Original Research

Epidemiological Studies on Bovine Tick-Borne Haemoparasitic Diseases in Chennai

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Abstract

The present study was aimed to assess the current epidemiological status of bovine tick-borne diseases (TBDs) in and around Chennai by conventional staining method and polymerase chain reaction (PCR). A total of 154 blood smear and whole blood samples were screened by Leishman staining and PCR respectively. PCR assay (50.65%) revealed significantly higher sensitivity in detection of TBDs in clinically suspected cattle than microscopic examination (35.06%). Results showed that Anaplasma spp. (20.78%) was the most prevalent parasite of cattle followed by Theileria spp. (11.69%) and Babesia spp. (2.6%) in Chennai. The infection of Anaplasma spp. (27.77 %), Babesia spp. (5.55 %) and Theileria spp. (11.11 %) were higher in less than 2 year of age group. The prevalence of anaplasmosis was relatively high (21.32%) in cross-bred cattle. However, higher prevalence of babesiosis (5.55%) and theileriosis (22.22%) was noticed in non-descript animals. Statistical analysis using chi-square test indicated a highly significant (P < 0.01) difference between these tests.

Key words: Epidemiology, Tick-borne diseases, Cattle, Active surveillance, PCR, Haemoparasites

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Introduction

Tick-borne pathogens (TBP) are considered to be one of the major hindrances to productivity and health of livestock, globally. The four main pathogens responsible for these losses are the tick-borne protozoa, Babesia and Theileria, and the tick-borne rickettsial disease pathogens, Anaplasma and Ehrlichia. In

developing countries like India, TBP spp. can impose considerable economic loss on large and smallholding livestock productivity farming systems: the resulting diseases causing high mortality rates, reduced milk production and loss of body condition (De Castro, 1997). The *Theileria* species that present in India are *T. annulata* and *T. orientalis* and both species are transmitted by *Hyalomma anatolicum* (Aparna *et al*., 2011) whereas babesiosis and anaplasmosis are transmitted by *Rhipicephalus (Boophilus) microplus. Theileria annulata* is widely considered as more pathogenic and associated with greater economic loss (Aparna *et al*., 2011). In India, bovine babesiosis, is predominantly caused by *Babesia bigemina.* The incidence of *B. bigemina* in native, cross-bred cattle and buffaloes has been frequently reported since long (Ghosh and Nagar, 2014). Additionally, *B. bovis* infection has also been reported in India (Muraleedharan *et al*., 1984; Add recent reports). Animals that recover from *Babesia* infection, becomes carriers and parasites go unnoticed on microscopy. Subclinical infections may endure for long periods with infected animals acting as reservoirs (Brown *et al.*, 2006). Cattle that recovered from acute infection of anaplasmosis may develop persistent infection and it is characterized by cyclic low-level rickettsaemia (French *et al.*, 1998). The pathogenic *Anaplasma marginale* has been reported in multiple states of India, including Odisha, Uttar Pradesh, Punjab, Haryana, Tamil Nadu and Karnataka (Ghosh and Nagar, 2014). Diagnosis of the tick-borne diseases (TBDs) can be challenging due to its different phases and multiple clinical manifestations. Traditional diagnostic techniques (peripheral blood smear examination, cytology and serology) are valuable diagnostic tools for TBDs. However, polymerase chain reaction (PCR) is more sensitive than other conventional methods. Keeping the above facts in view, the present study was undertaken to assess the current epidemiological status of tick-borne haemoparasitic diseases in cattle in Chennai, Tamil Nadu, India by conventional staining methods and PCR.

Materials and Methods

The present active surveillance study was conducted at Madras Veterinary College Teaching Hospital and Blue Cross of India, Chennai, Tamil Nadu, India to assess the current epidemiological status of bovine tick-borne diseases viz. anaplasmosis, babesiosis and theileriosis in and around Chennai. Cattle which were infested with ticks and showing clinical signs of fever (> 40°C), pale or icteric conjunctival and vaginal mucous membrane, panting, dark yellow or bloody urine and enlarged lymph nodes were selected for this active surveillance study. A total of 154 peripheral blood smear and whole blood samples were collected from cross bred (136) and non- descript (18) cattle. Of 154 cattle, 18 samples were received from cattle with less than two years of age, 61 from 1 to 3 calving, 67 from 4 to 7 calving and 8 from more than seven calving. The blood smears were subjected to Leishman staining (Kolawole *et al*., 2014). Whole blood samples (3 ml) from suspected animals were collected in EDTA vials and stored at – 20ºC until further use. The template DNA was extracted directly from fresh and frozen blood sample by the DNeasy® Blood and

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Tissue Kit (Qiagen, Germany) and the DNA were stored at -20°C until further use. The Oligo nucleotide primers used in this study are listed in Table 1.

