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Cytochrome b gene based molecular survey of *Theileria annulata* infection in cattle in Tamil Nadu, India

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Abstract

Theileria annulata is a haemoprotozoan parasite which causes Bovine Tropical Theileriosis in cattle. It is an important tick borne disease of tropical and subtropical countries. Conventional diagnostic methods are unreliable to diagnose the subclinical or carrier status of the disease. Polymerase chain reaction (PCR) assay based diagnosis using different target genes like 18SrRNA has been developed. In this study, an extra chromosomal DNA, cytochrome b gene has been utilized to screen *T. annulata* infection in cattle from two different agro climatic zones of Tamil Nadu. Significantly high level ($p > 0.01$) of *T. annulata* infection (25.66%) has been detected using cytochrome b gene based PCR whereas only 2.88% prevalence has been detected by conventional blood smear examination. North eastern agro climatic zone has shown a significantly ($p > 0.01$) higher prevalence of *T. annulata* (26.95%) by cytochrome b PCR than North western zone (22.96%). Cattle aged more than six years have shown a significantly ($p > 0.01$) higher prevalence (31.75%) than the cattle aged less than three years (11.11%). Present study clearly indicates that cytochrome b based PCR is a better target for epizootiological screening of bovine tropical theileriosis.

Keywords: *Theileria annulata*, Bovine Tropical Theileriosis, cytochrome b gene, PCR, molecular survey, sub clinical, carrier status

1. Introduction

Bovine tropical theileriosis caused by an apicomplexan protozoa *T. annulata* is a major vector borne hemoprotozoan infection in cattle. It is an economically important disease of bovine in tropical and subtropical countries not only because of clinical disease but also because of unaccounted losses due to carrier status of the disease.

Conventional diagnostic methods of clinical infection in cattle are Giemsa stained blood smear examination for piroplasms, lymphnode aspiration cytology for schizonts infected lymphocytes^[1]. Serological tests such as Indirect fluorescent antibody test (IFAT), enzyme linked immune sorbent assay (ELISA) etc. were used to detect the circulating antibodies against *T. annulata*^[2, 3, 4]. Usefulness of these methods is highly unreliable in carrier status of the disease exists and highly questionable in regions where pathogenic and non-pathogenic species of *Theileria* co-exists.

Molecular techniques like polymerase chain reaction have been developed for more sensitive and specific detection of *T. annulata* infection^[5, 6, 7, 8]. In these assays the primers specific for 18S rRNA gene^[9, 10], *T. annulata* major merozoite surface protein (Tams1)^[11, 12, 13] and heat shock protein 70 (HSP70) gene have been used for molecular diagnosis^[5]. However, PCR assays are highly dependent on the number of amplification targets present in the sample. Hence, a more repeated gene would be a better target to increase the sensitivity of PCR assays. Apart from sensitivity, gene with fast evolution and high mutations may help to understand the recent evolutionary events^[14].

Cytochrome b gene is a mitochondrial, extra chromosomal DNA with more than 100 copies per parasite genome^[15]. It is a fast evolving gene but not subject to a selection pressure for accumulating polymorphism^[16, 17].

Present study shows this interesting gene as a target for molecular survey of *T. annulata* infection in cattle from the North Eastern and North Western zones of Tamil Nadu state of India.

2. Materials and Methods

2.1 Study region and animal population

The study was carried out in dairy cattle reared in the areas of North Eastern and North Western agro climatic zones of Tamil Nadu between April 2015 and July 2018. The cattle selected randomly for *T. annulata* testing were aged between 6 months and 12 years. Blood samples were collected in EDTA anticoagulant tubes by jugular venipuncture from apparently healthy cross bred cattle with tick infestation. The peripheral blood smears were collected from capillary bed in the tip of the ear. The blood samples were stored at -20 °C until further use.

2.2 Blood Smear Staining

Blood smears were fixed with methanol and stained with Giemsa stain (Hi Media, India) and washed in distilled water and air dried smears were examined under a light microscope at 100X magnification for the presence of piroplasms of *T. annulata*.

2.3 DNA extraction

The DNA was extracted from whole blood using a Qiagen Blood DNA Kit. Briefly, 200 µL of blood was mixed with 20 µL proteinase K with this 200 µL of lysis buffer (AL) was added and mixed thoroughly by vortexing and incubated at 56 °C for 10 minutes. 200µL of ethanol (100%) was added. This mixture was transferred to the DNeasy Mini Spin Column placed in a 2ml collection tube and centrifuged at 8000 rpm for 1 minute. The flow through were discarded and the spin column was placed in the new 2ml collection tube. 500 µL of wash buffer 1 (AW1) was added to the column and again centrifuged at 8000 rpm for 1 minute. The spin column was again placed in a new 2ml collection tube and 500 µL wash buffer 2 (AW2) was added to the column and centrifuged at 13,000 rpm for 3 minutes. After these two washings, the spin column was transferred to a new 1.5 ml micro centrifuge tube. 30 µL of elution buffer (AE) was added to the spin column and was incubated for one minute at room temperature. The tubes were centrifuged at 8000 rpm for one minute. The flow through portions containing DNA were stored at -20°C until further use.

