Project ID 426

Competitive Research Grant

Sub-Project Completion Report

On

Molecular characterization of *Babesia*, *Theileria* and *Anaplasma* organisms in livestock in Bangladesh

Project Duration

May 2016 to September 2018

Department of Parasitology, Bangladesh Agricultural University

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
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Acknowledgement

The execution of CRG sub-project has successfully been completed by the Department of Parasitology, Bangladesh Agricultural University, Mymensingh using the research grant of USAID Trust Fund and GoB through Ministry of Agriculture. We would like to thanks to the World Bank for arranging the grand fund and supervising the CRGs by BARC. It is worthwhile to mention the cooperation and quick responses of PIU-BARC, NATP 2, in respect of field implementation of the sub-project in multiple sites. Preparing the project completion report required to contact a number of persons for collection of information and processing of research data. Without the help of those persons, the preparation of this document could not be made possible. All of them, who made it possible, deserve thanks. Our thanks are due to the Director PIU-BARC, NATP 2 and his team who has given their whole hearted support to prepare this document. We hope this publication would be helpful to the agricultural scientists of the country for designing their future research projects in order to technology generation as well as increasing production and productivity for sustainable food and nutrition security in Bangladesh. It would also assist the policy makers of the agricultural sub-sectors for setting their future research directions.

Published in: September 2018

Acronyms

A= *Anaplasma*
B= *Babesia*
bp= Base Pair
BLAST=Basic Local Alignment Search Tool
DNA= Deoxyribonucleic acid
EDTA= Ethylenediaminetetraacetic acid
nPCR= Nested Polymerase chain reaction
PCR= Polymerase chain reaction
RAP-1a= B. bigemina rhoptry-associated protein-1a
rRNA= Ribosomal ribonucleic acid
SBP-4= B. bovis spherical body protein

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Executive Summary

In Bangladesh, babesiosis and anaplasmosis are known to be the most common causes of economic losses in dairy and beef industry. Diagnosis of these diseases in Bangladesh relies mostly on clinical signs and microscopic examination of blood smears that may cause low chance of detection especially of subclinical cases leading to the misinterpretation of diagnosis as well as improper treatment of the disease. This project was therefore, undertaken for the establishment of molecular detection methods for identification of *Babesia*, *Theileria* and *Anaplasma* in cattle in Bangladesh and genetic analysis of the local isolates. A total 165 blood samples from the suspected cattle were collected from Mymensingh, Sirajganj, Rangpur, Kurigram and Dinajpur districts of Bangladesh during the month of May 2017 to September, 2018. The blood samples were collected in a 2 ml vial coated with EDTA as anticoagulant. Smear of the collected blood were examined under microscope at 40X. From the positive samples blood cells were concentrated, washed and stored at -20°C and later subjected to DNA extraction. PCR was performed targeting the 16S rRNA gene of *Anaplasma* spp and 18S rRNA gene for *Babesia* and *Theileria*. The microscopic and PCR positive samples were confirmed by sequencing analysis of nested PCR (nPCR) products except for *Anaplasma*.

A total of 45 suspected blood samples of cattle were examined microscopically for the presence of *Babesia* of which 26.7% (4/15) were found positive in Mymensingh and 36.7% (11/30) were positive in Sirajganj. All the 15 (33.33%) microscopically positive samples were then amplified by PCR using diagnostic primers that produced a band size of 619bp for *Babesia/Theileria* spp. Out of 15 positive samples only 5 were confirmed to be *B. bigemina* by nPCR targeting *B. bigemina* RAP-1a genes whereas no sample was found positive for *B. bovis* by nPCR targeting *B. bovis* spherical body protein-4 (SBP-4). A total of 50 suspected blood samples of cattle collected from Rangpur (n=25) and Sirajganj (n=25) districts were used for the detection of *Theileria*. Microscopic examination of blood smears revealed 28% (14/50) to be infected with *Theileria* spp. of which 36% (9/25) in Rangpur and 20% (5/25) in Sirajganj district. All the fourteen microscopically positive samples were found positive for *Theileria* on PCR analysis of 18S rRNA gene specific for *Theileria* spp. The overall occurrence of *Anaplasma* was 25.71% (18/70) in cattle. Out of the 70 suspected blood samples of cattle tested microscopically only 5 (16.67%) were found positive in Dinajpur (n=30) and 13 (32.5%) were positive in Sirajganj (n=40) districts. All the 18 microscopically positive samples when tested using PCR assay also showed positive results with the amplification of the gene (16S rRNA) products size of 781bp. An amplicon of 95bp obtained after secondary nested PCR targeting the MSP1β gene confirmed the presence of *Anaplasma merginale* in cattle of Dinajpur and Sirajganj districts of Bangladesh. Sequencing and phylogenetic analysis of *B. bigemina* RAP-1a gene revealed nucleotide sequence identities of 99-100% with Kenya, Egypt, USA, and Benin. The isolate, *Babesia bigemina* from Sirajganj was found to have no similarity with the isolate previously identified in Mymensingh district indicating the presence of a new isolate in Sirajaganj district area. Phylogenetic analysis of *Theileria* sp. demonstrated that the isolate from Rangpur cattle population is closely related to *Theileria annulata* from West Bengal, India with 92% sequence identity.

Through this study the molecular methods for detecting *Babesia, Theileria* and *Anaplasma* have been established. Further molecular characterization and genetic analysis of the detected organisms will help to better understanding the transmission dynamics and genetic diversity of these pathogens in Bangladesh.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the CRG sub-project: Molecular characterization of *Babesia, Theileria* and *Anaplasma* organisms in livestock in Bangladesh
2. Implementing organization: Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202
3. Name and full address with phone, cell and E-mail of PI/Co-PI (s): Prof. Dr. Md. Shahiduzzaman, Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202. Cell Phone: 01779094718. Email: szaman@bau.edu.bd
4. Sub-project budget (Tk):
   4.1 Total: 18,89,325.00
   4.2 Revised (if any): N/A
5. Duration of the sub-project:
   5.1 Start date (based on LoA signed): 09 May 2017
   5.2 End date : 30 September 2018
6. Justification of undertaking the sub-project:

Babesiosis, theileriosis and anaplasmosis are considered as the most important of all tick-borne diseases (TBDs) worldwide. These diseases cause significant economic losses to livestock farmers involved in dairy and beef production in the tropical and sub-tropical regions (Jongejan and Uilenberg, 2004). From India it is reported that due to clinical babesiosis in a crossbred cattle decreased milk production could be noticed for 30 days and during this period a total loss of 51.6 L of milk has been estimated (Laha *et al.,* 2012). In Bangladesh, babesiosis and anaplasmosis are ranked among the most common causes of economic losses in dairy and beef industry (Chowdhury *et al.,* 2006; Karim *et al.,* 2012; Siddiki *et al.,* 2010; Talukdar and Karim, 2001). The diagnosis of these diseases in Bangladesh has relied mostly on clinical signs, microscopic examination of blood smears (Chowdhury *et al.,* 2006) that may cause low chance of detection especially of subclinical cases leading to the misinterpretation of diagnosis as well as improper treatment of the disease. For confirmation of clinical or subclinical cases molecular detection of these organisms are necessary. It has been proved that some *Babesia*, *Theileria* and *Anaplasma* spp. share the same vector, and in most endemic areas cattle, goat and sheep are infected with these parasites. Thus, it would be practical to make use of a method that is able to simultaneously detect these genera in both animal and vector hosts. Polymerase chain reaction (PCR) has been increasingly applied to detect these pathogens in both blood and tick vectors instead of microscopy. Post-PCR detection methods are used to further enhance the sensitivity and confirm the specificity of the PCR technique such as nested PCR, real time PCR. Through the advent of PCR and automated sequencing methods, the analysis of gene sequences has led to an explosion of molecular phylogenetic comparisons of species. Phylogenetic analyses of *Babesia* and *Theileria* species are performed on the basis of 18S rRNA gene sequences; and 16S rRNA gene sequences for *Anaplasma* species (Lew and Jorgensen, 2005).