Table 1: List of oligonucleotide primers

Primer sequence (5' – 3')	Product size	Reference
MAR1Bb2 primer set for *Anaplasma marginale*	265 bp	Bilgic *et al*. (2013)
F: 5'- GCT CTA GCA GGT TAT GCG TC – 3'		
R: 5' - CTG CTT GGG AGA ATG CAC CT – 3'		
30-kDa gene for *Theileria annulata*	721 bp	d'Oliveria *et al*. (1995)
N516 F: 5' - GTA ACC TTT AAA AAC GT – 3'		
N517 R: 5' – GTT ACG AAC ATG GGT TT – 3'		
18S rRNA gene for *Babesia* spp.	339 bp	Birkenheuer *et al*. (2003)
5-22 F: 5' – GTT GAT CCT GCC AGT AGT – 3'		
1661 R: 5' – AAC CTT GTT ACG ACT TCT C – 3'		

The above-mentioned primers in Table 1 were custom synthesized and obtained from Sigma- Aldrich, Bangalore, India were used in this study. PCR analysis was carried out for the detection of blood parasites viz. *A. marginale*, *Babesia* spp., and *T. annulata* targeting MAR1Bb2, 18S rRNA and 30 kDa genes respectively. The PCR was performed in Biorad thermal cycler. All the reactions were carried out in volume of 25 µl in 0.2 ml PCR tubes. The reaction mixture contains 12.5 µl of PCR master mix-2X (Ampliqon®), 10 pmol of each, template DNA (mention the concentration of DNA instead of volume and 5.5 µl distilled water. Conditions of thermal cycling for different oligonucleotide primers used in this study are listed in Table 2.

Table 2: PCR cyclic conditions for tick-borne pathogens

Cycling conditions					
Final Extension	**Extension**	**Annealing**	**Denaturation**	**Initial denaturation**	**Organism**
65°C	65°C	56°C	94°C	94°C	*A. marginale*
10 min	1 min	30 sec	50 sec	5 min	
	Repeated for 35 cycles				
72°C	72°C	55°C	94°C	94°C	*T. annulata*
7 min	1 min	1 min	1 min	3 min	
	Repeated for 30 cycles				
72°C	72°C	58°C	94°C	94°C	*Babesia*
5 min	45 sec	45 sec	45 sec	5 min	
	Repeated for 35 cycles				

The amplified PCR products were electrophoresed in an ethidium bromide stained 1.5 per cent agarose gel and visualized in a transilluminator under UV light.

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Statistical Methods

The results of the both diagnostic tests were assessed statistically as per the procedure of Snedecor and Cochran (1994). The sensitivity and specificity of diagnostic tests were analyzed as per the methods described by Smith (1994). The concordance and Kappa value were calculated as per the methods delineated by Thrusfield (1995).

Results and Discussion

Peripheral Blood Smear

The overall prevalence of bovine TBDs in Chennai was observed as 35.06 per cent which is in agreement with Soundararajan and Rajavelu, (2006) and Chaudhri *et al*. (2013) who recorded 32.4 and 27.88 per cent of TBDs in Chennai and eastern Haryana respectively. However, high prevalence of TBDs with 43.1 to 76.85 per cent infection in cattle was also on record (Krishnamurthy *et al*. 2016 in Shimoga of Karnataka; Ananda *et al*., 2009 in North Bangalore; and Reetha *et al*., 2012 in Tamilnadu). In contrary, Bhatnagar *et al*. (2015) found lower positivity (9%) of tick-borne diseases in Southern Rajasthan. The differences in the overall prevalence rate might be due to variation in the tick infestations and relative potential of tick to transmit parasites, geography and climatic conditions.

