Indian J. Anim. Res., **48** (1) : 31-37, 2014
DOI- 10.5958/j.0976-0555.48.1.007

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com

DETECTION AND MANAGEMENT OF LATENT INFECTION OF *TRYPANOSOMA EVANSI* IN A CATTLE HERD

M.S. Bal, Amrita Sharma, Ashuma, Balwinder Kaur Batth, P. Kaur and L.D. Singla*

Department of Veterinary Parasitology,
Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, India

Received: 30-11-2012 Accepted: 01-09-2013

ABSTRACT

A study was conducted to evaluate the sensitivity of PCR based method for detection of *T. evansi* and to know its efficacy of corresponding trypanocidal treatment. A total of 101 random blood samples collected from the dairy farm were examined for the presence of *Trypanosoma evansi* infection. Polymerase Chain Reaction (PCR), using 21/22 mer primers, targeting repetitive sequence probe pMuTec 6.248 to yield 227 bp products was employed. The PCR detected 46.5% animals positive for *T. evansi* infection in comparison to only 6.9% detected by microscopy using Giemsa stained blood smears. Treatment of infected animals with Triquin (quinapyramine sulphate and chloride) showed improvement in general body condition within a period of two weeks. Results of PCR were also negative for *T. evansi* in all the post treatment blood samples. Subsequently all the 1400 animals at farm were treated with Triquin. After a period of one month, significant increase in levels of RBC, Hb and HCT and return of the values of BUN and TBIL of treated animals towards normal endorsed the effectiveness of the treatment.

Key words: Cattle, Detection, Latent, Management, Surra, *Trypanosoma evansi*

INTRODUCTION

Among all the trypanosomal infections caused by pathogenic trypanosomes, trypanosomosis ('Surra') caused by vector-borne haemoprotozoa, *Trypanosoma evansi* has the widest range of hosts and geographical distribution. The impact of clinical and sub-clinical form of this mechanically transmitted disease by biting flies such as tabanids (Claes *et al.*, 2004) is difficult to assess (Desquesnes *et al.*, 2013). Dairy animals, especially bovines, which are bearing great production stress along with other unknown and uninvited diseases, are potential viable host to the infection. Treatment of trypanosomosis in field conditions is done on the basis of clinical signs and demonstration of parasites in the blood, sometimes accompanied by haemato-biochemical tests. But the diagnosis of subclinical infection is far difficult as this disease is characterized by fluctuating parasitaemia with periods of paroxysms and intermission. Giemsa-stained blood smear examination (BSE) has a low sensitivity, equivalent to 10^5 trypanosomes/ml. Thus, in cases of subclinical infection and cryptic nature of the parasitaemia, it is often difficult to demonstrate parasites in the blood. Thus more than 50 to 80 % of the infections remain undetectable by direct microscopy. Even with the improved parasitological techniques for diagnosis, a high proportion of trypanosome infections remain undetected (Luckins, 1992). Clinical signs of the disease are also non-specific and not sufficiently pathognomonic (Juyal *et al.*, 2005). Although in endemic areas where the treatment is to be administered on herd basis, even a test with low sensitivity will suffice, yet it provides inadequate information about the epidemiology and magnitude of the disease in the particular geographical area, making monitoring and strategic control difficult.

As conventional parasitological techniques (CPT) cannot be relied upon for disease diagnosis and chemotherapy, therefore, it is imperative to use a test which is more sensitive and reliable. The polymerase chain reaction (PCR) based assays permit identification of haemoprotozoa at levels far

* Corresponding author's e-mail: ldsingla@gmail.com, ldsingla@rediffmail.com

below the detection limit of the commonly used parasitological techniques (Sharma *et al.*, 2013). Hence, in the present study, PCR assay has been successfully employed to reveal the incidence of subclinical *T. evansi* infection and to test the trypanocidal drug sensitivity in a dairy cattle herd of Punjab state using *T. evansi* specific primers.

