A study on prevalence, molecular identification and characterization of blood and tissue protozoa of domestic ruminants in Chittagong, Bangladesh

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Ab-ELISA	Antibody- enzyme linked immunosorbent assay
BA	Bovine anaplasmosis
bp	base pair
BRAC	Bangladesh rural advancement committee
CAT	Card agglutination test
c-ELISA	Competitive- enzyme linked immunosorbent assay
CFT	Complement fixation test
CMA	Chittagong metropolitan area
DAT	Direct agglutination test
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EFSA	European food safety authority
ELISA	Enzyme linked immunosorbent assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Gross domestic product
gDNA	Genomic deoxyribonucleic acid
gp45	Glycoprotein 45
GRA	Granule antigen
GST	Glutathione s-transferase
HCT	Haematocrit centrifugation technique
HE	Haematoxylin and eosin
HF	Holstein Friesian
i-ELISA	Indirect enzyme linked immunosorbent assay
IFA	Immunofluorescence antibody

IFAT	Immunofluorescence antibody test
IHA	Indirect haemagglutination
ITS	Internal transcribed spacer
LAT	Latex agglutination test
LM	Light microscopy
m-AECT	Miniature- anion exchange centrifugation technique
MAG	Matrix antigen
MAT	Modified agglutination test
mg	milligram
MIC	Microneme protein
ml	milliliter
MLST	Multilocus Sequence Typing
mM	millimolar
MSP	Membrane surface protein
nPCR	Nested polymerase chain reaction
OD	Optical density
OIE	Office international des epizooties
OR	Odd ratio
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCI	Phenol chloroform isoamyl alcohol
PCR	Polymerase chain reaction
RAP	Rhoptry-associated protein
RFLP	Restriction fragment length polymorphism
RLB	Reverse line blotting
RMG	Readymade garments
ROP	Rhoptry protein
rpm	rotation per minute
RRA	RAP-1 related antigen

rRNA	Ribosomal ribonucleic acid
SAG	Surface antigen
SDS	Sodium dodecyl sulphate
SG	Surface granule
Tg	*Toxoplasmagondii*
µl	microliter
µM	micromolar
µg	microgram

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Summary

Blood and tissue parasites infect both the small and large ruminants causing significant economic losses all over the world including Bangladesh. The actual extent of these parasitic diseases and their epidemiologic features is not yet comprehensively studied in Bangladesh. Accurate identification and characterization are essential for any epidemiologic investigation. With the availability of modern DNA based tools and resources, the capacity for accurate and reliable detection of any disease agents are now possible which was not available before. The present study was therefore designed to investigate the prevalence followed by molecular identification of selected hemoparasites (*Babesia, Anaplasma, Trypanosoma*) of cattle in different geographic areas of the country. In addition, an attempt was made towards the molecular characterization of tissue protozoan (*Toxoplasma gondii*) in small ruminants in the Chittagong Metropolitan area located in southern part of Bangladesh. Further analyses of the associated risk factors were also done to help to develop necessary prevention and control options against these important protozoa.

Blood samples collected from cattle were preserved in the laboratory for further examination with conventional microscopy and modern molecular tools like PCR and DNA sequencing. Sample size varied among different protozoa species and initial screening was aided by Giemsa stained thin smear examinations. A variable numbers of samples were assessed to detect hemoprotozoa including *Anaplasma* (n=1680), *Babesia* (n=1680) and *Trypanosoma* (n=480) where 8.21%, 1.43% and 0.63% prevalence was recorded respectively from different study areas inside Bangladesh.

Bovine anaplasmosis was found as an important rickettsial disease with limited reports related to its prevalence inside Bangladesh. Comparative prevalence of this hemoparasitewas recorded as 9.33% in the hilly areas, 9.00% in coastal areas and 5.83% in plain lands inside the country. Further analyses indicated a higher prevalence (P>0.05) in crossbred cattle compared to local or indigenous breeds. Variations in seasonal incidence were also observed with high case numbers recorded in winter season of the year. Age of the animals was also found an important epidemiologic factor where animals aged 18-30 months were more vulnerable to anaplasmosis than other age groups. Housing and floor types also found to be associated with variable prevalence and animals living in non-cemented or soil-type floors were more prone to suffer from anaplasmosis. Molecular characterization through PCR amplification of *16S rRNA* gene was achieved where an 80% cases (40 out of 50 samples) were found positive for *A. marginale* infections. Further amplification of *MSP4* gene fragments successfully identified all forty positive cases in *16S rRNA* screening. Further DNA sequencing and phylogenetic studies revealed that all the isolates of this present study were closely related to the isolates already reported from countries like Argentina, Australia and China.

Bovine babesiosis is an economic important protozoan disease with sporadic reports on its prevalence and incidence in Bangladesh. During this study, prevalence of babesiosis was found as 1.17%, 1.67% and 1.46% in the selected hilly, coastal and

plain areas respectively in the study areas. Further data analysis showed crossbred and female animals more vulnerable than local or indigenous cattle and their male counterparts. High number of babesiosis was recorded in summer season compared to other time of the year. Animals aged 6-18 months were more susceptible to babesiosis than other age groups. Molecular identification of the organism were achieved through PCR amplification of *18SrRNA* gene fragments where all positive cases (n=24) screened by conventional microscopy were successfully amplified. Further DNA sequencing and phylogenetic analysis revealed that two distinct isolates of *Babesia, Babesia bigemina* and *Babesia ovata* were present in the country.

Trypanosomiasis is one of the least studied blood parasites of cattle in Bangladesh and thought to be distributed in different parts where relevant vectors are available. During this study out of 480 blood samples, only three were found positive in a single dairy farm located in Chittagong Metropolitan area. All positive cases were recorded in female and Holstein Frisian crossbred cattle and in one farm where previous incidence and disease record was not available. The time of the year when these cases were found was in rainy season and all the animals were aged 6 years or more and none of the younger animals were found positive. Molecular identification was achieved through PCR amplification of *18SrRNA* gene fragment following previously published protocols. Sequencing of amplified DNA and further phylogenetic analysis revealed that *Trypanosoma evansi* were circulating in Bangladesh which is closely related to the isolates reported from countries like Taiwan, Thailand and Japan.

Toxoplasmosis is an important zoonotic tissue protozoan disease of small ruminants in Bangladesh and has been documented by a number of publications. Several studies reported variable prevalence rate of toxoplasmosis in different parts of the country. The present study revealed an overall prevalence of toxoplasmosis in sheep and goats as 30.43% and 41.30% respectively. Further statistical analysis of the data indicated that animals aged two years or more were highly seropositive to *Toxoplasma gondii*. History of abortion and sources of drinking water were found to be significantly associated with toxoplasmosis. Animals with previous history of abortion were highly seropositive compared to the other groups. High seroprevalence was recorded in farms where ponds and municipality supply water are used as water sources for animals.

Apart from serodiagnosis, acute and chronic cases of toxoplasmosis are always troublesome to detect hindering their prevention and control efforts. Combined histopathological and molecular approaches were applied during this study to detect the *Toxoplasma gondii* from several aborted tissues of sheep and goats. Prevalence of *T. gondii* was detected as high as 25% in sheep (n=4) and 36.84% (n=19) in goats. All the positive cases were detected in animals that aborted at 3.5 months of gestation. Further sequencing of amplified DNA and phylogenetic analysis of the sequences confirms the presence of *T. gondii* in a single cluster with minimum genetic variations among the four isolates which are closely related to the isolates recorded from countries like India and Iran.

Chapter-1
General introduction

1.1 Livestock sector in Bangladesh perspective

Bangladesh is a subtropical, agro based developing country covering an area of 55,125 square miles and located between 20°34-26º38N and 88°01-92º41E. Livestock is an emerging subsector of agriculture that plays a vital role in the economy of Bangladesh. The agricultural livestock species of the country mainly include cattle, buffalo, sheep and goats while other species have less economic significance. Livestock population in Bangladesh was recorded as cattle 23.17 million, buffalo 1.42 million, goats 24.75 million, sheep 3.02 million (Department of Livestock Services, Government of Bangladesh, 2012). While the number of population is thought to be increasing over the years, notable that their economic significance is also rising as well. These are mainly because of its role as a source of food, in generating employment, delivery of alternate energy sources (dung, biogas), bio fertilizer, weed control, use of marginal lands, investment and savings and transport etc. (Sansoucy, 1995). In the fiscal year 2014-15 the livestock sectors contribute approximate 2.07% in the national GDP of Bangladesh and in agriculture sector it was 16.95% (BBS, 2016). Moreover, the sector also plays a crucial role in poverty reduction in Bangladesh where around 75 percent rural people are directly or indirectly engaged on livestock for their livelihood. This subsector of agriculture pursues fulltime employment for 20% of total population and part-time employment for another 50% population (Rahman et al., 2014a). Generally people fulfill their nutritional requirement from the animal originated food at low cost. It was reported that this sector supply average 8% of total protein for human consumption and 42.5% of animal protein in the form of milk, meat and egg (BBS, 2006). Moreover, hides and skin of cattle, buffaloes, goats and sheep are valuable export items, ranked third in earnings after readymade garments (RMG) and shrimp and ultimately helping to fulfill the vision 2021, which is a national development agenda of current Government of Bangladesh.

1.2 Cattle are the largest population of ruminants in Bangladesh

Cattle, the largest group of ruminants in the country do provides the necessary draft power for ploughing and transportation in rural parts of Bangladesh. Most importantly, they provide the milk and meat that are rich in nutrient and essential for maintenance of human health. Cow dung is used as important source of nutrient for soil fertility maintenance by rural farmers. Dried cow dung is used as a fuel in some areas for cooking. The recent estimate of 25% of total yearly production of cow dung used as household fuel indicate how crucial they are for providing energy to rural people (Department of Livestock Services, Government of Bangladesh, 2000). Cattle hides, skin and bone are used as a raw material in industry that is earning huge revenue by exporting the leather and leather products. Dairying is also creating employment opportunity for many people in Bangladesh. Several giant agro-based farms such as PRAN, BRAC and Aftab running their operations on livestock sector which directly help in the developing different business options and nutrient supply to the huge and ever-growing population of Bangladesh.

1.3 Sheep and goat, the small ruminants of Bangladesh

Goats and sheep provide a wide range of products and socio-economic services and have played a significant role in the social life of many people (Peacock, 1996). They play a vital role in ensuring the economic gain and food security of a household, often being the only asset owned by many poor families. They are prolific and require low inputs for a moderate level of production, reach maturity early and are profitable to keep (Devendra and Burns, 1983). Farmers and villagers are increasingly relying on goats and sheep as means of survival and a way of boosting their income (Peacock, 2005).

1.4 Significance of blood parasites of ruminants

While a cattle farming plays a significant role in rural economy of Bangladesh, several factors directly and indirectly hinder the growth of livestock sector. Among them vector-borne blood parasitic diseases are highly crucial. These include diseases which are visible through light microscopy and remain in blood circulation to complete their life cycle (Demessie and Derso, 2015). Most of the blood parasitic diseases are caused by protozoa and rickettsia and are transmitted by various vectors.

The diseases are most prevalent in endemic areas and cause devastating and deadly diseases in the developing countries all over the world including Bangladesh. The diseases are transmitted through the respective vectors including ticks, flies and mosquitoes species which are very common in farm areas. The diseases cause devastating losses to the livestock industry in the form of death, decreased production, declined working efficiency (Uilenberg, 1995). In addition these are not only considered as a serious economic challenge but also involve in zoonoses in many countries of the world (Sparagano, 1999).

Anaplasmosis is an arthropod-borne rickettsial disease of cattle, sheep and goats and has a wide distribution. The disease in cattle is caused by *Anaplasma marginale* and *A. centrale*. Infection caused by *A. marginale* is characterized by fever, severe anemia, jaundice, weakness, decreased production, weight loss, abortion, hyper-excitability due to cerebral anoxia and death (Richey and Palmer, 1990) while infection with *A. centrale* induces subclinical to mild disease. Anaplasmosis poses important economic constraints to animal breeders. Besides the costs of the additional veterinary care, it causes abortion in animals, reduction of milk production, body weight and frequently leads to death (Stuen et al., 2003). The actual economic cost associated with anaplasmosis in Bangladesh has not yet reported by any study. However, one would assume substantial loss in small or large scale dairy farming considering the calculated loss in other countries of the world.

Bovine babesiosis is a tick borne blood parasitic disease caused by a number of *Babesia* species: *B. bigemina*, *B. divergens*, *B. bovis*, and *B. major*. Among them, *B. bigemina* and *B. bovis* have major impacts on cattle health and productivity in tropical and subtropical countries (Iseki et al., 2010). Infections in cattle are characterized by fever, anorexia, listlessness, dehydration and progressive anemia, and may be followed by hemoglobinuria and hemoglobinemia resulting in jaundice (Zintl et al., 2005). Babesiosis in cattle cause huge economic loss through mortality, loss of production, and cost of control measures along with possible impact on the international cattle trade embargo (Bock et al., 2004).

The trypanosomiasis is a devastating diseases caused by the salivarian group of trypanosome parasite. There are several species of trypanosomes in livestock which includes *Trypanosoma brucei, T. vivax, T. congolense, T. evansi* and *T. theileri* (Garcia

et al., 2011a; Nantulya, 1990; Sekoni et al., 2004; Soulsby, 1982). General signs are intermittent fever, pale mucous membrane, emaciation, enlarged lymphnode, occasional diarrhoea and a heavy mortality rate (Sekoni et al., 1988). However, the mortality and morbidity depends on the breed, immune status, flies prevalence and age of animals (OIE, 2003). The disease has great economic importance in the livestock industry due to high mortality and severe production losses in cattle with chronic trypanosomiasis.

1.5 Diagnosis of blood parasitic diseases

1.5.1 Anaplasmosis

Although challenging, accurate diagnosis is essential for controlling bovine anaplasmosis. There are several direct and indirect techniques for diagnosis of anaplasmosis. Direct methods include detection by Giemsa-stained peripheral blood smears by light microscopy (Aubry and Geale, 2011) and molecular test like polymerase chain reaction (PCR) based techniques (Molad et al., 2006). Indirect methods include different serological tests using different surface antigens (Madruga et al., 2001).

1.5.1.1 Clinical diagnosis

Bovine anaplasmosis is characterized by severe anemia and jaundice without hemoglobinemia and hemoglobinuria. Other clinical signs include weight loss, decreased milk production, abortion and sudden death (Richey and Palmer, 1990). However it is sometime difficult to differentiate from other diseases which develop similar symptoms and thereby need specialist opinion.

1.5.1.2 Conventional microscopy

The conventional microscopic examination of peripheral blood smears (thick and thin) is the most commonly used technique and is most sensitive when used to evaluate animals that are clinically ill, typically during the acute phase of disease (Eriks et al., 1989). However, parasites are seldom detected microscopically in chronic infection requiring alternative strategies.

1.5.1.3 Molecular diagnostic tools

Polymerase chain reaction (PCR) detects DNA of the parasite in peripheral blood and therefore recognizes active infection, and the relative amount of DNA detected correlates with the level of parasitemia (Eriks et al., 1989). PCR based diagnosis is very specific and sensitive molecular diagnostic method even at low parasitemia stage and is able to differentiation between species of *Anaplasma*. However the technique is costly, time consuming and requires modern equipments. Molecular techniques targeting different gene were used by various researcher at different part of world and some selected studies are summarized below (Table 1.1).

Table-1.1: **Selected studies where different types of primers targeting different genes used for molecular identification of *Anaplasma* spp.**

Target gene	Primers used	Amplicon size (bp)	References
MSP4	5-CCCATGAGTCACGAAGTGG-3 5-GCTGAACAGGAATCTTGCTCC-3	753	Joazeiro et al., 2015
16SrRNA	5-AAGCTTAACACATGCAAGTCGAA-3 5-AGTCACTGA CCCAACCTTAAATG-3	1406	Oh et al., 2009
MSP1α	5-TGTGCTTATGGCAGACATTTCC-3 5-AAACCTTGTAGCCCCAACTTATCC-3	1224	Baêta et al., 2015
MSP5	5-ATGAGAATTTTCAAGATTGTGTCTAACCTT-3 5-AGGAAAGCCCCCAAAGCCCCATACTT-3	714	Bacanelli et al., 2014
MSP 1b	For primary PCR 5-CCATCTCGGCCGTATTCCAGCGCA-3 5-CTGCCTTCGCGTCGATTGCTGTGC-3 For secondary PCR 5-CAGAGCATTGACGCACTA CC-3 5-TTCCAG ACCTTCCCTAACTA-3	347	Molad et al., 2006
MSP3	5-AACCCAACTTTCAACGGTATCAAGGACCT-3 5-ATCCCTACTTCAACCCTGGCTCCT-3	247	Hammac et al., 2014

1.5.2 Babesiosis

Different types of diagnostic techniques including conventional microscopy, serological tests, and molecular tools are currently available for diagnosis of bovine babesiosis. However, each of them has some limitations that should be considered beforehand.

1.5.2.1 Clinical diagnosis

Clinically, babesiosis can be confused with some other important diseases of cattle which develop similar symptoms (such as fever, anemia, hemolysis, jaundice, or reddish or coffee color urine). Therefore, confirmation of diagnosis by light microscopic examination through Giemsa-stained blood or organ smears is essential but not conclusive.

1.5.2.2 Conventional microscopy

Traditionally, the microscopic examination of thick and thin smears of peripheral blood for detection of *Babesia* parasites has always been considered as the gold standard method for the diagnosis of acute babesiosis (Böse et al., 1995). However, the major disadvantage of microscopic examination of blood parasites is the low sensitivity of the technique, thus making it difficult to detect parasites in blood smears during low parasitemia in carrier animals (Almeria et al., 2001). Again this is further complicated in cases of mixed infection (Jacobson, 2006).

1.5.2.3 Molecular diagnostic tools

PCR amplification for the diagnostic detection of babesiosis is considered as a powerful diagnostic tool both in the early phase of infection and in carrier animals. The sensitivity of a PCR assay can be increased by several folds by performing a semi-nested or nested PCR where two different sets of amplification primers are used (Mosqueda et al., 2012). Different investigators have used separate protocols and amplified different gene fragments for reliable molecular identification of *Babesia* organism. Some of these selected studies are summarized below (Table 1.2).

Table 1.2: **Selected studies where different types of primers targeting different genes used for molecular identification of *Babesia* spp.**

Target gene	Primers used	Amplicon size (bp)	References
SSrRNA gene	5-TGGCGGCGTTTATTAGTTCG- 3 5-CCACGCTTGAAGCACAGGA- 3	1124	Chaudhry et al., 2010
B. bigemina RAP-1 gene	5 - ATGATTCACTACGCTTGCCTC-3 5 - GTCTTGTAGTATATGGCGGTCATGTAG-3	600	Suarez et al., 2003
RRA gene	5 -CACGAGGAAGGAACTACCGATGTTGA-3 5 -CCAAGGAGCTTCAACGTACGAGGTCA-3 5 -TCAACAAGGTACTCTATATGGCTACC-3 5 -CTACCGAGCAGAACCTTCTTCACCAT-3	387	Suarez et al., 2011
Partial *18S rRNA* gene	5-TTTCTGMCCCATCAGCTTGAC-3 5-CAAGACAAAAGTCTGCTTGAAAC-3	422-440	Hilpertshauser et al., 2006
Rap-1c	5-AAGCAGCAGCCGTGGTACAAGCGTGG-3 5-TTACGACGATCGTTTGAAGTACTTC-3	657	Niu et al., 2015
Rap-1a	ACGCGAATGGTTGCGTTTCAGA GGCTCAGCAACATTGGCTTTCAG	570	Niu et al., 2015
gp45	For primary PCR 5-CATCTAATTTCTCTCCATACC-3 5-CCTCGGCTTCAACTCTGATGCCAAAG-3 For secondary PCR 5-CGCAAGCCCAGCACGCCC-3 5-CCGACCTGGATAGGCTGTGTGATG-3	170	Mtshali and Mtshali, 2013

1.5.3 Trypanosomiasis

Several approaches including microscopic and molecular diagnostics are available for accurate and reliable diagnosis of trypanosomiasis. These findings need to be incorporated with the relevent clinical features to confirm the diagnosis.

1.5.3.1 Clinical diagnosis

The clinical symptoms of trypanosomiasis in animals vary with host immunity, trypanosome species and respective strains. Common symptoms include intermittent fever, severe anemia, decreased body condition, rough hair coat along with enlargement of superficial lymph nodes, occasionally abortion with reduced milk yield which could be fatal if untreated. Affected calves show depression, weight loss, pale mucous membranes, enlarged lymphnodes, edema of the dewlap, cough, coryza along with diarrhoea (Batista et al., 2012). The clinical signs are not pathognomonic and not sufficient for diagnosis of trypanosomiasis.

1.5.3.2 Conventional microscopy

The most convenient and simple technique for detection of trypanosomes in peripheral blood is by direct examination of blood smears, either by the wet film method to detect motile trypanosomes or as Giemsa-stained thick and thin smears, by light microscopy (Nantulya, 1990). The direct microscopic examination is considered as gold standard method for diagnosis of trypanosomiasis although half of those infected could be missed by this techniques (Barnett, 1947). However, the sensitivity of direct microscopic examination was improved by using another two techniques namely (i) buffy coat techniques (Woo, 1970) by centrifugation of blood and (ii) miniature-anion exchange centrifugation technique (m-AECT) (Lumsden et al., 1979) that involve separation of blood cell prior to centrifugation. The disadvantage of these techniques are mainly their difficulties to apply in field condition (Lumsden et al., 1979).

1.5.3.3 Molecular diagnostic tools

Modern molecular techniques are capable of detecting the presence of parasites based on their antigenic components or DNA segments. It also can be used to differentiate the members of the genus *Trypanosoma* are preferably used because visual morphological discrimination of trypanosomes is always difficult (Jittapalapong et al., 2008). Developing of PCR for trypanosome is most reliable and highly sensitive technique for specific identification of natural animal infections by most trypanosome species and sub-species (Njiokou et al., 2004). The PCR is extremely sensitive as even minute amount of parasite DNA can be amplified into a detectable quantity to further characterize the species. Until now several target genes have been used to optimize PCR assayand some of the selected and the primer details are summarized in Table 1.3.

Table 1.3: **Selected studies where different types of primers targeting different genes used for molecular identification of *Trypanosoma* spp.**

Target gene	Primers used	Amplicon size (bp)	References
18S and *5.8S rRNA* genes	5-GCG TTC AAA GAT TGG GCA AT-3 5-CGC CCG AAA GTT CAC C-3	455	Desquesnes et al., 2001
18S gene	5-CAACGATGACACCCATGAATTGGGGA-3 5-TGCGCGACCAATAATTGCAATAC-3 5-GTGTCTTGTTCTCACTGACATTGTAGTG-3	Variable	Geysen et al., 2003
Universal Trypanosome Species	5- GCGTTCAAAGATTGGGCAAT-3 5-CGCCCGAAAGTTCACC-3	Variable	Mwandiringana et al., 2012
SG RoTat 1	5-GCGGGGTGTTTAAAGCAATA-3 5-ATTAGTGCTGCGTGTGTTCG-3	205	Claes et al., 2004
Minicircl	5-ACAGTCCGAGAGATAGAG-3 5-CTGTACTCTACATCTACCTC-3	436	Njiru et al., 2004
ITS-1	5-CCGAAGTTCACCGATATTG-3 5-GCTGCGTTCTTCAACGAA-3	Variable	Nirju et al., 2005
gGAPDH	5-CTYMTCGGNAMKGAGATYGAYG-3 5-GRTKSGARTADCCCCACTCG-3 5-GTTYTGCAGSGTCGCCTTGG-3	880-900	Hamilton et al., 2004

1.6 Tissue protozoa in small ruminants

Different types of tissue protozoan diseases including toxoplasmosis, sarcocystosis and neosporosis cause devastating loss in small ruminants. Among tissue protozoa, *Toxoplasma gondii* caused significant amount of loss in small ruminants and is considered as a major pathogen for abortion in small ruminants (Buxton et al., 2007; Dubey et al., 2011).

1.7 Effects of toxoplasmosis in small ruminants

Toxoplasmosis considered as one of the major causes of reproduction disorder in small ruminants (Dubey et al., 2011). Among food animals clinical toxoplasmosis in more common in goat with fatal outcome (Webster, 2010). Although asymptomatic in most cases, toxoplasmosis can cause abortion, mummification of fetus, still birth and weak lamb and kids (Anastasia et al., 2013). Infection acquired during perinatal period can lead to death of fetus and causing significant economic loss in livestock industries (Buxton, 1991). The majority of natural *T. gondii* infections in small ruminants are subclinical. Clinical sign include fever, anorexia, respiratory distress and sometimes diarrhoea. Central nervous system disorder is rarely reported. While causes significant economic loss in livestock industries, it also has zoonotic significance (Ghoneim et al., 2009).

1.8 Diagnosis of *Toxoplasma gondii* infection

Several techniques are available to detect toxoplasmosis in small ruminants that include serology, histopathology and molecular diagnostic techniques (Glor et al., 2013).

1.8.1 Serology

Serological diagnosis is more widely used technique to detect *T. gondii* infection both in human and animals (Hashemi-Fesharki, 1996). It is an important tool for diagnosis of abortion in small ruminants (Buxton, 1998). Presence of specific antibodies in body fluids indicates the tissue infection. There are so many serological tests used for the detection of antibodies against *T. gondii* including Dye test (Sabin and Feldman, 1948); IFAT, IHA (Jacobs and Lunde, 1957); CFT, MAT (Desmonts and Remington, 1980); LAT, DAT (Devada et al., 1998), ELISA (Li et al., 2000). Among all serological tests, some researcher concluded that IHA, IFAT and ELISA all are highly sensative for epidemiological survey in small ruminants (Figueiredo et al., 2001), while other researchers consider both IHAT and ELISA are efficient diagnostic tools for detection of *Toxoplasma* in small ruminants (Barakat et al., 2009). In the ELISA test, microtitre plates were precoated with inactivated antigen and diluted serum sample is added to bind with the antigen to form antigen–antibody complex. Numerous modification of the ELISA has been adapted to enhance the sensitivity and specificity of the conventional ELISA test (Dubey and Beattie, 1988). The test is very simple, economic, sensitive and easily adapted for field use (Spencer et al., 1980). The original ELISA use a soluble antigen made from *Toxoplasma* RH strain tachyzoites (Voller et al., 1976). Later on ELISA is used based on the recombinant antigen or combination of recombinant antigen H4/GST and H11/GST (Tenter et al., 1992). Various recombinant antigens including granule antigen GRA1, GRA2, GRA4, GRA6, GRA7 and GRA8; rhoptry protein ROP1 and ROP2; Matrix protein MAG1; micronems protein MIC2, MIC3, MIC4 and MIC 5 and surface antigen and SAG 2 were used for detection of specific antibodies against *T. gondii* (Bastien, 2002; Liu et al., 2015). Used of recombinant antigen or their combination showed higher sensitivity and specificity (Sudan et al., 2013). Sandwich ELISA with recombinant P35 is more specific for the acute infection than the traditional ELISA (Suzuki et al., 2000). Dot-ELISA, a modification of ELISA in which the antigen-antibody reaction is

performed on nitro-cellulose in place of the polystyrene plate, has been used to detect antigens and antibodies of *T. gondii by* some researchers (Pour Azami et al., 2011).