In the present study, the prevalence of anaplasmosis was recorded as 20.78 (32/154) per cent which is in accordance with Arunkumar and Nagarajan, (2013) and Reetha *et al.* (2012) who observed 19.3 and 21.73 per cent respectively, whereas Talukdar and Karim, (2001) have found highest positivity (33%) and Krishnamurthy *et al*. (2016), Bhatnagar *et al*. (2015) and Ananda *et al*. (2014) found lower positivity of 2.79, 3.5 and 12.5 per cent respectively. The higher prevalence of anaplasmosis in suspected cross-bred animals indicates the presence of sub-clinical infection or carrier status of this disease. *R. (B.) microplus* was reported as the commonest tick species in Tamil Nadu (Koshy *et al*., 1982). The abundance of biting flies (*Tabanus* spp. and *Stomoxys* spp.) due to hot and humid climatic conditions prevailing in the state may augment the mechanical transmission to the naive animals. Babesiosis was observed as 2.6 (4/154) per cent in this study which is in accordance with Bhatnagar *et al*. (2015) and Nair *et al.* (2011) who noticed 1.41 and 2.66 per cent respectively, whereas Krishnamurthy *et al*. (2016) and Vetrivel *et al*. (2017) found highest positivity of 6.9 and 20.27 per cent respectively. Soundararajan and Rajavelu, (2006) recorded lower prevalence of 0.53 per cent. The lower prevalence of babesiosis might be due to sub-clinical or carrier state of the disease, low number of parasites in circulation and seasonal variations.

In this present study, theileriosis was recorded as 11.69 (18/154) per cent which is in concurrence with Krishnamurthy *et al*. (2016) and Muraleedharan *et al.* (1994) who observed 12.5 and 17.7 per cent respectively, whereas Rakha and Sharma, (2003), Anandan, (1991), Nair *et al.* (2011) and Chaudhri *et al.* (2013) found higher prevalence of 76, 21.1, 43.33 and 22.88 per cent respectively. Bhatnagar *et al*. (2015)

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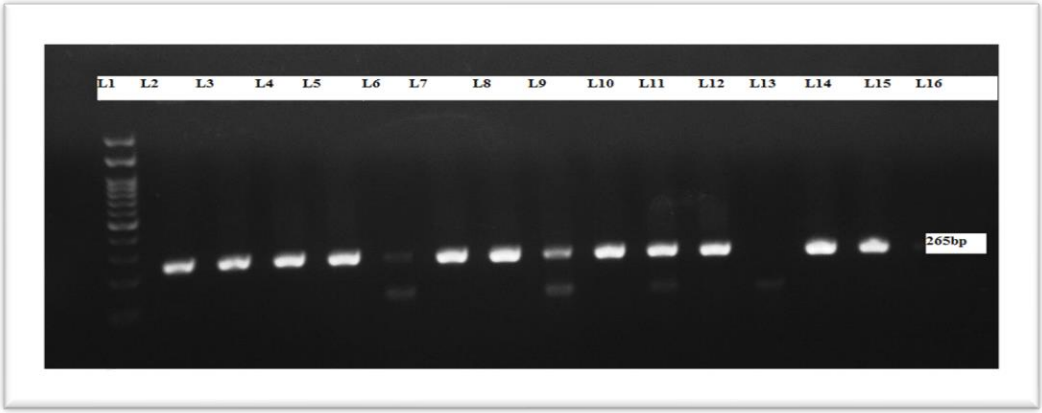
recorded lower prevalence of 3.8 per cent. The difference might be due to geographical location, climatic conditions, sample size and time period of the study.

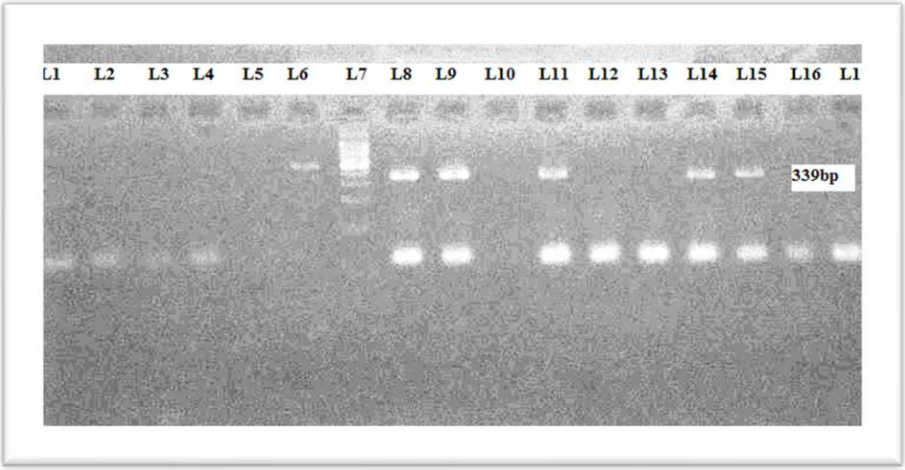
Polymerase Chain Reaction

The PCR assay revealed the overall positivity of bovine TBDs as 50.65 per cent in suspected cattle in Chennai.

