



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>

INTERNATIONAL JOURNAL  
OF ADVANCED RESEARCH

## RESEARCH ARTICLE

## Application of msp1β PCR and 16S rRNA semi nested PCR-RFLP for detection of persistent anaplasmosis in tick infested cattle

Hitesh Jaswal<sup>1</sup>, Mandeep Singh Bal<sup>1</sup>, L. D. Singla<sup>1</sup>, Amrita Sharma<sup>1</sup>, Paramjit Kaur<sup>1</sup>, C. S. Mukhopadhyay<sup>2</sup> and P. D. Juyal<sup>1</sup>

<sup>1</sup> Department of Veterinary Parasitology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, India

<sup>2</sup> School of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, India

### Manuscript Info

#### Manuscript History:

Received: 15 June 2014  
Final Accepted: 26 July 2014  
Published Online: August 2014

**Key words:** Anaplasma marginale, cattle, msp1β PCR, PCR-RFLP, semi nested PCR, ticks

#### \*Corresponding Author

Dr.L.D. Singla  
Email: ldsingla@gmail.com

### Abstract

Molecular diagnostic techniques viz; PCR targeting msp1β and semi nested PCR targeting 16SrRNA gene with subsequent restriction digestion of nested PCR products were employed for detection and identification of *Anaplasma marginale* DNA in tick infested cattle. A total of 120 blood samples along with ticks were collected from tick infested dairy cattle in and around Ludhiana district of Punjab, India. Prevalence of *Rhipicephalus (Boophilus) microplus* infestation was highest (89.16%), followed by *Hyalomma anatolicum anatolicum* (7.50%) and mixed infestation (3.33%). The detection percentage of *A. marginale* by stained thin blood smear examination of blood samples was found to be 15.83%. The BAP-2 and AL34S set of oligonucleotide primers used for amplifying msp1β gene of *A. marginale* detected DNA in 39.16% (47/120) cattle blood and detection percentage was more in adult cattle (42.30%) as compare to calves (18.75%). The semi nested PCR specific for *Anaplasma* genus revealed amplicon of 577 bp in 40.83 % (49/120) samples. Further restriction digestion of 577 bp semi-nested PCR products by Bst 1107I at *A. marginale* specific GTATAC site confirmed 42 out of 49 specific for *A. marginale* while remaining 7 undigested products indicate the presence of other species of *Anaplasma* in the region.

Copy Right, IJAR, 2014,. All rights reserved

### Introduction

Anaplasmosis caused by *Anaplasma marginale* an intra-erythrocytic rickettsial organism (Dumler et al., 2001), transmitted biologically by infected ticks or mechanically by biting flies or blood-contaminated fomites, is a common disease of cattle in Indian subcontinent. This rickettsial organism has some similarity to protozoa due to the lack of a traditional cell wall and is not capable of synthesizing lipopolysaccharide and peptidoglycan (Brayton et al., 2006). The economic losses due to tick and tick borne diseases in animals in India have been estimated to the tune of more than US\$ 498.7 millions per annum (Minjaw and Mcleod, 2003). Though exact estimate of economic losses due to anaplasmosis in India have not been documented but an estimated annual loss due to anaplasmosis in the US alone amounts to \$100 million and includes 50000 to 100000 cattle deaths (McCallon, 1973). Clinical signs of the disease include fever, anaemia, weakness, constipation, icterus, anorexia, dehydration, depression, laboured breathing, abortion and often death (Fowler and Swift, 1975; Richey and Palmer, 1990).

Anaplasmosis in cattle is traditionally diagnosis by microscopic examination of thin blood smears especially in acute form of infection. The sensitivity of this method is 10<sup>6</sup> infected erythrocytes per ml of blood

(Gale et al., 1996). Due to the low parasitemia in carrier cattle and difficulty to differentiate *Anaplasma* from other structures even by an experienced hand, this method is not recommended for the characterization of persistently infected cattle (Carelli et al., 2007). Sub-inoculation of *A. marginale* infected erythrocytes into susceptible, splenectomized calves has been considered as the 'gold standard' for detection of latent infection in cattle, but it is not practical for routine testing (Luther et al., 1980). Serological tests are may be sensitive or specific for antibody detection (OIE, 2008) but may not be able to reveal the current status of infection.

To know the presence of the active infection, specific and sensitive polymerase chain reaction was developed to detect *A. marginale* DNA from animal blood and ticks (Bekker et al., 2002, Molad et al., 2006, Carelli et al., 2007 and Ogo et al., 2012) which seems to be more practical for detection of the disease in bovines. Further, nested PCR has been devised to detect *A. marginale* in cattle increasing the specific and sensitive of the assay (Ybanez et al., 2013).

