Indian J. Anim. Res., 53 (3) 2019: 382-387
Print ISSN:0367-6722 / Online ISSN:0976-0555

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com

A comparative analysis of microscopy and PCR based detection methods for *Babesia* and *Trypanosoma* infecting bovines and assessment of risk factors

B.R. Maharana*, B. Kumar, J.P. Joseph and T.K. Patbandha

Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry,
Junagadh Agricultural University, Junagadh-362 001, Gujarat, India.
Received: 29-09-2017 Accepted: 15-03-2018 DOI: 10.18805/ijar.B-3507

ABSTRACT

The present study was carried out to evaluate and compare the status of infection and assessment of risk factors in 353 blood samples (144 cattle and 209 buffaloes) of bovines by PCR assay along with microscopic examinations (ME). ME revealed prevalence of *Babesia bigemina* and *Trypanosoma evansi* to be 22.91% and 0.69%, respectively in cattle and 12.44% and 0.95%, respectively in buffaloes. Conversely, PCR assay was able to detect 40.97% and 3.47 % prevalence of *B. bigemina* and *T. evansi* in cattle and 23.92% and 6.69% in buffaloes, respectively. The result revealed that the PCR assay was 100% sensitive and 82.9% specific when compared with ME for babesiosis and 100% sensitive and 95.42% specific for trypanosomosis. Multivariate logistic regression models showed that risk of babesiosis was significantly higher in cattle (Odds ratio (OR) =2.207, P=0.001) compared to buffaloes. The risk for surra in male buffaloes increased by 6.37 times (OR= 6.375, P=0.013). Conversely, risk of babesiosis was significantly lower in male cattle than females (OR= 0.467, P=0.044).

Key words: *Babesia*, Diagnosis, Microscopy, PCR, Risk factor, *Trypanosoma.*

INTRODUCTION

Babesia bigemina an intra erythrocytic apicomplexan protozoa is the causative agent for bovine babesiosis transmitted by a *Rhipicephalus* (*Boophilus*) *microplus* which causes extensive monetary loss in the form of morbidity and mortality in bovines (Sharma *et al*., 2013). The disease is characterized by fever, anaemia and haemoglobinuria. *Trypanosoma evansi*, a haemoprotozoan parasite of domestic livestock and wild animals mechanically transmitted by tabanid flies. It causes a wasting disease called 'Surra' (Soulsby, 1982). In India, babesiosis independently, accounts for annual losses to the tune of US$ 57.2 million (McLeod and Kristjanson, 1999); however, data is scanty on the economic losses due to *T. evansi*.

The haemoparasites are routinely diagnosed on the basis of clinical signs and ME. Further, both methods have the limitations of their inability to detect the subclinical and carrier animals which necessitates the use of highly sensitive and specific diagnostic tests. Specificity of serological methods are limited by cross reactivity (Passos *et al*., 1998). PCR assays allow diagnosis of parasite at levels far below the detection limit of the frequently used parasitological techniques (Almeria *et al.,* 2001). Thus, PCR is thought to be more convenient and extensively implemented technique for epidemiological investigations. Hence, the purpose of the present study was to standardize and employ PCR assay along with conventional method for diagnosis of *B. bigemina* and *T. evansi* infections.

MATERIALS AND METHODS

Animals and samples: Randomly 353 blood samples (144 cattle and 209 buffaloes) of bovines were collected and used for smear preparation, DNA isolation and other haematological examination. For age, animals were divided in to three groups *viz*. young (< 1 year), adults (1 to 5 yrs) and old (≥5 yrs). Data was also maintained in sex wise (male and female), breed wise like Gir, Kankrej cattle, Jaffrabadi buffalo and non-descriptive breed, and for seasons, the entire year is divided into three seasons like summer (March to June), Monsoon (July to October) and Winter (November to February).

Microscopic examinations (ME): Thin and thick blood smear were prepared and subjected to Giemsa staining method following the standard protocol (Soulsby, 1982).

DNA extraction: Genomic DNA was extracted from whole blood collected in EDTA vial using GENEJET whole blood genomic DNA purification mini kit as per the given protocol (Thermo Scientific, Lithuania).The genomic DNA of infection free leucocytes separated from the blood of a three-day-old neonatal bovine calf was included as negative control while genomic DNA isolated from *B. bigemina* infected erythrocytes of clinically infected cattle was used as positive control. Genomic DNA isolated from *T. evansi* infected cattle was used as positive control.

