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Molecular detection of three genotypes of *Theileria orientalis* in crossbred jersey cattle in Odisha, India

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Abstract

Theileria orientalis is an emerging disease in East Asia and Australasia which causes severe economic losses. The present study was aimed to investigate the presence of three genotypes of *T. orientalis* (Chitose type 1, Ikeda type 2 and Buffeli type 3) in the blood of crossbred jersey (CBJ) cattle in Odisha, India using the SYBR green-based quantitative PCR (qPCR). Out of 418 samples of examined blood smears, 344 (82.29%) animals were found positive for *Theileria* species. From 344 samples, 103 samples were further examined by PCR and 72 (69.9%) samples were found positive for *T. orientalis*. The results of 72 samples examined through qPCR were found positive for *T. orientalis* genotypes either in single or mixed infection. This study is the first report of the occurrence of three *T. orientalis* genotypes in CBJ cattle of Odisha with the presence of pathogenic Ikeda and chitose genotypes which can lead to clinical outbreaks.

Keywords: Blood, cattle, India, quantitative PCR, *Theileria*

Introduction

Theileria orientalis is a non-transforming strain producing its major pathogenic effects through damage to the erythrocyte. The infections with *T. orientalis* in cattle are considered benign however recently several clinical outbreaks of *T. orientalis* have occurred in Asia and Australia. Genotype Ikeda (Type 2) has been linked with the rapid spread of clinical theileriosis [1, 2, 3]. Clinical signs of oriental theileriosis characterized by fever, inappetence, ataxia, abortion, an increase in heart rate, anemia and jaundice. *T. orientalis* transmitted in India through tick *Haemaphysalis bispinosa* and *Rhipicephalus microplus* [4, 5].

PCR is the most sensitive diagnostic tool for the detection of *T. orientalis* using major piroplasm surface protein (MPSP) as a marker. MPSP is a protein present in huge numbers on the surface *T. orientalis* piroplasm in infected red blood cells. The genetic diversity of *T. orientalis* was determined using the sequence variations of the MPSP gene [6, 7]. PCR can identify *T. orientalis* infection in bovine up to two weeks before the piroplasm infected erythrocytes can be detected by microscopic examination [8, 9, 10]. However, conventional PCR assays cannot differentiate between clinically affected animals and subclinical carriers as they do not give any information about parasite load. To solve these problems quantitative PCR has been used for the detection of *T. orientalis* [11, 12, 13].

Sequencing of the major piroplasm surface protein (MPSP) gene revealed to date, at least 11 *Theileria orientalis* genotypes (Chitose or type 1, Ikeda or type 2, Buffeli or type 3, types 4 to 8, and N1 to N3) have been identified [14]. Several clinical outbreaks that occurred in cattle in the Asia-Pacific region are proposed to be related to genotypes Chitose and Ikeda [1, 4, 12, 15, 16]. To the best of the authors' knowledge, only three genotypes (out of the 11 types) of *T. orientalis* are reported in Indian cattle, type 1 (Chitose), type 3 (Buffeli) and type 7 [4, 5]. The Buffeli and Chitose types have been infrequently related to outbreaks of bovine anemia in New Zealand and Australia [17, 18, 19]. The effective control of blood parasites requires highly sensitive, specific and rapid techniques. The qPCR did not have been yet used to detect *T. orientalis* genotypes in Odisha. Although the qPCR technique is very expensive, which may prevent using it in poor economic situations; its rapid and accurate results guarantee its use as a very hopeful technique for effective diagnosis of parasitic infections.

Materials and Methods

Study area

The study was conducted in the coastal districts of Odisha between 18°2' and 22°6'N latitude and 82°8' and 87°6'E longitude where the climate is very hot and humid that provides suitable conditions for the development of tick-borne diseases.

Blood samples collection, microscopic examination and DNA extraction

The blood samples (n=418) were collected in tubes with EDTA. Thin blood films were prepared from blood samples and stained with Giemsa. The DNA was extracted from 200 µl blood using the QIAamp® DNA Extraction Kit (QIAGEN, USA) according to the manufacturers' protocols. By the aid of Nanodrop, the quantity and purity of the DNA samples were measured. The extracted DNA samples were kept at -20°C until further examinations.

