Indian Journal of Animal Sciences **82** (8): 812–817, August 2012

Examination of cross-reactivity among *Trypanosoma evansi, Theileria annulata* and *Babesia bigemina* by Te-mAB-LAT and PCR

REEMA[1], S S CHAUDHRI[2] and A SINGH[3]

Lala Lajpat Rai University of Veterinary and Animal Sciences, Uchani, Karnal, Haryana 132 001 India

Received: 22 October 2010; Accepted: 14 April 2012

ABSTRACT

Blood and serum samples from 55 crossbred cows detected positive for haemoprotozoan infections (*T. evansi*-11, *T. annulata*- 23 *and B. bigemina*- 21) on examination of stained blood smears were collected and stored for *T. evansi* antigen detection monoclonal antibody- based latex agglutination test (Te-mAb-LAT) and polymerase chain reaction (PCR). *Trypanosoma evansi* mAb-LAT revealed circulating antigens in serum samples of 18 animals, including 11 confirmed positive for *T. evansi*, 3 confirmed positive for *T. annulata* and 4 confirmed positive for *B. bigemina*. The DNA fragments of the size 227 bp, 175 bp and 721 bp were amplified by PCR assays using specific primers for *T. evansi, B. bigemina* and *T. annulata,* respectively. The animals confirmed positive by stained smear examination for specific haemoprotozoa (*T. evansi/ B. bigemina/ T. annulata*) were also positive with specific PCR assay (*T. evansi,* Te-PCR/ *B. bigemina*, Bb-PCR/ *T. annulata*, Ta-PCR). The Te-PCR was observed free from any cross-reactivity with *T. annulata/ B. bigemina* whereas Ta-PCR revealed 5 of 21 *B. bigemina* infected samples positive for *T. annulata* and Bb-PCR detected 1 out of 11 *T.evansi* infected samples and 12 of 23 *T. annulata* infected samples positive for *B. bigemina*. It indicated that some of the animals had mixed infections of *T. annulata* and *B. bigemina* which is a common occurrence due to mixed infection of their vectors feeding on animals. Secondly, it was possible to have missed detection of low level of infection on microscopic examination of the stained blood smears.

The detection of circulating antigens in 7 blood samples (3 of *T. annulata* and 4 of *B. bigemina* infected animals) with Te-mAb-LAT, without their detection by Te-PCR was possibly due to previous exposure of these animals with *T. evansi* and subsequent treatment with trypanocidal drug. It is inferred that Te-mAb-LAT, being simple to perform, rapid, convenient and cost-effective could be quite suitable for diagnosis of trypanosomosis at field level and PCR being sensitive and specific has its utility at the laboratory level.

Key words: *Babesia bigemina*, Cross-reactivity, *Theileria annulata*, *Trypanosoma evansi*, Monoclonal antibody-based antigen detection test, Polymerase chain reaction

Trypanosoma evansi causing trypanosomosis, commonly called 'Surra', is responsible for intermittent fever, oedema of dependent parts, loss of body weight, anaemia, decreased production and abortions in animals (Lohr *et al*. 1986 and Pathak and Singh 2005). Immunoassays have been developed to detect circulating antigens of *T. evansi* in the blood of infected animals and later the specificity of these assays improved with the use of monoclonal antibodies, mAb (Kashiwazaki and Thammasart 1998, Rayulu *et al.* 2009). Polymerase chain reaction (PCR) using specific primers was developed for the detection of specific genomic DNA fragments of haemoprozoan infections (D'Oliveira *et al.* 1995, Singh *et al.* 2007 and Shyma 2009) in cattle.

In India, an mAb based latex agglutination test (LAT) with immense field applicability was developed to detect *T. evansi* antigens (Rayulu *et al.* 2007 and 2009). The present study was carried out to detect cross-reactivity of mAb developed against cell membrane antigen of Indian isolate of *T. evansi* with *T. annulata* and *Babesia bigemina*.

