Himachal Journal of Agricultural Research 44(1&2) : 103-108 (2018)

Detection of haemoprotozoa in cows and buffaloes in Kangra district of Himachal Pradesh

Jyoti Jaryal, Ajay Katoch, Sanjeev Kumar, Kritika Dhial, Mandeep Sharma, Prasenjit Dhar, Rajesh Chahota and Subhash Verma*
Department of Veterinary Microbiology
DGCN College of Veterinary and Animal Sciences
CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur-176 062, India
*Corresponding author : sverma8@gmail.com
Manuscript Received: 06.02.2019; Accepted: 02.03.2019

Abstract

In this study, a total of 57 blood samples from cows and buffaloes showing clinical signs of high fever, anemia and /or swollen lymph nodeswere screened for haemoprotozoan infections using polymerase chain reaction (PCR). Some asymptomatic animals were also included in the study.Out of 57 blood samples, 14 samples were positive for either one or the other haemoprotozoa reflecting an overall prevalence of 24.56%. A total of nine samples of which six from cows and three from buffaloes were positive for *Theileria annulata* and five samples all from cows were found positive for mixed infection of *Babesia bovis* and *Anaplasma marginale*. This study shows that haemoprotozoan infections are common in cows and buffaloes population of Kangra District of Himachal Pradeshdue to conduciveenvironmental conditions for survival of vectors responsible for carrying these infectious agents. PCR technique showed high sensitivity and specificityup to species level, as it not only detected the haemoprotozoan infections in active cases but also in asymptomatic carrier animals.

Key words: *Anaplasma marginale*, *Babesia bovis*, Bovine haemoprotozoan, Himachal Pradesh, Kangra, PCR,

Haemoprotozoan infections are posing severe health problems not only in animals but also in humans, worldwide (Maharana *et al*. 2016). These infections pose a serious threat to cattle and buffaloes and causes serious losses to animal husbandry (Soundararajan and Rajavelu 2006; Shahnawaz 2011). Haemoprotozoan infections are transmitted by vectors usually ticks which feed on the blood. Various species of ticks are responsible for their transmission depending upon theirdistribution in that particular geographical condition. The warmness and humidity, the two key parameters of climate play critical role in the growth and proliferation of tick vectors which act as constant source of infection (Velusamy 2014). Amongst haemoprotozoan infectionsBabesiosis, Theileriosis, Trypanosomiosis and Anaplasmosis are most important (Tewari *et al*. 2011; Juyal *et al*. 2005; Singh *et al*. 2007). The climate of Kangra is warm and humid which is conducive for the growth and multiplication of natural vectors of these diseases (Tripathi *et al.* 1993).

Examination of a blood smear stained with Giemsa stain is most common method to identify the haemoprotozoan agent but is helpful only in acute cases and not in case of carrier animals (Altay *et al*. 2008). Microscopy based methods also suffer from specificity and sensitivity. Also, microscopy cannot identify the haemoprotozoans species on morphological basis. On the hand, molecular techniques such as PCR provide an alternate method which is highly sensitive and specific. PCR is very reliable for quick and confirmatory detection of haemoprotozoan agent in carrier as well as animals having acute infection upto species level (Gasser 2006).

The aim of present study was to utilize PCR to detect the haemprotozoan infections in bovine with the clinical signs or asymptomatic carriers of Kangra district of Himachal Pradesh, India.

Materials and Methods

Ethical approval

Samples were collected as per ethical committee guidelines of institution.

Climate

Kangra is located between 30º to 20´ and 32º to 59´ and 75º to 47´ and 77º to 45´ E among cis Himalayan ranges namely, Siwalik and Dhauladhar [8]. Variation in maximum, minimum and average temperature is 15.1 ºC to 35 ºC, 4.3 ºC to 21.3 ºC and 0.7 ºC to 26.8 ºC [8]. May and June months are hottest months of years [8]. Climate of Kangra ranges from subtropical to wet tropical, alpine and subalpine [8]. Summers are hot and humid which favours the growth of tick population.

Sample

In this study, a total of 57 blood samples from bovine collected in EDTA. Out of 57 samples, 48 were collected from cattle with clinical signs of fever upto 104º C, lymph node enlargement, anemic and diarrhea and nine samples came from asymptomatic animals These were processed forextraction of genomic DNA using blood genomic DNA isolation kit (HiMedia, India) for performing the PCR to detect the haemoprotozoan infections. Out of 57 blood samples, 46 samples from cows and 11 samples from buffaloes. These samples were screened for haemoprotozoan using PCR.