PCR Protocol: The PCR assay was initially optimized for genus level identification with primers specific for *Babesia*

*Corresponding author's e-mail and address : drbiswaranjanmaharana@gmail.com RVDEC LUVAS, Uchani, Karnal-132 001, Haryana, India.

Table 1: Primer sequences along with expected amplicon size.

Target organism	Nucleotide sequence	Region amplified	Product size (bp)	Reference
Babesia spp.	Ba F: 5'AATACCCAATCCTGACACAGGG3' Ba R: 5'TTAAATACGAATGCCCCCAAC 3'	ssu-r DNA	410 bp	Olmeda *et al.* (1997)
Babesia bigemina	BabF:5'CATCTAATTTCTCTCCATACCCCT CC 3' BabR:5'CCTCGGCTTCAACTCTGATGCCAAAG 3'	SpeI-AvaI restriction fragment of *B.bigemina* genomic DNA	278 bp	Figueroa *et al.* (1992)
Trypanosoma evansi	TEF: 5'-TGCAGACGACCTGACGCTACT-3' TER: 5'-CTCCTAGAAGCTTCGGTGTCCT-3'	Repetitive DNA	227 bp	Wyut *et al.* (1994)

spp. targeting a portion of ssu-r DNA (Olmeda *et al*., 1997) (Table 1).The PCR positive samples were further analyzed for species level identification with the primers specific for *B. bigemina* (Figueroa *et al*., 1992). PCR assay in a final volume of 25µl was carried out in a PCR thermal cycler (Applied Biosystem, USA). The master mix consisted of 2.5 µl of Dream *Taq* buffer (Thermo Scientific, USA), 0.5 µl of 10mM dNTP mix (Thermo Scientific, USA), 1 µl each (20pmol) of the primers, 0.2 µl of recombinant *Taq* DNA Polymerase (Thermo Scientific, USA) and 1 µl of template DNA isolated from infected bovine blood. The volume was made up to 25 µl with NFW. The PCR cycling conditions were set in automated thermal cycler: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 1 min and the final extension at 72°C for 10 min. The PCR cycling conditions for *B. bigemina* was similar except the annealing temperature at 57°C for 1 min.

Correspondingly, the PCR assay was optimized for detection of *T. evansi* with a set of primers specific to *T. evansi* repetitive DNA sequence probe pMUTec6.258 as described by Wuyts *et al.* 1994 (Wyuts *et al*., 1995). The cycles included an initial step at 95ºC for 4 minutes followed by 29 cycles of denaturing at 95ºC for 1 minute, primer annealing at 60ºC for 1 minute and primer extension at 72ºC for 1 minute. This is followed by final extension at 72ºC for 10 minutes and hold at 4ºC for indefinite time. The amplified PCR products were resolved by electrophoresis on a 1.2% agarose gel and visualized using gel documentation system (Vilverlourmat, Bioprint ST4, Germany).

Specificity of PCR primers: PCR amplification was employed on each individual positive DNA sample (*B. bigemina* and *T. evansi*) using their specific primers along with host leucocyte DNA to check cross reactivity, if any.

Statistical analysis: Chi-square (χ^{2}) test was used to see differences in haemoparasitic infections among age, sex, breed and season. Parameters which had significant effect on incidence of haemoprotozoan were further subjected to multivariate logistic regression models (SPSS Version 17.0, USA). Further, t-test was used to compare the various haematological parameters between the animals infected with *B. bigemina* and *T. evansi*.

Sensitivity and specificity of PCR and Microscopy: Sensitivity values for PCR were obtained using the correlations; Sensitivity= True Positive / (True Positive + False Negative) x100 and specificity using the formula; Specificity=True Negative/ (True Negative + False Positive) x100

RESULTS AND DISCUSSION

PCR primers specificity: PCR amplification employed on each individual positive DNA sample using their specific primers led to the detection of expected fragments of size 410bp (*Babesia* spp.), 278 bp (*B. bigemina*) and 227 bp (*T. evansi*), respectively. Non specific amplification from non target sequence was not observed.