Polymerase chain reaction and nucleotide sequencing

The conventional PCR protocol for detection of *T. orientalis* DNA was performed with a set of primers that amplifies (776

bp) of the MPSP gene of *T. orientalis* (Table 1). The PCR reaction was performed using the Thermal Cycler (T100TM, Bio-Rad, USA) with a total volume of 20 µl, consisting of 10 µl of 2× PCR TaqMixture (Himedia, India), 1.0 µl of each primer (10 pmol), 4 µl of nucleus free water and 4 µl of template DNA. Thermocycling conditions started with an initial denaturation step at 95°C for 3 min, followed by 37 cycles of denaturation at 95°C for 30 sec, 55°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The electrophoresis was performed in 1.5% agarose gel with ethidium bromide dye which was further visualized under UV light gel documentation system (Bio-Rad, Hercules, CA, USA).

Based on the conventional PCR results, positive samples for *T. orientalis* were sent for DNA sequencing to Life Sciences Institute, Bhubaneswar, India. PCR products were purified by ExoSAP-IT™ Express PCR Product Cleanup (ThermoFisher Scientific, USA). The sequencing reactions were done using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, USA) in Genetic Analyser ABI 3100 (Applied Biosystems, Germany).

Table 1: Oligonucleotide primers sequence used in the study

Primer Name	Sequence (5'→3')	Amplicon Size	Assay	Reference
MPSP -F	CTTTCCTAGGATACTTCCT	776 bp	<i>T. orientalis</i> Specific	[9]
MPSP-R	ACGGCAAGTGGTGAGAACT			
NZlike1-F	AGT TAA CGC CAC CGC AGC CG	86 bp	Ikeda Specific qPCR	[27]
NZlike1-R	ACG CGG TAT CCC TCT TCG GCA			
NZBuf-F	CGG ACC CTT CAA GGT TAA GAG TGT T	79 bp	Buffeli Specific qPCR	[27]
NZBuf-R	TGA CTG CAG TGT ATT TGG CA			
NZChi-F	TGG ATC TCC GAC TTC CTT ATC CTC G	87 bp	Chitose Specific qPCR	[27]
NZChi-R	TGC ATT CGG TAC CGG AAA GGT T			

Quantitative PCR (q PCR) to identify *T. orientalis* genotypes

Quantitative PCR was done in 96-well plates on a Bio-Rad CFX96 software qPCR thermal cycler (Bio-Rad, Hercules, CA, USA). All Cq values resulted from amplifications were calculated by the Bio-Rad CFX96 analysis software. The qPCR assays were performed in 10 µL reaction volume consisting of SYBR green qPCR master mix (Sigma- Aldrich) 5 µL, 5 pM each of forward and reverse primers of each type

(Table 2) and 1.5 µL of DNA. Thermal cycling conditions were standardized as 95°C for 2 min (initial denaturation), followed by 45 cycles of 95°C for 15 sec (denaturation) and 61.4°C for 30 sec (Chitose and Buffeli) and 60°C for 30 sec in case of Ikeda (annealing and extension) after that melt curve analysis from 72°C to 95°C with 0.2°C increments for 5 sec. The run quality was determined through using positive control, negative control and no template control (NTC). Three replicates were run for each concentration to document the reproducibility and stability of the qPCR.