Though anaplasmosis is highly prevalent in Punjab state of India (Singh et al., 2003 and Ashuma et al., 2013), yet there are scanty reports on the application of molecular techniques for diagnosis of disease in cattle especially latent infections. Hence the present study is envisaged to apply polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism (RFLP) in addition to conventional microscopy for diagnosis of anaplasmosis in tick infested cattle along with ticks in Ludhiana district of Punjab state.

## Materials and methods:

### Samples collection:

The study was carried out in Ludhiana (latitude 30.55° North; longitude 75.54° East) district of Punjab, India. Approximately 3-4 ml of blood samples were collected in EDTA coated vials collected from jugular vein of 120 tick infested dairy cattle from Ludhiana district, Punjab state. Along with blood samples, ticks were also collected from animal body. History of clinical condition of animal was recorded at time of sample collection.

### Conventional parasitological technique:

Thin blood smears of each blood sample were prepared and examined for *A. marginale* under oil immersion (100X) after staining with Leishman stain. A sample with heavy parasitemia was used as positive control for standardization of PCR assays.

### Identification of ticks:

Ticks collected were mounted in canada balsam after clearing as per standard procedure and identified microscopically according to keys given by Miranpuri (1979).

### DNA extraction:

DNA from blood samples was extracted using Himedia® HiPura™ Blood Genomic DNA Miniprep Purification Spin Kit as per the protocol of the manufacturer. DNA extraction was also carried out from ticks (*Rhiphicephalus (Boophilus) microplus*). Randomly, tick samples from 30 animals (20 from adult cattle and 10 from calves) were also processed for DNA extraction by using Himedia® HiPura™ Mammalian Genomic DNA Miniprep Purification Spin Kit as per the given protocol. Amount of extracted DNA and its purity was measured at OD260 and ratio of OD260 to OD280 respectively.

### PCR for *A. marginale* targeting *msp1β* gene:

The BAP-2 and AL34S pair of oligonucleotide primers were used to amplify *msp1β* gene of *A. marginale* (Ulrike et al., 2003) from DNA extracted from blood samples. The nucleotide sequence of the primer is as follows.

BAP-2: 5' GTA TGG CAC GTA GTC TTG GGA TCA 3'

AL34S: 5' CAG CAGCAG CAA GAC CTT CA 3'

The PCR reaction mixture (25 µl) was contained 12.5µl of KAPA 2G™ Fast Hotstart Ready Mix (2X containing KAPA2G fast hotstart DNA polymerase, KAPA 2G fast hotstart PCR buffer, 0.2 mM dNTP each, 1.5mM MgCl<sub>2</sub>) with 1.5 µl/0.6 µM of BAP-2/ AL34S primers (10 pmol), 4.5 µl nuclease free water and 5µl DNA template in automated Thermocycler (Eppendorf, master cyler personal) with the following programme: initial denaturation at 95°C (5min), 30 cycles of denaturation at 95°C (1min), annealing at 60°C (1 min), extension at 72° C (1.5 min) with final extension at 72 °C (5min). The amplified PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV Transilluminator for detection of 407 bp amplified product in positive cases.

### Semi nested PCR for *Anaplasma spp* targeting 16S rRNA gene:

Semi nested PCR was employed to DNA extracted from blood samples by using following primers:

P1: 5'AGAGTTTGATCCTGGCTCAG 3'

P2: 5'AGCACTCATCGTTTACAGCG 3'

P3: 5' GTTAAGCCCTGGTATTTTAC 3'

PCR reactions for semi nested PCR were standardized as per the conditions given by (Noaman et al., 2009) with minor modifications. P1 and P2 set of primers were used in the primary reaction and P1 and P3 set of primers were used in secondary reaction to amplify 16S rRNA gene of *Anaplasma*. The PCR reaction mixture (25 µl) for primary PCR was contained 12.5µl of KAPA 2G™ Fast Hotstart Ready Mix (2X containing KAPA2G fast hotstart DNA polymerase, KAPA 2G fast hotstart PCR buffer, 0.2 mM dNTP each, 1.5mM MgCl<sub>2</sub>) with 1.0 µl/0.4 µM of each primer (P1, P2, 10 pmol), 5.5µl of nuclease free water and 5µl DNA template in automated Thermocycler (Eppendorf, master cyler personal) with the following programme: initial denaturation at 95°C (5min), 35 cycles of denaturation at 94°C (45 seconds), annealing at 57°C (45 seconds), extension at 72° C (1.5 min) with final extension at 72 °C (10min). The amplified PCR products were separated by electrophoresis and visualized for 781 bp amplified product.

For secondary reaction, protocol was same as primary PCR with 0.5µl of primary PCR product as template. The amplified PCR products were separated by electrophoresis and visualized for 577 bp amplified product.

