Indian Journal of Animal Sciences **89**(4): 385–389, April 2019/Article
https://doi.org/10.56093/ijans.v89i4.89098

Direct PCR-RFLP based detection and differentiation of *Anaplasma* species in naturally infected goats of eastern Haryana, India

BISWA RANJAN MAHARANA[1], ANITA GANGULY[2], RANBIR SINGH BISLA[3], DEVAN ARORA[4], ANKIT KUMAR[5], SANDEEP POTLIYA[6], CHINMOY MISHRA[7] and INDRAJIT GANGULY[8]

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

Received: 17 April 2018; Accepted: 22 October 2018

ABSTRACT

The present investigation was designed to develop a novel, rapid and cost effective direct PCR-RFLP assay, as a specific diagnostic tool for detection and differentiation of two *Anaplasma* species (*A. ovis and A. marginale*). Blood samples were collected randomly from 83 goats. A direct blood polymerase chain reaction (DT-PCR) for amplifying a fragment of the major surface protein 5 (*msp5*) gene of *A. ovis/A. marginale* from whole blood was developed and standardized. Blood smear examination revealed 24 samples (28.91%) positive for *Anaplasma* inclusion bodies. While, 39 (47%) samples were positive by DT-PCR. The results revealed that DT- PCR was 100% sensitive and 74.57% specific compared to microscopy based detection (k =0.62). Additionally DT-PCR showed 94.44% sensitivity and 100% specificity compared to conventional PCR results with suspected blood samples (k=0.94). All DT-PCR positive samples were confirmed to be *A. ovis* by restriction fragments length polymorphism (RFLP) analysis. The phylogenetic tree and sequence analysis revealed *msp5* gene of *Anaplasma* species Indian isolate had maximum distance from *A. phagocytophilum* followed by *A. centrale* and *A. marginale* and 100% sequence identity with *A. ovis* isolates of Chinese origin which further confirmed the sequence identified in native goats to be of *A. ovis*. The simplified DT-PCR assay as a viable alternative to conventional PCR could be helpful for fast and accurate diagnosis of *Anaplasma* species and suitable for screening a large number of samples. Furthermore, results revealed that DT-PCR-RFLP of the *msp5* gene might be a useful method for simultaneous detection and differentiation of *A. ovis* and *A. marginale* in goats.

Key words: *Anaplasma marginale, Anaplasma ovis*, Goat, *msp5*, PCR-RFLP

Goat, among the main meat producing animals, has an important contribution in Indian rural economy. According to 19th Livestock Census, the total goat population in the country is 135.17 million that contributes around 26.40% of the total livestock population. However, the goat population has declined by 3.82% over the previous census due to various reasons. Haemoparasitism may be considered as one of the cause since small ruminants are highly susceptible to haemoprotozoan parasites posing a major constraint to livestock health and productivity (Velusamy *et al*. 2015). Among haemoparasites, anaplasmosis is considered as one of the economically important rickettsial diseases affecting ruminants across the country (PDADMAS 2005). It is an infectious, non contagious rickettsial disease characterized by progressive hemolytic anemia, fever, inappetance, weight loss, etc. (Maharana *et al*. 2016a). It spreads through infected tick bites or the mechanical transfer of fresh erythrocytes from biting flies or by blood contaminated infected fomites (Aubry and Geale 2010, Ganguly *et al*. 2017a). Among various *Anaplasma* species, *A. marginale* and *A. ovis* mostly affects cattle and goat, respectively. However, *A. marginale* can also cause latent infection in goats (Ahmadi-Hamedani *et al*. 2009). *A. ovis* can be differentiated from *A. marginale* based upon the intraerythrocytic location of inclusion bodies in stained blood smear. But when the level of parasitaemia is below 0.1 to 2%, morphological characteristics are not adequate to differentiate these *Anaplsma* species from each other and from Heinz bodies, Howell-Jolly bodies, staining artifacts etc. (Ndung'u *et al.* 1995). Additionally, serological tests lack adequate sensitivity for reliable detection of