There are no or very limited studies employing advanced molecular methods, reverse line blot hybridization, real time PCR and genome sequencing in Bangladesh. However, these studies have been limited to a few tick-borne diseases with most of the studies lacking adequate information on their genotypes or even knowledge on their molecular epidemiology, which is critical for the control and prevention of these diseases. Therefore, this study was done with the preliminary objective of determining and understanding the genetic diversities and molecular epidemiology of some species of

Babesia, Theileria and *Anaplasma* pathogens infecting cattle in Bangladesh. The sequences generated from these target genes were used to confirm the identity of the pathogens and establish phylogenies to aid in the understanding of their molecular epidemiology in Bangladesh in relation to other regions of the world. Finally it was hoped that the identified organisms and their genetic information will be used to proceed on development of effective drugs and vaccines.

7. **Sub-project goal:** Understanding molecular epidemiology of *Babesia* and *Anaplasma* in livestock in Bangladesh. However specific goal of this project is to identify previously known or unknown species of *Babesia*, *Theileria* and *Anaplasma* in Bangladesh.

8. Sub-project objective (s):

i) Establishment of methodologies for detection of *Babesia*, *Theileria* and *Anaplasma* in Bangladesh
ii) Molecular characterization and genetic analysis of *Babesia*, *Theileria* and *Anaplasma* spp. in cattle in Bangladesh

9. Implementing location (s): Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202

10. Methodology in brief:

10.1. Detection of *Babesia*, *Theileria* and *Anaplasma*

Sample collection and processing

The samples for investigating *Babesia*, *Theileria* and *Anaplasma* organisms were collected from Mymensingh (BAU teaching hospital and Dewkhola, Fulbaria, Fulpur), Sirajganj (Baghabari), Rangpur (Kaunia), Kurigram (Rowmari Upazilla) and Dinajpur (Sadar Upazila) districts of Bangladesh. A total of 165 blood samples from the suspected cattle were collected for this purpose. Blood samples were collected in 4 ml EDTA coated tubes from animals suspected of babesiosis infection and transported to the laboratory of the Department of Parasitology, Bangladesh Agricultural University, Mymensingh by maintaining a cool-chain. Blood samples were placed in cooler with ice during transportation and were refrigerated at 4°C until processed. The blood samples were further examined for identification of the organisms as described by (Solusby, 1982). The samples were stored at -20°C prior to DNA extraction.

Microscopic examination ??

At least two thin blood smears were prepared immediately after collection and fixed with 100% methanol. Haemoprotozan diseases (e.g. *Haemoproteus* spp, *Plasmodium* spp and *Leucocytozoon* spp) were identified by examining Giemsa stained thin blood smear according to published methods (Afifi *et al.*, 2014). Further confirmation of these blood protozoans were done using PCR assay according to previously described methods (Bensch*et al.*, 2000; Hallgren*et al.*, 2004).

PCR and Nested PCR

DNA was extracted directly from the whole blood collected in EDTA-coated tubes using PurelinkTM Genomic DNA Minikit (Invitrogen) following manufacturer's instructions. The DNA was stored at -20°C until further investigation.

Specific primers targeting *B. bovis* spherical body protein-4 (SBP-4), *B. bigemina* rhoptry-associated protein-1a (RAP-1a), *Theileria* spp. 18S rRNA and *A. marginale* major surface protein 5 (Msp5) genes were used to amplify the respective genes by using previously described nPCRs (Table 1).

PCR amplification for *Babesia* species

A PCR was performed for all microscopically positive samples for *Babesia*/*Theileria* initially with set diagnostic primers targeting the 18S rRNA gene of *Babesia*/*Theileria* (Kledmanee *et al.,* 2009). The PCR reaction condition was: 95°C for 5 minutes, 35 cycles at 95°C for 30 second, 61°C for 90 second, 72°C for 30 second and a final extension for 30 minutes at 60°C.

The amplified samples by diagnostic PCR primers were further amplified for detecting *B. bigemina* targeting RAP-1a gene (Terkawi *et al*., 2011). The PCR reaction condition was: 95°C for 5 min, 35 cycles of denaturation at 94°C for 1minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Nested PCR was performed to amplify 412 bp region of RAP-1a gene of *B. bigemina* (Terkawi *et a*l., 2011) with initial denaturation of 5minutes at 95^0C followed by 35 cycles (1 minute of denaturation at 94^0C, 1minute of annealing at 55^0C, and 1minute of extension at 72^0C) and final extension for 10minutes at 72^0C.

The PCR reaction conditions for detecting *B. bovis* were as follows: the initial denaturation was at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The conditions for nPCR were similar except for the annealing temperature, which was 53.5°C.

PCR amplification for *Theileria* species

A Primary PCR was performed by targeting the 18S rRNA gene of *Theileria* to confirm the detection of *Theileria* from blood samples according to the protocol described by Wamuyu *et al*. (2015) with some modification. The 18S rRNA gene of *Theileria* spp. was amplified using forward primer 5'-CGGTAATTCCAGCTCCAATAGCGT-3' and reverse primer 5'-TTTCTCTCAAAGGTGCTGAAGGAGT-3' (Wamuyu *et al.,* 2015). The PCR mixture was subjected to the following cyclic conditions using an Applied Biosystems™ 2720 thermal cycler: 95°C for 5 minutes, 30 cycles at 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute and a final extension of 72°C for 9 minutes.

PCR amplification for *Anaplasma* species

The presence of *Anaplasma* organism in the samples was confirmed targeting 16s rRNA gene by nested PCR as previously reported (Noaman *et al.,* 2009). Two primers that cover the hypervariable region of this locus were used. At first primary amplification was performed using the primers F1 (5'-AGAGTTTGATCCTGGCTCAG-3') and R1 (5'- AGCACTCATCGTTTACAGCG-3'). The PCR mixture was subjected to the following cyclic conditions using an Applied Biosystems™ 2720 thermal cycler: 95°C for 5 minutes, 35 cycles at 94°C for 45 seconds, 59°C for 45 seconds, 72°C for 45 seconds and a final extension for 10 minutes at 72°C.

To detect the presence of *A. merginale* organism in the samples the MSP1β gene was amplified using F3 (5′-TTGGCAAGGCAGCAGCTT-3′) and R3 (5′-TTCCGCGAGCATGTGCAT-3′) primers that generate a PCR product of 95bp (Carelli *et al.,* 2007). The mixture was subjected to the following cyclic conditions using an Applied Biosystems™ 2720 thermal cycler: 96°C for 1 minute, 35 cycles of denaturation at 96°C for 15 seconds, annealing at 53°C for 1 minute, extension at 72°C for 20 seconds, and a final extension at 72°C for 5 minutes.

All PCR reactions were performed in a 25µl volume containing 12.5µl of PCR master mix (Go Taq® Green Master, Promega, Madison, WI USA), 2µl of forward primer, 2µl of backward primer, 2µl DNA sample and 6.5µl of nuclease free water (Promega, Madison, WI USA). Double distilled water was used as a negative control for all PCR. The PCR was done in an Applied Biosystems™ 2720 thermal cycler. The amplified product of PCR assay was analyzed by gel electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Gel electrophoresis and visualization

The PCR products were analyzed by electrophoresis on 1.5% agarose gel at 100V for 55 minutes. EZ-Vision® In-Gel Solution, a non-mutagenic, non-toxic fluorescent DNA Dye was used as an in-gel stain to visualize DNA in agarose gel. Presence of *Babesia, Anaplasma* and *Theileria* was confirmed by identifying the 619 bp, 781bp, 480 bp band sizes of amplified target gene of the organisms.