#### **Restriction fragment length polymorphism (RFLP) for detection of *A. marginale* DNA:**

Restriction fragment length polymorphism (RFLP) technique was applied for confirmation of *A. marginale* DNA in the samples. The 577 bp product of semi-nested PCR for *Anaplasma* spp. was purified from enzyme and salts using Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit as per standard protocol of manufacturer. Ten µl of purified PCR product was then digested with 0.1 µl restriction endonuclease Bst1107I (BstZ17I; Thermo Scientific/Fermentas) (10U/ µl) in 2.5 µl 10X buffer and 12.5 µl DPEC water. This treatment was done at 37°C for 2 hour. The restriction endonuclease Bst1107I recognizes the sequence GTA↓TAC specific for *A. marginale* in the amplified PCR product (577 bp) and digests the template into 68 bp and 509 bp products in positive cases. The GTA↓TAC restriction site is specific for *A. marginale* genome and is not present in the corresponding PCR product of other *Anaplasma* species. As control, 10 µl PCR products were treated with 2.5 µl of 10X corresponding buffer and 12.5 µl H<sub>2</sub>O without adding of enzyme to differentiate the size of digested products from parent product.

#### **PCR for detection of *A. marginale* DNA from ticks:**

PCR targeting *msp1β* gene (as discussed above) was applied to DNA extracted from thirty tick samples (20 from adult cattle and 10 from calves). Analysis of Nucleotide Sequence

#### **Sequence analysis:**

PCR products each from *msp1β* and 16S rRNA semi nested PCR were custom sequenced from Xcelris Genomics, Ahmedabad, India. The nucleotide sequences were subjected to BLASTn analysis (Altschul et al., 1990) for determining the similarity with the sequences present in the nucleotide database. The final sequences were submitted to NCBI database and the accession numbers KF696857 and KF696858 were obtained.

The homologous sequences of *msp1β* belonging to different strains of *A. marginale* were retrieved from database using highly similar BLASTn. Phylogenetic analysis was done by the software MEGA5.2 (Tamura et al., 2011) using Maximum Likelihood with 500 bootstrap re-sampling.

#### **Statistical analysis:**

Data was analyzed using Win Episcope 2.0 software (Test of agreement) and Chi square (Test of association of factors).

## **Results**

The overall percent detection rate of stained thin blood smear examination for *A. marginale* infection in district Ludhiana was found to be 15.83% (19/120). In total, 17.30% (18/104) adults and 6.25% (1/16) calves were positive for *A. marginale* infection (Table 1) depicting apparently higher incidence of anaplasmosis in adults.

Results of tick identification from the collected specimen revealed more number of animals with *Rhipicephalus (Boophilus) microplus* infestation (89.16%) followed by *Hyalomma anatolicum anatolicum* (7.50%) and mixed infestation (3.33%). PCR amplification targeting *msp1β* gene revealed amplicon of 407 bp (Fig. 1) in positive cases with no amplification in negative controls. A total of 47 (39.16%) blood samples out of 120 were positive for *A. marginale* DNA. Detection percentage of *A. marginale* among adults and calves was recorded as 42.30% (44/104) and 18.75% (3/16) respectively (Table 1) with an apparent difference (P= 0.072308). Out of 120 animals under study, 21 animals had fever, rest 99 were apparently healthy at the time of sampling. Out of those 21 animals, 8 (38.09%) were positive for *A. marginale* by microscopic thin blood smear examination and 14 (66.67%) were found positive by conventional PCR. Among apparently healthy animals 11 (11.11%) were positive by microscopic thin stained blood smear examination and 33 (33.33%) were positive by conventional PCR (Table 2). There is significant difference in animals with the history of fever and detection by CPT (Chi square test, P Value=0.00209) and PCR (Chi square test, P Value=0.004477).

Primary nested PCR revealed only 10.83% (13/120) samples positive for *Anaplasma* genus with product size of 781 bp (Fig. 2). An amplicon of 577 bp (Fig. 3) was obtained in 40.83% (49/120) samples after secondary nested PCR confirming the presence of *Anaplasma* spp DNA. There was significant increase in sensitivity, in secondary/semi-nested PCR. Upon restriction digestion of secondary PCR products two DNA fragments of 509 bp (Fig. 4) and 68 bp were obtained confirming sample positivity for *A. marginale*. Results of PCR-RFLP revealed 42 out of 49 specific for *A. marginale* while remaining 7 undigested products indicate the presence of other species of *Anaplasma* in the region. (Table 3). However further studies are needed to confirm this aspect as the same may be due to heterogeneity in the restriction site of *A. marginale* sub species.