Present address: [1]Scientist (drbiswaranjanmaharana @gmail.com), Department of Veterinary Parasitology; [2]Senior Scientist (anitaganguly@gmail.com), Department of Veterinary Biochemistry; [3]Regional Director-cum-Principal Scientist (ranbir_bisla@yahoo.com), [6]Scientist (sandeeppotliya @gmail.com), Department of Veterinary Surgery and Radiology; [4]Scientist (devanarora7@gmail.com), Department of Veterinary Epidemiology and Public Health; [5]Scientist (ankitk813 @gmail.com), Department of Veterinary Medicine, Referral Veterinary Diagnostic and Extension Centre. [7]Assistant Professor (drchinmoymishra@gmail.com), Department of Animal Genetics and Breeding, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha. [7]Principal Scientist (drindrajit @gmail.com), Department of Animal Genetics and Breeding, ICAR-National Bureau of Animal Genetic Resources, Karnal, Haryana.

infection harbouring low parasitaemia and failure to differentiate between past and present infections (Sumbria *et al*. 2015). Nucleic acid based detection techniques like PCR assays allow diagnosis of parasite at levels far below the detection limit of the frequently used parasitological techniques and has an advantage in separating clinical and subclinical forms of parasitic infections (d'Oliveria *et al.* 1995, Almeria *et al*. 2001, Durrani and Kamal 2008, Seong *et al*. 2015, Yousefi *et al*. 2017). The present communication describes the use of a simple, rapid and economical DT-PCR-RFLP assay for specific detection and differentiation of two *Anaplasma* species in goats.

MATERIALS AND METHODS

Sample collection: A total of 83 blood samples (2 ml from each animal) were randomly collected from jugular vein of suspected goats with the help of sterile 5 ml syringe and reserved in a vial containing EDTA and kept on ice. This whole blood was used for smear preparation, DNA isolation and validation of DT-PCR assay. Ticks from body coat, ears etc. were collected in 70% alcohol and processed for identification.

Microscopic examination: Thin blood smear were prepared and subjected to Giemsa staining method following the standard protocol (Soulsby 1982) for the detection of inclusion bodies of *Anapalsma* species. Each slide was examined for 75 different microscopic fields with a magnification of 100×. All the slides were examined by the same technical personnel in order to avoid inter-observation variation.

DT-PCR protocol: Self designed oligonucleotide primers (AMO F:AGGGGGTCACCGTCAGTAG; AMO R: TGCAACTTATCGGCATGGT) were synthesized for establishment of direct blood PCR targeting the *msp5* gene of *A. marginale* and *A. ovis*. The specificity of primers was checked using primer BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis. The DT-PCR was standardised by taking 2 µl of whole blood sample. The assay was initially standardized using positive blood samples with ≥ 80% parasitaemia. The same DT-PCR was carried out on blood samples of 15 *Anaplasma* infected animals with varying degree of parasitaemia for validation and sensitivity detection of the assay. Briefly, DT-PCR was carried out in a thermal cycler (Veriti^{TM}, Applied Biosystem) with a final reaction volume of 20 µl containing 1 µl of whole blood, 10 µl of 2× PCR buffer, 0.5 µM of each primer and 0.5 µl of II DNA Polymerase. Both positive and negative controls were always included to exclude PCR carryover. The blood collected from *Anaplasma* infected erythrocytes of clinically positive goat (microscopically and DT-PCR positive) was used as positive control. Similarly, blood collected from the venous blood of a 3 day old kid without previous exposure to ticks, was used as negative control. The cyclic conditions were as follows: initial denaturation at 98°C for 5 min; followed by 35 cycles of final denaturation at 98°C for 1 sec, annealing at 55°C for 5 sec and extension at 72°C for 30 sec; with a final extension step of 72°C for 1 min. After completion of assay, tubes were centrifuged at 1000× g for 5 min to collect the clear supernatant for further analysis. DT-PCR products (supernatant) were analyzed by electrophoresis on 2.5% agarose gel, containing ethidium bromide, and documented under gel documentation system (Gel Doc XR+, Bio-Rad).

*RFLP analysis:*The RFLP analysis was done for differentiation of two species of *Anaplasma i.e A. ovis* and *A. marginale* in goats. Three sets of enzymes, viz. *Ssp*I, *Nde*I, *Hae*III were used for differentiation of these two species. The amplified products were digested with the restriction enzymes (Thermo Scientific, USA) as per manufacturer's protocol. Briefly, for each restriction enzyme, the reaction was set up in 30 µl reaction volumes in a 100 µl PCR tube. The digestion mixture consists of 2 µl of 10× buffer, 10 µl of PCR mixture, 2 µl of restriction enzyme (10 U/µl), and 18 µl of nuclease free water. The digestion mixture was incubated at 37ºC for 1 h. The analysis of the enzymatic digested PCR product (10 µl) was performed by ethidium bromide stained 4.0% agarose gel electrophoresis. Blood from *A. marginale* infected cattle (confirmed by PCR and microscopy) maintained in our laboratory was used for PCR-RFLP studies.