MATERIALS AND METHODS

Herd History: There were reports of death of four calves with general morbidity and reduced feed uptake at military dairy farm, Jalandhar, Punjab. Farm has a total of 1400 animals (cattle) including adult crossbred cows and calves. The farm was surrounded by open paddy fields. Body weight gain of the calves was not up to the farms standards. Additionally, three calves died just prior to the visit of experts from the University. The calves, reported undulating pyrexia ranging from 103-104º F, pale mucous membrane, diarrhoea, chacexia, pale mucus membranes and difficulty in respiration, were kept in separate sheds. Few adult animals were also reported with the history of pyrexia and respiration difficulty at the time of sampling. Two of the adult cows were exhibiting signs of corneal opacity and persistent pyrexia.

Stage 1 Sample Collection: Blood samples of the animals were collected from the month of May to September, 2011, the period of peak summer and monsoon. Random sampling of the animals in the farm was done to rule out the cryptic low grading daily performance of the farm animals and their occasional mortality. A total of 101 whole blood samples of 33 calves (less than one year of age) and 68 adults (more than one year of age), were randomly collected in EDTA and plain vacutainers, for haematological and biochemical evaluation.

Stage 2 sample collection: Animals found positive for *T. evansi* infection by both BSE and PCR were treated with Triquin (quinapyramine sulphate and chloride; Wockhardt Limited).Ten samples were collected post treatment to evaluate the status of infection by PCR in the treated animals.

Stage 3 sample collection: After the treatment of whole of the herd (1400 animals), 25 samples were again randomly collected for PCR assay. These samples were also evaluated for their haemato-biochemical profiles, which were then compared with those of stage 1 samples.

Microscopic examination: Giemsa stained thin blood smears were examined under microscope for the presence of any haemoprotozoan parasites (Kelly, 1979). Morphological and micrometric measurements of parasites were carried out on microscope fitted with micrometry unit by using Software DPZ-BSW (OLYMPUS). Parasitic load was calculated by counting number of trypanosomes/µl of blood for assessing the status of infection in the animals.

Molecular diagnosis

DNA Extraction: Genomic DNA was extracted from the blood samples by using the protocol of HiPura® Blood Genomic DNA Kit (HiMedia).

Polymerase Chain Reaction: The PCR reactions were performed by using 21mer forward primer (5'TGCAGACGACCTGACGCTACT3') and 22mer reverse primer (5' CTCC TAGAA GCTTCGGTGTCCT3') targeting repetitive sequence probe pMuTec 6.248 (Wuyts *et al.*, 1995) in genomic DNA of *T. evansi*. The reaction mixture contained 2U of Taq Polymerase, 1X PCR Buffer (Tris/HCl), 1.5 mM $MgCl_2$, 200 µM dNTPs and 0.5 µM of each primer in a 50 µl reaction mixture. The reactions were carried out in Eppendrof thermal cycler. Initial denaturation was carried out at 94 º C for 5 minutes to completely denature the DNA. It was followed by 30 cycles of denaturation at 94 º C for 30 seconds, annealing at 60º C for 30 seconds and extension at 72 º C for 30 seconds. Subsequent, extensive polymerization was done at 72 º C for 7 minutes.

Detection of PCR amplified product: The PCR products were analyzed on 1.5 % agarose gel stained with ethidium bromide (0.5 µg/ml). The electrophoresis was carried out at 70 V (45- 55 minutes). Along with the PCR products, gene ruler DNA™ ladder 100 bp plus (Fermentas) was also run. DNA bands were visualized under UV light and were photographed using gel documentation system (Bio Rad). The molecular size of PCR product was estimated.