Results of molecular diagnosis indicate positivity of *Anaplasma* by molecular tool in animals infested with *R. (Boophilus) microplus*. None of the animal with *H. anatolicum anatolicum* infestation was found positive for *A. marginale* infection. Study also revealed 26.67% (8/30) tick DNA samples positive for *A. marginale* after PCR amplification targeting *msp1β* gene. Detection percentage from ticks collected from adult cattle was 30% (6/20) as compare to 20% (2/10) in ticks collected from calves (Table 1) the difference being statistically non significant.

Phylogenetic analysis of *A. marginale* sequence showed that the strain of the present study is most closely related to strain Israeli and Florida as against strain PR1 and Habana (H) (Fig. 5).

**Table 1. Detection percentage of *Anaplasma marginale* in tick infested cattle.**

Age group	Blood Samples Examined	Detection Percentage (Positive Samples)	
		Conventional Microscopy	Conventional PCR ( <i>msp1β</i> )
Adult Cattle	104	17.30% (18)	42.31(44)
Cattle Calves	16	6.25%(1)	18.75(3)
<b>Total</b>	<b>120</b>	<b>15.83% (19)</b>	<b>39.16(47)</b>

$\chi^2$  test, P Value =0.259336 for Stained thin blood smear indicating detection rate non significantly higher in adult cattle as compare to calves

$\chi^2$  test, P Value: 0.072308 for conventional PCR indicating detection rate non significantly higher in adult cattle as compare to calves.

**Table 2. Detection rate of *Anaplasma marginale* infection in animals with history of fever and apparently healthy animals.**

Health status	Number of animals	Conventional microscopy	Conventional PCR
History of fever	21	8 (38.09%)	14 (66.67%)
Apparently healthy animals	99	11 (11.11%)	33 (33.33%)
Total	120	19	47

$\chi^2$  test, P Value=0.002093 ( in case of CPT with fever)

$\chi^2$  test, P Value=0.004477 (in case of PCR with fever)

**Table 3. Detection rate by semi-nested PCR for *Anaplasma* genus and PCR-RFLP for *A. marginale*.**

	Primary PCR ( <i>Anaplasma</i> spp.)	Semi nested PCR ( <i>Anaplasma</i> spp.)	PCR-RFLP ( <i>Anaplasma marginale</i> )
Number of Samples	120	120	120
Positive	13	49	42
Detection rate (%)	10.83	40.83	35

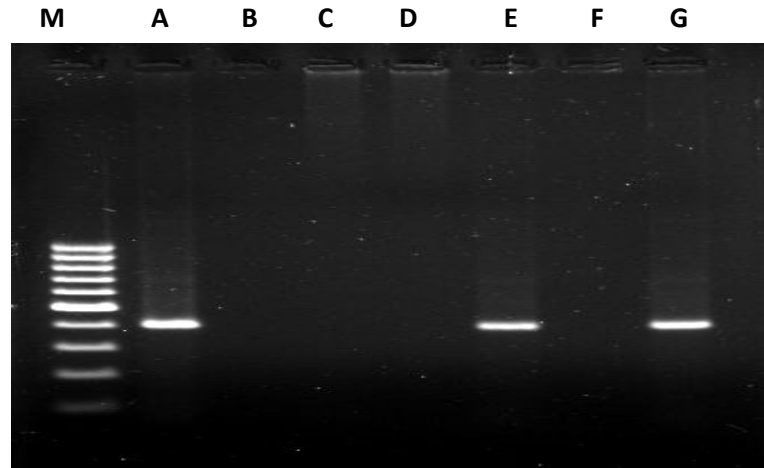


Fig. 1. Agarose gel (1.5%) electrophoresis showing amplified DNA (407bp) using primers BAP-2 & AL43S primers of *Anaplasma marginale* targeting *msp1 $\beta$*  sequence. M=100bp ladder, A=Positive control, B=Negative control, C-G=Field samples.

