Epidemiological studies on pathogenic Theileria infection in goat population of Assam, India

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Abstract
The present study was conducted to ascertain the burden of theileria infection in the goats population of Assam, a north-eastern state in India. Sick goats showing clinical symptoms of inappetence, anemia, weakness, fever and icterus were selected under the study design. Blood samples (n=543) were collected from 11 districts of Assam, irrespective of age, sex, and breed. Microscopic and molecular examination was conducted to detect Theileria spp. in the blood smear. An overall prevalence of 32.41%for Theileria infection was recorded. Age-wise, Theileria infection was recorded higher in the goats under 3 years of age (37.08%) and lowest over 1 year of age (25.4%). Higher prevalence percentage was recorded in females (35.82%) than in males (23.87%). Odds ratio analysis indicates that females have a significantly greater risk of developing Theileriosis than the males (p=0.007;OD=1.78;95% CI=1.16-2.72). A temporal study on the occurrence of the disease pattern revealed that the highest prevalence was recorded during monsoon. Optimization of buffered pH water with Giemsa's stain plays a crucial role in good staining, also immediate smear preparation shortly after collection, yielding better results when screened by conventional microscopy under field conditions to initiate specific treatment.

Keywords: Assam; epidemiology; goat; India; Theileria infection

Introduction

Livestock production system has been considered as one of the crucial activities towards sustainable human development through the provision of food, employment & economy in Assam. However, high prevalence of various infectious diseases has always been major constraint in livestock farming. Additionally, the high morbidity and mortality associated with these diseases limit the animals' ability to express their full genetic potential (Rehman et al., 2019). Among various diseases, various tick-borne blood parasitic infections like Babesiosis, Anaplasmosis, Trypanosomiasis, Ehrlichiosis and Theileriosis have been widely reported from time to time across various regions of India. However, Theileriosis in small ruminants stands out as one of the most prevalent yet insidious in nature. The disease in few cases is complicated with persistent infection phase resulting in chronic/wasting form in some species (Shruthi et al., 2017).

Theileriosis is a tick-borne hemoprotozoan disease prevalent across a broad geographical range, spanning from Southern Europe to Southern Russia, Northern Africa, Middle East, Central Asia, China, India and other tropical countries (Denizhan et al, 2017; Farhang etal 2017; Pal & Chakravarty, 2020). Small ruminant theileriosis or caprine theileriosi is a tick-borne hemoprotozoan disease caused by spp. of Theileria namely *T. luwenshuni* and *T.lestoquardi* which are highly pathogenic whereas *T. recondite*, *T. seperata* and T. ovis are less pathogenic (A'aiz & Dhaim, 2014). Pathogenic *T. luwenshuni* and *T. lestoquardi* have been reported from Iran, Iraq and India and are responsible for causing malignant ovine theileriosis (MOT) in sheep and goat (Namavari et al., 2011). It is similar to bovine tropical theileriosis which is transmitted by Hyaloma spp. but transmission by Haemaphysalis spp. has also been reported from China (Yu et al., 2015).

Small ruminant theileriosis develops in the erythrocytes and lymphoid cells. The disease may occur in acute, sub-acute or chronic form. The acute form of the disease is usually characterized by very high fever with high mortality rates within 3-6 days of infection. Other associated clinical manifestations include anaemia, icterus and enlargement of palpable lymph nodes (Zangana et al., 2011). Symptoms like malaise, anorexia, lacrimation, digestive disturbance, emaciation, dyspnoea, posterior weakness, lateral recumbency and transitory haemoglobinuria are also common. On contrast, parasitic diseases particularly, protozoan blood parasites are not extensively studied at the herd level, as their clinical effects are often overshadowed by more noticeable secondary infections (Rehman et al., 2010).

Detection of the haemo-parasites has been extremely advantageous in early diagnosis. Giemsa staining protocol of smear preparation from blood is considered to be “gold standard” for detection of infection particularly in acute cases. But it is difficult to detect in infection where there is less number of parasites causing low degree of parasitemia (Begum et al., 2019). In many cases, due to limited training, expertise, or the availability of equipment and solutions in our region, presumptive diagnosis, especially in the field, is often based on patient history, clinical signs, or response to treatment. Typically, drugs such as buparvaquone and oxytetracycline, combined with fluid therapy and other supportive medications to manage dehydration, weakness, and anemia, have been reported to cure malignant caprine theileriosis (Arif et al., 2024).

