Molecular and pathological studies on natural cases of bovine theileriosis

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Abstract The present communication is a part of study conducted on 32 cases of bovine lymphadenopathies. Out of which, six cases of bovine theileriosis were diagnosed on the basis of peripheral blood smear examination and gross lesions in lymph nodes and visceral organs. Nested PCR using two primer sets N516/N517 and 14136/294 was conducted on samples collected from affected lymph nodes confirms *Theileria annulata* infection in five out of six cases. Sequencing analysis of amplified product showed 92, 94 and 93 % homology of isolate TH1_Bovine, TH2_Bovine and THEN_Bovine for *T. annulata* with *T. annulata* Tamilnadu and Pant Nagar. An upregulation of Th2 cytokines in the cases of theileriosis was observed as the level of TNF- α in individual animals varies from higher value (1028 pg/100 μ g protein) to as low as 500 pg/100 μ g protein. An increase in level of Th2 cytokines IL-4 and IL-10 was also observed. The present study concluded that pathological studies and cytokine analysis of lymph nodes are of paramount importance in disease diagnosis and associated immune response of the animal with lymphadenopathies.

Keywords Bovine lymphadenopathies · Nested PCR · Th1/Th2 immune response · *Theileria annulata*

Introduction

Theileriosis is a tick borne blood protozoan disease caused by various *Theileria* species affecting cattle and buffaloes (Brown 1990). *Theileria annulata* is mainly responsible for heavy economic losses and results in high mortality, morbidity as well as production losses in domestic animals in tropical and subtropical regions (Brown 1990; Uilenberg 1995). In India, *T. annulata* is predominantly transmitted by *Hyalomma anatolicum anatolicum* in dairy animals (Tuli et al. 2015). The affected animals show fever, anaemia, enlargement of lymph nodes, anorexia and progressive loss of body weight (Gill et al. 1977; Aulakh and Singla 2006).

Theileria annulata has a biphasic life cycle. First phase involve arthropod vector, whereas second phase occur in domesticated and wild ruminants (Mehlhorn and Schein 1984). The protozoan parasite enters the bovine host during tick feeding as sporozoites, which multiply as schizonts in mononuclear leukocytes. The merozoites release from leukocyte and invade erythrocytes to develop into piroplasms (d'Oliveira et al. 1997). The diagnosis of theileriosis is usually made by direct detection of piroplasms and schizonts in blood smear and/or lymph node smears along with clinical signs of disease like high fever and enlargement of lymph nodes (Tuli et al. 2015). Molecular studies like conventional PCR and nested PCR are being used in these days to differentiate clinical and subclinical form of infection (d'Oliveira et al. 1995, 1997).

The present study was focused on diagnosis of natural cases of bovine theileriosis using conventional pathological

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and molecular techniques. Cytokine expression (Th1 and Th2) in affected lymph nodes was studied to find the correlation between Th1/Th2 immune responses with cytopathology of lymph nodes in bovine theileriosis.

Materials and methods

Present study was conducted on six suspected cases of theileriosis in cattle. These animals were presented to the clinics with history of fever over 104 °C, anemia, loss of body weight and alteration in various hematological parameters. Giemsa stained blood smears were examined for the presence of parasites. Due to grave condition these animals have died and brought to the post mortem hall of Department of Veterinary pathology, GADVASU, Ludhiana. Tissue samples of affected lymph nodes and associated visceral organs were collected from these animals. Impression smears were prepared from affected lymph nodes and stained with Wright's staining technique (Jain 1986) for cytological examination. A part of collected tissue samples was fixed in 10 % Neutral buffered formalin for routine histopathological examination and other part was kept at −20 °C for molecular studies and cytokine estimation. Formalin fixed tissues were processed for paraffin wax embedding, sectioned at 4–5 µm and stained with haematoxylin and eosin (H&E) for routine histopathology (Luna 1968).

DNA extraction

DNA was extracted from affected lymph nodes by using QI Amp DNA Tissue Kit (Qiagen). Micro tubes containing the extracted DNA were stored at −20 °C till further use.