Agarose gel electrophoresis showing PCR amplification of *A. marginale*



L1	**100 bp DNA ladder**	**L 3,4,5,6, 7,8, 9,10,11,12,14,15**	**Positive samples**
L2	**Positive control (265 bp)**	**L13**	**Negative samples**
L16	**Negative control**		



Agarose gel electrophoresis showing PCR amplification of Babesia genus specific

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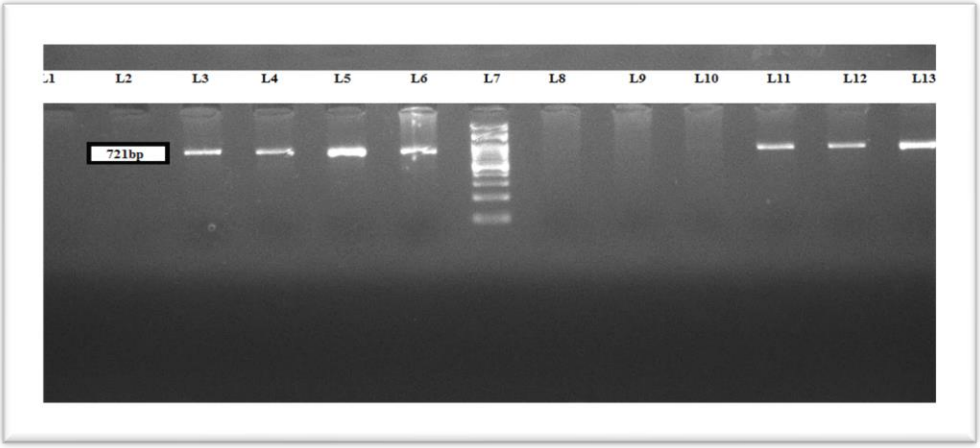
L7	**100 bp DNA ladder**	**L6,9,1, 14,15**	**Positive samples**
L8	**Positive control (339 bp)**	**L2,3,4, 10,12, 13,16,17**	**Negative samples**
L1	**Negative control**		
L8	**Negative control**		



The prevalence of anaplasmosis was 27.27 (42/154) per cent in the present study which is in concordance with Bilgic *et al*. (2013) who observed 21.91 per cent, whereas Singh *et al*. (2012) and Kolte *et al.* (2017) showed higher prevalence of 45.2 and 53.3 per cent respectively by semi-nested PCR. The variations in the results might be due to higher sensitivity of semi-nested PCR than conventional PCR assay and sampling size and assay protocols. The prevalence of babesiosis was 3.9 (6/154) per cent which is in accordance with Kolte *et al*. (2017) and Bhat *et al*. (2015) who observed 4.3 and 7.35 per cent respectively by conventional PCR methods.

The prevalence of theileriosis in this present study was 19.48 (30/154) per cent which is in concurrence Kolte *et al*. (2017) and Parthiban *et al*. (2010) who observed 15.8 and 18.18 per cent respectively, whereas Bilgic *et al*. (2013) observed higher prevalence of 69.8 per cent by multiplex PCR. The variations in the results might be due to sampling size.

Comparison of PCR with Microscopy for the Detection of Anaplasmosis, Babesiosis and Theileriosis

In PCR study, the overall positivity of bovine TBDs in Chennai was recorded as 35.06 and 50.65 per cent in peripheral blood smear and PCR assay respectively. Comparison of PCR with blood smear screening by microscopy for the detection of *Anaplasma*, *Babesia* and *Theileria* were evaluated and PCR showed sensitivity of 64.28, 50.00 and 46.67 per cent and specificity of 95.53 99.32 and 96.77 per cent respectively. The concordance between these tests was 87.01, 97.40 and 87.01 per cent with a kappa value of 0.64, 0.59 and 0.51(moderate agreement) in the diagnosis of anaplasmosis, babesiosis and theileriosis respectively. Statistical analysis using chi-square test indicated a highly significant ($P < 0.01$) difference between these

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tests. The results obtained by peripheral blood smear examination revealed a lower prevalence of TBDs when compared to PCR and was depicted in the table.