Conventional PCR: DNA was isolated from blood samples using standard phenol:chloroform extraction method (Sambrook and Russel 2001). Self designed primers targeting *msp5* gene were used to get the desired amplicon. The 25 µl volume PCR reactions were set up containing 12.5 µl Top Taq® PCR Master Mix (QIAGEN, India), 1 µl of each primer (10 pmol each of AMOF and AMOR), 1 µl of the DNA template, and the total volume was made up to 25 µl using nuclease-free water along with positive and negative control. The PCR cycling conditions were set in automated thermal cycler (Applied Biosystem, USA) with the following programme: initial denaturation at 94°C for 3 min, 32 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 30 sec and the final extension at 72°C for 7 min.

*Comparative sequence analysis of msp5 gene:*The amplified PCR products of three random positive samples (confirmed by microscopy, conventional PCR and DT-PCR)

Table 1. Kappa value prediction of DT-PCR with blood smear examination

Test		Blood Smear			Sensitivity (95% CI)	Specificity (95% CI)	Kappa value (κ)
		Positive	Negative	Total			
PCR	Positive	24	15	39	100%	74.57%	0.629
	Negative	0	44	44			
	Total	24	59	83			

were sequenced and BLAST analysis was performed for species identification from the genomic DNA of these hemoparasites. To check the quality of nucleotide sequences, chromatograms were analysed and verified in BioEdit program. Then the sequences were subjected to nucleotide BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) to acquire the homologous sequences of other strains and isolates. Using MEGA7 software, these nucleotide sequences were aligned based on the lowest Bayesian Information Criterion (BIC) score. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) with 1,000 bootstrap samplings (Tamura *et al*. 2013). The pair wise sequence distance was calculated with p-distance matrix.

Sensitivity and specificity of DT-PCR: The results of DT-PCR were compared with that of conventional PCR and blood smear examination. Sensitivity and specificity values for DT- PCR were obtained using the correlations considering microscopy and conventional PCR as gold standards. The Kappa value was estimated using the online software (http://graphpad.com/quickcalcs/kappa1.cfm). The kappa value thus calculated was compared and results were formulated.

RESULTS AND DISCUSSION

Clinical examination revealed that all animals were anaemic with pale mucous membrane and watery blood. Additionally, all animals were moderately infested with *Hyalomma anatolicum anatolicum*. Microscopy based examination revealed *Anaplasma* species in erythrocytes of 28.91% (24) samples of the total blood samples (83). Microscopy is considered as the simplest and most accessible diagnostic test, confirmed the clinical diagnosis of anaplasmosis (Rajasokkappan and Selvaraju 2016, Arunkumar 2014). Although microscopy is cheaper and easy to perform, it has limited sensitivity as it detects the organism when parasitaemia is above 10^9 infected erythrocytes/ml (Shkap *et al*. 2002) and requires technical expertise. Additionally, structures like Heinz bodies, Howell-Jolly bodies or staining artifacts, often make diagnosis difficult (Noaman *et al*. 2009). Further, the status of subclinical infection needs to be identified because this acts as a source of infection to other healthy animals and can become acutely clinical in individual animals under stress (Sharma *et al*. 2015). Specificity of serological techniques is limited by cross reactivity, false positive and negative results (Passos *et al*. 1998). Nucleic acid based assays like PCR permits identification of haemoprotozoa at levels far below the detection limit of the commonly used parasitological techniques (Sharma *et al*. 2013, Maharana *et al*. 2016b). In present study, in-house standardized rapid DT- PCR assay with specific primers for *A. marginale/ovis* in *msp5* gene was employed for detection of latent infections. Primer pairs were self designed (Fig. 1), analysed using Oligoanalyser software 3.1 (https://eu.idtdna.com/calc/analyzer) and used to amplify a 148 bp region of *msp5* gene specific to *Anaplasma* species. The BLAST analysis indicated that each oligonucleotide primer sequence was species-specific and did not possess additional local homology to the target sequence. Primer specific *Anaplasma* species did not produce PCR products from any of the other haemoparasite species and no such amplicons were detected in control negative samples. Total 39 (47%) blood samples were detected positive by DT-PCR. The entire samples positive by Giemsa stained method found positive by DT-PCR whereas no inclusion bodies were detected in 15 PCR positive samples

The results revealed that DT-PCR was 100% sensitive and 74.57% specific with a positive predictive value of 61.53% and negative predictive value of 100% when compared with optical compound microscopy based detection based on their kappa value estimation (Table 1). Additionally the sensitivity and specificity were found to be 94.44% and 100% compared to conventional PCR with a kappa value of 0.94 (Table 2).