1.8.2 Histopathology

The identification of characteristic histopatholotogical changes in the tissues are the indicative for toxoplasmosis (Buxton and Finlayson, 1986). The parasite had been detected in various tissues including brain, heart, skeletal muscle, small intestine, liver and diaphragm. Placenta, brain and heart are the most commonly affected organs as found in *T. gondii* induced abortion in small ruminants (Dubey and Beattie, 1988). In another study it was found that brain arethe most affected oran by toxoplasmosis in small ruminants and it was the most commonly used tissue for diagnosis of toxoplasmosis (Uggla et al., 1987). Detection of tachyzoite is very difficult to detect histopathologically except acute phase of toxoplasmosis (Remington et al., 2001) but bradyzoite cysts could be observed with the development of immunity.

Myocarditis, interstitial nephritis, non- suppurative encephalitis, hepatitis and diffuse interstitial pneumonia were the most histopathological changes in aborted feti (Ahmed et al., 2008; Dubey et al., 1990). Other histopathological changes include necrotic foci followed by mononuclear inflammatory reaction in *T. gondii* affected organs (Buxton and Finlayson, 1986). Periodic acid schiff (PAS), Giemsa stain, Modified Ziehl Nelson stain, Von Kossa stain etc. are also used as a special stain for toxoplasmosis (Sheehan and Hrapchak, 1980).

1.8.3 Molecular diagnostic tools

Molecular techniques such as PCR to detect *T. gondii* in fetal tissues and placenta were considered as important tool for diagnosis of toxoplasmosis (Hurtado et al., 2001; Kamani et al., 2010a). These technique have been commonly used for clinical diagnosis and epidemiological study of *T. gondii* (Su et al., 2010). Some of the molecular techniques include the PCR, nested PCR and quantitative PCR to detect DNA from biological samples along with PCR-RFLP, microsatellite and multilocus sequence typing (MLST) for identification of isolate of *T. gondii* (Su et al., 2010). PCR assays targeting different genes have been documented by researcher around the globe. Some of the selected studies using PCR approaches are summarized in Table 1.4.

Table 1.4: **Selected studies where different types of primers and the respective target genes used for molecular identification of *Toxoplasma gondii***

Target gene	Type of PCR	Primers used	Amplicon size (bp)	References
B1	Nested	For primary PCR: 5 -GGAACTGCATCCGTTCATGAG-3 5 -CTTTAAAGCGTTCGTGGTC-3 For secondary PCR: 5 -TGCATAGGTTGCAGTCACTG-3 5´-GGCGACCAATCTGCGAATACACC-3	94	Ahmed et al., 2008
18S-5.8S rRNA ITS-1	Nested	For primary PCR: 5-CCTTTGAATCCCAAGCAAAACATGAG-3 5-GCGAGCCAAGACATCCATTGCTGA-3 For secondary PCR: 5-GTGATAGTATCGAAAGGTAT-3 5-ACTCTCTCTCAAATGTTCCT- 3	227	Kamani et al., 2010a
ITS-1	Simple	5-GATTTGCATTCAAGAAGCGTGATAGTAT-3 5-AGTTTAGGAAGCAATCTGAAAGCACATC-3	333	Moazeni Jula et al., 2013
Universal *18S rRNA*		5-CGGCTACCACATCTAAGG-3 5-TATACGCTATTGGAGCTGG-3	205	Moazeni Jula et al., 2013
B1	Nested	For primary PCR: 5-GGAACTGCATCCGTTCATGAG-3 5-AATACGACTCACTATAGGGTGCATAGGTTG CA GT CACTG-3 For secondary PCR: 5'-GGCGACCAATCTGCGAATACACC-3 5'-TCTTTAAAGCGTTCGTGGTC-3	97	Burg et al., 1989
B1	Nested	For primary PCR: 5-TGTTCT GTC CTA TCG CAA CG-3 5-ACG GAT GCA GTT CCT TTC TG-3 For secondary PCR: 5-TCT TCC CAG ACGTGG ATT TC-3 5-CTC GAC AATACG CTG CTT GA-3	531	Habibi et al., 2012
B1	Nested	For primary PCR: 5 -TCA AGC AGC GTA TTG TCG AG-3 5 -CCG CAG CGA CTT CTA TCT CT-3 For secondary PCR: 5 -GGA ACT GCA TCCGTT CAT GAG-3 5 -TCT TTA AAG CGTTCG TGG TC-3	197	Habibi et al., 2012
P30	Simple	5-CACACGGTTGTATGTCGGTTTCGCT-3 5-TCAAGGAGCTCAATGTTACAGCCT-3	372	Hyman et al., 1995
529 bp repeat element	Simple	5-CGC TGC AGG GAG GAA GAC GAA AGT TG-3 5-CGC TGC AGA CAC AGT GCA TCT GGA TT -3	529	Homan et al., 2000
16S rRNA	Simple	5-CGCCTGTTTATCAAAAACAT-3 5-CCGGTCTGAACTCAGATCACGT-3	652	Hyman et al., 1995
ITS-1	Nested	For primary PCR: 5-CCTTTGAATCCCAAGCAAAACATGAG-3 5-GCGAGCCAAGACATCCATTGCTGA-3 For secondary PCR: 5-GTGATAGTATCGAAAGGTAT-3 5-ACTCTCTCTCAAATGTTCCT-3	227	Hurtado et al., 2001

1.9 Present status of blood protozoa of cattle in Bangladesh

Bangladesh has a tropical climate characterized by wide variations in rainfall, temperatures and humidity. This weather favors the growth and multiplication of many arthropods vectors like ticks, flies and mosquitoes etc. that are responsible for the transmission of a number of hemoprotozoan diseases. The most commonly found haemoparasitic diseases in the country include babesiosis, anaplasmosis along with less common theileriosis, leishmaniosis and trypanosomiasis. Researchers have long been studying the prevalence of different species of blood parasites in different parts of Bangladesh. Among them, *Trypanosoma theileri, B. bigemina*, *Theileria annulata, T. mutans* and blood rickettsia like *A. marginale* and *A. centrale* etc. were reported by some investigators (Ahmed, 1976; Alim et al., 2012; Banerjee et al., 1983; Rahman et al., 1982; Samad and Goutam, 1984; Siddiki et al., 2010; Talukdar and Karim, 2001). Among different hemoparsitic diseases in cattle, the prevalence of anaplasmosis and babesiosis was found to be comparatively higher (Alim et al., 2012; Chowdhury et al., 2006; Rahman et al., 1982; Samad et al., 1989). A comprehensive list of all reports on different hemoparasitic diseases prevalent in Bangladeshi cattle are summarized in Table 2.1, 2.3 and 2.4.

Several epidemiological studies have been conducted on vector-borne haemoprotozoan diseases in Bangladesh (Alim et al., 2012; Belal et al., 2015; Chowdhury et al., 2006; Rahman et al., 2015a; Samad et al., 1989; Siddiki et al., 2010; Talukdar and Karim, 2001). Most of these studies were based on microscopic examination of blood smear collected from peripheral blood circulation while some were based on molecular examination (Rahman et al., 2015a). To our knowledge, there is no published article on molecular characterization of blood protozoa in cattle in Bangladesh. Howover, previous report on vector-borne diseases in cattle in Chittagong area was very limited (Alim et al., 2012).

1.10 Present status of toxoplasmosis in Bangladesh

In Bangladesh, several earlier reports have been published on toxoplasmosis in animals and human (Biswas et al., 1993; Khatun et al., 1998; Rahman et al., 2015b; Samad et al., 1993; Samad et al., 1997; Shahiduzzaman et al., 2011). These studies were conducted in different regions of the country including northern Mymensingh and Rangpur areas. Most of the studies were based on serological techniques like

latex agglutination test (LAT) while one was based on histopathological study in naturally occurring abortion in Black Bengal goat (Bari et al., 1993).

In absence of modern molecular tools, until now researchers were solely dependent on serology to diagnose clinical cases. Further molecular investigation is therefore essential for their complete characterization.

The present research was designed with following objectives as described preferably in different dedicated Chapters:

1. Investigate the prevalence of selected vector-borne haemoprotozoan diseases (babesiosis, anaplasmosis and trypanosomiasis) in cattle in different selected areas of Chittagong region.
2. Determine different epidemiologic factors including breed, age, sex, seasons and their correlation with disease incidence.
3. Molecular characterization of the selected haemoparasites and their phylogenetics.
4. Determine the prevalence and identification of risk factors of toxoplasmosis in sheep and goats in Chittagong Metropolitan area of Bangladesh.
5. Histopathological identification and molecular characterization of *Toxoplasma gondii* from several cases of abortion in small ruminants.

Chapter-2

Review of Literature

2.1 Anaplasmosis

Bovine anaplasmosis is an important hemoparasitic disease of cattle caused by the rickettsial organism of the genus *Anaplasma* (Order Rickettsiales, Family Anaplasmataceae). The diseases is characterized by fever, weight loss, decreased milk production, pale mucous membranes, severe anemia, jaundice, brownish urine, hyper-excitability, abortion and mortality without showing any sign of hemoglobinemia and hemoglobinuria during acute form of the disease (Richey and Palmer, 1990). Clinical signs of disease are usually not apparent in persistently affected carrier animals. However, the persistent form is important for maintenance of *A. marginale* within a herd.

2.1.1 Etiology

There are several species of *Anaplasma* recorded to date. These include *A. marginale, A. centrale, A. ovis, A. phagocytophilum, A. bovis* and *A. platys.* Anaplasmosis in cattle is mainly caused by *A. marginale* and *A. centrale* (De La Fuente et al., 2008). Among the two, *A. marginale* is the most important one (Kumar and Sangwan, 2010). *A. centrale* is closely related to *A. marginale* and causes mild anaplasmosis in cattle. Although *A. bovis* usually found in cattle and small mammals are thought to act as reservoir of infection (Goethert and Telford, 2003). The protozoan infects the monocytes and responsible for causing monocytic anaplasmosis.

2.1.2 Susceptible host range

Bovine anaplasmosis has been reported in various domestic and wild animals including cattle, buffalo, bison, African antelopes and the mule deer (Dumler et al., 2001; Kocan et al., 2003). Clinical anaplasmosis is uncommon in wild animals, however two clinical cases of anaplasmosis were reported in giraffe (Augustyn and Bigalke, 1974). Serological occurrence of *Anaplasma* spp. has also been reported in impala *(Aepyceros melampus),* waterbuck (*Kobus ellipsiprymnus)* and plain zebra (*Equus quagga)* (Ngeranwa et al., 2008).

2.1.3 Transmission by tick vectors

Almost 20 species of ticks have been reported to transmit anaplasmosis experimentally (Kocan et al., 2004). The main vectors of bovine anaplasmosis are *Ixodid* ticks (mainly *Boophilus microplus*) while other ticks of the genera *Rhipicephalus, Dermacentor, Haemaphysalis, Hyalomma* and *Ixodes* can also transmit the ricketssia (Kumar and Sangwan, 2010). The infectious agent also transmitted mechanically via contaminated mouth parts of biting insects or by contaminated fomites such as needles, castrating knives, ear taggers and other surgical instruments (Ewing et al., 1997). In addition to mechanical and biological transmission, *A. marginale* can also be transmitted from cow to calf transplacentally during the time of gestation period (Kocan et al., 2003).

2.1.4 Geographic distribution

Anaplasmosis in cattle is prevalent worldwide and most commonly reported in South Africa, Australia, Asia, the former USSR, South America and the United States (Woldehiwet and Ristic, 1993). *A. bovis* has been most commonly reported in cattle and buffalo from Africa, the Middle East and South America (Ooshiro et al., 2008). In Bangladesh, *A. marginale*, *A. centrale* was previously reported in cattle (Ahmed, 1976).

2.1.5 Breed susceptibility

Several studies were conducted to determine the differences in susceptibility for *A. marginale* infection among local breeds (*Bos indicus*), European breeds (*Bos taurus*) and their crosses (Chowdhury et al., 2006; Jonsson et al., 2008). Among cattle breeds, *B. taurus* are more likely to develop acute anaplasmosis than crossbred Zebu cattle (Aguirre et al., 1987). However in experimental infection local and crossbred were equally susceptible to *A. marginale* and developed similar clinical signs (Bock et al., 1997).

2.1.6 Susceptibility among different sexes

Generally female animals are thought to be more susceptible to anaplasmosis than the male animals. Several published reports validate this statement (Alim et al., 2012; Atif et al., 2012; Rajput et al., 2005). The suppression of immunity in advanced

pregnancy due to physiological changes and or high energy loss in lactation in high yielding animals are thought to be the possible reasons for a higher prevalence of anaplasmosis in cows (Kocan et al., 2010).

2.1.7 Age susceptibility

Anaplasma spp. can cause infections in cattle of all age groups where severity and mortality rate depends on the age of animals. Generally animals more than two years of age develop clinical disease associated with a high mortality, contrary to calves that are less susceptible (Aubry and Geale, 2011). Calves under the age of 6 months rarely develop clinical signs and when older than 6 months are usually found to suffer from mild clinical disease (Kocan et al., 2010). Cattle between one and two years of age may develop more acute signs of disease, which is rarely fatal (Kocan et al., 2010).

2.1.8 Effect of climate change

Temperature plays an important role in the development and multiplication of the ticks that consequently play role in the epidemiology of anaplasmosis. Changes in the environment like climate change effects on the complex arthropod–pathogen–host epidemiological cycle through variety of complex mechanisms from the molecular to the population level (Tabachnick, 2010). Global warming may influence the movement of the tick vectors and accordingly the distribution of anaplasmosis varies in different places (Jonsson and Reid, 2000).The hot and humid climate is very favorable for the development and survival of tick vectors and is a constant and important source of infection to susceptible animals (Chowdhury et al., 2006).

2.1.9 Prevalence of anaplasmosis around the globe

Many sero-epidemiological and molecular studies have attempted to investigate the prevalence of anaplasmosis in different parts of the world. Some of the selected studies are summarized in Table 2.1. Molecular investigations were found to be less sensitive than serological studies where high case numbers were recorded by the researchers. The prevalence of anaplasmosis varies from 0.20% to 70.0% in different countries as reported through conventional tools like microscopy and staining. However, serological reports suggest a prevalence rate ranging from 16.57% to 79.40% possibly indicating higher sensitivity of the serological tests. Other reports

using molecular tools indicates a wide range of prevalence from 19.05 to 98.6% in some parts of the world. In Bangladesh several studies were conducted in northern, southern and north eastern part of the country based on parasitological approach and the prevalence was recorded from 0.20% to 70.0% with variable sample sizes. In neighboring India, the prevalence varied from 2.64% to 12.08% while in Pakistan the prevalence was ranged from 4.07% to 17.26%. A countrywide sero-surveillance is therefore necessary to determine the extent of this important blood parasite in Bangladesh.

Table 2.1: **Selected studies investigating the prevalence of anaplasmosis in cattle reported from different countries.** Different authors used different tools and sample size varied in each study.

Country	Regions	Sample size (n)	Methods applied	Percent Prevalence	References
Bangladesh	Sirajgonj	60	Microscopy	70.00	Chowdhury et al., 2006
Bangladesh	Sirajgonj	395	Microscopy	25.82	Belal et al., 2014
Bangladesh	Chittagong	166	Microscopy	1.00	Siddiki et al., 2010
Bangladesh	Chittagong	648	Microscopy	4.05	Alim et al., 2012
Bangladesh	Chittagong	2614	Retrospective study	0.20	Badruzzaman et al., 2014
Bangladesh	Hilly areas	475	Microscopy	14.94	Mohanta and Mondal, 2013
Pakistan	Sargodha	350	Microscopy	9.71	Atif et al., 2012
Pakistan	Islamabad and Attock	307	Microscopy	17.26	Khan et al., 2004
Pakistan	Punjab	836	Microcopy	4.07	Sajid et al., 2014
Pakistan	Punjab	1050	ELISA	31.05	Atif et al., 2013
India	Tamil Nadu	2637	Microcopy	2.64	Velusamy et al., 2014
India	Punjab	298	Microscopy PCR	12.08 51.01	Ashuma et al., 2013
India	Ludiana, Punjab	703	Microscopy	8.53	Singh et al., 2012
Malaysia	-	264	ELISA	79.40	Pong and Nik-Him, 2012
Iran	Khorasan Province	160	Microcopy	19.37	Razmi et al., 2006
Thailand	Nan, Nakhon Sawan and Ayutthaya	569	PCR	19.05	Saetiew et al., 2013
Morocco	North and Central Morocco	668	ELISA PCR	16.50 21.90	Hamou et al., 2012
Brazil	South western Amazonia	1650	PCR	98.6	Brito et al., 2010

2.2 Babesiosis

Babesiosis is an important vector borne disease caused by blood protozoa of the genus *Babesia* that affect many species of mammals including cattle (Bock et al., 2004). The disease was first reported in 1888 by Viktor Babes in Romania in erythrocytes of cattle associated with bovine hemoglobinuria or red water fever (Babes, 1888). Throughout the world, babesiosis is popularly known as piroplasmosis, tick fever, red water fever, Texas fever, splenic fever or tristeza etc. and are known to be transmitted only by *Ixodid* ticks (Ristic, 1988). In endemic areas major pathognomonic symptoms include high fever, haemolytic anemia, jaundice and haemoglobinuria (Soulsby, 1982). Other signs include fever, inappetence, depression, increased respiratory rate,

weakness, reluctant to move, muscle wasting, tremors and abortion (De Vos and Potgieter, 1994). In severe cases animals die within 24 hours.

2.2.1 Etiology

Bovine babesiosis is caused by several species of the genus *Babesia*. These include *B. bigemina, B. divergens, B. major, B. bovis, B. ovata* (Japan) and *B. occultans* (South Africa) (Bock et al., 2004; Uilenberg, 2006). Among them, *B. bigemina* and *B. bovis* have considerable impact on health and productivity of cattle in tropical and sub-tropical countries including Bangladesh (Iseki et al., 2010).

2.2.2 Susceptible host range

Babesia spp. is host-specific. *B. bovis* and *B. bigemina* usually infect cattle but capable of producing disease in water buffalo and some of wild ruminants (Kistner and Hayes, 1970). *B. bigemina* was recorded from roe deer, red deer and wild boar (Zanet et al., 2014). One study reported that white-tailed deer were responsible for reintroducing the tick vector of cattle Tick Fever in Central Texas, in USA (Holman et al., 2011). Fatal babesiosis due to *B. divergens* was reported in captive rein deer in United Kingdom (Langton et al., 2003) and captive woodland caribou in America (Petrini et al., 1995).

2.2.3 Transmission by tick vectors

Under natural condition, Babesia is transmitted by ticks which may become infected by feeding of infected blood. *Boophilus* ticks are major vector responsible for transmission of babesiosis. These vectors are commonly found in tropical and sub-tropical countries like Bangladesh (Rony et al., 2010). However other tick vectors are also capable of transmitting the disease. Selected *Babesia* organisms and their concerned vector ticks are presented in Table 2.2.

Table 2.2: **Major *Babesia* species infective to domestic animals and their *Ixodid* tick vectors** (Adapted from Uilenberg, 1995)

Species	Vector
Babesia bigemina	*Boophilus microplus* *B. decoloratus* *B. annulatus* *B. geigyi* *Rhipicephalus evertsi*
Babesia bovis	*B. microplus* *B. annulatus* *B. geigyi*
Babesia divergens	*I. ricinus* *I. persulcatus*
Babesia major	*Haemaphysalis punctate*
Babesia ovate	*H. longicornis*

B. microplus is the most important vector all over the world except in southern Africa, where *B. decoloratus* is available. *B. annulatus* is the principal vector of *B. bovis* and *B. bigemina* in Northern Africa (Sahibi et al., 1998), the Middle East (Pipano, 1997), Turkey (Sayin et al., 1994) and some areas of southern Europe (Caeiro, 1999). *B. divergens* is transmitted almost exclusively by *I. ricinus* in northern Europe (Friedhoff, 1988).

2.2.4 Geographic distribution

Babesia is a cosmopolitan parasite and the geographic distribution depends on the availability of tick vectors (AbouLaila et al., 2010). *B. bovis* and *B. bigemina*, are important blood parasite of cattle that present in many tropical and sub-tropical regions of the world including Africa, Asia, Australia, Central and South America, and the West Indies (Bock et al., 2004; Yin et al., 1997). *B. divergens* usually affect the cattle of north-western Europe (Telford III et al., 1993) and France (L'hostis et al., 1995). *B. major* have been reported mainly in European countries including the UK, the Netherlands, France, Germany and Spain (Hornok et al., 2014). *B. ovata* is reported from eastern Asia (Uilenberg, 1995). In Bangladesh the most prevalent *Babesia* species in cattle is *B. bigemina* that was recorded by several researcher (Banerjee et al., 1983; Chowdhury et al., 2006;Samad et al., 1989).

2.2.5 Breed susceptibility

The zebu cattle (*Bos indicus*) are known to exihibit high levels of innate resistance to *B. bovis* and *B. bigemina* infections when compared to *Bos taurus* and their crosses (Bock et al., 1997). This resistance is manifested by lower parasitemia in *Bos indicus* compared to *B. taurus* kept under identical pasture conditions (Aguirre et al., 1990). The high resistance of zebu cattle to ticks and to babesiosis is a widely known phenomenon, which motivated the farm owners to replace pure *B. taurus* by *B. indicus* cattle and their crosses in areas of high tick infestation in Australia (Jonsson, 2006). Differences in susceptibility to *B. bigemina* in between indigenous/local cattle and crossbred cattle have also been demonstrated in Bangladesh (Al Mahmud et al., 2015; Alim et al., 2012; Chowdhury et al., 2006). All these studies indicated that the prevalence of babesiosis is higher in crossbred cattle when compared to the local or indigenous group.

2.2.6 Susceptibility among different sexes

Several studies have been reported to compare the susceptibility of male and female cattle to babesiosis (Alim et al., 2012; Hamsho et al., 2015; Swai et al., 2007). These studies indicate that the prevalence was higher in female animals than male. The higher prevalence of babesiosis in female animals might be linked to the fact that female animals are kept longer for breeding and milk production purposes (Kamani et al., 2010b). Other possible reasons are thought to be hormonal disturbances due to its use in milk production and breeding system which lowers the immune system of the animal (Hamsho et al., 2015).

2.2.7 Age susceptibility

There is an inverse relation between severity of clinical signs, speed of recovery and mortality rates with age of the host in babesiosis (Florin-Christensen et al., 2014). Calves generally resist acute infection because of innate immunity through colostrum and this colostral antibody confers protection to calves that may persist for up to 6 months for *B. bovis* and 3 to 4 months for *B. bigemina* (Wright, 1990). After 6 months of age, the number of infected animals in enzootic areas increases. The highest infection rate in animal are usually recorded at the age of 6-12 months. Infection is uncommon in animals over 5 years. Animal under 1 year of age are infected

predominantly with *B. bigemina* and those over 2 years of age by *B. bovis* (Radostits et al., 2006*)*. However there are many case reports of babesiosis in calves less than a month of age (Vairamuthu et al., 2012; Venu et al., 2015).

2.2.8 Environmental factors

Many reports are published on seasonal variations in the prevalence of clinical babesiosis. Highest incidence occurs soon after the peak of the tick population. Temperature, humidity, rainfall, air are the most important factors which regulate the activity of the tick populations (Radostits et al., 1994). In endemic areas, where there are many infected ticks, the immunity of the host is maintained at a high level through repeated challenge and overt disease is rare. In contrast, where there are few ticks or when hosts are confined to limited areas, the immune status of the population is low, clinical outbreak of babesiosis takes place (Zintl et al., 2005).

2.2.9 Other miscellaneous factors

Babesiosis is higher in areas where the climatic conditions and environmental management allow the presence of the tick vectors throughout the year. Calves become infected during the first months of life, at an age whene they are relatively resistant to the clinical disease and develop immunity. Breed, management strategies, tick infestation rates and regular acaricide treatment play an important role in the occurrence of babesiosis (Andrade et al., 2004). Other factors like transportation, parturition, malnutrition and superimposed infection with different parasites especially *A. marginale* may contribute to this infection (Radostits et al., 1994).

2.2.10 Prevalence of babesiosis around the globe

Several serological (e. g. ELISA, IFAT, IFA), molecular (e. g. PCR assay), parasitological (microscopy with staining) and reviews have been reported from different parts of the world (Table 2.3). In Bangladesh, a low prevalence was recorded in a retrospective study while overall serological prevalence ranged from 14.0 to 72.3%. However, prevalence was ranged from 3.6 to 62.5% using more sensitive molecular tools.

Among studies reported from Bangladesh, the prevalence ranged from 0.80% to 16.63% in different parts of the country. In neighboring India, the prevalence ranged

from 0.65% to 29.00% while in Pakistan the prevalence ranged from 1.0% to 3.60%. In absence of modern diagnostic tools only microscopic identification of *Babesia bigemina* has been confirmed in Bangladesh to date. Further technological developments need to be adapted among researchers and farming community for reliable and accurate diagnosis of this important hemoprotozoa which is responsible for significant economic losses in livestock sector.

Table 2.3: **Selected studies investigating the prevalence of babesiosis in cattle in different countries.** Different authors used different tools and sample size varied in each study.

Country	**Regions**	**Sample size (n)**	**Methodology applied**	**Percent Prevalence**	**References**
Bangladesh	Sirajgonj	395	Microscopy	2.27	Al-Mahmud et al., 2015
Bangladesh	Sirajgonj	60	Microscopy	3.30	Chowdhury et al., 2006
Bangladesh	Sylhet	100	Microscopy	16.00	Nath and Bhuiyan, 2013
Bangladesh	Chittagong	166	Microscopy	1.00	Siddiki et al., 2010
Bangladesh	Chittagong	648	Microscopy	4.05	Alim et al., 2012
Bangladesh	Chittagong	2614	Retrospective study	0.80	Badruzzaman et al., 2014
Bangladesh	Hilly areas	475	Microscopy	16.63	Mohanta and Mondal, 2013
Bangladesh	Rangpur	400	Microscopy Molecular	1.50	Rahman et al., 2015a
Pakistan	Qadirabad	100	Microscopy PCR	18.00 29.00	Chaudhry et al., 2010
Pakistan	Islamabad and Attock	307	Microscopy	0.65	Khan et al., 2004
Pakistan	Sargodha	350	Microscopy	6.57	Atif et al., 2012
India	Tamil Nadu	2637	Microscopy	1.00	Velusamy et al., 2014
India	North eastern part	333	PCR	3.60	Laha et al., 2015
India	Punjab	703	Microscopy	1.56	Singh et al., 2012
Iran	Kurdistan	6469	Microscopy	2.10	Fakhar et al., 2012
Srilanka	-	316	PCR	30.1	Sivakumar et al., 2012
Myanmar	-	713	PCR	15.75	Bawm et al., 2016
Thailand	Northern part	700	ELISA IFAT	71.45 72.30	Iseki et al., 2010
Malaysia	-	100	IFAT	14.00	Rahman et al., 2010
Italy	Southern part	117	ELISA	23.10	Cringoli et al., 2002
Philippines	Five province	250	PCR	9.40	Yu et al., 2013
Portugal	Central and southern part	406	ELISA PCR	65.50 62.50	Silva et al., 2009
Nigeria	-	168	Microscopy	9.50	Onoja et al., 2013
Gambia	-	184	IFA	65.00	Kuttler et al., 1988

2.3 Trypanosomiasis

Trypanosomiasis is caused by blood and tissue dwelling protozoan parasites of the genus *Trypanosoma* and transmitted cyclically by the tsetse fly *Glossina*, the most common vector for trypanosomiasis (Fasanmi et al., 2014). Trypanosomes can infect all species of domesticated livestock including cattle throughout many of the tropical and subtropical regions of the world. The disease is characterized by intermittent fever, edema, occasional diarrhoea, abortion, decreased fertility and emaciation. In some instances, infected animals show no overt signs of disease but can succumb if stressed, for example, by work, pregnancy, milking or adverse environmental conditions (Lucking, 1988). The disease has negative impact on overall profits of the farmer by reducing the productivity of animals and by reducing the quality of animals, lowering the feed conversion ratio and by eventual death of the animal (Luckins, 1988).

