DOI: https://doi.org/10.54393/fbt.v4i02.112

FUTURISTIC BIOTECHNOLOGY

https://fbtjournal.com/index.php/fbt
ISSN (E): 2959-0981, (P): 2959-0973
Volume 4, Issue 2 (April-June 2024)

Original Article

OPEN ACCESS

First Evidence of Haplotypes of *Babesia bigemina* from District Sialkot Pakistan and their Relation to Other Countries

Asma Waheed Qureshi[1*] and Duaa Mir[1]

[1]Department of Zoology, Government College Women University, Sialkot, Pakistan

ARTICLE INFO

Keywords:
Haplotype Analysis, Tick-Borne Disease, Phylogenetic, 18S rRNA Gene

How to Cite:
Qureshi, A. W., & Mir, D. (2024). First Evidence of Haplotypes of *Babesia bigemina* From District Sialkot Pakistan and Their Relation to Other Countries: Haplotypes of *Babesia bigemina*. Futuristic Biotechnology, 4(02). https://doi.org/10.54393/fbt.v4i02.112

***Corresponding Author:**
Asma Waheed Qureshi
Department of Zoology, Government College Women University, Sialkot, Pakistan
asma.qureshi@gcwus.edu.pk

Received Date: 3rd April, 2024
Acceptance Date: 18th May, 2024
Published Date: 30th June, 2024

ABSTRACT

Babesiosis is a tick borne disease of animals and causes significant losses to livestock sector and in turn to the economy of Pakistan. **Objective:** To analyze the molecular and phylogenetic analysis of *Babesia* species for inter and intra specific genetic variations in district Sialkot, Pakistan. **Methods:** Total 150 (Cow=120, Buffalo=20 and Sheep=10) blood samples were collected. Microscopic examination of blood smears stained by field stain method was performed. DNA was extracted by phenol chloroform extraction method and 18S rRNA gene was targeted for PCR amplification. MEGAX software was used to perform phylogenetic analysis. Haplotype analysis was performed with Popart software by using median joining network method. **Results:** Out of 150 blood samples, 50 (33%) were positive for *Babesia bigemina (B. bigemina).* Out of these, 36 showed 446bp bands specific for 18S rRNA gene of *B. bigemina.* In Phylogenetic tree one sequence from cow had 98% similarity with sequence from Uganda while all other sequences from cow had 98% similarity with sequence from China. Isolate from buffalo showed 98% similarity with sequence of buffalo previously reported from Pakistan and sequence of sheep appeared as more related to buffalo samples. Haplotype analysis from current study revealed four haplotypes, two from cow, one each from buffalo and sheep. **Conclusions:** This study was helpful for molecular and phylogenetic analysis of *Babesia* isolates from district Sialkot, Pakistan. Haplotyping has revealed the genetic variants among different and same host species.

INTRODUCTION

Babesiosis is one of the most significant Tick-Borne Diseases (TBDs) that affects Bovids in Pakistan [1]. It is a deadly disease which is caused by an intra-erythrocytic protozoan parasite belonging to genus *Babesia.* The important vectors for transmission of this disease is *Boophilus* genus of ticks which is extensively available in tropical and subtropical countries [2]. *Babesiosis* is also known as the red-water disease which is caused by different species of genus *Babesia.* Both subclinical and acute infections are caused by *Babesia* spp. [3]. The parasite multiplies in red blood cells that results in the demolition of a huge number of red blood cells. The most important characteristic feature of this disease is coffee colored urine. Affected animals experience the noticeable increase in body temperature (40-41°C), struggle for breathing, loss of appetite, termination of rumination, jaundice of various degrees from paleness in case of mild infection to severe yellow coloration of mucous membranes of vagina and conjunctiva in more progressive cases, abnormal thinness of body (emaciation), progressive hemolytic anemia, increased heart rate, increased respiratory rate, haemoglobinuria, weakness in body, unwillingness to move and eye problems. The high fever in infection causes abortion to pregnant females in some cases [4]. It also reduces the fertility in males specially bulls [5]. Traditionally, the microscopic examination is used for identification of parasites in stained blood smears. The sensitivity of this method is low and it cannot differentiate morphologically indistinguishable organisms [6]. Polymerase Chain

DOI: https://doi.org/10.54393/fbt.v4i02.112

Reaction (PCR) is a nucleic acid based assay that is far more sensitive than conventional methods for the identification of parasites including *B. bigemina* [7, 8]. Polymerase Chain Reaction (PCR) is sensitive to the extent that, it can detect parasite even if there is only one parasitic cell present in the sample. The PCR based methods have been proved as almost 100 times more specific and sensitive than microscopic examination. This method is highly specific and consumes less time which makes it more satisfactory for diagnostic purposes [8-10].