MATERIALS AND METHODS

Blood samples for specific diagnosis were collected from crossbred cows showing clinical signs suggestive of haemoprotozoan infections and brought for treatment to Veterinary Unit, CCS Haryana Agricultural University, Regional Research Station, Karnal from April 2009– January 2010. Blood samples were collected in vials with and without anticoagulant (EDTA, 2mg/ml of blood). The samples with

Present address:[1]Veterinary Assistant Surgeon (reemayourfriend @yahoo.co.in), 284/21, Bhagat Singh Colony, Narwana, Jind, Haryana.

[2]Senior Scientist cum Incharge (chaudhri_sumer3 @rediffmail.com), [3]Professor and Head (adjitprinik@msn.com), Department of Veterinary Microbiology.

anticoagulant were used for the detection of *T. evansi*, *T. annulata* and *B. bigemina* parasites by blood smear examination. The parasite-positive blood and serum samples (*T. evansi*-11; *T. annulata*- 23 and *B. bigemina*- 17) were stored in sterilized vials at –70°C until further use for Te-mAb-LAT and PCR.

Parasitological examination: Blood smears were prepared from blood with anticoagulant and stained with Giemsa (1:10) for 20 min. The smears were examined using microscope under 400× and 1000× magnification for identification of haemoprotozoan parasites (*T. evansi, T. annulata* and *B. bigemina*). The blood samples with anticoagulant were also examined for the presence of *T. evansi* by microhematocrit test (MHCT) as per Woo and Rogers (1974).

Monoclonal antibody based latex agglutination test for detection of T. evansi *antigens*

The mAb, which were prepared and cryopreserved by Rayulu *et al*. (2007), were obtained from Immunology Section, Department of Veterinary Microbiology, CCS HAU, Hisar. These antibodies were used in different dilutions (1:2 and 1:10) in PBS, pH 8.0, to find out the optimum dilution that was able to agglutinate the *T. evansi* antigens after coating with latex beads. This test was optimized and carried out as per Rayulu *et al*. (2009).

Formation of agglutination within 10 min were categorized as positive for circulating antigens of *T. evansi* and the positive reactions were graded as follows:

++++ agglutination within 2 min; +++ agglutination within 5 min; ++ agglutination within 8 min; + agglutination within 10 min.

Polymerase chain reaction for detection of T. evansi, T. annulata *and* B. bigemina

Polymerase chain reaction (PCR) was standardized for detection of T. evansi, T. annulata and B. bigemina in blood samples by DNA amplification by using HPLC purified specific primers, with details shown in Table 1.

Extraction of genomic DNA of parasites: The parasite-positive blood samples were used for extraction of genomic DNA with some modification of the method described by Sambrook and Russell (2001).

Briefly, 1 ml of the blood sample was thoroughly mixed with 10 ml lysis buffer (10 mM Tris-HCl, pH 8.0– 5mM EDTA, pH 8.0 –0.2% SDS, 20 µg/ml RNase in ultra pure water) and incubated for 1 h at 37°C. After incubation, proteinase K at a final concentration of 100 µg/ml was added and incubated at 50°C for 3 h in a water bath with intermittent shaking. Equal volume of phenol: chloroform: isoamyl alcohol (P: C: I, 25:24:1) was added in the above solution and thoroughly mixed for one minute. The tube was centrifuged at 3000 × g for 10 min at room temp (RT) and upper aqueous phase was collected in a clean centrifuge tube. The aqueous phase was once more treated with equal volume of PCI and then once with equal volumes of chloroform: isoamyl alcohol (C:I, 24:1). Upper aqueous phase was finally collected after centrifugation at 3000 × g for 5 min at RT. One-tenth volume of 3M sodium acetate pH 5.2 was added to upper aqueous phase and DNA was precipitated by adding equal volume of ice-cold isopropanol and incubating it at 4°C overnight. The DNA pellet was obtained by centrifugation at 4000 × g for 15 min at RT. The pellet was then mixed with 250 µl of tris-HCl buffer, pH8.0 and 250 µl of CI and stirred for 1 min. Later, it was centrifuged at 10 000× g for 3 min. The aqueous phase was removed and taken in an eppendorf tube (1.5 ml). One-tenth volume of 3M sodium acetate, pH 5.2 was added in aqueous phase and DNA was precipitated by adding twice the volume of absolute alcohol for 15 min at 4°C. Later, it was centrifuged at 10 000 × g for 10 min. The pellet was washed with 300 µl of 70% ethanol and re-centrifuged at 10 000 × g for 5 min. The pellet so obtained was dried under vacuum for 5 min and suspended in 50 µl of 10 mM tris-HCl, pH8.5 buffer and stored at –70°C until further use. DNA from field blood samples was also extracted with commercially available genomic DNA extraction kit according to the manufacturer's instructions.