PCR assay

Molecular detection of theileriosis was done by nested PCR (d'Oliveira et al. 1995, 1997) (Table 1). Briefly, the PCR reaction mixture (25 µl) was made by using 12.5 µl of ready to use Taq PCR Master Mix, 2× (that contains 2.5 units Taq DNA Polymerase, 200 µM of each dNTPs, 1.5 mM MgCl₂, 1× QIAGEN PCR buffer), A reaction volume of 25 µl was made containing 12.5 µl of Taq PCR

Master Mix, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 0.5 µl of nuclease free water and 10 µl of DNA template for primary PCR. In nested PCR, reaction volume of 25 µl was made containing 12.5 µl of Taq PCR Master Mix, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 7.5 µl of nuclease free water and 2 µl of the primary PCR product. Thermal cycling conditions was performed using an automatic thermal cycler (Eppendorf, Germany) with an initial denaturation of 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. PCR products were analysed by agarose gel electrophoresis and visualized in gel-documentation system (Alpha Innotech, San Leandro, CA).

Nucleotide sequence analysis for *Theileria annulata*

Amplicon of the Nested PCR products were custom sequenced from Xcelris Genomics, Ahmedabad, India. The nucleotide sequences were subjected to blast analysis (Altschul et al. 1990) for determining the similarity with the sequence present in the nucleotide database. Phylogenetic analysis was conducted by using DNA STAR software.

Cytokine estimation

Cytokine ELISA was done on lymph node tissue samples which were kept at −20 °C by using commercially available ELISA kits as shown in Table 2. Tissue pieces of affected lymph nodes from suspected cases of theileriosis along with three normal animals, which served as control, was thawed, triturated, mixed with 1 ml PBS and centrifuged at 10,000 rpm for 20 min (4 °C). The supernatant was collected and stored it at −20 °C till further use.

Results

The diagnosis of theileriosis was confirmed by observing various forms of theileria piroplasms in the erythrocytes (Fig. 1). On necropsy, the peripheral and visceral lymph nodes were generally found enlarged and punched out

Table 1 Primers used in PCR for detection of *Theileria annulata* from affected lymph nodes (d'Oliveira et al. 1995, 1997)

PCR	Primer	Sequence	Product size (bp)
Primary PCR	N516 (forward)	5'GTAACCTTTAAAAACGT3'	721
	N517 (reverse)	5'GTTACGAACATGGGTTT3'	
Nested PCR	14136 (forward)	5'AAGACCCTTAAGGTCGGAGACAAGA3'	572
	294 (reverse)	5'GTCGACAACCTGGTTTGTATC3'	

Table 2 Various ELISA kits used for estimation of IL-2, IL-4, IL-10, INF- γ and TNF- α

Cytokine	ELISA kit
IL-2	Raybio [®] Bovine IL-2 ELISA Kit
IL-4	Thermo Scientific Bovine IL-4 Screening Kit
IL-10	Cusabio ELISA Kit
INF- γ	Thermo Scientific Bovine INF- γ ELISA Kit
TNF- α	Raybio [®] Bovine TNF- α ELISA Kit

Fig. 1 Blood smear (inlet) showing *Theileria annulata* piroplasms in RBC's. Giemsa stain, Bar 20 μ m

ulcers along with haemorrhages and necrotic foci were observed in abomasal mucous membrane (Figs. 2, 3) besides pale mucous membranes and petechial haemorrhages on various visceral organs. Impression smears from affected lymphnodes showed presence of normal lymphocytes along with lymphoblasts and occasional plasma cell (Fig. 4).

Histopathologically, lymph nodes from affected animals revealed lymphocytic proliferation (Fig. 5) along with infiltration of plasma cells and macrophages (Fig. 6). In addition, lymph node tissue section showed presence of schizonts of *T. annulata* either extracellularly or intracellularly within many lymphocytes and macrophages (Fig. 7). The mucosal epithelium of abomasum showed multifocal areas of erosion, necrosis and ulceration along with infiltration of lymphocytes, plasma cells and macrophages in the lamina propria adjacent to these lesions (Fig. 8).

Due to low sensitivity of primary PCR using primer set N516/N517, the required amplification was not achieved in the present study. However, second round of amplification using internal primer set 14136/294 yielded product of size

Fig. 2 Photomicrograph showing enlargement of prescapular lymph node **Fig. 3** Photomicrograph showing characteristic punched out ulcers in abomasum

572 bp (Figs. 9, 10). The nested PCR was able to detect *T. annulata* in five out of six cases diagnosed clinically.

