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Original Article

Molecular epidemiology, risk factors and hematological evaluation of asymptomatic *Theileria annulata* infected cattle in Odisha, India

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Abstract

Background: *Theileria annulata* is a tick-borne apicomplexan parasite that affects bovine and causes severe economic losses. **Aims:** Our study aimed to determine the molecular prevalence of *T. annulata* infection in asymptomatic carrier cattle in Odisha, India, to study the association of potential risk factors with theileriosis, and to investigate the effect of the parasite infection on hematological parameters in naturally affected animals. **Methods:** A total of 226 cattle blood samples were collected from seven districts of Odisha, India. Molecular diagnoses of tropical theileriosis by polymerase chain reaction (PCR), cloning, sequencing, and phylogenetic analysis of isolated parasites were performed. Potential risk factors were investigated by univariable and multivariable logistic regression statistical analysis. Hematological parameters were compared between positive and negative animals. **Results:** All animals included in our study were clinically normal, however, 54.86% (124/226) of examined animals were positive by PCR for *T. annulata*. The multivariable logistic regression showed that contact with other cattle from different herds during grazing (P<0.0001; OR: 12.75; 95% CI: 5.21-31.21), previous history of clinical signs (P=0.002; OR: 3.31; 95% CI: 1.53-6.31), and frequency of a ectoparasiticides application pre year (P<0.0001; OR: 9.22; 95% CI: 3.03-28.09) were the potential risk factors for the occurrence of tropical theileriosis. Nucleotide sequence identity data demonstrated that *T. annulata* strain (MN818858) Odisha shared homology of 99.6%, 99.49%, and 99.36% with Uttar Pradesh, India (MF346035), Bahrain (AF214797), and Hyderabad, India (MK034702), respectively. **Conclusion:** This is the first study to gain insight into the molecular epidemiology, risk factors, phylogeny, and hematological analysis of asymptomatic *T. annulata* infected cattle from India.

Key words: Cattle, Epidemiology, India, PCR, *Theileria annulata*

Introduction

Theileria species (Apicomplexa: Piroplasmida; Theileriidae) are vector born parasites that infect domestic ruminants worldwide (Dobbelaere and McKeever, 2002). *Theileria* is an intracellular protozoan parasite and its life cycle is completed in both vertebrate and invertebrate hosts (Sitotaw *et al*., 2014). Tropical theileriosis leads to severe economic losses in livestock production due to their morbidity and mortality rates (Jabbar *et al*., 2015). Clinical signs begin with high fever, swelling of the superficial lymph nodes, increased pulse rate, increased respiratory rate, anorexia, decreased milk production, constipation, followed by dark tarry diarrhea in severe cases along with hemoglobinuria and anemia (Radostitis *et al*., 2006; Oryan *et al*., 2013).

The diagnosis of the disease is mostly based on the examination of blood smears. The lymph node biopsy cannot be used in carrier animals and low parasitemia (Kundave *et al*., 2014). In contrast, the polymerase chain reaction (PCR) based assays were found to be the most effective tool for the detection of *Theileria* in clinically and subclinically infected animals (Eamens *et al*., 2013). The effective control of blood parasites needs rapid and high sensitive diagnostic tests, which can also examine the efficiency of therapeutic and prophylactic measures. Molecular techniques have been developed for accurate and rapid identification of *Theileria* spp. in animals having negative serological tests while can infect ticks (Ghaemi *et al*., 2012).

Animals recovered from an acute infection of tropical theileriosis become a persistent carrier, which plays a

significant role in the continuance of the parasite life cycle, especially in endemic regions (d'Oliveira *et al*., 1995; Sharifiyazdi *et al*., 2012). Our study is the first study in Odisha (India) to determine the molecular prevalence of *Theileria annulata* infection in asymptomatic carrier cattle, and to study the association of potential risk factors with theileriosis and to investigate the effect of theileriosis on hematological parameters in naturally affected animals.

Materials and Methods

Ethics statement

There is no precise law for blood sample collection and so no consent was obligatory. In this study 5 ml blood collected from the jugular vein under aseptic conditions from cattle after taking the permission of the farm owners.

