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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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1567-1348/

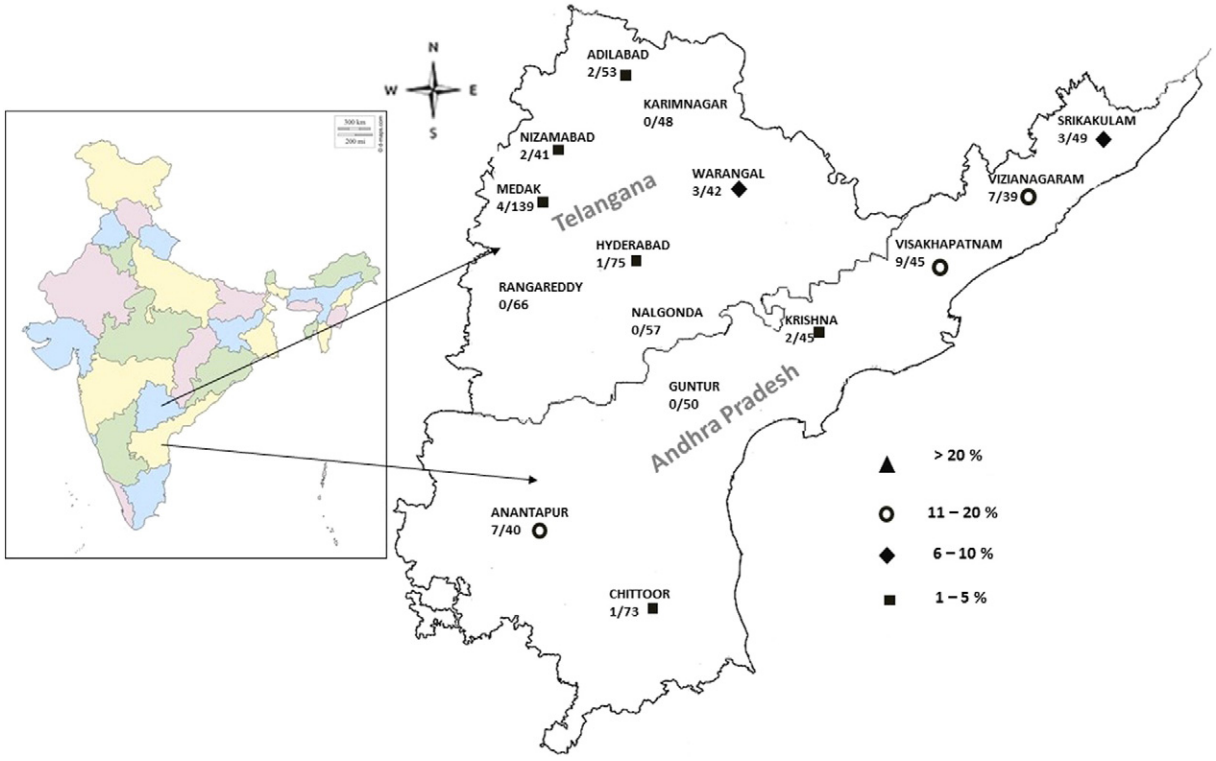

Fig. 1. A map of India showing the states of Andhra Pradesh and Telangana. On the map of India, Telangana and Andhra Pradesh were highlighted showing different districts (n = 15) from where samples were collected. Below each district name, the number of positive samples over total numbers is indicated. 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

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TOM12           CTTTGCCTAGGATACTTCCTTATCTTCGCTGCAGCTGCAGAGGAGAAAAAAGAGCCAGCT
TOM14           CTTTGCCTAGGATACTTCCTTATCTTCGCTGCAGCTGCAGAGGAGAAAAAAGAGCCAGCT
TOM5            CTTTGCCTAGGATACTTCCTTATCTTCGCTGCAGCTGCAGAGGAGAAAAAAGAGCCAGCT
TOM16           CTTTGCCTAGGATACTTCCTTATCTTCGCTGCAGCTGCAGAGGAGAAAAAAGAGCCAGCT
TOM2            CTTTGCCTAGGATACTTCCTTATCTTCGCTGCAGCTGCAGAGGAGAAAAAAGAGCCAGCT
TOM6            CTTTGCCTAGGATACTTCCTTATCTTCGCTGCAGCTGCAGAGGAGAAAAAAGAGCCAGCT
TOM4            CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAAGATNGCA
TOM1            CTTTGCCTAGGATACTTA---AACG-CTCTGCAACTGCAGAGGA-AAAAAA-AGATG-CA
TOM10           CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAG-AGGAAAAAAAAGATNGCA
TOM9            CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM17           CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM15           CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM11           CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM18           CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM3            CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM13           CTTTGCCTAGGATACTTCCTTATCGTCTCTGCAACTGCAGAGAGGAAAAAAAAGATNGCA
TOM7            CTTTGCCTAGGATACTTCCTCATCGCCACTGCAACAGCAGAGGAGAAGAAAGAACCAGCA
TOM8            CTTTGCCTAGGATACTTCCTCATCGCCACTGCAACAGCAGAGGAGAAGAAAGAACCAGCA
                **********************    * *  * ***** * ******    * *** *     *