In Assam, goat serves as the 2nd largest livestock in terms of population among all of the livestock population which is around 4,315,173 (20th Assam Livestock Census, 2019).The disease is economically important and considered one of the major threats to the animal production sector (Mohammadi et al., 2017) especially in a developing nation like India, where small ruminants are often acknowledged as “poor man’s cow” for serving as source of income to landless or marginal farmers. The challenge is more when we discuss about a state like Assam where adjoining northeastern-states surrounded by porous international geographical boundaries.

The climatic condition of Assam is moderately hot and very humid receiving an annual precipitation of about 2800 mm. This favors propagation of different vector species for which many vector- borne diseases are found in man and animal. Till date, numerous studies have been undertaken concerning haemoprotozoan infections in large ruminants from Assam but limited information is available on epidemiology of theileriosis in goats. However, only a single study exits regarding comparison of detection of Theileria infection in goats using staining and molecular technique (Begam et al., 2019). But detail epidemiological study on caprine theileriosis from this part of India is still lacking. Hence, the current study was envisaged to understand the Theileria infection within caprine population from this region of the country.

Materials and methods

Study design and area

Eleven (11) districts of Assam namely,Baksa, Darrang, Dhemaji, Dhubri, Golaghat, Jorhat, Kamrup (metro), Kamrup (rural), Lakhimpur, Nagaon and Sonitpur in unorganized goat farms which were mostly based in rural areas were included in the present study (Figure 1).The selected district have been well known for goat rearing practices especially as a source of livelihood for poor/ marginal farmers.

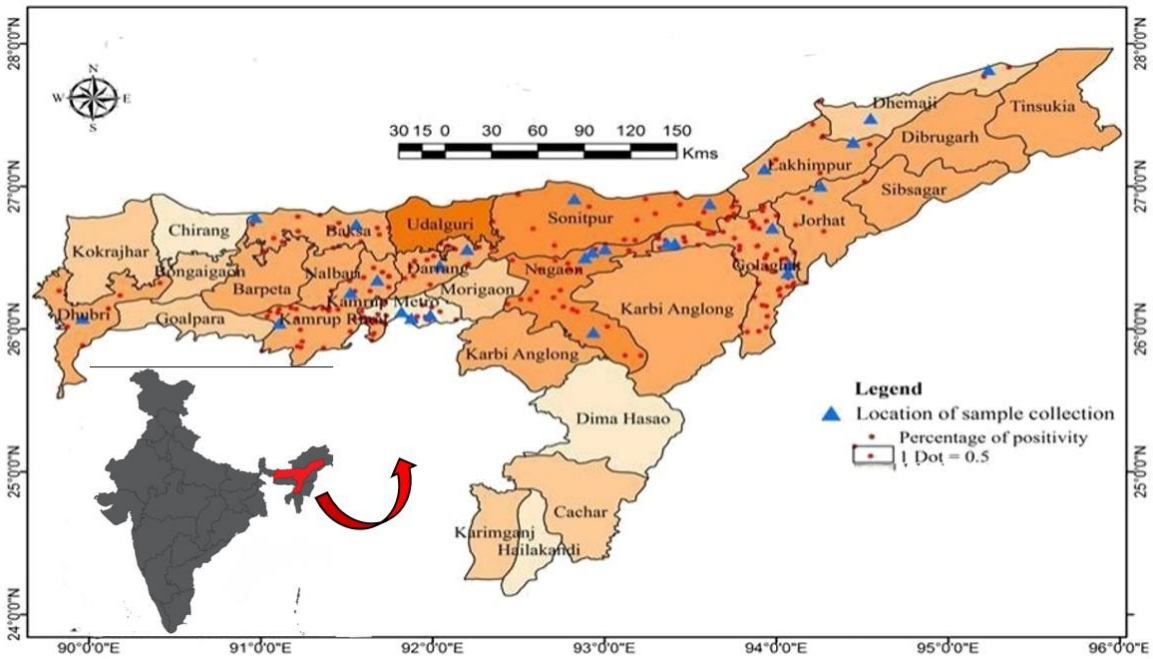

Figure 1. Thematic map showing Sampling area (Arc® Map GIS software version 10.2)