Study areas and animals

An epidemiologic study was done in seven districts of Odisha (Cuttack, Ganjam, Jagatsinghapur, Kendrapada, Khorda, Nayagarh, and Puri), (Fig. 1) during the rainy seasons from June to September, 2019. Odisha is situated alongside the eastern coast of India between 18°2′ and 22°6′ N latitude and 82°8′ and 87°6′ E longitude. The weather of Odisha is hot and humid, which provides suitable conditions for developing tick-borne diseases in the state. Two-hundred and twenty-six cattle from 28 farms, with size varying from 5 to 20 animals, and the age range of 3 months to 13 years were included for the study. A questionnaire was prepared to collect data on age, breed, sex, contact with other cattle from different herds during grazing, tick infestation, lactation and pregnancy status, previous history of clinical signs, and frequency of ectoparasiticides application per year. According to the census of the Odisha state government, the number of cattle was 1300000. The total number of required animal samples was determined using a random sampling design on Win Episcope 2 program. The accepted error and the expected prevalence were set at 5.5% and 20.5% (Sahoo *et al*., 2017), respectively. The sample size dertermined by the program was 207 cattle which was then considered to 226 samples (Thrusfield *et al*., 2001).

Sample collection and hematological analysis

Blood samples were collected from the jugular vein of each animal in a vacutainer K_2 EDTA tube (B.D. Bioscience, Germany). Hematological parameters i.e. red blood cells (RBC) count, hemoglobin concentration (Hb), hematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (TLC), neutrophil, lymphocyte, monocyte, eosinophil, were measured using an automatic blood analyzer ADVIA 2120i Hematology System (Siemens, USA).

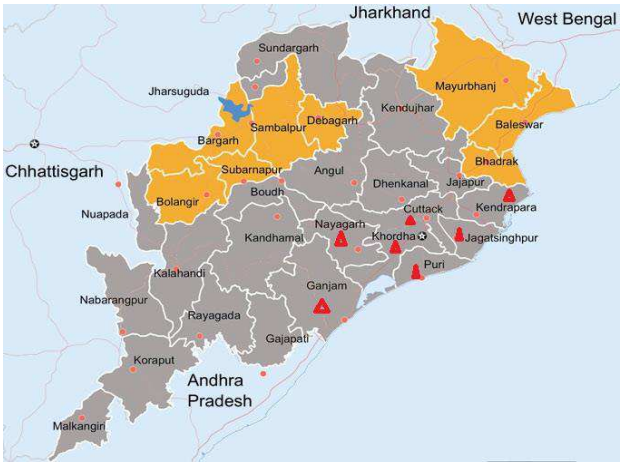

Fig. 1: Sample collection sites of Odisha indicated by red triangles

DNA isolation and amplification of the *Tams 1* gene

The DNA was extracted from the blood samples using the QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The quality and quantity of DNA were measured using a Nanodrop. Samples were stored at -80°C until further studies. The PCR amplification was done in a 25 µL reaction volume, containing 16.25 µL of nucleus free water, 2.5 µL of 10 × PCR Buffer, 2.0 µL of dNTPs, 1.0 µL of 10 pmol of each primer (Tams1-forward ATG CTG CAA ATG AGG AT-3´ and Tams1-reverse GGA CTG ATG AGA AGA CGA TGA G-3´), 1.25 U of Taq polymerase (Himedia, India) and 2.0 µL of DNA template. The PCR condition was performed using a thermal cycler (Bio-Rad, Hercules, CA, USA) that included an initial denaturation at 95°C for 5 min, 37 cycles of denaturing step at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 5 min, with a final hold at 4°C. A negative control containing nucleus-free water was used instead of template DNA. Positive control DNA isolates from previous work were used and confirmed by sequencing. The amplified sequence size was 783 bp fragment (Kirvar *et al*., 2000). The PCR products were visualized on 1.5% agarose gel stained with ethidium bromide under ultra violet (UV) light gel documentation system (Bio-Rad, Hercules, CA, USA).