2.3.1 Etiology

Trypanosomes are protozoan parasites of the genus *Trypanosoma* belonging to the family Trypanosomatidae. The most important tsetse fly-transmitted trypanosomes are *Trypanosoma brucei, T. congolense* and *T. vivax* and cause diseasein cattle and other ruminants. *T. evansi*, the etiological agent of surra which is also known as “bayawak”or “higpit” infects numerous domestic and wild animals in warm climates (Baticados et al., 2011). *T. theileri*, a large stercorarian trypanosome occurs extremely rarely and has been reported to be present in cattle and buffaloes in Bangladesh (Samad and Shahid-Ullah, 1985).

2.3.2 Susceptible host range

Trypanosomes can infect all domesticated animals; clinical cases have been described in cattle, sheep, goats, pigs, horses, camels and human. The organism was also detected from the wild animals can but generally do not produce clinical form of disease to them. They are the source (reservoir) of infection for domestic animals (Menon and Mathew, 2008). The effect of the trypanosomiasis varies with the host. African native humpless or taurine cattle (N'Dama, Baoule) are known as naturally trypanotolerant (Radostits et al., 1994).

2.3.3 Transmission by fly vectors

Trypanosomiasis is an important disease of cattle which is transmitted cyclically by the tsetse fly *Glossina* (Fasanmi et al., 2014). There are 23 species of *Glossina* belonging to different groups according to their preferred habitats as forest species, riverine species and savannah species. *T. evansi* is mainly transmitted by *Tabanid* flies. In addition, the disease can also be transmitted mechanically by several blood-sucking flies like stable fly (*Stomoxys calcitrans*), buffalo fly (*Haematobia* spp.), deer fly (*Chrysops* spp.) and mosquitoes (Soulsby, 1982). Some reports indicate that mechanical transmission of *T. vivax* and *T. congolense* occurred by various blood feeding dipteran (Desquesnes et al., 2009a).

2.3.4 Geographic distribution

The geographical distribution of trypanosomiasis rely on abundance and infection rate of vector, the livestock management system, the level of drug resistance and drug use in a particular area and the degree of livestock to resist to tsetse fly bites as well as to trypanosome strains circulating in the area. The disease occurs throughout the tropical regions of Africa and in large areas of Asia and South America (Menon and Mathew, 2008). Geographical distribution of *T. evansi* is continuous from the northern part of Africa through the Middle East to South-East Asia (Desquesnes et al., 2013). *T. vivax* is an important trypanosome species that infect in cattle herds in tropical areas of Africa, Central America and South America (Osório et al., 2008). *T. theileri* is reported in cattle in Bangladesh (Samad and Shahid-Ullah, 1985).

2.3.5 Effect of climate change events

Global warming is thought to influence the epidemiology of vector-borne diseases like trypanosomiasis by altering pathogen and vector development rates and generation times, shifting the geographical distribution of vector or reservoir host populations, altering transmission dynamics or modifying host susceptibility to infection (Patz et al., 2000). The population density of tsetse fly population in an area and their interaction with the host will determine the level of infection. Suitable habitat like forest area favours the breeding of tsetse flies rather than agricultural and industrial areas (Radostits et al., 1994).

2.3.6 Immune mechanisms

Animals having exposure to one strain or species of trypanosome are not immune to infection with another strain or species. This is due to variation in antigenicity among different trypanosome species. Cattle breed were classified into two distinct groups based on the susceptibility to *Trypanosoma* infection. Trypanotolerant and trypanosusceptible breeds of cattle have distinct antibody responses. Trypanosusceptible cattle produce high titres of polyspecific IgM but fail to produce IgG to specific trypanosome antigens. In contrast, although T cell and macrophage/ monocyte responses of infected cattle are depressed, significant differences have not been described between tolerant and susceptible breeds of cattle (Taylor, 1998).

2.3.7 Prevalence of trypanosomiasis around the globe

Various parasitological and serological examinations including IFAT, agglutination test, Complement Fixation test, Card Agglutination Test (CAT), ELISA along with molecular techniques like PCR, Reverse Line Blot (RLB) etc. have been attempted by the researchers to determine the prevalence and molecular characterization of trypanosomiasis in animals.

The overall parasitological prevalence of trypanosomiasis globally varied from 0.18% to 43.0%. Highest prevalence of trypanosomiasis (56.7%) was recorded in Blue Nile State of Sudan (through ELISA) and lowest prevalence (0.13%) was recorded in Thailand (through PCR and microscopy).

Although not comprehensively studied, few reports have indicated the existence of trypanosomes in domestic animals in Bangladesh. The very first report on trypanosomiasis was recorded as merely 0.58% (n=857) in cattle where *T. theileri* was identified by morphological analyses (Rahman et al., 1982). In another study, the same parasite species was reported by other investigators (Samad and Shahid-Ullah, 1985). We are not aware of any other published report on trypanosomes in Bangladesh to date. This requires further comprehensive investigation using modern DNA based diagnostic tools to detect and characterize this important protozoan. The molecular study can be complementary to the microscopic investigation and may help develop necessary prevention plan to combat the infection.

Table 2.4: **Selected studies investigating the prevalence of trypanosomiasis reported from different countries.** Different authors used different tools and sample size varied in each study.

Country	Regions	Sample size (n)	Methods applied	Percent prevalence	References
Bangladesh	Different parts	857	Microscopy	0.58	Rahman et al., 1982
India	Karnataka	215	Microscopy	6.90	Krishna Murthy et al., 2014
India	Punjab	703	Microscopy	0.28	Singh et al., 2012
India	Madhya prodesh	97	Microscopy	18.60	Agarwal et al., 2003
India	Northern Kerala	150	PCR	34.60	Nair et al., 2011
Thailand	Country wide	1979	ELISA	25.00	Desquesnes et al., 2009b
Philippines	Luzon	145	PCR Microscopy	0.13 0.13	Baticados et al., 2011
Malaysia	Johore	2229	Microscopy	5.92	Cheah et al., 1999
Tanzania	Northern part	239	Microscopy	5.00	Swai and Kaaya, 2012
Spain	Canary Island	1228	Serology	5.00	Rodríguez et al., 2012
South Africa	KwaZulu-Natal	473	Microscopy PCR	6.60 50.00	Gillingwater et al., 2010
South Africa	Hluhluwe-iMfolozi Park	60	PCR RLB	5.00	Yusufmia et al., 2010
Nigeria	North- Central	637	Microscopy	2.80	Kamani et al., 2010b
Nigeria	Kaduna State	1293	Microscopy	8.40	Enwezor et al., 2009
Sudan	Blue nile state	210 141	Microscopy PCR	43.00 56.70	Salim et al., 2011
Kenya	Busia	2773	PCR	11.90	Von wissmann et al., 2011
Ethiopia	Oromia Region	388	Microscopy	12.40	Fentahun et al., 2012
Senegal	Western	1141	Microscopy	2.40	Seck et al., 2010
Cameron	Western	83	CATT	16.82	Lako et al., 2010
Uganda	Eastern	203 203	Microscopy ELISA	8.90 45.30	Jing et al., 2009
Peru	Lima and Pucallpa	303	PCR	4.15	Mekata et al., 2009

2.4 *Toxoplasma gondii*

2.4.1 Brief History

Toxoplasma gondii was first discovered by Nicolle and Manceaux in a rodent. In the same year Splendore independently discover similar parasite in rabbit (Dubey, 2008). The importance of *T. gondii* was highlighted when it was proven that it is responsible for storm of abortion in sheep in 1957 (Dubey, 2008). In the year 1970, sexual stage life cycle of *T. gondii* was discovered in the small intestine of cat (Dubey, 2008; Jackson and Hutchison, 1989). Gold standard serological test for detection of antibody of *T. gondii* was described by Sabin and Feldman in 1948 (Dubey, 2008). First molecular test, PCR for specific detection of the organism using *B1* gene was developed soon (Burg et al., 1989).

2.4.2 Etiology

Toxoplasmosis is an important disease caused by the obligate intracellular protozoan named *T. gondii* (Barakat et al., 2009; Tenter et al., 2000). *T. gondii* belongs to the phylum Apicomplexa, class Protozoa, order Eucoccidia, Family Sarcocystidae and genus *Toxoplasma* (Kim and Weiss, 2004; Webster, 2010). *Toxoplasma*s have many strains and are grouped into three genetic types (I, II and III). Type I is highly virulent in mice, type II is the most common type in persistently infected animals (sheep and goats) and type III is defined as avirulent strain. Clinical human infections are more often associated with type II strains (Sibley, 2003). It is one of the most common and important parasitic zoonotic disease in all over the world (Dubey, 2007). *T. gondii* infects as wide range of animals including mammals and birds (Negash et al., 2004). The infection in animals usuallyoccured by ingestion of raw or undercooked meat containing viable tissue or by ingesting food or water contaminated with sporulated oocysts form faeces of infected felids (Dubey, 2004; Gebremedhin and Gizaw, 2014).

2.4.3 Transmission

In the life cycle of *T. gondii*, along with the sporulated oocysts (containing sporozoites), tachyzoites and bradyzoites are the three infective stages responsible for infection in both intermediate and final host (Tenter, 2009). Final host get infection by ingesting bradyzoite containing tissue cyst. Sheep and goat which are intermediate host of *T. gondii* get infection by ingestion of oocyst containing tissue or food or

drinking water contaminated with sporulated oocysts and occasionally by transplacental transmission (Radostits et al., 2006; Tenter et al., 2000). The most important source of *T. gondii* infection for sheep and goats that kept in pasture is the oocysts shed by cats in their faeces (Blewett and Watson, 1982). Transplacental transmission is also an important route of infection in animal (Mamidi et al., 2002). The available report indicate that <2% of sheep become congenitally-infected with *T. gondii*, and less than 4% of persistently infected sheep transmit it to the next generation (Buxton et al., 2007; Dubey, 2009; Higa et al., 2010). Congenital transmission occurs when female animal get infection during pregnancy and it cross the placenta and multiply in fetal tissues (Ahmad et al., 2015). Isolation of *T. gondii* from the semen of experimentally infected male indicate that it also transmitted by coitus in sheep (Lopes et al., 2009) and goats (Dubey and Sharma, 1980). Human can get infection by consumption of raw or undercooked meat (Kijlstra and Jongert, 2008) while in exceptional cases it may also be transmitted by consumption of dairy products (originating from sheep and goats) (EFSA, 2007) and occasionally by blood transfusion (Samad et al., 1997).

2.4.4 Risk factors of *Toxoplasma gondii* in small ruminants

Limited epidemiological information regarding caprine and ovine toxoplasmosis risk factors are available in literature. Among small ruminant, sheep had relatively higher prevalence than goats (Gebremedhin and Gizaw, 2014). It may be due to the differences in feeding habits since sheep are more likely to get infection from the pasture as they graze close to the ground than goats which prefer browsing. One study showed that sheep has higher prevalence that goat but have no significant association between them (Tzanidakis et al., 2012).

Age of the animals was positively associated with the seroprevalence of toxoplasmosis in sheep and goat. Adult animals had comparatively higher prevalence of toxoplasmosis infection compared to young animals (Ahmad et al., 2015; Ramzan et al., 2009; Rossi et al., 2011). It is due to exposure of adult animals to the risk factors for longer period of time than the younger ones (Van der Puije et al., 2000). But there is also exception that there was no significant difference between age group of less than one year of age and more than one year age in goat (Djokić et al., 2014).

There were so many research reports which indicate that female sheep and goats are more susceptible than males to *Toxoplasma* infections (Ahmad et al., 2015; Alexander and Stimson, 1988). It may be explained by the fact that immunity in females is reduced by various factors such as pregnancy, nutrition and lactation (Messingham et al., 2001). Although there are so many published reports they reported that there were no significant correlation between *Toxoplasma* infection and the gender of the animals (Cavalcante et al., 2008; Gebremedhin and Gizaw, 2014).

Cats are the definitive hosts of the parasite and play a vital role in infecting other animals by shedding oocysts in the environment (Lopes et al., 2010). Presence of cat specially the free roaming cats increase the risk of transmission of the infection in animals. Cats increase the oocysts load on nearby pastures resulting in contamination of environment. These oocysts when ingested along with food and water result in postnatal infection.

There were so many available reports that presence of cat plays a vital role in transmission of toxoplasmosis in sheep and goat (Ahmad et al., 2015; Lopes et al., 2010). The number of cats in the farm premises also plays a role in epidemiology of toxoplasmosis. It was reported that greater risk of infection was observed in farms with more than 10 cats was presence in farm (Cavalcante et al., 2008). Similarly there were so many report that cat were not influencing factor in the prevalence of toxoplasmosis in sheep and goats (Gazzonis et al., 2015)**.**

The prevalence of toxoplasmosis was comparatively higher in animals that are reared in unhygienic condition compared to that reared in hygienic condition. If the feed and water were contaminated with cat faeces it increases the risk of diseases. Using of public supply water (Tzanidakis et al., 2012) and usage of outdoor water source (Ahmad et al., 2015) in the farm is an important risk factor of toxoplasmosis. Types of feeding trough used in the farm were also an important factor in the epidemiology of toxoplasmosis. Using of wooden trough increases the risk of *Toxoplasma* infection (Cavalcante et al., 2008).

2.4.5 Prevalence of *Toxoplasma gondii* in small ruminants around the world

Different types of serological methods (such as ELISA, DAT, MAT, LAT, IFAT, IHA, IHAT and IFA) were used to evaluate the seroprevalence of *T. gondii* in sheep

and goats. The reports indicate a wide variation in the prevalence of toxoplasmosis in different continents and are summarized below (Table 2.5, 2.6, 2.7 and 2.8).

The seroprevalence of toxoplasmosis in Asian countries varies from 2.50- 69.00% and 5.10-61.00% in sheep and goats respectively. Apparently lower prevalence was observed in countries like Korea, China, while a higher prevalence was observed in countries like Indonesia, Saudi Arabia, Malaysia and Bangladesh. However, in India and Pakistan the prevalence varied from region to region where variable prevalence was recorded. In Bangladesh only few studies were conducted in central (Mymensingh) and northern part (Rajshahi) of the country. The prevalence ranged from 12.09 -61.00% and 17.65-69.00% in goats and sheep respectively. Notable that in all these studies, the prevalence of toxoplasmosis in sheep was comparatively higher than in goats (Samad et al., 1993; Shahiduzzaman et al., 2011).

In Africa, a huge variation in prevalence was observed among different countries. Preliminary literature review from African countries (Table 2.6) suggests an overall prevalence ranging from 6.70-74.80% and 4.60-98.40% in sheep and goats respectively. No statistically significant relationships between sheep and goats were revealed and susceptibility might be associated with the species of the host. A relatively higher prevalence was observed in countries like Tunisia, Sudan, Libya and Ethiopia whole lower prevalence were recorded in Algeria, Nigeria and South Africa.

A similar observation was reported in European countries regarding the prevalence of toxoplasmosis in small ruminants. The prevalence ranged from 17.00-73.40% and 19.00-87.4% in goats and sheep respectively. Apparently higher prevalence was recorded in countries like Serbia, Poland, Belgium, France and Romania while lower prevalence of toxoplasmosis was recorded in Scandinavian countries like Sweden, Finland and Norway.

In other parts of the world excluding Asia, Africa and Europe the overall prevalence ranged from 15.2-59.0%. The higher prevalence was observed in Caribbean islands and most parts of Brazil while lower prevalence was observed in Mexico and some parts of Brazil.

Table 2.5: **Selected studies investigating the prevalence of toxoplasmosis in small ruminants in Asia.** Different authors used different serological tools and sample size varied in each study.

Country	Regions	Animal species	Sample size (n)	Methods applied	Percent prevalence	References
Bangladesh	Mymensingh	Goats	528	LAT	12.88	Samad et al., 1997
Bangladesh	Mymensingh	Sheep Goats	17 306	LAT LAT	17.65 12.09	Samad et al., 1993
Bangladesh	Mymensingh	Sheep Goats	25 25	LAT LAT	40.00 32.00	Shahiduzzaman et al., 2011
Bangladesh	Rajshahi	Goats	145	ELISA	55.10	Rahman et al., 2015b
Bangladesh	Rajshahi	Sheep Goats	83 146	LAT LAT	69.90 61.00	Rahman et al., 2014b
Pakistan	Northern Punjab	Goats Sheep	419 413	ELISA ELISA	14.32 18.16	Ahmad et al., 2015
Pakistan	Mardan	Sheep Goats	290 350	IHA IHA	44.13 42.28	Shah et al., 2013
Pakistan	Punjab	Sheep Goats	100 100	LAT LAT	11.20 25.40	Ramzan et al., 2009
Pakistan	South-East Pakistan	Sheep Goats	40 58	LAT LAT	2.50 0.00	Zaki, 1995
India	Northern part	Sheep Goats	1227 961	IHA IHA	25.30 30.30	Chhabra et al., 1985
India	Punjab	Sheep	186	ELISA	3.76	Sharma et al., 2008
India	-	Sheep Goats	40 107	LAT LAT	25.00 19.60	Mirdha et al., 1998
India	-	Sheep Goats	60 63	ELISA ELISA	50.00 41.26	Singh et al., 2015
Iran	East Azerbaijan	Sheep Goats	101 85	ELISA ELISA	24.80 10.60	Moazeni Jula et al., 2013
Iran	South eastern Iran	Sheep Goats	562 778	MAT MAT	24.70 15.80	Bahrieni et al., 2008
Kuwait	-	Sheep	528	IHAT	17.80	Alazemi, 2014
Iraq	Erbil	Sheep Goats	259 88	LAT LAT	25.40 28.40	Kader and Al-Khayat, 2013
Myanmar	Central regions	Goats	119	LAT	11.40	Bawm et al., 2016
China	Yunnan	Sheep Goats	154 392	IHA IHA	17.60 9.70	Zou et al., 2015
China	Jinzhou	Sheep Goats	402 216	MAT MAT	17.90 14.80	Xu et al., 2014
Korea		Goats	610	ELISA	5.10	Jung et al., 2014
Libya	Different regions	Sheep	5806	LAT	71.0	Al-Mubarak et al., 2013.
Saudi Arabia	-	Sheep Goats	891 555	IFAT IFAT	36.40 35.30	Alanazi, 2013
Saudi Arabia	Najran	Sheep Goats	85 88	IFAT IFAT	43.50 31.80	Mosa et al., 2015
Malaysia	-	Goats	200	IFAT	35.50	Chandrawathani et al., 2008
Thailand	Satun	Goats	631	LAT	27.90	Jittapalapong et al., 2005
Indonesia	Lampung	Goats	160	LAT	47.50	Matsuo and Husin, 1996
Palestine	Northern Palestine	Goats	151	ELISA	13.40	Othman and Alzuheir, 2014

Table 2.6**: Selected studies investigating the prevalence of toxoplasmosis in small ruminants in Africa.** Different authors used different serological tools and sample size varied in each study.

Country	Regions	Animal species	Sample size (n)	Methods applied	Percent Prevalence (%)	References
Ethiopia	Central Ethiopia	Sheep Goats	305 323	DAT DAT	20.00 15.48	Gebremedhin et al., 2014
Tunisia	Southern	Sheep Goats	204 32	MAT MAT	40.20 34.50	Lahmar et al., 2015
Algeria	-	Sheep Goats	276 108	IFAT IFAT	11.59 13.21	Dechicha et al., 2015
Ethiopia	Southern and central	Goats	641	MAT	74.80	Teshale et al., 2007
Tanzania	Southern	Goats	337	LAT	19.30	Swai and Kaaya, 2012
Nigeria	Borno	Sheep Goats	372 372	ELISA ELISA	4.60 6.70	Kamani et al., 2010c
Ghana	-	Sheep Goats	732 526	ELISA ELISA	33.20 26.80	Van der Puije et al., 2000
Uganda	-	Goats	784	ELISA	31.00	Bisson et al., 2000
South Africa	Western Cape	Sheep	292	ELISA	8.00	Hammond-Aryee et al., 2015
Sudan	Khartoum	Sheep	80	LAT	57.50	Khalil and Elrayah, 2011
Libya	Different regions	Sheep	5806	LAT	71.00	Al-Mubarak et al., 2013

Table 2.7: **Selected studies investigating the prevalence of toxoplasmosis in small ruminants in Europe.** Different authors used different serological tools and sample size varied in each study.

Country	Regions	Animal species	Sample size (n)	Methods applied	Percent Prevalence	References
Italy	Lombardy	Sheep Goats	505 474	IFAT IFAT	59.30 41.70	Gazzonis et al., 2015
Italy	Tuscany	Sheep	630	IFAT	38.62	Cenci-Goga et al., 2013
Greece	-	Sheep Goats	1501 541	ELISA ELISA	48.60 30.70	Tzanidakis et al., 2012
Spain	Southern	Sheep Goats	503 494	ELISA ELISA	49.30 25.10	García-Bocanegra et al., 2012
Belgium	-	Sheep	3170	ELISA	87.40	Verhelst et al., 2014
Serbia	-	Goats	431	MAT	73.30	Djokic et al., 2014
Norway	-	Goats	2188	DAT	17.00	Stormoen et al., 2012
Turkey	Istanbul	Sheep	181	ELISA	31.00	Oncel and Vural, 2006
Bulgaria	Stara zagora	Sheep Goats	380 364	IHAT IHAT	48.20 59.80	Prelezov et al., 2008
Czech Republic	-	Goats	251	ELISA	66.00	Bartova and Sedlack, 2012
France	Haute-Vienne	Sheep	257	DAT	43.80	Dumetre et al., 2006
Poland	Pomerania	Sheep	1646	ELISA	55.90	Holec-Gasior et al., 2015
Portugal	Northern Part	Sheep Goats	119 184	MAT MAT	33.60 18.50	Lopes et al., 2013
Romania	4 historical areas	Goats	735	ELISA	52.80	Iovu et al., 2012
Finland	Nation wide	Sheep	1940	DAT	24.60	Jokelainen et al., 2010
Sweden	-	Sheep	704	ELISA	19.00	Lunden et al., 1991

Table 2.8: **Selected studies investigating the prevalence of toxoplasmosis in small ruminants in different parts of the world.** Different authors used different serological tools and sample size varied in each study.

Country	Regions	Animal species	Sample size (n)	Methods applied	Percent prevalence	References
Brazil	Ceara	Goats	2362	ELISA	25.10	Cavalcante et al., 2008
Brazil	Gurguéia microregion	Sheep Goats	374 580	ELISA ELISA	48.70 40.50	Rego et al., 2016
Brazil	Santa Catarina	Goats	654	IFA	33.02	De Moura et al., 2016
West indies	St. Kitts and Nevis	Sheep Goats	116 66	ELISA ELISA	26.00 34.00	Hamilton et al., 2015
Caribbean Island	Dominica, Grenada, Montserrat, St. Kitts and Nevis	Sheep Goats	305 442	ELISA ELISA	59.00 52.90	Hamilton et al., 2014
Mexico	Michoacán State	Goats	341	MAT	15.20	Alvarado-Esquivel et al., 2013

2.4.6 Molecular studies on toxoplasmosis in aborted animals

Several molecular studies using the different fetal tissues and placenta of aborted ovine and caprine samples have been reported from different countries (Table 2.9). Most of these studies were based on targeting the *Toxoplasma B1* gene and considered to be highly sensitive and reliable in detecting the protozoa. However few studies were based on targeting other genes of *Toxoplasma* including *18SrRNA*, *ITS-1* and *18S–5.8S ITS-1*.

Diagnosis of toxoplasmosis from different tissue stages need careful sample collection and processing steps. The success rate of *Toxoplasma* diagnosis also varies according to type of fetal tissues and placenta from where DNA will be extracted for molecular studies. However, previously published reports suggest that the most preferable samples for toxoplasmosis would be fetal brain, heart and placenta in small ruminants (Moazeni Jula et al., 2013; De Moraes et al., 2011).

Table 2.9: **Selected molecular studies on toxoplasmosis in aborted sheep and goats.** Researcher uses different target genes and tissue samples for PCR and nested PCR assays.

Country	**Animal Species**	**Target gene**	**Types of PCR**	**Type of tissue**	**Detection rate (%)**	**References**
Iran	Sheep	*B1*	Nested	Brain (37)	54.00	Danehchin et al., 2016
Iran	Sheep goats	*18SrRNA , ITS-1*	Nested	Brain (13) Cotyledon (13) Blood (34)	69.00 23.00 14.70	Moazeni Jula et al., 2013
Iran	Sheep	*B1*	Nested	Fetal tissues (18)	66.67	Habibi et al., 2012
Italy	Sheep	*ITS-1*	Nested	Placenta (142) Brain (16) Liver (3)	3.52 87.50 66.67	Chessa et al., 2014
Italy	Sheep goats	*18S–5.8S ITS1*	Nested	Fetal sample (2471) Fetal sample (362)	11.10 6.40	Masala et al., 2003
Nigeria	Sheep Goats	*18S-5.8S rRNA ITS1*	Single tube nested	Brain (76) Liver (70) Lung (76) Hearts (65) Placenta (40)	0.00 0.00 0.00 0.00 0.00	Kamani et al., 2010a
Egypt	Sheep Goats	*B1*	Nested	Sheep (8) Goats (4)	100.00 100.00	Ahmed et al., 2008
Jordan	Sheep Goats	*B1*	Simple	Fetal tissues (106) Blood (149)	29.25 30.20	Abu-Dalbouh et al., 2011
Brazil	Sheep	*B1*	Nested	heart+ Placenta Brain +Liver +Lung+ Spleen Medulla +Cerebellum	100.00 80.00 60.00	De Moraes et al., 2011
Spain	Sheep Goats	*ITS-1*	Nested	Sheep (74) Goats (26)	5.40 3.84	Moreno et al., 2012

CHAPTER-3

Study on prevalence, molecular identification and characterization of *Anaplasma* infection in cattle in selected hilly, coastal and plain areas of Chittagong Division in Bangladesh

Study on prevalence, molecular identification and characterization of *Anaplasma* infection in cattle in selected hilly, coastal and plain areas of Chittagong Division in Bangladesh

3.1 Abstract

Bovine anaplasmosis is an important haemoparasitic disease of farm animals caused by *Anaplasma* spp. Although reported from all over the world, it is common in tropical and subtropical countries including Bangladesh. The present study was conducted in selected hilly, coastal and plain areas of Bangladesh to determine the comparative prevalence and risk factors along with molecular characterization of *Anaplasma* spp. During this study, classical microscopic technique (through Giemsa stained blood smear examination) revealed a total of 8.21% cattle (n=1680) were found positive (n=138) for *A. marginale* infection. The prevalence of anaplasmosis in the hilly area was 9.33% (56 out of 600); in the coastal area it was 9.00% (54 out of 600) and in the plain area it was 5.83% (28 out of 480). Further analyses revealed that the prevalence of *Anaplasma* spp. was higher ($P>0.05$) in crossbred cattle when compared to local cattle. Considering the seasonal prevalence, the infection was significantly higher ($P<0.05$) in winter (11.09%) followed by rainy (7.13%) and summer (6.43%). Again, animals aged between 18-30 months were found to be more susceptible to anaplasmosis than other age groups. Animals kept on un-cemented floor (mati floor/ soil floor) were more prone to anaplasmosis than the animals that are kept on partially-cemented and cemented floor (Paka floor). The prevalence of anaplasmosis in this study was found somewhat higher in female animals than male animals but this was statistically insignificant. We attempted using modern molecular tools and fifty randomly selected (microscopically positive) samples were analysed by PCR. After amplification of *16SrRNA* gene, 80% (40 out of 50 samples) of the samples were confirmed as positive for *A. marginale* infections. Besides, all these 40 positive samples were further amplified using previously described primer of *MSP4* gene. Further sequencing of representative samples and phylogenetic studies revealed that all the isolates of this present study were closely related to the isolates already reported from Argentina, Australia and China.