The current study was for molecular characterization of *Babesia* species in District Sialkot, Pakistan and to investigate about the evolutionary relationship of isolated species with reported *Babesia* spp. throughout the world. This type of study has not been previously done from District Sialkot, Pakistan. Hence, this study on molecular analysis of *B. bigemina* from Bovids may be contemplated as first report of this kind in District Sialkot, Pakistan.

METHODS

Sample Collection

Blood samples for detection of *Babesia spp.* were collected from January 2019 - June 2020 from district disease diagnostic laboratory, Sialkot. Total 150 blood samples were collected from different host animals in sterilized vacutainers containing EDTA. Out of 150 blood samples 120 were collected from cows, 20 from buffalos and 10 samples were collected from sheep.

Microscopic Examination

The microscopic examination of *Babesia spp.* was performed at District disease diagnostic laboratory, Sialkot. Blood samples were analyzed for identification and morphological differentiation of piroplasms of *Babesia spp.* by field stain method by following the standard protocol. Finally, the stained slide was observed under oil immersion lens of light microscope.

DNA Extraction

The genomic DNA extraction was performed by Phenol: Chloroform: Isoamyl alcohol (PCI) method as previously described [11]. The extracted DNA was stored at -20ºC till further utilization. DNA extraction was confirmed by running the samples on 1% agarose gel.

PCR Amplification

A pair of primers specific for 18S rRNA gene of *B. bigemina* i.e., for forward reaction **F**-5 CCCAATCCTGACACAGGGAG3' and for reverse reaction **R**-5 GCAAATGCTTTCGCAGTGGT3' was used to amplify the amplicon of 446bp [12]. PCR amplification was performed by using GeneDireX, Inc. amaR OnePCR kit. A 50µl PCR mixture was prepared by adding 30µl PCR mix from kit, 3µl of each forward and reverse primers, 10µl of distilled water and 4µl of template DNA. Thermal cycler (BIO-RAD T100™) was set for 35 cycles under conditions of thermal cycler for PCR amplifications as given in table 1.

Table 1: Thermal Cycler Conditions for PCR Amplification

Steps	Temperature (°C)	Time	Cycles
Initial Denaturation	95°C	5 min	-
Denaturation	94°C	5 sec	35 Cycles
Annealing	57°C	1 min	
Extension	72°C	1 min	
Final Extension	72°C	10 min	
Infinite Hold	4°C	∞	

PCR products were run on 1% agarose gel. PCR products in gel were visualized in gel documentation system for the desired amplicon size specific for species under examination.

Sequencing and Bioinformatics Analysis

PCR products were sent for sequencing to 1st BASE, Singapore and Malaysia. Purification of PCR products was also performed. PCR products were sequenced in forward direction. Nucleotide BLAST was used for the analysis of 18S rRNA gene sequences for similarity and accuracy level. Nucleotide sequence from different animal hosts showing genetic diversity were submitted to Genbank and accession numbers was received. MEGA X software was used for construction of phylogenetic tree by neighbor joining method with Kimura 2 parameter model and bootstrap support from 1000 replicates.

Haplotype Analysis

A haplotype network to show the relationship of different haplotypes derived from 18S rRNA gene sequence of B. *bigemina* generated from samples of present study and those reported from different selected countries was done by using Popart software 4.1. Haplotypes were generated by median joining network method which is a character based method of haplotype analysis.

RESULTS

Microscopic Examination

Microscopic identification of this species was accomplished according to the morphological characteristics reported by [13] which indicated the presence of *B. bigemina* species in samples of current study as shown in figure 1.