Optimization of Te-PCR, Bb-PCR, Ta-PCR and Th-PCR

PCR using specific primers was performed for

Table 1. Details of *T. evansi, T. annulata* and *B. bigemina* specific primers for PCR

Primer designation	Sequence	Tm (°C)
TE-FOR (Forward)	5'-TGCAGACGACCTGACGCTACT-3'	64.52
TE- REV (Reverse)	5'-CTCCTAGAAGCTTCGGTGTCCT-3'	64.54
TA-FOR (Forward)	5'-GTAACCTTTAAAAACGT-3'	46.00
TA-REV (Reverse)	5'-GTTACGAACATGGTTT-3'	52.70
TH-FOR (Forward)	5'-AGTTTCTGACCTATCAG-3'	45.70
TH-REV (Reverse)	5'-TTGCCTTAAACTTCCTTG-3'	55.30
BB-FOR (Forward)	5'-TGTCCTCGTTTGCTTCTTAGGGGACTCCT-3'	73.80
BB-REV (Reverse)	5'-CCGACACGATGCACACTAACATTACCCAA-3'	75.60

amplification of 227, 175 and 721 bp fragments for *T. evansi, B. bigemina* and *T. annulata* as per Wuyts *et al.* (1994) and standardized by Shyma (2009), Figueroa *et al*. (1992) and D'Oliveira *et al*. (1995), respectively.

Primers annealing temperatures for Bb-PCR and Ta-PCR were optimized. Primers concentration of 1.0 µM, template DNA 10 µl, 200 µM each dNTP and 2 units of Taq DNA polymerase with 10 × PCR buffer containing 15 mM $MgCl_2$ were used. The reaction was carried out in 50 µl volume in thin-walled 0.2 ml PCR tube for 30 cycles in a thermocycler using three different annealing temperatures with the following steps in thermal cycling conditions.

I. DNA denaturation, 95°C for 4 min; *II*. DNA denaturation at 95°C for 1 min; *III*. Primer annealing (Te and Bb), 55°C–60–65°C for 1 min and for (Ta) 45°C-50–55°C for 1 min and 45°C-55–65°C for 1 min; *IV.* Primer extension, 72°C for 1 min; *V.* Denaturation, 95°C for 1 min; *VI.* Annealing**-** (Te and Bb) 55°C-60–65°C for 1 min and for (Ta) 45°C-50–55°C for 1 min and (Ta, Te and Bb) 45°C-55–65°C for 1 min; *VII.* Extension, 72°C for 10 min; *VIII.* Hold, 4°C for indefinite time. The steps II to IV were repeated for 29 cycles and step V to VII once as the last cycle (i.e. 30^{th}) and then the tubes were held at 4°C. The PCR tubes were stored at –70°C until analysis of PCR products by agarose gel electrophoresis (AGE).