3.2 Introduction

Livestock is an integral component of the complex farming system in Bangladesh as it is not only a source of animal protein but also a major source of draught power as well as employment (Rahman et al., 2014a). The productivity of animals in Bangladesh is greatly hampered by haemoparasitic diseases including anaplasmosis. Moreover the agro-ecological and geo climatic conditions of Bangladesh are highly favorable for growth and multiplication of different vectors likes ticks which playan important role in the transmission of the anaplasmosis (Chowdhury et al., 2006). The prevalence of anaplasmosis in cattle has been reported from different areas of Bangladesh (Alim et al., 2012; Samad et al., 1989; Siddiki et al., 2010) over the years.

Bovine anaplasmosis (BA) is an important hemoparasitic disease of cattle caused by the rickettsial organism of the genus *Anaplasma* (Order Rickettsiales, Family Anaplasmataceae) (Kocan et al., 2004). The diseases is characterized by fever, weight loss, decreased milk production, pale mucus membranes, severe anemia, jaundice, brownish urine, hyper- excitability abortion and mortality without showing any sign of hemoglobinemia and hemoglobinuria during acute form of the anaplasmosis (Richey and Palmer, 1990). Among all different species, *A. marginale* is responsible for significant economic losses in the livestock industries around the world including Bangladesh, as it confers high morbidity and mortality of animals (Kocan et al., 2003).

Epidemiological investigation, which is a prerequisite of prevention and control of a disease, largely relies on efficient diagnosis of blood parasites. There are several diagnostic methods recommended for the diagnosis of anaplasmosis such as classical Giemsa stained blood smear examination, serological tests and molecular test. In acute phase of disease the organism are easily detectable in Giemsa stained blood smear (Farias, 1995) however in carrier animals or in chronic stage it is quite difficult to detect. On the contrary, molecular techniques such as polymerase chain reaction (PCR) based approach have been developed with high sensitivity and specificity (Corona and Martinez, 2011; Palmer et al., 1986). Earlier, many researchers used PCR, targeting single or multiple species specific genes, to determine *Anaplasma* spp. In case of *A. marginale*, the amplification and detection of *16SrRNA* (Zhou et al., 2010), *heat-shock protein* (groEL) (Lew et al., 2003) and *major surface protein*

(MSP) (Molad et al., 2004) gene was found effective in previous studies. Major surface proteins (*MSP*) gene of *A. marginale* was categorized into six group based on their further characterization details (Kocan et al., 2010). Among six major surface proteins, *MSP5*, *MSP4* and *MSP1a* have been frequently used for molecular characterization of *A. marginale* (Aubry and Geale, 2011). *MSP4* genes have been used for phylogenetic studies providing information on biogeography and evolution of *Anaplasma* spp. In this experiment we used routine parasitological examination of peripheral blood along with molecular techniques (based on PCR amplification of *16s ribosomal subunit* gene and *MSP4* gene) to identify positive cases of anaplasmosis in study areas and to perform further genomic and phylogenetic analyses.

The present study was undertaken with the following objectives:

1. To determine the prevalence and risk factors of anaplasmosis in hilly, coastal and plain area of Chittagong division.
2. To achieve molecular identification and phylogenetic investigation of identified *Anaplasma* spp.

3.3 Materials and Methods

3.3.1 Description of study areas

The study was conducted in the hilly and the coastal areas of 4 different southern districts of Bangladesh. These include Chittagong Metropolitan area (the plain area), Bandarban sadar and Ruma Upazilla of Bandarban district, Rangamati sadar and Kaokhali upzilla of Rangamati district (the hilly area); Noakhali sadar Upazilla and Suburnochar upazilla of Noakhali district and Laxmipur sadar and Ramgoti upazilla of Laxmipur district (the coastal areas).

3.3.2 Study periods

The study was conducted for a period of 12 months. The field works started in January, 2013 and ended in December, 2013 and the total period was divided into viz., i) winter (November to February), ii) summer (March to June) and iii) rainy (July to October).

3.3.3 Target animals

Holstein Friesian (HF) crossbred and local cattle (Red Chittagong /Indigenous/Non-descript) were selected as target animals.

3.3.4 Target sampling

Blood samples were collected at one point of every month from each selected areas. Samples were collected from different age groups, from both sexes and from the Holstein Friesian crossbred and local cattle in three consecutive seasons. A questionnaire (Annex -1) was used to record relevant information comprising owner's name and address, animal Identification (ID), farm size, breed, age, sex, housing history and farmer's economic status etc. Farmer's economic status were categorized into viz i) Poor ii) Moderate and iii) ultra poor. Housing history was treated as floor type and categorized into paka (cemented) and mati (soil) floor.

3.3.5 Sample collection

Blood samples were collected during the study period and individual animal was considered as a sampling unit. About 3-5ml of peripheral blood was collected from the jugular vein in vials containing EDTA (7.2 mg). The vials were carried to the

Parasitology laboratory of Chittagong Veterinary and Animal Sciences University (CVASU) in a cool box and kept in refrigerator at 4°C until further use. During examining the samples, two thin blood smears were prepared and subsequently air-dried and fixed by 100% methyl alcohol for 3-5 min. The prepared thin blood smears was stained with the Giemsa stain and allowed to stay for 25-30 min following previously described methods. After rinsing with running tap water, the stained blood smears were air-dried and examined under binocular microscope (1000X) for the identification of blood parasites (Urquhart et al., 1996).

3.3.6 DNA extraction

Total genomic deoxyribonucleic acid (gDNA) was extracted from the whole blood samples by using PCI method (Barbaro et al., 2004). Briefly, the frozen blood samples were first thawed and 50µl of blood was added to a microcentrifuge tube containing 500µl of Lysis buffer (100mM Tris Hcl pH 8.5-5ml + 0.5M EDTA - 0.5ml + 10% SDS -1ml +5M NaCl-2ml). Later, 50µl of 10% SDS was added and mixed thoroughly for few min prior tothe addition of 3µl of proteinase-k. The buffers were mixed thoroughly by inverting the micocentrifuge tube for few min. Later the centrifuge tubes were incubated at 56ºC for 30 min in hot water bath followed by incubation with equal volume of PCI (Phenol: Chloroform: Isoamyl alcohol (25:24:1). The mixing was aided by vortexing for few min. After centrifugation of the mixtures for 10 min at 12,000 rpm, upper aqueous layer was carefully removed to a new sterilized microcentrifuge tube. Then an additional 500µl of Chloroform: Isoamyl alcohol (24:1) solution was added with the upper aqueous solution and mixed thoroughly by repeated inverting for few min. Further centrifugation at 12,000 rpm for 10 min wasdone followed bythe removing the upper aqueous layer in a fresh sterilized microcentrifuge tube. Chilled absolute ethanol was added to double the volume and sample was left at -20ºC for overnight for precipitation of genomic DNA. On the following day, the mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was decanted carefully to retain the pellet. To the pellet, 500µl of 70% ethanol was added and centrifuged at 13,000 rpm for 10 min and the supernatant was removed to collect the pellet. The pellet was kept for air dry under laminar air flow and later resuspended in nuclease free water and stored in -20ºC for further use.

3.3.7 DNA amplification

The diluted DNA template was transferred into PCR tube and the reaction volume contained 12.5µl of GoTaq® G2 hot start green master mix (2X Green GoTaq®Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM MgCl2), 1.5µl of each primer pairs (10 picomole), 2µl of template and 7.5µl of nuclease free water. PCR amplification was carried out using a 2720 thermal cycler (Applied Biosystems, USA). To identify the *Anaplasma* spp. from DNA samples PCR amplifications were performed at the following thermal conditions:

3.3.7.1 Amplification of *16S ribosomal sub unit* gene

An initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min. The mixture was examined for the presence of DNA fragment by loading 5µl of PCR product into each well of 1.5% agarose gel stained with Ethidium bromide. A 100bp plus DNA ladder was included into the first well. The electrophoresis was run for 30 min at 130 volts and the gel was visualized under UV illumination.

3.3.7.2 Amplification of *MSP4* gene

Only positive sample to *16S ribosomal subunit* gene were amplified by using *MSP4* gene primer and thermal profile were given as below:

An initial denaturation step at 94°C for 5 min was followed by 30 cycles at 94°C for 30s, 55.5°C for 30s and 72°C for 45s with a final extension step of 72°C for 5 min.

Table 3.1: **Details of the primers used for amplification of gene fragments of *Anaplasma* sp.**

Target gene	Primer name	Sequence	Amplicon size (bp)	References
16SrRNA	AE-F AE-R	5'-AAGCTTAACACATGCAAGTCGAA-3' 5'-AGTCACTGA CCCAACCTTAAATG-3'	1406	Oh et al., 2009
MSP4	AM-F AM-R	5'-TTGTTTACAGGGGGCCTGTC-3' 5'-GAACAGGAATCTTGCTCCAAG-3'	831	Ahmadi-Hamedani et al., 2009

3.3.8 PCR products purification

Amplified DNA products after each PCR run was purified using commercial PCR purification Kit (Favorgen, Korea®). The procedures followed were as described by the manufacturer. Briefly, with 40µl of PCR product 5 volumes of FADF buffer (supplied with the kit) was added and mixed thoroughly by vortexing. The mixture was then transferred to a FADF column and centrifuged for 1 min and the flow through was discarded. Now 750µl of wash buffer (supplied) was added to the column and later centrifuged for 1 min. After discarding the flowthrough the column was centrifuged again for 3 min to dry and placing on to a new microcentrifuge tube. Forty microlitre of elution buffer (10 mM Tris-HCl, pH 8.5) was then added to the column and after incubating at room temparature for 2 min the column was centrifuged for 2 min to collect the eluted DNA. The purified DNA was then measured by fluorimeter for concentration (µg/µl) before sending for DNA sequencing through commercial sources.

3.3.9 DNA Sequencing

Purified PCR products were sent for sequencing by commercial suppliers (Bioneer Corp, South Korea) Sanger sequencing methods.

3.3.10 Phylogenetic analyses

Once the sequences were available from the suppliers, the sequences were initially checked using a BLAST sequence similarity search through NCBI (the National Center for Biotechnology Information: http://blast.ncbi.nlm.nih.gov/Blast.cgi) website. By the BLASTN homology search the nucleotide sequences were determined that is corresponding with *A. marginale* sequences published in GenBank. The multiple alignment analysis was performed using the Clustal W program (Thompson et al., 1994) while the phylogenetic analysis was performed by Neighbor joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) using the MEGA software, version-5 (Tamura et al., 2011). The tree stability was estimated by a bootstrap analysis for 1,000 replications (Felsenstein, 1985).

3.3.11 Statistical analysis

The obtained information was imported, stored and coded accordingly using Microsoft Excel-2003 to STATA/IC-11.0 (Stata Corporation College Station, TX, USA) for analysis. Descriptive statistics was expressed as proportion with confidence interval. The results were expressed in percentage with P-value for chi-square test. Significance was determined when $P<0.05$.

3.4 Results

3.4.1 Parasitological examination

The present study was designed to analyze samples from different geographic locations in southern Bangladeshi Division, Chittagong. A total of 1680 whole blood samples (600 from each of hilly and coastal areas and 480 from plain areas) were collected from cattle randomly irrespective of the clinical signs. Using conventional blood smear examination, it was observed that 138 were found positive (out of 1680 cattle) for *A. marginale* infection with a prevalence rate of 8.21%. Organisms in blood smears appeared as spherical dot like bodies located in periphery of the infected RBCs (Fig-3.1). Further observation based on origin of samples indicated considerable variations among the prevalence of anaplasmosis in different geographic areas (Fig-3.2). The prevalence of anaplasmosis in the hilly area was 9.33% (n= 600) while in the coastal areas, it was 9.00% (n= 600) and in the plain areas 5.83% (n= 480). However these variations indicated no significant differences in these observations.

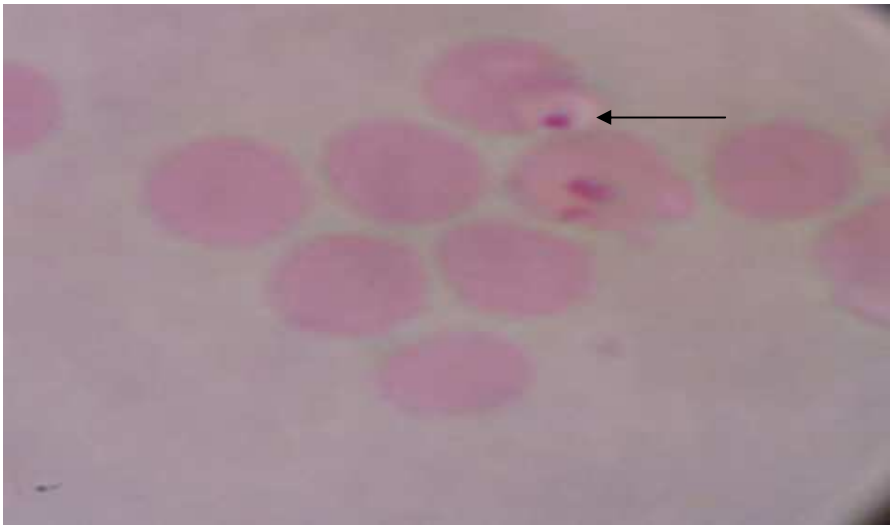

Fig 3.1: **Representative figure from Giemsa- stained thin blood smear** indicating *A. marginale* located inside the RBCs (Arrow) X100.

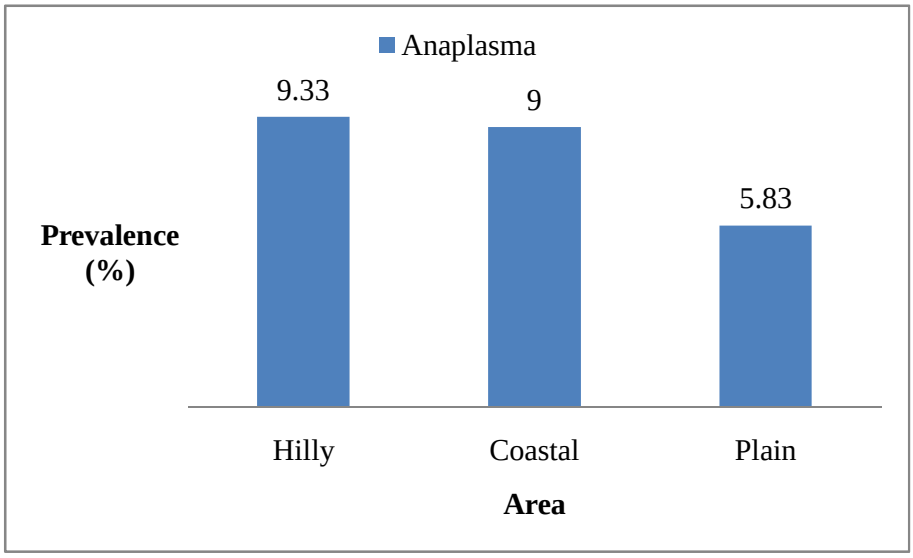

Fig 3.2: **Area-wise prevalence of anaplasmosis** (Here X axis indicates the study areas and Y axis indicate the prevalence). The prevalence was observed higher in hilly area in compared to coastal and plain area.

The study also attempted to identify the association of different categorical variables such as season, breed, age, sex; cattle shed floor category and economic capacity of the animal owners with the incidence of anaplasmosis (Table 3.2). The data analyses revealed higher ($P>0.05$) prevalence of *Anaplasma* spp. in crossbred cattle when compared to local cattle. Considering the seasonal occurrence, the disease was found significantly higher ($P<0.05$) in winter compared to rainy and summer seasons. When considering the age of animal, it was found that most of the *Anaplasma* infection occurred in age of 18-30 month (Fig. 3.3). Animals kept on un-cemented floor (soil type/ mati floor) seem to be more susceptible to anaplasmosis than the animals that were kept on partially-cemented and cemented floor (Paka floor). Again, female animals were found more susceptible than male counterparts but the difference was not statistically significant. No specific significant difference was found based on animal owner`s economic condition that may relate to the variable prevalence of anaplasmosis in three different groups.

Table 3.2: **Association of different categorical variables with the incidence of anaplasmosis (by using Chi-square test),** where significantly higher prevalence was recorded in winter season.

Variables	**Categories (N)**	**Prevalence**
Season	Summer (560)	6.43 (36)
	Rainy (561)	7.13 (40)
	Winter (559)	11.09 (62)***
Sex	Male (400)	6.50 (26)
	Female (1278)	8.75 (112)
Economic condition	Poor (523)	8.41 (44)
	Moderate (1087)	8.28 (90)
	Ultra poor (70)	5.71 (4)
Floor	Paka (1011)	7.42 (75)
	Mati (669)	9.42 (63)
Breed	Cross (455)	9.23 (42)
	Local (1225)	7.84 (96)

*** Significance at P<0.01

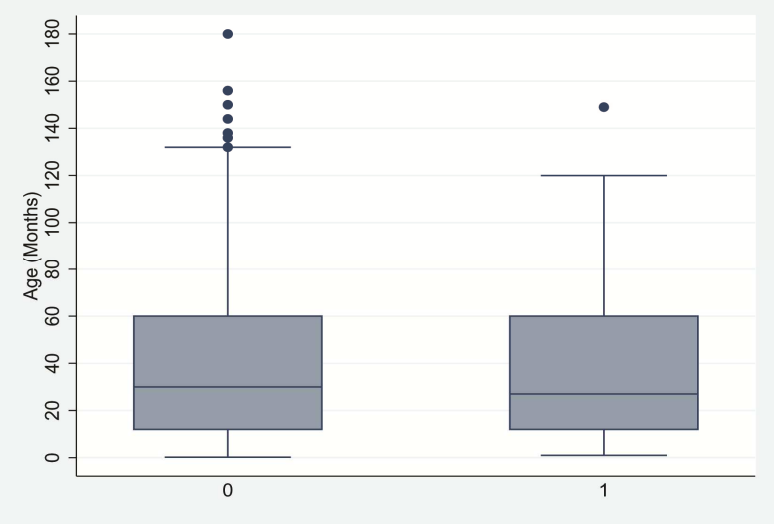

Fig 3.3: **Box plot showing association between anaplasmosis with age [**where zero "0" indicate negative and "1" indicate positive to anaplasmosis. The box plot indicates that 50% of infected animals were within the age group of 18-30 months].

3.4.2 Molecular examination

Molecular techniques such as PCR were applied to randomly select 50 positive animals to parasitological examination based on the *16SrRNA* of *Anaplasma* gene. The positive sample on agar gel electrophoresis was given a clear band of 1406 bp (fig 3.4). Out of 50 samples 40 were found positive to molecular examination and give an incidence rate of 80%. Again molecular techniques applied to all positive samples to *16SrRNA* using *MSP4* gene amplification and gives positive result on 831bp (fig 3.5).

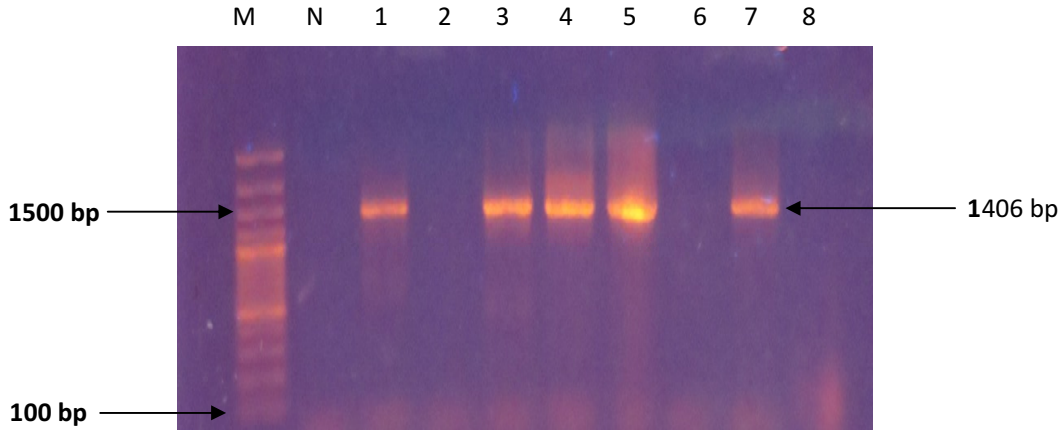

Fig 3.4: **Amplification of the genomic DNA of *A. marginale* from blood of cattle by using *16SrRNA* gene**. Lane M is for 100 bp plus DNA ladder; N is for negative control; Lanes 1-8 is suspected samples; Lanes 1, 3, 4, 5, and 7 having amplicons of ~ 1406 bp indicated presence of *A. marginale* organisms.

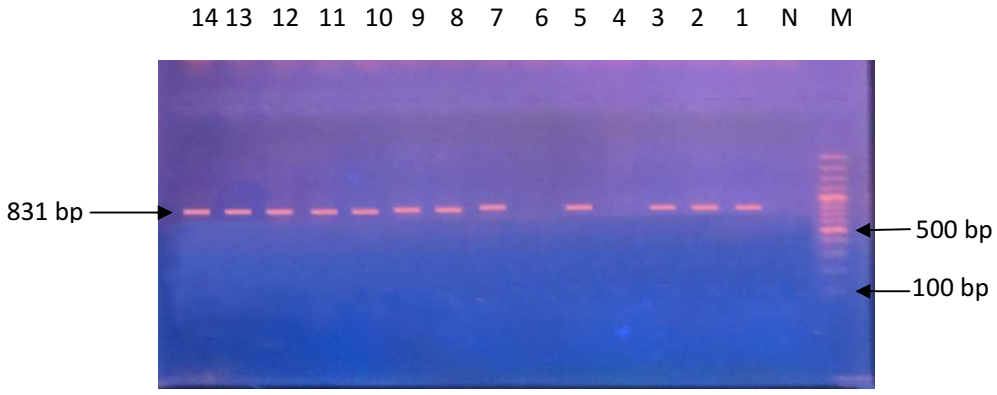

Fig 3.5: **Amplification of the genomic DNA of *A. marginale* from blood of cattle by using *MSP4* gene.** Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-14 is suspected samples; Lanes 1, 2, 3, 5, 7-14 having amplicons of 831 bp indicated presence of *A. marginale* organisms.

3.4.3 Phylogenetic analyses

A phylogenetic tree inferred based on *MSP4* gene sequences of *A. marginale* isolates are shown in Fig. 3.6. The *MSP4* nucleotide sequences of *A. marginale* were compared with other known sequences published in GenBank, and revealing 99% identities to *A. marginale* isolates from Australia (AY665999), Mexico (JN564651), Argentina (AF428086), Venezuela (AF030059), China (HM 640938), Brazil (JN022562), Taiwan (EU677383), Israel(AY787172), Italy (DQ000620), Tunisia (KJ512174), Nigeria (EU106082), Zimbabwe (AY666009), North America (AY010250) and Spain (AY456001). In the phylogenetic analysis, CVASU *A. marginale* isolates showed a separate branch but cluster together with *A. marginale* strains originating from Argentina, Australia, Mexico, Venezuela and China.

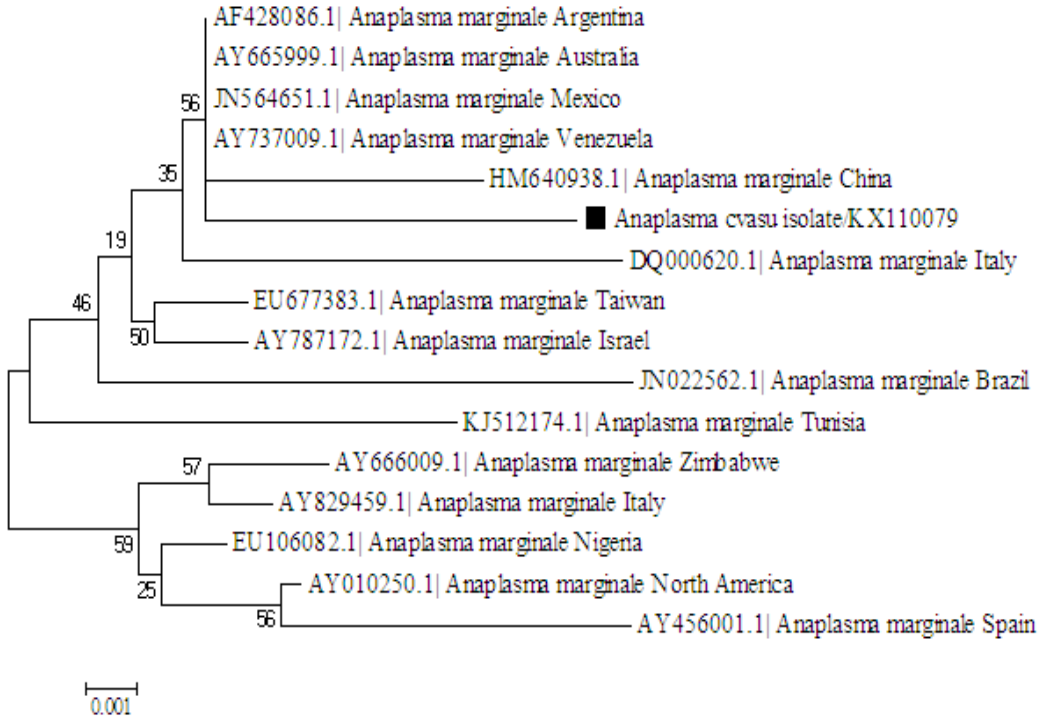

Fig 3.6: **Phylogenetic tree constructed using nucleotide sequences of *Anaplasma marginale MSP4* gene fragment and all relevant sequences of this gene submitted in GenBank.** The accession numbers and countries are shown before isolate name. The fragment of *MSP4* gene sequences obtained in this study were indicated with bold triangle. The tree was inferred using the neighbor joining method of MEGA 5.2, bootstrap values are shown at each branch point. Number next to the branch demonstrates bootstrap support from 1000 replications. All sites of the alignment containing insertions-deletions, missing datawere eliminated from the analysis (option "complete deletion").

3.5 Discussion

Anaplasmosis is one of the important rickettsial diseases of cattle reported from all around the world. Researchers have tried to identify various contributing factors associated with the prevalence of this important hemoparasitic and vector-borne disease. Diagnostics tools mostly employed in developing countries like Bangladesh to detect anaplasmosis mainly include staining peripheral blood samples after preparation of thin blood smear (microscopy). With the advancement of modern DNA based diagnostic tools it is now hightime to employ state-of-the-art procedures like PCR for comprehensive molecular investigation. During the present study, classical microscopy technique was complemented by PCR amplification of two different genes of *Anaplasma* spp. and further validation of species was confirmed by DNA sequence analyses and phylogenetics.

In this study, the overall prevalence of anaplasmosis was recorded as 8.21% (n=1680), which is lower than the findings (33-70%) of other previous studies (Chowdhury et al., 2006; Samad et al., 1989; Nath and Bhuyian., 2013). It may be due to the differences in geographical locations of studies conducted. Again, the current prevalence rate was little higher than the findings of some investigators who had different sample sizes (Alim et al., 2012), (Siddiki et al., 2010), (Rahman et al., 2015a) and (Sajid et al., 2014). Very early reports indicated the occurrence of subclinical *Anaplasma* infection in 5.93% cattle has been reported from Bangladesh (Samad et al., 1989). A somewhat similar observation (9.71% prevalence) was reported later by other researchers in neighboring Pakistan (Atif et al., 2012). The variations of the results could be due to random selection of apparently healthy animals rather than the clinically suspected animals. Moreover geographical variation play vital role in the prevalence of anaplasmosis inside Bangladesh.