Although in present study, there is limited variation in the degree of sensitivity, there are many advantages of using DT-PCR over conventional PCR assay. DT-PCR eliminates

Fig. 1. *msp5* sequence comparision between *A. ovis* and *A. marginale*. The highlighted portion depicts the base differences. The primers designed as illustrated by lines and arrow indicates the direction of polymerization.

Table 2. Kappa value prediction of DT-PCR with conventional PCR

Test		Conventional PCR			Sensitivity (95% CI)	Specificity (95% CI)	Kappa value (κ)
		Positive	Negative	Total			
DT-PCR	Positive	39	0	39	94.44%	100%	0.94
	Negative	2	42	44			
	Total	41	42	83			

*Kappa value > 0.81, almost perfect agreement; 0.61–0.80, substantial agreement; 0.41–0.60, moderate agreement; 0.21–0.40, fair agreement; 0.01–0.20, slight agreement; 0.00, poor agreement.

DNA extraction and purification steps and reduces the risk of contamination. Less than 5 µl blood is required to perform the assay. The assay radically reduces resources, time and cost compared to other PCR assay making it suitable for screening large number of asymptomatic cases (Echeverry *et al*. 2016). Additionally, field veterinarians and clinicians require an inexpensive, simple and rapid method for diagnosing infections (Nishimori *et al*. 2016, Ganguly *et al*. 2017b). Further, DT-PCR employs a modified high fidelity DNA polymerase with rapid performances in the presence of strong inhibitors of blood (Wikman *et al*. 2004).

Additionally, PCR RFLP technique was employed for differentiation of *A. ovis* and *A. marginale*. Three restriction enzymes were selected based on available sequence in NCBI database to differentiate these two species of *Anaplasma*. The restriction digestion of PCR products yielded products of size 66 bp, 82 bp with *Hae* III and 40 bp, 108 bp with *Nde*I. All the samples were found positive for *A. ovis*. DNA of *A. marginale* (maintained in our laboratory) when digested with *Ssp*I, yielded 52 and 96 bp on 2% agarose gel.

PCR-RFLP revealed all positive PCR products to be *A. ovis*. Any sample positive for *A. marginale* could not be identified. The results obtained from blood samples collected from goats showed that the DT-PCR-RFLP used in this study is more sensitive than detection by light microscopy, routinely in our laboratories. Our findings are in line with the earlier reports (Ahmadi-Hamedani *et al*. 2009, Hornok *et al*. 2007). Further, the amplified fragment of *msp5* gene in Indian isolates was subjected to sequencing by Sanger's di-deoxy chain termination method (Genetic analyser 3130XL, Applied Biosystem, USA). After BLAST analysis, nucleotide sequences from 8 different strains/ isolates were retrieved. The nucleotide sequences were aligned using Kimura 2-parameter model having lowest BIC score of 1118.72. Among the analysed nucleotide sequences, 248 variable sites were identified which included 196 parsim-informative sites and 52 singleton sites. When the pair-wise sequence distance was calculated, *msp5* gene from Indian isolate showed close sequence homology with strain isolated from other sheep and goat (Table 3). The *msp5* gene isolate from *A. marginale, A. centrale* and *A. phagocytophilum* form a different clade in phylogenetic tree (Fig. 2). The analysis of sequence distance revealed *msp5* gene of Indian isolate had maximum distance from *A. phagocytophilum* followed by *A. centrale* and *A. marginale* (Table 3). These findings support the fact that *msp5* gene is hypervariable among the strain/isolates. In the current study, sequence heterogeneity was not observed between the goats of the same flock.