Table 2: Results of microscopic examination and PCR amplification of MPSP gene of *T. orientalis*

Microscopic examination			PCR		
Examined	Positive	Negative	Examined	Positive <i>T. orientalis</i>	Negative <i>T. orientalis</i>
418	344(82.3%)	74(17.7%)	103	72 (69.9%)	31(30.1%)

Table 3 Number of blood samples that were positive for *T. orientalis* genotypes Chitose, Ikeda, Buffeli and mixed genotypes

Chitose Specific qPCR	Ikeda Specific qPCR	Buffeli Specific qPCR	Mixed Ikeda +Chitose	Mixed Ikeda +Buffeli	Mixed Buffeli+ Chitose	Mixed Chitose+ Ikeda+ Buffeli	Total
Positive	Positive	Positive	Positive	Positive	Positive	Positive	
6(8.3%)	18(25%)	5(6.9%)	9(12.5%)	7(9.7%)	6(8.3%)	21(29.3%)	72(100%)

Statistical analysis

Descriptive statistical analysis was performed using SPSS 22 statistical software program windows version.

Results

Examination of blood smears revealed the presence of

elongate intraerythrocytic bodies in the case of *T. orientalis* infection. Out of 418 samples of examined blood smears, 344 (82.29%) animals were positive for *Theileria* species (Table 2). Out of 344 samples, 103 samples were further examined on polymerase chain reaction (PCR). The product size of 776 bp was considered positive *T. orientalis* in 72 samples (Figure

1). The nucleotide sequence analysis (nBLAST) of 776 bp fragment of *T. orientalis* showed 99.87% similarity with the corresponding gene sequence available in GeneBank.

Positive *T. orientalis* samples further examined by qPCR. A Cq (quantification cycle) value was obtained, corresponding to the PCR cycle at which the concentration of the target DNA crossed the arbitrary threshold determined by the Software (Bio-Rad CFX96 analysis software) supplied with the PCR amplification platform.

The results of 72 samples examined through qPCR were found positive as the following 6 (8.3%) Chitose, 18 (25%) Ikeda, 5 (6.9%) Buffeli, 9 (12.5%) mixed (Ikeda+Chitose), 7 (9.7%) mixed (Ikeda+Buffeli), 6 (8.3%) mixed (Buffeli+Chitose) and 21 (29.3%) mixed (Chitose+Ikeda+Buffeli) (Table 3). Amplification plots of *T. orientalis* genotypes (Chitose, Ikeda and Buffeli) were generated (Figure 2, 3, 4). Melt curve analysis revealed no detectable peaks in no template control (NTC) and the identity of amplicons size proved the specificity of the protocol.

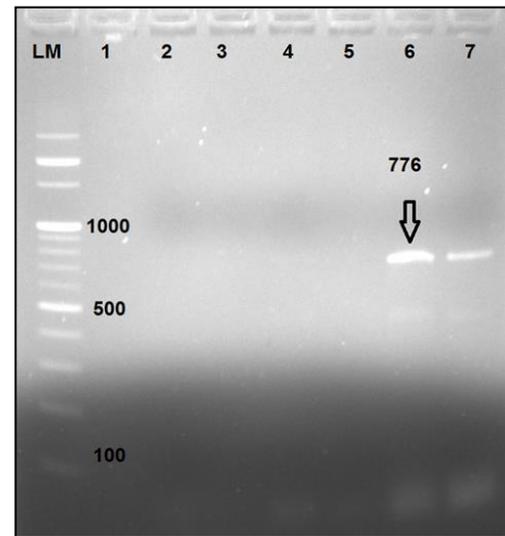


Fig 1: Amplified PCR product of MPSP gene of *T. orientalis* Lanes 6 and 7 were positive samples (776 bp) LM- 100 bp ladder