During this study, the highest prevalence of anaplasmosis was recorded in hilly areas (9.33%) followed by coastal areas (9.00%) and plain areas (5.83%). The prevalence of anaplasmosis in the hilly areas was not consistent with previous other published report who recorded 14.94% of prevalence of anaplasmosis in three hilly district of Chittagong (Mohanta and Mondal, 2013). However a far lower prevalence rate (3.7%) was recorded in indigenous cattle of Khagrachori hilly district in Chittagong by a separate report (Alim et al., 2012).

Present study was unable to find any statistically significant differences among male and female animals infected with *Anaplasma* spp. Still, the percent prevalence was found to be higher in female animals compared to the male animals. This was consistent with the observation by other investigators (Alim et al., 2012) and (Atif et al., 2012). The suppression of immunity in advanced pregnancy due to physiological change and or huge energy loss in lactation in high yielding animals might be the possible reasons for the higher prevalence of anaplasmosis in female cattle (Kocan et al., 2010). In addition, some do believe that high incidence in female could be linked with the fact that contaminated needles are commonly used for injecting drugs for milk let down (Atif et al., 2012).

The present study indicates that animals aged 18-30 months are more susceptible to anaplasmosis. This observation is in consistent with previous report (Rahman et al., 2015a) who recorded high prevalence in animals aged between 12-30 months. However one report in another part of Bangladesh indicated 2-3 years older animals to be more vulnerable to anaplasmosis (Atif et al., 2012) while two other group indicated adult animals are more susceptible than younger animals (Alim et al., 2012; Chowdhury et al., 2006).

Seasonal variation in disease incidence is considered to be one of the most influencing factors on the prevalence of blood parasitic diseases. This is largely due to environmental factors that regulate tick population in the farm or adjacent areas. Besides tick vectors, some biting insects and mechanical instruments also play roles in transmission of the disease. During the present study no meteorological data (such as temperature, rainfall, humidity etc.) were collected and analyzed. However, we attempted to identify the variations in prevalence of the protozoan diseases throughout different seasons. The prevalence of anaplasmosis was found to be significantly higher in winter season followed by rainy and summer season. Similar seasonal variation was observed by previous investigators who reported higher incidence in winter season in cattle in northern Sylhet district of Bangladesh (Nath and Bhuyian, 2013). However, on the contrary to this observation, in southern Chittagong district of Bangladesh couple of other studies has been reported where higher prevalence was found in both rainy and summer season (Alim et al., 2012; Belal et al., 2015). All these variations are thought to be due to changes in macroclimate that is essential for breeding of ticks (Vairamuthu et al., 2012). Moreover contaminated fomites and some

biting insects (flies) are also capable of transmission of the disease and the prevalence of insect probably higher in late winter to rainy season.

The breed of animal is thought to have some impact in the incidence of bovine anaplasmosis. High incidence of anaplasmosis in crossbred cattle was recorded during this study compared to the indigenous or local cattle. However this was not statistically significant. The finding was consistent with the observation by other investigators who used animal samples from different other parts of Bangladesh (Alim et al., 2012; Atif et al., 2012; Chowdhury et al., 2006; Rahman et al., 2015a). The literature suggests that *Bos taurus* are more likely to develop acute anaplasmosis than crossbred Zebu cattle (Aguirre et al., 1987). However, experimentally infected local and crossbred cattle were reported to be equally susceptible to *A. marginale* and developed similar clinical signs (Bock et al., 1997). This might be due to the lack of immunity during high milk yielding stage in crossbred along with characteristic genetic makeup and unusual seasonal stress.

The housing and floor type in animal house was considered as an important factor related to incidence of anaplasmosis in animals. High incidence was recorded in conventional soil type farm houses compared to animals that were kept in cemented (paka) floor with better management options. Similar finding was recorded by other researchers who suggested that the variation could be due to higher vector load in muddy floor and improper use of insecticide (Nath and Bhuyian, 2013; Sajid et al., 2014). This also can be linked with the financial condition of the farm owner where most poor farmers are unable to spend more to build a cemented floor.

The present study is the first attempt of molecular study for detection of *Anaplasma* spp. in cattle in Bangladesh. Although selective samples screened through microscopy were further analysed for molecular study 80% of samples (40 Out of 50) were found positive in PCR assay based on amplification of partial *16SrRNA* gene. Remaining negative samples might include false positive cases where parasitic stages are frequently confused with other microscopic structures (inside RBCs) such as Heinz bodies, Howell-Jolly bodies or staining artifacts. However, notable that further PCR assay of *MSP4* gene fragments also successfully validate the PCR results as both the genes were amplified in same sample sets. Further validation of the genus was

confirmed by DNA sequencing followed by sequence-similarity based BLASTn search through NCBI website.

The *MSP4* gene-specific primer pair routinely used for identification of *A. marginale* was used during this study. The *MSP4* gene is highly conserved among different strains of *A. marginale* and also between different *Anaplasma* spp. (De La Fuente et al., 2005a, b). Phylogenetic analyses of the *MSP4* gene fragment sequences demonstrated that the Bangladeshi *A. marginale* isolates shares a separate branch but clustered with those from other countries. They were also positioned closer to the isolates from China, Argentina, Australia and Mexico as supported by low bootstrap value that indicates that there is a huge genetic variation among them.

3.6 Conclusion

The present study attempted to investigate the prevalence of anaplasmosis in cattle along with identification of possible epidemiological factors of disease incidence. A high overall prevalence rate (8.21%) was found during the study. While babesiosis was also sreened in the same area (as described in Chapter 4 of this thesis) with a overall prevalence of 1.43%, it is clear that anaplasmosis is more important in the study areas.

We also analyzed the questionnaire based data for the identification of risk factors of anaplasmosis in the study areas. Significant relationship was found with time of the year and the disease prevalence while age or breeds were not found to be the risk factors.

Molecular studies were also performed which is first of its type to diagnose the bovine anaplasmosis in Bangladesh. This was achived by modern PCR based assay where two specific genes (*16S rRNA* and *MSP4*) were partially amplified followed by DNA sequencing. Further bioinformatics analysis and phylogenetic investigations revealed that isolate of the study was closely related to the isolates reported from countries like China, Argentina and Mexico.

In the light of current study it will recommended for the farmers of the study areas to use acaricide and regular strategic prophylactic treatment especially in the winter season for tick control as well as disease control.

Further studies can be concentrated on identification of tick vectors along with molecular detection of organisms from both vectors and hosts from all different districts of the country. This will ultimately develop national database of vector and hosts and will help policymakers to develop suitable control strategies to combat bovine anaplasmosis.

CHAPTER-4

Study on prevalence, molecular identification and characterization of *Babesia* infection in cattle in selected hilly, coastal and plain areas of Chittagong Division of Bangladesh

Study on prevalence, molecular identification and characterization of *Babesia* infection in cattle in selected hilly, coastal and plain areas of Chittagong Division of Bangladesh

4.1 Abstract

Bovine babesiosis is the most economically important haemoprotozoan disease caused by *Babesia* spp. The present study was conducted in selected hilly, coastal and plain areas of Bangladesh to determine the prevalence and identification of risk factors along with molecular characterization of *Babesia* spp. Using classical blood smear examination and Giemsa staining, 1.43% (n=1680) cattle was found positive for *Babesia* infection during this study. The prevalence of babesiosis in the hilly areas was 1.17% (7 out of 600 cases); in the coastal areas 1.67% (10 out of 600 cases) and in the plain areas 1.46% (7 out of 480 cases). Further data analyses based on the predetermined questionnaire revealed additional epidemiological information. The prevalence of *Babesia* spp. was found higher ($P>0.05$) in crossbred cattle when compared with the local or indigenous group of cattle. Seasonal variation of disease prevalence indicated that babesiosis was significantly higher ($P<0.05$) in summer season followed by rainy and winter season. Animals at the age of 6-18 months were found more susceptible to the infection based on collected samples from three different geographic areas of Bangladesh. Animals kept on natural soil-type floor (mati floor) were found to be more affected by babesiosis than the animals that are kept on partially-cemented and cemented floor (Paka floor). No statistical difference was found between male and female animals indicating no variation in prevalence due to sex of the animals. Further molecular studies through PCR and DNA sequencing of all microscopically positive samples (by amplification of *18SrRNA* gene) confirmed the genus and their phylogenetic relationship. Two distinct clusters were found through phylogenetic analyses of which one was closely related to *Babesia ovata* and another cluster was closely related to *Babesia bigemina*. Further study using samples from other different parts of Bangladesh and using different genes for PCR assay can highlight the actual evolutionary origin of *Babesia* spp. affecting cattle in the country. The knowledge will ultimately help develop effective control strategies which mostly depend on vector control and farm management.

4.2 Introduction

Bovine babesiosis is an economically important vector borne disease of cattle caused by the blood protozoa of the genus *Babesia* (McCosker, 1981). The clinical form of the disease is characterized by anemia, fever, hemoglobinuria, jaundice, abortion in female animals and occasional nervous symptoms and death. Among different species of *Babesia* that affect the cattle, *B. bovis* and *B. bigemina* are the most economically important species globally. The economic losses comprise of mortality, loss of milk/meat production, associated costs of control measures etc. However the disease can make significant impact on the international cattle trade for any country exporting meat and meat products (Bock et al., 2004).

Reliable and accurate diagnosis is important for effective monitoring, treatment and control of babesiosis (Bashir et al., 2009). The routine diagnosis is usually accomplished by routine microscopic examination (Giemsa or Wrights stained) of blood smears collected from the peripheral circulation. However, the classical staining techniques are suitable for detection of acute and heavy infections but unable to identify sub-clinical infections where the parasitemia is usually much lower (Almería et al., 2001; Aziz et al., 2014). Different serological techniques such as IFAT and ELISA are commonly used for the diagnosisof subclinical infection of babesiosis (Bock et al., 2004; Molloy et al., 1998). One of the major limitations of these techniques is the occurrence of false positive and false negative results due to cross-reactions (Sharma et al., 2013) and the lack of discrimination between previous exposure and current infections (Wagner et al., 1992). The application of molecular tests such as PCR based tools have been therefore developed which has high sensitivity and specificity (Almería et al., 2001). The technique has been proven to provide reliable results in detection of *Babesia* spp. in blood, particularly when the parasitemia is very low or not detectable in microscopic examination (Figueroa et al., 1992).

In Bangladesh, the prevalence of babesiosis in cattle has been reported in different areas by some investigators. Most of the published studies were based on the classical blood smear examination (Giemsa stain) and the prevalence was recorded as 1-14% with a variable sample size (Samad et al., 1989; Siddiki et al., 2010). Those reports also investigated the risk factors associated with *Babesia* prevalence in Bangladesh

based on age, sex, climate and seasons. The present study was designed to get further comprehensive investigation to investigate the prevalence of babesiosis at different locations of Chittagong Division with special emphasis on molecular characterization of the organism for the first time in the country. This is the first attempt to identify specific species and strains of *Babesia* through PCR based tools and their further sequencing and phylogenetic investigation.

The present study was conducted with the following specific objectives:-

1. To determine the prevalence and risk factors of babesiosis in cattle in selected areas of Bangladesh.
2. Molecular identification and characterization of *Babesia* spp. along with the phylogenetic investigations.

4.3 Materials and Methods

4.3.1 Description of the study areas

The study area of this experiment was same and described in section 3.3.1.

4.3.2 Study periods

The study periods were similar as described in section 3.3.2.

4.3.3 Target animals

Target animals of this study were similar as described in section 3.3.3.

4.3.4 Target sampling

Target animals were similar as mentioned in section 3.3.4.

4.3.5 DNA extraction

The protocol for DNA extraction was followed as mentioned in the PCI method and the protocols have been briefly described in section 3.3.6 earlier.

4.3.6 PCR assay

The diluted DNA template was transferred into PCR tube and the reaction volume composes of 12.5µl of GoTaq® G2 hot start green master mix (2X Green GoTaq® reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM MgCl2), 1.5µl of each primer (10 picomole), 2µl of template and 7.5µl of nuclease free water. PCR amplification was carried out using a 2720 thermal cycler (Applied Biosystems). To identify the *Babesia* spp. from DNA samples PCR amplifications were performed at the following thermal conditions: 94ºC for 2 min followed by 35 cycles of 94ºC for 30 sec, 61ºC for 45 sec, 72ºC for 1 min and followed the final extension step at 72ºC for 10 min. The mixture was examined for the presence of DNA fragment by loading 5µl of PCR product into each well of 1.5% agar rose gel stained with Ethidium bromide. A 100 bp DNA ladder was included into the first well. The samples were fun for 30 min at 130 volts and then finally washed under running tape water and visualized under UV illumination.

Table-4.1: **Details of the primers used for amplification of gene fragments of *Babesia* sp.**

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
18S rRNA	Baf-F Baf-R	5' -TTTCTGMCCCATCAGCTTGAC -3' 5'- CAAGACAAAAGTCTGCTTGAAA -3'	422-440	Hilpertshauser et al., 2006

4.3.7 PCR products purification

The protocol for PCR product purification was followed as mentioned in the manufacturer's instruction. The commercially available PCR purification kit (Favorgen, Korea®) was used and protocol was mentioned earlier in section 3.3.8.

4.3.8 DNA sequencing

Purified PCR products were used for sequencing by commercial suppliers (Bioneer Corp, South Korea) for DNA sequencing.

4.3.9 Phylogenetic analyses

The phylogenetic analyses of partial sequence of *18SrRNA* gene of *Babesia* organism according to the protocol that was mentioned in section 3.3.10.

4.3.10 Statistical analyses

The statistical analyses of epidemiological data were carried out by following similar methodology which was mentioned earlier in section 3.3.11.

4.4 Results

4.4.1 Parasitological examination

The present study was designed to investigate the prevalence of babesiosis using modern molecular tools. However, classical microscopic technique was used for screening field samples before molecular tests were performed. Altogether, 1680 whole blood samples (600 from hilly areas, 600 from coastal areas and 480 from plain areas of Chittagong Division) were collected from cattle randomly without considering the clinical signs. On classical blood smear examination (Giemsa staining technique), 1.43% animals (n=1680) were found positive for *Babesia* spp. infection where the organisms in thin blood smears appeared as pear-shaped bodies usually located in periphery of the infected RBCs (Fig 4.1). As expected, a substantial variation in prevalence of babesiosis in different geographic areas was observed in this study (Fig 4.2). The prevalence of babesiosis in the hilly area was 1.17% (7 out of 600); in the coastal areas 1.67% (10 out of 600) and in the plain areas 1.46% (7 out of 480). However these were not statistically significant.

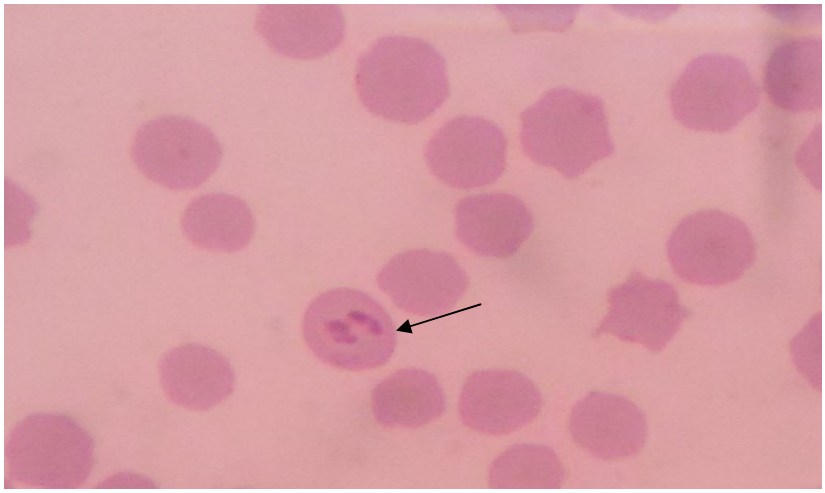

Fig 4.1: **Representative figure from Giemsa stained thin blood smear** indicating *Babesia* spp. located inside the RBCs (Arrow) × 100.

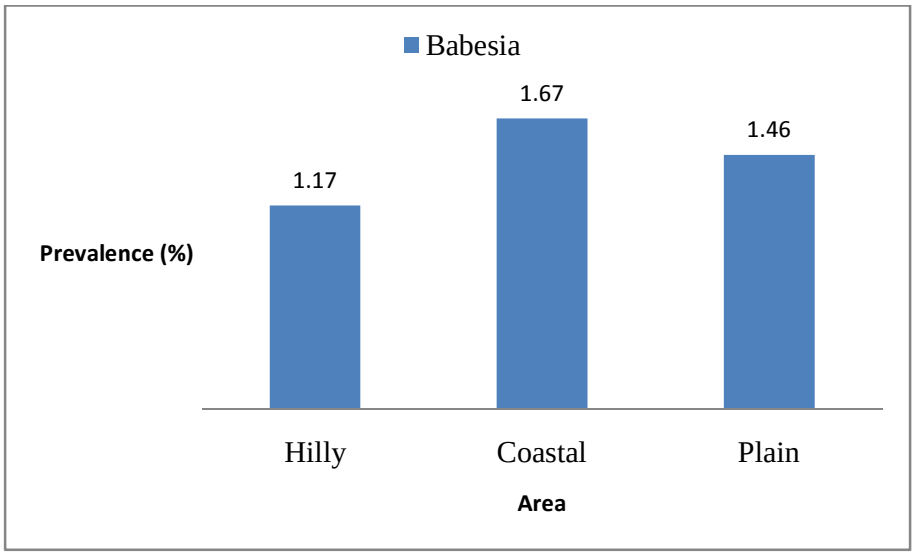

Fig 4.2: **Area wise prevalence of *Babesia*** (Here X axis indicates the areas and Y axis indicate the prevalence. Higher prevalence was recorded in coastal area in compared to hilly and plain area).

During this study an attempt was made to investigate the association of different categorical variables such as season, breed, age, sex, floor type at cattle sheds and economic condition of the animal-owners with the prevalence of babesiosis (Table 4.2). As predicted, crossbred cattle were highly susceptible to babesiosis compared to local or indigenous cattle. Animals were more affected in summer ($P<0.05$) followed by rainy and winter seasons. Cattle aged between 6-18 months were more prone to suffer from the disease compared to other age groups (Fig 4.3). Animals kept on soil-type floor (mati floor) were most susceptible to babesiosis followed by those on partially-cemented and cemented floor (Paka floor). No significant differences were found between male and female animals or the economic status of the animal owners with the relative prevalence of babesiosis.

Table 4.2: **Association of different categorical variables with the incidence of babesiosis (by using Chi-square test).** The prevalence was significantly higher in summer season.

Variables	Categories (N)	Prevalence (%)
Season	Summer (560)	2.50 (14)***
	Rainy (561)	1.25 (7)
	Winter (559)	0.54 (3)
Sex	Male (400)	1.00 (4)
	Female (1280)	1.56 (20)
Economic condition	Poor (523)	1.72 (9)
	Moderate (1087)	1.38 (15)
	Ultra poor (70)	0.00 (0)
Floor	Paka (1011)	1.09 (11)
	Mati (669)	1.94 (13)
Breed	Cross (455)	1.53 (7)
	Local (1225)	1.39 (17)

*** Significance at P<0.001.

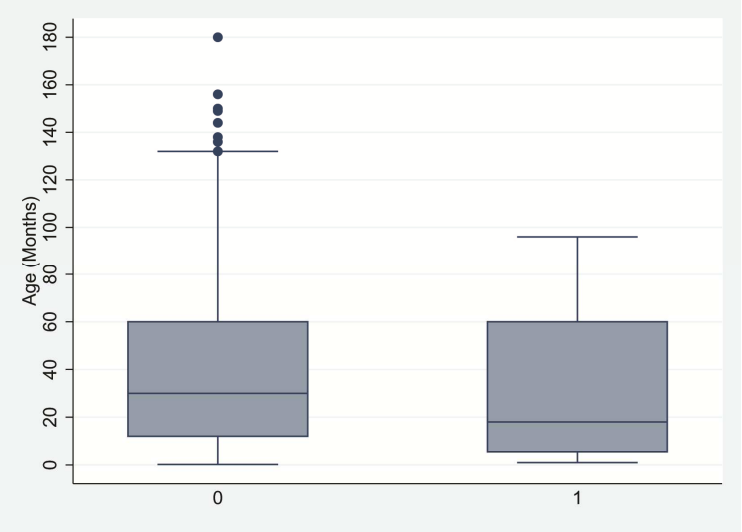

Fig 4.3: **Box plot showing association between babesiosis with age** [where zero"0" indicate negative and "1" indicate positive to babesiosis. The analyses indicate that 50% of infected animals were within the age group of 6-18 months].

4.4.2 Molecular examination

Further molecular investigation using PCR based analyses were applied to all 24 positive samples (verified by microscopic) examination based on the amplification of *18SrRNA* gene. All these amplicons produced characteristic bands of 421-440 bp on

agar gel electrophoresis (Fig 4.4). Thereby PCR assay complemented our findings from classical thin blood smear technique.

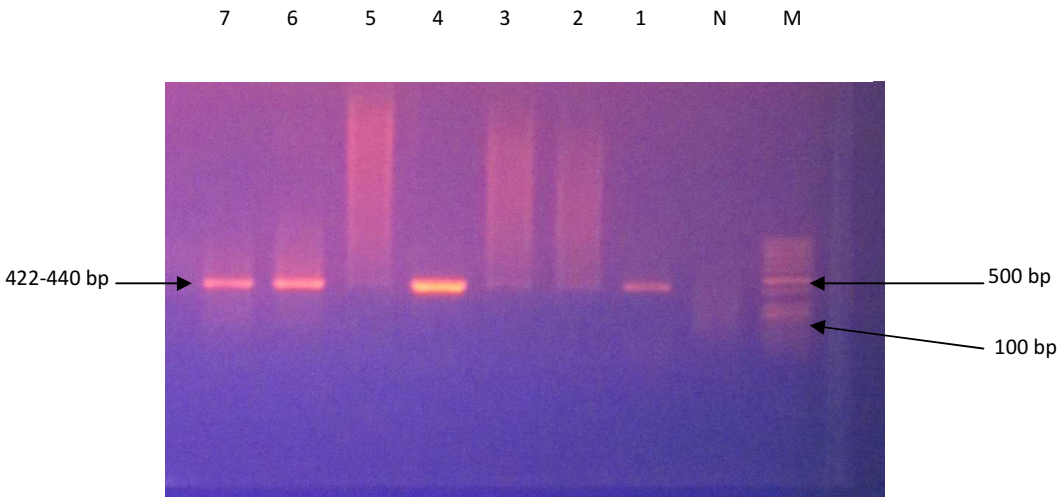

Fig 4.4: **Amplification of the genomic DNA of *Babesia* sp. from blood of cattle by using *18SrRNA* gene.** Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-7 is suspected samples; Lanes 1, 4, 6 and 7 having amplicons of 422~440 bp indicated the presence of *Babesia* organisms.

4.4.3 Phylogenetic analyses

A phylogenetic tree inferred based on *18SrRNA*gene sequences of *Babesia* spp. isolates are shown in Fig. 4.5. The *18SrRNA* nucleotide sequences of *Babesia* spp. obtained from six randomly selected samples (designed as bab1, bab2, bab3, bab4, bab5 and bab6) shared 100% identity when compared bab1 to bab3 and bab6; 99% identity when compare bab1 to bab2; 95% identity when compared bab1 to bab5 and 91% identity when compared bab4. The sequences (bab1, bab2, bab3, bab5 and bab6) were also compared with other known sequences published in GenBank, and revealing 97% identities to *B. ovata* isolates from China (AY603400 and AY603403) and Korea (AY081192). On the other hand, sequence bab4 were revealing 94% identities to *B. bigemina* isolates from India, China, Brazil and Australia.

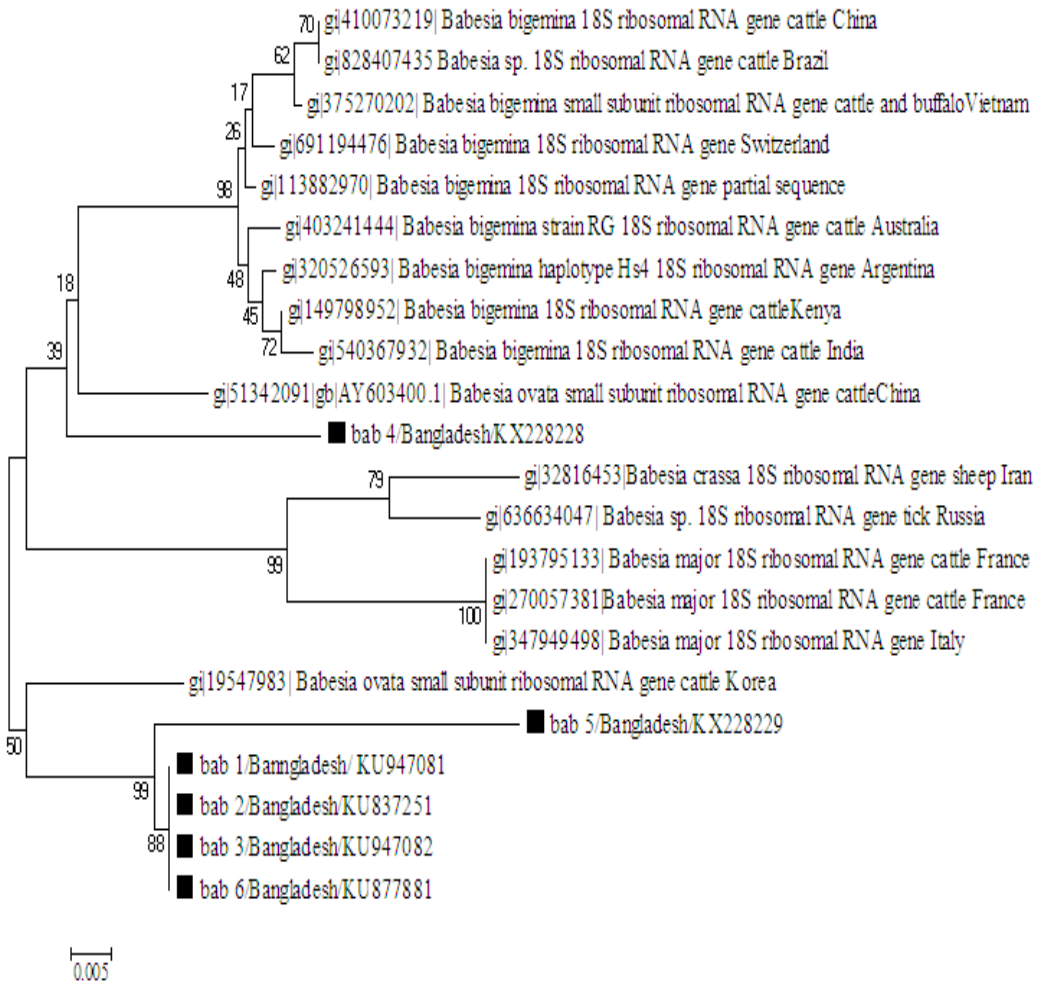

Fig 4.5: **Phylogenetic tree generated from the nucleotide sequences of *Babesia 18SrRNA*gene and all relevantsequences of this gene submitted in GenBank,** the accession numbers and countries are shown with isolate names. The *18SrRNA* sequences obtained in this study were indicated with bold triangle. The tree was inferred using the neighbor joining method of MEGA 5.2, bootstrap values are shown at each branch point. Number next to the branch demonstrates bootstrap support from 1000 replications. All sites of the alignment containing insertions-deletions, missing data were eliminated from the analysis (option “complete deletion”).