Sampling and selection of animals

For the current study, goats of both sexes were selected, ranging in age from under 1 year to over 3 years. Household/farmers predominantly rear a single indigenous breed (Assam Hill goat), and rarely other exotic breeds/cross bred, thereby eliminating the need for breed-specific epidemiology in this study.Goats (n= 543) including 155 males and 388 females were selected on random basis from the study area, irrespective of age and sex.Further, age wise distribution of goats in the study includes 173 goats that were less than1 year of age, 247 were between 1-3 years old and 123 aged 3 years and above. Prevalence studybased on sex and season (as per state meteorological department) were also performed.

Blood samples

Samples were obtained by venipuncture of the ear vein (for on spot smear preparation) and jugular vein (for molecular testing) of sick goats in EDTA vials and then shipped to the laboratory facility under cold pack for parasitological and molecular studies to the department of Veterinary Medicine, College of Veterinary Science, Guwahati, Assam. Parasitological examinations of the samples were conducted on the same day. Subsequently, the samples preserved in a deep freeze at -20°C until further use. In most of the instances, slides were promptly prepared in the field condition followed by fixing in methanol. These slides were preserved in a dust-free slide box for transportation to the laboratory.

Microscopic examination

Giemsa-stained thin blood smears (GSBS) were prepared to investigate the presence of any blood parasite following standard procedure. A small drop of well mixed blood was poured over a clean glass slide and was made to fix for 30 seconds by dipping in methanol after proper drying. Post fixation, the smears were stained with commercial Giemsa's stain diluted in SorenSen Buffer for 45 minutes, washed thoroughly under running tap water thoroughly. After proper washing, these slides were allowed to dry and subsequently were observed under 100 X lens using oil immersion for presence of Theileria parasite (Soulsby, 1982). The parasites were recognized through their distinctive morphological characteristics (Soulsby, 1982). A sample was recorded as negative if no parasites were detected after examining at least 50 oil immersion fields for duration of 20-30 minutes.

Optimisation of Buffer water pH (SorenSen Buffer)

Monitoring reagent performance is crucial for achieving accurate haemoprotozoan diagnosis via microscopy and minimizing discrepancies among microscopists examining blood smears. The Giemsa staining technique is the gold standard for haemoprotozoan detection, requiring high-quality Giemsa working solutions buffered to an optimal pH of 7.0–7.2. Since distilled water is slightly acidic, buffering is necessary, with contamination-free solutions ensuring reliable results. Properly stained blood films typically appear purplish; bluish staining indicates an alkaline buffer, while pinkish staining suggests acidity. To correct pH deviations, we adjusted the buffered water by adding 2% disodium hydrogen phosphate (Na_2HPO_4) for pH below 7.0 or 2% potassium dihydrogen phosphate (KH_2HPO_4) for pH above 7.2.

Prevalence

The prevalence of Theileria was assessed based on factors as stated in Tables 1 to 3. The prevalence (p) was calculated following established methodology outlined by Thrusfield (2007) using following the formula.

$$p = \frac{\text{Total infected goats during specified period}}{\text{Total goats examined}} \times 100$$

Molecular detection of Theileria species

Samples were subjected to molecular testing using polymerase chain reaction (PCR) for detection of *Theileria* spp. in the samples. Genus specific primers were used as per the standard technique. DNA extraction was carried out from the blood samples using DNeasy Blood and Tissue Kit (Quiagen®, Hilden, Germany) as per manufacturer's instructions. Extracted DNA's were subjected to PCR reaction using nucleotide sequence 989 5'-AGTTTCTGACCTATCAG-3' as a forward primer and 990 5'-TTGCCTTAAACTTCCTTG-3' as a reverse primer in order to get a product size of 1098 base pairs.