Conclusively, the present investigation describes an in-house standardized DT-PCR-RFLP assay as a novel, simple, economical and rapid diagnostic technique for simultaneous detection and differentiation of *Anaplasma* species in goats. The present study seems to be the first molecular based report of *A. ovis* in notable number of carrier goats in eastern Haryana, India which can serve most probably as the reservoir of infection for vector ticks. Further studies are needed to confirm the presence of *A. ovis* and its capacity to cause disease in sheep, wild animals in several parts of India.

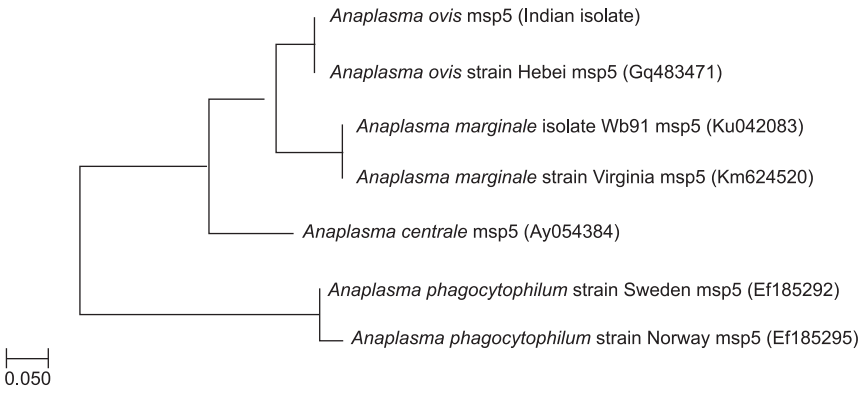

Fig. 2. Phylogenetic analysis of *msp5* gene sequences derived from various *Anaplasma* species.

Table 3. Sequence pair wise distances of *msp5* gene (nucleotide sequence)

A	B	C	D	E	F	G	H
*Anaplasma_ovis_msp5*_(Indian_isolate)							
*Anaplasma_ovis*_strain_Jingtai_*msp5*_(HM195102)	0.00						
*Anaplasma_ovis*_strain_Hebei_*msp5*_(GQ483471)	0.00	0.00					
*Anaplasma_marginale*_isolate_Wb91_*msp5*_(KU042083)	0.13	0.13	0.13				
*Anaplasma_marginale*_strain_Virginia_*msp5*_(KM624520)	0.13	0.13	0.13	0.00			
*Anaplasma_centrale_msp5*_(AY054384)	0.23	0.23	0.23	0.25	0.25		
*Anaplasma_phagocytophilum*_strain_Sweden_*msp5*_(EF185292)	0.45	0.45	0.45	0.55	0.55	0.47	
*Anaplasma_phagocytophilum*_strain_Norway_*msp5*_(EF185295)	0.47	0.47	0.47	0.60	0.60	0.50	0.03

ACKNOWLEDGEMENTS

The authors express their sincere sense of gratitude to the Vice Chancellor, LUVAS, Hisar, for providing research facilities and financial support.

REFERENCES

Ahmadi-Hamedani M, Khaki Z, Rahbari S, Kazemi B and Bandehpour M. 2009. Molecular identification of anaplasmosis in goats using a new PCR-RFLP method. *Iranian Journal of Veterinary Research* **10**(4): 367–72.

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**: 249–59.

Arunkumar S. 2014. A report on the incidence of *Anaplasma ovis* infection in sheep. *International Journal of Food, Agriculture and Veterinary Science* **4**(1): 71–72.

Aubry P and Geale D W. 2011. A review of bovine anaplasmosis. *Transboundary and Emerging Disease* **58**(1): 1–30.

d'Oliveira C, Van der Weide M, 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.

Durrani A Z and Kamal N. 2008. Identification of ticks and detection of blood protozoa in Friesian cattle by polymerase chain reaction test and estimation of blood parameters in district Kasur, Pakistan. *Tropical Animal Health and Production* **40**(6): 441–47.

Echeverry D F, Deason N A, Davidson J, Makuru V, Xiao H, Niedbalski J, Kern M, Russell T L, Burkot T R, Collins F H and Lobo N F. 2016. Human malaria diagnosis using a single-step direct-PCR based on the *Plasmodium* cytochrome oxidase III gene. *Malaria Journal* **15**: 128.

Ganguly A, Bisla R S, Singh H, Bhanot V, Kumar A, Kumari S, Maharana B R and Ganguly I. 2017a. Prevalence and haematobiochemical changes of tick borne haemoparasitic diseases in cross bred cattle of Haryana, India. *Indian Journal Animal Sciences* **87**(5): 552–57.