Nucleotide sequencing and phylogenetic analysis of *Tams 1* gene

The amplified *Tams 1* gene from each positive sample was chosen and sequenced. In brief, purification of PCR amplicons was performed with an illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK), cloned into a TOPO® TA Cloning® kit vector (Invitrogen, USA), and then transformed into *Escherichia coli* DH5α competent cells supplemented with ampicillin. Positive colonies were examined by PCR and then digested with *Eco*R1 restriction enzyme (Thermofisher Scientific, USA). Plasmids from positive colonies were collected and purified using the GeneJET™ Plasmid Miniprep kit (Thermofisher

Scientific, USA). Two recombinant clones of each sample were selected and sequenced using the *M13* gene primers through Sanger sequencing by BigDye™ Terminator v3.1 Cycle Sequencing kit (Thermofisher Scientific, USA). The nucleotide sequence was compared with previously available *Tams 1* gene sequences of *T. annulata* parasites in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) followed by multiple alignments using ClustalW2. Our isolated nucleotide sequence was deposited in the NCBI GenBank database with the accession number MN818858. The phylogenetic tree was constructed based on a maximum likelihood phylogeny, Tamura 3-parameter model and 1000 bootstrap replicate using MEGA 7 (Tamura, 2013).

Statistical analysis

Statistical analysis was performed using SPSS 22 statistical software program windows version. Descriptive statistical analysis and the distribution of potential risk factors between the cases of tropical theileriosis were done. Logistic regression analysis was performed to check the relationship between the prevalence of infection and the potential risk factors. The univariable logistic regression statistics followed by the independent factors with significant P-value (P<0.1) were incorporated into the multivariable logistic regression analysis. For each variable, the results were P-value, confidence interval (CI: 95%), and odds ratio (OR). Results were considered to be significant at P<0.05 (Rizk *et al*., 2017).

Results

Amplification of *T. annulata* DNA revealed a product size of 783 bp, considered positive for *T. annulata* (Fig. 2). Out of the 226 cows incorporated in this study, *T. annulata* DNA was detected in 124 animals (54.86%); notably, Puri district was recognized as a relatively high-risk area for *T. annulata* infection, with a prevalence of 64.10% (Table 1).

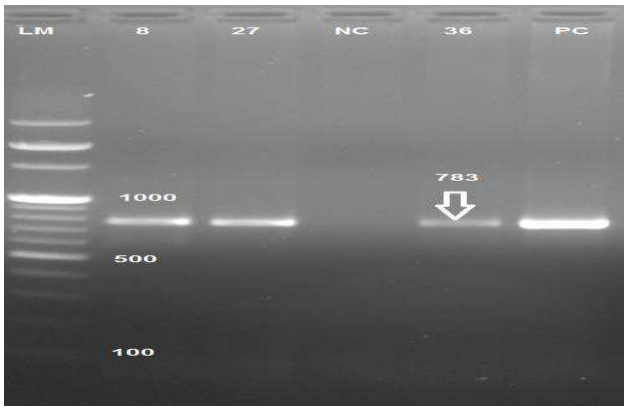

Fig. 2: PCR amplification of *Tams 1* gene of *T. annulata* showing an amplified target of 783 bp. Lanes with sample number 8, 27, and 36 are positive samples. LM: Ladder marker, NC: Negative control, and PC: Positive control

The univariable statistical analysis of epidemiological data (Table 2) revealed that the association of sex and breed with prevalence was statistically insignificant P>0.05. On the other hand, age, contact with other cattle from different herds during grazing, tick infestation, lactation and pregnancy status, previous history of clinical signs, and the frequency of ectoparasiticides application per year were the potential risk factors for tropical theileriosis. The highest prevalence (85.71%, P=0.024; OR: 6.00; 95% CI: 1.26-28.42) of infection was recorded in cattle above eight years of age, while the lowest prevalence (50%) of infection was observed in the animals catogorized in the age group of lower than 1-3 years old. Additionally, the higher prevalence (55.7%) was recorded in crossbred jersey cattle while the lowest prevalence (33.33%) was recorded in indigenous breeds. We found the highest prevalence (82.81%, P<0.0001; OR: 9.67; 95% CI: 4.33-21.6) in cattle contact with other cattle from different herds during grazing.