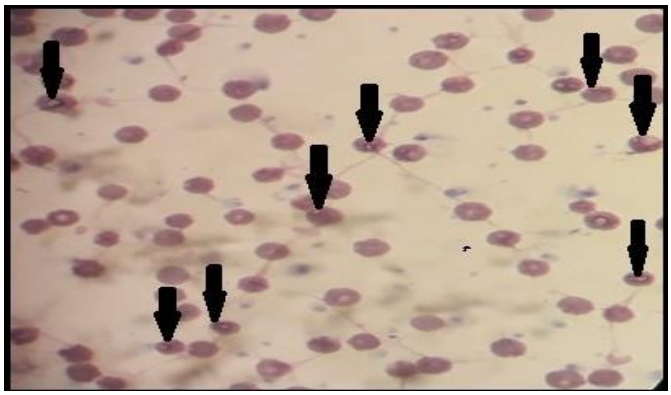

Figure 1: Field Stained Blood Smear Showing the *B. Bigemina* (Black arrows) Under 100X Oil Immersion Lens

DOI: https://doi.org/10.54393/fbt.v4i02.112

Out of 150 blood samples 50 blood samples were positive by microscopic examination indicating the 33% rate of prevalence and frequency in District Sialkot as shown in table 2.

Table 2: Result of Screening of Blood Smears by Microscopic Examination

Total Number of Blood Samples Collected	Number of Positive Samples N (%)	Positive Samples From Different Host Animals	
		Host	N (%)
150	50 (33%)	Cow	45 (90%)
		Buffalo	3 (6%)
		Sheep	2 (4%)

PCR Amplification

DNA was successfully extracted by Phenol chloroform extraction method. PCR was applied on all 50 samples that were confirmed as positive by microscopic examination. Out of 50 samples 36 produced the characteristic band of 446bp in 1% agarose gel. No amplification was seen in case of negative control as shown in figure 2.

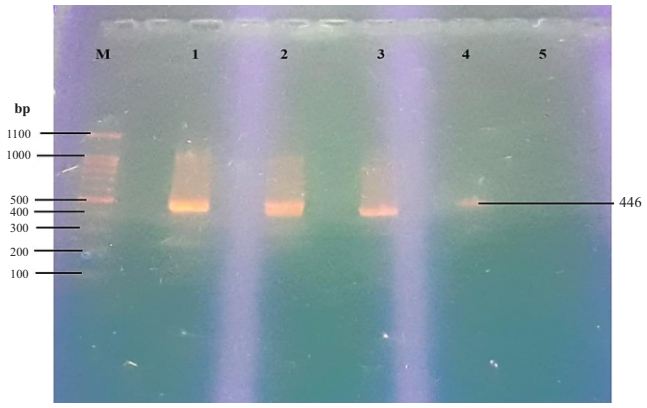

Figure 2: PCR amplification of 18S rRNA gene of *B. bigemina*. Lane M=DNA marker (100bp), Lanes 1, 2 (Cow), 3 (Buffalo), 4 (Sheep) showing positive samples and Lane 5=negative control

Sequencing Analysis

Nucleotide BLAST results confirmed that sequences of our samples were of *B. bigemina*. All the 18S gene sequences generated were aligned by using CLC viewer software to observe the nucleotide polymorphism. Multiple sequence alignment showed both single and multiple nucleotide polymorphism at various positions along the whole length of sequences. Inter and Intra-specific variations were also noted in sequences as shown in figure 3.

