lymphocytes and monocytes and develop into schizonts. These schizonts were present within the cytoplasm of the infected lymphocytes and appear as Koch blue bodies and were pathognomonic in tropical theileriosis (Agarwal *et al*. 2022). In the current study macroschizonts were detected with in the lymphocytes. This was in agreement with the findings of Abou-El-Naga *et al*. (2005) and Khatoon *et al*. (2013).

The pleomorphic nature of the theileria piroplasm will not allow for differentiation of Theileria spp. by conventional blood smear examination. This limitation can be overcome by molecular methods like PCR which was most preferred method for detection of Theileria spp. in epidemiological studies. Hence, all the 24 blood samples that were positive by blood smear examination in the current study, were subjected species specific PCR for confirmation by targeting the cytochrome b1 gene of *T. annulata*. All samples yielded amplicon size of 312bp specific for *T. annulata* by PCR. Mohmad *et al*. (2022) and Parveen *et al*. (2021) also used cytochrome b1 primer set for diagnosis of tropical theileriosis caused by T. *annualata*. Verma *et al*. (2023) and Parveen *et al*. (2021) stated that PCR based on cytochrome b1 gene was highly specific and sensitive for detection of T.*annulata*.

The clinical signs observed in tropical theileriosis infected cattle in the present study were anorexia, pyrexia, lymph node enlargement, pale mucus membrane, reduced milk yield, diarrhoea, weight loss and icterus. Similar clinical findings were reported by Gupta *et al.* (2019), Saravanan *et al.* (2017) and Al-Emarah *et al*. (2012) in their studies.

Pyrexia recorded in theileriosis infected cattle in the present study was attributed to the release of inflammatory cytokines by the infected cells. In theileriosis infected cattle, the production of interferon-gamma (IFN-γ) by activated lymphocytes stimulates the macrophages to produce cytokines IL-1α, IL1-β, IL-6, IL-10 and tumor necrosis factor-alpha (TNF-α). The TNF-α and cytokines are potent inducers of fever and other inflammatory changes (Shimpi Kumari *et al*., 2022).

It was well established that the schizont stage of the *T. annulata* interfere with the host cell signalling pathways that regulate the cell proliferation and survival. The schizont stage also inhibits the apoptosis of the infected cells and interacts with the microtubules of the host cells to ensure its persistence with in the lymphocytes. Further, *Theileria annulata* induces the clonal proliferation of the infected lymphocytes leading to uncontrolled proliferation and clonal expansion of the parasitized host cell population (Al-Emarah *et al*., 2012 and Woods *et al*., 2013) and this resulted in lymphadenopathy observed in theileriosis infected cattle in the present study.

The effect of two treatment combinations adopted in the present study was assessed based on the remission of clinical signs.

The cattle in G-I were treated with Inj. Buparvoquone @ 2.5 mg/kg body weight I/M as single injection. Buparvaquone is a second generation hydroxynaphthoquinone and the drug of choice for treatment theileriosis (Ibrahim et al., 2020). Buparvoquone exerts its anti-theilerial activity by competitively inhibiting the binding of ubiquinone (coenzyme Q) to cytochrome b of mitochondrial cytochrome bc1 complex leading to inhibition of electron transport chain in the parasite but not the host (Pudney et al., 1992).

Treatment with buparvaquone was effective in nine animals which resulted in remission of clinical signs and the animals became smear negative after 48 hours of treatment. This was in agreement with Aparna *et al*. (2011) and Kakati *et al*. (2015) who also reported that single dose of buparvaquone was effective in the treatment of *T. annulata* infection.

The remaining three cattle in G-I, showed no improvement after 48 hours and theileria piroplasms were detected in the blood smear. So these three cattle were treated with second dose of buparvaquone and marked clinical improvement was noticed after second dose of buparvaquone. The detection of theileria piroplasms in the blood smears of three cattle in G-I, after treatment with first dose of buparvaquone might be due to the development of resistance to buparvaquone by theileria parasites as reported by field veterinarians. They observed persistent high level of parasitaemia and degenerative forms of *T.annulata* in the blood smears of cattle after treatment in some animals and in some cases leading to fatality (Mhadhbi *et al*., 2015). Mutations in the cytochrome b gene of *T.annulata* were associated with treatment failures and resistance to buparvaquone (Hacılarlıoglu *et al*., 2023 and Chatanga *et al*., 2019). This might be the reason for detection of theileria piroplasms in the blood smears of three cattle in G-I, after treatment with first dose of buparvaquone.

In G-I cattle improvement in appetite was noticed from 3 to 5 days after initiation of treatment and restored to normal on 8th to 10th day. The body temperature returned to normal after 5th day onwards as assessed by improvement in appetite, body temperature returned to normal.

In G-II cattle improvement in clinical signs was noticed from 3 to 7 days after initiation of treatment

and improvement in appetite was noticed from 3 to 5 days after initiation of treatment and restored to normal in all the animals by 7th day, body temperature returned to normal by 5th day of treatment.

Conclusion

In present study, all the G-II cattle were recovered by giving single dose of inj buparvaquone along with with oral anti-oxidant preparation (Vitamin E 100 mg, Selenium 0.5 mg and Ocimum sanctum extract per ml), where as in G-I few cattle had taken second dose of inj buparvaquone. Hence G-II cattle was recovered better in comparison with G-I cattle.

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