*Tropical Biomedicine* 40(2): 236-240 (2023)
https://doi.org/10.47665/tb.40.2.015


RESEARCH ARTICLE

# *Theileria orientalis* Buffeli pathotype in cows in a theileriosis-endemic region of India

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**ARTICLE HISTORY**

Received: 3 February 2023
Revised: 11 May 2023
Accepted: 12 May 2023
Published: 30 June 2023

**ABSTRACT**

Oriental theileriosis caused by *Theileria orientalis* is a growing health concern of lactating cows in its endemic areas. Rapid and sensitive diagnostic tests are demand areas for appropriate and effective prophylactic and therapeutic measures. Quantitative polymerase chain reaction (qPCR) is the answer for both detection and quantification of parasites. Present study deals with qPCR for detection of parasitemia level of *T. orientalis* in apparently healthy and clinically affected cows. Major piroplasm surface protein (MPSP) gene present in *T. orientalis* was cloned in pUC57 vector and transformed into *E. coli* Top 10 cells. Single and mixed infections of hemoprotozoa other than *T. orientalis,* causing anemia were differentiated through blood smear examination and PCR tests. *T. orientalis* was detected in 108 (63.15%) ill and 48 (26.66%) healthy cows. Piroplasms detected per 1000 red blood cells (RBCs) was 0-1 in the healthy group as compared to 3-22 in those showing clinical signs. Parasitemia in ill cows ranged between $6.9 \times 10^2$ and $4.5 \times 10^3$ parasites / µl of blood which was significantly higher ($p<0.05$) than healthy group ($2.6 \times 10^2$ - $5.7 \times 10^2$ parasites / µl of blood). Phylogenetic study of the isolates showed similarity with Buffeli type that unfolded its pathogenic form in apparently healthy and ill cows.

**Keywords**: Parasitemia; major piroplasm surface protein; hemoprotozoa; piroplasms.

## INTRODUCTION

Among ticks and tick-borne diseases (TBDs), theileriosis affects bovines in tropical and sub-tropical countries across the world. Annual losses due to TBDs in India are estimated to be around 787 million USD (Singh *et al*., 2022). Oriental theileriosis caused by the intracellular protistan parasite *Theileria orientalis* has gained significant clinical relevance in the Asia-Pacific region (Sivakumar *et al*., 2014). Numerous *T. orientalis*-associated outbreaks have been reported in the last decade in India, United States, Australia, New Zealand, Mongolia, and Japan (Stockham *et al*., 2000, Kamau *et al*., 2011, McFadden *et al*., 2011; George *et al*., 2015). *T. orientalis* is predominantly transmitted by ticks belonging to the genera *Ixodid* and *Haemaphysalis* spp. Additionally, other blood sucking insects (tabanids), sucking lice and mosquitoes have also been recognized as potential vectors (Hammer *et al*., 2015, 2016; Watts *et al*., 2016). *T. orientalis* has a total of 11 genotypes: type 1 (Chitose), 2 (Ikeda), 3 (Buffeli), types 4–8 and types N1–N3 (Perera *et al*., 2014; Dinkel *et al*., 2021). Among these genotypes, Ikeda is primarily associated with clinical form of the disease (Eamens *et al*., 2013; Telionis *et al*., 2022). Sivakumar *et al.* (2013) recorded types 1, 3, 5 and 7 in Sri Lanka. Out of the 11 genotypes, 8 have been identified in India (George *et al*., 2015). Aparna *et al.* (2011, 2013) demonstrated the presence of types 1, 3 and 7 in South India and George *et al.* (2015) reported types 2, 4 and 5 along with two new types (TOM 7 and TOM 8) in Andhra Pradesh, India. Sahoo *et al*. (2017) reported the prevalence of *T. orientalis* among apparently healthy and ill lactating cows of Odisha.

The traditional diagnostic methods for *T. orientalis* rely upon clinical signs, serology and microscopic analyses. Although these methods are useful in disease diagnosis and estimation of parasite burden, they have certain limitations. Serology is unsuitable for discriminating the different genotypes of *T. orientalis* (Mans *et al*., 2015). Microscopic detection of macroschizont-infected lymphocytes and/or piroplasm-infected erythrocytes by Giemsa stain commonly practiced in field situations has low sensitivity and specificity. Moreover, disease detection in carrier cows becomes difficult due to the low degree of parasitemia (d' Oliveira *et al*., 1995). Such occult cases remain a potential source of infection for healthy and susceptible stock. The carrier state of *T. orientalis* in the endemic districts of Odisha has been reported previously by Sahoo *et al*. (2017). Molecular diagnostics have overcome most of the limitations of the traditional techniques. Studies have evaluated real time polymerase chain reaction (RT - PCR) assay for identification and quantification of *T. orientalis*. TaqMan quantitative polymerase chain reaction (qPCR) results have shown *T. orientalis* concentration

in clinical cases between $5.6 \times 10^4$ and $3.3 \times 10^6$ genomes per µl of blood (Pulford *et al*., 2016). In the present study, we describe detection and quantification of *T. orientalis* pathotype in both clinically infected and carrier cows of Odisha where theileriosis is endemic.