2.4. *Theileria annulata* Cytochrome b gene amplification

Extracted DNA was used to amplify 1092bp cytochrome b

whole gene cytoF (5'CAGGGCTTTAACCTACAAATTAAC3') and cytoR (5'CCCCTCCACTAAGCGTCTTTTCGACAC 3') primers [8] with following cycling conditions: an initial denaturation step at 94 °C for 5 min., 30 cycles at 94 °C for 1 min., 54 °C for 1 min. and 72 °C for 1min. and a final elongation step at 72 °C for 10min. Amplified products were electrophoresed in 1% agarose gel with ethidium bromide and visualized under Geldoc® system (Fig. 1).

2.5. Statistical analysis

The observed prevalence was estimated as follows:

Prevalence (%) = {(number of infected animals)/(total number of examined animals)} X 100.

The Pearson's chi-squared test was used to determine whether there is a significant difference between the expected frequencies and the observed frequencies in terms of agro climatic zones, testing methods and age groups categories by using SPSS 20 statistical software.

Results and Discussion

Out of 417 crossbred cattle screened, only 12 animals were positive for *T. annulata* infection by examination of blood smears stained with Giemsa stain (2.88%). Whereas cytochrome b gene based PCR showed *T. annulata* infection in 107 animals (25.66%). North Eastern agro climatic zone had shown significantly ($p>0.01$) higher prevalence of *T. annulata* (26.95%) than North Western agro climatic zone (22.96%) by cytochrome b gene based PCR (Table-1 and Fig. 2). The molecular prevalence of theileriosis in crossbred cattle aged more than 6 years (31.75%) was significantly ($p>0.01$) higher than cattle aged 3-6 years (20.43%) and cattle aged less than 3 years (11.11%) (Table- 2 and Fig.3).

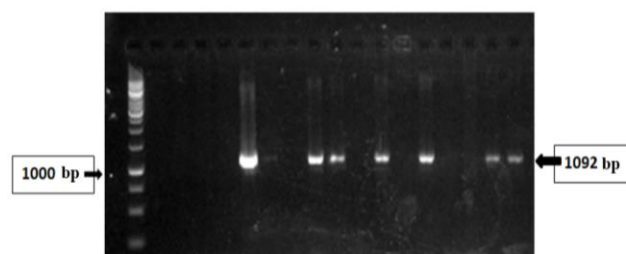


Fig 1: Cytochrome b gene (1092 bp) of *Theileria annulata*

Table 1: Molecular prevalence of *Theileria annulata* in crossbred cattle in North Eastern and North Western agro climatic zones of Tamil Nadu

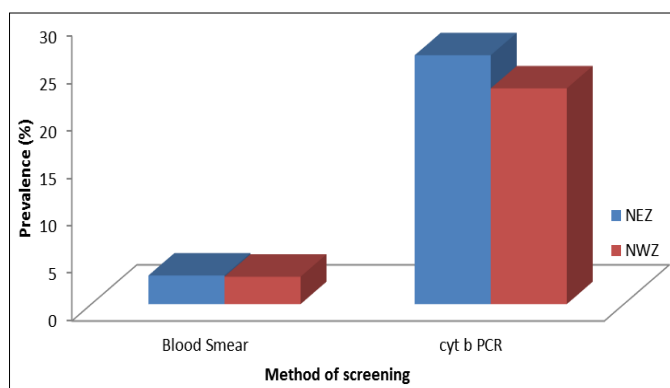
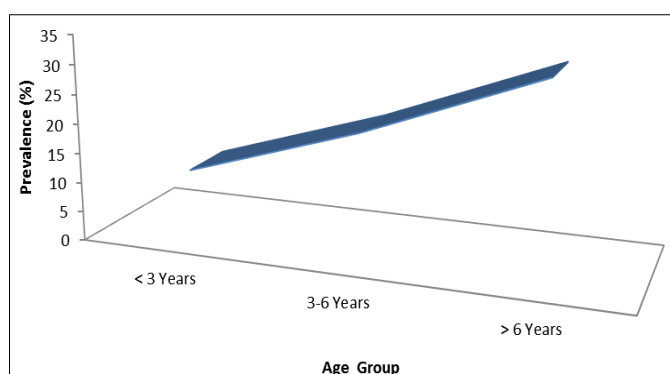
Agroclimatic Zones	Places	Number of Crossbred Cattle screened	Number of Cattle Positive for <i>T. annulata</i>		Percent prevalence of <i>T. annulata</i>	
			Blood Smear	Cyt b PCR	Blood Smear	Cyt b PCR
North Eastern Zone (NEZ)	Chennai	126	3	37	2.38	29.36
	Tiruvallur	47	2	13	4.25	27.66
	Kancheepuram	53	1	15	1.87	28.30
	Thiruvannamalai	56	2	11	3.57	19.64
	Total	282	8	76		
	NEZ Average Prevalence (%)				2.84	26.95
North Western Zone (NWZ)	Krishnagiri	39	1	8	2.56	20.51
	Dharmapuri	44	1	10	2.27	22.73
	Salem	52	2	13	3.85	25.00
	Total	135	4	31		
		NWZ Average Prevalence (%)				2.96
Over all		417	12	107	2.88	25.66
Pearson's Chi-Squared (χ^2) Value					0.94	57.28
Significance (p)					NS	0.001