TOM12           AAGGCTGAAGAGAAGAAGGATTTAGCTCTTGAAGTTAATGCTACCCAGGCTGAAAATGTT
TOM14           AAGGCTGAAGAGAAGAAGGATTTAGCTCTTGAAGTTAATGCTACCCAGGCTGAAAATGTT
TOM5            AAGGCTGAAGAGAAGAAGGATTTAGCTCTTGAAGTTAATGCTACCCAGGCTGAAAATGTT
TOM16           AAGGCTGAAGAGAAGAAGGATTTAGCTCTTGAAGTTAATGCTACCCAGGCTGAAAATGTT
TOM2            AAGGCTGAAGAGAAGAAGGATTTAGCTCTTGAAGTTAATGCTACCCAGGCTGAAAATGTT
TOM6            AAGGCTGAAGAGAAGAAGGATTTAGCTCTTGAAGTTAATGCTACCCAGGCTGAAAATGTT
TOM4            AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM1            AAGGCTGAAGAAAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM10           AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM9            AAGGCTGACGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM17           AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM15           AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM11           AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM18           AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM3            AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM13           AAGGCTGAAGAGAAGAAAGATTTAACTCTCGAAGTTAACGCCACCCAAGCCGAACATGTT
TOM7            AAGGCAGAAGAGAAGAAAGACTTAACTCTTGAAGTTAACGCAACTGCTTCTGAACATTTT
TOM8            AAGGCAGAAGAGAAGAAAGACTTAACTCTTGAAGTTAACGCAACTGCTTCTGAACATTTT
                ***** ** ** ***** ** *** **** ******** ** **     * *** ** **

TOM12           ACAGTCAATGCAACAAAACCCGATGACGTCGTTTTTACTGCTAATGATGGATATCGCTTC
TOM14           ACAGTCAATGCAACAAAACCCGATGACGTCGTTTTTACTGCTAATGATGGATATCGCTTC
TOM5            ACAGTCAATGCAACAAAACCCGATGACGTCGTTTTTACTGCTAATGATGGATATCGTTTC
TOM16           ACAGTCAATGCAACAAAACCCGATGACGTCGTTTTTACTGCTAATGATGGATATCGTTTC
TOM2            ACAGTCAATGCAACAAAACCCGATGACGTCGTTTTTACTGCTAATGATGGATATCGTTTC
TOM6            ACAGTCAATGCAACAAAACCCGATGACGTCGTTTTTACTGCTAATGATGGATATCGCTTC
TOM4            ACAGTCAGCGCAACCAACGCCAACGACGTCGTTTTTACTGCCGAGGATGGATACCGCTTC
TOM1            ACAGTCAACGCAACCAACGCCAACGACGTCGTTTTTACTGTTGAGGACGGATACCGCTTC
TOM10           ACAGTCAACGCAACCAACGCCAACGACGTCGTTTTTACTGTTGAGGACGGATACCGCTTC
TOM9            ACAGTCAACGCAACCAACGCCAACGACGTCGTTTTTACTGTTGAGGACGGATACCGCTTC
TOM17           ACAGTCAACGCAACCAACGCCAACGACGTCGTTTTTACTGTTGAGGACGGATACCGCTTC
TOM15           ACAGTCAACGCAACCAACGCCAACGACGTCGTTTTTACTGTCGAGGATGGATACCGCTTC
TOM11           ACAGTCAGCGCAACCAACGCCAACGACGTCGTTTTTACTGCCGAGGATGGATACCGCTTC
TOM18           ACAGTCAGCGCAACCAACGCCAACGACGTCGTTTTTACTGCCGAGGATGGATACCGCTTC
TOM3            ACAGTCAGCGCAACCAACGCCAACGACGTCGTTTTTACTGCCGAGGATGGATACCGCCTC
TOM13           ACAGTCAGCGCAACCAACGCCAACGACGTCGTTTTTACTGCCGAGGATGGATACCGCTTC
TOM7            AAAGTCAACGCAACCAACTCTAATGACGTCGTTTTTACTGCTGAGGAGGGATACCGTATT
TOM8            AAAGTCAACGCAACCAACTCTAATGACGTCGTTTTTACTGCTGAGGAGGGATACCGTATT
                * *****  ***** **  *  * ****************   * ** ***** **  *