The PCR was carried out in a 25µl reaction mixture consisting of 5µl DNA template, 12.5µl DyNAzyme II PCR mix, 5.5µl nuclease-free water, and 1µl (10 pmol) of each Theileria-specific primer. The amplification process involved 30 cycles: denaturation at 94ºC for 1 minute, annealing at 56ºC for 1 minute, and final extension at 72ºC for 1 minute, followed by holding at 4ºC. The reactions were conducted using a semi-automated thermal cycler (TC-5000; Bibby Scientific, Burlington, USA).The positive control for *Theileria* spp was provided by the Department of Veterinary Parasitology, CVSc., AAU, Khanapara, Guwahati, India. Distilled water was used as a negative control. The PCR product was subjected to agarose gel electrophoresis followed by visualization GelDoc XR+ system (Bio-Rad, USA).

Statistical analysis

Confidence interval limits for infection rates of *Theileria* species were calculated using the methodology described by Bawm et al. (2016). In this formula, p-hat represents the proportion of positive cases, obtained by dividing the number of infection events by the total sample size, while the z-score corresponds to the critical value from standard tables, and n denotes the total number of samples analyzed.

$$p \pm z.\sqrt{P\,\frac{p(1-p)}{n}}$$

Results

A total of 166 samples were confirmed to be affected with intra-erythrocytic forms of *Theileria* species under microscopic examination (ME) of blood smears. Intra-erythrocytic pleomorphic forms of *Theileria* species, occurring as a single entity measuring 4.00–5.00µm were detected by ME (Fig 2).However, PCR conducted on all the blood samples could able to detect 10 (ten) more positive cases, as seen by the amplification of expected product size of 1098 bp in *gel doc* system (Fig 3) in addition to the 166 cases detected by ME. Hence, the overall prevalence of 32.41% (176/543) was recorded for Theileria infection in goats of Assam, India (Table 1).

Further, higher infection rate was observed in goats of > 3 years age group (37.08 %) followed by 1-3 years (34.70 %) and (25.4 %) in < 1 year of age (Table 1). Similarly, a gender-wise, prevalence of Theileria infection was recorded significantly higher (p<0.05) in females (Table 3). Additionally, odd ratio analysis of positive cases revealed significant association (p< .01) (Table 2). A significant difference (p < 0.05) was observed in the seasonal prevalence of Theileria infection among goats. Monsoon months were recorded with higher prevalence compared to both summer and winter months (Table 3).

Table 1. Prevalence of Theileria infection in goats by age group and sex

Category	Blood samples screened	Goats infected	Infected (%)	95% CI	Chi-sq	P-value
1. Overall	543	176	32.41%	-	-	-
2. Age Group						
<1 year	173	44	25.4%	18.95- 31.85	5.833	P<0.05
1–3 years	219	76	34.7%	28.41-40.99		
>3 years	151	56	37.08%	29.40-44.76		
3. Sex						
Male	155	37	23.87%	17.17-30.57	7.2243*	P<0.05
Female	388	139	35.82%)	31.06-40.58		

*Significant association (P<0.05), χ^2=7.2243

Table 2. Odd-ratio analysis for positive cases of Theileria infection in goats

Sex	Samples		Total	Odd of outcome	Odd Ratio	P-value	95% CI
	Positive	Negative					
Male	37	118	155	31.356%	1.78*	0.007	1.165-2.72
Female	139	249	388	55.823%			
Total	176	367	543				
*Significant association (P<0.01), *OD=1.78							

Table 3: Season wise prevalence of Theileria infection in goats

Season	No. of blood samples examined	goats infected (%) (95% CI)	Chi.sq
Summer (April-June)	121	33	10.18* (df=2; P<.0.05)
Monsoon (July-October)	235	97	
Winter (November-March)	187	46	
***Significant association (P<0.05), *χ^2=10.18**			