Relative Efficacy of conventional parasitological technique and PCR assay and consequent clinical picture: Overall, microscopic examination revealed Fig 1,2 the prevalence of *B. bigemina* and *T. evansi* to be 22.91% and 0.69%, respectively, in cattle and 12.44% and 0.95% respectively in buffaloes (Table 2). Microscopy did not reveal any mixed infection. Out of the total sample size (353), 24.64% (87/353) of animals portrayed consistent signs of anaemia, general weakness, history of reduced feed conversion, and occasional episodes of fever. Ticks were

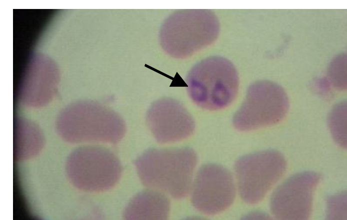

Fig 1: Intra erythrocytic *Babesia bigemina* organism (in pair) under oil immersion lens(x1000).

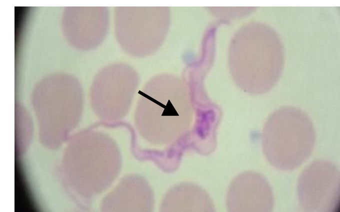

Fig 2: Inter erythrocytic *Trypanosoma evansi* organism under oil immersion lens (x1000).

Table 2: Infections recorded according to sex, age, season and breed in bovines.

Animal species	Parameter	Category	No. of samples tested	Positive for *B. bigemina*	Positive for *T. evansi*	Overall Prevalence
Cattle	Sex	Male	48	14 (29.16%)	2 (4.16%)	16(33.33%)
		Female	96	45 (46.87%)	3 (3.12%)	48(50%)
χ^2 value				**4.149, p=0.042**	**0.104, p=0.748**	
Buffalo	Sex	Male	11	2 (18.18%)	3 (27.27%)	5(45.45%)
		Female	198	48 (24.24%)	11 (5.55%)	59(29.79%)
χ^2 value				**0.210, p=0.647**	**7.864, p=0.005**	
Cattle	Age	< 1 year	14	3 (21.42%)	0 (0%)	3(21.42%)
		1-5years	39	15 (38.46%)	2 (5.12%)	17(43.58%)
		>5years	91	41 (45.05%)	3 (3.29%)	44(48.35%)
χ^2 value				**2.940, p=0.230**	**0.831, p=0.660**	
Buffalo	Age	< 1 year	14	3 (21.42%)	0 (0%)	3(21.42%)
		1-5years	30	3(10%)	3 (10%)	6(20%)
		>5years	165	44 (26.66%)	11 (6.66%)	55(33.33%)
χ^2 value				**3.926, p=0.140**	**1.529, p=0.466**	
Cattle	Season	Winter	21	8 (38.09%)	0 (0%)	8(38.09%)
		Summer	68	30 (44.11%)	3 (4.41%)	33(48.52%)
		Rainy	55	21 (38.18%)	2 (3.63%)	23(41.81%)
χ^2 value				**0.527, p=0.768**	**0.939, p=0.625**	
Buffalo	Season	Winter	47	11 (23.40%)	3 (6.38%)	14(29.78%)
		Summer	100	25 (25%)	6 (6%)	31(31%)
		Rainy	62	14 (22.58%)	5 (8.06%)	19(30.64%)
χ^2 value				**0.132, p=0.936**	**0.271, p=0.873**	
Cattle	Breed	ND	34	14 (41.17%)	3 (8.82%)	17(50%)
		Gir	78	34 (43.58%)	2 (2.56%)	36(46.15%)
		Kankrej	32	11 (3.43%)	0 (0%)	11(34.37%)
χ^2 value				**0.797, p=0.671**	**4.248, p=0.120**	
Buffalo	Breed	Jaffrabadi	144	33 (22.91%)	9 (6.25%)	42(21.64%)
		ND	65	17 (26.15%)	5 (7.69%)	22(33.84%)
χ^2 value				**0.258, p=0.612**	**0.149, p=0.699**	

observed on 40.28% (58/144) of the affected cattle and 22.01% (46/209) of the affected buffaloes. Based on morphology, population of the ticks was mainly identified as *R. (Boophilus) microplus.* Parasitaemia was observed in 59 cases for *B. bigemina* and only in 3 cases for *T. evansi* piroplasms by ME (Table 2). Only one cattle was showing intermittent fever from the clinically positive cases of trypanosomosis. Eighty cases positive for babesiosis showed history of fever and haemoglobinuria.