TOM12           AAGACTCTCAAAGTTGGAGATAAAACATTGTATACCGTTGATACAACCAAATTCACTCCA
TOM14           AAGACTCTCAAAGTTGGAGATAAAACATTGTATACCGTTGATACAGCCAAATTCACTCCA
TOM5            AAGACTCTCAAAGTTGGAGATAAAACATTGTATACCGTTGATACATCCAAATTCACTCCA
TOM16           AAGACTCTCAAAGTTGGAGATAAAACATTGTATACCGTTGATACATCCAAATTCACTCCA
TOM2            AAGACTCTCAAAGTTGGAGATAAAACATTGTATACCGTTGATACATCCAAATTCACTCCA
TOM6            AAGACTCTCAAAGTTGGAGAAAAAACATTGTATACCGTTGATACAACCAAATTCACTCCA
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Fig. 2. Multiple alignment of MPSP gene sequence. Comparison of the MPSP nucleotide sequence of TOM 1, TOM 2, TOM 3, TOM 4, TOM 5, TOM 6, TOM 7, TOM 8, TOM 9, TOM 10, TOM 11, TOM 12, TOM 13, TOM 14, TOM 15, TOM 16, TOM 17, and TOM 18 collected from random animals from 15 different districts. TOM 1, 2, 3 belongs to Anantpur, TOM 4 from Chittoor, TOM 5 from Hyderabad, TOM 6 from Warangal, TOM 7 and 8 from Krishna, TOM 9 and 10 from Medak, TOM 11 from Nizamabad, TOM 12 from Srikakulam, TOM 13, 14 and 15 from Visakhapatnam, TOM 16 and 17 from Vizianagaram and TOM 18 from Adilabad, DNA were isolated from the bovine animals from the respective districts. Sequences were aligned using ClustalW-alignment program. Box represents residues that differ from the consensus sequence.

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TOM4            AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM1            AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM10           AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM9            AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM17           AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM15           AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM11           AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM18           AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM3            AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM13           AAGACTCTCAAGGTTGGAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCA
TOM7            AAGACTCTCAAGGTCGGAGACAAGAATTTGTATACTGTTGATACATCCAAATTCACTCCA
TOM8            AAGACTCTCAAGGTCGGAGACAAGAATTTGTATACTGTTGATACATCCAAATTCACTCCA
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TOM12           ACAGTCGCCCACAGACTTAAGCATGGTGATNGCATTGTTCTTCAAGCTTGACCTTTCTCC
TOM14           ACAGTCGCCCACAGACTTAAGCATGGTGATNGCATTGTTCTTCAAGCTTGAACTTTCTCC
TOM5            ACAGTCGCCCACAGACTTAAGCATGGTGATNGCATTGTTCTTCAAGCTTGACCTTTCTCA
TOM16           ACAGTCGCCCACAGACTTAAGCATGGTGATNGCATTGTTCTTCAAGCTTGACCTTTCTCA
TOM2            ACAGTCGCCCACAGACTTAAGCATGGTGATNGCATTGTTCTTCAAGCTTGACCTTTCTCA
TOM6            ACAGTCGCCCACAGACTTAAGCATGGTGATNGCATTGTTCTTCAAGCTTGACCTTTCCCA
TOM4            ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTTGACCTTTCTCC
TOM1            ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM10           ACTGTCGCCCACAGACTGAAGCATGATGATNGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM9            ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM17           ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM15           ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM11           ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM18           ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM3            ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM13           ACTGTCGCCCACAGACTGAAGCATGATGANAGACCTGTTCTTCAAGCTCAACCTGTCCCA
TOM7            ACAGTTGCCCACAGACTGAAGCACGCTGATNGACCTGTTCTTCAAGCTTGACCTTTCTCA
TOM8            ACAGTTGCCCACAGACTGAAGCACGCTGATNGACCTGTTCTTCAAGCTTGACCTTTCTCA
                ** ** *********** ***** * ***  *   ************  * ** ** * *