## MATERIALS AND METHODS

**Sample collection**

The study was conducted in Odisha (Coordinates 20.1411° N, 86.0606° E) from April 2019 to August 2021. Crossbred Jersey cows of this area that showed signs of lack of appetite, reduced milk yield, anemia, fever, enlarged of prescapular lymph nodes, coughing, panting, salivation and lachrymation were categorized as clinically ill or *Theileria* suspect (n=171). One hundred and eighty apparently healthy cows were included as control whose individual daily recorded milk yield was more than 10L and they were maintained under completely stall-fed management with provision of locally cultivated fodder and commercial cattle feed.

**Primer designing and screening of samples**

Blood samples of both categories of cows were screened for detection of *T. annulata* and/or *T. orientalis* using Rotor-Gene Q (QIAGEN, Hilden Germany) by SYBR Green-based real time PCR. Cows having only *T. orientalis* infection were identified for studies on genotype and degree of parasitemia by microscopic examination and qPCR. Real time primer design was conducted by Integrated DNA Technologies (Coralville, United States). Sequences of local isolates were taken and primers were designed from specific regions within major piroplasm surface protein (MPSP) gene of *T. orientalis* (5' GACTGACAAGGATTGGGTTCAG 3' and 5' CCTGCCTCTGTAAACTTGGATG 3') and major merozoite/ piroplasm surface antigen (Tams 1) gene of *T. annulata* (5' TCGTCAGACTCGACTACTTCT 3' and 5' GCATCTGCCTGTGACATTTG 3').

**Hematology**

Hematological examination using standard procedures (Coles, 1986), with respect to hemoglobin (Hb) and packed cell volume (PCV), was performed both in ill and healthy cows. Ten lactating cows from each category were randomly selected for correlation with selection criteria.

**Microscopic examination**

The *T. orientalis* load in blood samples detected by the qPCR was correlated with the number of piroplasms found in 100X (4 high power fields correspond to 1000 red blood cells [RBCs]). The cows with absence of any clinical signs but detected with *T. orientalis* in blood samples were considered as asymptomatic carriers. Apart from *Theileria* spp., other hemoparasites, such as *Babesia*, *Anaplasma*, and *Trypanosoma* spp. were screened through blood smear examination using Giemsa stain.

**SYBR green-based qPCR assay**

pUC57 vector and *E. coli* Top 10 cells were used for cloning the MPSP gene of *T. orientalis* (M/S Biotech Desk, Hyderabad, India). The cloned MPSP gene was subjected to 10-fold serial dilution, ranging from $10^{10}$ to $10^3$ copy/µl. Standard curve denoted copy number and quantification cycle (Cq) value. Genomic DNA extraction from blood was performed using QIAamp DNA kit (QIAGEN, Hilden, Germany). The amplification reactions were performed on Rotor Gene Q (5PLEX HRM, QIAGEN). The total volume of 25µl consisted of 12.5µl of 5x SYBR green master mix, 1µl of both primers (5'GACTGACAAGGATTGGGTTCAG3' and 5'CCTGCCTCTGTAAACTTGGATG3') and 3µl of template DNA. The cycling protocol was 95°C for 10 min, and 40 cycles each of 95°C for 15s and 60°C for 30s. The load of *T. orientalis* was calculated based on formulae described by Dandasena *et al*. (2018) and Hammer *et al*. (2016).

**Phylogenetic study**

Phylogenetic study was performed by Molecular Evolutionary Genetic Analysis (MEGA) 7 software using Neighbor-Joining method. The analysis involved 43 nucleotide sequences that represent Ikeda, Chitose and Buffeli isolate of *Theileria* including the isolates from present study. The representing sequences were downloaded from NCBI database and aligned using Clustal W method and finally 417 bp length of sequence were used for phylogenetic analysis. Total 1000 replicates of bootstrap test were used to compute evolutionary distances through Kimura 2-parameter method.