In the present study, the higher prevalence of bovine babesiosis can be closely correlated to the abundance of the vector i.e. prevalence of *R. microplus.* Lower prevalence of trypanosomosis in bovines as compared to babesiosis can be well corroborated with the fact that due to antigenic variations, the parasite, *T. evansi* has a cryptic infection leading to paroxysm and intermission phases and non-detectable parasitaemia during the infection processes. Our observations correlate with the results of previous studies (Maharana *et al.*, 2016; Vohra *et al.*, 2012). In contrast, Kumar *et al.* (2016), reported significantly higher prevalence of haemoparasites in bovines in south-western Gujarat. This may be due to collection of samples specifically from clinically suspected animals.

ME frequently fail to detect latent infections owing to scanty parasitaemia in peripheral blood in the chronic forms and only reliable in the acute phase of the disease. Further, the status of subclinical infection needs to be identified because this type of infection acts as a source to other healthy animals (Sharma *et al.*, 2015). PCR permits identification of haemoprotozoa at levels far below the detection limit of the commonly used parasitological techniques (Sharma *et al.*, 2013). In case of trypanosomosis, microscopy has limited sensitivity as infection is detected when parasitaemia is above 10^5 trypanosomes/ml (Desquesnes and Davila, 2002). PCR can detect 1 trypanosome/ml of blood (Davila *et al.*, 2003) or even 1 pg of *Trypanosoma* DNA in presence of host DNA (Clausen *et al.*, 1998).

PCR reported to be as much as 1000 times more sensitive than microscopy for detection of *Babesia* spp., with detection at parasitaemia levels ranging from 0.001% to 0.0000001% (1 parasite in 109 RBCs) (Criado-Fornelio, 2007). This property makes the PCR a very useful method for the prepatent period and chronic phase of the disease (Sharma *et al.*, 2013).

In the present investigation, PCR detected 40.97% and 3.47% prevalence rates of *B. bigemina* and *T. evansi*, in cattle respectively. In case of buffaloes, the prevalence recorded was 23.92% and 6.69% respectively Fig.3-5. The assay could effectively diagnose *B. bigemina* infection in 54.12% clinical, 19.26% subclinical (with clinical signs but negative by ME), and 26.60% carrier cases (ME negative and with no clinical signs). Correspondingly similar PCR assay report was observed in case of *T. evansi* infection in bovines under study revealing 21.05% of subclinical cases and 63.15% of carrier cases (Table 3, 4). PCR revealed higher prevalence of *B. bigemina* and *T. evansi* in cattle and buffaloes as compared to much lower prevalence observed by ME. These results defend the greater sensitivity of PCR in detecting the subclinical and latent infections. The molecular prevalence of *T. evansi* was reasonably lower than that of *B. bigemina* because there is inconsistent occurrence

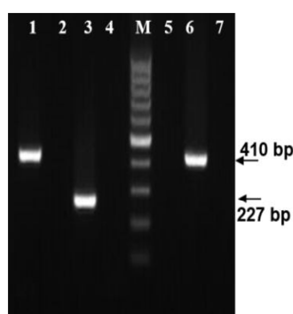

Fig 3: Agarose gel (1.2%) electrophoresis showing amplified DNA for *Babesia* (410 bp) and *Trypanosoma* (227 bp)
Lane 1: Amplification of *Babesia* spp. genomic DNA
Lane 2: No amplification of *Babesia* spp. genomic DNA
Lane 3: Amplification of *T. evansi* genomic DNA
Lane 4: No amplification of *T. evansi* genomic DNA
Lane M: Molecular Size marker 100 bp
Lane 5: No amplification for host leucocyte DNA
Lane 6: Amplification of *Babesia* spp. genomic DNA from test sample
Lane 7: No amplification *Babesia* species from test sample