Haemato-biochemical analyses: The sera from blood were separated after centrifugation at 5000 rpm for 10 min and were stored at- 20°C until use for estimation of biochemical parameters. Serum biochemistry *viz* ALKP(U/L), GLU(mg/dl), AST(U/L), ALB (g/dl), BUN (mg/dl), TP (g/dl), TBIL (mg/dl), ALT(U/L), CRSC (mg/dl) and GLO (g/dl) was done with the help of Vet Scan VS2 chemistry analyzer. The complete haemtological profile of blood collected in EDTA coated vacutainers *viz,* WBC (X

10^3cells/ µl), RBC (X 10^6 cells/ µl), Hb (g/dl), HCT (%), MCV (fL), MCH (pg), MCHC (g/dl) and PLT (X 10^3cells/ µl)) was obtained by automatic haemocytometer (ADVIA 2120, SIEMENS Heath Care Diagnostics TNc. Deerfield, IL, USA).

Statistical analysis: Data was analyzed using Win Episcope 2.0 software, (Diagnostic test agreement) and Statistical Package for Social Sciences (SPSS for windows version 11.0.1 SPSS INC, Chicago, Illinois) to work out the agreement between blood smear examination technique and PCR based molecular technique. ANOVA was applied to find out the variance in the data obtained in different groups of animals.

RESULTS AND DISCUSSION

Stage 1:

Microscopy: Microscopic examination of 101 thin blood smears revealed presence of *T. evansi* in only 7 cases depicting a detection rate of 6.9% (Table1). All the affected animals were adults having average 15% trypanosomes/High Power Field with an average parasitaemia of 49.01 X 10^4 tryps/µl. Micrometric measurements revealed average size of parasite to be 25.16± 1.4 µm. One of the cows having typical sign of bilateral corneal opacity (Fig.1a and b) had little stumpy and coiled trypanosomes in the blood smear. Average size of parasite in this case was also within the average length range of *T. evansi* 15-34 µm and clinical signs are similar as documented by Bhatia *et al.*, (2006).

Polymerase Chain Reaction (PCR): Analysis of PCR products revealed specific 227 bp band in positive cases (Fig. 2). PCR results revealed 47 samples (46.5 %) positive for *T. evansi* infection (Table-1). Based on intensity/brightness/thickness of the product band (227 bp), samples could be categorized as strong positive, moderate positive and weak positives (Fig. 3). However, most of the samples tested showed weak band of 227 bp for *T. evansi* by PCR, especially the parasitologically negative samples. All parasitologically positive samples exhibit mild to strong positive band. Shyma (2009) observed weak positive bands by PCR (21/22-mer primers) in case of some parasitologically positive samples from cattle and buffaloes probably due to PCR inhibitors in the reaction. There was slight agreement between two tests *viz.* blood smear examination (BSE) and PCR (kappa value 0.158) indicating PCR to be much sensitive test as compared to the former (Table-1). PCR was able to detect more positive samples as compared to CPT. Omanwar *et al.*, (1999) detected 3 (15%) positive out of 20 parasitological negative blood samples from camel in Rajasthan by using 21/22-mer primers. Mugittu *et al.*, (2001) compared PCR and CPT (buffy coat and blood smears examination) for diagnosis of trypanosomosis in cattle in Tanzania. PCR was able to detect trypanosomal DNA in 27 (43%) of the 62 parasitologically negative samples. Holland *et al.* (2001) reported PCR to be highly sensitive test as compared to parasitological tests in experimentally infected water buffaloes. Various research workers observed high sensitivity of PCR as compared to CPT in case of camels, donkeys, dogs and mice (Ravindran *et al.*, 2008; Shyma, 2009; Fernandez *et al.*, (2009). observed a very high