TOM12           TGCCAAGCCACTTTTGTTCAAAATGAAGTCGGACAAGGAATGGGTTCAGTTTGGATATGC
TOM14           TGCCAAGCCACTTTTGTTCAAAATGAAGTCGGACAAGGAATGGGTTCAGTTTGGATATGC
TOM5            TGCCAAGCCACTCTTGTTCAAAATGAAGTCGGACAAGGAATGGGTTCAGTTTGGATATGC
TOM16           TGCCAAGCCACTCTTGTTCAAAATGAAGTCGGACAAGGAATGGGTTCAGTTTGGATATGC
TOM2            TGCCAAGCCACTCTTGTTCAAAATGAAGTCGGACAAGGAATGGGTTCAGTTTGGATATGC
TOM6            TGCCAAGCCACTCTTGTTCAAGAAGAAGACTGACAAGGATTGGGTTCAGTTTGGATATGC
TOM4            TGCCAAGCCGCTTTTGTTCAAAATGAAGTCGGACAAGGAATGGGTTCAGTTTGGATATGC
TOM1            CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM10           CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM9            CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM17           CGCCAAGCCCCTTCTGTTCAAGAGGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM15           CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM11           CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM18           CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM3            CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM13           CGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTTAACTATGC
TOM7            TGCTAAGCCATTGTTGTTCAAGAAGAAGACCGACAAGGATTGGGCACAGTTCAGCTATGC
TOM8            TGCTAAGCCATTGTTGTTCAAGAAGAAGACCGACAAGGATTGGGCACAGTTCAGCTATGC
                 ** *****  *  ******* * ****   ******** ****  *****    *****

TOM12           ACAGTATCTCGATGAAGTAGTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM14           ACAGTATCTCGATGAAGTACTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM5            ACAGTATCTCGATGAAGTACTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM16           ACAGTATCTCGATGAAGTACTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM2            ACAGTATCTCGATGAAGTACTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM6            ACAGTATCTCGATGAAGTACTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM4            ACAGTATCTCGATGAAGTACTTTGGAAGGAGAAGAAGGAAACCAAGGATCTCGATGCATC
TOM1            CCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM10           CCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM9            CCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM17           CCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM15           CCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTC
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Fig. 2 (*continued*).

percentage similarity within the MPSP gene sequences from different districts were studied using MegAlign program (DNASTAR). Phylogenetic tree was made using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.58485429 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)

