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Molecular Studies on Bovine Benign Theileriosis (*Theileria orientalis*) in Cattle of Puducherry Region

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ABSTRACT

A work was conducted during the months of January–August 2014 in Pondicherry in order to find the species of *Theileria* prevalent in the cross bred and Non-descript cattle. The selection of cases was based on the clinical signs and confirmative diagnosis by Polymerase Chain Reaction (PCR) for genus and species specific. The PCR analysis of the bovine blood revealed that 100 out of 110 animals were infected with *Theileria*, while seventy eight of those 100 samples were positive for *T. orientalis*. The primers used for detection of *Theileria* specific was Small Sub-unit r-RNA (SSrRNA) primer of 1098 bp and for *T. orientalis*- Major Piroplasm Surface Protein (MPSP) primer of 849 bp.

Keywords: PCR, *T. orientalis*, cattle, Pondicherry

Theileriosis is a tick-borne protozoan parasite that multiplies in lymphocyte and erythrocytes, causing hyperthermia and anemia. Bovine benign theileriosis is caused by *T. orientalis* complex, when infected animals were under stress, such as mixed infection with other parasites or viruses; they show severe symptoms and occasionally die. Theileriosis is one of the most economically important diseases of cattle by reducing the milk production and working ability in bullock. Based on DNA sequencing of PCR-amplified regions of the major piroplasm surface protein (MPSP) and/or small subunit of nuclear ribosomal RNA (SSU) genes, the *T. orientalis* complex was subdivided into eight genotypes (type 1- chitose, type 2- ikeda, type 3- buffeli and types 4–8) (Kakuda *et al*., 1998; Gubbels *et al*., 2000). Ikeda and/or chitose genotypes were known to be associated with pathogenic form, whereas other genotypes appear to relate to benign infections in cattle.

MATERIALS AND METHODS

Cattle brought to Large Animal Medicine Unit, TVCC, RIVER, with clinical signs suggestive of theileriosis like anorexia, fever, lethargy, congested / pale mucosa, lymphadenomegaly, bruxism, lameness and tick infestation were screened and included under study group.

Five milliliter of whole blood was collected in 0.5 ml of Acid Citrate Dextrose (ACD) and subjected for DNA isolation. DNA was isolated from the blood collected in Acid citrate dextrose solution as per the standard DNA extraction procedure described below (Sambrook and Russel, 2001). An aliquot of 300 µl of whole blood from each sample was transferred to 2 ml microfuge tubes. Red blood cell lysis buffer (900 µl) was added to each tube and the contents were mixed by inversion. The solution was incubated at room temperature for 10 minutes and was centrifuged (Eppendoff) at 10,000 rpm for 3 minutes. The supernatant was discarded. The pellet was subjected to further lysis till maximum RBC's were removed and the WBC pellets were seen. The supernatant was discarded and the WBC pellet was resuspended in 600 µl of ice cold cell lysis buffer and the suspension was vortexed for 30 seconds. 200 µl of sodium acetate (Sigma) was added to the solution and the content was mixed by vortexing vigourously for 20 seconds. Then it was incubated at

4˚C for 5 minutes and the precipitated protein/ SDS complex was pelleted by centrifugation at 10000 rpm for 3 minutes in room temperature. The supernatant was transferred to a fresh 2 ml centrifuge tube containing 600 µl of isopropanol. The solution was mixed by gentle inversion and then the precipitated DNA was recovered by centrifugation at 10000 rpm for 1 minute at room temperature. The supernatant was removed by aspiration and 600 µl of 70% ethanol was added to DNA pellet and mixed by inversion. The tubes were centrifuged at 10000 rpm for 1 minute at room temperature. The above steps were repeated twice. Finally, the supernatant was removed and the DNA pellet was air dried to remove the traces of alcohol. DNA pellet was dissolved in 100 µl of Tris EDTA buffer (M/s HiMedia, India) and incubated at 65˚C for 15 minutes. The integrity of the isolated DNA was assessed by 1.0 % Agarose Gel Electrophorisis as per the method of Helling *et al*. (1974). The purity and quantity of the DNA was checked as per the method of Sambrook and Russell (2011). Fifty micro-liter of DNA sample was diluted with 450 µl of ultra pure water and its absorbance at 260 and 280 nm (Hitach) were measured. The purity of DNA was assessed from the ratio of absorbance at 260 and 280nm. The samples with acceptable purity (ratio of 1.8 to 2.0) were quantified (µg/ml) by multiplying the absorbance value at 260nm by factor 50. The good quality DNA was stored at -80˚C until use.

PCR was standardized using genus (*Theileria)* specific and species (*T. orientalis)* specific primers as described by Oliveira *et al*. (1995) and Tanaka *et al*. (1993), respectively. The primers were synthesized from M/s Synergy Scientific Pvt. Ltd., Chennai, India and the details of the primers were listed in Table 1.

Since positive control was not available for *Theileria*, field sample which was positive for piroplasms in RBC and Koch blue bodies in the cytoplasm of lymphocytes by peripheral blood smear and lymph node aspiration smear examination respectively was used for standardization. The reaction mixture for genus specific PCR was prepared by adding 20 ng of DNA (~5 µl) and 50 picomoles of each primer with PCR master mix (Amplicon) in a total volume of 20 µl as given in Table 2. Whereas 25 picomoles of each primers (*T. orientalis* specific) were used in the species specific PCR. The reaction mixture was subjected to initial denaturation at 94˚C for 5 minutes followed by 30 cycles of amplification with denaturation at 94˚C for 1 minute, annealing at 55˚C for 1 minute for genus specific PCR and 63˚C for 1 minute for *T. orientalis* specific PCR and extension at 72˚C for 1 minute with final extension at 72˚C for 10 minutes. The amplified products were analyzed in 1.0 % agarose gel electrophoresis using 10 µl of PCR product at 100 volts for 45 minutes along with 100 bp ladder (Invitrogen). The amplicon with 1098 bp and 849 bp for genus specific and *T. orientalis* specific PCR were custom sequenced (n=3) (M/s Synergy Scientific Pvt. Ltd., Chennai, India), respectively. The specificity of the sequence obtained were assessed by align the sequence with that in the Genbank using 'BLAST' (Basic Local Alignment Search Tool) Altschul *et al*. (1990).

Table 1: Details of primer used in PCR assay for detection of theileriosis

Primer	Primer position	Primer sequence	Expected product size (bp)
SSU rRNA gene	278-294	AGTTTCT-GACCTATCAG	1098
Theileria specific (Oliveira *et al*., 1995)	1376-1359	TTGCCTTA-AACTTCCTTG	
MPSP	167-183	CACGCTATGTT-GTCCAAGAG	849
Theileria orientalis (Tanaka *et al*., 1993)	1019-1038	TGTGAGACT-CAATGCGCCTA	

Table 2: Details of reaction mixture used in PCR assay for detection of theileriosis

Composition	Volume
DNA template	20 ng to 50ng
Forward Primer	50 picomol for *Theileria* spp. 25 picomol for *T.orientalis*
Reverse Primer	50 picomol for *Theileria* spp. 25 picomol for *T.orientalis*
Master mix	10 microlitre
Ultrapure water	3 microlitre
Total volume	20 microlitre

One hundred and ten field samples suspected for theileriosis based upon the clinical symptoms were screened for with genus specific primers and those found positive were further subjected to another round of PCR for the identification of species *T. orientalis.*

RESULTS AND DISCUSSION

Out of 110 cattle suspected for theileriosis, 33 cases (33%) showed piroplasm in the peripheral blood smear and/or schizonts in the lymph node aspiration smear.

Agarose gel electrophoresis of PCR product revealed the amplicon of 1098 bp for genus (*Theileria*) specific PCR (Fig. 1) and amplicon of 849 bp for *Theileria orientalis* specific (Fig. 2).

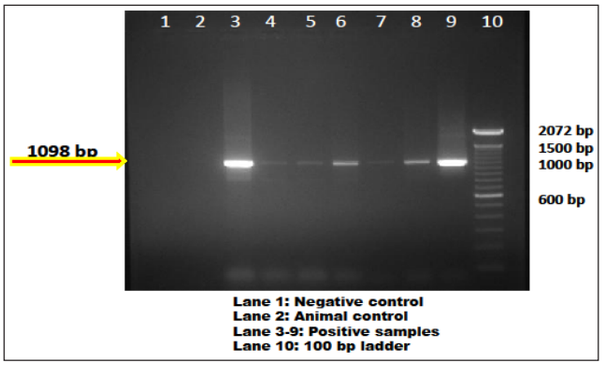

Fig. 1: Agarose gel electrophoresis of PCR product of Theileria spp. Lane 1-Negative control (containing distilled water and Lane 2- Animal control (Healthy animal free of theileriosis), Lane 3 to 9- Positive samples (Animals positive for *Theileria spp***.** showing bands with a base pair size of 1098), Lane 10-100 Base pair ladder (ladder size from 100bp to 2072bp size)