**Statistical analysis**

Independent values were analyzed through two sample t-test assuming unequal variances to know whether there is any significant difference in load of *T. orientalis* between healthy and ill group.

## RESULTS

**Detection of haemoparasites through microscopic and molecular analyses**

Three hundred and fifty-one Jersey cross bred cows were screened through microscopic analysis as well as PCR to segregate infections due to *T. orientalis* alone. Among 171 ill cows, 75(43.85%) were positive for *Theileria* spp. on microscopic examination using Giemsa-stained blood smears that showed the presence of intra-erythrocytic round, oval, rod-, or ring-shaped merozoites. However, upon subsequent analysis of such samples by qPCR, 108 (63.15%) cows were found positive for *T. orientalis*. *T. annulata*, either alone or in combination with *T. orientalis*, was detected in 22 (12.86%) and 37 (21.63%) cows, respectively. The remaining four (2.33%) ill cows were positive for haemoparasites other than *Theileria* spp. The concentration of *T. orientalis* measured by SYBR green-based real-time PCR in clinically affected lactating cows was 2555±952.75 ($6.9 \times 10^2 - 4.5 \times 10^3$) parasites / µl of blood and the number of piroplams per 1000 RBCs was in the range of three to twenty-two.

Similarly, on examination of 180 blood smears from apparently healthy cows, three (1.66%) were positive for *Theileria* spp. by microscopic examination, and 48 (26.66%) were positive for *T. orientalis* by qPCR. One hundred and thirty-two (73.33%) healthy cows were free from *T. orientalis* infection. The degree of parasitemia in apparently healthy cows (asymptomatic carrier) was 396±76.43 ($2.6 \times 10^2$ to $5.7 \times 10^2$) parasites / µl of blood and 0-1 piroplasm was detected per 1000 RBCs.

**Hematological examination**

Hematological examination of *T. orientalis*-infected ill cows (n=10) revealed Hb in the range of 6.2–8.8 g% and a PCV of 18-26%, as compared to 8.6–10.8 g% and 26–30% in healthy cows (n=10).

**Phylogenetic study**

Evolutionary analysis of MPSP gene sequences showed that the isolated *T. orientalis* strains (MN098314 and MN098315) had similarity with *T. orientalis* buffeli type found in different parts of the world (Figure 1).

**Statistical analysis**

Results of statistical analysis showed that the load of *T. orientalis* in blood samples from clinically affected cows (2555±952.75) was significantly higher ($p < 0.05$) than healthy counterpart (396±76.43). It signifies the term *T. orientalis* buffeli pathotype.