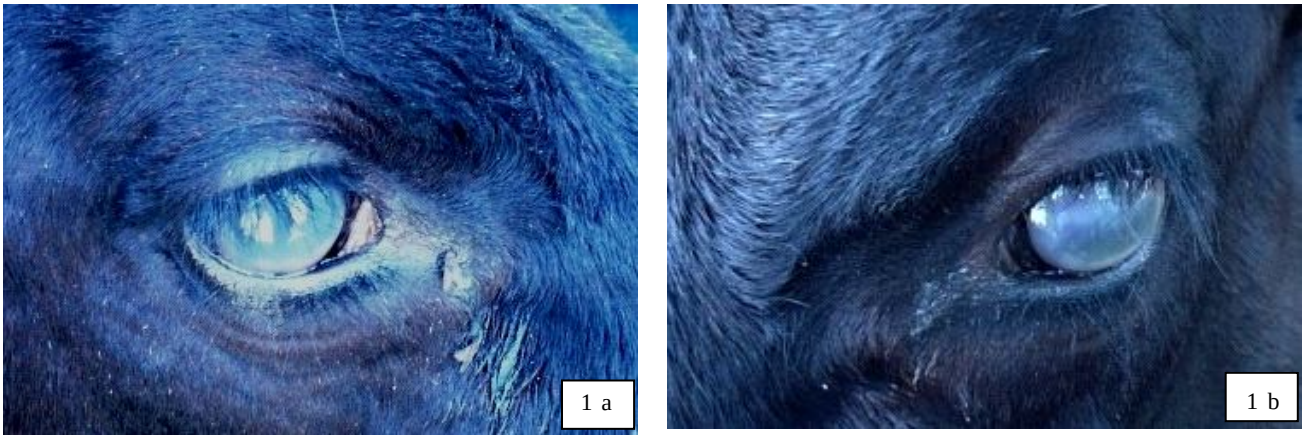

FIG. 1: Cows with typical sign of bilateral corneal opacity: 1 a. Right eye; 1 b Left eye

TABLE 1: Prevalence of *T. evansi* by conventional blood smear examination (BSE) and Polymerase Chain Reaction (PCR) based molecular diagnosis.

Test	Total Samples	Positive samples	Detection percentage %
Blood smear Examination (BSE)	101	7 (0^{C}+ 7^{A})	6.9
PCR	101	47(19^{C}+ 28 ^{A})	46.53 (40.4% ^{C} + 59.6% ^{A})

^{C}= calves; ^{A}= adults

Agreement between blood smear examination (BSE) test with PCR: Kappa value = 0.158 (0.058 to 0.158) at 95% CI (Slight Agreement)

prevalence (60.49%) of *T. evansi* by PCR as compared to 1.95 % by wet blood film in domestic animals *viz.* cattle, buffaloes and equines. So, molecular diagnosis especially PCR based tests should be employed in combination with conventional methods for screening of animals for trypanosomosis. Early detection of subclinical/carrier stage of infection and subsequent treatment helps in prevention of any serious outbreak at the farm especially during months of year with abundance of vector population for spread of disease.

Out of the 47 positive cases, 19 were calves and 28 were adult cows, revealing age wise prevalence of 40.4% and 59.6%, respectively. Most of the animals (70.2%) were harboring subclinical form of trypanosomosis as depicted by general decrease in body condition and weakness, only 27.7% depicted intermittent/low grade fever, weakness, emaciation and oedema; and 2.1 % showed corneal opacity. The clinical symptoms are in agreement with Hilali *et al.* (2006) and Singla *et al.* (1996**)**. The farm is located beside open field and there was dense vegetation around it. This epizootology is remarkably suitable for the breeding of tabaniid flies, one of the important vectors in trypanosome transmission.