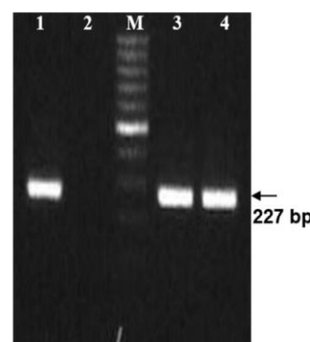

Fig 4: Agarose gel (1.2%) electrophoresis showing amplified DNA *Trypanosoma* (227 bp)
Lane 1: Positive control for *Trypanosoma evansi*
Lane 2: Negative control
Lane M: Molecular size marker 100 bp
Lane 3, 4: Amplification of *T. evansi* genomic DNA from test sample

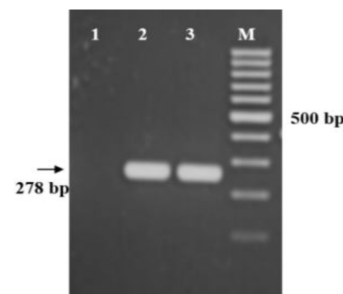

Fig 5: Agarose gel (1.2%) electrophoresis showing amplified DNA from *B. bigemina* (278 bp)
Lane M: Molecular marker 100 bp
Lane 1: Negative control
Lane 2: positive control
Lane 3: Amplification of *B. bigemina* genomic DNA from the blood of test sample positive for *Babesia* spp.

Table 3: Prevalence of *B. bigemina* and *T. evansi* by Light Microscopy and PCR.

Animal species	No. of samples tested	Positive for *B. bigemina* (109) Light Microscopy	By PCR	Positive for *T. evansi* (19) Light Microscopy	PCR
Overall	353	59 (16.71%)	109 (30.88%)	3 (0.85%)	19 (5.38%)
Cattle	144	33 (22.91%)	59 (40.97%)	1 (0.69%)	5 (3.47%)
Buffalo	209	26 (12.44%)	50 (23.92%)	2 (0.95%)	14 (6.69%)
χ^2 value		6.722, p=0.010	11.611, p<0.001	0.07, p=0.791	1.743, p=0.187

Table 4: Correlation of infection of *Babesia bigemina* and *Trypanosoma evansi* with sensitivity of PCR.

	Total *Babesia bigemina* positive(109)			Total *Trypanosoma evansi* positive (19)		
	+ve/CS	-ve/CS	-ve/NCS	+ve/CS	-ve/CS	-ve/NCS
PCR positive	59(54.12%)	21(19.26%)	29(26.60%)	3(15.78%)	4(21.05%)	12(63.15%)

+ve/CS: Sample positive by light microscopy and animal exhibited clinical signs (Clinical Cases)
-ve/CS: Sample negative by light microscopy and animal exhibited clinical signs (Subclinical cases)
-ve/NCS: Sample negative by light microscopy and animal exhibited no clinical signs (Latent cases)

Table 5: Risk factors associated with overall haemoprotozoan infection in bovines.

Parameters		Estimates	SEM	P value	OR	95% CI
Overall (PCR based)						
Babesia	(Cattle vs Buffalo)	-1.157	0.162	0.001	2.207	1.394-3.495
Trypanosoma	(Cattle vs Buffalo)	-2.634	0.277	0.194	0.501	0.176-1.423
Overall (Giemsa based)						
Babesia	(Cattle vs Buffalo)	-1.951	0.210	0.010	2.093	1.189-3.683
Trypanosoma	(Cattle vs Buffalo)	-4.640	0.711	0.793	0.724	0.065-8.058
Sex: Cattle						
Babesia	Male vs Female	-0.125	0.205	0.044	0.467	0.223-0.978
Trypanosoma	Male vs Female	-3.434	0.587	0.748	1.348	0.218-8.350
Sex: Buffalo						
Babesia	Male vs Female	-1.139	0.166	0.648	0.694	0.145-3.326
Trypanosoma	Male vs Female	-2.833	0.310	0.013	6.375	1.481-27.440

of the tabanid flies as compared to the ticks. Moreover, the incidence of co-infection of both protozoa was nil owing to different vectors (tick and hematophagus flies) responsible for their transmission (Sharma *et al.*, 2013). Higher overall prevalence rate of haemoparasitic infection in cattle than buffaloes (44.44% vs. 30.62%) might be due to great variation in the sample size of cattle and buffaloes (Yadav *et al.*, 1985). Additionally, buffaloes are considered to be more resistant towards the haemoprotozoan diseases. Hence buffaloes act as reservoir hosts to other animals and may become clinically ill under stress conditions.