Agarose gel electrophoresis (AGE) for PCR products: The PCR products were analyzed by AGE in 2% agarose in tris-borate-EDTA, pH 8.3 (TBE) buffer and run at 75 volts according to Sambrook and Russell (2001). The DNA fragments were stained with 0.5 µg/ml ethidium bromide, visualized in a UV-transilluminator for DNA band of 227 bp (*T. evansi*), 175 bp (*B. bigemina*), 721 bp (*T. annulata*) size and photographed using a digital camera.

Depending on the intensity of ethium bromide stained PCR products visible in the agarose gel, samples were categorized as follows: ++++ very strong positive; +++ strong positive; ++ weak positive; + very weak positive.

Table 2. Optimized conditions for Ta-PCR, Te-PCR, Bb-PCR

Reagents	Final concentration	Volume/ 50 µl
Nuclease free water	-	31.6
I0 × PCR buffer containing 15 mM $MgC1_2$	1 ×	5
dNTPs mix, each 10 mM	200µM	1
Primers		
Forward: Te-FOR (10µM)	200nM	1
Reverse : Te-REV (10µM)	200nM	1
Forward: Ta-FOR (10µM)	200nM	1
Reverse : Ta-REV (10µM)	200nM	1
Forward: Bb-FOR (10µM)	200nM	1
Reverse : Bb-REV (10µM)	200nM	1
Template DNA	-	10
Dream Taq DNA polymerase (5U/µl) (fermentas)	2 Units	0.4

RESULTS AND DISCUSSION

The examination of sera samples of 55 confirmed parasite-positive crossbred cows for *T. evansi* (n,11)*, T. annulata* (n, 23) and *B. bigemina* (n,21) with mAb based Te-LAT revealed the presence of circulating antigens of *T. evansi* in all *T. evansi* infected animals. In addition, 3 *T. annulata* (T-1, 4 and 11) and 4 *B. bigemina* (B-1, 2, 5 and 10) infected cattle were declared positive for *T. evansi* circulating antigen by Te-LAT (Tables 3, 4 and 5). Possible reasons for this observation could be the limits of diagnostic specificity of Te-LAT or probability that these *T. annulata* and *B. bigemina* -positive sera samples were collected from the animals having *T. evansi* circulating antigen because of actual *T. evansi* infection sometime in the past and the parasites cleared with trypanocidal drug treatment before sample collection. Earlier studies have shown that *T. evansi* antigen remained in circulation even after 4 weeks of treatment with specific trypanocidal drug (Thammasart *et al*. 2001, Wernery *et al*. 2001, Singh and Chaudhri 2002). Therefore, to make Te-LAT and all other Ag- detecting tests more dependable, it would be necessary to obtain reliable history of anti-trypanosome treatment of the animal during few weeks prior to sample collection.

The PCR of *T. evansi* positive samples by using all the, 3 sets of Te, Ta and Bb specific primers revealed that all the 11 samples were positive with Te-specific primers (Fig. 1, Lane

Table 3. Screening of *Trypanosma evansi* parasite- positive crossbred cattle with latex agglutination test (Te-LAT) and polymerase chain reaction (PCR) using *T. evansi, T. annulata* and *B. bigemina* specific primers

Sample no.	Diagnostic tests			
	Te-LAT	Te-PCR	Bb-PCR	Ta-PCR
Tr-1	+++	+++	-ve	-ve
Tr-2	++++	++++	-ve	-ve
Tr-3	++	+++	-ve	-ve
Tr-4	++	++++	-ve	-ve
Tr-5	+++	+++	-ve	-ve
Tr-6	++	++	-ve	-ve
Tr-7	+	++	-ve	-ve
Tr-8	+	++	-ve	-ve
Tr-9	+++	++	-ve	-ve
Tr-10	+++	++++	-ve	-ve
Tr-11	+	++	++	-ve
Total	11	11	11	1
0				

Te-LAT, *T. evansi*, specific monoclonal antibody based latex agglutination test; Te-PCR, PCR using T. *evansi*- specific primers; Bb-PCR, PCR using *B. bigemina*-specific primers and Ta-PCR, PCR using *T. annulata*, specific primers.