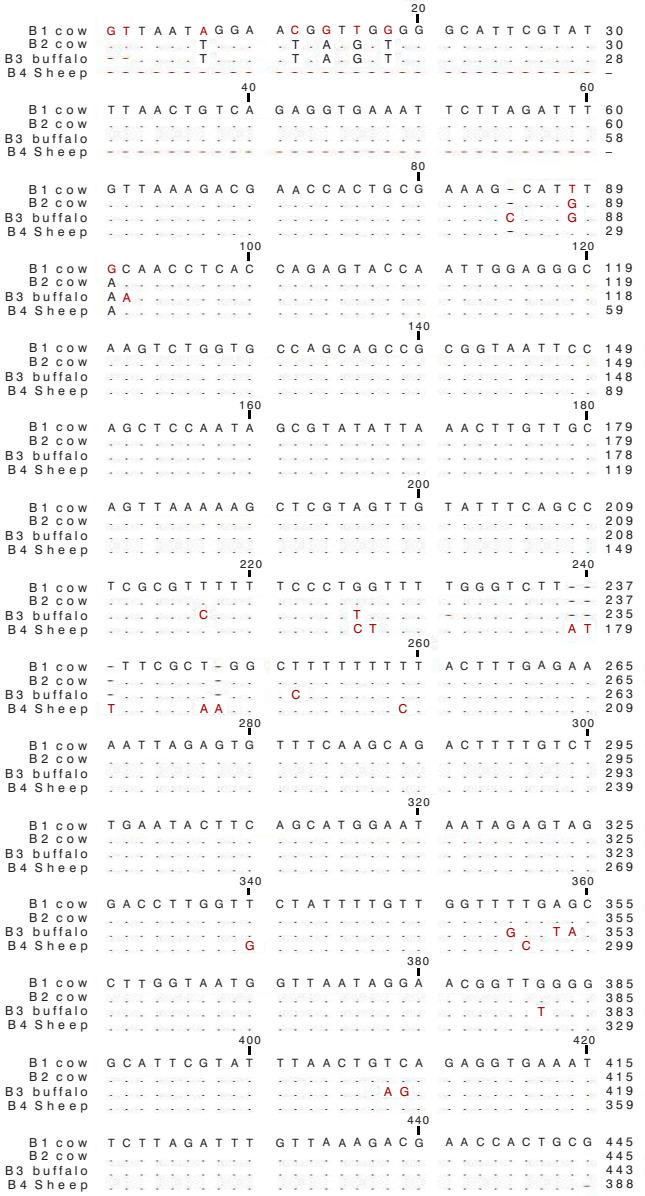

Figure 3: Multiple Sequence Alignment of 18S rRNA gene of Selected Samples of *B. bigemina* Showing Genetic Variability are Designated as B1(Cow), B2(Cow), B3(Buffalo) and B4(Sheep)

Phylogenetic Analysis

Phylogenetic analysis of 18S rRNA gene sequence of *B. bigemina* revealed that all the sequences of cow (B1-MZ817010) showed similarity with sequences from China (KY805824) except one B2-MZ817011 showed 98% with Uganda (KU206297). The sequence B3-MZ817012 from Buffalo showed 98% identity with sequence of 18S rRNA gene sequence reported from Pakistan (KY765562). While the sample from sheep i.e. B4-MZ817013 did not shared any clade with reported sequences as shown in figure 4.

DOI: https://doi.org/10.54393/fbt.v4i02.112

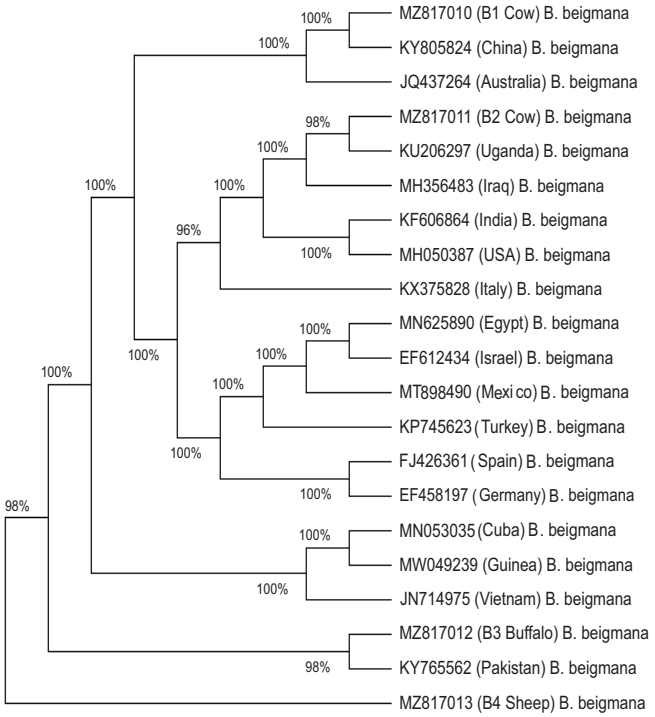

Figure 4: Phylogenetic Tree Constructed by Neighbor Joining Method from 18S rRNA Gene Sequence of *b. Bigemina*

Haplotype Analysis

Four haplotypes were recognized in current study. In haplotype network, Hap 2 haplotype was dominant as indicated by the size of circle. Hap 2 and Hap 1 represented the sequences from cow, Hap 3 from buffalo while Hap 4 from Sheep (figure 5A) [14].