4.5 Discussion

Babesiosis is known to be an important protozoan disease causing huge economic loss in livestock sector of Bangladesh. Previously, a few reports were published on this disease in cattle showing a variable rate of prevalence (1-16.63%) in some selected areas of Bangladesh (Siddiki et al., 2010; Mohanta and Mondal, 2013). Unfortunately, in all cases, researchers used conventional tools of diagnosis which have significant drawbacks in terms of accuracy. The study is therefore complementing other previous reports and highlights the need for further molecular investigation with a view to formulate effective control strategies.

The overall prevalence of babesiosis recorded during this study was 1.43% (n=1680). This observation was consistent with other investigator who has reported 1.52% to 2.29% prevalence in different parts of the country (Al Mahmud et al., 2015; Rahman et al., 2015a; Siddiki et al., 2010). In neighboring Pakistan, the prevalence was recorded as 1.75% for *B. bigemina* which is the most common species affecting the cattle population (Afridi and Ahmad, 2005). Compared to this reports involving Bangladeshi cattle have indicated higher prevalence ranging from 3.28% (in subclinical cases) to 7.17% in some areas (Alim et al., 2012; Samad et al., 1989). Further higher prevalence of upto 16% was also reported by other reports where samples were collected from northern districts of Bangladesh (such as Mymensingh and Sylhet) (Banerjee et al., 1983; Nath and Bhuyian., 2013). One would assume that these variations in the prevalence of babesiosis may be due to the climate-associated factors and the distribution of relevant vector ticks who might be active and available in different seasons.

During this study, maximum prevalence of babesiosis was recorded in coastal areas (1.67%) followed by plain areas (1.46%) and hilly areas (1.17%). These were not consistent with other previous reports who recorded as high as 16.67% prevalence in three hilly district of Chittagong and Khagrachori hilly districts in Chittagong (Alim et al., 2012; Mohanta and Mondal, 2013). Again, the prevalence of babesiosis in coastal areas were reported as high as 4.62% (in Noakhali coastal area of Bangladesh) by previous investigators which is not consistent with this present study (Alim et al., 2012).

No significant differences were recorded among male and female animals with the incidence of babesiosis. The similar observation was reported earlier by other investigators (Al Mahmud et al., 2015; Alim et al., 2012; Kamani et al., 2010b). Animals aged 6-18 months were found to be more susceptible compared to other age groups. Similar observation was reported by other researchers who mentioned high prevalence in animals aged >1-2.5 years (Chakraborti, 2002; Chowdhury et al., 2006; Rahman et al., 2015a). However severity of the disease increased with ageing, but the infection rate were detected higher in young animals. It may be due to declining of immunity with the increasing of age (Wright, 1990).

Season is one of the most influencing factors on the prevalence of blood parasitic diseases. During the present study, the prevalence of babesiosis was significantly higher in summer seasons followed by rainy and winter seasons. Similar seasonal influence was observed by other researcher (Al Mahmud et al., 2015; Alim et al., 2012) who reported higher prevalence in summer season in some northern parts of Bangladesh. These seasonal influences could be linked to the variations in vector load which depends on the humidity, rainfall and overall climatic condition (Radostits et al., 2006). Further analyses of meteorological data and the geographic information of the study areas in Bangladesh can provide substantial information which was not conducted during this study.

The prevalence of babesiosis in this study was comparatively higher in crossbred cattle than local cattle although the observations were statistically insignificant. The findings were similar to the observation by previous investigators (Al Mahmud et al., 2015; Alim et al., 2012; Chowdhury et al., 2006). The zebu cattle, *B. indicus* reportedlyshow strong innate resistance to *B. bovis* and *B. bigemina* infections when compared to *B. taurus* and their crosses (Bock et al., 1997). The lower prevalence in local cattle might be linked to constant minimum exposure to infections and development of passive immunity along with the genetic makeup of each different cattle breed (Siddiki et al., 2010).

When farm housing conditions were assessed, the prevalence of babesiosis was recorded higher in animals that are kept in uncemented (mati) floor in compared to animals that were kept in cemented (paka) floor. Similar finding was observed earlier

and thought to be linked with higher vector load in muddy floor (Nath and Bhuyian, 2013).

Along with classical thin blood smear and Giemsa staining technique, molecular techniques were applied during this study. All the positive samples (from microscopy) were successfully amplified by PCR assay using specific primers of *Babesia 18SrRNA* gene. Further sequencing confirmed the genus and species of the protozoa which were analysed for phylogenetics. The analyses revealed that five isolate are separately branched but clustered together with *Babesia ovata* which was not previously reported in Bangladesh (Alim et al., 2012; Samad et al., 1989). Notable that all those reports in Bangladesh were based on morphological examination and accurate identification was not always possible based on microscopy. Earlier, *Babesia ovata* were reported in Japan (Minami and Ishihara, 1980), China (Bai et al., 1990), South Korea (Suh, 1987) and the present report is the first record of this species in Bangladesh. All the five isolates from the present study are clustered together with *Babesia ovata* isolates recorded from Korea. The bab 4 isolate are separately branches in phylogenetic tree and form cluster with *B. bigemina* which also show highest nucleotide identity as found through BLASTn analyses. This bab 4 isolates cluster together with the *B. bigemina* isolates of India, China and Vietnam with very short branch length indicating little genetic diversity among the species.

The results from the present study (Partial sequencing of *18SrRNA* gene) indicate that both *B. bigemina* and *B. ovata* are present in Bangladeshi cattle sporadically. Further comprehensive study with wide sample number and geographic areas is essential to understand the molecular epidemiology of this important hemoparasite.

4.6 Conclusion

Present study was aimed to investigate the prevalence of babesiosis in Bangladeshi cattle along with identification of epidemiological factors of the disease incidence. Microscopic examination was complemented with molecular analyses and babesiosis was detected from different geographic areas of Bangladesh. A number of risk factors including age, sex, season, housing condition etc., were assessed with variable results. Seasonal incidence and prevalence was found significant while age and sex of animals, farm floor type were not found to be related to occurrence of the bovine babesiosis. Further analyses with more sensitive and reliable diagnostic tools like PCR assay with subsequent sequencing and phylogenetics revealed novel species *B. ovata* as the causal agent in some animals.

The present study successfully characterized the prevailing Babesia sp including *B. bigemina* from study areas. This is supported by previous several reports indicating the availability of tick vectors such as *Rhipicephalus appendiculatus, R. sanguineus, Haemaphysalis bispinosa* and *Boophilus microplus*.

The study indicates that regular strategic prophylactic treatment and use of acaricides should be ensured especially in summer season in order to the control of babesiosis and other vector borne parasitic diseases.

The present study was first molecular investigation of cattle babesiosis in Bangladesh. The phylogenetic analyses successfully categorized the isolates in three different clades indicating existence of genetic diversity. Further comprehensive study with large geographic areas and increased number of samples is therefore essential to understand the actual extent of babesiosis in the country. The vector biology, geography and other epidemiological information highlight the significance of babesiosis and its control.

CHAPTER-5

Study on prevalence, molecular identification and characterization of *Trypanosoma* infection in cattle in Chittagong Metropolitan area, Bangladesh

Study on prevalence, molecular identification and characterization of *Trypanosoma* infection in cattle in Chittagong Metropolitan area, Bangladesh

5.1 Abstract

Trypanosomiasis is an important haemoparasitic disease of animals including cattle. The present study attempted to ascertain the prevalence of trypanosomiasis and relevant risk factors associated with the disease. All the samples for this study were collected from different dairy farms inside Chittagong Metropolitan Area (CMA) under Chittagong district of Bangladesh. The preliminary screening was aided by conventional thick smear technique (Giemsa stain) while modern molecular tools like PCR was used for accurate and reliable identification and characterization. Only three samples (out of 480 samples tested) were found positive through thick blood smear and PCR assay. All these three samples were from Holstein Friesian crossbred cow and none of the local indigenous breed was found positive. Data analyses revealed highest prevalence of trypanosomiasis in rainy season when compared to summer and winter season. Animals older than six years were found to be infected by trypanosomiasis. PCR assay was applied to all three screened positive samples by amplifying gene fragment of *18SrRNA* gene and were successfully amplified. Further sequencing of PCR products and phylogenetic analyses revealed that the isolates recovered during this study were closely related to the isolates previously reported from Egypt, Taiwan, Thailand and Japan.

5.2 Introduction

Trypanosomiasis is a chronic devastating diseases that affect human and all worm-blooded animals and caused by flagellated protozoans of the genus *Trypanosoma* (Laohasinnarong et al., 2011). It has a great economic importance in the livestock industry as it causes high mortality and severe production losses in cattle. Cattle infected with trypanosome result in chronic debilitating, emaciating with fatal outcome which is characterized by the high fever, anemia, jaundice and abortion in female animals. Trypanosomiasis not only caused the direct loss in the form of mortality, morbidity, infertility of the infected animals and costs of treatment or controlling the disease but also caused the indirect losses through exclusion of livestock and animal power from the huge fertile tsetse infected areas (Kidane-Mariam, 2000). There are several species of trypanosomes in livestock which includes *Trypanosoma brucei*, *T. vivax*, *T. congolense*, *T. evansi* and *T. theileri* (Garcia et al., 2011a; Nantulya, 1990; Sekoni et al., 1988; Soulsby, 1982). Several species of Tsetse fly play the major role in the the transmission of the disease (Ford and Katondo, 1977).

Different techniques are used for the detection of trypanosomes from the blood. Although parasitological approach remains the most appropriate method for the clinical diagnosis in field condition, it lacks sensitivity and specificity (Thumbi et al., 2008). Serological tests such as complement fixation test (CFT), indirect fluorescent antibody test (IFAT), card agglutination test (CAT) and enzyme linked immunosorbent assay (ELISA) are not reliable for differentiating current or post treatment infections (Ahmed et al., 2013; Luckins, 1977). Now, these techniques are being replaced by modern molecular diagnostic tools which have high sensitivity and specificity (Malele et al., 2003; Solano et al., 1999). For molecular analysis, various target sequences such as *kinetoplast DNA, ribosomal DNA, internal transcribed spacer region* and *VSG* genes are recommended for the detection of *Trypanosoma* spp. (Sengupta et al., 2010).

Though livestock contribute in national economy of Bangladesh, its development is hindered by different constraints. The most important constraints of livestock sectors are widespread diseases, including trypanosomiasis. Moreover the climatic condition of Bangladesh favor the growth and multiplication of vector and uncontrolled cattle

trading systems also play role in disease production. Until now, a number of epidemiological and parasitological investigations were carried out on commonly available blood parasites in different regions of Bangladesh. However, no molecular investigation of haemoprotozoan diseases especially trypanosomes were reported. Earlier report on prevalence (5 out of 857 cattle) of *T. theileri* was the very first of its type where microscopic identification was performed (Rahman et al., 1982; Samad and Shahid-Ullah, 1985). The present study was conducted with the following objectives:

1. To determine the prevalence and identification of risk factors associated with trypanosomiasis.
2. Molecular identification and characterization of *Trypanosoma* spp. with subsequent phylogenetic investigation.

5.3 Materials and Methods

5.3.1 Study area

The study was conducted in the selected farms located in Chittagong metropolitan area of Bangladesh. The survey was done to the randomly selected commercial dairy farms as well as individually reared animals.

5.3.2 Study periods

The study was conducted during January, 2013 till December, 2013 and the total period was divided into three different seasonal categories such as i) winter (November to February), ii) summer (March to June) and iii) rainy (July to October).

5.3.3 Target animals

Holstein Friesian (HF) crossbred and local cattle (Red Chittagong breed /Indigenous/Non-descript types of cattle) were selected as study animals.

5.3.4 Target sampling

In each month blood samples were collected from different selected areas at random basis. Samples were collected from different age group, from both sex and from the Holstein Friesian crossbred and local cattle in three consecutive seasons. A questionnaire (Annex-1) was used to record information like owner's name and address, animal Identification (ID), breed, age, sex, season, housing history and farmer's status etc. Farmer's status were categorized into viz i) Poor ii) Moderate and iii) ultra-poor. Housing history was treated as floor type and categorized into paka and mati floor.

5.3.5 Sample collection, preservation and examination

Biological samples (blood) were collected during the study period and an individual animal was considered as a sampling unit. About 3-5ml of blood was collected from the jugular vein in an EDTA vial. After that the tube was kept in a cool box and then transferred in to the Parasitology laboratory, Chittagong Veterinary and Animal Sciences University (CVASU). Examination was done immediately after coming into the Parasitology laboratory and rest of the blood samples were stored at -20°C until

required for molecular processing. Three wet smear and two thick smears were made. Wet blood smear were examined at low power (10X). To observe the Trypanosome, two thick blood smears were prepared and subsequently they were air dried and fixed by 100% methyl alcohol for 3-5 min. The prepared thick blood smears (Hendrix and Robinson, 2006) was stained with the Giemsa stain for 25-30 min. After rinsing with water, the stained blood smears were air dried and examined under binocular microscope (1000X) with immersion oil for the identification of blood parasites (Soulsby, 1982; Urquhart et al., 1996).

5.3.6 DNA extraction

The protocol for DNA extraction was followed as mentioned in the PCI method and the protocols have been briefly described in section 3.3.6 earlier.

5.3.7 PCR assay

The diluted DNA template was transferred into PCR tube and the reaction volume composes of 10µl master mix (Taq DNA polymerase, dNTPs, MgCl2 and reaction buffer, Promega®, catalog # M7122), 1µl (20 Pico mole) of each primer, 2µl template and 6 µl deionized water. PCR amplification was carried out using a thermal cycler. The samples were programmed to a temperature step cycles at 94^0C for 4 min (min) initial denaturation followed by 40 cycles consisted of 60s at 94^0C, 90s at 58^0C and 120s at 72^0C. Five microliter volume of each sample was electrophoreses in 1.5% agar rose gel with 1X TAE buffer (40mM Tris, 20mM Acetate and 1mM EDTA with pH around 8.6). The gel was immersed in Ethidium bromide solution for 30 min. In case of semi nested PCR, 2µl of amplified products from the first run was added to the PCR tube as a template and other ingredients was same except for primer pair. The amplification programme was same to the first run of PCR except for 25 cycles.

The mixture was examined for the presence of DNA fragment by loading 5µl of PCR product into each well of 1.5% agar rose gel stained with Ethidium bromide. A 100 bp DNA ladder was included into the first well. The samples were run for 30 min at 130 volts and then finally washed under running tape water and visualized under UV illumination.

Table 5.1: **Details of the primers used for amplification of gene fragments of *Trypanosoma* sp.**

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
18S rRNA	18 ST n F2 18 ST n R3	5'- CAACGATGACACCCATGAATTGGGGA-3' 5'- TGCGCGACCAATAATTGCAATAC-3'	700 – 800	Gaysen et al., 2003
18S rRNA	18 ST n F2 18 ST n R2	5'- CAACGATGACACCCATGAATTGGGGA-3' 5'- GTGTCTTGTTCTCACTGACATTGTAGTG-3'	600 – 700	

5.3.8 PCR products purification

The protocol for PCR product purification was followed as mentioned in the manufacturers instruction. The commercially available PCR purification kit (Favorgen, Korea®) was used and the protocols have been briefly described in section 3.3.8 earlier.

5.3.9 DNA Sequencing

Purified PCR products were sent for sequencing by commercial suppliers (Bioneer Corp, South Korea) by Sanger sequencing method.

5.3.10 Phylogenetic analyses

The phylogenetic analyses of partial sequence of *18SrRNA* gene of *Trypanosoma* organism according to the protocol that was mentioned in section 3.3.10.

5.3.11 Statistical analyses

The statistical analyses of epidemiological data were carried out by following similar methodology which was mentioned earlier in section 3.3.11.

5.4 Results

5.4.1 Parasitological examination

The study was conducted in selected farms in Chittagong with a view to identify trypanosome parasites. After initial screening by microscopy, only three samples were found positive (n=480) which was further confirmed by molecular investigation using PCR assay and subsequent DNA sequencing. The characteristic morphological features of the trypanosomes were validated by taking photographs. Several organisms were found in each microscopic field, under high power objective (Fig.5.1). The organism appeared leaf-like with a single flagellum under oil immersion. Further data analyses also revealed epidemiological information which was not available in Bangladesh context earlier. No statistically significant differences between different age groups, however all three affected animals were older and more than six years of age (Table 5.2). The prevalence of trypanosomiasis was only recorded in crossbred cattle. The prevalence of trypanosomiasis was recorded in animals that kept in paka (cemented floor) where as no infections were recorded in animals that kept in mati floor (uncemented floor). The management of the farm (from where positive cases were found) was not satisfactory as found by poor drainage system and high incidence of flies that might be responsible as vectors of the protozoa.

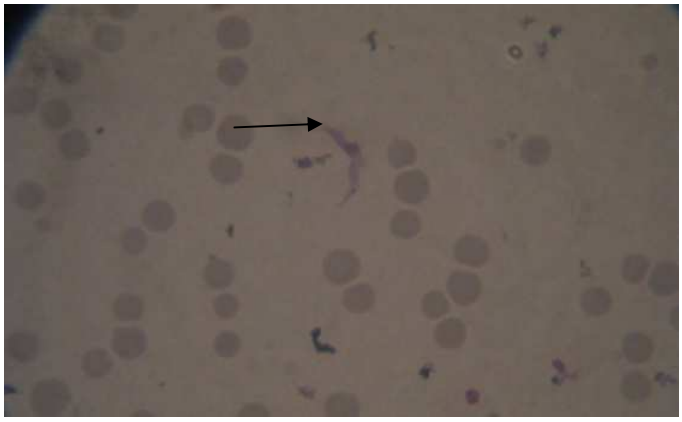

Fig 5.1: **Snapshot from Giemsa stained thin blood smear indicating *Trypanosoma* sp.** located outside the RBCs (Arrow) × 100

Table 5.2: **Association of different categorical variables with the incidence trypanosomiasis (Chi-square test),** all positive caseswas detected in rainy season.

Factors	**Factor level**	**Prevalence (%)**
Breed	Cross (309)	0.97 (3)
	Local (171)	0.00 (0)
Season	Summer (160)	0.00 (0)
	Rainy (160)	1.88 (3)*
	Winter (160)	0.00 (0)
Sex	Male (91)	0.00 (0)
	Female (389)	0.78 (3)
Floor category	Paka (395)	0.76 (3)
	Mati (85)	0.00 (0)
Farmers economic condition	Poor (146)	0.00 (0)
	Moderate (272)	1.10 (3)
	Ultra poor (62)	0.00 (0)

*Significance at P<0.05

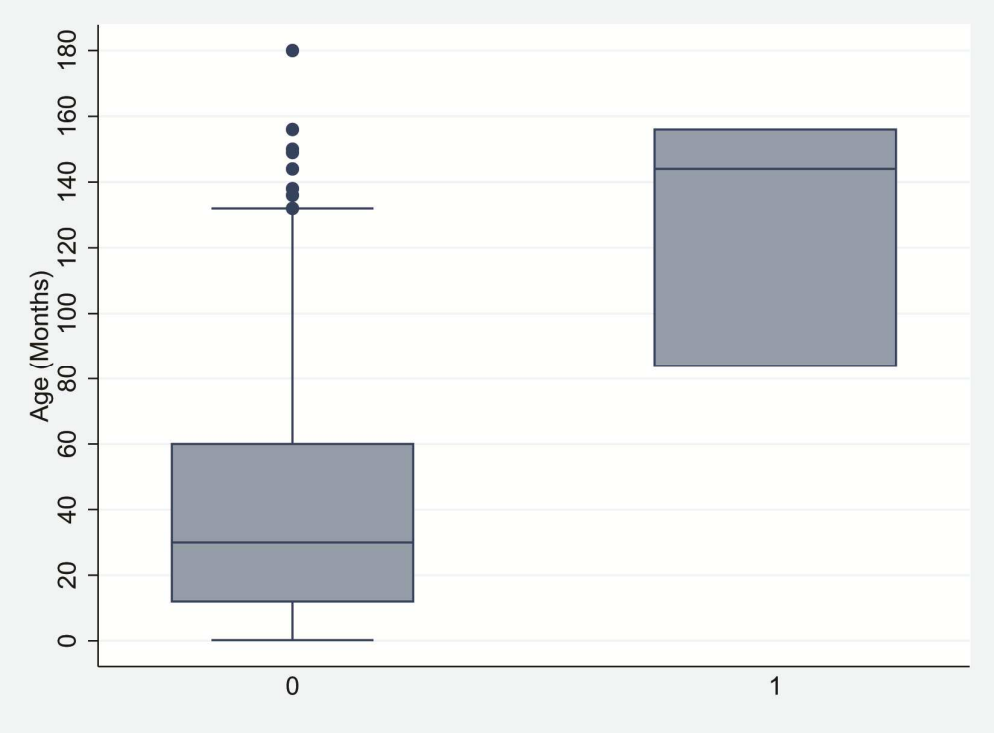

Fig 5.2: **Box plot showing association between trypanosomiasis with age** [where zero "0" indicate negative and "1" indicate positive to trypanosomiasis. Analyses indicate that 50% of infected animals were within the age group of 82-142 months].

5.4.2 Molecular examination

The PCR assay confirmed the parasite as *Trypanosoma*. The PCR amplification using the first run primer pair gave trypanosome DNA tested a major product between 700 and 800 bp (Fig 5.3), as predicted from the GenBankTM sequences. Second run amplification gave clear major amplicons between 600 and 700 bp with small size differences among most trypanosome species.

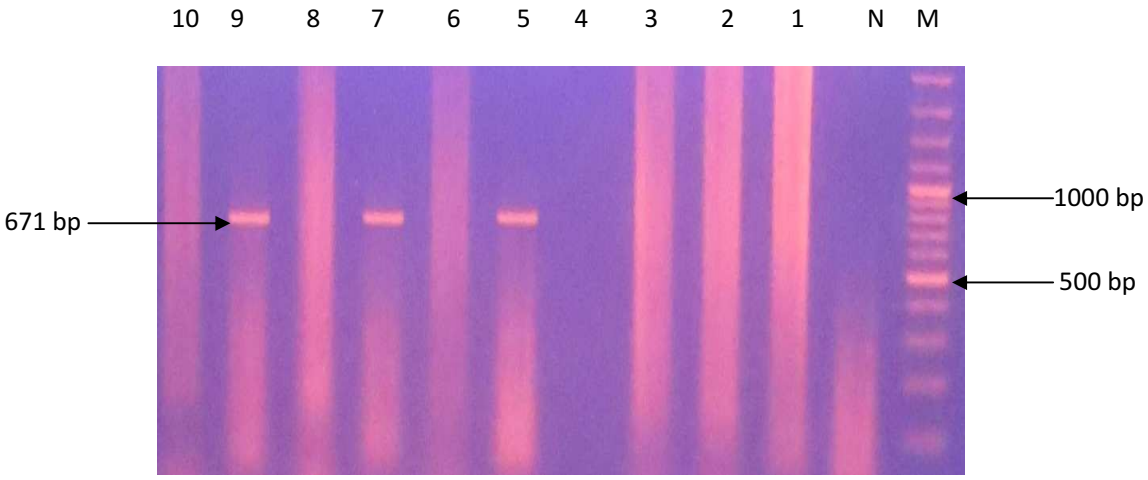

Fig. 5.3: **Amplification of the genomic DNA of *Trypanosoma* sp. from blood of cattle by using *18SrRNA* gene.** Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-9 is suspected samples; Lanes 5, 7 and 9 having amplicons of 671 bp indicating the presence of *Trypanosoma* specific gene fragment.

5.4.3 Phylogenetic analyses

A phylogenetic tree inferred based on *18SrRNA* gene sequences of *Trypanosoma* spp. isolates are shown in Fig. 5.4. The *18SrRNA* nucleotide sequences of *Trypanosoma* spp.were compared with other known sequences published in GenBank, and revealing 99% identities to Thailand (AY912269) and 97% identities to *Trypanosoma* spp. isolates from Egypt (AB551921 and AB551922), Thailand (AY904050), Taiwan (D89527) and Japan (AB301937). In the phylogenetic analysis, CVASU *Trypnosoma evansi* isolates (KC675213) showed a separate branch but cluster together with *Trypanosoma evansi* strains originating from Thailand, Egypt, Taiwan and Japan.

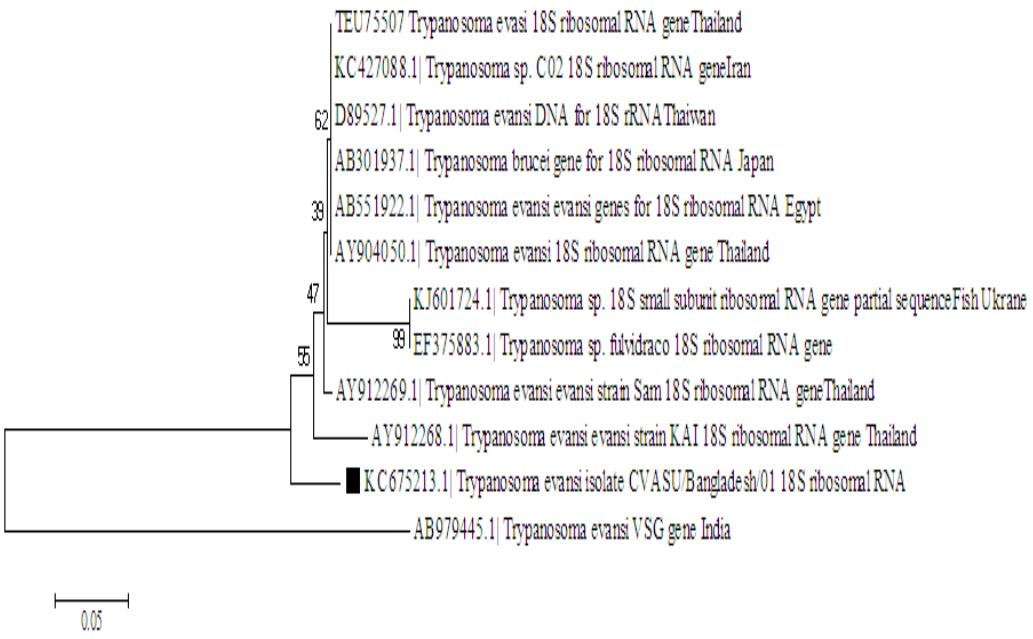

Fig 5.4: **Phylogenetic tree constructed using nucleotide sequences of *Trypanosoma* spp. *18SrRNA* gene and relevantsequences of this gene submitted in GenBank,** the accession numbers and countries are shown beside isolate names. The *18SrRNA* sequences obtained in this study were indicated with accession number KC 675213. The tree was inferred using the neighbor joining method of MEGA 5.2, bootstrap values are shown at each branch point. Number next to the branch demonstrates bootstrap support from 1000 replications. All sites of the alignment containing insertions-deletions, missing data were eliminated from the analysis (option "complete deletion").

5.5 Discussion

Trypanosomiasis is a devastating disease of cattle with potential zoonotic significance in some parts of the world. Sustainable control strategies against trypanosomiasis require an organized epidemiological study and molecular studies can complement existing microscopic studies based on staining followed by classical smear of peripheral bloods. With little studies reported to date on trypanosomiasis in the country, present study was based on identification and characterization of trypanosome parasites using both these approaches. After initial screening by conventional microscopy samples were further analysed by molecular tools like PCR, DNA sequencing and phylogenetics etc. To our knowledge this is the first molecular level study of these important protozoa involving samples from Bangladesh. However several reports have already published in neighboring India and Pakistan where trypanosomiasis is reported more frequently.