Haematobiochemical Changes: Biochemical changes in the infected animals (Table-2) depicted increase in the level of AST in *T. evansi* infected animals with 8 animals (24.24%) having much higher range than the normal (Kahn *et al.*, 2005b). BUN value of the treated animals also increased significantly. TBIL of 66.6% animals was in higher range than the normal (Kahn *et al.*, 2005b). The increase in BUN and TBIL is due to the damage caused by the trypanosomes during pathogenesis that results in degeneration of tissue of the visceral organs. The total bilirubin of the infected calves was increased as a result of enhanced erythrocytic destruction due to hemolysin and membrane injury. Increase in BUN may be due to tubular degeneration, interstitial nephritis and mononuclear infiltration of the renal glomeruli (Hilali *et al.*, 2006). Normocytic normochromic type anemia was observed due to decrease in values of Hb, RBC and

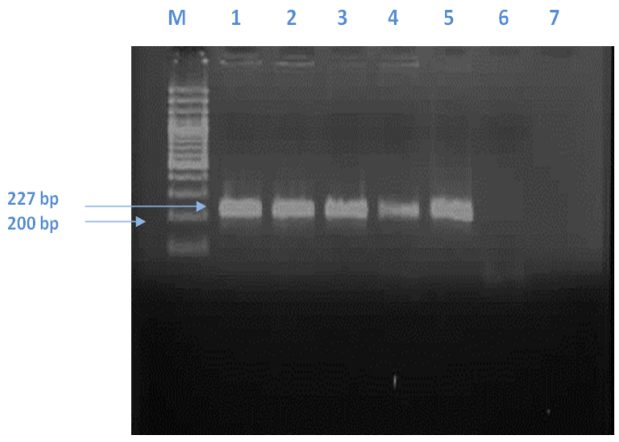

FIG.2: Gel electrophoresis of PCR products. Lane M: Gene Ruler/molecular weight marker (100 bp), 1,2,3,4: Positive samples, 5: Positive control (DNA from host cell free trypanosomes), 6: Negative control and 7: Negative sample using 21/22mer primer (product size 227 bp).

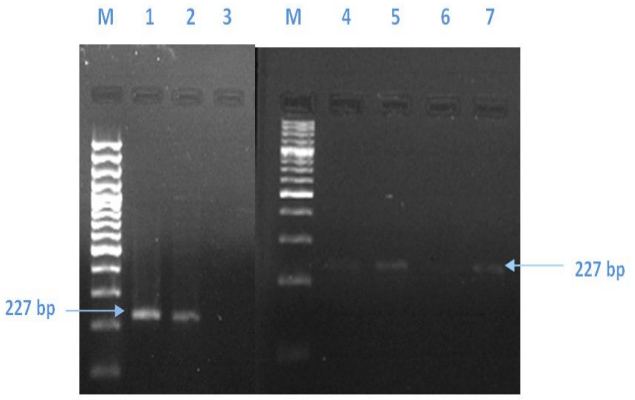

FIG. 3: Band patterns of strong positive (1), mild positive samples(2) and weak positive (4,5,7) samples after gel electrophoresis using 21/22 mer set of primers, 3,6: Negative samples, M: Gene Ruler/molecular weight marker-100 bp.

TABLE 2: Biochemical parameters in *T. evansi* infected animals (as detected by PCR) before and after treatment

	Parameter	ALKP	GLU	AST	ALB	BUN	TP	TBIL	ALT	CRSC	GLO
Before treatment (a)	Total samples tested = 33	65.84 ±40.26	37.36 ±19.71	110.09 ±126.9	2.51 ±0.49	16.63* ±4.92	7.76 ±1.41	1.01* ±0.44	24.90 ±13.12	0.66 ±0.243	5.25 ±1.145
	No. of animals below NR	4	20	4	20	0	3	0	1	13	2
	No. of animals within NR	28	13	21	13	30	12	11	28	20	8
	No. of animals above NR	1	0	8	0	3	18	22	4	0	22
After treatment (b)	Total samples tested= 8	51.625 ±21.71	34.37 ±10.01	64.62 ±26.07	2.7 ±0.38	10.25* ±3.84	6.96 [a] ±1.90	0.63* ±0.49	22.3 ±9.85	0.58 ±0.294	4.26 ±2.09
	No. of animals below NR	0	6	2	3	2	2	0	0	4	2
	No. of animals within NR	8	2	6	5	6	4	6	7	4	2
	No. of animals above NR	0	0	0	0	0	2	2	1	0	4
Kahn *et al.*, 2005b	Normal range (NR)	18-153	42-75	45-110	2.8-3.9	7.8-25	6.2-8.2	0.0-.8	6.9-35	0.6-1.8	2.9-4.9

