DIVERSITY ANALYSIS OF THEILERIA SPECIES IN LOCAL BUFFALOES OF ODISHA, INDIA

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ABSTRACT

Buffaloes were screened for theileriosis by routine microscopic examination and also subjected for characterisation by PCR technique. Blood samples were collected from lactating buffaloes in post partum period from endemic areas of Athagarh block of Cuttack district, Odisha, India. Genomic DNA of Theileria piroplasm was isolated and genus specific primers were used for amplification of small subunit ribosomal RNA sequences. The amplified PCR products of *Theileria* spp. were sequenced. Out of 86 cases examined, 21 and 31 samples were found positive by Giemsa stained blood smear method and PCR technique respectively. The PCR product was sequenced and analysed for homology. The identified nucleotide sequence had close sequence homology with *Theileria orientalis* and *Theileria buffeli*. These findings also support the fact that 18S small subunit rRNA gene is hyper variable among the species. The nucleotide sequence was submitted to NCBI and a new accession number (MN262069) was assigned.

Keywords: *Bubalus bubalis*, buffaloes, DNA, PCR, *Theileria* sp.

INTRODUCTION

Buffaloes are integral part of Indian dairy industry as they contribute more than 55% of total milk production of the nation. The Indian climate favours easy maintenance and multiplication of vectors and thus, the animals suffer from arthropod born diseases (Dhar *et al*., 1987). Tick born diseases including theileriosis cause major economic losses due to significant effect on growth and production of animals (Swaid *et al*., 2013).

The two most pathogenic and economically important theileria parasite affecting buffaloes are *Theileria parva* and *Theileria annulata*. *Theileria parva* occurs in Eastern and Southern Africa and causes east coast fever. *Theileria annulata* causes tropical theileriosis, also known as Mediterranean theileriosis and occurs in North Africa, Southern Europe and Asia. The mortality rate for tropical theileriosis can also vary from 3% to nearly 90%, depending on the strain of parasite and the susceptibility of the animals (scientific.dept@oie.int, 2009). There are only few systematic studies on the occurrence and pathology of theileriosis in buffaloes in India except few sporadic case reports or molecular study and characterisation of the parasite rather than the disease. Therefore,

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the current research work was aimed at study the incidence of the disease in native buffaloes of coastal Odisha using routine blood smear examination and compare with molecular diagnosis.

MATERIALS AND METHODS

Blood samples (86 nos) of lactating buffaloes in postpartum period were collected in 5 ml EDTA vials (Schalm, 1965) from randomly selected five buffalo herds in endemic area of Athagarh block of Cuttack district, Odisha, India. The selected animals were apparently healthy so that the presence of Theileria piroplasm can be studied. The blood smears were prepared on grease free clean dry slides from EDTA mixed blood. The smears were air dried and stained with Giemsa stain solution and examined under oil immersion lens for detecting piroplasms inside erythrocytes.The Genomic DNA was isolated from the blood samples by 'Phenol: chloroform isolation' method (Sambrook and Russel, 2001). Theileria genus specific primers (Forward 5`-AGTTTCTGACCTATCAG-3`, and Reverse - 5`-TTGCCTTAAACTTCCTTG-3`) were used for amplification of small subunit ribosomal RNA sequences (Durrani *et al*., 2008). The amplified PCR product was run in 1.2% Agarose gel electrophoresis and bands were viewed in Gel Documentation system (BIO-RAD, USA). The PCR product was eluted and submitted for nucleotide sequencing. To check the quality of nucleotide sequences, chromatograms were analyzed and verified in BioEdit programme. These nucleotide sequences were aligned in MEGA X. The alignment was done based on the lowest Bayesian Information Criterion (BIC) score. The pair wise sequence distance and phylogenetic tree were derived using the neighbor-joining method (Saitou and Nei, 1987) with 1,000 bootstrap samplings (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

A total of 86 fresh blood samples including 21 cases found positive in blood smear examination (Figure 1) were subjected to PCR, it was observed that all 21 positive cases in blood smear examination were also positive in PCR.