Polymerase Chain Reaction

The PCR reaction was carried out in a total reaction volume of 50 µl comprisingof 1 µl of each 10 µM primer, 1 µl of 10µM dNTPs mix, 5 µl of 10X PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.125 µl of 5U/ml Taq DNA polymerase (Promega) and 2 µl of template DNA. The final volume was adjusted using nuclease-free water. PCR amplification was performed in a BioRad thermal cycler at 95 °C for 2 min followed by 30 amplification cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min with a final extension at 72 °C for 5 min. Primers used for generation of amplimers are shown in table 1 (Bilgic2010; Bilgic2013).

Analysis of PCR product

The amplimers were electrophoresed on 1.5% agarose (HiMedia, India) gel at a constant voltage of 10V/cm, visualized and photographed under UV light using Alpha Digidoc Gel documentation system after staining with ethidium bromide.

Results and Discussion

Out of 57 blood samples, nine were positive for *Theileria annulata* and 5 for mixed infection of *Babesia bovis* and *Anaplasma marginale*. Representative results of PCR were electrophoresed on 1.5% agarose and have been shown in figures 1 & 2. Out of nine samples, six from cows and three from buffaloes were positive for *Theileria annulata* and five samples all from cows were found positive for mixed infection of *Babesiabovis* and *Anaplasma marginale*. Overall, the prevalence of 24.56% was recorded for haemoprotozoa under investigation. *Theileria annulata* infection had the highest incidence of 15.79% followed by mixed infection of *Babesia bovis* and *Anaplasma marginale* at incidence rate of 8.77% A prevalence of 78.57% was recorded for haemoprotozoan infections in cows, while a prevalence of 21.43% was recorded in buffaloes.Of the nine samples, which were from asymptomatic cattle, twoyielded positive PCR resulted for Theileria annulata Haemoprotozoan infections are tick borne diseases. Haemoprotozoan infections cause serious economical losses to animal husbandry in term of reduced milk yield, reduced draught power, mortality and treatment cost (Guan *et al.* 2010).

Table 1. Details of primers used for specific amplification of various haemoprotozoa

Primers	Sequence 5'-3'	Product size (bp)
BOVTacyt1F	ACTTTGGCCGTAATGTTAAAC	312
BOVTacyt1R	CTCTGGACCAACTGTTTGG	
BOVAmF	GCTCTAGCAGGTTATGCGTC	265
BOVAmR	CTGCTTGGGAGAATGCACCT	
BOVBbF	CAAGCATACAACCAGGTGG	166
BOVBbR	ACCCCAGGCACATCCAGCTA	

Veterinary important blood parasites are *Theileria* spp., *Babesia* spp. and *Anaplasma* spp. (Rickettsiale) (Uilenberg 2006). Microscopy techniques are still considered as benchmark for diagnosis of haemoprotozoan infections, but microscopic techniques are unable to distinguish the type of species and subspecies of haemoprotozoa in the blood smear on morphological basis (Norval 1992). Animal affected with acute theileriosis after recovery become carrier which could not be detected using microscopy (Brown 1990). Staining of blood smears and use of microscopy for the diagnosis of haemoprotozoan infections is found to be less sensitive and unreliable in cases of carrier animals and chronic cases (Callow *et al*. 1993; Bose *et al*. 1995;Terkawi *et al*. 2011). It has been found that nucleic acid based assays like DNA hybridization, PCR, multiplex- PCR, random amplification of polymorphic DNA-PCR, restriction fragment length polymorphism- PCR, real time-PCR etc. diagnose not only active cases of haemoprotozoan infections but also chronic and carrier asymptomatic animals (Salih *et al*. 2015). PCR is found to be highly specific and sensitive (Ala and Wayne 2005; Ashuma *et al*. 2013; Sharma *et al* 2012; Ybanez *et al*. 2013). Sensitivity and specificity of PCR can be increased by using nested-PCR as observed in the case of *A. marginale* (Laha *et al*. 2015). One of the studies carried out in late nineties on bloodprotista of cattle and buffaloes in Kangra Valley, Himachal Pradesh by Jithendran(1997)found that *Theileria annulata* prevalence at 29.5%, much higher than what has been recorded in the current study. This study recorded the incidence of *B. bovis* at 8.77%which was not reported by Jithendran(1997). The incidence of *A. marginale* as reported by Jithendran (1997) was 4.5% which is lower than that recorded in our study. This study did not screen for *Babesia bigemina* for which the prevalence had been reported very high at 18.5% by