Table 1. The nucleotide sequences of the primers used for PCR analysis and the PCR product profile

Pathogen target gene	Assay	Oligonucleotide sequences (5' > 3')	Product size (bp)	References
Babesia/ Theileria 18S rRNA	PCR	F -CCAATCCTGACACAGGGAGGTAGTGACA R -CCCCAGAACCCAAAGACTTTGATTTCTCTCAAG	619	Kledmanee *et al.,* 2009
Theileria 18S rRNA	PCR	F-CGGTAATTCCAGCTCCAATAGCGT R-TTTCTCTCAAAGGTGCTGAAGGAGT	480	Wamuyu *et al.,* (2015)
B. bigemina RAP-1a	PCR	F- GAGTCTGCCAAATCCTTAC R- 5'-TCCTCTACAGCTGCTTCG	879	Terkawi *et al.,* 2011
	nPCR	F 5'-AGCTTGCTTTCACAACTCGCC R 5'-TTGGTGCTTTGACCGACGACAT	412	
B. bovis SBP-4	PCR	F- AGTTGTTGGAGGAGGCTAAT R- TCCTTCTCGGCGTCCTTTTC	907	Terkawi *et al.,* 2011
	nPCR	F- GAAATCCCTGTTCCAGAG R- TCGTTGATAACACTGCAA	503	
Anaplasma spp 16S rRNA	PCR	F- AGAGTTTGATCCTGGCTCAG R- AGCACTCATCGTTTACAGCG-3	781	Noaman *et al.,* 2009
A. merginale MSP1β gene	nPCR	F-TTGGCAAGGCAGCAGCTT R- TTCCGCGAGCATGTGCAT	95	Carelli *et al.,* 2008 and Molad *et al.,* 2006

10.2. Molecular characterization and genetic analysis of *Babesia*, *Theileria* and *Anaplasma* spp.
DNA sequencing and phylogenetic analysis

To further confirm and validate the nested-PCR results, the representative samples of *Babesia* and *Anaplasam* species with most clear fragments were sequenced at DNA solution ltd, Dhaka, Bangladesh where DNA sequencing of PCR products were performed by using Bigdye terminator Sequencing kit in ABI 3500 Dx Genetic analyzer (Applied Bio-systems, USA). Primers used for the sequencing of the PCR products were the same as for the amplification. The purified PCR products were sequenced in both directions (forward and reverse) to resolve any potentially ambiguous sites. The obtained sequence was edited using MEGA 6.0 program (Tamur *et al.,* 2013) by combining the forward and reverse sequences and deleting unresolved noisy nucleotide sites at both ends of sequence. Multiple alignments were then performed on the edited sequence by Clustal W which is integrated in the MEGA 6.0 program. The edited sequence was compared with the available sequence in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

A phylogenetic tree was created from the sequences of *Babesia bigemina* RAP-1a gene identified in this study and those available on the GenBank, using the neighbor-joining method in MEGA version 6.0 (Tamura *et al.,* 2013). The sequence was compared with available sequences in GenBank database with the following accession number: KP347559, AF017296, AF017286-AF017288, AF0174486, KF192811, KX685379, KX685381, KX685383, KX685384, KX685386, KY484520, KU042084, KT220513, KC515388, KC515387, AB617643 and AB 594817. *Babesia canis* HM590440 was included as an out group. The representative sample sequence of *Babesia bigemina* was sent for accession number.

Sequences of the 18S rRNA genes of *Theileria* sp. Isolated from Rangpur have been allocated the accession number LC419995 and from Sirajganj LC439356 by the GenBank. A phylogenetic tree was created with the Rngpur and Sirajganj isolates and those available on the GenBank, using the neighbor-joining method in MEGA version 6.0 (Tamura *et al*., 2013). The sequences were compared with the following accession numbers: KX375820 for *Theileria sergenti* Italy, AB000272 for *Theileria buffeli* Australia, DQ287959 for *Theileria buffeli* Spain and MF287950 for *Theileria annulata* West Bengal. *Toxoplasma gondii* X68523 was included as an out group.

11. Results and discussion:
11.1. Detection of *Babesia*, *Theileria* and *Anaplasma*

Results:
Microscopic examination
Babesia- A total of 45 blood samples from cattle were examined microscopically (Figure 1 and Table 2) among which 4 (26.7%) out of 15 blood samples were *Babesia* positive in Mymensingh and 11 (36.7%) out of 30 were *Babesia* positive in Sirajganj districts. The study showed that the occurrence of *Babesia* in Sirjaganj was higher than Mymensingh.

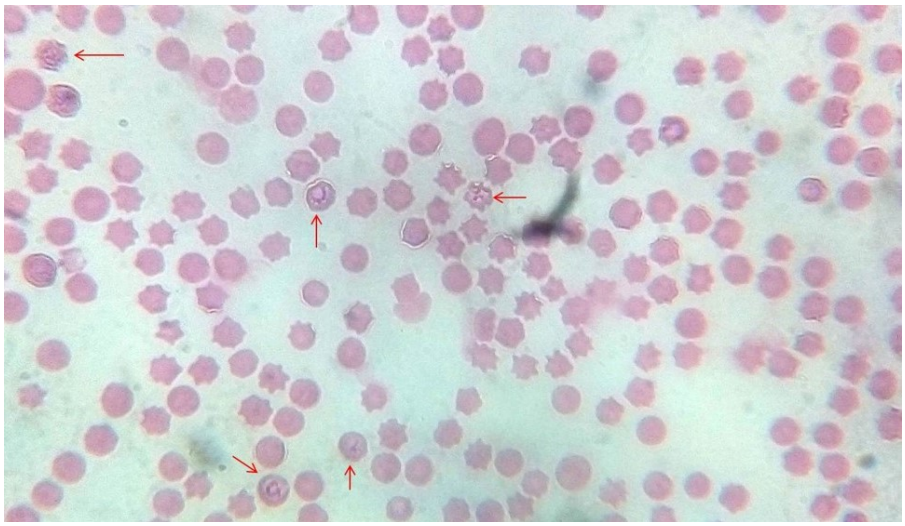

Figure 1: *Babesia* spp. (arrow) within RBC of cattle (40X Microscopy)

Theileria- A total of 50 blood samples from cattle were examined microscopically of which 9 (36%) were positive in Rangpur out of 25 samples and 5 (20%) were positive in Sirajganj out of 25 samples (Figure 2 and Table 2) with an overall occurrence rate of 28% (14/50). The study shows that the occurrence of *Theileria* in Rangpur was higher than Sirajganj.

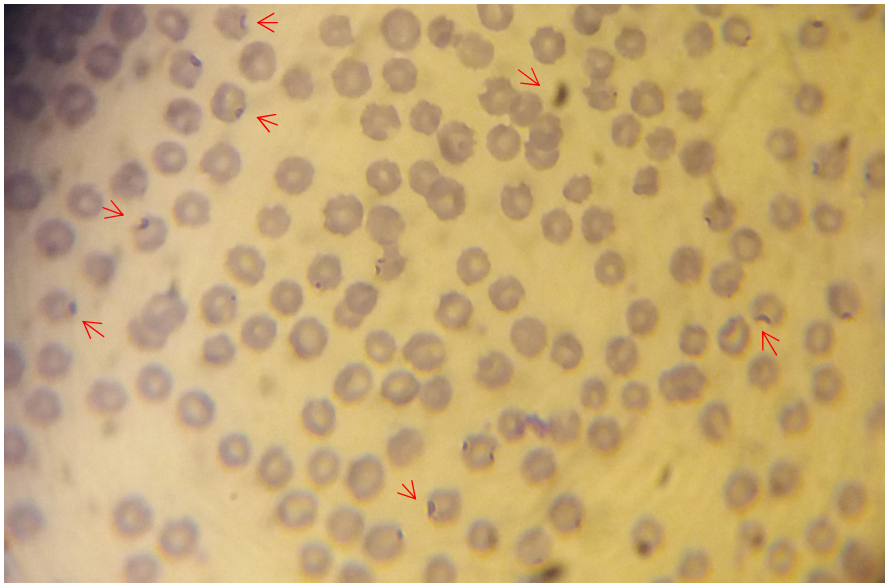

Figure 2: *Theileria* spp. (arrow) within blood cell of cattle (blood smear), 100x Microscopy, Giemsa staining

Anaplasma- A total of 70 blood samples from cattle were examined microscopically of which 5 (16.67%) were positive for *Anaplasma* infection (Figure 3 and Table 2) out of 30 samples from Dinajpur and 13 (32.5%) were found positive out of 40 samples from Sirajganj District with an overall occurrence of

25.71% (18/70). The study depicted apparently a higher occurrence of anaplasmosis in cattle in Sirajganj than Dinajpur district.

