Molecular Prevalence of *Theileria annulata* in Cattle from Different Agroclimatic Zones of Tamil Nadu, India

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**ABSTRACT**

Bovine tropical theileriosis caused by *Theileria annulata* is an economically important disease of cattle in tropical and subtropical countries. Conventional diagnostic methods are unable to identify the subclinical and carrier status of this disease. Molecular diagnostic methods are more sensitive in detection of subclinical and carrier condition on the disease. In this study 817 blood samples were collected from cattle of seven different agroclimatic zone of Tamil Nadu state. DNA extracted from these blood samples were screened by nested Polymerase Chain reaction using primers targeting the partial region of 18S rRNA gene of *T. annulata*. 114 samples were positive out of 817 samples. North eastern zone showed more prevalence (20.57 %) followed by North western zone (18.80%) whereas hilly zone (3.23%) showed least prevalence of *T. annulata* in cattle. Present study shows that the 18S rRNA based molecular screening of *T. annulata* infection in cattle is useful to assess the prevalence of bovine tropical theileriosis.

**Keywords**

Cattle, Molecular prevalence, *Theileria annulata*, 18S rRNA, Agroclimatic zones, Nested PCR, Tamil Nadu, India

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Introduction

*Theileria annulata* is an apicomplexan tick borne protozoan which causes Tropical Bovine Theileriosis. In tropical countries, it is an economically important disease and one of the major obstacles for the improved livestock production particularly in exotic and cross bred dairy cattle in tropical and subtropical countries including India (Minjauw and McLeod, 2003).

The losses are not only due to clinical disease and mortality but also due to carrier status of the disease associated production losses in terms of delay in growth, reproduction and milk production (Gharbi et al., 2011). The adverse effects of the disease are more
prominent in crossbred cattle compared to the indigenous cattle population.

In India, several reports documented bovine tropical theileriosis from subclinical to severe clinical outbreaks (Shastri et al., 1980; Bansal et al., 1987). The prevalence of this disease in cross bred cattle was reported from Karnataka (Ananda et al., 2009), Kerala (Nair et al., 2011), odhisa (Acharya et al., 2017), Punjab (Shahnawaz et al., 2011), Tamil Nadu (Velusamy et al., 2014) and Uttrakhand (Kohli et al., 2014) based upon blood smear studies and serological studies.

The conventional diagnosis of theileriosis is based on history of tick infestation, clinical signs and examination of Giemsa stained blood and lymphnode aspiration smears (Mans et al., 2015). These methods become unreliable when there is subclinical and / or carrier status of the disease. The advent of molecular techniques like polymerase chain reaction has revolutionized the scenario from unreliable to more sensitive and specific detection of infections including the carrier status.

In this study, a qualitative PCR has been used to study the molecular epidemiology of tropical bovine theileriosis in the different agroclimatic zones of Tamil Nadu.

**Materials and Methods**

**Study region and animal population**

The present study was carried out in seven agro climatic regions of Tamil Nadu state of India (Fig. 1 and Table 1). Tamil Nadu is the Southernmost state of India. It is located between 8.05’ and 13.34’ North latitudes and 76.14’ and 80.21 East longitudes. It covers an area of about 13 Mha. and accounts for about 4 per cent of the total geographical area of the country. The Tamil Nadu State forms part of the peninsular shield and composed of geologically ancient rock of diverse origins (i.e different soils). About three fourth of the area of the state is unclassified crystalline rocks of Archaean age and the rest is sedimentary rocks.

The State can broadly be divided into seven agro-climatic zones. The climate is semi-arid in the plains and humid to sub-humid in the hills with annual rainfall from 750 mm in some parts of the plains to over 2400 mm in the high hills.

The study was carried out in dairy cattle between April 2015 and September 2018. Blood samples were collected in EDTA anticoagulant tubes by jugular venipuncture from cattle selected randomly. The cattle selected for *Theileria annulata* testing were aged between 6 months and 12 years. The blood samples were stored at -20°C until further use.

**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 8000rpm for 1 minute. The flow through were discarded and the spin column was placed in new 2ml collection tube. 500μL 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 500μL 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 flows through portion containing DNA were stored at -20°C until further use.