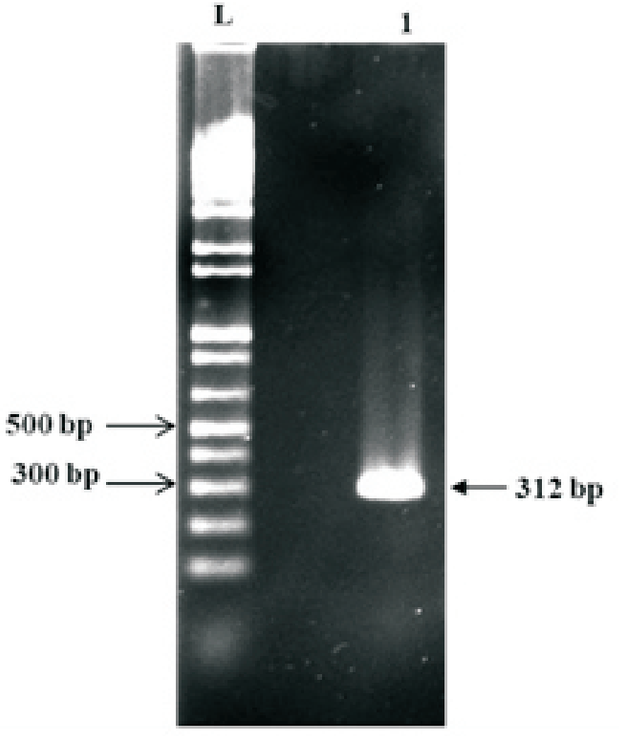

Figure 1. Positive PCR result (Lane 1) for *Theileria annulata* (312 bp). (L indicates molecular marker)

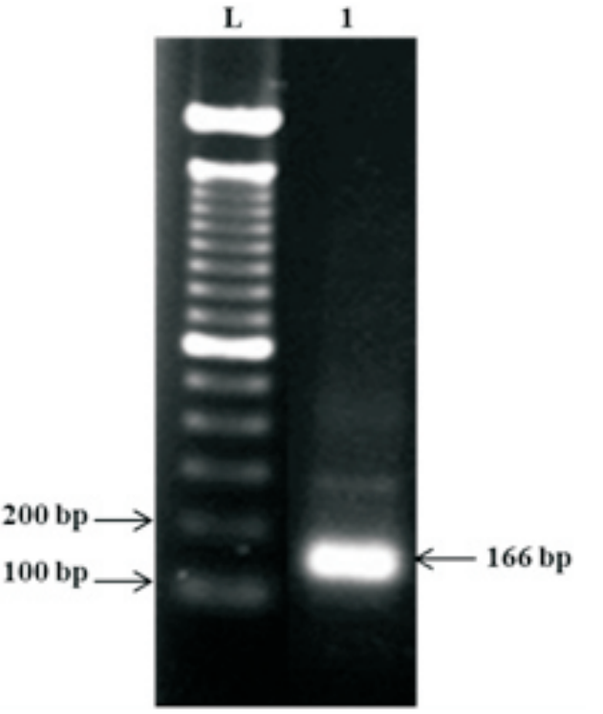

Figure 2. Positive PCR result (Lane 1) for *Babesia bovis* (166 bp). (L indicates molecular marker)