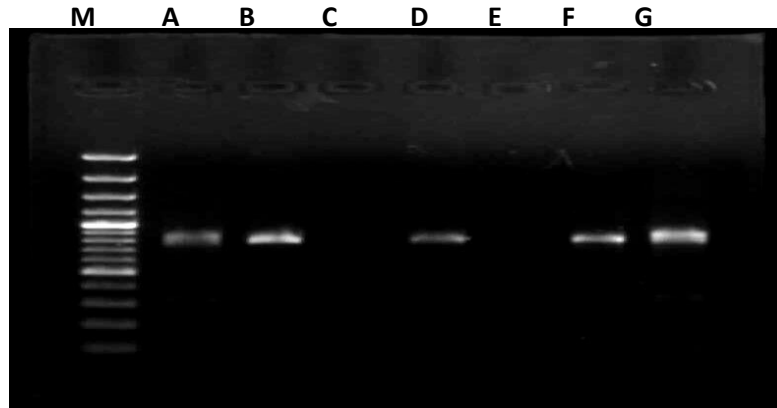
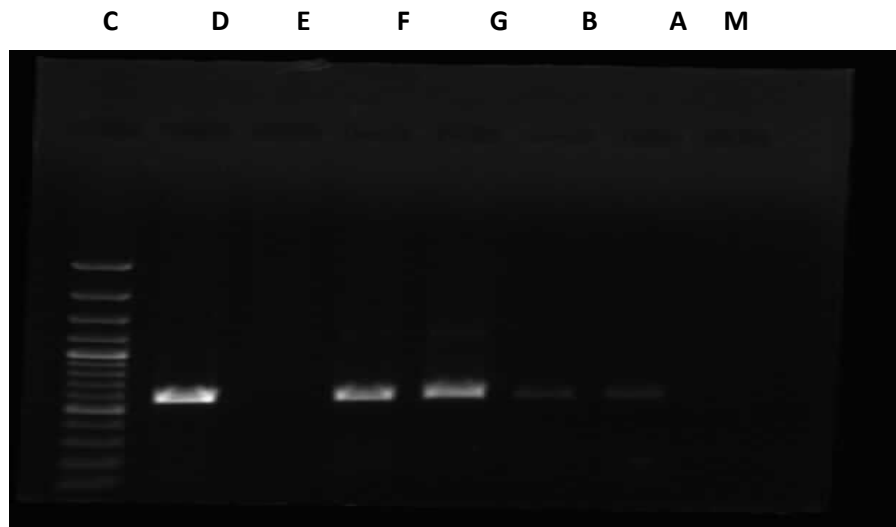
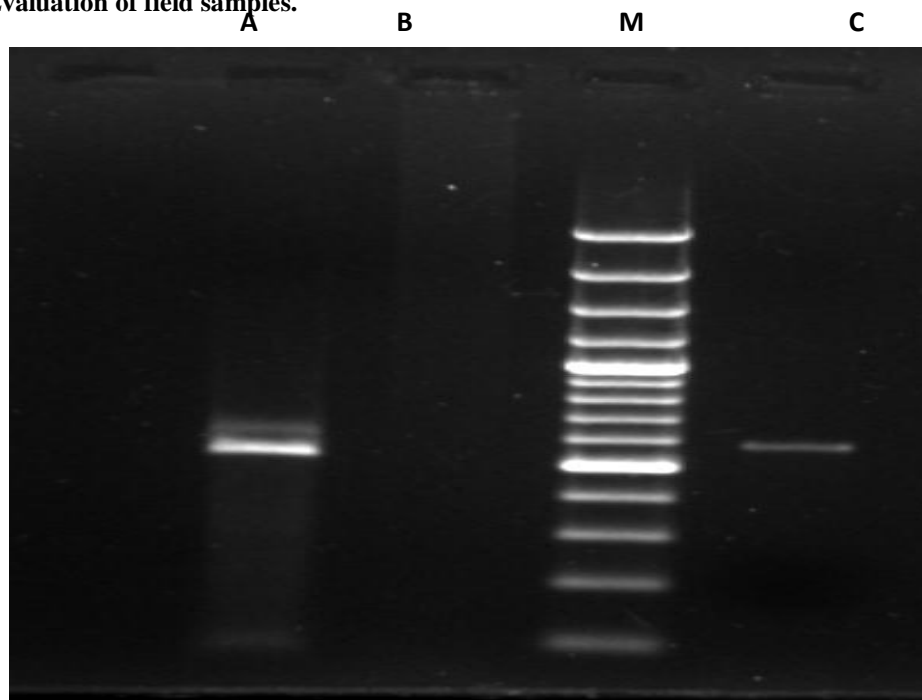


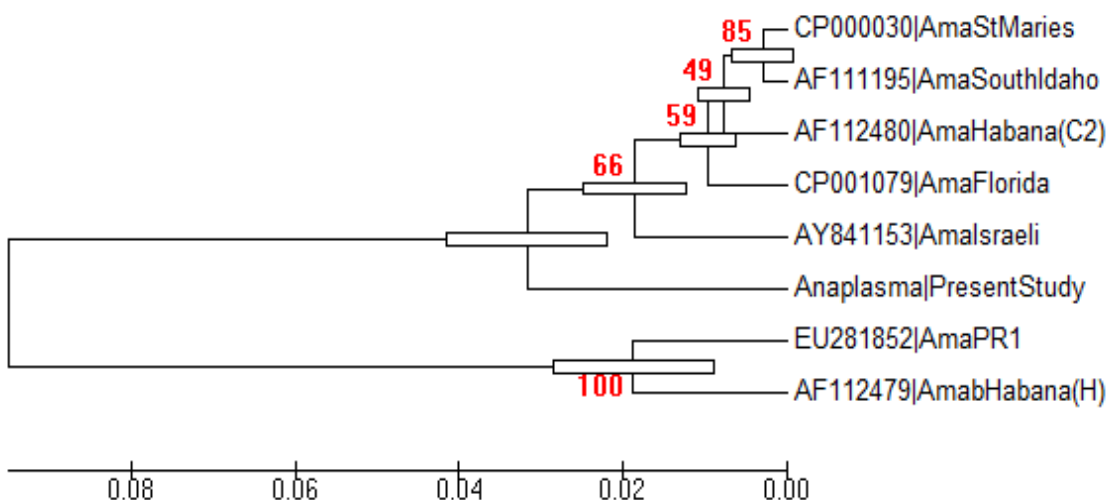
Fig. 2. Agarose gel (1.5%) electrophoresis showing amplified DNA (781 bp) using primers P1 & P2 primers of *Anaplasma* spp. targeting 16srRNA gene sequence. M=100bp ladder, A= Positive control, C= Negative Control, B,D,E,F,G= Evaluation of field samples.