The sequence of three isolates was analysed by DNA STAR and Clustal Omega Software for multiple sequence alignment and phylogenetic tree (dendrogram). Sequence analysis was carried out with the help of Edit Seq programme (DNA STAR Lasergene version 6 Software). The phylogeny analysis, homology and divergence with other

Fig. 4 Prescapular lymph node: impression smear showing presence of normal lymphocytes and lymphoblasts. Wright stain, Bar 20 μm

Fig. 7 Prescapular lymph node: photomicrograph showing Koch blue bodies in lymphocytes. H&E stain, Bar 20 μm (color figure online)

Fig. 5 Prescapular lymph node: photomicrograph showing depletion with focal lymphocytic proliferation. H&E stain, Bar 100 μm

Fig. 8 Abomasum: photomicrograph showing superficial necrosis with deep ulcer H&E stain, Bar 200 μm

Fig. 6 Prescapular lymph node: photomicrograph showing lymphocytic proliferation. H&E stain, Bar 20 μm

reported sequences from India and elsewhere was done by DNA STAR software. The genomic sequence over a 348, 259 and 291 nucleotide region of the amplicon that was obtained from bovine lymph node tissue specimen (TH1_Bovine, TH2_Bovine and THEN_Bovine) has been deposited with GenBank (accession no: KT275245, KT275246 and KT275247).

Phylogenetic analysis suggests that these isolates (accession no: KT275245, KT275246 and KT275247) belonged to *T. annulata* Tams1 gene. Further, these isolates were genetically most closely related to bovine theileriosis isolates previously reported from Tamilnadu (JX648210.1) and DDN Pant Nagar (KM061794.1) nucleotide sequence similarity (Fig. 11).

In the present study, there was significantly ($p \leq 0.05$) decrease in TNF- α and INF- γ also showed decrease

Fig. 9 Amplification in nested PCR for *T. annulata*. Lane 1 DNA ladder 100 bp plus. Lane 2 negative control. Lane 3–8 samples

Fig. 10 Amplification in nested PCR for *T. annulata*. Lane 1 DNA ladder 100 bp plus. Lane 2–6 samples. Lane 7 negative control

response, whereas an increase in IL-2, IL-4 and IL-10 was observed (Fig. 12). The level of TNF- α in individual animals varies from higher value (1028 pg/100 μ g protein) to as low as 500 pg/100 μ g protein.

Discussion

Giemsa stained blood smear from infected animals revealed the presence of *T. annulata* piroplasms in erythrocytes. The gross and cytopathological changes observed in lymph nodes, abomasum and other visceral organs in the present investigation of field cases of bovine theileriosis were similar to those describe previously by

Gill et al. (1977), Sandhu (1996) and Tuli et al. (2015). Tuli et al. (2015) also examined Giemsa stained thin blood smears immediately after blood collection and observed microscopically different forms of *Theileria* parasite in the erythrocytes. However, blood picture showed mixed infection with *Babesia* and *Anaplasma* species from similar bovine blood samples which occurred most frequently in bovines. Similarly, a mixed infection of *T. annulata* with *Anaplasma marginale* was observed in few cases in the present investigation.

The necropsy findings observed in the affected animals in the present investigation were in agreement with Oryan et al. (2013). It has been reported that abomasal epithelium showed multifocal necrotic areas with erosions and ulcers. Enlarged, edematous and haemorrhagic lymph nodes along with presence of petechial and ecchymotic haemorrhages in the mucosal and serosal surfaces were the consistent findings in the present investigation. Oryan et al. (2013) also reported histological change i.e. infiltration of lymphocytes, plasma cells and macrophages in lymph node, peyer's patches and spleen as observed in the present study in affected lymph nodes. Abomasal mucous membrane showed numerous multifocal areas of erosion, necrosis and ulceration. Submucosal areas and lamina propria showed hyperemia, haemorrhages and were heavily infiltrated with lymphocytes, plasma cells, macrophages and eosinophils. Similarly, Hassan et al. (2012) reported reticulo-endothelial hyperplasia, reactive follicular hyperplasia, marked enlargement of germinal centers and slight increase in interfollicular lymphoid tissue within cortical and paracortical regions and attributed these lesions to proliferation of both infected and non-infected T-cells. Similarly in the present study, Koch's blue bodies were observed in the proliferating lymphocytes as well as extracellularly in the affected lymph nodes. The molecular studies were carried out for detection of *T. annulata* using nested PCR as described by d'Oliveira et al. (1997) that yielded an amplicon at 572 bp, confirmatory to the presence of *T. annulata* infection in the lymph nodes of five of the six affected animals.