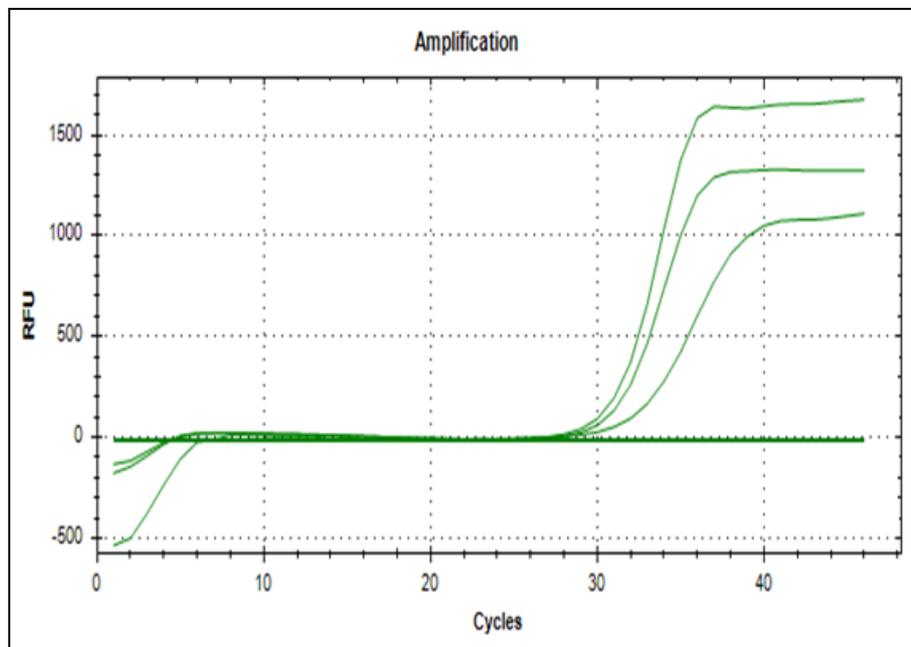


Fig 2: Amplification plot for *T. orientalis* (type 1 Chitose)

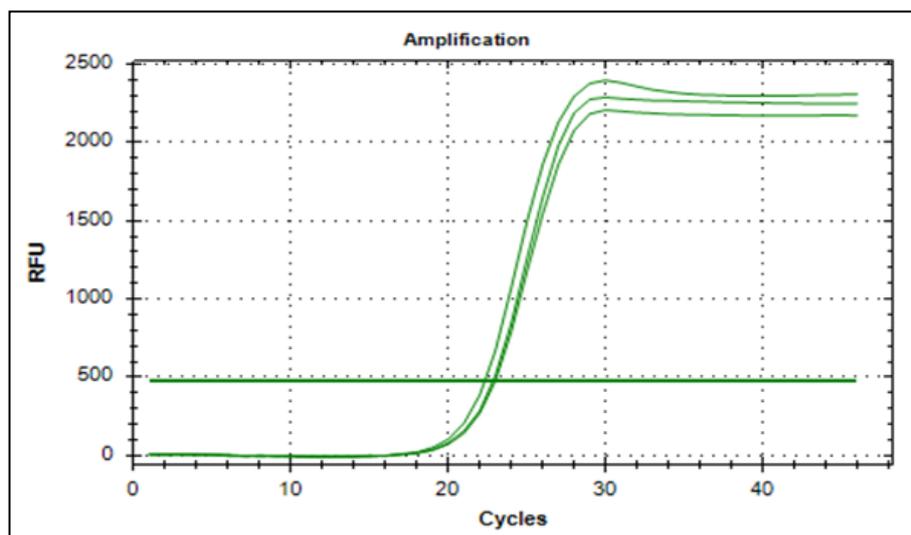


Fig 3: Amplification plot for *T. orientalis* (type 2 Ikeda)

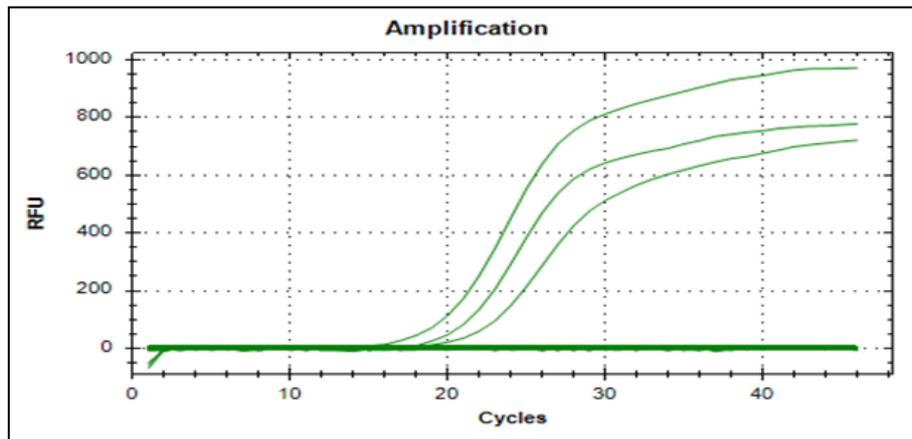


Fig 4: Amplification plot for *T. orientalis* (type 3 Buffeli)

