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Emergence of new genotype and diversity of *Theileria orientalis* parasites from bovines in India

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ABSTRACT

Bovine theileriosis is a serious threat to livestock worldwide. Uncertainty around species prevalence, antigenic diversity and genotypes of strains make it difficult to assess the impact of this parasite and to provide necessary treatment. We aimed to characterize genotypic diversity, phylogeny and prevalence of *Theileria orientalis* parasites from the states of Telangana and Andhra Pradesh, India by collecting bovine blood samples from the major districts of the two states. Bioinformatic analysis identified antigenic diversity among the prevalent parasite strains using major piroplasm surface protein (MPSP) gene. Our study revealed a prevalence rate of 4.8% (n = 41/862) of *T. orientalis* parasites in bovine animals and a new genotype of *T. orientalis* parasite which was not previously reported in India. The emergence of these new genotypes could be an explanation for the frequent outbreaks of bovine theileriosis. Further, whole genome sequencing of *T. orientalis* strains will help to elucidate the genetic factors relevant for transmissibility and virulence as well as vaccine and new drug development.

1. Introduction

Protozoan parasite belonging to genus *Theileria* causes huge economic loss to livestock industry worldwide. *Theileria orientalis* species compared to *Theileria annulata* and *Theileria parva*, causes less mortality, however, it has the capacity to become lethal and in the past has been the cause of enormous loss of livestock (Seddon and Albiston, 1966; Shimizu et al., 1992; Onuma et al., 1998; Chae et al., 1999; Stockham et al., 2000; Cossio et al., 2002). Further, several reports from India have highlighted *T. annulata* as the causative agent of theileriosis (Manuja et al., 2006; Aparna et al., 2013; Shweta et al., 2014; Kundave et al., 2014; Tiwari et al., 2015), however, reports from Kerala, India (Aparna et al., 2011, 2013) have revealed the existence of *T. orientalis* parasite in the field. During the life cycle, *T. orientalis* is unique in that it proliferates inside erythrocytes as piroplasms and does not transform leucocytes like *T. annulata* and *T. parva*.

Available vaccine contains live attenuated *T. annulata* parasite (Tait and Hall, 1990) and so can only control *T. annulata* specific infections. They are ineffective if disease is caused by other *Theileria* species or in cases of mixed infections. Parasite escape mechanisms are not well understood and studies focusing on antigenic diversity using parasite surface genes or immunodominant genes can be helpful in understanding the disease mechanism. Epidemiological and phylogenetic studies have reported 8 different genetic types of *T. orientalis* parasites in the field on basis of major piroplasm surface protein (MPSP) gene polymorphism (Kim et al., 2004; Ota et al., 2009; Jeong et al., 2010). MPSP gene is expressed abundantly on the surface of the piroplasm inside the infected erythrocyte and has proven to be a good marker for phylogenetic and diversity studies (Kim et al., 2004; Ota et al., 2009; Altangerel et al., 2011; Kamau et al., 2011; Yokoyama et al., 2011; Aparna et al., 2013; Sivakumar et al., 2013, 2014; Bawm et al., 2014).

Surface gene play an important role in host parasite interaction and evolve under different evolutionary conditions or pressure (Shirakata et al., 1989; Onuma et al., 1998). This current study was intended to investigate the prevalence, genetic diversity and identification of different types of *T. orientalis* parasites in India using MPSP. In total, 862 bovine blood samples were collected randomly from 15 different districts belonging to states of Andhra Pradesh and Telangana, India from a period of year 2013 to 2015. This is a first study in India focused on understanding epidemiological and phylogenetic basis of *T. orientalis* infections which has led to the identification of parasite belonging to unique type of group based on MPSP sequencing. It has further highlighted antigenic polymorphism within Indian strains which will be helpful in designing control measures.

2. Materials and methods

2.1. Sample collection

Random blood samples of bovine population were collected from the major districts of Telangana and Andhra Pradesh (Fig. 1). Blood was drawn from jugular vein by a trained veterinarian into BD vacuum

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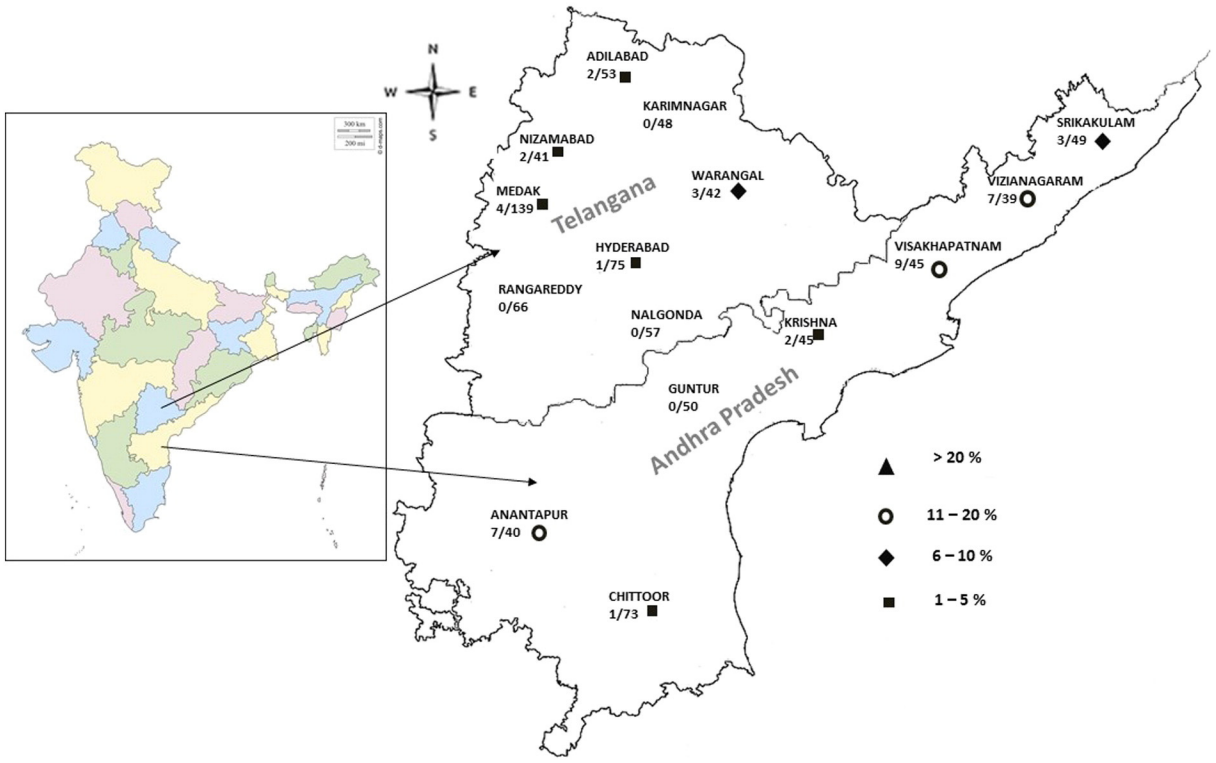