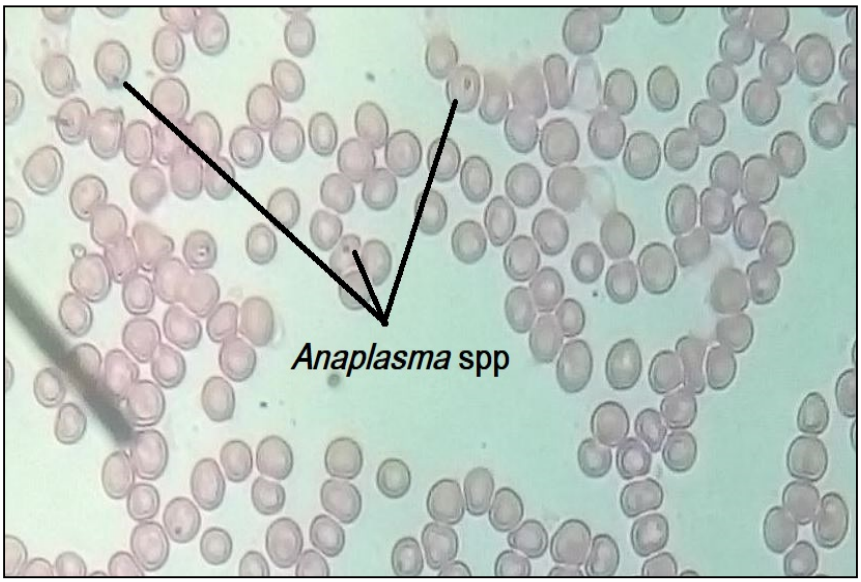

Figure 3: *Anaplasma* spp. under microscope (40X)

Table 2: Occurrence of *Babesia, Theileria* and *Anaplasma* spp in cattle in the study areas

Pathogens	Method of examination	Occurrence (%) of pathogens	
		Mymensingh	Sirajganj
Babesia/Theileria	Microscopy/PCR	26.7 (4/15)	36.7 (11/30)
		Overall 33.33% (15/45)	
Babesia bigemina	nPCR	6.67 (1/15)	13.33 (4/30)
		Overall 11.11% (5/45)	
Theileria spp	Microscopy/PCR/nPCR	Rangpur	Sirajganj
		36 (9/25)	20 (5/25)
		Overall 28% (14/50)	
Anaplasma merginale	Microscopy/PCR/nPCR	Dinajpur	Sirajganj
		16.67 (5/30)	32.5 (13/40)
		Overall 25.71 (18/70)	

PCR analysis

All microscopic positive blood samples were first analyzed by PCR that confirmed the presence of *Babesia/Theileria* in the samples with a band size of 619bp (Figure 4). Primary PCR for *Babesia bigemina* targeting RAP-1a gene confirmed the amplified product size of 879bp (Figure 5). Further analyzing the samples by PCR and nPCR detected *B. bigemina* in 5 samples (1 from Fulpur, Mymensigh and 4 from Baghabari, Sirajganj) with a band size of 415bp (Figure 6) but no *B. bovis* were detected in those samples. Analysis of the 45 blood samples from cattle by nPCR revealed an overall occurrence of 11.11%

for *B. bigemina* infections with 6.67% in Mymensigh and 13.33% in Sirajganj areas. The occurrence of *Babesia/Theileria* infection was not significantly different for those two study areas ($P > 0.5$).

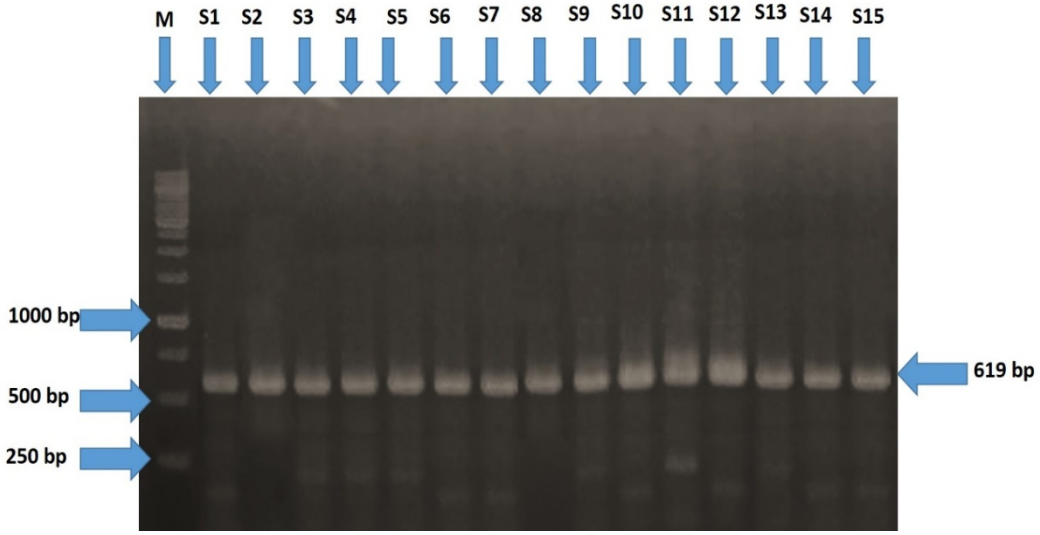

Figure 4: Ethidium-bromide-stained agarose gel image of PCR-amplified fragments using primer set specific for *Babesia/Theileria* genera. Target amplified product size is 619bp. M (Ladder-1kb) and S1-S15 are samples.

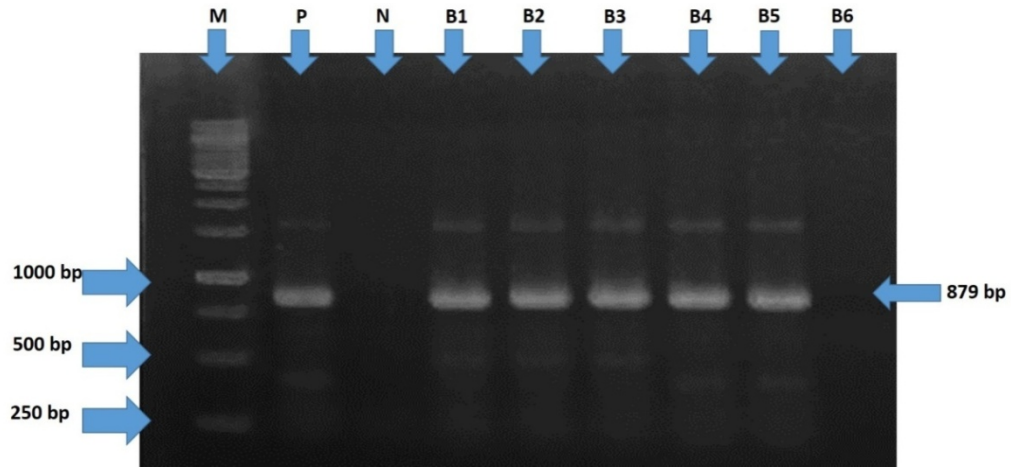

Figure 5: Agarose gel image of amplified product by primary PCR for *Babesia bigemina RAP-1a* gene. Amplified product size is 879bp. M (Ladder-1kbp), P (Positive sample), N (Negative sample) and B1-B6 are samples.

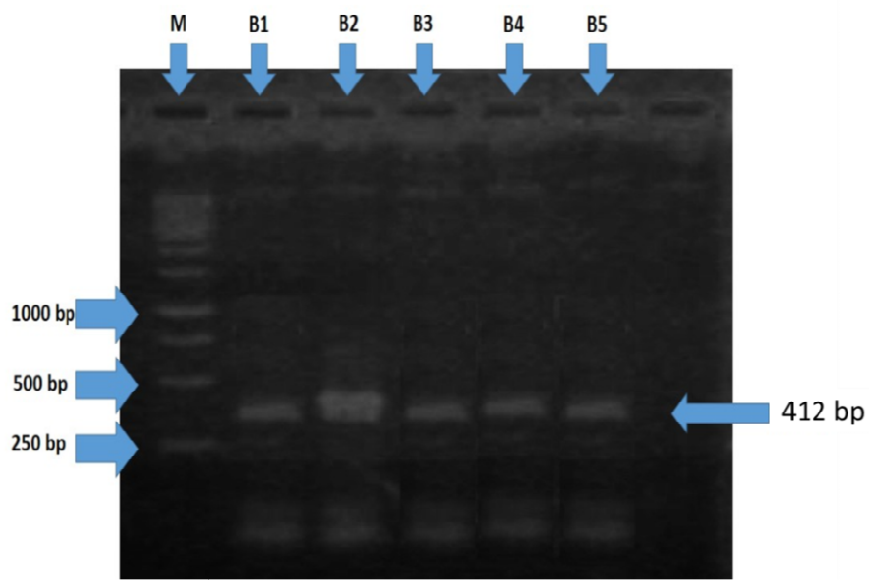

Figure 6: Agarose gel image of amplified nPCR product from primary PCR of *Babesia* organism. The target sequence amplified product size is 412bp. M (Ladder-1kbp), and B1-B5 are samples.