Alkaline phosphatase (ALKP), Glucose(GLU), Aspartate aminotransferase (AST), Albumin(ALB), Blood Urea Nitrogen(BUN), Total Protein (TP), Total Bilirubin (TBIL), Alanine aminotransferase (ALT), Creatinine(CRSC) and Globulin (GLO)
Values are shown as Mean ± standard deviation
* indicates values varying significantly($P< 0.05$)

PCV (Table-3). The injury to the erythrocytes may be due to the lashing action of the trypanosomes. The increase in the level of total protein was also observed. Hyperproteinemia may be due to increase in immunoglobulin level, which may be attributed to the development of the antibodies. Similar biochemical findings were reported by Singla *et al.* (2000) and Kaur and Juyal (2003) in cow calves. It may be due to release of potentially pathogenic biological and chemical toxins by trypanosomes which are responsible for lysis of erythrocytes or due to the inhibition of haemopoetic system.

Treatment: Although, parasitologically only 6.9 per cent animals were found positive, however, situation was alarming when PCR was applied. By the time dairy in-charge could be informed and the treatment could be attempted, trypanosomosis led to mortality of 5 adult cows and 3 calves. Consequently, all the animals positive either by PCR or BSE were treated with trypanocidal drug- Triquin (quinapyramine sulphate and chloride**;** Wockhardt Limited) @ 5mg/kg body weight s/c.

Stage 2: All animals positive for *T. evansi* infection were treated with Triquin. A total of 10 post treatment blood samples were randomly collected to evaluate the efficacy of the drug. The parasitemia was cleared within a period of two weeks, as all the 10 samples were found negative by PCR (Fig. 4) showed that Triquin is effective. After successful treatment and improvement in condition of diseased animals, same treatment was recommended for all the animals at the farm. Subsequently all the 1400 animals of the farm were treated with Triquin (quinapyramine sulphate and chloride) @ 5mg/kg body weight s/c. In one of our previous studies, quinapyramine sulphate and chloride combination (Triquin) was found to be more effective drug against trypanosomiasis as compared to isometamedium chloride (Kumar *et al.*, 2012)

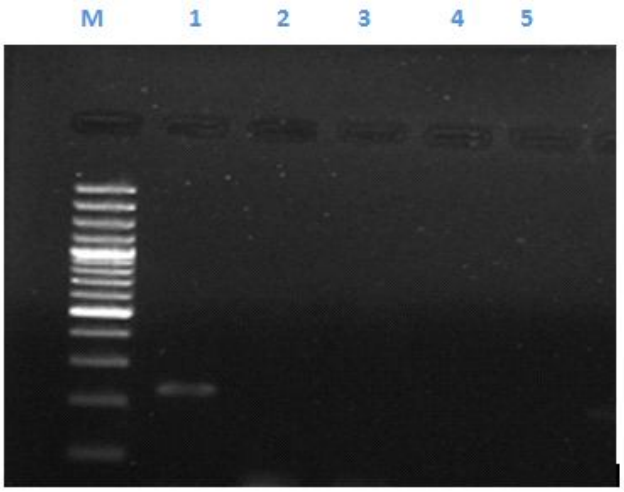

FIG. 4: Showing negative samples (2, 3, 4, 5) after gel electrophoresis of PCR products from blood samples of treated animals. Lane M: Gene Ruler/molecular weight marker (100 bp), 1: Positive control (DNA from host cell free trypanosomes), 5: Negative control using 21/22mer primer (product size 227 bp

Stage 3: After one month, twenty five samples were again randomly collected and were subjected to haemato-biochemical parameters and PCR assays. All the tested samples were negative by PCR assay in treated animals.