Ganguly A, Bisla R S, Ganguly I, Singh H, Bhanot V and Chaudhri S S. 2017b. Direct blood PCR detection of *Babesia bigemina* and its effect on haematological and biochemical profile in crossbred cattle of eastern Haryana. *Indian Journal of Animal Research* **51**(1): 141–45.

Hornok S, Elek V, de la Fuente J, Naranjo V, Farkas R, Majoros G and Földvári G. 2007. First serological and molecular evidence on the endemicity of *Anaplasma ovis* and *A. marginale* in Hungary. *Veterinary Microbiology* **122**: 316–22.

Maharana B R, Kumar B, Prasad A, Patbandha T K, Sudhakar N R, Joseph J P and Patel B R. 2016a. Prevalence and assessment of risk factors for haemoprotozoan infections in cattle and buffaloes of South-West Gujarat, India. *Indian Journal of Animal Research* **50**(5): 733–39.

Maharana B R, Tewari A K, Saravanan B C and Sudhakar N R. 2016b. Important hemoprotozoan diseases of livestock: Challenges in current diagnostics and therapeutics: An update. *Veterinary World* **9**(5): 487–95.

Ndung'u L W, Aguirre C, Rurangirwa F R, McElwain T F, McGuire T C, Knowles D P and Palmer G H. 1995. Detection of *Anaplasma ovis* infection in goats by major surface protein5 competitive inhibition enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **33**: 675–79.

Nishimori A, Konnai Satoru, Ikebuchi Ryoyo, Okagawa T, Nakahara A, Murata S and Ohashi K. 2016. Direct polymerase chain reaction from blood and tissue samples for rapid diagnosis of bovine leukemia virus infection. *Journal of Veterinary Medical Science* **78**(5): 791–96.

Noaman V and Shayan P. 2009. Molecular detection of *Anaplasma phagocytophilum* in carrier cattle of Iran- first documented report. *Iranian Journal of Microbiology* **1**(2): 37–42.

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**: 239–49.

PDADMAS. 2005. Project Directorate on Animal Disease Monitoring and Surveillance – Annual report. ICAR. pp 1–12.

Rajasokkappan S and Selvaraju G. 2016. Prevalence of anaplasmosis in goats in Ramanathapuram district of Tamil Nadu. *International Journal of Environmental Science and Technology* **5**(2): 511–14.

Saitou N and Nei M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**(4): 406–25.

Sambrook J and Russel DW. 2001. *Molecular cloning: A laboratory manual.* 3rd edn. Cold spring Harbor Laboratory Press, NY, USA.

Seong G, Han Y J, Chae J B, Chae J S, Yu D H, Lee Y S, Park J, Park B K, Yoo J G and Choi K S. 2015. Detection of *Anaplasma* sp. in Korean native goats (*Capra aegagrus hircus*) on Jeju Island, Korea. *Korean Journal of Parasitology* **53**(6): 765–69.

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**: 251–57.

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.

Shkap V, Molad T, Fish L and Palmer G H. 2002. Detection of the *Anaplasma centrale* vaccine strain and specific differentiation from *Anaplasma marginale* in vaccinated and infected cattle. *Parasitology Research* **88**: 546–52.

Sumbria D, Singla L D, Sharma A, Bal M S and Kumar S. 2015. Multiplex PCR for detection of *Trypanosoma evansi* and *Theileria equi* in equids of Punjab, India. *Veterinary Parasitology* **211**: 293–99.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30**(12): 2725–29.

Velusamy R, Rani N, Ponnudurai G and Anbarasi P. 2015. Prevalence of intestinal and haemoprotozoan parasites of small ruminants in Tamil Nadu, India. *Veterinary World* **8**(10): 1205–09.

Wikman M, Steffen A C, Gunneriusson E, Tolmachev V, Adams G P, Carlsson J and Stahl S. 2004. Selection and characterization of HER2/neu-binding affibody ligands. *Protein Engineering Design and Selection* **17**(5): 455–562.

Yousefi A, Rahbari S, Shayan P, Sadeshi-dehkordi Z and Bahonar A. 2017. Molecular detection of *Anaplasma marginale* and *Anaplasma ovis* in sheep and goat in west highland pasture of Iran. *Asian Pacific Journal of Tropical Biomedicine* **7**(5): 455–59.