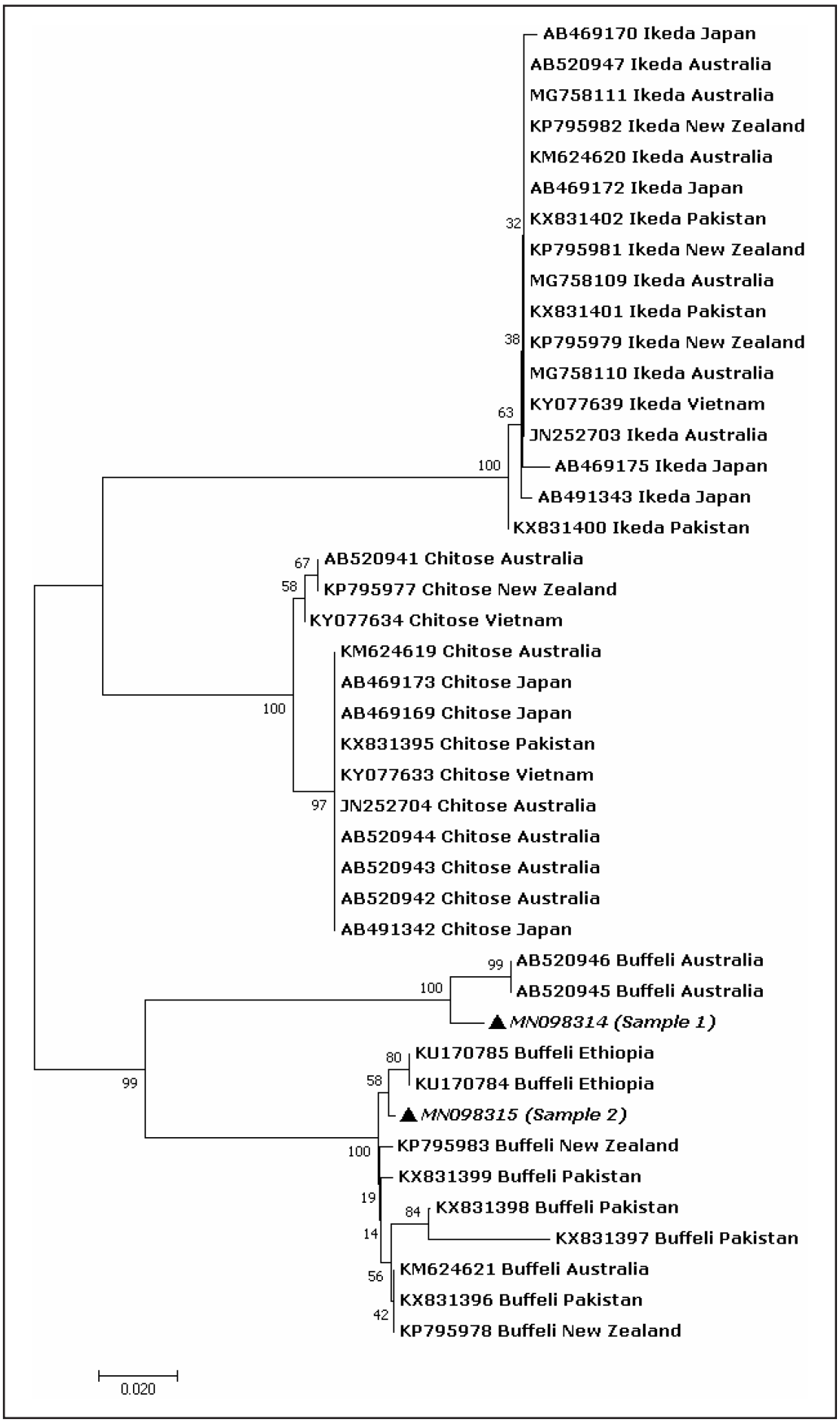


**Figure 1.** Evolutionary analysis of MPSP gene sequences showing phylogenetic relationship of *T. orientalis* (Samples 1 & 2 - MN098314 and MN098315) with other known *T. orientalis* strains isolated from different parts of the world.

## DISCUSSION

Earlier investigations in the study region have focused on *T. annulata*. We investigated to unveil the pathotype and circulating load of *T. orientalis* pathotype in asymptomatic or apparently healthy (n=180) and clinically affected (n= 171) cows raised in a region of India in which the parasite is considered endemic. Study revealed that more than a quarter of the apparently healthy cows carried this parasite, while almost two-thirds of the clinically ill cows showed infection with *T. orientalis* by qPCR. The parasitic load in blood samples from cows with and without clinical signs of theileriosis differed statistically.

Obligate intracellular hemoprotozoan parasites belonging to genus *Theileria* cause substantial economic losses to the livestock sector. Such impact is more pronounced in lactating cows, in the form of decreased milk yield and poor reproductive performance. Odisha is a region situated in the tropical zone and experiences vector-borne diseases due to extreme climatic conditions of heat and humidity that favor multiplication of the vectors (Ogre, 1999). The present study was undertaken in this region where dairy farmers have been lodging complaints of increased morbidity related to theileriosis in high yielders (yielding capacity $\geq$ 10L/day) during the period of high environmental temperature and humidity. Data about the quantification of *T. annulata* in India was sparse (Kundave *et al*., 2014; Dandasena *et al*., 2018).

In this study, we used differential diagnosis to uncover the probable causes leading to anemia. Diseases such as bovine tropical theileriosis, babesiosis, anaplasmosis and trypanosomiasis were eliminated based on microscopy and epidemiological data. The diagnosis of *T. orientalis* infection was confirmed by the process of elimination.

Blood smear examination facilitates identification of intraerythrocytic and schizont stages of *Theileria* spp. Although popular, this method is rarely successful in detecting carriers. Moreover, it is unreliable for differentiating pathogenic from non-pathogenic forms, based on their morphology. This method is also not helpful for tagging subtypes of *T. orientalis*. Pulford *et al*., 2016 categorized the number of piroplasms in 1000 erythrocytes as negative (0), low (1–9), moderate (10–100) and high (>100). Serological tests are less dependable due to their cross-reactivity and inability to distinguish carriers from clinical cases (Roy *et al*., 2000). To overcome these constraints, real-time PCR was preferred test due to its higher sensitivity for detecting carrier-state infection in endemic locations.