An overall prevalence of bovine trypanosomiasis in Chittagong region was recorded as 0.63%, during this study which was consistent with the previous findings by other researchers who reported 0.58% prevalence in north western part of the country (Rahman et al., 1982; Samad and Shahid-Ullah, 1985). The low prevalence of trypanosomes are thought to be due to variation of parasitic stages in peripheral blood as sample collection time could impact the microscopic test as a whole. Another reason of low prevalence may be due to the availability and frequent use of trypanocidal drug such as Berenil® (Intervet), Babcop® (Square, Bangladesh) which is available throughout the country.

Although we did have enough focus on vectors in this but it was observed that tabanid flies were present in the farm areas where positive case were detected. It is generally known that the distribution of arthropod-borne diseases is greatly associated with the availability and abundance of its vector host. The arthropods fly involved in the transmission of *Trypanosoma evansi* in the Bangladesh are still not reported but this study indicated that it may be one of the vectors of trypanosomiasis of cattle in Bangladesh.

The three animals affected with trypanosomiasis were older than 6 years. This might not indicate any relationship of age of animals with the susceptibility. However similar findings were reported by other investigator elsewhere where cattle of 2-4

years of age were found affected (Baticados et al., 2012). Another study by other research group documented a high susceptibility of cattle to trypanosomes at the age of >5 years (Tasew and Duguma, 2012). One would assume that this age related susceptibility might be linked with lowered immunity of adult animals and other stressors such persistence of antibodies following treatment, chronic nature of infection and intermittent parasitaemia, stress, poor management, draught etc.

Infection was also recorded in cow during this study. This does not mean that male animals are less susceptible to trypanosomiasis. These variations may be linked with livestock management system in the farming animals, in which large numbers of males are removed and sold at any early age; the rest being used either for breeding. Another factors of higher prevalence in adult females might be due to pregnancy and lactation, which may reduce resistance in female camels and render them more susceptible to infection (Bhutto et al., 2010).

Climate associated factors are significant for transmission of trypanosomiasis. The prevalence was significantly higher in rainy season in compared to summer and winter season. Similar results were observed by many other investigators (Agarwal et al., 2003; Krishnappa et al., 2002; Rani et al., 2015). The disease was encountered more after the onset of monsoons due to high prevalence of flies from July to November with a peak from August to October (Prasad et al., 1997). This is therefore essential to understand fly biology to efficiently control the infection to prevent further outbreak.

Although, erosion on different body parts of the animals are very common in cemented or partially cemented floor which subsequently attracting the flies and increasing the risk of the trypanosomiasis. But in this study we did not found any significant association between the floor type and the prevalence of the diseases.

Further sensitive molecular tools, PCR were applied for molecular detection of the organism. Although only positive case of microscopically examination was subjected to molecular techniques in this studies despite all three positive cases of microscopic screening techniques were also detected in molecular techniques. However PCR is sensitive enough to detect even one trypanosome per ml of blood (Masiga et al., 1996; Solano et al., 1999). PCR was also suitable for tracing of carrier animals and

provides a quantitative validated measure that is beneficial for epidemiological survey and the following of drug treatment in animals.

Trypanosomes were basically identified by its morphological structure and host competency prior to the advent of modern genetic methods. Identification of different molecular markers and their uses for more precise identification approaches for this organism (Garcia et al., 2011b; Auty et al., 2012). Molecular tools including phylogenetic studies of trypanosomes and the integration of genetic information and morphological characters has helped to better our understanding of evolutionary and taxonomic relationships (Hamilton et al., 2004; Garcia et al., 2011b).

The amplified DNA from PCR analyses was subjected to sequencing through commercial sources. The sequence data confirm the presence of *Trypanosoma* spp. as inferred from sequence similarity based NCBI BLAST searching. Bioinformatics analyses revealed that the isolate of this study showed 97% identity with *T. evansi*. The phylogenetic tree was prepared based on the partial sequence of *18SrRNA* gene and by using neighbor joining methods indicating that the isolates reported from this experiment given a separate branch but clustered together with the isolates reported from Japan, Thailand and Taiwan. The phylogenetic tree showed the evolutionary relationship of the sequences in which the length of the horizontal line was proportional to the estimated genetic distance between the sequences. This suggests that considerable polymorphism occurred within the species although originating from different geographic regions. Again, only *T. theileri* has been recorded in Bangladesh by previous investigators through morphological studies (Rahman et al., 1982; Samad and Shahid-Ullah, 1985). On the basis of phylogeny we identified the protozoan as *T. evansi* during this study which is yet to be reported from cattle in Bangladesh.

5.6 Conclusion

Trypanosoma is one of the least known protozoa affecting dometic ruminants in Bangladesh. To our knowledge this is the first report of its incidence in Chittagong region of Bangladesh. Earlier two separate reports have been published although no molecular studies were employed.

Only three positive cases were recorded during this study where all were cows aged more than 6 years. All cases were detected in rainy season and farms were located in areas frequented by cattles imported from neighboring India and Myanmar.

The sequence analysis and subsequent phylogenetic investigation successfully identified the protozoa, *Trypanosoma* spp. which were closely relate to *Trypanosoma evansi*. This was the first report of trypanosome from Chittagong region and third from Bangladesh.

Hence, the necessary attention should be given (especially during the rainy season) to this disease so as to improve livestock production and subsequent agricultural development in Chittagong area.

Further study regarding epidemiological aspects and use of sensitive detection techniques is highly recommended to determine the prevalence and risk factors of trypanosomiasis in the country. A complete understanding of the fly vectors, their distribution, farm biosecurity records etc. is essential to determine different associated risk factors.

CHAPTER-6

Sero-epidemiological study of toxoplasmosis in small ruminants in Chittagong Metropolitan area of Bangladesh

Sero-epidemiological study of toxoplasmosis in small ruminants in Chittagong Metropolitan area of Bangladesh

6.1 Abstract

Toxoplasmosis is an important zoonotic disease that affects all worm blooded animals including sheep and goats. The current study was undertaken involving blood samples from small ruminants (sheep and goats) to estimate the sero-prevalence of toxoplasmosis along with identification of associated risk factors using indirect enzyme linked immunosorbent assay (ELISA) techniques. The overall seroprevalence of toxoplasmosis was recorded as 35.87% (n=184) where the prevalence in goat (41.30%) was little higher than that of the sheep (30.43%). The prevalence of toxoplasmosis was significantly ($P<0.05$) higher in the age group of >2 years (50.56%) compared to the younger age group of 1- 2 years (28.12%) and <1year (17.86%). The prevalence of toxoplasmosis was significantly higher in animals having previous history of abortion (67.86%) when compared to animals that had no abortion (30.13%) history bygone. The prevalence was significantly higher in animals having municipal supplied (WASA) drinking water and pond water in compared to animals that were offered river and deep tube well water. Further investigation to correlate management issues like disposal techniques of placenta was found not a significant issue with the higher prevalence of disease. The study highlighted the need for further in-depth sero-epidemiological studies to assess the impact of toxoplasmosis in small ruminants and risk of transmission of toxoplasmosis in humans as zoonoses.

6.2 Introduction

Toxoplasma gondii is an obligate intracellular protozoan that causes anthropozoonic diseases in man and animals (Kasper, 2005). This protozoan is capable of infecting all worm-blooded and some cold-blooded animals worldwide. It is thought that a third population of the planet is infected with *T. gondii* which is ubiquitous (Tenter et al., 2000). Human can get infection through ingestion of contaminated food and water (through cysts and oocysts) (Ferguson, 2009). Cats play an important role in the spreading of the disease as a final host (Dubey, 2009). Toxoplasmosis has significant impact on both medical and veterinary aspect along with significance on small ruminant farming (Moreno et al., 2012). It is not only responsible factor for abortion and stillbirth in small ruminants but also responsible for transmission of the diseases to human via contaminated meat and milk (Dubey, 1996; Ghoneim et al., 2009). Moreover toxoplasmosis has detrimental effects on health and productive performance of does and ewes and sometimes causing death after parturition (Radostits et al., 2006). Therefore toxoplasmosis was considered as one of the major economically important disease of livestock production system (Freyre et al., 1999).

The prevalence of toxoplasmosis varies greatly among different geographical areas due to different farming practices and environmental factors (Tenter et al., 2000). Surveillance and monitoring of toxoplasmosis in human and animals is required for necessary control and prevention options but not yet available (Spišák et al., 2010).

The diagnosis of toxoplasmosis is usually made by the direct demonstration of the parasite from biopsy or autopsy material (tissues), but such techniques are time consuming and unsuitable for large scale survey. To overcome this problem serological test were used for diagnosis of toxoplasmosis (Hashemi-Fesharki, 1996). Among all serological test developed for serodiagnosis of toxoplasmosis, ELISA is very simple, economical, sensitive and easily adapted for field use (Spencer et al., 1980).

Despite of high economic return from ever growing small ruminants farming in Bangladesh, this enterprise faces several problems of production related diseases including toxoplasmosis. Though toxoplasmosis has huge impact on health of small ruminants and other production animals, it was not properly addressed in Bangladesh till now. Until now only few studies was reported by investigators especially in the northern Mymensingh district of Bangladesh based on MAT and LAT (Samad et al.,

1997; Shahiduzzaman et al., 2011). The prevalence of toxoplasmosis was recorded as 16.10% in cattle, 17.60% in sheep and 12.0% in goat in an earlier study (Samad et al., 1993). However in southern region of the country such as Chittagong, no reports were available on the prevalence and incidence of toxoplasmosis in small ruminants to date.

The present study was conducted with following specific objectives:-

1. Sero-epidemiological investigation of *Toxoplasma gondii* infection.
2. Identification of toxoplasmosis associated risk factors in sheep and goat in study areas.

6.3 Material and methods

6.3.1 Study animals

The study was conducted in sheep and goats that were reared in farming condition in different areas of Chittagong metropolitan area (CMA). Sheep and goats of different breeds and aged over six months were included in this study. To determine the age susceptibility to toxoplasmosis, animals were classified into three sub group on viz i) >2 years ii) 1-2 years and iii) <1 year. Age was determined by interviewing of the owner. This cross-sectional study was carried out from July, 2014 to December, 2014.

6.3.2 Target sampling

The farm that had a previous history of abortion or still birth or weak kid or lamb was considered for this study. Blood samples were collected from a total of 92 sheep and 92 goats. A questionnaire (Annex-2) was used to record information like owner's name and address, animal Identification (ID), farm size, breed, age, sex, history of abortion, presence of cat in the farm premises, sources of drinking water, disposal techniques of placenta etc.

6.3.3 Blood collection

Blood samples were collected directly from the jugular vein using vacutainer tubes without anticoagulant, properly labeled and the sera were separated by centrifugation at 3200 RPM for 10 min (Gebremedhin and Gizaw, 2014). The extracted sera were transferred to other sterile vials and kept at −20°C until serologically assayed.

6.3.4 Serological examination

Serum sample were tested for the presence of IgG antibodies against *Toxoplasma gondii* using a commercial indirect ELISA kit (IDEXX® toxo test, IDEXX Switzerland AG, Switzerland) according to manufacturer instruction. The kit was used for detection of antibodies against *T. gondii* in small ruminants. The test is validated if: -the mean value of the positive control O.D should not exceed 2.00 and the mean value of the negative control should not exceed 0.500. The ratio of the mean O.D values of the positive and negative controls (ODPC and ODNC) is greater than equal to 0.300 would also criterion to validate the sample. All the serological tests were done in serological lab, Poultry Research and Training Centre (PRTC),

Chittagong Veterinary and Animal Sciences University (CVASU). For interpretation of the result S/P% was calculated as: S/P% = (OD 450 value of the sample - OD450 value of the negative control) / (mean OD 450 value of the positive control - OD450 value of the negative control) x 100. Any samples with an S/P less than or equal to 20% were considered as negative, the samples with an S/P between 20% and 30% were considered as suspected, the samples with an S/P between 30% and 100% were considered as weak positive and the samples with an S/P greater than or equal to 100% considered as positive.

6.3.5 Statistical Analyses

The statistical analyses of epidemiological data were carried out by following similar methodology which was mentioned earlier in section 3.3.11.

6.4 Results

6.4.1 Overall prevalence

The overall seroprevalence of anti-*Toxoplasma gondii* IgG antibodies in small ruminants was recorded as 35.87% (n=184). Among the equal number of sheep and goat tested (n=92), the prevalence was found as 30.43% and 41.30% respectively (Table 6.1). However, no statistically significant differences were observed between two species (Table-6.1).

Table 6.1: **Overall prevalence of toxoplasmosis in sheep and goat.** Non significant association was observed in between the prevalence and species but the prevalence was comparative higher in goat than sheep.

Type of host	No. tested	No. positive	Prevalence (%)	P-value
Sheep	92	38	30.43	0.124
Goat	92	28	41.30	

6.4.2 Analyses of risk factors

The collected data through questionnaire were analyzed to identify the possible risk factors of *Toxoplasma gondii* infection in sheep and goat in the selected study areas. It was revealed that the prevalence was significantly higher in animals under the age group of >2 years (50.56%) compared to the younger age group of 1-2 years (28.21%) and <1 years (17.86%) (Table-6.2). Female animals had comparatively higher prevalence than the male but this observation was not statistically significant. History of earlier abortion of study animals were found as an important risk factor as the prevalence was significantly higher in animals with previous history of abortion. The body condition of animal and the presence of cat population in and around farms played no important role in the prevalence of toxoplasmosis as found from the statistical analyses. Sources of drinking water were found as an important risk factor for higher incidence of toxoplasmosis in the study areas. The prevalence was found significantly higher in the farms where water was supplied from open pond (with environmentally contaminated cysts/oocysts). On the contrary, farms using ground water (through shallow and deep tube well) as water sources were having less prevalence of the disease. Other management factors considered during this study

such as disposal practices of aborted materials like placenta were not found as risk factor for toxoplasmosis in the study area through statistical findings.

Table 6.2: **Association of different categorical variable with the incidence of toxoplasmosis (by using chi-square test).** Age of animals and sources of drinking water were significantly associated with toxoplasmosis.

Variables	**Categories (Sample size)**	**No. of positive cases and percent prevalence**
Disposal of placenta	Burial (68)	26 (38.24 %)
	Nearby field (76)	28 (36.84)
	Both (40)	12 (30.00%)
Age	>2 years (89)	45 (50.56%) ***
	1-2 years (39)	11 (28.12%)
	<1year (56)	10 (17.86%)
Sources of drinking water	WASA (36)	21 (58.33 %) ***
	Pond (15)	8 (53.33 %) ***
	River (40)	12 (30.00%)
	Tube well (93)	25 (26.88%)
Access of cat in the farm	Yes (106)	43 (40.57 %)
	No (78)	23 (29.49%)
BCS	<3 (101)	35 (34.65 %)
	3 (74)	27 (36.49 %)
	>3 (9)	4 (44.44 %)
History of abortion	Yes (28)	19 (67.86 %) ***
	No (156)	47 (30.13%)
Sex	Male (47)	13 (27.66 %)
	Female (137)	53 (38.69 %)

*** Significance at P<0.001

6.5 Discussion

Toxoplasmosis in small ruminants is considered as significant due to its role on transmitting the disease to the human through direct contact or consumption of animals originated foods. Numerous studies have reported possible transmission of toxoplasmosis from animal to human hosts. While in developed countries cat plays an important role in transmission of toxoplasmosis, small ruminants are thought to be important sources of transmission in developing countries. In Bangladesh, two previous reports involving serological studies indicated sheep and goats harboring the antigens. The seroprevalence recorded in these studies ranged from 17-42% and 12-32% in sheep and goats respectively (Shahiduzzaman et al., 2011; Samad et al., 1993). Another report based on latex agglutination test (LAT) and modified agglutination test (MAT) estimated the relative prevalence and investigated the effect of age with toxoplasmosis (Rahman et al., 2015b). During this study we employed more sensitive enzyme linked immunosorbent assay (ELISA) approach for serological investigation of toxoplasmosis in sheep and goats in Chittagong metropolitan area (CMA) and attempted to identify possible risk factors.

The overall seroprevalence of *T. gondii* in sheep in the study area was recorded as high as 30.43%. This finding is consistent with the previously published report in the Mymensingh area of northern Bangladesh where 40% of sheep were tested positive for toxoplasmosis (Shahiduzzaman et al., 2011). When compared with other countries of the world, a similar pattern in *Toxoplasma* prevalence in small ruminants were recorded in Brazil 31.1% (Carneiro et al., 2009), China 29.8% (Liu et al., 2010), Iran 31.2% (Youssefi et al., 2007) and Turkey 31.0% (Oncel and Vural, 2006). However, a higher prevalence was recorded in West indies which was 44.1% (Chikweto et al., 2011). The variation of the results may be due to the use of different serological methods, sample size, host species, management factors and most importantly the climate conditions.

Notable that the findings of prevalence of goats of this study (41.30%) varies with that of (Samad et al., 1997) and (Shahiduzzaman et al., 2011) who reported 12.88% and 32% prevalence in other parts of Bangladesh. Further study can highlight the regional influence and there correlation with the incidence of toxoplasmosis in Bangladesh perspective.

Type of animal species is important to assess the impact of toxoplasmosis in terms of susceptibility. During this study, no significant differences were observed between sheep and goat on the prevalence of toxoplasmosis while the prevalence in goat were little higher than the sheep. The finding was consistent with the findings of some other investigators in other parts of the world (Barakat et al., 2009; Gondim et al., 1999). Age is the most widely focused variable in literature and its impact on prevalence of the disease has been recorded in numerous studies (Do and Na, 1994). In this study there was found correlation between the age and prevalence of toxoplasmosis. Adult are significantly more susceptible to *T. gondii* infection than younger one. This correlation also supported by the findings of (Balea et al., 2012; Gebremedhin et al., 2014). It is due to the higher probability of exposure to the source of with the increases of age. This difference in the prevalence of toxoplasmosis is the cumulative effect of age which was previously reported by (Dubey, 2009). On the contrary, there are some available reports which indicate that gender was not significantly associated with the *T. gondii* infection (Guimares et al., 2013; Soares et al., 2009).

In this study we recorded comparatively higher prevalence of toxoplasmosis in female than male animal. This finding was supported by the observation of (Ahmad et al., 2015). It is due to the pregnancy, lactation which reduced the immunity of female animal (Messingham et al., 2001). Another reason for higher prevalence in female animals may be due to keeping of female animals for longer period of time for breeding and milk production purposes than males. Moreover farmers frequently sold the male animals for cash return and only fewer bucks are found per flock which kept for breeding purposes.

Infection by *T. gondii* in sheep and goats is usually associated with reproductive problems such as abortion, stillborn, stillbirth and mummified fetus (Weissmann, 2003). Although, reproductive failure in sheep and goats not only associated with *T. gondii* infection but it may also occurred by various etiological agents (Costa et al., 2012). We also recorded that the prevalence of toxoplasmosis were significantly higher in animals that had previous history of abortion than the animals that had such type of history. Similar finding was recorded by (Si et al., 2011). It may be due to long term remaining as a carrier. Therefore our study indicated that reproductive failure in small ruminants in the study area was mainly due to the toxoplasmosis.

In this study we did not found any significant association between the prevalence of toxoplasmosis and accessibility of cat in farm premises which was not expected. It may be due to the fact that most of the farmers of the study area did not possess cat but frequented with stray cats. However, slightly higher prevalence was recorded in farm that had access by cats. Similar findings were recorded by other investigators (Guimaraes et al., 2013; Lopes et al., 2009) and might be due to high number of stray cats leading to increased contamination of soil, food and water with the infective cysts.

Sources of drinking water might play important role in the epidemiology of toxoplasmosis. Few earlier reports indicate that cat faeces contaminated with oocysts of the parasite can be more easily ingested in the diet with natural water sources (Frenkel et al., 1995). In this study we also observed that running supply water (WASA supply water) and pond water is an important risk factor of toxoplasmosis in the study area. (Andrade et al., 2013) and (Tzanidakis et al., 2012) also found running water as an important factor of toxoplasmosis. The higher prevalence in animals that drank pond water is expected because of higher chance of contamination with oocyst. Common accessibility of cat to farm premises and types of waterer (which are difficult to clean) increase the contamination of water sources. The study also suggests that water purification and chlorination processes are either ineffective against oocysts or non-existent (Bowie et al., 1997).

Abortion are commonly occurred when it infect the animals during pregnancy. Placenta is the common site of infection of *T. gondii*. Improper diagnosis and treatment of infected animals and the improper disposal of aborted fetuses and placentas which in turn increase the chances of infection (Liu et al., 2010). However in our study we did not get any significant association in between disposal techniques and prevalence.

During the present study, it appears that the percentage of infection was comparatively higher in animals with poor body condition compared to healthy animals. This might be associated with the reduced immunity in animals with compromised nutrition that makes the animals more vulnerable to acquired infection.

6.6 Conclusion

The sero-surveillance study revealed that toxoplasmosis is common among sheep and goat in selected areas of southern Bangladesh. We analyzed the association of different epidemiological factors with the prevalence rate of toxoplasmosis in small ruminants. The study indicated a very high prevalence of anti- *T. gondii* antibodies in goats and sheep sera from Chittagong metropolitan areas of Bangladesh.

Several risk factors were considered to be associated with the prevalence of toxoplasmosis. The questionnaire based data analysis showed animal older than two years of age and those with history of abortion were mostly seropositive. In addition animal offered pond water and municipal supply water highly seropositive.

This important zoonotic disease can be prevented if proper surveillance and monitoring can be implemented. The poor people who rear small ruminants like sheep and goats are quite vulnerable to get toxoplasmosis which is one of the worst zoonotic illnesses in human capable of causing abortion. In the study areas such information can be useful for field veterinarians as well as for farm owners in order to develop strategic control plan of toxoplasmosis in farm level in the study areas. In particular, accessibility of cats to their farm premises should be s avoided and supply of safe drinking water should be strictly follow. In addition to serious impact on human health, the disease is responsible for significant loss of productive performance in small ruminants and thus causing significant economic losses. Further countrywide surveillance and analyses with modern molecular tools can increase our understanding of different serotypes of *Toxoplasma gondii* which is crucial to develop necessary regarding control and prevention approaches of this important protozoan.

CHAPTER-7

Molecular identification and characterization of *Toxoplasma gondii* in small ruminants in Chittagong Metropolitan Area.

Molecular identification and characterization of *Toxoplasma gondii* in small ruminants in Chittagong Metropolitan Area.

7.1 Abstract

Toxoplasma gondii is an important zoonotic parasite that causes abortion in small ruminants. The present study was carried out involving small ruminants (sheep and goat) that are admitted to Teaching Veterinary Hospital of Chittagong Veterinary and Animal Sciences University (CVASU) in Bangladesh with history of abortion. Placenta and fetal brain were collected for subsequent histopathological and molecular investigations. Following DNA extraction, a nested PCR analyses targeting *Toxoplasma B1* gene was applied where all positive samples were identified by amplification of 197 bp gene fragment. Histopathological examination of H&E stained tissue slides showed bradyzoite stages which complemented molecular identification. Prevalence of *T. gondii* was detected as high as 25% in sheep (n=4) and 36.84% (n=19) in goats. However, this does not indicate that goats are more susceptible to toxoplasmosis as the sample size was not substantial. All the positive cases were detected in animals that were aborted at theage of 3.5 months of age ($P<0.05$). No correlation was found between age of animals, population of cat in farm areas and animal nutritional status (body condition) with the prevalence of toxoplasmosis. The presence of *T. gondii* in fetal tissues and neonatal specimens along with characteristics histopathological changes indicate congenital infection. Further analyses using larger sample number would be useful to detect different risk factors associated with toxoplasmosis in small ruminants.

7.2 Introduction

Toxoplasmosis is an important devastating diseases caused by an intracellular protozoan *Toxoplasma gondii*. This protozoan is capable of infecting all worm-blooded animals and had worldwide distribution (Dubey and Beattie, 1988). It is one of the major causal agents of reproductive failure in sheep and goat (Tenter et al., 2000). The infection is responsible for substantial economic losses in sheep and goat industry by causing frequent abortions and thereby reducing the farm income (Buxton et al., 2007). The pathology of toxoplasmosis is associated with the invasion of the organism in the placenta and fetus approximately two weeks after having an infection. Infection during the early stages of gestation can result in fetal death, resorption and abortion, while infection in the latter stage of gestation (Aitken, 2007). While detailed histopathology of toxoplasmosis has been investigated in developed countries, further molecular diagnostic tools are not yet attempted in Bangladesh.

Reliable and early diagnosis of toxoplasmosis is necessary to develop effective control and prevention options. Conventional diagnosis of abortion due to toxoplasmosis is based on detection of antibodies in fetal fluid by serological tests (Dubey et al., 1987), demonstration of parasites by immunohistochemistry (Uggla et al., 1987) and observation of characteristic histopathological change in placenta and brain tissues (Buxton and Finlayson, 1986). The isolation of *Toxoplasma* from aborted tissue samples is the widely accepted gold standard for diagnosis of toxoplasmosis (Losson and Buxton, 2007). Recently molecular assay such as PCR amplification of different genes is routinely used as an effective tool for diagnosis of toxoplasmosis (Hurtado et al., 2001). A number of reports have been published where different gene fragments were amplified through PCR assay. These *T. gondii* specific genes include *Toxoplasma B1 repetitive* gene (Burg et al., 1989), *P30 surface antigen* gene (Savva et al., 1990), *small ribosomal rRNA* gene (Tenter et al., 1994) and *529bp repeated element* (Homan et al., 2000). Along with PCR analyses, classical histopathological examination can be used as complementary tool to effectively diagnose toxoplasmosis. However collection of sample is always critical and may lead to false negative result if any contamination takes place. Therefore careful sample collection, preservation and processing are crucial.

Worldwide a number of studies have been reported that involved both histopathological and molecular diagnostics. The sample types also varied that include blood, heart, brain, lung, liver and cotyledon from aborted fetus. Among all these sampling methods, detection rate were higher in brain and cotyledon compared to other samples (Dubey and Beattie, 1988). Among all targeting gene that are used for molecular identification of the *T. gondii*, higher sensitivity of PCR assay targeting *B1* gene was reported by some investigators. However, on the contrary, *B1* element was reportedly absent in 4.8% of human *T. gondii*-positive samples (Menotti et al., 2010).

Considering an important zoonoses, toxoplasmosis has enormous significance in human health. Most rural people especially women are engaged in rearing sheep and goat in Bangladesh which has substantial impact in short term economic return and eventual poverty alleviation. In addition to the health risks of women (suspected abortion), economic loss are also encountered through frequent abortion of small ruminants due to toxoplasmosis. The rate of abortion in farm animals is again correlated to the large number of animals, too high animal's density, inappropriate breeding conditions, absence of early and accurate diagnostics etc. Alongside other production related issues, abortion is one of the important constraints in the development of sheep and goat industries in Bangladesh. While abortions in small ruminants are more frequent in different areas of the country, yet no specific and sensitive diagnostic tools have been developed or used to identify the causal agents of abortion. Previous serological survey indicated that the prevalence of toxoplasmosis in sheep and goat was 42 and 32% respectively in central part of Bangladesh (Mymensingh) (Shahiduzzaman et al., 2011).

The present study was conducted with following objectives:-

1. To identify the *T. gondii* as a causative agent of abortion in sheep and goat by using the histopathological examination and nested PCR assay.
2. Molecular characterization of *Toxoplasma gondii* by sequencing and phylogenetic investigations.