A statistically significant relationship between the existence of tick and the presence of infection was also revealed, the highest prevalence was in the group of animals suffering from tick infestation (62.66%, P=0.001; OR: 2.57; 95% CI: 1.46-4.53). Moreover, a relationship between the frequency of ectoparasiticides application per year and the prevalence of *Theileria* infection was observed. The highest occurrence of infection (70%, P<0.0001; OR: 6.06; 95% CI: 2.43-15.14) was reported in the group which had not used ectoparasiticides followed by (56.25%, P=0.005; OR: 3.34; 95% CI: 1.45-7.96) the group using ectoparasiticides once a year. The lowest prevalence (27.77%) was also shown in the group applying ectoparasiticides treatment 3 or 4 times per year (P=0.034). The higher prevalence was observed (68.75%, P<0.0001; OR: 2.73; 95% CI: 1.57-4.74) in animals with the previous history of the disease. Furthermore, lactation and pregnancy status was a potential risk factor of occurrence of bovine theileriosis. The higher prevalence observed in periparturient cows within two months of the postpartum period (65.38%, P=0.004; OR: 2.56; 95% CI: 1.36-4.83) followed by pregnant cows (50%) and non-pregnant cows (42.42%).

Multivariable logistic regression model (Table 3) showed that contact with other cattle from different herds during grazing, previous history of clinical signs, and frequency of ectoparasiticides application per year were the potential risk factors for the occurrence of tropical theileriosis. Contact with other cattle from different herds during grazing was significantly associated with a high prevalence of the infection (P<0.0001; OR: 12.75; 95% CI: 5.21-31.21). The previous history of clinical signs also revealed a significant association with a high prevalence of the infection (P=0.002; OR: 3.31; 95% CI: 1.53-6.31). Additionally, the higher prevalence was observed in the group which not used ectoparasiticides (P<0.0001; OR: 9.22; 95% CI: 3.03-28.09).

Hematological parameters were compared between negative and positive groups for *T. annulata* infection through *Tams 1* gene for which the amplification was

Table 1: Collection of blood samples from different districts of Odisha

SL. No.	District name	No. of blood samples	PCR positives	Prevalence (%)
1	Cuttack	22	9	40.90
2	Ganjam	16	8	50
3	Jagatsinghapur	14	6	42.85
4	Kendrapada	12	5	41.66
5	Khorda	68	40	58.82
6	Nayagarh	16	6	37.5
7	Puri	78	50	64.10
Total		226	124	54.86

SL: Serial number

Table 2: Univariable logistic regression model for the potential risk factors associated with tropical theileroisis in cattle

Variables	No. of examined animals	No. positive samples by PCR (%)	β	SE	OR	CI	P-value
Age	226	124 (54.86)					
Older cow (>8 years old)	14	12 (85.71)	1.79	0.79	6.00	1.26-28.42	0.024*
Adult (3-8 years old)	126	69 (54.76)	0.19	0.28	1.21	0.69-2.09	0.495
Young (<1-3 years old)	86	43 (50%)	-	-	-	-	-
Sex							
Female	215	118 (54.88)	0.14	0.64	1.01	0.30-3.39	0.98
Male	11	6 (54.54)	-	-	-	-	-
Breed							
Crossbred Jersey	210	117 (55.7)	0.923	0.87	2.516	0.45-14.03	0.29
Crossbred Holstein	10	5 (50)	0.692	1.07	2.0	0.24-16.36	0.51
Other native breeds	6	2 (33.33)	-	-	-	-	-
Contact with other cattle from different herds during grazing							
Yes	64	53 (82.81)	2.27	0.4	9.67	4.33-21.6	<0.0001*
No	162	71 (43.8)	-	-	-	-	-
Tick infestation							
Yes	150	94 (62.66)	0.9	0.28	2.57	1.46-4.53	0.001*
No	76	30 (39.47)	-	-	-	-	-
Previous history of clinical signs							
Yes	96	66 (68.75)	1.05	0.28	2.73	1.57-4.74	<0.0001*
No	130	58 (44.61)	-	-	-	-	-
Lactation and pregnancy periparturient	104	68 (65.38)	0.94	0.32	2.56	1.36-4.83	0.004*
Pregnant	56	28 (50)	0.30	0.36	1.35	0.66-2.77	0.4
Non-pregnant	66	28 (42.42)	-	-	-	-	-
Frequency of ectoparasiticides application per year							
Without treatment	60	42 (70)	1.8	0.64	6.06	2.43-15.14	<0.0001*
One	96	54 (56.25)	1.2	0.42	3.34	1.45-7.96	0.005*
Two	34	18 (52.9)	1.07	0.50	2.92	1.08-7.89	0.034*
Three or four	36	10 (27.77)	-	-	-	-	-