Haematobiochemical Changes: Biochemical changes in the treated animals depicted that the level of BUN and TBIL started decreasing and were within normal range, one month post treatment. Similarly other parameters also turned towards normal, except for glucose, which was towards lower side in 6 (75%) animals, however glucose level was lower in 20 (60.6%) animals before treatment (Table 3). Decrease in glucose levels in the sub clinically infected animals may be due to utilization of glucose by parasite. Significant increase in the levels of Hb, RBC and PCV was observed one month post treatment. Along with this, platelet count was towards lower side in 7 (14.9%) animals where as it was within normal range in all the 24 (100%) animals after treatment.

TABLE 3: Hematological parameters in *T. evansi* infected animals (as detected by PCR) before and after treatment.

	Parameter	RBC (10^6cells/ µl)	HB (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	Plt (x 10^3cells/ µl)
Infected animals (a)	Total samples tested = 47	4.71* ±1.63	6.61* ±1.77	20.36* ±4.34	47.6* ±17.93	14.62 ±2.55	33.29 ±8.57	297.21* ±217.5
	No. of animals below NR	26	35	37	23	19	16	7
	No. of animals within NR	21	12	10	17	25	7	38
	No. of animals above NR	0	0	0	7	3	24	2
Treated animals (b)	Total samples tested = 24	5.99* ±1.27	7.81* ±1.09	22.42*± 3.05	38.59* ±7.64	13.5 ±2.4	35.94 ±5.73	432.29* ±165.2
	No. of animals below NR	6	13	16	15	13	2	0
	No. of animals within NR	18	11	8	9	11	6	24
	No. of animals above NR	0	0	0	0	0	16	0
Kahn *et al.*, 2005 a	Normal range (NR)	5-10	8-15	24-46	40-60	14.4-18.6	26-34	100-800

Values are shown as Mean ± standard deviation; $P < 0.05$

* indicates values varying significantly($P < 0.05$)

RBC = red blood cells, HB = Haemoglobin, PCV = Packed cell volume, MCV = Mean corpuscular volume, MCH = mean corpuscular heamoglobin, MCHC = Mean corpuscular haemoglobin concentration, Plt = Platelets

REFERENCES

Bhatia, B.B., Pathak K.M.L. and Banerjee D.P. (2006). Text Book of Veterinary Parasitology. Second edition. Kalyani Publishers, Ludhiana – New Delhi: 304-315p.

Claes, F., Radwanska M., Urakawa T., Majiwa P.A., Goddeeris B. and Buscher P. (2004). Variable Surface Glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. *Kinetoplastid. Biol. Dis.*, **3**:1"6.

Desquesnes M., Holzmuller P., Lai D., Dargantes A., Lun Z. and Jittaplapong S. (2013). *Trypanosoma evansi* and Surra: A review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. *BioMed Research International*, vol. 2013, Article ID 194176, 22 pages, 2013. doi:10.1155/2013/194176.

Fernandez, D., Baradat B.G., Eleizalde M., Marcano E.G., Perrone T. and Mendoza M. (2009). *Trypanosoma evansi*: A comparison of PCR and parasitological diagnostic tests in experimentally infected mice. *Exp. Parasitol.*, **121**: 1-7.

Hilali M., Abdel-Gawad A., Nassar A. and Abdel-Wahab A. (2006). Hematological and biochemical changes in water buffalo calves (*Bubalus bubalis*) infected with *Trypanosoma evansi*. *Vet. Parasitol.*, **139** : 237–243.

Holland, W.G., Claes F., My L.N., Thanh N.G., Tam P.T., Verloo D., Buscher P., Goddeeris B. and Vercruysse J.A. (2001). Comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. *Vet. Parasitol.*, **97**: 23–33.