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TOM11           CCAGTACCTAGAGGATGTCCTATGGAAAGGGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM18           CCAGTACCTAGAGGATGTCCTATGGAAAGGGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM3            CCAGTACCTAGAGGATGTCCTATGGAAAGAGGAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM13           CCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTC
TOM7            TCAGTACCTTGAGGATGTTCTCTGGAAGGAAAAGAAAGAGCTCAAAGATCTCGATGCATC
TOM8            TCAGTACCTTGAGGATGTTCTCTGGAAGGAAAAGAAAGAGCTCAAAGATCTCGATGCATC
                 ***** ** ** ** **  * ***** *   **** **    ** ** *******  **
TOM12           CAAGTTTGCAGAGGCAGGTCTTTTTGCTGCTGATGCCTTCGGTACTGGTAAGGTTTATGA
TOM14           CAAGTTTGCAGAGGCAGGTCTTTTTGCTGCTGAGGCCTTCGGTACTGGTAAGGTTTATGA
TOM5            CAAGTTTGCAGAGGCAGGTCTTTTTGCTGCTGATGCCTTCGGTACTGGTAAGGTTTATGA
TOM16           CAAGTTTGCAGAGGCAGGTCTTTTTGCTGCTGAAGCCTTCGGTACTGGTAAGGTTTATGA
TOM2            CAAGTTTGCAGAGGCAGGTCTTTTTGCTTCTGAAGCCTTCGGTACTGGTAAGGTTTATGA
TOM6            CAAGTTTGCAGAGGCAGGTCTTTTTGCTGCTGATGCCTTCGGTACTGGTAAGGTTTATGA
TOM4            CAAGTTTGCAGAGGCAGGTCTTTTTGCTGCTGAGGCCTTCGGTACTGGTAAGGTTTATGA
TOM1            GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM10           GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM9            GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM17           GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM15           GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM11           GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM18           GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM3            GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM13           GAAGTTCTCAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAGGTGCACGA
TOM7            CAAGTATACAGAGGCTGGTCTTTTTGCACCTGACACTTTTGGTACTGGAAAGGTATATGA
TOM8            CAAGTATACAGAGGCTGGTCTTTTTGCACCTGACACTTTTGGTACTGGAAAGGTATATGA
                 ****   **** ** ******** **  ****  * ** ***** ** *****  * **
TOM12           CTTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATTC
TOM14           CTTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATTC
TOM5            CCTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATCC
TOM16           CTTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATCC
TOM2            CTTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATCN
TOM6            CTTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATTC
TOM4            CTTCGTCGGACCATTCAAAATCCAGNAAGTCAAATTCGAAAATCTGGATGTCGGTGATTC
TOM1            CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAGTTTGAGGAGAAGGTAGTCAAAGATGN
TOM10           CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAGTTTGAGGAGAAGGTAGTCAAAGATGN
TOM9            CTTCGTAGGAAACTTCAGGGTCANGCAAGTCAAGTTTGAGGAGAAGGTAGTCAAAGATGN
TOM17           CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAGTTTGAGGAGAAGGTAGTCAAAGATGN
TOM15           CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAGTTTGAGGAGAAGGTAGTCAAAGATGN
TOM11           CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAATTTGAGGAGAAGGTAGTCAAAGATGN
TOM18           CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAATTTGAGGAGAAGGTAGTCAAAGATGN
TOM3            CTTCGTAGGAAACTTCAAGGTCANGCAAGTCAAGTTTGAGGATAAGGTAGTCAAAGATGN
TOM13           CTTCGTAGGAAACTTCAAGGTCACCAAGGTCAAGTTCGAGGATAAGGAAGTCGGACATCC
TOM7            TTTTGTTGGAAACTTCAAGGTCAAGNAAGTAAAGTTCGAGGATAAGGATGTAGGAAAGCC
TOM8            TTTTGTTGGAAACTTCAAGGTCAAGNAAGTAAAGTTCGAGGATAAGGATGTAGGAAAGCC
                  * ** ***   ****   **    * ** ** ** **  *   **  **     *
TOM12           TAAAAAGGCTAAATACACCGCAGTCAAAGTATATGTCGGTACCGATGATAAGAAGATTGT
TOM14           TAAAAAGGCTAAATACACCGCAGTCAAAGTATATGTCGGTACCGATGATAAGAAGATTGT
TOM5            TAAAAAGGCTAAATACACCGCAGTCAAAGTATATGTCGGTACCGATGATAAGAAGATTGT
TOM16           TAAAAAGGCTAAATACACCGCAGTCAAAGTATATGTCGGTACCGATGATAAGAAGATTGT
TOM2            ATAAAAGGCTAAATACACCGCAGTCAAAGTATATGTCGGTACCGATGATAAGAAGATTGT
TOM6            TAAAAAGGCTAAATACACCGCAGTCAAAGTATATGTCGGTACCGATGATAAGAAGATTGT
TOM4            TAAAAACGCTAAATACACCGCAGTCAAAGTATATGTCGGTACTGATGATAAGAAGATTGT
TOM1            AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM10           AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM9            AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM17           AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM15           AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM11           AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM18           AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM3            AATCAAGGCCAAATACACCGCTGTCAAAGTTTACGTCGGTACCGATGACAAGAAGGTCGT
TOM13           AAAGAAGGCCAAATACACCGCAGTCAAAGTTTACGTCGGTACCGATGACAAGAAAATCGT
TOM7            AGATAATGCCAAATACACTGCTGTCAAAGTTTATGTCGGTTCTGATGATAAGAAAGTCGT
TOM8            AGATAATGCCAAATACACTGCTGTCAAAGTTTATGTCGGTTCTGATGATAAGAAAGTCGT
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Fig. 2 (*continued*).

were shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 65 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 604 positions in the final dataset. Evolutionary analyses were conducted using MEGA6 (Tamura et al., 2013).

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                    ** ** ******** ** ******** ** ****** * ***** *****  * **
TOM12           AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAAGAGGTTTACTTTAAATTGGT
TOM14           AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAAGAGGTTTACTTCAAATTGGT
TOM5            AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAAGAGGTTTACTTCAAATTGGT
TOM16           AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAAGAGGTTTACTTCAAATTGGT
TOM2            AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAAGAGGTTTACTTCAAATTGGT
TOM6            AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAAGAGGTTTACTTCAAATTGGT
TOM4            AAGACTCGACTACTTCTATACCCGTGATGAGAGATTCAAAGAGGTTTACTCCAAATTGGT
TOM1            AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM10           AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM9            AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM17           AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM15           AAGACTGGACTACTTTTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM11           AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM18           AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM3            AAGACTGGACTACTTCTACACGGGTGACGAGAGGTTCAAGGAAGTATACTTCAAATTGGT
TOM13           AAGACTCGACTACTTCTATACCGGTGATGAGAGATTCAAGGAAGTATACTTCAAATTGAT
TOM7            GAGACTTGACTACTTTTATACCAAGGACGAAAGATTCAAGGAAGTTTACTTCAAATTAGT
TOM8            GAGACTTGACTACTTTTATACCAAGGACGAAAGATTCAAGGAAGTTTACTTCAAATTAGT
                 ***** ******** ** **    ** ** ** ***** ** ** **** *****  *
TOM12           TGACGGAAAATGGAAGAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM14           TGATGGAAAATGGAAGAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM5            TGATGGAAAATGGAAGAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM16           TGATGGAAAATGGAAGAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM2            TGATGGAAAATGGAAGAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM6            TGATGGAAAATGGAAGAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM4            TGATGGAAAATGGAAGAAGCTTGAGCAGAGTGAGGCAAACAAGGATTTGCACGCTATGAA
TOM1            CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM10           CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM9            CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM17           CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM15           CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM11           CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM18           CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM3            CGATGGAAAATGGAAGAAGGTCGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM13           CGATGGAAAGTGGAAAAAGCTTGAGCAGAGCGAGGCAAACAAGGATTTGCACGCTATGAA
TOM7            TGACGGAAAGTGGAAAAAGTTGGAACAGACTGAGGCAAACAAGGACTTACATGCCATGAA
TOM8            TGACGGAAAGTGGAAAAAGTTGGAACAGACTGAGGCAAACAAGGACTTACATGCCATGAA
                 ** ***** ***** *** * ** ****  ************** ** ** ** *****
TOM12           CGATGCTTGGTCTTTGGAATACAAACCTCTCGTCGACAAGTTCTC
TOM14           CGATGCTTGGTCTTTGGAATACAAACCTCTCGTCGACAAGTTCTC
TOM5            CGATGCTTGGTCTTTGGAATACAAACCTCTCGTCGACAAGTTCTC
TOM16           CGATGCTTGGTCTTTGGACTACAAACCTCTCGTCGACAAGTTCTC
TOM2            CGATGCTTGGTCTTTGGACTACAAACCTCTCGTCGACAAGTTCTC
TOM6            CGATGCTTGGTCTTTGGAATACAAACCTCTCGTCGACAAGTTCTC
TOM4            CGATGGATGGTCTTTGGACTACAAACCTCTTGTCGACAAGTTCTC
TOM1            CAGTGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM10           CAGTGCTTGACCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM9            CAGTGCTTGGCCTTCGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM17           CAGTGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM15           CAGTGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM11           CAGTGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM18           CAGTGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM3            CAGTGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM13           CAATGCTTGGCCTTTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM7            CACAGCATGGCCTCTGGACTACAAGCCTCTTGTCGACAAGTTCTC
TOM8            CACAGCATGGCCTCTGGACTACAAGCCTCTTGTCGACAAGTTCTC
                *   *  **  **  *** ***** ***** **************
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Fig. 2 (*continued*).

3. Results

3.1. Molecular prevalence of T. orientalis infection using MPSP gene based PCR

DNA extracted from blood samples of bovines (n = 862) from 15 different districts of the states, Andhra Pradesh and Telangana, India were screened for the presence of *T. orientalis* parasites using MPSP gene based PCR amplification. A total of 41 blood samples out of 862 were positive for *T. orientalis*, with an individual prevalence of 8.5% (29/341) and 2.3% (12/521) in Andhra Pradesh and Telangana, respectively. PCR result indicated presence of parasites in 11 districts, with a high incidence of infection in the districts of Anantpur (17.5%), Visakhapatnam (20%) and Vizianagaram (17.9%). In districts of Rangareddy, Nalgonda, Guntur and Karimnagar, amplification of MPSP gene was not observed (Fig. 1).