β: Regression coefficient, SE: Standard error, OR: Odds ratio, CI: Confidence interval at 95%, and P<0.05

Table 3: Multivariable logistic regression form for the potential risk factors associated with tropical theileroisis in cattle

Variable	β	SE	P-value	OR	CI
Contact with other cattle from different herds during grazing					
Yes	2.5	0.45	<0.0001*	12.75	5.21-31.21
No	-	-	-	-	-
Previous history of clinical signs					
Yes	1.13	0.36	0.002*	3.31	1.53-6.31
No	-	-	-	-	-
Frequency of ectoparasiticides application per year					
Without treatment	2.222	0.56	<0.0001*	9.22	3.03-28.09
One	1.52	052	0.004*	4.57	1.64-12.72
Two	0.88	0.62	0.15	2.42	0.71-8.26
Three or four	-	-	-	-	-

β: Regression coefficient, SE: Standard error, OR: Odds ratio, CI: Confidence interval at 95%, and P<0.05

Table 4: Results of hematological parameters in infected and non infected animals according to PCR amplification of *Tams 1* gene

Parameters	Cattle with *Tams 1* gene amplification (n=124)	Cattle without *Tams 1* gene amplification (n=102)	P-value
Hb (g/dl)	8.31 ± 2.61	10.35 ± 1.68	<0.001*
RBC (M/µL)	5.10 ± 1.72	6.31 ± 1.37	<0.001*
PCV (%)	24.30 ± 7.61	29.31 ± 5.24	<0.001*
MCV (fl)	49.27 ± 6.25	47.02 ± 4.67	0.005*
MCH (pg)	16.73 ± 2.18	16.79 ± 2.32	0.95
MCHC (g/dl)	34.13 ± 2.67	35.13 ± 2.33	0.001*
TLC (10^3/µL)	7.79 ± 3.35	8.496 ± 2.86	<0.001*
Neutrophils (10^3/µL)	3.98 ± 1.90	4.31 ± 1.51	<0.001*
Lymphocytes (10^3/µL)	5.66 ± 1.83	5.28 ± 1.45	<0.001*
Monocytes (10^3/µL)	0.12 ± 0.47	0.12 ± 0.60	0.112
Eosinophils (10^3/µL)	0.36 ± 0.30	0.23 ± 0.22	0.004*

Hb: Hemoglobin, RBC: Red blood cells, PCV: Packed cell volume, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, TLC: Total leukocyte count, and P<0.05

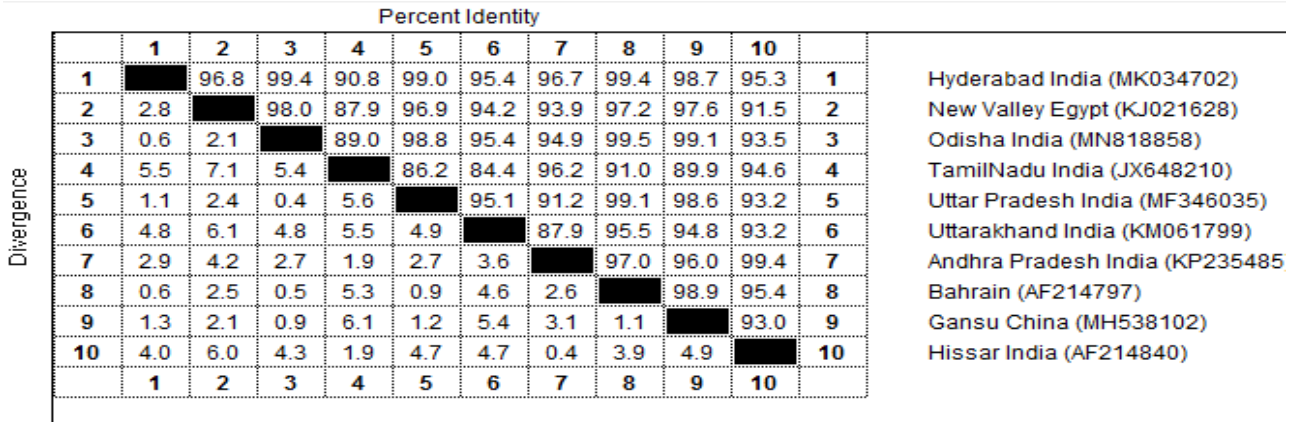

Fig. 3: *Tam 1* nucleotide difference estimation (lower left triangle) and percentage identity (upper right triangle) for *T. annulata* isolate from Odisha, India