**Fig. 3.** Agarose gel (1.5%) electrophoresis showing amplified DNA (577bp) using primers P1 & P3 primers of *Anaplasma* spp. targeting 16srRNA gene sequence. M=100bp ladder, A=Positive control, B=Negative control, C,D,E,F,G=Evaluation of field samples.



**Fig. 4.** Agarose gel (1.5%) electrophoresis showing PCR-RFLP product DNA and undigested Product DNA using primers P1 & P3 of *Anaplasma marginale* targeting Restriction site. M=100bp ladder, A=P1 & P3 digested product (509 & 68 bp), B=Negative control, C=Undigested Product.



**Fig. 5.** Phylogenetic tree constructed by maximum likelihood method (with 500 bootstrap resampling) from the partial *msp1b* coding sequences of different strains of *Anaplasma marginale*.

### Discussion:

In the current study PCR based detection rate of *A. marginale* in tick infested cattle was found to be 39.16 % as compared to 15.83% detection rate by conventional stained blood smear examination. PCR is found to be more sensitive as compared to conventional microscopy for diagnosis of infection in cattle which has been previously

reported by many authors (Carelli et al., 2007, Gale et al., 1996 and Ashuma et al., 2013). Incidence of *A. marginale* was found apparently more among adults (42.30%) as compared to calves (18.75%). Anaplasmosis is essentially a disease of adult cattle while younger animals may remain susceptible but exhibit little detectable signs. Adult animals are considered to harbor carrier phase of the infection resulting from their prior exposures in life to *A. marginale* infection (Soulsby, 1982 and Singh et al., 2003). In the present study, animals with history of fever were found to have higher chances of *A. marginale* infection both by CPT and PCR. Usually pyrexia is the first recorded sign of anaplasmosis and may occur prior to the infection of 1% of the erythrocytes (Jones and Brock 1966).

Semi nested PCR targeting 16S rRNA gene for detection of *Anaplasma* spp. DNA and further restriction digestion (RFLP) of PCR products for *A. marginale* specific site in cattle blood was applied. Studies by Echaide et al. (1998) revealed nested PCR ability to detect as few as 30 infected erythrocytes per ml to diagnose low levels of rickettsemia. Present study revealed that after digestion of 49 PCR products only 42 found positive for *A. marginale*, however 7 remained undigested.

Statistically there is substantial agreement (kappa value=0.661) between detection percentage of conventional PCR with primers BAP-2/ AL34S and PCR RFLP for accurate diagnosis of *A. marginale* infection in blood of cattle.

The difference in detection percentage of conventional PCR and PCR-RFLP may be due to alterations in cut site of enzyme, specificity of enzyme for the restriction site and inherited sensitivity of individual primers. However, Ahmadi-Hamedani et al. (2009) depicted PCR-RFLP assay as a specific and sensitive diagnostic tool enabling direct and concurrent identification of two *Anaplasma* species. Jefferies et al. (2007) reported that PCR in combination with restriction fragment length polymorphism (RFLP) analysis offers an effective and rapid means of discriminating between species without the need for DNA sequencing. Caccio et al. (2002) reported detection of unusual species with universal primers followed by sequencing or RFLP. PCR-RFLP can be considered being more accurate than simple PCR, nevertheless due to very small difference in digested and undigested PCR RFLP products, few samples may have been remained undigested. As restriction site is specific to *A. marginale* in 16S rRNA gene, PCR RFLP technique can be used to detect the *A. marginale* after restriction endonuclease digestion (Noaman and Shayan 2010a, 2010b). Present study revealed significantly higher sensitivity of msp1 $\beta$  PCR and 16S rRNA PCR RFLP as compared to conventional microscopy in detecting latent infection of *A. marginale* in cattle.