Correlation of the Infection Status according to sex, age and seasons: Overall incidence of babesiosis in female cattle (46.87%) was significantly (P=0.042) higher than male (29.16%). However, in buffaloes, though the incidence of babesiosis was higher in females than males the data did not differ significantly ((24.24% vs. 18.18%), P=0.647). This may be due to hormonal disturbances in females which pretence it to weakened immune system. Conversely, higher prevalence of trypanosomosis was observed in male cattle and buffaloes compared to females but the difference was statistically significant in buffaloes only (4.16 vs. 3.12%, P=0.748 and 27.27% vs. 5.55%, P=0.005, respectively). In general, male cattle and buffaloes are given less care and management in comparison to productive females. So, stressful conditions might be a contributing factor for increasing incidence of surra in male bovines.

Comparative distribution of babesiosis and trypanosomosis in bovines did not reveal any statistical association with the age of the host (Table 2). The overall prevalence of haemoprasaitic infections in bovines was moderately higher in summer and monsoon, the data being statistically insignificant (p>0.05) which might be due to high abundance of vectors in these seasons of the year (Ganguly *et al.*, 2017). This is in accordance with the observations made by earlier workers (Roy *et al.*, 2004). Difference in infection rates were statistically non significant between breeds of both cattle and buffalo (Table 2).

Sensitivity and specificity of PCR and Microscopy: The result revealed that the PCR assay is 100% sensitive and 82.9% specific when compared with ME for babesiosis. Similarly, for trypanosomosis, the PCR assay is found to be 100% sensitive and 95.42% specific. Our findings are in line with observations of earlier workers who have reported the sensitivity of PCR spanning around 95 to 100% (Sharma *et al.*, 2013; Shams *et al.*, 2013).

Correlation of occurrence of haemoprotozoan infection with various risk factors: Multivariate logistic regression models (Table 5) showed that the risk of babesiosis was significantly higher in cattle (OR=2.207, P=0.001) compared to buffaloes. Cattle and buffaloes were at similar risk of getting the infection of surra (OR=0.5, P=0.194). The risk for surra in male buffaloes increased by 6.37 times (OR= 6.375, P=0.013) compared to female counterpart but such association was not observed for babesiosis (OR= 0.694, P=0.648). Though risk of overall prevalence of surra in male cattle was higher than that of female (OR=1.348, p=0.748), risk of babesiosis significantly reduced in male cattle compared to female (OR= 0.467, P=0.044). Our findings are not indifferent from those reported earlier (Sharma *et al.*, 2013; Vohra *et al.*, 2012).

The current investigation reveals the latent infection of babesiosis and trypanosomosis in bovines of south western Gujarat. The various risk factors discussed above significantly associated with the occurrence of disease. The PCR based detection will be of immense help in determining the molecular epidemiology of haemoparasites.

ACKNOWLEDGEMENT

Authors are also thankful to Principal and Dean, College of Veterinary Science & A.H., Junagadh for providing the necessary facilities.

REFERENCES

Almeria, S., Castella, J., Ferrer, D., Ortuno, A., Estrada-Pena, A. and Gutierrez, J. F. (2001). Ovine piroplasms in Minorca (Balearic Islands, Spain): a comparison of PCR-based and light microscopy detection. *Veterinary Parasitology,* **99**: 249-259.

Clausen, P.H, Wiemann, A., Patzelt, R., Kakaire, D., Poetzsch, C., Peregrine, A. and Mehlitz, D. (1998). Use of a PCR assay for the specific and sensitive detection of *Trypanosoma* spp. in naturally infected dairy cattle in peri-urban Kampala,Uganda. *Annals of the New York Academy of Sciences,* **849**: 21–31.