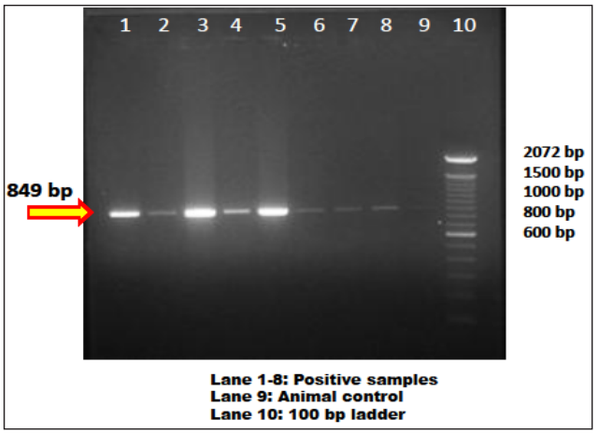

Fig. 2: Agarose gel electrophoresis of PCR product of *Theileria orientalis*. Lane 1 to 8- Positive samples (Animals positive for *Theileria orientalis* showing bands with a base pair size of 849), Lane 9- Animal control (Healthy animal free of theileriosis), Lane 10-100 Base pair ladder (ladder size from 100bp to 2072bp size)

The sequence obtained was subjected to BLAST analysis to confirm the specificity of the amplified as positive control are not available. The genus specific sequence had 94% identity with corresponding sequences for *Theileria* isolates of Shannxi province, China JW-2014 clone 9-7 18S ribosomal RNA gene, partial sequence (GenBank accession number KJ020545.1), Japan-18S ribosomal RNA, partial sequence, isolate: Clifton-41 (GenBank accession number AB520956.1), USA 18S ribosomal RNA type C gene, complete sequence (GenBank accession number U97051.1). *T. orientalis* specific sequence had 99 % identity with corresponding sequences from *T. orientalis* isolates in India (Kerela) Wayanad 1 major piroplasm surface protein-like (MPSP) gene, partial sequence (GenBank accession number JX648207.1 and GenBank accession number HQ444179.1), India (Kerela) Wayanad 4 major piroplasm surface protein-like (MPSP) gene, partial sequence (GenBank accession number JX648209.1

Four hundred and thirteen cases were registered in LAM Unit, TVCC, RIVER during the study period. Out of 110 theileriosis suspected samples, 100 were found positive by *Theileria* specific PCR. Seventy eight of those 100 samples were positive for *T. orientalis*.

Theileriosis caused by *Theileria orientalis* is the most common infectious disease affecting cattle which is of veterinary importance worldwide (Radostits *et al.*, 2010). *T. orientalis* infection can be diagnosed routinely by direct microscopic examination of Leishman-stained blood smears and lymph node aspiration smear which requires expertise and time consuming (more number of samples). By this method detection of organism (*T. orientalis*) in carrier cattle was difficult. So advances molecular technique (PCR) used to detect pathogens directly, specifically, and sensitively. Among 413 animals that were enrolled to Large Animal Medicine Unit of TVCC, one hundred and ten animals were presented with clinical manifestations suggestive of theileriosis. A total of 100 (24.21%) cattle were found positive either by peripheral blood smear and/or polymerase chain reaction which is in agreement with Keles *et al*. (2001), Kamau *et al*. (2011) and Yu *et al*. (2011) who reported that theileriosis in cattle was endemic in tropical regions, parts of Australia and north east China, whereas Chaisi *et al*. (2013) and Aktas *et al*. (2006) reported an incidence of *Theileria orientalis* was 5.8% and 7% in South Africa and eastern Turkey respectively, in exotic cattle. The 100 samples found

positive for theileriosis, 78 % were caused by *T. orientalis*. This shows that *T. orientalis* was found to be the most prevalent species of *Theileria* in Pondicherry region. Those samples found negative by PCR were also negative by smear examination (n=10) and at the same time those sample found positive by smear examination were also positive by PCR. This shows that PCR was found to be more sensitive and specific for the detection of *Theileria* in the present study which is in concurrence with the findings of Dumanli *et al*. (2005) and Ghanem *et al*. (2013) who reported that PCR is more reliable, accurate and sensitive method for detection of theileriosis than other methods like blood film examination and serological methods (ELISA and IFAT). Similarly, Aktas *et al*. (2006) and Fard *et al*. (2013) reported PCR assay in diagnosing *Theileria* parasites in carrier cattle was more sensitive ($P<0.05$) than smear preparation method and suggested that it can be used in epidemiological studies. However, Parthiban *et al*. (2010) and Shahnawaz *et al*. (2011) reported that PCR is used for detection of *Theileria* both in blood samples and ticks than the smear method.

CONCLUSION

In conclusion, this study has revealed that the existing species of *Theileria* in Pondicherry was found to be *Theileria orientalis* and the incidence of theileriosis in the present study was 24.21 per cent. PCR was found to be more sensitive method in the diagnosis of theileriosis in cattle compared with blood smear and lymph node aspiration smear examination.

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