Discussion

Despite the high prevalence and the economic losses caused by *T. orientalis* infection among the cattle in Odisha, no attempts have been made to detect the types of *T. orientalis* genotypes in Odisha. The rapid development of molecular techniques made the detection of vector-borne diseases more accurate and sensitive in a particular geographic area. The qPCR assay depends on genotype-specific primers for identification of Buffeli, Chitose and Ikeda genotypes.

Rapid diagnosis of haemoparasite infections can be done through examination of blood smears, abnormal erythrocyte morphology with the presence of schizonts in infected mononuclear cells and erythrocytes. The infected erythrocytes appeared with thorn-like protrusions on its surface and these abnormalities in erythrocytic morphology are mainly due to presence of schizonts and immune-mediated processes [20]. The limitation of microscopic examination is its inability to differentiate the types due to the high pleomorphism among the piroplasms. Moreover, it is more likely to give misdiagnosis of theileriosis therefore, PCR is the highly sensitive and specific technique for detection of the parasite DNA in blood [21, 22, 23]. In veterinary parasitology, PCR is mostly used to identify piroplasm-carrier animals using species-specific primers [24, 25].

DNA from animals suffering from anemia usually contained PCR inhibitors therefore; development of DNA extraction protocols was a challenging aspect. Dilution of blood in double distilled water before DNA extraction was done to decrease the viscosity of the sample and also, to dilute PCR inhibitors. This dilution technique was reliable but reduces the sensitivity of the assay. However, in a recent outbreak in New Zealand the sample dilution and column-based DNA extraction technology was performed, as the sensitivity of the assay was satisfactory and provided reliable results [10, 15].

Methods for diagnosing *T. orientalis* genotypes in Indian cattle have become very important for monitoring suspected cases of infection. The qPCR assay described here was used to provide supporting data to help type Ikeda or other types of *T. orientalis*. The application of qPCR in Odisha to detect *T. orientalis* genotypes revealed that Ikeda genotype is the main pathogenic genotype involved in oriental theileriosis in cattle which was similar to findings in New Zealand. Furthermore, a relationship was found between the severity of infection of the genotype Ikeda and anemia during outbreaks of bovine theileriosis in New Zealand [1, 4, 12, 16].

Severe infections of the pathogenic genotypes, Ikeda and Chitose have been associated with the clinical cases of oriental theileriosis in Australia and New Zealand. Hence, it is

important to estimate the burden of different genotypes of *T. orientalis* to detect, differentiate, or quantitate these genotypes [11, 12].

The advantage of SYBR green real-time its low cost in comparison to TaqMan probes, but the problem false positive signal with non-specific amplicons can be formed [26] however during melt curve analysis by CFX96 analysis software only single peak was formed and also specific amplicons were confirmed through gel electrophoresis on 3% agarose and visualized under UV light.

This is the first report of *T. orientalis* Ikeda type, Chitose and Buffeli in cattle in Odisha. Further studies will be needed to investigate the clinical association with different genotypes of *T. orientalis* infection in cattle to setup effective prevention and control strategies. The mutations, single nucleotide polymorphism and epigenetic differences cannot be detected through qRT-PCR so further examination needed through using High resolution melting (HRM) curve analysis.

Conclusion

The results of the present study revealed the first report on the occurrence of three genotypes of *T. orientalis* (Chitose, Ikeda and Buffeli) in Odisha, India. Detection of Ikeda and chitose genotypes that consider pathogenic genotypes lead to many outbreaks in other countries. Therefore, the present study will help to establish a protocol for disease control.

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