Haplotype Networking of 18S rRNA Sequences from Current Along With Sequences from Other Countries

Haplotype network generated between current study sample and other selected sample from 24 countries represented total 16 haplotypes. Hap 1 haplotype of our study from cow and China shared the same circle indicating same evolutionary lineage. Hap 2 from cow made network with the haplotype reported from eight other countries while Hap 3 from buffalo form network with another Haplotype reported from Pakistan from same host and Hap 4 from sheep showed large number of mutation before making network with haplotype from Cuba and West Africa (figure 5B). Nucleotide diversity and neutrality test statistics is given in table 3, where Tjima's D represented the negative value which indicates population growth [14].

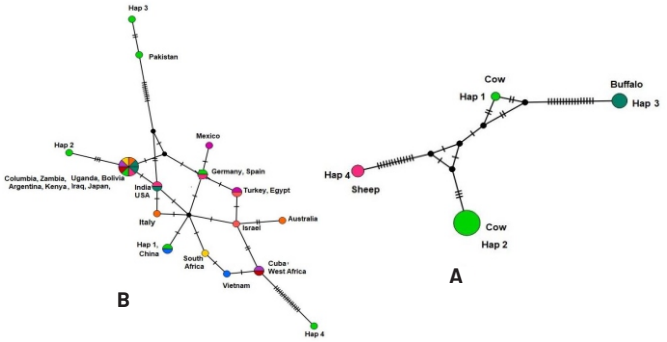

Figure 5: Haplotype Network of 18S rRNA: a) Sequences Generated from Samples of Current Study. b) Haplotype Network of Sequences from Current Study and Those Reported from other Countries

Size of circles is proportional to haplotype frequency and no. of dashes on lines exhibit the number of mutations between nodes representing different sequences. Nucleotide diversity and neutrality test statistics of *B. bigemina* is given in table 3, where the positive value of Tajima's D shows reduction in population size.

Table 3: Nucleotide Diversity and Neutrality Test Statistics of *B. bigemina*

Variables	Analysis of Sequences from Current Study	Current Study with Reported Sequences
Nucleotide Diversity (N)	0.0160036	0.154468
No. of Segregation Sites	14	36
No. of Parsimony-Informative Sites	0	16
Tajima's D	0.691885	-1.47792

DISCUSSION

Babesiosis is well known tick borne disease that causes enormous losses to livestock sector in Pakistan. In present study microscopic examination indicated 33% rate of prevalence in District Sialkot, Pakistan. Our results are in agreement with the study reported (34%) from Southern Punjab [15]. Other workers also reported prevalence of *Babesia* from some areas of Pakistan. Prevalence of *Babesia* in Kasur was reported 33.33% in 2008 [1]. From Khyber Pakhtunkhawa 27.5% prevalence was reported by PCR analysis and 9.83% by microscopic examination [16]. Another report from Khyber Pakhtunkhawa showed overall prevalence of 20.66% [17]. A report from Qadirabad revealed 18% positive by microscopic examination while 29% were positive by PCR [18]. While from Malakamd Agency lower prevalence was reported i.e., 12.49 % [19]. These variations in rate of prevalence can be attributed to the differences in climatic zones and environmental conditions of areas under study and rate of exposure to vector ticks and breeds of animals. The animals which are mostly at risk are exotic and their different breeds than local animal breeds [20]. For molecular and phylogenetic analysis 18S rRNA gene of *B. bigemina* was amplified as it is