**Theileria annulata DNA amplification**

Primary PCR was performed with a set of gene specific primers to amplify 416bp partial region of 18S rRNA gene of Theileria genus. Using this primary PCR product as template a nested PCR was performed with a species specific primers to amplify 193bp partial region of 18S rRNA gene of *Theileria annulata*. The PCR was carried out in 20 μL volume for each reaction consisting of 10 μL Red Eye Master Mix, 1 μL of forward primer and 1 μL of reverse primer, 1 μL template and 7 μL nuclease free water. The reactions were performed in a thermal cycler. For primary PCR the cyclical conditions were initial denaturation at 94°C for 5 Minutes followed by 30 cycles of denaturation, annealing and extension (94°C for 45 sec., 67°C for 1 min. 72°C for 1 min) and a final extension at 72°C for 5 min. The cyclical condition for nested PCR were initial denaturation at 94°C for 5 min. followed by 30 cycles (94°C for 45 Sec, 62°C for 1 Min. and 72°C for 1 min.) and a final extension at 72°C for 5 Min. Electrophoresis was performed in 1% agarose gel with ethidium bromides and visualized under Geldoc® system (Fig. 2).

**Statistical analysis**

The observed prevalence was estimated as follows:

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\text{Prevalence (%) } = \frac{\text{number of infected animals}}{\text{total number of examined animals}} \times 100
\]

**Results and Discussion**

Out of 817 blood sample screened for *Theileria annulata* infection, 114 samples were positive by PCR (13.95%, Table 1). Among the seven agroclimatic zones of Tamil Nadu Northeastern zone had shown highest prevalence (20.57 %) followed by North western zone (18.80%) whereas the high hilly fall zone had shown least prevalence (3.23 %) (Fig. 3). The high prevalence in the northeastern zone might be due to abundance of tick vectors and poor housing conditions.

Variation in the molecular prevalence of infection in cattle from different agroclimatic zones is directly related to the factors like vector prevalence, temperature and humidity.

The prevalence was higher in cattle aged >6 years (64/114) than cattle aged <3 years (19/114) and 3-6 years (31/114). *T. annulata* infection of cattle was shown to be positively associated with age (Weir *et al.*, 2011). The 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 (Sutherst *et al.*, 1983; Kabir *et al.*, 2011).

Polymerase chain reaction assays are more specific and sensitive than microscopy and serological methods to study the epidemiological status of any protozoan infection.18S rRNA gene based PCR assays have been successfully applied by many researchers for quick and accurate diagnosis of *T. annulata* infection (Khan *et al.*, 2013; George *et al.*, 2015). 18S rRNA based quantitative PCR assay also has been done to quantify *T. annulata* infection (Chaisi *et al.*, 2013). Though diagnosis of Bovine Tropical Theileriosis can be done with conventional microscope based methods, it is unreliable when the parasite load in blood is less.
Fig.1 Agroclimatic zones of Tamil Nadu

Fig.2 *Theileria annulata* nested PCR gel showing 193bp
**Fig. 3** Molecular prevalence of *T. annulata* infection in Tamil Nadu

| Table 1 Molecular prevalence of *Theileria annulata* in cattle from various agroclimatic zones of Tamil Nadu

| Agroclimatic Zones | Places | Total Number of samples screened | Number of Samples Positive for *T. annulata* by PCR | Percent prevalence |
|--------------------|--------|----------------------------------|-----------------------------------------------|-------------------|
| North Eastern Zone | Chennai, Tiruvallur, Kancheepuram, Thiruvannamalai, Cauddalore | 209 | 43 | 20.57 |
| North Western Zone | Salem, Namakkal, Dharmapuri, Krishnagiri | 117 | 22 | 18.80 |
| Western Zone | Erode, Coimbatore | 97 | 12 | 12.37 |
| Cauvery Delta Zone | Karur, Trichy, Thiruvarur, Ariyalur | 103 | 15 | 14.56 |
| Southern Zone | Madurai, Tirunelveli | 91 | 11 | 12.09 |
| Hilly Zone | The Nilgiris, Kodaikkkanal, Pannaikadu, Vathalagundu | 93 | 3 | 3.23 |
| High Rainfall Zone | Nagerkoil | 107 | 8 | 7.47 |
| **Total** | | 817 | 114 | 13.95 |
The carrier status of infection is the major source of spreading the infection to healthy population. Prevalence studies based on molecular methods will help in accurate diagnosis of any state of infection is need of the hour for planning better control and prevention of the disease.

The result of the present study has proved that PCR amplification of 18S rRNA gene of *T. annulata* from blood of bovine is a useful tool to assess the molecular epidemiological status of the bovine tropical theileriosis.

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