Table 4. Screening of *Theileria annulata*- positive crossbred cattle samples with Te-LAT and PCR

Sample no.	Diagnostic tests			
	Te-LAT	Te-PCR	Bb-PCR	Ta-PCR
T-1	+	++	+	-ve
T-2	-ve	++	-ve	-ve
T-3	-ve	++++	-ve	-ve
T-4	++	+++	-ve	-ve
T-5	-ve	++	+	-ve
T-6	-ve	+++	-ve	-ve
T-7	-ve	+	+++	-ve
T-8	-ve	++++	-ve	-ve
T-9	-ve	+++	-ve	-ve
T-10	-ve	+++	-ve	-ve
T-11	+	++++	++	-ve
T-12	-ve	++++	-ve	-ve
T-13	-ve	++	-ve	-ve
T-14	-ve	+	+++	-ve
T-15	-ve	+++	-ve	-ve
T-16	-ve	+	+++	-ve
T-17	-ve	+	+++	-ve
T-18	-ve	++	++++	-ve
T-19	-ve	++	-ve	-ve
T-20	-ve	++	++	-ve
T-21	-ve	++	++	-ve
T-22	-ve	+	++++	-ve
T-23	-ve	++++	++	-ve
23	3	23	12	0

Te-LAT: *T. evansi,* specific monoclonal antibody based latex agglutination test; Te-PCR, PCR using T. *evansi*- specific primers; Bb-PCR, PCR using *B. bigemina*-specific primers and Ta-PCR, PCR using *T. annulata*, specific primers.

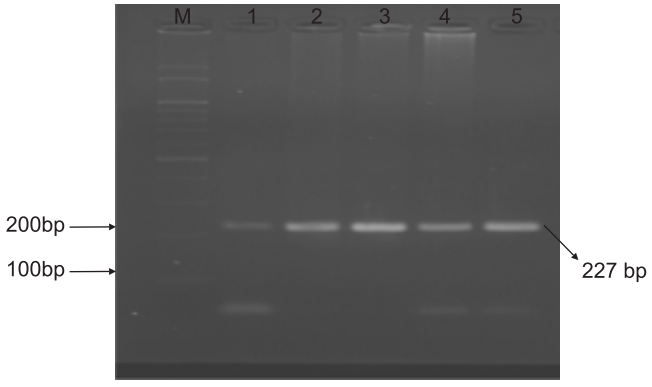

Fig. 1. Te-PCR product of 227 bp size as resolved by agarose gel electrophoresis using 2% agarose in 0.5X TBE. [Lane 1: 100 bp DNA ladder (Fermentas, USA); Lane 1: Positive control showing 227 bp product; Lane 2–5: positive field samples]

2 to 5) and none positive with Ta- specific and only 1 (Tr-11) was positive with Bb-specific primers. It means that all *T.evansi* infected animals were free from *T. annulata* and *B.bigemina* except 1 animal, which was positive for *B.bigemina* specific band. The positivity of this animal with

Table 5. Screening of *B. bigemina*- positive crossbred cattle samples with Te-LAT and PCR

Sample no.	Diagnostic tests			
	Te-LAT	Te-PCR	Bb-PCR	Ta-PCR
B-1	+	++	- ve	- ve
B-2	+	+++	+	- ve
B-3	-ve	++	- ve	- ve
B-4	-ve	++	- ve	- ve
B-5	++++	++++	+	- ve
B-6	-ve	++++	- ve	- ve
B-7	- ve	++++	- ve	- ve
B-8	- ve	++++	- ve	-ve
B-9	- ve	++++	+	- ve
B-10	++	++++	- ve	- ve
B-11	- ve	++++	+	- ve
B-12	- ve	++++	++	-ve
B-13	- ve	++++	- ve	- ve
B-14	- ve	+	- ve	- ve
B-15	- ve	++	- ve	- ve
B-16	- ve	++++	- ve	- ve
B-17	- ve	++	- ve	- ve
B-18	- ve	++	- ve	- ve
B-19	- ve	++	- ve	- ve
B-20	- ve	++	- ve	- ve
B-21	- ve	+++	- ve	- ve
21	4	21	5	0

Te-LAT, *T. evansi,* specific monoclonal antibody based latex agglutination test; Te-PCR, PCR using T. *evansi*- specific primers; Bb-PCR, PCR using *B. bigemina*-specific primers and Ta-PCR, PCR using *T. annulata*, specific primers.