DOI: https://doi.org/10.54393/fbt.v4i02.112

a part of functional core of ribosomes and it is also exposed to same selection pressure in every living organism [21]. Various studies have reported 18S rRNA gene as useful marker for molecular detection of *Babesiosis* [12, 22-26]. Sequence analysis showed genetic variations between samples from different hosts and even intra-specific variations in case of cow. The bioinformatics analysis revealed that samples of our study were also distinct from sequences reported from other countries and Pakistan, although, samples from cow showed close relationship with China, Uganda and Iraq, from buffalo to other samples of buffalo from Pakistan and sheep samples were not closely related genetically to other samples. The *B. bigemina* species with similar sequences having slight or more variations correlates to increase geographical distribution of this parasite species where it has potential disease causing effect in Mammals [27]. Migration of hosts between different areas and colonization of new areas has potential effect on dynamics of parasite [28]. Seasonal migrations and trade has an impact on diversity, epidemiology and load of parasites [29]. Long distance migration of host animals increases the risk of spread of pathogenic organisms and facilitate their transmission. When organisms invade new areas different traits and environmental conditions affect their survival. The mutation and adaptive radiations play their role which makes organisms able to survive by adaptation and also causes these species to diverge from their ancestral species [30]. In present study four haplotypes were recognized from 18S rRNA gene sequences from District Sialkot, Pakistan while overall 16 haplotypes were recognized from analysis of 18S rRNA gene sequences from selected countries of world and current study. Presence of haplotypes in 18S rRNA gene sequence of *B. bigemina* can be attributed to the lack of bottleneck effect and unequal rate of crossing over [31]. In addition to the prevalence of emergent strains of *B. bigemina* in District Sialkot, Pakistan, existence of haplotypes can also be attributed to the pathogenicity of Babesia species and resistance of B. *bigemina* against immune response of host [32, 33].

CONCLUSIONS

It can be concluded that this study provides insight into molecular and phylogenetic analysis of *B. bigemina* to assess the relationship between isolated strains and previously reported strains. In District Sialkot, *B. bigemina* was detected as dominant species responsible for *babesiosis* which was confirmed by PCR and sequencing of 18S rRNA gene of *B. bigemina.* This study was helpful in tracking *babesiosis* in district Sialkot, Pakistan. Haplotyping has revealed the presence of genetic variants among different and same host species.

ACKNOWLEDGEMENT

I would like to thank Dr. Rizwan Bashir from District Disease Diagnostic Center, Sialkot for permission and assistance for sample collection.

Author's Contribution

Conceptualization: AWQ
Methodology: DM
Formal analysis: AWQ
Writing, review and editing: AWQ, DM

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

Source of Funding

The author received no financial support for the research, authorship and/or publication of this article.

REFERENCES

[1] Durrani AZ and Kamal N. 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. 2008 Aug; 40: 441-7. doi: 10.1007/s11250-007-9117-y.

[2] Shah SS, Khan MI, Rahman HU. Epidemiological and hematological investigations of tick-borne diseases in small ruminants in Peshawar and Khyber Agency. Pakistan Journal of Advances in Parasitology. 2017 Feb; 4(1): 15-22.

[3] Aziz KA, Khalil W, Mahmoud M, Hassan N, Mabrouk DM, Suarez CE. Molecular characterization of babesiosis infected cattle: Improvement of diagnosis and profiling of the immune response genes expression. Global Veterinaria. 2014 Mar; 12(2): 197-206.

[4] Mohamed G and Ebied M. Epidemiological studies on bovine Babesiosis and Theileriosis in Qalubia governorate. Bharati Vidyapeeth Medical Journal. 2014 Sep; 27(1): 36-48.

[5] Zulfiqar S, Shahnawaz S, Ali M, Bhutta AM, Iqbal S, Hayat S *et al.* Detection of Babesia bovis in blood samples and its effect on the hematological and serum biochemical profile in large ruminants from Southern Punjab. Asian Pacific Journal of Tropical Biomedicine. 2012 Feb; 2(2): 104-8. doi: 10.1016/S2221-1691(11)60202-5.

[6] Maharana BR, Tewari AK, Saravanan BC, Sudhakar NR. Important hemoprotozoan diseases of livestock: Challenges in current diagnostics and therapeutics: An update. Veterinary World. 2016 May; 9(5): 487. doi: 10.14202/vetworld.2016.487-495.