Fourteen cattle sampled in this study were found positive for *Theileria* spp. on PCR amplification and gel electrophoresis. The positive samples formed a band at approximately 480bp which was the expected fragment size for this piroplasm (Figure 7). All microscopically positive samples were confirmed by PCR. No *Theileria* piroplasms were seen on blood smears of samples that were negative in PCR.

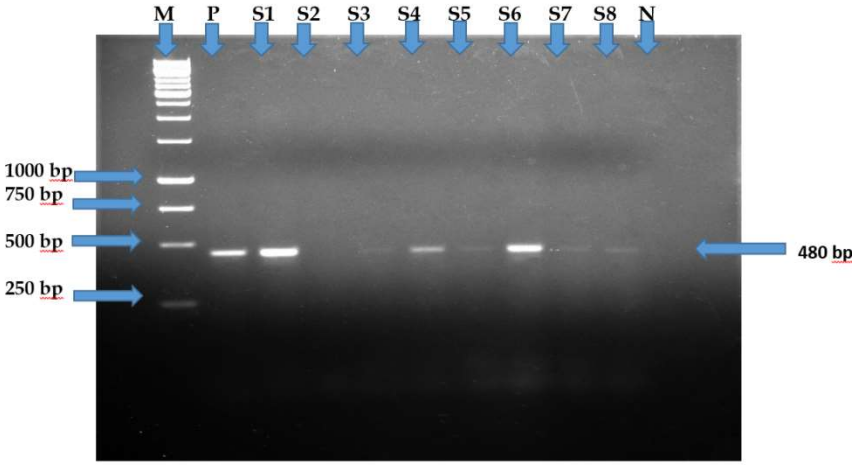

Figure 7: Gel electrophoresis PCR amplification of a partial sequence of 18S rRNA of *Theileria* spp. gene, product size approximately 480bp. Lanes (From Left): (M) Molecular weight marker = 1kbp; (p) Positive control; (S1-S8) are samples; (N) Negative control

Out of the 70 cattle blood samples tested in this study, *Anaplasma* DNA was amplified from 18 (25.71%) samples. The highest detection rate was noted in the cattle blood samples collected from Sirajganj. The target sequence of *Anaplasma* spp was confirmed by obtaining PCR amplification products size of 781bp (Figure 8). An amplicon of 95bp (Figure 9) was obtained after secondary nested PCR targeting the MSP1β gene confirming the presence of *Anaplasma merginale* DNA in the samples.

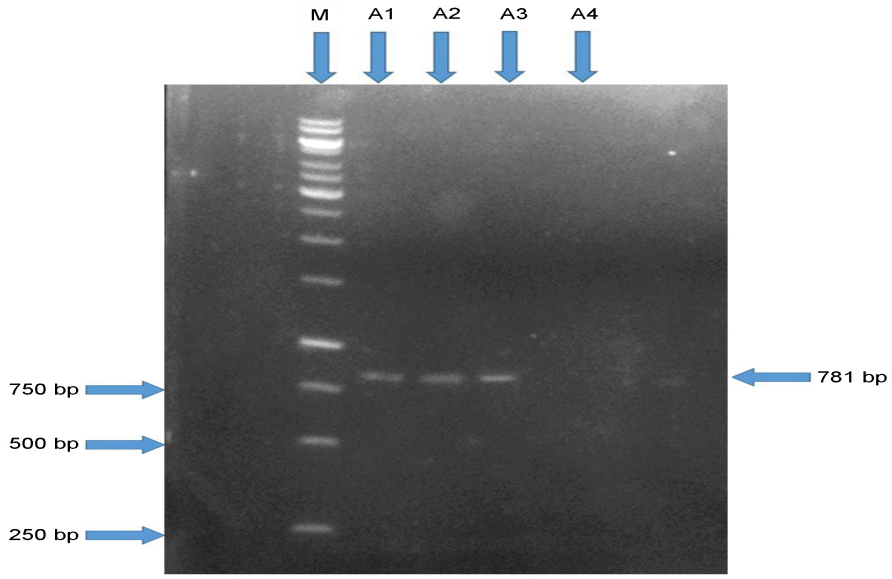

Figure 8: Detection of *Anaplasma* spp. by primary PCR: PCR product size for the target sequence of *Anaplasma* spp. is 781bp. M, Ladder-1kbp; A1-A4 are samples.

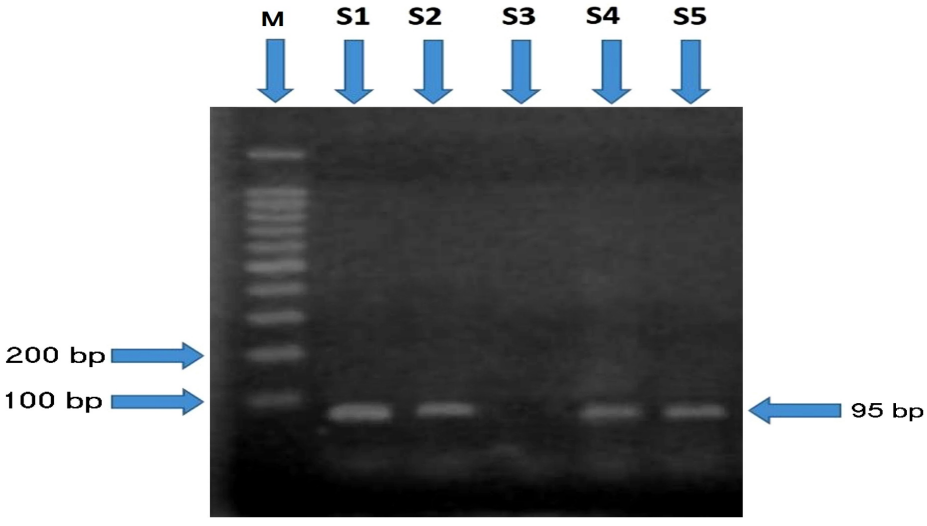

Figure 9: Confirmation of *Anaplasma merginale* by PCR: The amplified products for the target sequence of *Anaplasma merginale* is 95bp. M, Ladder-100bp; S1-S5, sample.

Sequencing and phylogenetic analysis

***Babesia*:** Three nested-PCR positive samples (B1, B4 and B5 of figure 6) with most clear fragments for *B. bigemina* were selected and sequenced for homology analysis of *B. bigemina* rhoptry-associated protein-1a (*Babesia bigemina* RAP-1a gene). The representative sequence of RAP-1a submitted to GenBank can be retrieved with accession number MH790974 (Sirajganj, Bangladesh). The sequences (MH790974) showed all nucleotide sequence identities of 99-100% with Kenya (KP347559), Egypt (KF192811), USA (AF017296), AF017286-AF017288, AF0174486 and 99% identity with KX685379, KX685381, KX685383, KX685384, KX685386 (Benin), KY484520, KU042084, KT220513, KC515388, KC515387, AB617643 and AB 594817 (Figure 10).

Babesia bigemina Sirajganj (MH790974.1) has no similarity with the *Babesia* sp. Mymensingh (MF576177.1) (Roy *et al.,* 2018) indicating the presence of a new isolate in Sirajaganj area.