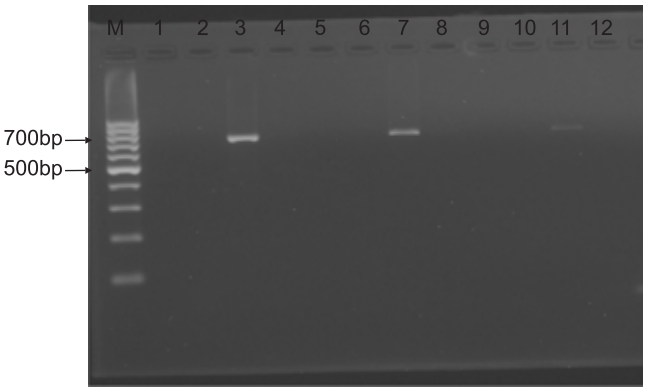

Fig. 2. Ta-PCR product of 721 bp size as resolved by agarose gel electrophoresis using 2% agarose in 0.5X TBE; [Lane 1: 100 bp DNA ladder; Lane 3, 7 and 11 positive field samples showing 721 bp products]

B. bigemina was due to mixed infection of *T. evansi* and *B. bigemina*, which was detected on a later stage when this animal exhibited typical clinical signs of babesiosis.

All the 23 blood samples obtained from *T. annulata* positive crossbred cows were declared positive using Ta-specific primers in PCR (Table 4, Fig. 2) and none was positive by Te-specific primers. However, analysis of these

Table 6. Cross-reactivity examination of hemoprotozoa by Te-LAT and PCR

Type of infection	Sample no.	Diagnostic tests		
		Te-PCR	Bb-PCR	Ta-PCR
T. annulata	T-1	-ve	+	++
T. annulata	T-4	- ve	- ve	+++
T. annulata	T-11	- ve	+	++++
B. bigemina	B-1	- ve	++	- ve
B. bigemina	B-2	- ve	+++	+
B. bigemina	B-5	- ve	++++	+
B. bigemina	B-10	- ve	++++	- ve
	7	0	6	5

Te-LAT, *T. evansi,* specific monoclonal antibody based latex agglutination test; Te-PCR, PCR using T. *evansi*- specific primers; Bb-PCR, PCR using *B. bigemina*-specific primers and Ta-PCR, PCR using *T. annulata*, specific primers.

samples with Bb-specific primers in PCR exhibited *B. bigemina* specific band of 175bp size in 12 samples (T-1, 5, 7, 11, 14, 16 to 18, and 20 to 23). It could be possibly due to mixed infection of *T. annulata* and *B. bigemina* missed on microscopic examination.

The blood samples from 21 *B. bigemina*-positive cross-bred cattle were confirmed positive by Bb-specific primers in PCR (Table 5; Fig. 3) and none was positive by Te-specific PCR, indicating lack of any cross-reaction between these 2 parasites. However, 5 of these samples were positive by Ta-specific primers for *T. annulata* (B-2, B-5, B-9, B-11, B-12). The above reason of mixed infections could also be valid for this observation. Mixed infections of *T. annulata* and *B. bigemina* are common and have been reported from several countries where vectors of both the parasite species co-infest the animals (García-Sanmartin *et al.* 2006, Ica 2007).