Theileria annulata causes often fatal leuco-proliferative disorder, tropical theileriosis in the ruminant host animals, which is the result of widespread dissemination and proliferation of cytokine secreting, parasite-infected cells. In the present study, as compared to control, there was significantly decreased in TNF- α and INF- γ also showed decrease response whereas increase in IL-2, IL-4 and IL-10. There is increasing evidence to suggest that proinflammatory cytokines produced by *T. annulata* infected cells play a central role in both pathology and immune evasion. *T. annulata* induced production of various T cell stimulatory and inflammatory cytokines which may be responsible for immunopathological symptoms observed

Fig. 11 Phylogenetic analysis of *T. annulata*

during infection. McKeever et al. (1997) also showed upregulation of IL-10 and IL-2 which may influence immune response of cattle to challenge. Cytokines produced early in immune response determine the nature of ultimate T-cell response (Dayne's et al. 1990; O'Garra and Murphy 1994). However, Brown et al. (1995) showed increased response of IL-2, IL-4, IL-10, TNF- α and INF- γ which showed contrary results with our TNF- α and INF- γ results.

In the present study the level of TNF- α in individual animals varies from higher value (1028 pg/100 μ g protein) to as low as 500 pg/100 μ g protein. Brown et al. (1995) suggested that cells infected with *T. annulata* produce high levels of inflammatory cytokines especially TNF- α which

was a potent source of fever. INF- γ mainly produced from *T. annulata* stimulated T-cells and not from infected cells.

Further, in the present study there were increased level of Th2 cytokines IL-4 and IL-10. Th2 cytokines confer protection to infections. IL-10 can have a direct effect on monocyte-macrophages, limiting the production of proinflammatory cytokines, and can act directly on CD4+ T cells, inhibiting proliferation and production of IL-2, INF- γ , IL-4, IL-5, and TNF- α . As IL-10 was found to stimulate growth of parasitised cells, and responding lymph node cells have upregulated mRNA, IL-10 production may result in potentiation of the growth of parasitised cells in vivo (McKeever et al. 1997). IL-10 has also been shown to suppress Th1 T cell responses in other species.

Fig. 12 Correlation of Th1 and Th2 cytokines with cytopathology of lymph nodes in bovine theileriosis

TNF- α and IL-2 could play a role in the pathogenesis of *Theileria* infections by promoting the proliferation of macroschizont-infected cells and the associated lymphoid hyperplasia (Preston et al. 1992). The ulcerative lesions associated with tropical theileriosis may be due to this proinflammatory cytokines (Forsyth et al. 1999). It has also been stated that the clinical symptoms of tropical theileriosis (anorexia, pyrexia, cachexia and anaemia) are characteristic of those induced by TNF- α .

Yamada et al. (2009) indicated that high protozoan load and resulting intense inflammatory responses might be involved in the severity of clinical signs observed in *T. parva*-infection. They observed that mRNA expressions of IL-1 β , IL-6 and IL-10 were up-regulated with protozoan DNA load increasing while the down-regulation or no detectable levels of the expression of type-1 cytokines, such as IL-2 and interferon (IFN)-gamma were observed in *T. parva*-infected animals.

The difference in the pattern of cytokine profile in the present study from that of other may be due to either the use of different techniques to estimate cytokines, samples used like blood/tissues/cell line, the chronicity of the disease, experimental/natural or in vitro infection, time and condition of sample collection (living/dead animal).

Theileria species appeared unique among various intracellular protozoan parasite, as it existed within cytoplasm of macrophages and induced its replication. In contrary with our results Brown et al. (1995) showed that cells infected with *T. annulata* produce high levels of inflammatory cytokines especially TNF- α , which is a potent inducer of fever and also leads to anemia, muscle wasting and necrosis. *T. annulata* leads to production of

various T cell-stimulatory and inflammatory cytokines which are responsible for immunopathological lesions observed during infection. Increased production of IL-10 which showed similar findings with our results leads to reduced efficiency of immune response against infected cells (Brown et al. 1995).