Fig. 1. A map of India showing the states of Andhra Pradesh and Telangana. On the map of India, Telangana and Andhra Pradesh were highlighted showing different districts (n = 15) from where samples were collected. Below each district name, the number of positive samples over total numbers is indicated. Circles indicate the prevalence (%) of *T. orientalis* parasites in different districts of two states. The sketch of India's map was obtained from @ d-maps.com.

tubes with EDTA. A total of 862 blood samples were collected from both cross breed (n = 504) and native breed (n = 358) animals (Table 1). Approximately 5 mL of blood was collected from each animal and stored at 4 °C. DNA was extracted within 24 h of blood collection using Qiagen kit as per manufacturer's instructions. DNA was quantified using Nanodrop (Thermo Scientific) instrument and freezed at −80 °C.

2.2. PCR amplification

Extracted DNA underwent PCR amplification of *T. orientalis* MPSP gene primers (Forward primer — 5′ CTTTGCCTAGGATACTTCCT 3′ and Reverse primer — 5′ ACGGCAAGTGGTGAGAACT 3′) which gave an amplified product of 776 base pairs (bp) as described elsewhere (Ota et al., 2009). PCR was done with SpeedStar *Taq* DNA polymerase (Clonetech, Takara) using thermocycler (BIO-RAD T100 Thermal cycler). PCR conditions were: initial denaturation at 95 °C for 1 min, followed by denaturation at 95 °C, for 10 s; annealing at 61 °C for 20 s; and extension at 72 °C for 10 s for 35 cycles with final extension at 72 °C for 1 min. The PCR products were confirmed by electrophoresis on 1.5% agarose gel, visualized under UV light using Gel Documentation system (G:BOX, SYNGENE) with 1 Kb DNA ladder (Thermoscientific) as reference.

Table 1
List of samples, place, breed of animals and prevalence district wise in two states of Telangana and Andhra Pradesh identified by specific PCR.

S. no.	Districts	Sample number	Breed (no. of samples)	Prevalence (%)
1	Anantapur	40	CB (18),IN (22)	17.5
2	Chittoor	73	CB (54),IN (19)	1.4
3	Guntur	50	CB (16),IN (34)	0.0
4	Krishna	45	CB (01),IN (44)	4.4
5	Srikakulam	49	CB (43),IN (06)	6.1
6	Visakhapatnam	45	CB (43),IN (02)	20.0
7	Vizianagaram	39	CB (23),IN (16)	17.9
8	Adilabad	53	CB (02),IN (51)	3.8
9	Hyderabad	75	CB (65),IN (10)	1.3
10	Karimnagar	48	CB (22),IN (26)	0.0
11	Medak	139	CB (110),IN (29)	2.9
12	Nalgonda	57	CB (23),IN (34)	0.0
13	Nizamabad	41	CB (24),IN (17)	4.9
14	Rangareddy	66	CB (58),IN (08)	0.0
15	Warangal	42	CB (02),IN (40)	7.1

Footnote: CB represents Cross breed animals which includes Jersey and Holstein Friesian cattle. IN represents Indian native breed animals. The digits in parentheses represent the number of animals.

2.3. Cloning and sequencing of MPSP gene

One representative positive PCR sample from each district (n = 15) was selected for cloning MPSP gene and sequence analysis. Amplified PCR products were purified by Nucleospin Gel and PCR cleanup kit (Macherey Nagel, Germany) following the manufacturer's instructions. After purification, the MPSP gene products were cloned into a TOPO cloning vector (Invitrogen, Life Technologies) followed by transformation into Top 10 cells with ampicillin as a marker. Positive colonies were selected by PCR and restriction digestion with *Eco*R1 enzyme followed by plasmid isolation using Nucleospin plasmid kit (Macherey Nagel, Germany). Sequencing of the positive clones (minimum five clones per sample) was performed using universal primers of M13 gene present on the backbone of the TOPO vector. Sequencing services were outsourced to Bioserve Biotechnologies Pvt Ltd, Hyderabad, India. The nucleotide sequences were applied to BLAST: Basic Local Alignment Search Tool available with the National Center for Biotechnology Information (NCBI) database for the homology analysis against known MPSP gene.

2.4. Bioinformatics analysis

Multiple alignment was done using ClustalW alignment method on MegAlign program (DNASTAR) using MPSP gene sequences based on Ota et al. (2009) and Jeong et al. (2010). Antigenic diversity and