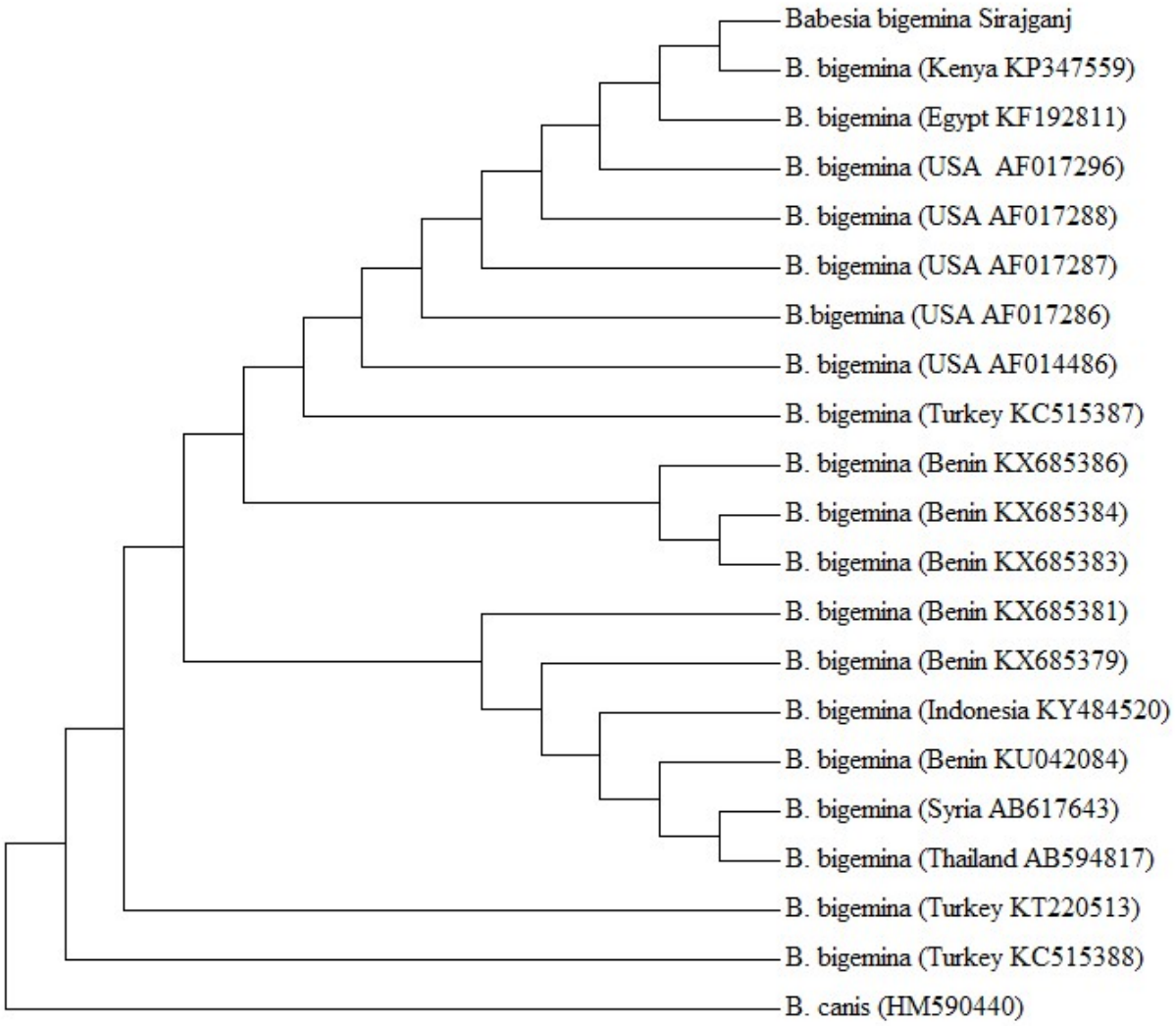

Figure 10: The Neighbor-Joining analysis of RAP-1 gene of *Babesia bigemina* identified in this study and those present in the GenBank database (accession numbers in parentheses). The numbers at the branching points are bootstrap values expressed as percentages of 1,000 replications. The horizontal scale bar gives an indication of the number of nucleotide substitutions per site. The origin of published sequences is indicated after the isolate name. *Babesia bigemina* Sirajganj is the sequence identified in this study. *Babesia canis* was used as out group.

Theileria: Sequence analysis revealed two identical isolates LC419995 (Rangpur) and LC439356 (Sirajganj). Phylogenetic analysis demonstrated that *Theileria* sp. present in the Rangpur cattle population is closely related to *Theileria annulata* from West Bengal, India. The identified sequence shared 92% identity with MF287950 (Figure 11).

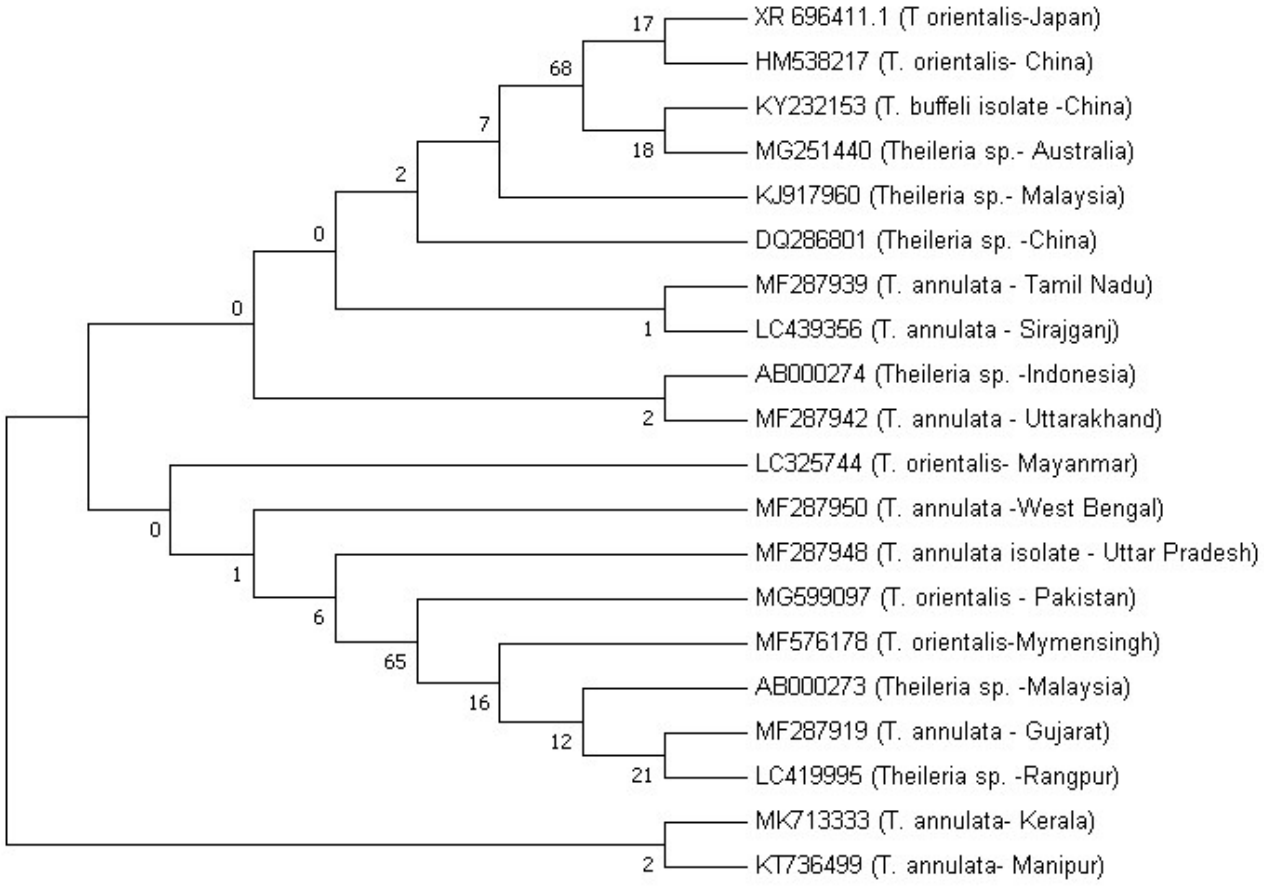

Figure 11: The Neighbor-Joining analysis of 18S rRNA gene of *Theileria* sp. Rangpur identified in this study and those present in the GenBank database (accession numbers in parentheses). The numbers at the branching points are bootstrap values expressed as percentages of 1,000 replications. The horizontal scale bar gives an indication of the number of nucleotide substitutions per site. The origin of published sequences is indicated after the isolate name. *Theileria* sp. Rangpur is the sequence identified in this study.