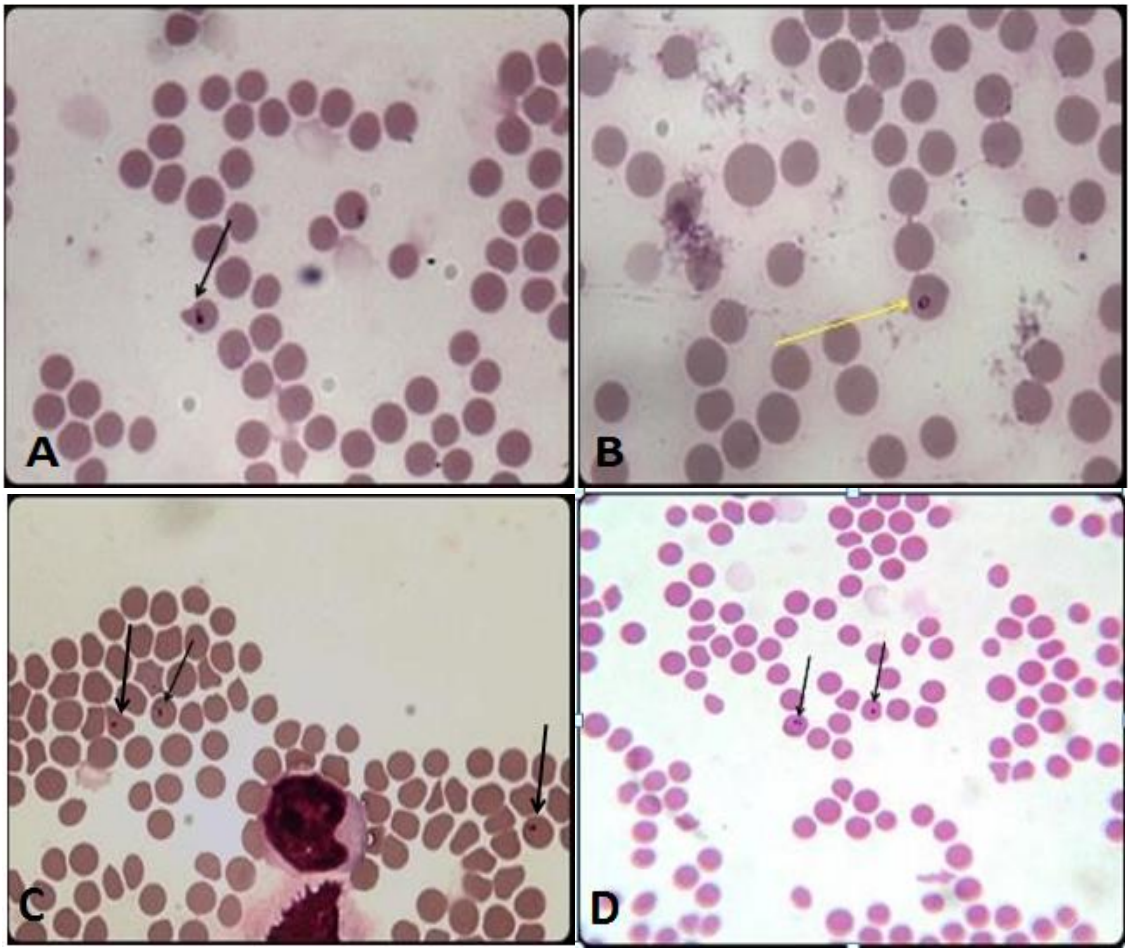

Figure 2. Giemsa-stained blood smear from goat showing intra-erythrocytic pleomorphic piroplasm of *Theileria*spp. A) Ring form; B) Nail Form; C) Comma-shaped; D) Dot shaped. (100X, arrows)