Jithendran(1997) along with prevalence of mixed *T. annulata* and *B. bigemina* infections were diagnosed in 3.5% of positive animals. However, Jithendran's work was based on microscopic exmaination of blood smears obtained from bovine. Manystudies have evaluated the utility of PCR as a tool to detect hemoprotozoan infections and have been reported in the ensuing discussion highlighting its importance in recording prevalence. In northern Kerala, highest prevalence of *Trypanosoma evansi* (34.6%) followed by *Theileria* sp. (16%) other than *T. annulata* and *B. bigemina* (0.6%) was found in cattle using polymerase chain reaction (Nair *et al.* 2011). Kakati *et al.* (2015) reported 3.6% *B. bigemina* infections in cattle using PCR in Assam. In a recent study, *B. bigemina* infection has been found in 64.91% and *A. marginale* infection in 14.03% cattle of Assam (Kakati *et al*. 2015). Although, Giemsa staining/microscopy was not used, ample studies carried out in the past had placed on record their advantages and limitation. Noaman and Shayan (2010) compared PCR-RFLP and microscopy and concluded that PCR-RFLP is more sensitive, specific and robust technique than microscopy to detect *A. marginale in cattle.* Another study by Noaman (2014)comparing microscopy with PCR has shown 57% sensitivity and 99% specificity vis-à-vis 100% sensitivity and specificity for detection of *Theileria* spp. in cattle. Hence, low sensitivity is primary limitation of microscopy, particularly in animals which are persistently affected or are latent carriers.In the current study, blood samples used were from bovine having a history of high (about up to 105 ºC) and persistent fever, (more than one week) and some were anaemic and passing dark brown to blackish watery diarrhea. Few animals have enlarged superficial lymph nodes as well. Some samples were from asymptomatic animals. PCR based detection of haemoprotozoan infection was carried out for the first time in Himachal Pradesh, albeit on a small number of animals, but it establishes beyond doubt its utility in terms of specificity, sensitivity and speed in delivering an early confirmed diagnosis of blood protozoan infection for the veterinarians to institute early rational therapy and alleviate economic losses and suffering of animals.

Conclusion

From this preliminary study, it is concluded that haemoprotozoan infection in cow and buffalo population of Kangra District of Himachal Pradesh is common in both clinically symptomatic and asymptomatic animals. Such infected animals have a poor immune system and are prone to super infection. Mixed infection corroborates this observation. Animals infected with these protozoa may also remain carrier for rest of their life if not treated well and could pose heightened threat to other healthy population in tick infested areas. It is therefore prudent to undertake proper investigation to diagnose such infection early on for institution of rational treatment and also undertake measures to get rid of vector population.

Acknowledgements

Authors thank CSKHPKV, Palampur and Head, Department Veterinary Microbiology, DGCN COVAS, Palampur for their support to complete this study. .

References

Ala L and Wayne J. 2005. Molecular approaches to detect and study the organisms causing bovine tick borne diseases: babesiosis and anaplasmosis. African Journal of Biotechnology **4**: 292-302.

Altay K, Aydin MF, Dumanli N and Aktas M. 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. Veterinary Parasitolology **158**: 295–301.

Ashuma SA, Singla LD, Kaur P, Bal MS, Batth BK and Juyal PD. 2013. Prevalence and haemato-biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India). Asian Pacific Journal of Tropical Medicine **6**: 139-144.

Bilgic HB, Karagenc T, Shiels B, Tait A, Eren H and Weir W. 2010. Evaluation of *cytochromeb* as a sensitive target for PCR based detection of *T. annulata* carrier animals. Veterinary

Parasitolology **174**: 341–347.

Bilgic HB, Karagenç T, Simuunza M,Shiels B, Tait A, Eren H and Weir W. 2013.Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. Experimental Parasitology **133** (2): 222–229.

Bose R, Jorgensen WK, Dalgliesh RJ, Friedhoff KT and de Vos AJ. 1995. Current state and future trends in the diagnosis of babesiosis. Veterinary Parasitolology **57**: 61-74.

Brown CG. 1990. Control of tropical theileriosis (*Theileria annulata* infection) of cattle. Parasitologia **32** (1): 23-31.

Callow LL, Rogers RJ, de Vos AJ. 1993. Tick-borne diseases: cattle pathology and serology. In: Corner LA, Bagust TJ (eds.), Australian Standard Diagnostic Techniques for Animal Diseases. CSIRO Information Services, East Melbourne: 1-16.

Gale KR, Dimmock CM, Gartside M and Leatch G. 1996. *Anaplasma marginale*: detection of carrier cattle by PCR ELISA. International Journal of Parasitolology **26**: 1103-1109.

Gasser RB. 2006. Molecular tools - Advances, opportunities and prospects.Veterinary Parasitolology **136** (2): 69-89.

Guan G, Moreau E, Liu J, Hao X and Luo J. 2010. Molecular evidence of experimental transmission to sheep by *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis*. Parasitology International **59**: 265–267.

JithendranKP. 1997. Blood protista of cattle and buffaloes in Kangra Valley, Himachal Pradesh. Indian Journal of Animal Sciences **67** (3): 207-208.

Juyal PD, Singla LD, Kaur P,Tandon V andDhawan BN. 2005. Management of surra due to *Trypanosoma evansi* in India: An overview. *Infectious diseases of domestic animals and zoonosis in India.* Proceedings of the National Academy of Science, India **75**: 109–120.