[7] Desquesnes M and Dávila AM. Applications of PCR-based tools for detection and identification of animal

DOI: https://doi.org/10.54393/fbt.v4i02.112

trypanosomes: a review and perspectives. Veterinary Parasitology. 2002 Nov; 109(3-4): 213-31. doi: 10.1016/S0304-4017(02)00270-4.

[8] Figueroa JV, Chieves LP, Johnson GS, Buening GM. Multiplex polymerase chain reaction based assay for the detection of Babesia bigemina, Babesia bovis and Anaplasma marginale DNA in bovine blood. Veterinary Parasitology. 1993 Oct; 50(1-2): 69-81. doi: 10.1016/0304-4017(93)90008-B.

[9] Fahrimal Y, Goff WL, Jasmer DP. Detection of Babesia bovis carrier cattle by using polymerase chain reaction amplification of parasite DNA. Journal of Clinical Microbiology. 1992 Jun; 30(6): 1374-9. doi: 10.1128/jcm.30.6.1374-1379.1992.

[10] Persing DH, Mathiesen D, Marshall WF, Telford S, Spielman A, Thomford JW *et al.* Detection of Babesia microti by polymerase chain reaction. Journal of Clinical Microbiology. 1992 Aug; 30(8): 2097-103. doi: 10.1128/jcm.30.8.2097-2103.1992.

[11] Mannan A. A study on prevalence, molecular identification and characterization of blood and tissue protozoa of domestic ruminants in Chittagong, Bangladesh. [Doctoral Dissertation]. A thesis submitted in the total fulfillment of the requirements for the degree of Doctor of Philosophy from Department of Pathology and Parasitology Department of Pathology and Parasitology Faculty of Veterinary Medicine Chittagong Veterinary and Animal Sciences University Chittagong-4225, Bangladesh.2017.

[12] Sudan V, Shankar D, Sharma B, Jaiswal AK, Singh A. Molecular characterization and sequence phylogenetic analysis of Babesia bigemina cattle isolate from Mathura based on 18S ribosomal DNA gene. The Indian Journal of Animal Sciences. 2017 Aug; 87(8): 977-9. doi: 10.56093/ijans.v87i8.73470.

[13] Levine ND. Protozoan parasites of domestic animals and of man. In: Protozoan Parasites of Domestic Animals and of Man; 1962. 412. doi: 10.5962/bhl.title.7000.

[14] Parmaksız A and Eksi E. Genetic diversity of the cyprinid fish Capoeta trutta (Heckel, 1843) populations from Euphrates and Tigris rivers in Turkey based on mtDNA COI sequences. Indian Journal of Fisheries. 2017 Jan; 64(1): 18-22. doi: 10.21077/ijf.2017.64.1.62396-03.

[15] Iqbal F, Fatima M, Shahnawaz S, Naeem M, Shaikh RS, Ali M *et al.* A study on the determination of risk factors associated with babesiosis and prevalence of Babesia sp., by PCR amplification, in small ruminants from Southern Punjab (Pakistan). Parasite: Journal De La Société Française De Parasitologie. 2011 Aug; 18(3): 229. doi: 10.1051/parasite/2011183229.

[16] Shams S, Ayaz S, Ali I, Khan S, Gul I, Gul N *et al.* Sensitivity and specificity of PCR & microscopy in detection of Babesiosis in domesticated cattle of Khyber Pakhtunkhwa, Pakistan. International Journal of Advanced Research and Technology. 2013; 2: 37.

[17] Faryal Saad FS, Muhammad Khaisroon MK, Kalimullah Khan KK, Noor-ul-Akbar NU. Prevalence and molecular detection of Babesiosis in the slaughter animals of Peshawar Khyber Pakhunkhawa Pakistan. International Journal of Current Microbiology and Applied Sciences. 2015; 4(8): 1030-6.

[18] Chaudhry ZI, Suleman M, Younus M, Aslim A. Molecular detection of Babesia bigemina and Babesia bovis in crossbred carrier cattle through PCR. Pakistan Journal of Zoology. 2010 Apr; 42(2): 201-4.

[19] Ahmad N and Hashmi HA. A comparative study on the incidence of ticks and ticks borne diseases on local and crossbred cattle in Malakand agency. Journal of Animal and Plant Sciences. 2007; 17(3-4): 56-8.