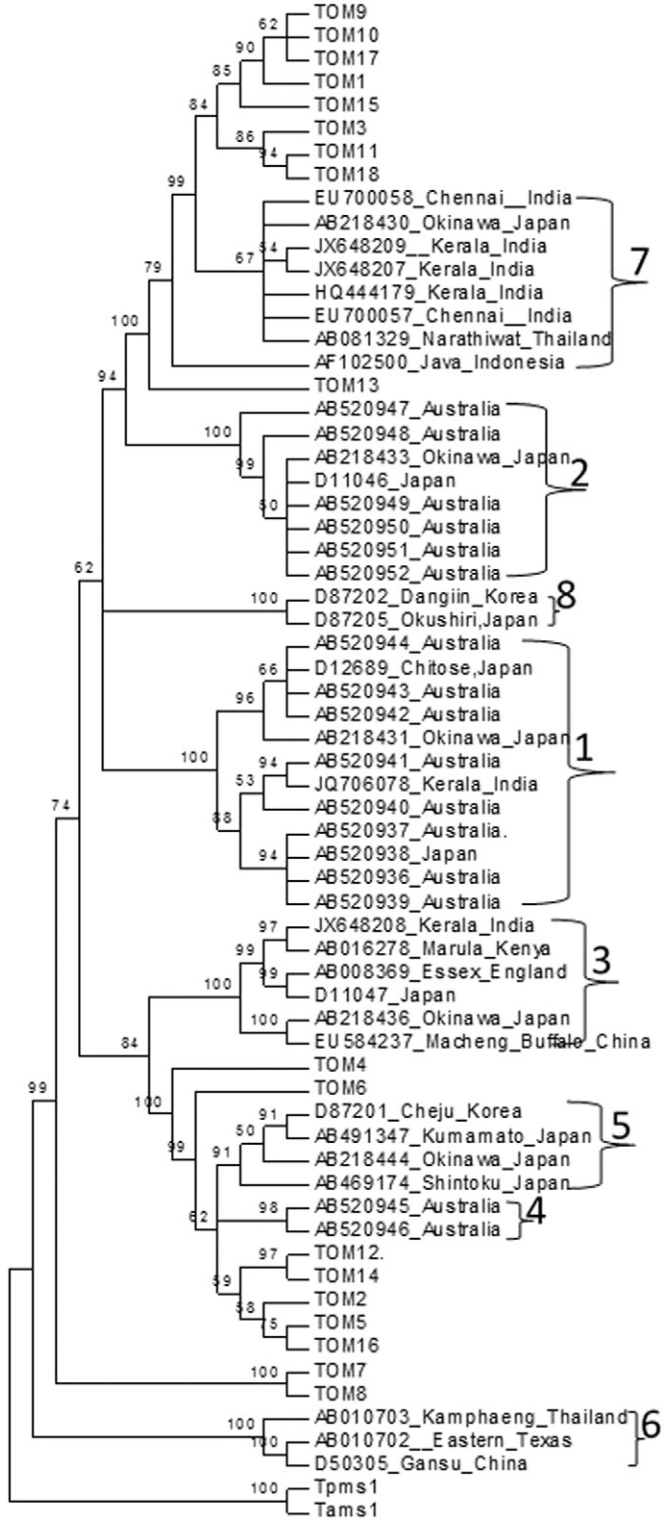

Fig. 3. Phylogenetic tree of the MPSP genes. Phylogenetic relationship among the nucleotide sequences of MPSP genes of samples collected from Andhra Pradesh and Telangana states (TOM 1, TOM 2, TOM 3, TOM 4, TOM 5, TOM 6, TOM 7, TOM 8, TOM 9, TOM 10, TOM 11, TOM 12, TOM 13, TOM 14, TOM 15, TOM 16, TOM 17, TOM 18), together with previously registered MPSP gene sequences available at NCBI database. *T. parva* and *T. annulata* served as outgroup. Numbers (1–8) represent different types of *T. orientalis* genotypes reported previously. TOM 1, 2, 3 belongs to Anantpur, TOM 4 from Chittoor, TOM 5 from Hyderabad, TOM 6 from Warangal, TOM 7 and 8 from Krishna, TOM 9 and 10 from Medak, TOM 11 from Nizamabad, TOM 12 from Srikakulam, TOM 13, 14 and 15 from Visakhapatnam, TOM 16 and 17 from Vizianagaram and TOM 18 from Adilabad, DNA were isolated from the bovine animals from the respective districts.

3.2. Genetic variation analysis of *T. orientalis* based on MPSP gene

DNA of parasite strains belonging to 11 districts were positive for MPSP gene. Further, minimum of one positive representative DNA sample from the 11 districts were cloned and sequenced to study genetic variation. Minimum five positive clones of each sample were sequenced using forward and reverse M13 universal primers and sequence analysis revealed no differences within the clones. Multiple alignment and divergence analysis of MPSP gene sequences revealed variable level of antigenic diversity ranging from 0.3 to 23.6% within the districts examined (Fig. 2). Maximum antigenic diversity was seen in isolates (TOM 7 and TOM 8) from Krishna district of Andhra Pradesh.