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Aulakh GS, Singla LD (2006) Clinico-haematobiochemical observations on bovines naturally infected with *Theileria annulata*. *J Vet Parasitol* 20:49–52
- Brown CGD (1990) Control of tropical theileriosis (*Theileria annulata* infection) of cattle. *Parassitologia* 32:23–31
- Brown DJ, Campbell JD, Russell GC, Hopkins J, Glass EJ (1995) T-cell activation by *Theileria annulata*-infected macrophages correlates with cytokine production. *Clin Exp Immunol* 102:507–514
- d'Oliveira C, van der Weide M, Jacquet P, Jongejan F (1997) Detection of *Theileria annulata* by the PCR in ticks (Acari: Ixodidae) collected from cattle in Mauritania. *Exp Appl Acarol* 21:279–291
- d'Oliveira CM, van der Weide M, Habela MA, Jacquet P, Jongejan F (1995) Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *J Clin Microbiol* 3:2665–2669
- Daynes RA, Araneo BA, Dowell TA (1990) Regulation of murine lymphokine production in vivo. III. The lymphoid tissue microenvironment exerts regulatory influences over T helper cell function. *J Exp Med* 171:979–996
- Forsyth LMG, Minns FC, Kirvar E, Adamson RE, Hall FR, McOrist S, Brown CGD, Preston PM (1999) Tissue damage in cattle infected with *Theileria annulata* accompanied by metastasis of cytokine-producing, schizont-infected mononuclear phagocytes. *J Comp Pathol* 120:39–57

- Gill BS, Bhattacharyulu Y, Kaur D (1977) Symptoms and pathology of experimental bovine tropical theileriosis (*Theileria annulata* infection). *Ann Parasitol Hum Comp* 52:597–608
- Hassan AH, Salmo NA, Jabbar, Ahmed S (2012) Pathological and molecular diagnostic study of theileriosis in cattle in Sulaimaniyah province, Iraq. 2012. In: Proceeding of the eleventh veterinary scientific conference, pp 306–314
- Jain NC (1986) Veterinary hematology. In: Jain NC (ed) Schalm's veterinary hematology, 4th edn. Lea and Febiger, Philadelphia
- Luna LG (1968) Manual of histologic staining methods of Armed Forces Institute of Pathology, 3rd edn. The Blakiston Div., McGraw – Hill Book Company, New York
- McKeever DJ, Nyanjui J, Ballingall KT (1997) In vitro infection with *Theileria parva* is associated with IL-10 expression in all bovine lymphocyte lineages. *Parasite Immunol* 19:319–324
- Mehlhorn H, Schein E (1984) The piroplasms: life cycle and sexual stages. *Adv Parasitol* 23:37–103
- O'Garra A, Murphy K (1994) Role of cytokines in determining T-lymphocyte function. *Curr Opin Immunol* 6:458–466
- Oryan A, Namazi F, Sharifiyazdi H, Razavi M, Shahriari R (2013) Clinicopathological findings of a natural outbreak of *Theileria annulata* in cattle: an emerging disease in southern Iran. *Parasitol Res* 112:123–127
- Preston PM, Brown CG, Richardson W (1992) Cytokines inhibit the development of trophozoite-infected cells of *Theileria annulata* and *Theileria parva* but enhance the proliferation of macroschizont-infected cell lines. *Parasite Immunol* 14:125–141
- Sandhu GS (1996) Histopathological, biochemical and haematological studies in crossbred calves suffering from experimental tropical theileriosis. MSc thesis, Punjab Agricultural University, Ludhiana, India
- Tuli A, Singla LD, Sharma A, Bal MS, Filia G, Kaur P (2015) Molecular epidemiology, risk factors and hematochemical alterations induced by *Theileria annulata* in bovines of Punjab (India). *Acta Parasitol* 60:378–390
- Uilenberg G (1995) International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol* 57:19–41
- Yamada S, Konnai S, Imamura S, Simuunza M, Chembensofu M, Chota A, Nambota A, Onuma M, Ohashi K (2009) Quantitative analysis of cytokine mRNA expression and protozoan DNA load in *Theileria parva*-infected cattle. *J Vet Med Sci* 71:49–54