However, out of 65 Negative cases in blood smear examination 10 samples were positive by PCR (Figure 2). Thirty one samples showed clear bands of about 1098 bp in gel electrophoresis. This result indicates PCR is more sensitive for detection of *Theileria* than routine microscopic examination of blood smear. Similar observations were found in earlier studies (Sanchez *et al*., 1998; Bayugar *et al*., 2002; Dumanli *et al*., 2005; Azizi *et al*., 2008; Durrani *et al*., 2008; Kundave *et al*., 2009; Ghanem *et al*., 2013; Khatoon *et al*., 2015; Tiwari *et al*., 2015; Acharya *et al*., 2017; Singh *et al*., 2017).

The positive samples showed clear cut band of 1098 base pair in agarose gel (Figure 3). The positive samples were eluted and submitted for nucleotide sequencing. The quality of nucleotide sequence of *Theileria* spp. was found to be good quality. The sequencing result was submitted to NCBI and a new accession number (MN262069) was obtained. The BLAST analysis identified 6 similar nucleotide sequences from different species. The alignment was based on the model described by Tamura *et al*. (2013) with gamma distribution having BIC score 6679.35. When the pair-wise sequence distance was calculated, nucleotide sequence of our isolate showed maximum (6%) sequence distance from *Theileria mutans* (Table 1). In phylogenetic analysis, it was observed that

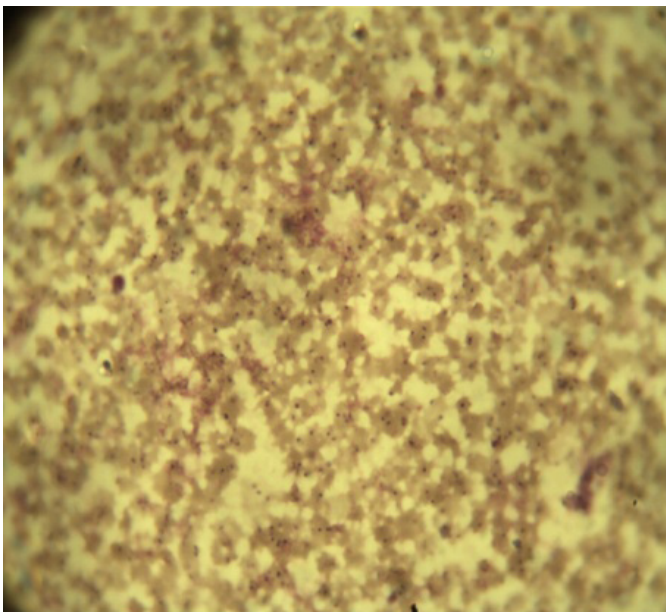

Figure 1. Blood smear with Giemsa stain showing presence of *Theileria piroplasms.*

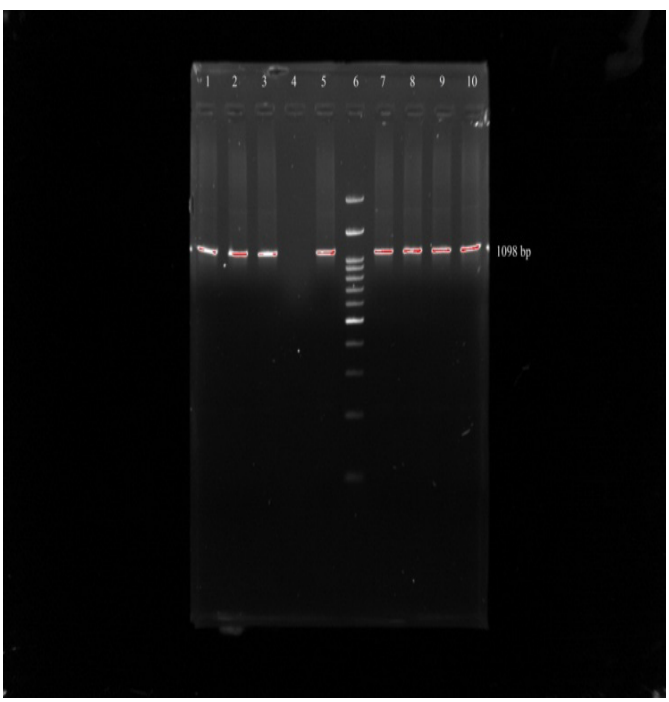

Figure 2. Gel electrophoresis of PCR product.
Lane 1, 2, 3, 7, 8, 9 and 10: Positive for *Theileria* spp.
Lane 4: Negative control for *Theileria*
Lane 5: Positive control for *Theileria*
Lane 6: 100bp Ladder