3.3. Phylogenetic analysis and genotype distribution

Phylogenetic analysis of all the MPSP gene sequences were compared with reference genotypes published previously, resulted in identification of two new and four known genotypes (type 2, 4, 5 and 7). The neighbor joining tree prepared from MPSP gene sequences suggested differences in the isolates of Andhra Pradesh and Telangana that formed a separate cluster from the previously reported Indian *T. orientalis* strains. Phylogenetic tree suggested presence of two new genotypes of parasite strains (TOM 7 and TOM 8) from Krishna district creating a separate clade in the tree. Strains from districts of Anantpur (TOM 1 and 3), Medak (TOM 9 and 10), Nizamabad (TOM 11), Visakhapatnam (TOM 15), Vizianagaram (TOM 17) and Adilabad (TOM 18) clustered near type 7 parasites but in a separate clade. Cases belonging to type 4 were found from districts of Anantapur (TOM 2), Hyderabad (TOM 5), Srikakulam (TOM 12), Visakhapatnam (TOM 14) and Vizianagaram (TOM16).

Further, type 2 strain also known as Ikeda group was found in Visakhapatnam district (TOM 13) and strains from Chittoor (TOM 4) and Warangal (TOM 6) districts falls under Type 5 (Fig. 3).

4. Discussion

This is the first study that revealed epidemiological, phylogenetic and antigenic diversity within the prevailing *T. orientalis* parasite population from the states of Andhra Pradesh and Telangana in India. Our data indicated the existence of type 2, type 4, type 5 and a new genotype belonging to *T. orientalis* parasite in India. Due to the scarcity of data concerning the prevalence and the incidence of other species causing theileriosis, most treatment and vaccination regimens were designed against *T. annulata*. Prevalence of *T. orientalis* parasites in the states of Andhra Pradesh and Telangana were evaluated with the help of MPSP gene based PCR. An overall prevalence rate of *T. orientalis* infection was 4.8% with a higher occurrence rate in Andhra Pradesh as compared to Telangana state.

Further, characterization of the *T. orientalis* parasites was done from each district by sequencing the MPSP gene. MPSP is thought to be involved in immune evasion due to its high polymorphism and location as a surface protein (Shirakata et al., 1989; Onuma et al., 1998). Phylogenetic analysis using MPSP gene sequence revealed that parasite strains identified were closer to type 7, type 5, type 4 and type 2 and also recognized a new genotype of *T. orientalis* parasites when compared with major 8 types of parasites reported from worldwide. Out of 8 genotypes of *T. orientalis* reported in Asia, reports from India have shown presence of 1, 3 and 7 types of parasites (Aparna et al., 2011, 2013) while a recent report has shown types 1, 3, 5, and 7 in Srilanka which is one of the close neighbors of India (Sivakumar et al., 2013). Other than parasite genotype strains earlier reported from India, we have identified type 2, type 4 and type 5 along with a new type (TOM 7 and TOM 8) from Krishna district in Andhra Pradesh using phylogenetic analysis. MPSP gene based genotypes, type 5 and 2 of *T. orientalis* have been reported in Japan, Australia and Korea causing outbreaks for theileriosis. The genotype type 2 also known as Ikeda type is considered to be most

pathogenic (Jeong et al., 2010; Ota et al., 2009; Kamau et al., 2011). Presence of the type 2 genotype and the new type of strain, demonstrate the need for ongoing epidemiological surveys to monitor the evolution or emergence of new strains. Further, an animal simultaneously infected with two types of strains can put stress on the host immune system by making it fight against different types of parasite at the same time. Despite of high amino acid similarity, type 1 and type 2 genotypes of *T. orientalis* parasite showed antigenic diversity (Kubota et al., 1995). Multiple alignment of MPSP gene revealed major differences between the isolates from Krishna district (with divergence rate of 23.5%) as compared to parasite strains from other districts (Fig. 2).

Our study not only provides evidence for the presence of *T. orientalis* parasites in the states of AP and Telangana, but, also sheds light on the new type and antigenic diversity of prevalent *T. orientalis* parasites in the field. Prevalence of *T. orientalis* in India provides challenge as well as opportunity to focus on designing vaccines and medicines for treating *T. orientalis* infections which otherwise could present serious problem in near future to livestock industry in India.

5. Conclusion

Our study has led to the identification of an altogether new genotype of *T. orientalis* parasite strains from India and as well as few genotypes (type 2, 4 and 5) which were not previously reported. It will be interesting to study how these strains have evolved in India. Further, our results emphasize on the need to study the prevalence and diversity of parasites within the *Theileria* genus beyond *T. annulata*. The results showing strains having high antigenic diversity can help to devise a better infection control policy, treatment regimen and vaccine development.

Competing interests

No.

Authors contribution

NG, DPR and VB carried out the experiments, PS and VB conceptualized the study, PS and VB contributed in writing the manuscript, DPR contributed in sample collection.

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