[20] Atif FA, Khan MS, Iqbal HJ, Arshad GM, Ashraf E, Ullah S. Prevalence of Anaplasma marginale, Babesia bigemina and Theileria annulata infections among cattle in Sargodha District, Pakistan. African Journal of Agricultural Research. 2012 Jun; 7(22): 3302-7. doi: 10.5897/AJAR11.2051.

[21] Criado-Fornelio A, Buling A, Pingret JL, Etievant M, Boucraut-Baralon C, Alongi A *et al.* Hemoprotozoa of domestic animals in France: prevalence and molecular characterization. Veterinary Parasitology. 2009 Jan; 159(1): 73-6. doi: 10.1016/j.vetpar.2008.10.012.

[22] Razmi G, Pourhosseini M, Yaghfouri S, Rashidi A, Seidabadi M. Molecular detection of Theileria spp. and Babesia spp. in sheep and ixodid ticks from the northeast of Iran. The Journal of Parasitology. 2013 Feb; 99(1): 77-81. doi: 10.1645/GE-3202.1.

[23] Ayaz S, Shams S, Abdel-Reheem MA, Khan S, Ullah R. Epidemiology and molecular detection of babesiosis in household dairies in districts Kohat and Karak, Khyber Pakhtunkhwa Pakistan. Life Science Journal. 2013 Jan; 10(10): 188-93.

[24] Laha R, Mondal B, Biswas SK, Chand K, Das M, Sarma D *et al.* 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. 2015 Mar; 47: 633-6. doi: 10.1007/s11250-015-0769-8.

[25] Elhaig MM, Selim A, Mahmoud MM, El-Gayar EK. Molecular confirmation of Trypanosoma evansi and Babesia bigemina in cattle from Lower Egypt.

DOI: https://doi.org/10.54393/fbt.v4i02.112

Pakistan Veterinary Journal. 2016 Oct; 36(4): 409-14.

[26] Hashem M, Neamat-Allah AN, Gheith MA. A study on bovine babesiosis and treatment with reference to hematobiochemical and molecular diagnosis. Slovenian Veterinary Research. 2018 Nov; 55(20). doi: 10.26873/SVR-643-2018.

[27] Bonnet S, Michelet L, Moutailler S, Cheval J, Hebert C, Vayssier-Taussat M *et al.* Identification of parasitic communities within European ticks using next-generation sequencing. PLoS Neglected Tropical Diseases. 2014 Mar; 8(3): e2753. doi: 10.1371/journal.pntd.0002753.

[28] Peacock SJ, Bouhours J, Lewis MA, Molnár PK. Macroparasite dynamics of migratory host populations. Theoretical Population Biology. 2018 Mar; 120: 29-41. doi: 10.1016/j.tpb.2017.12.005.

[29] Mysterud A, Qviller L, Meisingset EL, Viljugrein H. Parasite load and seasonal migration in red deer. Oecologia. 2016 Feb; 180: 401-7. doi: 10.1007/s00442-015-3465-5.

[30] Altizer S, Bartel R, Han BA. Animal migration and infectious disease risk. Science. 2011 Jan; 331(6015): 296-302. doi: 10.1126/science.1194694.

[31] Van Herwerden L, Gasser RB, Blair D. ITS-1 ribosomal DNA sequence variants are maintained in different species and strains of Echinococcus. International Journal for Parasitology. 2000 Feb; 30(2): 157-69. doi: 10.1016/S0020-7519(00)00002-3.

[32] Amirmajdi MM, Sankian M, Mashhadi IE, Varasteh A, Vahedi F, Sadrizadeh A *et al.* Apoptosis of human lymphocytes after exposure to hydatid fluid. Iranian Journal of Parasitology. 2011 Jun; 6(2): 9–16.

[33] Spotin A, Majdi MM, Sankian M, Varasteh A. The study of apoptotic bifunctional effects in relationship between host and parasite in cystic echinococcosis: a new approach to suppression and survival of hydatid cyst. Parasitology Research. 2012 May; 110: 1979-84. doi: 10.1007/s00436-011-2726-4.