Seven serum samples, 3 *T. annulata* positive and 4 *B. bigemina* positive, were declared positive by *T. evansi* antigen-detecting Te-LAT (Table 6), but were negative by Te-specific primers in PCR (Fig. 4A). These Te-mAb-LAT-positives, Te-PCR negative samples were most probably free

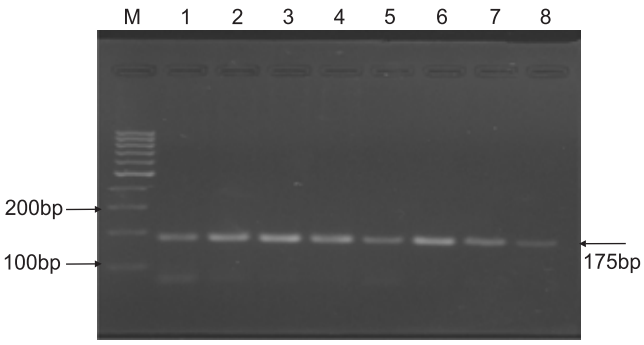

Fig. 3. Bb-PCR product of 175bp size as resolved by agarose gel electrophoresis using 2% agarose in 0.5X TBE; [Lane M: 100 bp DNA ladder; (Lane 1: *B. bigemina* positive control), 2–8: positive field samples with Bb -primers showing 175 bp product]

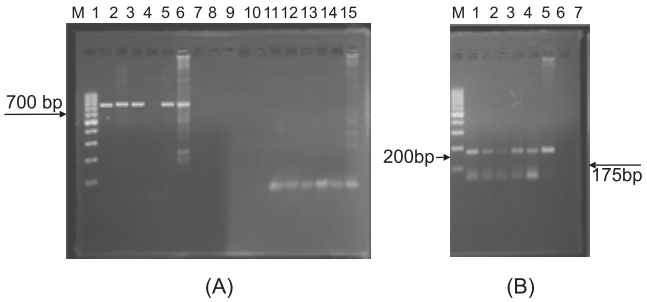

Fig. 4. Agar gel electrophoresis with 2% agarose in 0.5X TBE, at 75V for 2hrs for *B. bigemina* and *T. annulata* positive samples with *B. bigemina*, *T. annulata* and *T. evansi* primers; [Lane M: 100 bp DNA ladder; Lane: 1-7: Te-LAT positive field sample with Ta primers showing 721 bp product, Lane 9–15: Te-LAT positive field sample with Te- primers, (B) Lane M: 100 bp DNA ladder (Fermentas, USA); Lane 1-7: Te-LAT positive samples with Bb-primers showing 175 bp product.

from *T. evansi* parasites. Further PCR assay of DNA samples from these animals by using Ta-specific primers revealed *T. annulata* specific bands in 5 (3 *T. annulata* and 2 *B. bigemina* infected animals) (Fig. 4A). The PCR assay of these animals by using Bb-specific primers showed the presence of *B. bigemina* specific band of 175 bp size in all the animals except one (i.e. T–4) infected with *T. annulata* (Table 6, Fig. 4 B). It indicated that 4 out of 7 animals had mixed infection with both the hemoprotozoa, which were either missed on microscopic examination of stained blood smears or 1 of the 2 infections were subclinical at the time of collection of blood.

It could therefore be concluded that blood samples from 55 crossbred cows with Te-PCR, Ta-PCR and Bb-PCR by using specific primers did not reveal any cross-reactivity of *T. evansi* with *T. annulata* and *B. bigemina*. The cross-reactivity observed between *T. annulata* and *B. bigemina* was suggestive of the mixed infections which seemed to have been missed on microscopic examination or due to subclinical infection. Secondly, *T. annulata* and *B. bigemina* samples detected positive by Te-LAT were probably due to actual presence of circulating antigen because of previous exposure of these animals with *T. evansi* and treatment with trypanocidal drug prior to sample collection.

REFERENCES

D'Oliveira C, Weide MV, Habela M A, Jacquiet P and Jongejan F. 1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *Journal of Clinical Microbiology* **33**: 2665–69.