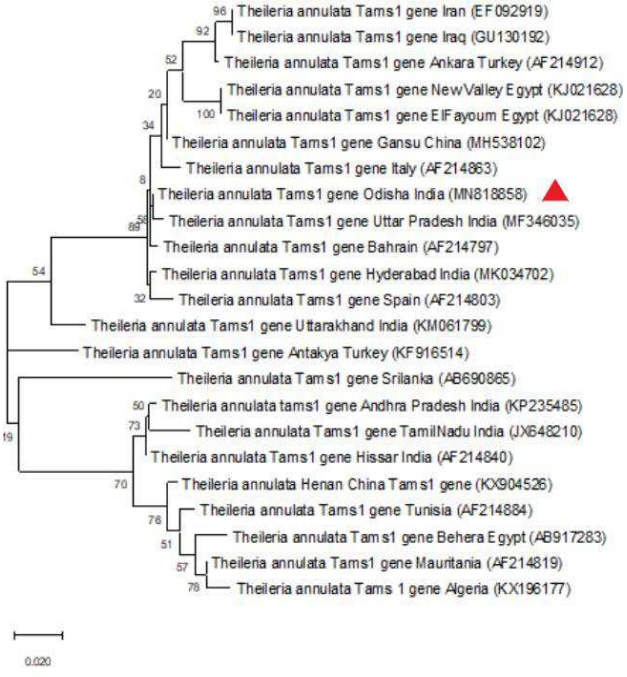

Fig. 4: Phylogenetic analysis of the partial nucleotide sequences (783 bp) of the *Tams 1* gene and other variants of *T. annulata* available in GenBank. Sequence isolated in Odisha is indicated with the red triangle

identified (Table 4). Although the mean values were within the normal range in both groups, the RBC, Hb, PCV, MCHC, TLC, and neutrophil values obtained in the *T. annulata* positive animals were significantly (P<0.05) lower than those found in the *T. annulata* negative group. In addition, the MCV, lymphocyte and eosinophil values were significantly (P<0.05) higher in infected animals.

A higher genetic divergence was observed between Odisha isolate and Hissar, India and Tamil Nadu isolates. The lower genetic diversity was between Odisha isolate and Uttar Pradesh, India and Bahrain isolates (Fig. 3). The amplicon size of the partial *Tams 1* gene of *T. annulata* revealed 783 bp. Analysis of the *Tams 1* gene partial nucleotide sequence of *T. annulata* (MN818858) through online BLAST showed a similarity of 99.6%, 99.49%, 99.36%, and 99.11% with Uttar Pradesh, India (MF346035), Bahrain (AF214797), Hyderabad, India (MK034702) and China (MH538102), respectively (Fig. 4).

Discussion

Tropical theileriosis causes severe economic loss because of high mortality, a decrease in milk production, decrease in growth rate and increase in treatment cost

(Brown, 1997). The scarcity of available information regarding the infection rate of tropical theileriosis results in the poor control measures. The present study recorded a high prevalence (54.86%) of tropical theileriosis in cattle of Odisha using the *Tams 1* gene PCR assay. The samples were randomly collected from cattle in seven different regions of Odisha. The highest prevalence was observed in Puri area (64.10%) attributed to its location adjacent to the Bay of Bengal with sweltering and humid agroclimatic conditions which is suitable for ticks proliferation (Kohli *et al*., 2014). Moreover, extensive cattle breeding and inadequate hygienic measures are responsible for higher prevalence in this district. The results of this research are similar to Gujarat, where 74/113 samples were identified as positive (Kundave *et al*., 2014), and to a study from Banaskantha showing a prevalence of 46.15% (Chauhan *et al*., 2015). This variation in prevalence is undoubtedly associated with the environmental changes and management measures on vectors.