Kakati P, Sarmah PC, Bhattacharjee K, Bhuyan D and Baishya BC. 2015. Molecular detection and associated pathogenesis in a fatal case of *Theileria orientalis* infection in India: Probable circulation of a virulent strain and stress associated factors. International Journal of Recent Scientific Research **6**: 4235-4239.

Laha R, Mondal B, Biswas SK, Chand K, Das M, Sarma D, Goswami A and Sen A. 2015. Detection of *Babesia bigemina* infection in cattle from north-eastern India by polymerase chain reaction and its genetic relatedness with other isolates. Tropical Animal Health and Production **47** (3): 633-636.

Maharana BR, Tewari AK, Saravanan BC and Sudhakar NR. 2016.Important haemoprotozoan diseases of livestock: Challenges in current diagnostics and therapeutics: An update. Veterinary World **9** (5): 487-495.

Nair AS, Ravindran R, Lakshmanan B, Kumar SS, Tresamol PV, Saseendranath MR, Senthilvel K, Rao JR, Tewari AK and Ghosh S. 2011.Haemoprotozoa of cattle in northern Kerala, India. Tropical Biomedicine **28** (1): 68-75.

Noaman V and Shayan P. 2010. Comparison of microscopy and PCR-RFLP for detection of *Anaplasma marginale* in carrier cattle. Iranian Journal of Microbiology **2** (2): 89.

Noaman V. 2014. Comparison of molecular and microscopic technique for detection of *Theileria spp.* in carrier cattle. J. parasit. Dis.**38** (1): 64-67.

Norval RAI, Perry BD and Young AS. 1992.*The epidemiology of theileriosis in Africa*. ILRI (aka ILCA and ILRAD).

Salih DA, El Hussein AM and Singla LD. 2015. Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. Journal Veterinary Medicine Animal Health **7** (2): 45-56.

Shahnawaz S, Ali M, Aslam MA, Fatima R, Chaudhry ZI, Hassan MU, Ali M and Iqbal F. 2011. A study on the prevalence of a tick transmitted pathogen, the hematological profile of cattle from Southern Punjab (Pakistan). Parasitology Research **109** (4): 1155– 1160.

Sharma P, Juyal PD, Singla LD, Chachra D and Pawar H. 2012.Diagnosis of *Trypanosoma evansi* in cattle and buffaloes by employing real time PCR using TaqMan assay. Veterinary Parasitology **190**: 375-382.

Singh H, Mishra AK, Rao JR and Tewari AK. 2007. A PCR assay for detection of *Babesia bigemina* infection using clotted blood in bovines. Journal of Applied Animal Research **32** (2): 201-202.

Soundararajan C and RajaveluG. 2006. Prevalence of haemoprotozoan among cattle and buffaloes. Indian Veterinary Journal **83** (12):1258-1260.

Terkawi MA, Thekisoe OM, Katsande C, Latiff AA, Mans BJ, Matthee O, Mkize N, Mabogoane N, Marais F, Yokoyama N, Xuan X and Igarashi I. 2011. Serological survey of *Babesia bovis* and *Babesia bigemina* in cattle in South Africa. Veterinary Parasitolology **182**: 337-342.

Tewari AK, Ray D, Mishra AK and Bansal GC. 2011. Identification of immunodominant polypeptides common between *Babesia bigemina* and *Theileria annulata*. Indian Journal of Animal Sciences **71**: 679–680.

TripathiAK, Srivastava AK andPandey SN. 1993. *Advances in Environmental Sciences*. APH Publishing pp 111-128.

Uilenberg G. 2006. Babesia-a historical overview. Veterinary Parasitology **138** *(*1-2): 3-10.

Velusamy R, Rani N, Ponnudurai G, Harikrishnan TJ, Anna T, Arunachalam K, Senthilvel K and Anbarasi P. 2014. Influence of season, age and breed on prevalence of haemoprotozoan diseases in cattle of Tamil Nadu, India. Veterinary World **7**: 574–578.

Ybanez AP, Sivakumar T, Ybanez RD, Ratilla JC, Perez ZO, Gabotero SR, Hakimi H, KawazuS, Matsumoto K, Yokoyama N and Inokuma H. 2013. First Molecular Characterization of *Anaplasma marginale* in Cattle and *Rhipicephalus* (*Boophilus*) microplus Ticks in Cebu Philippines. Journal of Veterinary & Medical Science **75**: 27-36.