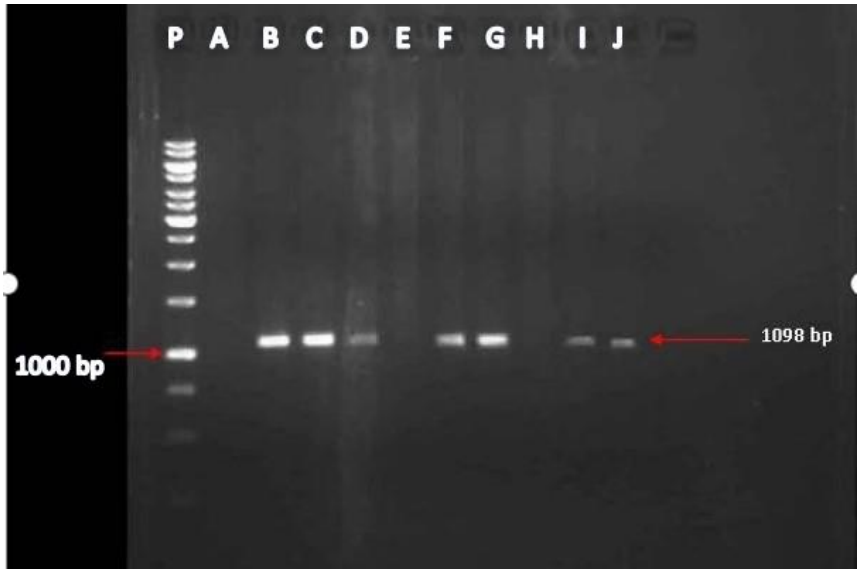

Figure 3. Agarose gel electrophoresis of 1098 bpfragmentsof*Theileria*spp. DNA Lane P: 1Kb DNA ladder; Lane A: Negative control; Lane B: Positive control; Lane C,D.F and G: Positive sample;Lane H: Negative sample

Discussion

In India, limited research has been conducted on haemoparasitic infection of small ruminant especially in goats (Patra et al., 2018). Also, the detail studies regarding the prevalence and occurrence of caprine theileriosis from this part of country is very scanty and only handful of interim work on caprine theileriosis from field condition is available.

Overall prevalence

In our study 32.41% goats were recorded positive for Theileria infection. The present study is in accordance with A'aiz & Dhaim (2014) who also reported higher infection of *T. ovis* and *T. lestoquardi* in small ruminants. However, Jegede et al. (2015) and Rather et al. (2015) reported a lower prevalence of caprine theileriosis i.e. 9.50% and 0.40% respectively. The high prevalence observed in the current study might be attributed to the hot and humid climate of the northeastern state, characterized by short winters, long summers, and heavy rainfall. These conditions favor the growth and multiplication of ticks, which act as natural vectors for haemoparasitic diseases. The seropositivity of theileriosis was reported 30 to 60% in India, except in Himalayan region due to unfavourable climate for tick survival (Kumar et al., 2015).

Age wise

Prevalence of Theileria infection was recorded to be highest in goats that were >3 years of age (37.08 %), followed by goats that were within 1-3 years of age (34.70 %) and lowest in the age group of <1 year of age (24.5 %) but chi square test revealed no statistical significant relationship exists between age groups and prevalence of infection at 0.05 level of significance. The lower prevalence of Theileria infection in kids of less than 1 year could be attributed due to transfer of passive immunity. These results are in line with the reports published by Naz et al. (2012); Dhaim et al. (2014) and Rather et al. (2015). However, Radostits et al. (2009) reported that the organism present inside the leukocyte may get transfer from mother to kid during suckling, which may provide pre-immunity to natural infection and also another reasin could be the presence of fetal haemoglobin in fetal erythrocytes that makes the intracellular environment of RBC's less conducive for multiplication of some haemoparasitic species (Selim et al., 2022). In this study the higher prevalence recorded adult goats might be due to increased migratory activity in search of fodder, breeding or marketing. Besides, these adult goats may act as carrier for a considerable period of time without showing any clinical manifestation. Similar observation were also documented by Dhaim et al. (2014), Jegede et al. (2015). Whereas, Naz et al. (2012) reported no direct relation of age on prevalence of Theileria infection in caprine exists.

Sex wise

In this study, Chi-square test equals to 7.2243 with p-value < 0.05and revealed statistically significant relationship exist between sex and Theileria infection in goats. The higher rate of prevalence recorded among females might be due to the additional gender associated physiological stress factors like pregnancy, parturition and lactation as suggested by Jegede et al. (2015) and Patra et al. (2018). Similar statement was also opined by Lisle and Rowe (2015) who stated that the higher susceptibility to parasites might be also linked with the size or sexual dimorphism, where females are typically larger than males, could potentially results in a greater exposure

by providing more attachment area for incoming parasites. On contrary, Ezenwa et al. (2012) documented that males tend to have a higher susceptibility to parasitic infection, which is attributed to immune suppression linked with androgens, particularly testosterone. Conversely, female estrogens may potentially enhance humoral immunity. Additionally, in the present study, Odd ratio analysis (P=.007) for positive cases of Theileria infection indicates that female goat has significantly higher risk of developing Theileria infection compared to male.