Prevalence of Bovine Tick-Borne Disease by Blood Smear and PCR Assay in Chennai

Prevalence by Tests	*Anaplasma marginale*	*Babesia*	*Theileria annulata*	Total
Total no of sample screened	154	154	154	154
% positive by blood smear	32 (20.78%)	4 (2.60%)	18 (11.69%)	54 (35.06%)
% positive by PCR	42 (27.27%)	6 (3.90%)	30 (19.48%)	78 (50.65%)
Chi square test	66.4**	55.45**	44.16**	55.33**

*Chi square interpretation: ** - Highly significant, * - Significant, NS – Non significant*

Microscopic examination techniques are best suited to diagnose the acute infections but have limited application for detection of the parasite in carrier animals or sub-clinically infected animals exhibiting low level parasitaemia in the peripheral blood (reference). In the present study, PCR assay revealed significantly higher sensitivity in detection of tick-borne parasites in clinically suspected animals. Thus, the higher sensitivity of PCR over microscopic examination method is well established (Almeria *et al.*, 2001; Khaminsou *et al.*, 2007).

Epidemiology of Bovine Tick-Borne Diseases

Age-Wise

In the present study, 27.77 (5/18), 22.95 (14/61), 17.91 (12/67) and 12.5 (1/8) per cent samples were found positive for anaplasmosis in the age group of less than 2 year, 1-3 calving, 4-6 calving and above 7 calving respectively and chi-square test indicated a highly significant correlation ($P < 0.01$) between ages. In Babesiosis, 5.55 (1/18), 3.27 (2/61) and 1.49 (1/67) per cent samples were found positive in the age group of less than 2year, 1-3 calving and 4-6 calving respectively. In this present study, 11.11 (2/18), 13.11(8/61), 10.44 (7/67) and 12.5 (1/8) per cent samples were found positive for theileriosis in the age group of less than 2year, 1-3 calving, 4-6 calving and above 7 calving respectively. The data is depicted in the following table-

Determinant	Sampling details	No. of blood samples	No of positive cases in peripheral blood smear			No. of positive cases in PCR		
			Anaplasma	*Babesia*	*Theileria*	*A. marginale*	*Babesia*	*T. annulata*
Age	< 2 year	18	5 (27.77%)	1 (5.55%)	2 (11.11%)	7 (38.89%)	1 (5.55%)	4 (22.22%)
	1-3 calving	61	14 (22.95%)	2 (3.27%)	8 (13.11%)	16 (26.22%)	4 (6.55%)	12 (19.67%)
	4-6 calving	67	12 (17.91%)	1 (1.49%)	7 (10.44%)	16 (23.88%)	1 (1.49%)	11 (16.42%)
	> 7 calving	8	1 (12.50%)	-	1 (12.50%)	3 (37.50%)	-	3 (37.50%)
Total		**154**	**32 (20.78%)**	**4 (2.60%)**	**18 (11.69%)**	**42 (27.27%)**	**6 (3.90%)**	**30 (19.48%)**

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In this study, higher prevalence of 27.77 (5/18), 5.55 (1/18) and 11.11 (2/18) of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. were observed in less than 2 year of age respectively compared to other age groups. Similar study has been conducted by Kolte *et al*. (2017) who found that 24.42 per cent of immature cross-bred animals (< 2 years old) were more susceptible to tick-borne diseases than younger native animals. In contrary, Ananda *et al*. (2009) found 63.15 per cent of positive cases for TBDs in 4-6 years of age. The variation in age-wise prevalence might be due to sampling protocol, sample size, selection of animals and geographical location.

Breed-wise

The higher positivity of anaplasmosis was noticed in cross-bred cattle (21.32%) than in non-descript cattle (16.66%). The positivity of babesiosis was observed in 2.20 (3/136) and 5.55 (1/18) per cent of samples in cross-bred and non-descript cattle respectively. Theileriosis was noticed in 10.29 (14/136) and 22.22 (4/18) per cent of samples in cross-bred and non-descript cattle respectively. Similar study was reported by Anandan, (1991) who observed 27.31 per cent of cross-bred animals exhibit higher infectivity of TBDs when compared to native zebu cattle. In contradiction, Zahid *et al*. (2005) reported the higher incidence of theileriosis in Holstein-Friesian (24%) and Jersey cows (15%) than native breeds of Pakistan. In this present study the sample size is not equal. The variation in result might be due to sampling protocol, sample size, innate resistance of animals and selection of animals.

Conclusion

The current prevalence rate of bovine TBDs in Chennai was recorded as 35.06 and 50.65 per cent by microscopy and PCR respectively. PCR assay showed high sensitivity and specificity than microscopy in detecting tick-borne parasites. The proper knowledge of epidemiology is the prerequisite for the prevention and control of TBDs in cattle.

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Conflict of Interest

The authors declare that no conflict of interest.

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