According to the findings of current study, *R. (Boophilus) microplus* found to be more in the region as compare to *Hyalomma anatolicum anatolicum*. Singh (2012) also reported more prevalence of *R. (Boophilus) microplus* as compare to *Hyalomma anatolicum anatolicum* in various agroclimatic zones of Punjab state may be due to the effects of macroclimatic factors (Singh et al., 2000). Current study found none of the animal with *H. anatolicum anatolicum* infestation positive for *A. marginale* infection both by conventional microscopy and molecular methods. Studies conducted by Esteves et al. (2009) demonstrated that *R. (Boophilus) microplus* and *R. annulatus* may be the major tick vectors of *A. marginale* in tropical and subtropical regions of the world. Study revealed non significantly higher detection percentage of *A. marginale* DNA by PCR in ticks collected from adult cattle (30%) than those from calves (20%). Ogo et al. (2012) worked on molecular prevalence of *A. marginale* in *R. microplus* ticks and reported 20% (9/45) ticks positive for *A. marginale* DNA by PCR. Ybanez et al. (2013) also conducted similar studies on cattle ticks and found 13.33% (8/60) *R. (Boophilus) microplus* ticks positive for *A. marginale* DNA by PCR.

Relatively less prevalence of *A. marginale* was seen in tick samples (26.67%) as compared to blood samples (39.16 %). However, Eriks et al. (1993) observed positive correlation between rickettsemia levels in cattle and the resulting infection rates of ticks. They also stated that if persistent low level of parasitemia is maintained in cattle then transmission rates of infection to ticks can be reduced.

Results of present study explored the status of anaplasmosis in tick infested cattle of Ludhiana district of Punjab, India. The molecular tools viz: PCR, PCR-RFLP will be helpful for detection of latent infections of *A. marginale* in tick infesting cattle which may act as nidus for spread of disease to susceptible animals.

### Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

### Acknowledgments

First author is thankful to the Dean Postgraduate Studies-cum-Director Research, GADVASU, for permission to carry out Master's research work. The work was carried out under RKVY project entitled, 'Employment of immune-molecular diagnostic tools for haemoprotozoans in Livestock of Punjab'.

## 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. *Iran.J. Vet. Res.* 4: 367-372.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Ashuma, Sharma, A., Singla, L. D., Kaur, P., Bal, M. S., Bath, B. K. and Juyal, P. D. (2013). Prevalence and haemato-biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India). *Asian Pac. J. Trop. Biomed.* 6: 139-144.
- Bekker, C. P., De Vos, A., Taoufik, A., Sparagano, O. A. and Jongejan, F. (2002). Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Vet. Microbiol.* 89: 223-238.
- Brayton, K. A., Palmer, G. H. and Brown, W. C. (2006). Genomic and pro-teomic approaches to vaccine candidate identification for *Anaplasma marginale*. *Expert Rev. Vaccines.* 5:95-110.
- Caccio, S. M., Antunovic, B., Moretti, A., Mangili, V., Marinculic, A., Baric, R., Slemenda, S. and Pieniazek, N. (2002). Molecular characterisation of *Babesia canis canis* and *Babesia canis vogeli* from naturally infected European dogs. *Vet. Parasitol.* 106: 285-292.
- Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L. and Buonavoglia, C. (2007). Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet. Microbiol.* 124: 104-107.
- Dumler, J. S., Barbet, A. F., Bekker, C. P., Dasch, G. A., Palmer, G. H., Ray, S. C., Rikihisia, Y. and Rurangirwa, F. R. (2001). Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int. J. Syst. Evol. Micr.* 51: 2145-2165.
- Echaide, S. T. D., Knowles, D. P., McGuire, T. C., Palmer, G. H., Suarez, C. E. and McElwain, T. F. (1998). Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J. clin. Microbiol.* 36: 777-782.
- Eriks, I. S., Stiller, D. and Palmer, G. H. (1993). Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. *J. clin. Microbiol.* 31: 2091-2096.
- Esteves, E., Bastos, C. V., Zivkovic, Z., De la fuente, J., Kocan, K. D., Blouind, E., Ribeiro, M. F., Passosf, L. M. F. and Daffre, S. (2009). Propagation of a brazilian isolate of *Anaplasma marginale* with appendage in a tick cell line (bme26) derived from *Rhipicephalus (boophilus) microplus*. *Vet. Parasitol.* 161: 150-153.
- Fowler, D. and Swift, B. L. 1975. Abortion in cows inoculated with *Anaplasma marginale*. *Theriogenology.* 4: 59-67.
- Gale, K. R., Dimmock, C. M., Gartside, M. and Leatch, G. (1996). *Anaplasma marginale*: Detection of carrier cattle by PCR. *Int. J. Parasitol.* 26: 1103-1109.
- Jefferies, R., Ryan, U. M. and Irwin, P. J. (2007). PCR-RFLP for the detection and differentiation of the canine Piroplasm species and its use with filter paper-based technologies. *Vet. Parasitol.* 144: 20-27.
- Jones, E. W. and Brock, W. E. 1966. Bovine anaplasmosis: its diagnosis, treatment and Control. *J. Am. Vet. Med. Assoc.* 149: 1624-33.
- Luther, D.G., Cox, H.U. and Nelson, W.O. (1980). Comparisons of sero tests with calf inoculations for detection of carriers in anaplasmosis-vaccinated cattle. *Am. J. Vet. Res.* 41: 2085-86.
- McCallon, B. R. (1973). Prevalence and economic aspects of anaplasmosis. Proceedings of the Sixth National Anaplasmosis Conference. pp 1-3. Heritage Press, Still water, Okla.
- Minjauw, B. and McLeod, A. (2003). Tick-borne diseases and poverty: the impact of ticks and tick-borne diseases on the livelihood of small scale and marginal livestock owners in India and eastern and southern Africa. Research report, DFID Animal Health Programme. Edinburg: Centre for Tropical Veterinary Medicine, University of Edinburgh, UK.