Criado-Fornelio, A. (2007). A review of nucleic acid-based diagnostic tests for *Babesia* and *Theileria*, with emphasis on bovine piroplasms. *Parassitologia* (Rome), **49**: 39–44.

Dávila, A.M., Herrera, H.M., Schlebinger, T., Souza, S. S. and Traub-Cseko, Y. M. (2003). Using PCR for unraveling the cryptic epizootiology of livestock trypanosomosisin the Pantanal, Brazil. *Veterinary Parasitology,* **117**: 1–13.

Desquesnes, M. and D´avila, A. M. R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology,* **109**(3-4): 213–231.

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**: 2576–2582.

Ganguly, A., Bisla, R.S., Singh, H., Bhanot, V., Kumar, A., Kumari, S., Maharana, B.R. and Ganguly, I. (2017). Prevalence and hematobiochemical changes of tickborne haemoparasitic diseases in crossbred cattle of Haryana, India. *Indian Journal of Animal Sciences*, **87**(5):552-557.

Kumar, B., Maharana, B. R., Prasad, A., Joseph, J. P., Patel, B. and Patel, J.S. (2016). Seasonal incidence of parasitic diseases in bovines of south western Gujarat (Junagadh), India. *Journal of Parasitic Disease,* **40**(4):1342-1346.

Maharana, B.R., Kumar, B., Prasad, A., Patbandha, T.K., Sudhakar, N.R., Joseph, J.P. and Patel, B.R. (2016). Prevalence and assessment of risk factors for haemoprotozoan infections in cattle and buffaloes of South-West Gujarat, India. *Indian Journal of Animal Res*earch, **50** (5): 733-739.

McLeod, R. and Kristjanson, P. (1999). Final Report of Joint ESYS/International Livestock Research Institute/Australian Centre for International Agricultural Research Tick Cost Project-Economic Impact of Ticks and Tick-Borne Diseases to Livestock in Africa, Asia and Australia, International Livestock Research Institute, Nairobi, Kenya.

Olmeda, A.S., Armstrong, P.M., Rosenthal, B.M., Valladares, B., del Castillo, A., de Armas, F., Miguelez, M., Gonzalez, A., Rodriguez., J.A. and Spielman, A. (1997. Telford 3rd SR. A subtropical case of human babesiosis. *Acta Tropica,* **67**: 229–234.

Passos, L.M., Bell-Sakyi, L. and Brown, C.G. (1998). Immunochemical characterization of in vitro culture-derived antigens of *Babesia bovis* and *Babesia bigemina*. *Veterinary Parasitology,* **76**: 239–249.

Roy, S., Tiwari, A., Galdhar, C.N., Upadhyay, S.R., Ratre, H.K., Sahu, S.K. and Maiti, S.K. (2004). *Indian Journal of Veterinary Medicine,* **24**:5-7.

Shams, S., Ayaz, S., Ali, I., Khan, S., Gul, I., Naila, Gul. and Khan, S. N. (2013). Sensitivity and Specificity of PCR & Microscopy in detection of Babesiosis in domesticated cattle of Khyber Pakhtunkhwa, Pakistan. *International Journal of Advancement in Research and Technology,* **5**(2):37-41.

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,* doi: 10.1155/2013/893862.

Sharma, A., Singla, L.D., Tuli, A., Kaur, P. and Bal, M.S. (2015). Detection and assessment of risk factors associated with natural concurrent infection of *Trypanosoma evansi* and *Anaplasma marginale* in dairy animals by duplex PCR in eastern Punjab. *Tropical Animal Health and production,* **47**:251–257.

Soulsby, E. J. L. (1982). Helminths, Arthropods and Protozoa of Demesticated Animals. 7th ed. ELBS, Bailliers *Tindall and Cassel, London.*

Vohra, S.P., Patel, J.V., Patel, B.B., Patel, S.B. and Umale, R.H. (2012). Seasonal incidence of haemoprotozoan diseases in cross bred cattle and buffaloes in Kaira and Anand districts of Gujarat, India. *Veterinary World,* **5**: 223-224.

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.

Yadav, C.L., Gupta, R.P. and Ruprah, N.S. (1985). The prevalence of haemoprotozoan infections in cattle and buffaloes. *Journal of Indian Veterinary Medicine,* **9**: 205–209.