Three hundred and fifty-one lactating cows with or without clinical signs of theileriosis from an endemic region of Odisha were included in the study. Among the clinically ill cows, 63.15% (108/171) were tested positive for *T. orientalis.* A higher percentage of clinically ill cattle i.e., 90% (916/1022) were tested positive for *T. orientalis* by quantitative PCR in an earlier study (Pulford *et al*., 2016). Present study revealed that 26.66% (48/180) apparently healthy cows harbored the hemoprotozoa. In another study, five healthy cows harbored *T. orientalis* out of 30 heads (Anupama *et al*., 2015). Variation in the prevalence rate recorded by various authors across the globe could be attributed to one or more of the factors such as geographical region, density of ticks, climatic conditions, age group, management practices and immunity. In addition, the study period, difference in sample size, and sensitivity and specificity of the diagnostic tests used in the study could contribute to the unequal prevalence rates. In our study area, farm management systems with inadequate ventilation and floor space in shed might contribute to intrinsic or extrinsic stress, which were likely to increase the prevalence rate of the disease.

Earlier, *T. orientalis* load was measured using hydrolysis probe qPCR, where cattle with clinical signs at the time of sampling were either in the moderate or high level group, with parasitemia exceeding $1.5 \times 10^4$ gene copies (GC)/µl. Lower concentrations were noticed in convalescent, in-contact cows, and those from herds without clinical cases of infection (Bogema *et al*., 2015). In another probe-based (TaqMan) qPCR assay, concentration of parasites in clinical cases varied between $5.6 \times 10^4$ and $3.3 \times 10^6$ genomes per µl of blood (Pulford *et al*., 2016). Concentration of the parasitic genome varied from $3.5 \times 10^1$ to $3.6 \times 10^4$ GC/µl (Hammer *et al*., 2016). The higher limit of parasitic load observed by these investigators, both in clinically affected cows and carrier state cows may be due to the host's immune status, variation in the parasite genotypes, and periods of endemicity.

Two gene targets commonly used for detecting *T. orientalis* infections are ssu rRNA and MPSP. In the current study, MPSP was chosen as the target because it is expressed abundantly on the surface of piroplasm infected erythrocytes and serves as a good marker for phylogenetic and diversity studies (George *et al*., 2015). This surface gene also plays an important role in host parasite interaction and evolves under different circumstances or pressure (Shirakata *et al*., 1989).

*T. orientalis* has 11 subtypes, based on molecular markers such as MPSP, 23-kDa piroplasm membrane protein (p23), ssu rRNA and / or first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2), as previously reported (Gubbels *et al*., 2000, Aktas *et al*., 2006; Ota *et al*., 2009). At this stage, it could be inferred that infection due to *T. orientalis* Buffeli type is distributed in the endemic region of India included in the present study. Furthermore, the strains were identical to those from Australia and Ethiopia. Although Buffeli type is normally considered to be non-pathogenic, in our study cows infected with *T. orientalis* Buffeli type alone exhibited clinical signs of theileriosis, such as anorexia, high rectal temperature, anemia, reduced milk yield, panting, reduced body condition, enlarged superficial lymph nodes, and coughing. Our findings were supported by reports of earlier investigators who recorded life-threatening cases due to *T. orientalis* Buffeli (Chae *et al*., 1999; Stockham *et al*., 2000, Cossio-Bayagur *et al*., 2002). Pathogenic form of parasite has also been detected in asymptomatic animals in Eastern Asia and Australia (Kang *et al*., 2012; Sivakumar *et al*., 2013).

Buparvaquone with or without oxytetracycline has been the drug of choice for the past few decades. Prophylaxis is achieved through administration of locally available live attenuated vaccine (Rakshavac–T by Indian Immunologicals, India) that provides protection only against *T. annulata*. Improper health management practices, multiple use of unsterilized hypodermic needle and inefficient tick control measures in theileriosis endemic regions predispose cross-bred and exotic cattle to this infection, which poses a future threat to sustainable growth of dairy farming.

## CONCLUSION

Our study provided information about the prevalence of *T. orientalis* Buffeli genotype along with its variable degree of parasitemia in apparently healthy and ill cross bred Jersey lactating cows from theileriosis endemic region in Odisha, India. The study could be extended to other regions with inclusion of all genotypes of *T. orientalis* for effective control and containment of the disease.

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

## ACKNOWLEDGMENTS

Authors are thankful to the field veterinarians and farmers who cooperated during the entire study.

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