- Miranpuri, G. S. (1979). Tick taxonomy in India- a review (including notes on their biology, ecology, geographical distribution, host-relationship, ticks and tick-borne diseases and keys for species identification). Paper presented at workshop at Advances in Insect Taxonomy in India and the Orient, Manali, Himachal Pradesh, from Oct 9 -12. India.
- Molad, T., Mazuz, M. L., Fleiderovitz, L., Fish, L., Savitsky, I., Krigel, Y. and Leibovitz, B. (2006). Molecular and serological detection of *A. centrale*- and *A. marginale*-infected cattle grazing within an endemic area. *Vet. Microbiol.* 113: 55–62.
- Noaman, V. and Shayan, P. (2010a). A new PCR-RFLP method for detection of *Anaplasma marginale* based on 16SrRNA. *Vet. Res. Comm.* 34: 43–50.
- Noaman, V. and Shayan, P. (2010b). Comparison of microscopy and PCR-RFLP for detection of *Anaplasma marginale* in carrier cattle. *Iran. J. Microbiol.* 2: 89-94.
- Noaman, V., Shayan, P. and Amininia, N. (2009). Molecular diagnostic of *Anaplasma marginale* in carrier cattle. *Iranian J. Parasitol.* 4: 26-33.
- Ogo, N. I., de Mera, I. G. F., Galindo, R. C., Okubanjo, O. O., Inuwa, H. M., Agbede, R. I. S., Torina, A., Alongi, A., Vicente J., Gortázar, C. and De La Fuente, J. (2012). Molecular identification of tick-borne pathogens in Nigerian ticks. *Vet. Parasito.* 187: 572– 577.
- O I E. (2008). Bovine Anaplasmosis. “Terrestrial Manual” O.I.E. 1: 599-610.
- Richey, E. J. and Palmer, G. H. (1990). Bovine anaplasmosis. *Comp. Cont. Educ. Pract.* 12: 1661–1668.
- Singh A.P., Singla L.D. and Singh A. (2000). A study on the effects of macroclimatic factors on the seasonal population dynamics of *Boophilus microplus* (Canes, 1888) infesting the cross-bred cattle of Ludhiana district. *Int. J. Anim. Sci.* 15(1): 29-31.
- Singh, J, Tuli, A. and Singla, L.D. (2003). Mixed *Anaplasma* and *Babesia* infection outbreak in crossbred cattle. *Punjab Vet. J.* 3: 73-74.
- Singh, N. K., 2012. Epidemiology of Ixodid ticks in dairy animals and detection of resistance against synthetic pyrethroids in *Rhipicephalus microplus* in Punjab. PhD dissertation. Guru Angad Dev Veterinary and Animal Sciences, Ludhiana, Punjab.
- Soulsby, E.J. L. (ed). (1982). Helminths, arthropods and protozoa of domesticated animals. 7<sup>th</sup> Edn. Lea and Febiger, Philadelphia.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28: 2731-2739.
- Ulrike, G. M., Cynthia, M. T., Meghan, J. L., Elizabeth, W. H., Timothy, J. K., and William, R. D. (2003). Isolation of an *Anaplasma* sp. organism from White-Tailed Deer by tick cell culture. *J. clin. Microbiol.* 41: 4328-4335.
- Ybanez, A. P., Sivakumar, T., Ybanez, R. D., Ratilla, J. C., Perez, Z. O., Gabotero, S. R., Hakimi, H., Kawazu, S., Matsumoto, K., Yokoyama, N. and Inokuma, H. (2013). First Molecular Characterization of *Anaplasma marginale* in Cattle and *Rhipicephalus (Boophilus) microplus* Ticks in Cebu Philippines. *J. Vet. Med. Sci.* 75:27-36.