Figueroa J V, Chieves L P, Johnson G S and Buening G M. 1992. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. *Journal of Clinical Microbiology* **30**: 2576–582.

García-Sanmartín J, Nagore D, García-Pérez A L, Juste R and Hurtado. 2006. Molecular diagnosis of *Theileria* and *Babesia* species infecting cattle in Northern Spain using reverse line blot macroarrays. *BMC Veterinary Research* **2**: 16.

Ica A, Vatansever Z, Yildirim A, Duzlu O and Inci A. 2007.

Detection of *Theileria* and *Babesia* species in ticks collected from cattle. *Veterinary Parasitology* **148**: 156–60.

Kashiwazaki Y and Thammasart S. 1998. Effect of anti-immunoglobulin antibodies produced in cattle infected with *Trypanosoma evansi* on antigen detection ELISA. *International Journal of Parasitology* **28**: 1353–60.

Lohr K F, Pholpark S, Siriwan P, Leesirikul N, Srikitjakarn L and Staak C. 1986. *Trypanosoma evansi* infection in buffaloes in north-east Thailand. 11. Abortions. *Tropical Animal Health and Production* **18**: 103–08.

Mugittu K N, Silayo R S, Majiwa P A O, Kimbita E K, Mutayoba B M and Miscue R. 2001. Application of PCR and DNA probes in the characterization of trypanosomes in the blood of cattle in farms in Morogoro, Tanzania. *Veterinary Parasitology* **94**: 177–89.

Pathak K M L and Singh N. 2005. Animal trypanosomosis. *Intas Polivet* **6**: 194–99.

Rayulu V C, Singh A and Chaudhri S. S. 2007. Monoclonal antibody based immunoassays for detection of circulating antigens of *Trypanosoma evansi* in buffaloes. *Italian Journal of Animal Sciences* **6**(8): 907–10.

Rayulu V C, Chaudhri S S and Singh A. 2009. Evaluation of parasitological and monoclonal antibody based assays in detection of *Trypanosoma evansi* infection in animals. *Indian Journal of Animal Sciences* **63**(9): 978–81.

Sambrook J and Russell D W. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd edn. Vol. 1 Cold Spring Harbour Lab Press. New York, USA.

Shyma, K.P. 2009. 'Comparison of monoclonal antibody based latex agglutination test with PCR for diagnosis of *Trypanosoma evansi* in domestic animals.' M V Sc. thesis, CCS Haryana Agricultural University, Hisar, India.

Singh A and Chaudhri S S. 2002. Comparison of efficiency of parasitological methods with Ag-ELISA in *Trypanosoma evansi* infected crossbred calves. *Indian Journal of Animal Sciences* **72** (1)*:* 117–19.

Singh H, Mishra A K, Rao J R and Tewari A K. 2007. A PCR assay for detection of *Babesia bigemina i*nfection using clotted blood in bovines. *Journal of Applied Animal Research* **32**: 201–02.

Thammasart S, Kanitpun R, Saithasao M and Kashiwazaki Y. 2001. Preliminary studies by ELISA on the antigen and antibody with *Trypanosoma evansi* in cattle. *Tropical Animal Health and Production* **33**: 189–99.

Wernery U, Zachariah R, Mumford J A and Luckins T. 2001. Preliminary evaluation of diagnostic tests using horses experimentally infected with *Trypanosoma evansi. Veterinary Journal* **161**: 287–300.

Woo P T K and Rogers D. 1974. A statistical study on the sensitivity of the haematocrit centrifugation technique in the detection of trypanosomes in blood. *Transactions of Royal Society of Tropical Medicine and Hygiene* 68: 319–26

Wuyts N, Chodesajjawatee, N and Panyim S. 1994. A simplified and highly sensitive detection of *Trypanosoma evansi* by DNA amplification. *Southeast Asian Journal of Tropical Medicine and Public Health* **25**: 266–71.