NS- Not Significant

Table 2: Age wise molecular prevalence of theileriosis in crossbred cattle in North Eastern and North Western agro climatic zones of Tamil Nadu

Age group of the Crossbred Cattle screened	Number of Crossbred Cattle screened	Number of Cattle Positive for <i>T. annulata</i>		Molecular Prevalence (%)	
		Blood Smear	Cyt b PCR	Blood Smear	Cyt b PCR
< 3 Years	72	2	8	2.78	11.11
3-6 Years	93	3	19	3.23	20.43
> 6 Years	252	7	80	2.78	31.75
Total	417	12	107		
Prevalence (%)				2.88	25.66
Pearson's Chi-Squared (χ^2) Value				3.63	12.39
Significance (p)				NS	0.001

NS- Not Significant

**Fig 2:** Prevalence (%) of *Theileria annulata* infection in crossbred cattle from North Eastern and North western agro climatic zones of Tamil Nadu**Fig 3:** Molecular Prevalence (%) of *T. annulata* infection in different age groups of crossbred cattle from North Eastern and North Western agro climatic zones of Tamil Nadu

Epizootiological studies on tropical theileriosis based on conventional methods like blood smear examination and serological tests may have less sensitivity when there is a low level of *T. annulata* organisms in the circulation. Detection of *T. annulata* in blood smears will be influenced by several factors like quality of the stain, duration of staining, quality of the smear etc. Apart from this, it is very difficult and needs expertise to differentiate pathogenic *T. annulata* and non or less pathogenic *T. orientalis* based on morphology alone using microscope.

Screening of *T. annulata* infection based on the serological tests like enzyme linked immunosorbant assay and indirect fluorescent antibody test will have problem of cross reactivity across other *Theileria* species and also *Babesia* species. More importantly, because of the waning of antibody titers in longtime carrier animals which are serologically negative but

these carrier animals can be potentially infective for feeding ticks and transmission of the disease to the naïve animals [11, 18].

The findings of this study is in agreement with the studies conducted in Turkey, Spain, South Africa and Australia which also revealed that the cytochrome b gene based molecular diagnosis is more sensitive and specific than conventional methods of screening [16, 17, 19]. Cytochrome b gene based PCR can detect very low level *T. annulata* parasitemia and can also discriminate non-pathogenic *T. orientalis*. Increased sensitivity of cytochrome b gene might be due to the increased copy number of this gene with fast evolution and high mutations hence, specific target for detection of blood protozoan infection [16, 17]. It has also been proved that the cytochrome b, an extra chromosomal gene, based PCR is 20% more sensitive than ribosomal DNA for detection of *Babesia bovis* and *B. bigemina* infection in cattle [19].

A significantly ($p > 0.01$) higher prevalence of *T. annulata* infection in North Eastern agro climatic zone than the North Western agro climatic zone of Tamil Nadu might be due to the abundance of tick vector, macroclimatic factors and poor housing facilities which favours the tick breeding in North Eastern zone. This finding is in accordance with the molecular epidemiological study on *T. annulata* conducted in different agroclimatic zones of Punjab, India [12]. The temperature, humidity and climate of this zone would have also played role in higher infection exposure risk to the animals of this zone and increased prevalence of *T. annulata* infection.

The molecular prevalence (%) of *T. annulata* by cytochrome b gene based PCR in crossbred cattle screened from North Eastern and North Western agro climatic zones of Tamil Nadu showed a steady increase as the age advances (Fig.3). This finding is supported by the Tunisian study on *T. annulata* infection of cattle which also revealed that the prevalence rate is positively correlated with increasing age of the cattle [20]. The significantly ($p > 0.01$) higher incidence of *T. annulata* in cattle aged > 6 years might be due to increased attractiveness for ticks, multiple infections, hormonal changes, and high production stress due to calvings. This is in accordance with the earlier reports published from Bangladesh and Australia indicating higher prevalence in aged adult cattle exposed for the tick vectors for a longer periods [21, 22].

Results of this study have revealed that the *T. annulata* infection could be detected with higher sensitivity and specificity using extra chromosomal DNA, the cytochrome b gene of *T. annulata* even in cattle harbouring the *T. annulata* organism and acting as a potential source of infection but looking apparently healthy without showing any clinical signs of theileriosis.

Conclusions

The extra chromosomal DNA gene, cytochrome b gene based PCR can be a better target for epizootiological screening of *T. annulata* infection in cattle in different geographical areas and also in carrier animals compared to conventional blood smear examination. Climatic conditions and abundance of tick vector of each zone are important factors for higher infection rate. Prevalence of bovine tropical theileriosis increases as age of the animal advances.

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