Score	Expect	Identities	Gaps	Strand
440 bits(238)	1e-127	280/301(93%)	0/301(0%)	Plus/Plus

```
Query  1    TATCTTTCCGGATGATTACTTTGAGAAAATTAGAGTGCTCAAAGCAGGCTTTTGCCTTGA  60
Sbjct  77   ..............................C..........T.................  136

Query  61   ATAGTTTAGCATGGAATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTAGGTACC  120
Sbjct  137  ...C....T............A....C...................C.............  196

Query  121  AAAGTAATGGTTAATAGGAACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATT  180
Sbjct  197  CT....CC............C.............C...........T...........  256

Query  181  CTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATC  240
Sbjct  257  ....A.....................................TC.................  316

Query  241  AAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCCTAACCATAAACT  300
Sbjct  317  ........................A..T....C............A.............  376

Query  301  A  301
Sbjct  377  .  377
```

Figure 12 a: Sirajganj (LC439356-*Theileria annulata*) VS. Rangpur (LC419995-*Theileria* sp) isolate

Score	Expect	Identities	Gaps	Strand
551 bits(298)	6e-161	300/301(99%)	0/301(0%)	Plus/Plus

```
Query  1    TATCTTTCCGGATGATTACTTTGAGAAAATTAGAGTGCTCAAAGCAGGCTTTTGCCTTGA  60
Sbjct  605  ..............................................................  664

Query  61   ATAGTTTAGCATGGAATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTAGGTACC  120
Sbjct  665  ....................A.....................................  724

Query  121  AAAGTAATGGTTAATAGGAACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATT  180
Sbjct  725  ..............................................................  784

Query  181  CTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATC  240
Sbjct  785  ..............................................................  844

Query  241  AAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCCTAACCATAAACT  300
Sbjct  845  ..............................................................  904

Query  301  A  301
Sbjct  905  .  905
```

Figure 12 b: Sirajganj (LC439356- *Theileria annulata*) Vs Mymensingh-(MF576177.1- *Babesia* sp) isolates

Figure 12 c: Rangpur (LC419995- Theileria sp) Vs. Mymensingh (MF576177.1- Babesia sp) isolates

Figure 12 a, b and c: Sequence comparison among indentified isolates of this study and previously identified isolates in Mymensingh district.

The two strains identified in Sirajganj and Rangpur region have 93% similarities with no gap (Figure 12-a).

Sirajgonj strains are 99% similar with previously identified strain in Mymensingh district with no gap and only 82 position of Sirajagonj isolate was replaced by A nucleotide of Mymensingh isolates (Figure 12-b)

Comparison between Rangpur strain (LC419995) and Mymensingh strain (MF57617) (Roy *et al.,* 2018) of this study and there are 3 gaps and 92% similarities in identity with 1 gap for T nucleotide of Mymensingh strain at 1008 position of Rangpur strain (Figure 12 c).

Discussion:

Detection and characterization of Babesia species

The overall occurrence of *Babesia* in this study was higher (33.33%) than the study of Roy *et al.* (2018) as he found only 1%, but Banerjee *et al.,* (1983) reported higher prevalence (14.53%) of subclinical babesiosis in dairy cattle of Mymensingh and Dhaka districts of Bangladesh. The occurrence of *Babesia* in Sirajganj was 36.7% which is almost similar with the study of Chowdhury *et al.* (2006). However, some study recorded a 3.30% prevalence of *Babesia bigemina* infection in cattle of the selected Milk vita project areas of Bangladesh and Sirajganj sadar area of Bangladesh and the prevalence of babesiosis is 3.10% in Sirajganj but the results was only based on microscopic examination of the organism using Giemsa stained blood smears and it is difficult to differentiate the *Babesia* spp and mixed infection with *Theileria* spp. Mahmud *et al.* (2015). However, higher occurrence of Babesiosis in this study may be due to the collection of prospective samples from symptomatic animal. Abundant access to grazing land may

increases the probability of cattle coming into contact with ticks and thereafter may increase the chance of infection with *Babesia* (Roy *et al.,* 2018). The results indicated that the animals in the study areas were infected with *B. bigemina* not by *Babesia bovis*. Although the low sample numbers in each location may not allow a fair comparison of positive infection rates between locations, it was noted that Fulbaria of Mymensingh had the lowest percentage (5.3%) of cattle infected with *B. bigemina*, indicating a situation of minimal disease.

The area of Sirajganj from where blood samples were collected is known as "bathan" (low-lying large grazing areas) with thousands of farms (small to medium) where mainly Shahiwal and Jersey are reared for milking purpose and during the time of spring or immediately after monsoon when the water compel the farmer to graze their cows in bathan. Although the treatment with anti-Babesial drug are commonly used to *Babesia* sysmptomatic animals due to availability of the hard ticks (Boophilus, Rhipicephalus) the *Babesia* infection are more common in this area. In other study area (Fulbaria, Mymensingh) the samples were collected from the farmer house having small farm of cross breed.

Tick fever in cross bred animals is high (Veterinary Epidemiological Bulletin Sri Lanka, 2012). In a study it is indicated almost 80% of suspected crossbred cows acting as carriers were Shahiwal or Jersey (Chakraborti, 2002). However, occurrence of babesiosis in the study population suggested a continuous challenge of such infection in those areas. The occurrence of babesiosis is strongly based on the prevalence of ticks therefore, the farming practices in those places should observe routine disease diagnostic procedures, isolation of carrier animals from herd and best management practices with clean environment. This is necessary to eliminate the tick infestation and in turn plays a vital role in eliminating the *Babesia* spp. from a herd.

By sequencing the 18S rRNA gene of the *Babesia* isolates in this study, a phylogenetic tree was created (Fig 10). It showed that the identified *Babesia bigemina* Sirajganj was 100% identical with *Babesia bigemina* isolated from cattle in Kenya (Moumouni PFA *et al.,* 2015; KP347559), from cattle and water buffaloes Egypt (Ibrahim, H.M *et al.,* 2013 KF192811) and USA (Hotzel *et al.,* 1997 AF017296, AF017286-AF017288, AF0174486) and shared 99% identity with the isolates from *Rhipicephalus microplus*-invaded and non-invaded ticks in cattle Benin (Moumouni, PFA *et al.,* 2018 KX685379, KX685381, KX685383, KX685384, KX685386), from cattle in Indonesia (Guswanto *et al.,* 2017 KY484520), from Amblyomma variegatum ticks in Benin (Moumouni, PFA 2011 KU042084), in Turkey (Zhou, M *et al.,* 2016 KT220513), from cattle Turkey (Duzlu O *et al.,* 2015 KC515388, KC515387), cattle in Syria (Terkawi MA *et al.,* 2012 AB617643) and from water buffaloes in Thailand (Terkawi MA *et al.,* 2011 AB 594817). These results demonstrated that *B. bigemina*, is present in cattle in Sirajganj areas. The presence of *B. bigemina* was expected, since the parasite has been reported previously (Islam *et al.,* 2009). However, *B. bigemina* was detected at molecular level for the first time in Sirajganj.

The comparison of available RAP-1 nucleotide sequences from Bangladesh displayed a high level of sequence identity when compared to other published *B. bigemina* strains of countries other than Bangladesh including neighboring countries in Asia. The mismatches observed in figure 12 between two aligned sequences can be interpreted as point mutations and gaps can be interpreted as indels (insertion or deletion mutations) introduced in one or both lineages in the time since they diverged from one another. However, from our knowledge, no previous information was available on the polymorphism of *B. bigemina* RAP-1 sequences of Bangladesh, suggesting the need to collect more geographical *B. bigemina* isolates of Bangladesh for sequencing in order to test the level of diversity among RAP-1 sequences of *B. bigemina* isolates.

Detection and characterization of species

In our study using 18S rRNA based PCR, we found the overall prevalence rate of *Theileria* spp. infections in cattle to be 28% with the prevalence of 36% at Rangpur and 20% at Sirajganj district. The samples were randomly collected from cattle, either crossbred or native breed from different places of Rangpur and Sirajganj districts. Microscopically detected *Theileria* organism was confirmed by PCR using genus specific primers (Wamuyu *et al*., 2015). All the 14 positive samples of *Theileria* organisms on microscopic examination generated an amplicon of 480bp, which suggests the animals were infected with *Theileria* spp. The existence of *Theileria* spp. had also been reported earlier by Samad *et al.* (1984), and Mahmud *et al*. (2015) who recorded 8.47% and 5.82% respectively which differ from present study. This may be due to that the prevalence of *Theileria* spp. infection in cattle, in those studies, were determined based on microscopic examination of peripheral blood smears. The present study supports with the report of Samad *et al.* (1984) who observed that the overall prevalence of theileriosis in Complement Fixation to be 22.03% in dairy cattle of Bangladesh. Similar results were also observed by Ali *et al.* (2015) who found that the prevalence of *Theileria* species infection in cattle was 80.0% (36/45) in Natore and 20.4% (30/147) in Rajshahi. Ahmed (1996) found that the prevalence of *Theileria mutans* infection in cattle and buffalo in the district of Dhaka was 64.86% which was very high. Alim *et al.* (2012) observed that the prevalence of theileriosis was 4.62% in Noakhali district, and 8.33% in Khagrachori district which is far less than the present study.