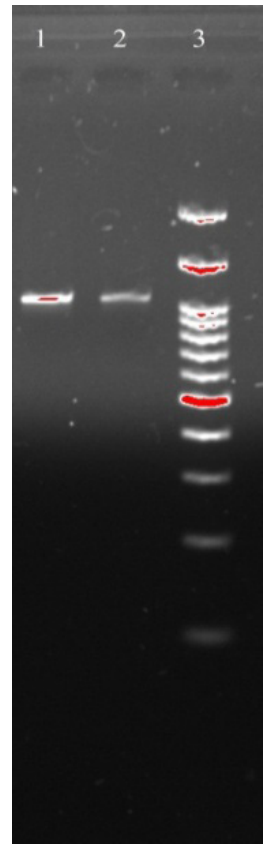

Figure 3. Gel electrophoresis of eluted PCR product.
Lane 1 and 2: Eluted PCR product of 18S rRNA of *Theileria* spp.
Lane 3: 100bp Ladder

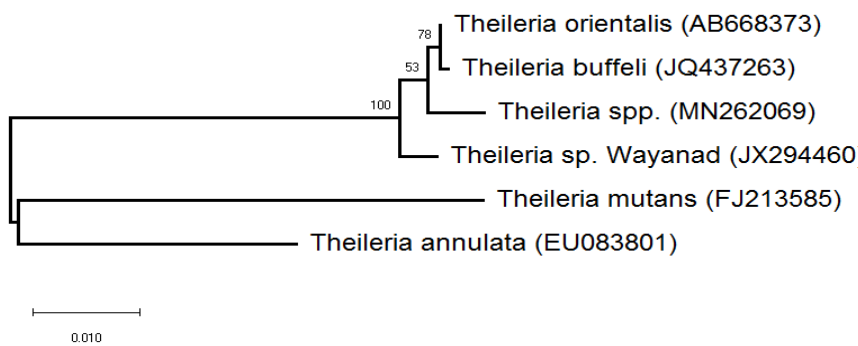

Figure 4. Phylogenetic tree constructed using nucleotide sequence of 18S rRNA gene in *Theileria*.

Table 1. Pair wise sequence distance of *Theileria* species.

***Theileria* species**	**Sequence distance**					
Theileria sp. Wayanad - (JX294460)		0.00	0.01	0.01	0.00	0.00
Theileria orientalis - (AB668373)	0.01		0.01	0.01	0.00	0.00
Theileria mutans - (FJ213585)	0.06	0.07		0.01	0.01	0.01
Theileria annulata - (EU083801)	0.04	0.05	0.05		0.01	0.01
Theileria buffeli - (JQ437263)	0.01	0.00	0.07	0.05		0.00
Theileria species Odisha isolate -	0.01	0.01	0.06	0.04	0.01	

our isolate of *Theileria* spp. remained in one cluster along with *Theileria buffeli* and *Theileria orientalis* (Figure 4).

The nucleotide sequence of our *Theileria* species had close sequence homology with *Theileria orientalis* and *Theileria buffeli*. This is in accordance of host pathology (buffalo is primary host for *Theileria orientalis* and *Theileria buffeli*. So our isolated *Theileria* spp. may belong to *Theileria orientalis* or *Theileria buffeli*. The nucleotide sequence was submitted to NCBI and a new accession number (MN262069) was assigned. These findings support the fact that 18S small subunit rRNA gene is hyper variable among the species. However, it remains conserved in the different species of same genus. But it is premature to comment further on relationships within Theilaeria group due to limited availability of data. Intra-specific variation is limited to a few nucleotide substitutions in the 18s small subunit rRNA gene.

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