Juyal, P.D., Singla L.D. and Kaur P. (2005). Management of Surra due to *Trypanosoma evansi* in India: an overview. In: V. Tandon and B.N. Dhawan (ed) Infectious diseases of domestic animals and zoonosis in India. *Proceedings of the National Academy of Sciences of India* 75(B)-special issue: 109-120p.

Kahn, C.M., Line S. and Aiello S.E. (ed) (2005b). Reference guide Table 6 in Merck manual 9th Edition Edition Merck & Co., Inc. Whitehousestation, N.J., U.S.A.: 2584-2585p.

Kahn, C. M., Line, S. and Aiello, S. E. (2005a). Reference guide Table 7 in Merck manual 9th Edition Merck & Co., Inc. Whitehousestation, N.J., U.S.A.: 2586-2587p.

Kaur, P., and Juyal P.D. (2003). Haematobiochemical changes in experimental surra in cow calves treated with diminazene aceturate alongwith antipyrine and procaine. *Indian Vet. J.*, 8**: 975-978.**

Kelly, W.R. (1979). Veterinary Clinical Diagnosis, 2nd edition, Bailliere Tindall, London: 261-300p.

Kumar H., Gupta M.P., Sidhu P.K., Mahajan V., Bal M.S., Kaur K., Ashuma, Verma S. and Singla L.D. (2012) Outbreak of *Trypanosoma evansi* infection in cross bred cattle in Punjab, India. *J. Applied Anim Res.*, **40**: 256-259

Luckins, A.G. (1992). Protozoal diseases of camels. In Allen, W. R., Higgins, A.J., Mayhew, I.G., Snow, D.H. and Wade J.F. (eds.) Proceedings of the Ist International conference. R. and W. Publication New Market Limited, Suffolk, U.K.: 23-27p.

Mugittu, K.N., Silayo R.S., Majiwa P.A.O., Kimbita E.K., Mutayoba B.M. and Maselle R. (2001). Application of PCR and DNA probes in the characterization of trypanosomes in the blood of cattle in farms in Morogro, Tanzania. *Vet. Parasitol.*, **94**: 177-189.

Omanwar, S., Rao J.R., Basagoudanavar S.H., Singh R.K. and Butchaiah G. (1999). Direct and sensitive detection of *Trypanosoma evansi* by polymerase chain reaction. *Acta Veterinaria Hungarica*, **47**: 351-359.

Ravindran R., Rao J.R., Mishra A.K., Pathak K.M.L., Babu N., Satheesh C. and Rahul S. (2008). *Trypanosoma evansi* in camels, donkeys and dogs in India: comparison of PCR and light microscopy for detection- short communication. *Veterinarski Arhiv.*, **78**: 89-94.

Sharma A., Singla L.D., Tuli A. Kaur P., Batth B.K. Javed M. and Juyal P.D. (2013). Molecular prevalence of *Babesia bigemina* and *Trypanosoma evansi* in dairy animals from Punjab, India, by Duplex PCR: A step forward to the detection and management of concurrent latent infections. *BioMed Research International*, vol. 2013: Article ID 893862, 8 pages, 2013. doi:10.1155/2013/893862.

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, CCSHAU, Hisar.

Singla, L.D., Juyal P.D. and Ahuja S.P. (2000). Serum protein changes of cross-bred calves experimentally infected with *Trypanosoma evansi* and immuno-modulated with levamisole. *Indian Vet. J.*, **77**: 172-174.

Singla, L.D., Juyal P.D. and Kalra I.S. (1996). Effects of levamisole on the clinical response of *Trypanosoma evansi* infection in cow-calves. *Indian Vet. J.*, **73***:* 11-15.

Wuyts, N., Chokesajjawatee N., Sarataphan N. and Panyim S. (1995). PCR amplification of crude blood on microscopic slides in diagnosis of *Trypanosoma evansi* infection in dairy cattle. *Annales de la Societe Belge de Medecine Tropicale*, **75**: 229-37.