Concerning the age of the animals, the higher prevalence was observed in animals older than eight years. This was consistent with the previous studies (Lydia *et al*., 2017; Rizk *et al*., 2017). Additionally, lower prevalence was observed in animals less than one year and this is in agreement with (Kala *et al*., 2018). This may be due to the recurrent infections of older animals several times during their life and the low tick-infestation of calves (Gharbi and Darghouth, 2014).

Contact with other cattle during grazing appeared to be a critical risk factor in the occurrence of tropical theileriosis. This agreed with a previous study (Lydia *et al*., 2017). Some farmers in Odisha let their animals free grazing causing more contact with other animals of different herds that can increases the prevalence of theileriosis. Furthermore, the higher prevalence was observed in periparturient cows (65.38%) within two months of the postpartum period followed by pregnant cows (50%) and non-pregnant cows (42.42%). This may be due to stress as a main reason of the disease occurrence; therefore, recently calved and pregnant cows are at a high-risk for the development of clinical signs (Watts *et al*., 2016).

Transmission of *T. annulata* occurs rapidly when specific tick vectors and infected animals are found together. In India, *Hyalomma anatolicum anatolicum* is the vector for *T. annulata* transmission (Ponnudurai *et al*., 2017). Elimination of tick with acaricides is the most usual method used for theileriosis control. The accurate tick control method can lead to a decrease in the prevalence of the disease; so, more treatments lead to less disease occurrence (Demeneghi *et al*., 2016). This is in agreement with our results, the herds which use ectoparasiticides treatment 3 or 4 times per year have the lowest infection rate of tropical theileriosis. In addition, the study of the previous history of clinical signs is very significant for the establishment of accurate and strict control measures. All animals included in our study were clinically normal; however, 54.86% (124/226) of examined animals were positive by PCR for *T. annulata*. This a prominent epidermiologically found in which asymptomatic carriers play a crucial role in transmission of the disease to susceptible animals, leading to severe economic losses (d'Oliveira *et al*., 1995). An earlier study made in Tunisia revealed that 50.8% of the estimated costs were due to tropical theileriosis (Gharbi *et al*., 2006).

The hematological parameters of cattle affected clinically with tropical theileriosis had considerably lower values of RBC, Hb, and PCV than healthy animals (Abd Ellah, 2015). On the contrary, MCV values were increased with an increase in parasite load (Nazifi *et al*., 2010). Eosinophilia, and neutropenia were also occured in *T. annulata* infected cattle as compared to healthy animals (Ganguly *et al*., 2015). In this study, although some cases suffered from low RBC values, the mean values were within the normal range and this was in agreement with the results obtained from another study in Spain (Lydia *et al*., 2017).

Whole blood PCR examination revealed a high prevalence of *T. annulata* infection and this is not surprising as Odisha is considered an endemic region for tropical theileriosis. Our findings will help to focus on the prevalence of *T. annulata* infection in Odisha and the development of an accurate control policy leading to a reduction of the economic losses of the disease. Phylogenetic analysis of *T. annulata* isolated from Odisha was successfully applied and showed relationships with other local and global isolates. Amplification of the genetic marker (*Tams 1* gene) was successfully performed and the molecular phylogeny of *T. annulata* was constructed. The current isolate was closely related to *T. annulata* of Uttar Pradesh, India isolate (MF346035), Bahrain isolate (AF214797), Hyderabad India (MK034702) and China (MH538102) with nucleotide sequence identity 99.6%, 99.49%, 99.36%, and 99.11%, respectively. Our findings were in agreement with the published findings of the molecular phylogeny of *T. annulata* based on the *Tams 1* gene marker from India and Srilanka (Aparna *et al*., 2013; Sivakumar *et al*., 2014). This study completely supported earlier work that successfully cloned and sequenced *Tams 1* gene of *T. annulata* as an accurate technique for identification and performed its molecular phylogeny from different states of India (Aparna *et al*., 2013).

This is the first study to give insight into the molecular epidemiology, risk factors, phylogeny and hematological analysis of asymptomatic *T. annulata* infected cattle from Odisha, India. The current study revealed a high prevalence of *T. annulata* infection in the cattle population which supports that Odisha is an endemic area for tropical theileriosis, and is considered an overwhelming tick-borne disease of the cattle industry. These findings will help in setting up effective and urgent control policies against tropical theileriosis.

Conflict of interest

The authors of this paper have declared that no

competing interests exist.

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