Seasonal prevalence

Seasonal variation is a recognized key factor influencing the prevalence of blood-borne parasites. The occurrence of Theileria infection fluctuated across different seasons, with a statistically significant association ($p< 0.05$) observed between seasonality and infection rates. Prevalence of Theileria infection was found to be much higher during monsoon season (42.84%) as compared to winter season (15.10%). The higher prevalence observed during this season may be related to the enhanced reproduction and spread of ticks, which act as vectors for this infection. This increase is likely influenced by factors such as temperature, rainfall, and relative humidity, which create favorable conditions for tick activity. (Patra et al., 2018). Similar observation were also opined by Ullah et al. (2018) and Islam et al. (2021) whereas, contradictory to the present findings, higher prevalence of theileriosis in summer season have also been documented by Rather et al. (2015).

Microscopic examination (ME)

ME revealed the presence of various forms of intra erythrocytic piroplasm Theileria species (Figure 2). Similar shapes have also been stated by Shruthi et al. (2017) and Arif et al. (2024). However, association of these different forms in pathogenesis of disease is not yet known.

Molecular detection

PCR conducted on blood samples using Theileria genus specific primer, confirmed the presence of *Theileria* spp. at 1098 bp in *gel doc* system (Figure 3). ME revealed 164 positive cases of Theileria infection out the total blood samples examined. However, 12 more samples were detected positive for *Theleria* spp in addition to 164 by PCR, thereby taking the total positive samples to 176. This discrepancy in result might be due to low parasitemia in carrier animals which remains undetected under microscope. The present findings are parallel with reports of Shahzad et al. (2015) who opined that traditional microscopic method based on Giemsa staining protocol is reliable to detect infection may give false negative result in some cases. Failing to detect parasite under microscopic examination from old samples/preserved samples is due to the fact that positive blood samples when kept at room temperature for a few hours results in clumping of infected erythrocytes. The erythrocyte that clumps either settled at the bottom of the samples and thereby the parasites did not appear in the smears made after preservation or they got washed out during the staining process rendering the smears as negative. Therefore, to avoid this situation, we have prepared the blood smears immediately after collection at field condition. Moreover, in the present study, the use of staining solution prepared with optimized buffered distilled water and Giemsa's stain yielded more homogeneous staining and better background clarity during microscopic examination of blood smears.

PCR technique is being more sensitive and specific than microscopic that can detect the infection in carrier animals and facilitates better identification of the parasite within the erythrocytes thereby, ensuring precisediagnosis (Begum et al. 2019). Serological techniques were also proposed by many scholars for detecting the circulating antibodies against these parasites, particularly in subclinical infection during epidemiological investigation (Yang et al., 2022; Nasreen et al., 2020). One major drawback of such test is occurrence of false positive/or false negative results due to cross reactivity between Theileria spp which is also cumbersome and time demanding to perform (Mans et al., 2015).

Conclusion

The present study discusses about the certain epidemiological attributes of Theileria infection in goat from the state of Assam, India. The present findings strongly indicate that the Theileria infection in the goats should be considered in the differential diagnosis with other diseases of caprine. Moreover, we recommend use of the correctly maintained buffered distilled water-Giemsa stain solution to yield better results while detecting *Theileria* spp. by conventional microscopy in routine diagnostic and also firmly opined on the practice of immediate preparation of blood smears from sick goats, especially at field level to enable prompt and precise diagnosis for initiating rational treatment. Further, due to chronic and insidious nature of this infection, routine monitoring and further comprehensive studies are necessary to understand the on-going status and to curb any future outbreak.

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Ethical Statement

The study was approved by the Institutional Animal Ethics Committee, College of Veterinary Science, Assam, India (770/ac/CPCSEA/FVSc/AAU/IAEC/16-17/402).

Disclosure statement

No conflict of interest was reported by the authors.

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