In India, there are reports showing the presence of *T. annulata* infection using molecular based assay for e.g., a study from Gujarat where 74 samples were detected positive out of 113 (65.48%) (Kundave *et al*., 2014), in Bangalore 41 cases were positive out of 132 (31.06%) (Ananda *et al.,* 2009) while a study from Punjab showed a prevalence rate of 14.65 % using microscopic examination (Singh *et al.,* 2012). The present study shows higher prevalence of *Theileria* spp. than the report of Khattak *et al*. (2012) who observed that the overall prevalence of theileriosis on Giemsa stain was 5.20% in Khyber Pukhtoon Khwa province (Pakistan). The present study also differ from Safieldin *et al*. (2011) who observed that the prevalence of *Theileria* species infection in dairy cattle in Omdurman locality, Sudan was 7% for dry cool season. These differences may due to species difference, climatic and geographical variations and methods applied in the study. Through this study it could be concluded that PCR is more sensitive method of detection of infection than microscopic examination. However, occurrence of the theileriosis in the study population suggested a continuous challenge of such infection in those areas.

DNA sequence of Rangpur and Sirajganj isolates of *Theileria* 18S rRNA gene recorded in GenBank as accession number of LC419995 and LC439356 respectively. Sequence showed high similarity between the Rangpur isolates of *Theileria* 18S rRNA gene sequence and *Theileria annulata* isolate of West Bengal 2 18S ribosomal RNA gene registered in the GenBank. This indicates that the Rangpur strain may come from west **Bengal** due to animal movement to this area of Bangladesh as this region is not far from west **Bengal**. But the sequence differed from other *Theileria* spp. isolated from Italy, Spain and Australia. Sirajgonj strain was found highly similar with the previously isolated strain from Mymensingh district but genetically differed from Rangpur strain.

***Anaplasma*:**

By microscopic method and in the PCR assessment, overall 25.71% of cattle were positive for *Anaplasma* infection. The findings of the study conform to those of Belal *et al.* (2014) who found 25.82% overall prevalence of anaplasmosis on Geimsa stained blood smear examination in Sirajganj district. Chowdhury *et al.* (2006) recorded much higher prevalence (70%) of anaplasmosis in clinically suspected cattle of

Sirajganj district than those of other inland reports. The present study revealed that *Anaplasma* infection was higher in cattle in Sirajganj (32.5%) than Dinajpur (16.67%), conferring to the finding of Sandip *et al.,* (2016) in Dinajpur (18.5%). Talukdar and Karim (2001) who reported that 33% cattle of Baghabari Milk Shed Area had *Anaplasma* infection. Chowdhury *et al.* (2006) recorded much higher prevalence (70%) of anaplasmosis in clinically suspected cattle of Sirajganj district than those of other inland reports. Occurrence of anaplasmosis of this study was different from the reports of Siddiki *et al.* (2009) and Samad *et al.* (1989), who recorded 3% and 5.93% in the different areas of Bangladesh. Bary *et al.* (2018) recorded highest prevalence of anaplasmosis 12.0% in crossbred cattle followed by 6.0% in local cattle in summer. *A. merginale* organism was recorded in the samples by nPCR (Carelli *et al.,* 2007).

Conclusion

The results confirm that the methods for detecting *Babesia, Theileria* and *Anaplasma* by Microscopy and PCR are in place. Further molecular characterization and genetic analysis of the detected organisms will help to better understanding the transmission dynamics and genetic diversity of these pathogens in Bangladesh

12. Research highlight/findings:

Highlights

- The overall occurrences of *Anaplasma, Babesia* and *Theileria* spp. among the samples collected from cattle of different districts were 25.71%, 11.11% and 28.00% respectively

- The protocols for PCR analysis of *Babesia, Theileria* and *Anaplasma* have been established and this is thought to be the first molecular detection and genetic characterization of these pathogens in Bangladesh.

- *Babesia bigemina* was found in both Sirajganj and Mymensingh districts. Using phylogenetic analysis the isolate from Sirajganj was found to have no similarity with the isolate previously identified in Mymensingh district indicating the presence of a new isolate of Babesia bigemina in Sirajaganj district area.

- Sequence analysis revealed two identical isolates LC419995 (Rangpur) and LC439356 (Sirajganj). Phylogenetic analysis demonstrated that *Theileria* sp. present in the Rangpur cattle population is closely related to *Theileria annulata* from West Bengal, India with 92% sequence sharing.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	Table & chair	25,000	Table & chair	25,000	
(b) Lab &field equipment	Gel doc system	3,25,000	Gel doc system	3,25,000	
(c) Other capital items	Chemical & apparatus	2,92,000	Chemical & apparatus	2,92,000	

2. Establishment/renovation facilities: Not applicable

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: Not applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	584132	584132	584132	00	100%	
B. Field research/lab expenses and supplies	659301	658084	658084	00	100%	
C. Operating expenses	111292	111292	111292	00	100%	
D. Vehicle hire and fuel, oil & maintenance	75000	75000	75000	00	100%	
E. Training/workshop/seminar etc.	00	00	00	00	100%	
F. Publications and printing	80000	20000	20000	00	100%	
G. Miscellaneous	30000	30000	30000	00	100%	
H. Capital expenses	349600	349600	349600	00	100%	
Total	1889325	1828108	1828108	00	100%	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	**Major technical activities performed in respect of the set objectives**	**Output (i.e. product obtained, visible, measurable)**	**Outcome (short term effect of the research)**
Establishment of methodologies for detection of *Babesia, Theileria* and *Anaplasma*	Sample collection, microscopic examination of blood smears and PCR and nPCR	Reliable and reproducible methods of using PCR and nPCR for detection of *Babesia, Theileria* and *Anaplasma* have been established. The overall occurrences of *Anaplasma, Babesia* and *Theileria* spp. among the samples collected from cattle of different districts were 25.71%, 11.11% and 28.00% respectively	Methods for molecular detection of *Babesia, Theileria* and *Anaplasma* orgaqnisms established is expected to be used by other laboratories
Molecular characterization and genetic analysis *Babesia, Theilear* and *Anaplasma*	Sequencing and phylogenetic analysis	The isolate, *Babesia bigemina* from Sirajganj was found to have no similarity with the isolate previously identified in Mymensingh district indicating the presence of a new isolate in Sirajaganj district area. Phylogenetic analysis of *Theileria* sp. Isolated from the cattle of Rangpur district was found to be closely related to *Theileria annulata* from West Bengal, India with 92% sequence sharing.	The knowledge of phylogenetic analysis is expected to help policy makers in taking appropriate control measures of the diseases.

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication	2		
Information development			
Other publications, if any		**Thesis 3**	**MS thesis**

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

None

ii. **Generation of new knowledge that help in developing more technology in future**

The overall occurrences of *Anaplasma, Babesia* and *Theileria* spp. among the samples collected from cattle of different districts were found to be 25.71%, 11.11% and 28.00% respectively

The isolate, *Babesia bigemina* from Sirajganj was found to have no similarity with the isolate previously identified in Mymensingh district indicating the presence of a new isolate in Sirajaganj district area.

Phylogenetic analysis of *Theileria* sp. Isolated from the cattle of Rangpur district has found to be closely related to *Theileria annulata* from West Bengal, India with 92% sequence sharing.

iii. Technology transferred that help increased agricultural productivity and farmers' income

None

iv. Policy Support

The knowledge of phylogenetic analysis is expected to help policy makers in taking appropriate control measures of the diseases.

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring [description & output of consultation meeting, monitoring workshops/seminars etc.):

a) A desk monitoring was done by PIU-BARC-NATP-2 team on 07.03.2018 that was headed by Dr. Mian Sayeed Hassan, director, project implementation unit (PIU), BARC. The comments of monitoring team helped executing the project in right direction.
b) Research progress was presented in the six month progress workshop (15-16 May, 2018) that was presented on 16 May, 2018 at BARC auditorium.
c) One year research progress was presented in the Annual Workshop (10-13, September), 2018 at BARC auditorium.

ii) Field Monitoring (time& No. of visit, Team visit and output):

Two internal field monitoring was done by Bangladesh Agricultural University Research System (BAURES) team on March 03, 2018 and 17 May, 2018. Members of the monitoring team expressed their high satisfaction.

H. Lesson Learned/Challenges (if any)

I. Challenges (if any)

Signature of the Principal Investigator
Date ……..
Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

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