7.3 Material and methods

7.3.1 Study area

The study was conducted in Sahedul Alam Quadery Teaching Veterinary Hospital (SAQTVH) of Chittagong Veterinary and Animal Sciences University (CVASU).

7.3.2 Study duration

This study was carried out for the periods of one year starting from January, 2014 and ended at December, 2014.

7.3.3 Target animals

Target animals are sheep and goats that were admitted to SAQTVH with history of bloody discharge through vagina. A pre set questionnaire were used collection of data regarding age of the animals, gestation age, presence or absence of cat, body condition of animals etc.

7.3.4 Sample collection

Tissue samples were taken from the 23 aborted feti (19 goats and 4 sheep) that were admitted to SAQTVH, CVASU by maintaining appropriate techniques. Samples were divided into two portion- one portion for histopathological study and another portion for molecular study.

7.3.5 DNA extraction

DNA was extracted only from tissue sample. From Tissue samples DNA was extracted by using commercial DNA extraction kit (Favorgen) by following their instruction. Briefly, 25mg tissue sample was taken into a sterilized mortar and pestle and then grinded it to make a homogenous mixture by using PBS. Then the mixture was transferred into a centrifuge tube. 200µl FATG1 buffer and 20µl proteinase k (10mg/ml) added to the sample mixture and mixed thoroughly by vortexing. The mixture was incubated at 60ºC until the tissue is lysed completely and vortexing was done in every 10-15 min during incubation. To remove the drops from the inside of the lid the tube was briefly spine. 4µl of RNase A (100 mg/ml) was added and incubated for 2 min at room temperature. After that 200µl FATG2 buffer was added to the sample mixture, mixed thoroughly by pulse-vortexing and incubated at 70ºC for

10 min. To remove the drops from the inside of the lid the tube was briefly spine. 200 µl ethanol (96 ~ 100%) was added to the sample and mixed thoroughly by pulse-vortexing. To remove the drops from the inside of the lid the tube was briefly spine. A FATG mini column was placed in a collection tube and then the sample mixture (including any precipitate) was transferred carefully to FATG column. Centrifugation was done at 14000 rpm for 1 min and the flow-through was discarded. Then the same FATG column placed into a new collection tube. FATG column was washed with 500µl W1 buffer by centrifugation for 1 min and then the flow-through was discarded. Again FATG column washed with 750µl wash buffer by centrifugation for 1 min then discarded the flow-through. Centrifugation was done for an additional 3 min to dry the column. Finally FATG column was placed to elution Tube. 50µl elution buffers was added to the membrane center of FATG column and FATG column was stand for 3 min. Final centrifugation was done for 2 min to elute total DNA. DNA was stored at 4ºC or -20ºC.

7.3.6 Histopathology

Tissues were collected by maintaining proper techniques. Only brain tissue of fetus and cotyledon were collected for investigation of toxoplasmosis. Half portion of tissues was kept for molecular study and half portion were processed for histopathological study. Firstly tissues were preserved in Bouin's solution for 2-3 days. After that the samples were made smaller (5mm thickness). Again fixation was done in Bouin’s solution (10 folds of the tissue size and weight) and tissues were fixed for 3- 5 days in the solution. The tissues were trimmed into a thin section and washed over night in running tape water to remove formalin. After that the tissues were dehydrated by ascending ethanol series to prevent shrinkage of cells as per following schedule. The tissues were dehydrated in 50%, 70%, 80%, 95%, 100%, 100%, 100% ethanol, one hour in each. Impregnation was done in melted paraffin (56-60°c) for 3 hours. Then the tissues were sectioned with a microtome at 5µm thickness. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The sections were allowed to spread on warm water bath at 40-42°C. Then the sections were taken on grease free clear slides. The slides containing section were air dried and kept in cool place. Then the slide was ready for routine hematoxylin and eosin staining.

The sectioned tissues were deparaffinized in three changes of xyline (three min in each). Then the sectioned tissues were rehydrated through descending grades of alcohol (three changes in absolute alcohol, three min in each; 95% alcohol for two min; 80% alcohol for two min; 70% alcohol for two min) followed by distilled water for five min. After that the tissues were stained with Harris hematoxylin for fifteen min and washed in running tap water for 10-15 min. Then the tissues were differentiated in acid alcohol by 2 to 4 quick dips in acid alcohol (1 part HCL and 99 parts 70% alcohol). Again washed in running tap water for five min followed by 2-4 dips in ammonia water until sections were bright blue. After washing the sections were stained with eosin for one minute. Differentiated and dehydrated in alcohol (95% alcohol: three changes, 2-4 dips each; absolute alcohol: three changes 2-3 min for each). Then cleared in xyline: three changes (five min each). In final step tissues were mounted with cover slip by using DPX. Then slides were dried at room temperature and examined under a low (10X) and high (40X, 100X) power microscopic fields.

7.3.7 DNA amplification

PCR was performed in a 2720 thermal cycler® (Applied Biosystem) in a total reaction volume of 25µl containing 12.5µl of GoTaq® G2 hot start green master mix (2X Green GoTaq®reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM MgCl2), 1.5µl of each of the primers (10 pico mole) derived from B1 gene, 2µl of template (sample DNA) and 7.5µl nuclease free water. For the PCR amplification, initial denaturation was performed at 94ºC for 30 seconds, followed by 50 cycles of denaturation at 94ºC for 15 seconds, annealing at 45ºC for 30 seconds, extension at 72ºC for 45 seconds and final extension at 72ºC for 10 min. For the nested PCR, The PCR reaction was performed at similar temperature to the primary PCR, but the amplification cycles was carried out for 35 cycles. The PCR amplified products were visualized by electrophoresis on agar rose gel 1.5% stained with Ethidium bromide. Under a transilluminator with a 100 bp DNA ladder, the product length of the positive result was 197 bp.

Table 7.1: **Details of primer used for PCR (*Toxoplasma gondii*)**

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
B1 (primary)	Toxo- F1 Toxo-R1	5´-TCA AGC AGC GTA TTG TCG AG-3´ 5´-CCG CAG CGA CTT CTA TCT CT-3´	_	Wiengcharoen et al., 2004
B1(secondary)	Toxo- F2 Toxo-R2	5´-GGA ACT GCA TCCGTT CAT GAG-3´ 5´-TCT TTA AAG CGTTCG TGG TC-3´.	197 bp	

7.3.8 PCR products purification

The protocol for PCR product purification was followed as mentioned in the manufacturer's instruction. The commercially available PCR purification kit (Favorgen, Korea®) was used and protocol was mentioned earlier in section 3.3.8.

7.3.9 DNA Sequencing

Purified PCR products were sent for sequencing by commercial suppliers (Bioneer Corp, South Korea) by Sanger sequencing method.

7.3.10 Phylogenetic analyses of sequences

The phylogenetic analyses of partial sequence of *B1* gene of *Toxoplasma gondii* according to the protocol that was mentioned in section 3.3.10.

7.3.11 Statistical analyses

The statistical analyses of epidemiological data were carried out by following similar methodology which was mentioned earlier in section 3.3.11.

7.4 Results

7.4.1 Clinical observation

All cases were admitted to the SAQ teaching veterinary hospital with history of bloody discharge through vagina. Clinical and ultrasonographical examination was done to detect the fetus was either live or death. The aborted dam showed the signs of anorexia but no nervous symptoms were detected during the time of examination. No detectable gross lesions were observed in fetal tissue during the time of sample collection (Fig. 7.1 and 7.2). Among all cases (n=23), 12 case were at <3.5 month gestation and 11 cases were >3.5 month of gestation.

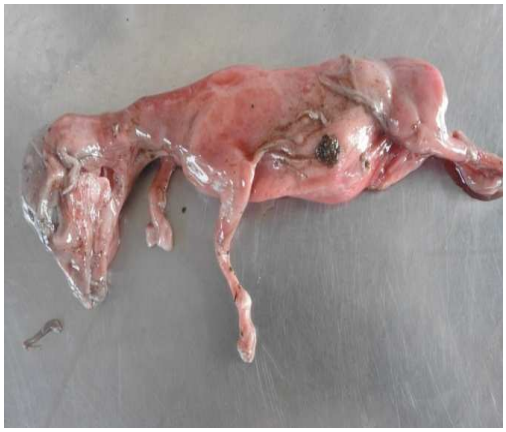

Fig 7.1: Representative picture of aborted fetus

Fig 7.2: Representative picture of macerate fetus

7.4.2 Molecular examination

DNA amplification of *Toxoplasma gondii* using the mostly used *B1* gene primer produced a 197bp fragment in nested PCR (Fig 7.3) and showed the parasites in 36.84% goat and 25% sheep. The associations of different categorical variable with toxoplasmosis positive samples were shown in Table 7.2. Abortion rate were comparatively higher in animals age between 2-3 years (41.18%) in compared to the animals age greater than 3 years. However, the detection of positivity of toxoplasmosis was higher in poor body conditioned animals compared to good body condition animals but it was not significant. The prevalence was significantly higher in animals that abort at >3.5 months of age in compared to animals that abort at < 3.5 month of age. Presence of cat in the farm premises were not found as a significant factor of toxoplasmosis.

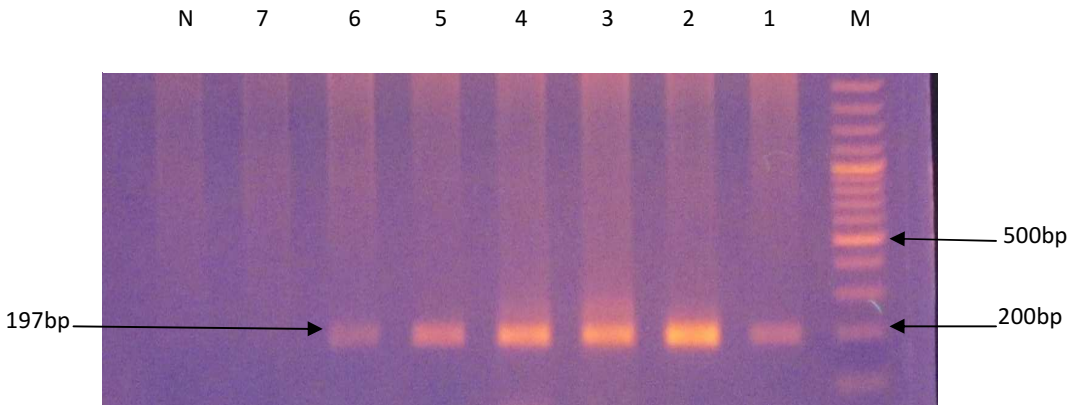

Fig 7.3: **Amplification of the genomic DNA of *Toxoplasma gondii* from tissue of aborted small ruminants by using *B1* gene.** Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-7 is suspected samples; Lanes 1-6 having amplicons of 197 bp indicated presence of *Toxoplasma* organisms.

Table 7.2: **Association of categorical variable with incidence of *Toxoplasma* abortion (by using Chi-square test). Significant association were observed in between toxoplasmosis and gestation age.**

Factor	**Factor level**	**Percent of positive cases**
Species	Goat (19)	36.84
	Sheep (4)	25.00
Age category	2-3 years (17)	41.18
	>3 years (6)	16.67
Body condition	Good (12)	25.00
	Poor (11)	45.45
Presence of cat	Yes (22)	36.36
	No (1)	0.00
Abortion at	<3.5 month (12)	0.00
	>3.5 month (11)	72.73***

*** Significance at P<0.001

7.4.3 Histopathological findings

There is lymphocytic infiltration, the inflammatory reactions induced in the fetal brain tissue. Accumulation of lymphocytes in the brain tissue with presence of bradyzoites cyst (Fig 7.4) of *T. gondii* indicates the positivity of toxoplasmosis.

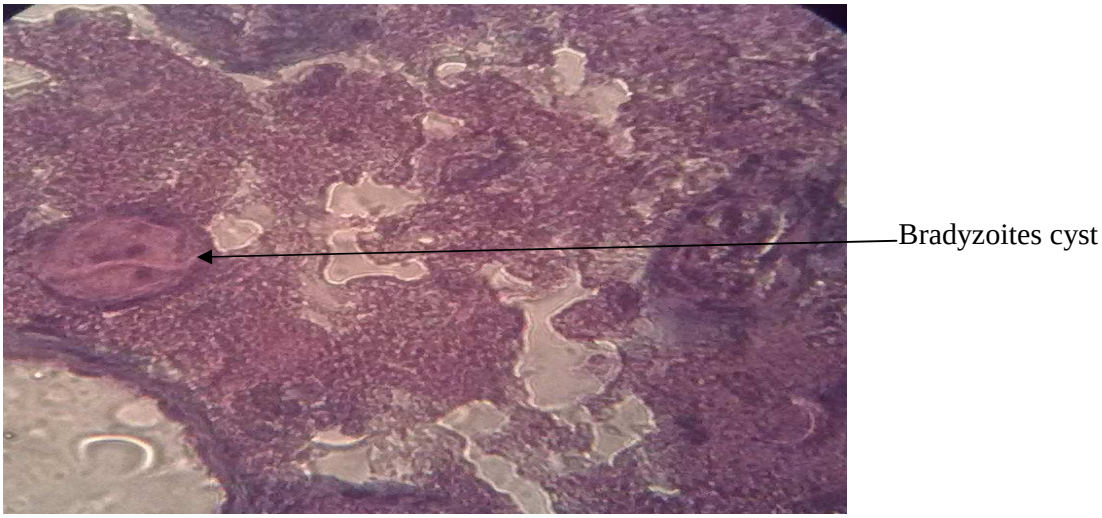

Fig 7.4: **Histopathological study of brain tissue characterized by presence of bradyzoite cysts** (Indicated by arrow)

7.4.4 Phylogenetic analyses

Phylogenetic tree (Fig. 7.5) was constructed by using sequences isolated from this experiment and all related isolates deposited in gene bank by using MEGA 5.1 software and followed the Neighbour joining method. All isolate of this experiment cluster together with isolate of India and Iran.

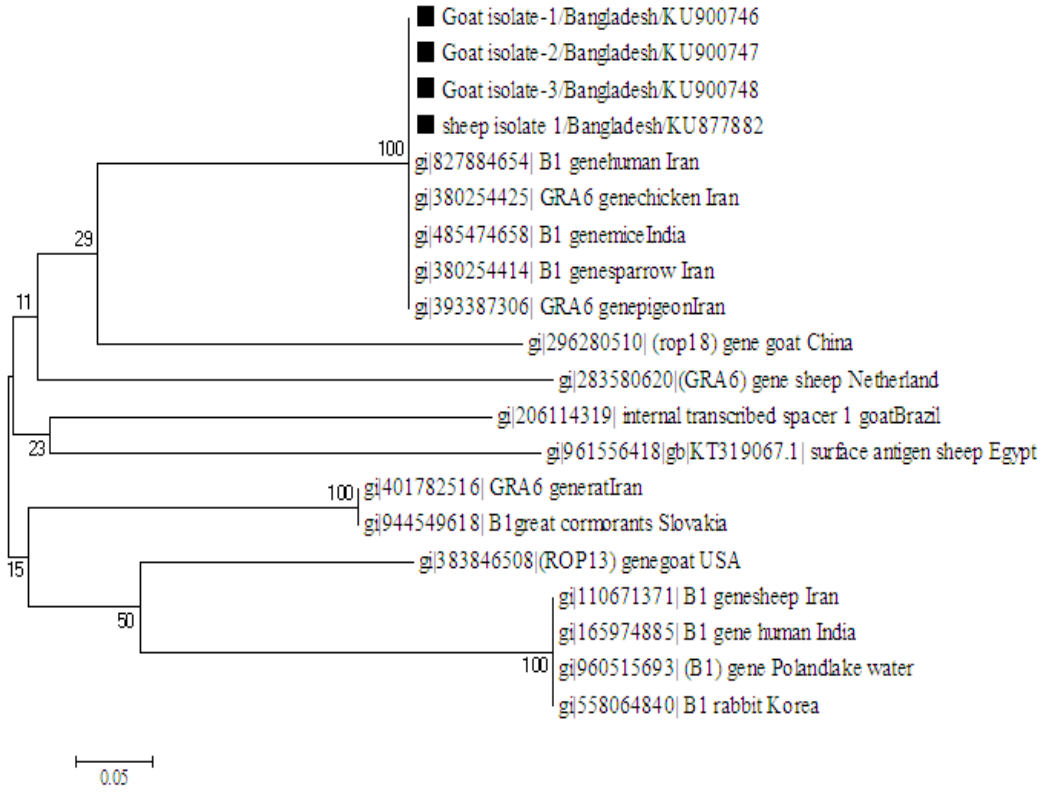

Fig 7.5: **Phylogenetic tree constructed from the nucleotide sequences of *T. gondii B1* gene and all relevant sequences derived from NCBI BLASTn search results**. These sequences were deposited in GenBank from different countries and the accession numbers and countries are shown before isolate name. The *B1* sequences obtained in this study were indicated as sheep isolate and goat isolate. The tree was inferred using the neighbor joining method of MEGA 5.2, bootstrap values are shown next to the branch point. Number next to the branch demonstrates bootstrap support from 1000 replications. All sites of the alignment containing insertions-deletions, missing data were eliminated from the analysis (option "complete deletion").

7.5 Discussion

Abortions are a common problem in sheep and goat farming industries worldwide and can lead to substantial economic loss. Among many other infectious diseases, toxoplasmosis is considered as responsible for mostly late abortion in small ruminants. In Bangladesh, few studies have been published reporting the seroprevalence of toxoplasmosis in sheep and goats. According to these reports, the prevalence ranged from 17-42% and 12-32% in sheep and goats respectively (Shahiduzzaman et al., 2011; Samad et al., 1993). However no specific data were available on the actual number of cases linking *T. gondii* associated abortion and stillbirth in these animals. Further diagnosis of different causes of abortion is therefore necessary to explore the actual economic losses associated with toxoplasmosis in small ruminant industry in the country.

To our knowledge this is the first molecular study of ovine and caprine toxoplasmosis in Bangladesh that has detected *T. gondii* using PCR, estimated its prevalence rate among aborted animals and evaluated the possible risk factors. One of the objectives of this study was to use a more sensitive and reliable molecular assay to detect *T. gondii* from aborted fetal samples. Following previously published protocols we have amplified the *B1* gene fragment which is thought to be highly sensitive and specific for *Toxoplasma* (Filisetti et al., 2003). For DNA extraction we have used two different sample types (aborted fetal brain and placenta) according to published protocols. While the tissue specific stages are usually available in a number of tissues, available literatures suggest a higher prevalence of *Toxoplasma* in brain, heart and placenta (De Moraes et al., 2011; Moazeni Jula et al., 2013).

The type of the host is also important as the infection rate varies from species to species. During this study the rate of abortion due to toxoplasmosis was recorded as 25% and 36.84% in sheep and goat respectively. This was consistent with the observation by previous investigators who recorded late abortion rate as high as 35.6% and 43.7% for sheep and goats, respectively (Ahmed et al., 2008). The comparative analyses of the species-specific susceptibility are not yet explored. However, some authors reported goat species more susceptible to sheep but this is not well accepted as various risk factors are associated with the disease prevalence (Tibary, 2009). Despite that it was assumed that it may be due to higher activity and

movement of goats in compare to sheep which increase the probability to get more exposure to contaminated source.

During this study, the association of different categorical variables with the incidence of toxoplasmosis was also assessed based on predefined questionnaire. The general conception is that the severity of toxoplasmosis depends on the stage of gestation when infection occurs in the host animals (Innes et al., 2009). We recorded higher incidence in animals that abort >3.5 months of gestation compared to animals that abort <3.5 months of gestation. Previous report also indicated that infection prior to 40 days of gestation may cause embryonic death and fetal resorption, while infection between 40 and 120 days of gestation results in fetal maceration, mummification or abortion and infection after 120 days of gestation produces stillbirth or birth of weak kids or lamb (Menzies and Miller, 1997; Dubey et al., 1987).

In this study all the positive cases were recorded in the farms that are frequented with stray cats or kittens. However, although important for transmission, this was not statistically significant. Still one would assume that cats play an important role through shedding millions of *Toxoplasma* oocysts that survive in the environment for variable periods of time (Tenter et al., 2000) and animals may be infected by ingestion of contaminated feed and water (Dubey, 2009).

The percentage of abortion was comparatively higher in animals with poor body condition compared to healthy animals. This might be associated with the reduced immunity in animals with compromised nutrition that makes the animals more vulnerable to acquire infection.

The prevalence of toxoplasmosis detected from the aborted animals were comparatively higher in age group of 2-3 years of age in compared to age group of more than 3 years of age. The findings are somewhat controversy to serological findings that was described in previous experiment. It may be due to development of resistance of animals against clinical toxoplasmosis. Our study also indicates that special care needed to pregnant animals at the age of 2-3 years of age.

Until now, confirmatory diagnosis of abortion due to toxoplasmosis is based on characteristics histopathological findings, isolation of organisms from fetal tissues and modern molecular diagnostic tools (Losson and Buxton 2007). The most common

lesion observed in abortion by *T. gondii* is necrotizing placentitis exclusively in the cotyledonary areas and non suppurative encephalitis (Caldeira et al., 2011). In our study we have done the histopathological study of fetal brain and cotyledon tissues. We found only one positive histopathological change in brain tissue which was the lymphocyte infiltration along with the presence of bradyzoite cysts. Non-suppurative encephalitis with necrotic areas was also described by some other investigators (Uggla et al. 1987; Bari et al., 1993).

Although there are only one species under the genus *Toxoplasma*, significant intraspecific differences with respect to disease presentation were observed (Sibley et al., 2002). This strain variation is based on pathogenicity in mice as well as variability in specific genetic markers, including the *B1* gene. In this study phylogenetic analyses following DNA sequencing revealed that all three confirmed isolates of goats and one confirmed isolate of sheep were clustered together in a same clade which was similar to the sequence of *B1* gene human, Iran; *B1* gene mice, India; *B1* gene sparrow, Iran which was supported by high bootstrap value. Although this present study determined the coding sequences of the *B1* genes and revealed relatively considerable sequence variability within this locus among *T. gondii* isolates from different hosts and geographical regions. There is some variation in alignment when comparing each isolate to other it may be due to there is a 35 copies of *B1* gene in locus which are highly variable (Wahab et al., 2010).

7.6 Conclusion

Diagnoses of causal agents of abortion in small ruminants are always difficult using conventional approaches. During this study, positive cases of toxoplasmosis were further verified using histopathology and PCR based molecular identification.

While characteristics histopathological changes were detected in tissue like fetal brain, the source of transmission need to be identified to unravel the transmission dynamics of toxoplasmosis in small ruminants. Diagnosis based on histopathology could be a reliable option but selection of samples for parsite concentration is an important concern and may lead to false negative.

The PCR assay was able to successfully amplify partial fragment of *Toxoplasma B1* gene. Further study using other marker gene can complement the findings. Phylogenetic analysis of the sequence data indicated clustering of all the Bangladeshi isolates in a single clade indication less genetic diversity among the isolates.

The epidemiological factors associated with small ruminants toxoplasmosis indicates gestation age (greater than 3.5 months) and congenital infection was suspected. During periods special care like restrict the movement of final host at farm premises will needed by the farmers to avoid the unwanted abortion. Further analysis with larger aborted samples and in depth molecular investigation can comprehensively explore the transmission pattern of these important zoonotic protozoa.

Chapter-8

General conclusion and recommendations

Bangladesh is a developing country where livestock industries play important role in rural economy. Various diseases including parasitic infections are responsible for substantial morbidity and mortality leading to unexpected economic loss by the marginal farmers. The geographic landscapes and other different factors are thought to be related with the relative incidence and prevalence of these parasitic diseases in different parts of the country. Keeping this in mind, present study was conducted to increase our understanding of the current scenario of hemoprotozoan diseases in southern part of Bangladesh. The samples were collected exclusively from Chittagong regions while some other districts were also visited for comparative prevalence studies. Although vector borne diseases are known to have significant impact in livestock farming and substantial economic losses are presumed, no economic impact has been published till date. We attempted to measure the prevalence of important protozoan illness including anaplasmosis, babesiosis, trypanosomiasis and toxoplasmosis along with validation of the diagnostic protocols (including microscopic, molecular and histopathological techniques) that has been reported previously. The study was the first molecular investigation in Bangladesh using DNA based molecular characterization which was followed by sequencing by Sanger method and subsequent phylogenetic analyses. We also assessed the various risk factors associated with the variation of prevalence of these diseases by using classical statistical tools. Altogether the present study highlighted the need for further in depth analyses and country-wide surveillance of vector-borne hemoparasitic diseases which is crucial to prevent future outbreak as well as restoring the economic losses associated with these diseases.

Anaplasmosis is thought to be an important parasitic infection in cattle in Bangladesh where different seasons are prevailing. Seasonal variation was found as important factor associated with incidence of anaplasmosis in the country during this study. Significant higher prevalence of anaplasmosis was observed in winter compared to summer and rainy seasons. Sequencing of partial gene fragment was found useful in molecular characterization of different isolates. Further geographic analyses revealed

their phylogenetic position closer to the isolates reported earlier from other countries including China, Argentina, Australia and Mexico.

Babesiosis is also an important parasitic infection in cattle in Bangladesh which hinder the development of livestock development in Bangladesh. Different factors playing role in the disease prevalence where season were found as an important factors associated with the incidence of babesiosis. Significant higher prevalence was observed in summer season in compared to rainy and winter season. The disease commonly affects the animals aged 6-18 months. Sequencing of partial gene fragment was found useful in molecular characterization of different isolates. Through phylogenetic analysis, two distinct clusters were found of which one was closely related to *Babesia ovata* and another cluster was closely related to *Babesia bigemina*. Further study can highlight any other species existing in this country where many tick vectors are available and no or minimum preventive measures are followed by the farmers.

Trypanosomiasis is one of the important diseases less frequently reported in Bangladesh. During this study only one farm was found to have positive cases indicating sporadic nature of this protozoan. Phylogenetic analyses revealed that the isolates recovered (*Trypanosoma evansi*) during this study were closely related to the isolates previously reported from countries like Taiwan, Thailand, Japan and Egypt.

Toxoplasmosis is an important disease of small ruminants causing significant economic losses by causing abortion, still birth and other congenital disturbances. Different factors are thought to be associated with the toxoplasmosis. The seroprevalence was observed higher in goat than sheep. The prevalence of toxoplasmosis was significantly ($P<0.05$) higher in the age group of >2 years compared to the younger age group of 1- 2 years and <1 year. Several factors like history of abortion, use of clean water etc. were found as possible risk factor that might be contributory to toxoplasmosis in the study areas.

Toxoplasmosis was found as a possible cause for abortion in the sheep and goats in study areas. The present study attempted to identify all the different factors associated with these abortions that might be linked to toxoplasmosis.

Based on the above findings and reviewing the relevant literatures, following recommendations are suggested as the output of the present study:-

a) The year-long and countrywide surveillance of vector-borne hemoparasitic diseases are necessary to assess seasonal variations of the infections and predict necessary control measures.

b) Alongside age, sex, breed, species farm management is an important factor towards the prevalence of hemoparasitic diseases in small and large ruminants.

c) Necessary database for vector and their geographic locations need to be available by farmers in different parts of the country to plan effective containment strategies.

d) Classical diagnostic protocols are useful for field level diagnosis by thin blood smear and can be adopted for regular surveillance.

e) Advanced molecular diagnostics are available in city areas which should be made available to remote areas by concerned livestock service personnel. These can be achieved by developing sample collection and dispatch to the nearest molecular test lab and result of the test can be shared by digital devices (e.g. Email or mobile phone etc.).

f) