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RT PCR Assay to Determine Efficacy of Buparvaquone in Reducing Degree of Parasitemia of *Theileria orientalis*

Shraddha Sinha[1], Sonali Sahoo[1], Bikash Kumar Behera[2], Soumesh K. Padhi[2], Niranjana Sahoo[1], Manaswini Dehuri[3], Sangram Biswal[1], Priyadarshini Sahoo[1]

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ABSTRACT

Background: Theileriosis is a haemoprotozoan illness that poses severe economic loss to dairy farmers due to its negative impact on mortality and productivity, especially in tropical and subtropical areas of the world.

Methods: The present study was conducted for a period of one year (June 2020 to July 2021) during which 55 blood samples of cattle showing clinical signs suggestive of theileriosis were examined and quantified by using multiplex real time PCR for *Theileria orientalis* and *Theileria annulata* using Taq-Man probe.

Result: Prevalence of theileriosis in three *Theileria*-endemic districts of Odisha due to *Theileria annulata* and *Theileria orientalis* were 26.11 per cent (11/42) and 57.14 per cent (24/42), respectively. Mixed infection were reported in 16.6 percent (7/42) cases. Parasite load for *T. annulata* in clinically affected cases ranged from 2.85×10^3 to 1.51×10^5 parasites per ml of blood while for *T. orientalis,* it ranged from 1.94×10^4 to 5.88×10^5 parasites per ml of blood. Ten cattle positive for *T. orientalis* were administered two doses of buparvaquone (Butalex) @2.5 mg/kg b.w. at an interval of 48 hours after which degree of parasitemia were quantified ten days post treatment. The parasitic load in blood ten days post treatment decreased significantly from 4.04×10^4 to 2.21×10^2 per ml of blood.

Key words: Buparvaquone, RT-PCR, *T. orientalis*.

INTRODUCTION

Bovine theileriosis, a tick-borne haemoprotozoan disease of cattle is distributed all over the world. Tropical and sub-tropical regions, especially experience the loss of productivity in bovines due to tick and tick-borne diseases is mostly experienced by the tropical and subtropical countries due to the climatic conditions favourable for the propagation of ticks. The estimated annual loss in productivity worldwide ranges from 14 to 19 billion US dollars (De Castro, 1997). The impact was found higher in developing countries due to unaffordable tick control and treatment costs. Tropical theileriosis due to *Theileria annulata*, the aetiological agent of tropical theileriosis' is recognised as pathogenic species since long owing to its ability to multiply extensively during intraleukocyte development (Morrison, 2015). Oriental Theilerosis has also been reported from different countries including India and Odisha (Sahoo *et al*., 2017) and is gaining significance since the last decade.

Since *T. orientalis* is non-lymphoproliferative, they are traditionally recognised as benign parasites of bovine. Outbreaks are mainly limited to immunosuppressed or stressed cattle, associated with poor farm management (Watts *et al.,* 2016). However, the major economic losses include loss in milk production, abortions and severe morbidity. Clinical signs include fever, anaemia, in appetence, dyspnoea, swollen supra-scapular lymph node, and rarely haemoglobinuria is also observed (Fukushima *et al*., 2021). Examination of Blood smear, lymph node aspiration fluid examinations and polymerase chain reaction (PCR) tests have been developed and standardised for detection of haemoprotozoans, with the PCR tests being the most sensitive and accurate amongst them (Perera *et al*., 2015). Although Giemsa-stained blood and lymph smears are still the most often used diagnostic tools, particularly during the acute stage as generally enough infected cells can be seen through a microscope (Al-Hosary *et al*., 2020). The lymph node biopsy smear examination had the lowest diagnostic efficacy as compared to microscopic examination of the peripheral blood smear and found that, even at low

[1]Department of Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.
[2]Centre for Wildlife Health, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.
[3]Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.

Corresponding Author: Shraddha Sinha, Department of Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.
Email: sinhashraddha220@gmail.com

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parasitemia, PCR is a more precise and specific diagnostic technique for the identification of *Theileria* spp in cattle (Farooq *et al*., 2019). The Multiplex PCR was found as useful approach to identify co-infection of tick-transmitted illnesses in cattle in endemic locations where, there are potential co-infections (Kumar *et al*., 2021).

Buparvaquone is the most common drug for treatment of theileriosis due to its low toxicity and short plasma half-life of seven days. There is, however, a prepatent period of ten to thirteen days between the time a tick feeds on cow blood and the onset of fever and clinical symptoms. The invention of buparvaquone in 1980s has changed the treatment scenario, however it only reduces parasitaemia but animal remains as carrier (Dandsena *et al*., 2018). Despite the high prevalence of *T. orientalis* infection and the resulting economic losses in cattle of Odisha, no attempts have been made earlier to determine the efficacy of buparvaquone in lowering parasitemia of *T. orientalis* genotypes. The present study aims to assess the efficacy of Buparvaquone in reducing degree of parasitemia of *Theileria orientalis* by molecular method.

MATERIALS AND METHODS

Source of sample

The blood samples in commercially available EDTA vials presented at Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Bhubaneswar, Odisha by the cattle owners from three different districts of Odisha (Cuttack, Puri and Khurda) for haemoparasite investigation were included in the study. The samples were collected from animals showing the signs of inappetence, fever, (>103°F), pale mucous membrane, dyspnoea and decreased milk yield were the basis for collection of samples from the period of April 2019 to July 2020.

Isolation of DNA from whole blood and qPCR

Genomic DNA extraction was carried out by using commercially available DNA mini kit (Qiagen, USA) from each blood sample. 200 µl of whole blood from each sample was utilized for DNA extraction by following the manufacturer's instructions. The primers Tann and Tori were used for *Theileria annulata* and *Theileria orientalis* respectively (Table 1). Real-time Polymerised chain reaction was performed in a final reaction volume of 25 µl of reaction mixture containing 15 µl Taq-Man probe-based master mix (rotor gene Q), 1 µl forward and reverse primer each and 0.25 µl Taq-Man probe both for *Theileria orientalis* and *Theileria annulata* with cycling conditions as described in Table 2. The graphical representation in q PCR for *Theileria annulata*-FAM (Fluorescein, a dye label) channel was by green colour while for *Theileria orientalis*- JOE (Xanthene Fluorophore) channel, it was yellow colour.

Quantification of *Theileria* spp in blood samples

In 9 consecutive plate runs with three replicate reactions per dilution step, tenfold serial dilutions (10^1-10^9) of the linearised *Theileria* spp recombinant plasmid were studied. The results of these dilution series were used to determine the analytical sensitivity and linearity of the procedure for real-time PCR quantification. The analytical sensitivity of the assay, also known as the limit of detection (LoD), was defined as the lowest concentration (target gene copy) that can be detected. These serial dilutions were used to estimate the number of test runs (number per response) where 95 per cent of test runs yielded positive results.

Calculation of parasitic load

The standard deviation (SD) of the quantification cycle values determined for the ten-fold serial dilutions of plasmid PCR standards was used to indicate intra-assay and inter-assay reproducibility of the *Theileria* spp in TaqMan qPCR assay. The amount of parasitemia was determined by converting Cq values into an estimate of copy number (Q) per reaction tube.10^2 means lowest parasite concentration, 10^9 means highest parasite concentration.

By nanodrop spectrophotometer the concentration of genomic DNA was detected. Two standard curves, one for *Theileria annulata* and one for *Theileria orientalis* were created. Calculation of the gene copy number of plasmids was done by given formula (Dandsena *et al.,* 2018).

$$GCN = \frac{6.02\times10^{23}\ (\text{copy/mol}) \times \text{Amount of DNA (ng)}}{\text{DNA length (bp)} \times 660\ (\text{g/mol/bp})}$$

Plotting standard curve for *Theileria* spp

The isolated plasmid DNA was diluted with nuclease-free water to a final concentration of 100 ng/µl (copy 10^{10}) before being serially diluted seven times by ten-fold dilution to a final concentration of 10 fg/µl (copy 10^3). Real-time Polymerised chain reaction was performed by using three different concentrations for quantification. 12.5 µl master mix (Rotor Gene Q), 1 µl of primers both forward and reverse, 0.3 µl probe, 5 µl DNA and 5.2 µl RT-PCR grade nuclease free water was used to make it 25 µl for both *Theileria annulata* and *Theileria orientalis*.

RESULTS AND DISCUSSION

Detection *Theileria* spp by real time PCR

Bovine theileriosis caused by *T. annulata* and *T. orientalis* are transmitted by ixodid ticks that significantly affects animal productivity. On eastern coast of India, the hot and humid climatic condition encourage vector multiplication and thereby results in higher incidence of such ailment. For effective control of bovine theileriosis, a rapid, sensitive and specific diagnostic method is required. Multiplex qPCR has proven to be an effective approach for detecting and quantifying parasites (Perera *et al*., 2015). Using a variety of molecular markers including major piroplasm surface protein (MPSP), 11 genotypes of *T. orientalis* complex have been identified (Gebrekidan *et al.,* 2020).

Out of 55 samples, 42 (76.36%) samples were found positive for *Theileria* spp, of which, 11 (26.11%) samples were positive to *Theileria annulata*, 24 (57.14%) samples to

Theileria orientalis and 7 (16.66%) samples were positive for mixed infections. Rest 13 (23.63%) blood samples were tested negative for any of the *Theileria* spp (Fig 1).

Concentration of *Theileria orientalis* in affected cattle

A standard linear curve was plotted taking ten-fold serial dilution of plasmid DNA for seven times with maximum (100 ng/µl or copy 10^{10}) and minimum (10 fg/µl or copy 10^{3}) concentrations of *T. orientalis*. The concentration of haemoparasites in blood samples tested were deduced in clinically affected cows that ranged from 2.85×10^{3} to 1.51×10^{5} parasites per ml of blood. Parasitic load for *T. orientalis* in clinically affected cases were measured against the above standard curve that ranged from 1.94×10^{4} to 5.88×10^{5} parasites per ml of blood (Fig 2 a and b). The prevalence study revealed the dominance of *T. orientalis* over *T. annulata*. The higher presence of *T. orientalis* could be due to the possible role of the most abundant tick vector in India, *Rhipicephalus* sp in transmission (Patial *et al*., 2021). Parasitemia of *T. orientalis* assessed through real time PCR in lactating cows of Odisha by RT PCR targeting MPSP gene from *T. orientalis* cloned into pUC57 estimated the parasitic load to be ranging from $6.9\text{-}16.8\times10^{3}$/µl of blood (Sahoo *et al*., 2020). Detection of *T. orientalis* from *Haemaphyslis longicornis* ticks using qPCR assay in South Australia reported genotypic concentration of different strains ranging from $3.5\times10^{1}\text{-}3.6\times10^{4}$ per ml of blood (Hammer *et al*., 2016). The detection of *T. orientalis* conducted by hydrolysis quantitative probe assay and quantified parasitemia ($>3\times10^{5}$ GC/µl) showed strong correlation with clinical signs (Boegma *et al.,* 2015).

Efficacy of buparvaquone on Theileria spp.

In the 1980s, the development of new chemotherapeutic medicines like buparvaquone (BPQ) expanded therapy options. Due to its low toxicity and seven day plasma half-life, buparvaquone (BPQ) is the medicine of choice for treating theileriosis. However, point mutations in the parasite's mitochondrial cytochrome b gene have resulted in resistance, which has become a source of concern (Mhadhbi *et al*., 2015). Ten cows with clinical signs of fever (>104°F), anaemia, dyspnoea, salivation, swelling of pre-

Table 1: Primers and probes used for multiplex qPCR.

Oligo name	Sequence (5′-3′)	Product size	Target genome
Tann18SF	AGACCTTAACCTGCTAAATAGG	120 bp at 60°C	*Theileria annulata*
Tann18SR	CATCACA-GACCT-GTTATTGC		
Tann probe	FAM5'AAGTTTCTACTGTCCCGTT3'BHQ1 (Ros-Garcia *et al.,* 2012).		
Tori F	GGAAACCAAGGATCTCGATG	100 bp at 60°C	*Theileria orientalis*
Tori R	GAATGGTCCGACGAAGTCAT		
Tori probe	JOE-TTGCAGAGGCAGGTCTTTTT-BHQ1		

Table 2: *Theileria annulata* and *Theileria orientalis* multiplex real-time temperature protocol.

Events	Temperature	Time	Cycle
Initial denaturation	95°C	5 min	1 cycle
Denaturation	95°C	10 sec	40 cycles
Annealing and extension	60°C	20 sec	(Green and Orange channel)

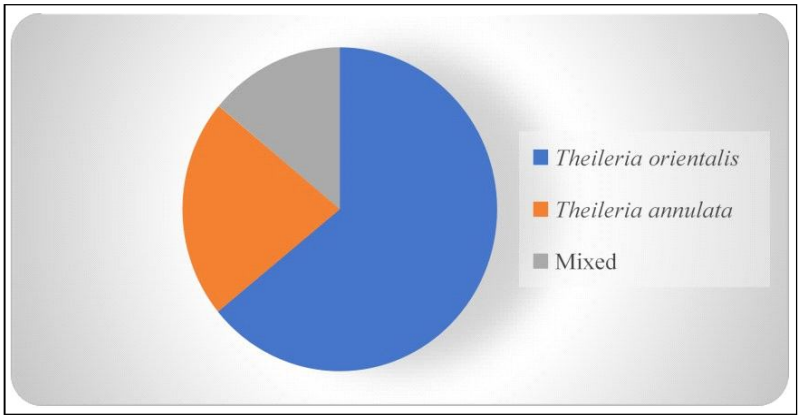
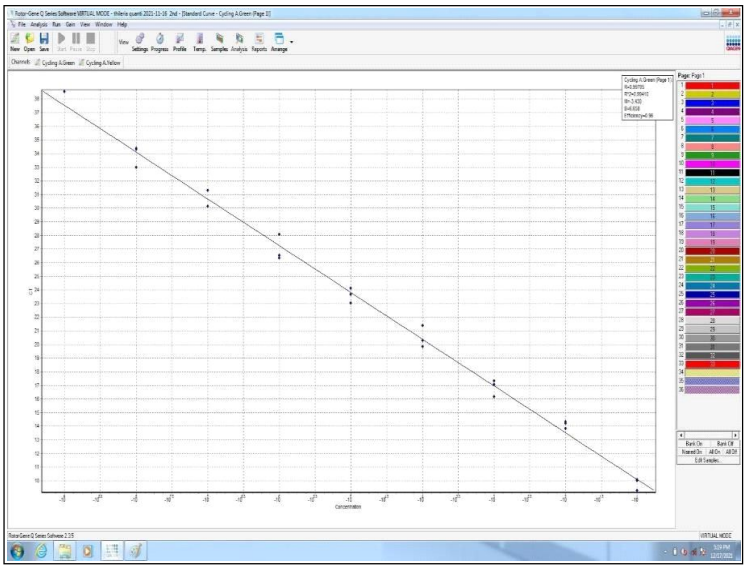
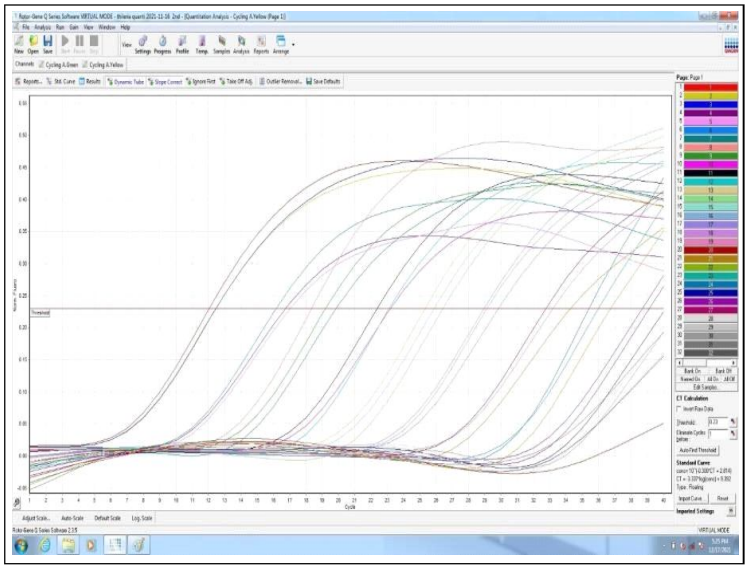


Fig 1: Prevalence of bovine theileriosis in clinically affected cattle.
Theileria annulata (26.11%) *Theileria orientalis* (57.14%) and mixed (16.66%).

scapular lymph node, decreased milk yield and positive for *T. orientalis* (through molecular test) were treated with buparvaquone (Butalex) @ 2.5 mg/kg bw at an interval of 48 hrs. The degree of parasitemia decreased 10 days post-treatment from 4.04×10^4 to 2.21×10^2 per ml of blood indicating significant reduction in parasitemia (Table 3). However, none of treated animal showed complete reduction of the *T. orientalis* within such period as confirmed by and quantified by real time PCR for presence of *T. orientalis*. While previous studies have documented efficacy of the drug against *T.annulata*, the efficacy studies against *T.orientalis* is scanty. Previous studies detected a significant drop in parasite DNA from 72.54+4.55% to 0.01+0.003%. during RT-PCR assay indicates its efficacy in treatment of *T. annulata* with buparvaquone (Dandsena *et al*., 2018). In the present investigation, reduction of the parasite load of *T. orientalis* after treatment with two doses of buparvaquone at an interval of 48 hours indicates efficacy of the drug at the recommended dose and frequency. There has been previous reports of treatment of single dose of buparvaquone and repeat dose of buparvaqoune with oxytetracycline, where remission of clinical signs were observed (Goud *et al*., 2020). The results of the study will help support the clinician to implement suitable therapeutic approach against prevailing theileriosis. Therapeutic trial on *T. annulata* could not be included because of less number of positive cases. However, studies can be planned against mixed infection with *T. annulata*.



(a)



(b)

Fig 2 (a and b): Standard curve and melting curve of test samples for *Theileria orientalis*.

Table 3: Gene copy number of *Theileria orientalis* before and after treatment.

Cow Sl. no.	Gene copy number of *Theileria orientalis*/ml of blood	
	Pre-treatment ($N\times10^4$)	Post-treatment ($N\times10^2$)
1	2.34	1.95
2	1.62	2.42
3	5.88	1.92
4	1.94	2.46
5	3.07	1.96
6	7.31	1.95
7	1.22	1.89
8	2.47	2.02
9	3.99	1.96
10	5.92	1.92
Mean	4.04	2.21

CONCLUSION

The study concluded that Buparvaquone (Bulatex) administered to clinically affected cows @2.5 mg/kg bw intramuscularly twice at an interval of 48 hrs proved efficacious in reducing *T. orientalis* concentration and complete disappearance of clinical signs ten days post treatment. Further studies with different treatment regimen and associated risk factors can be undertaken in different regions to throw more light on level of drug resistance or efficacy rate.

Conflict of interest

All authors declared that there is no conflict of interest.

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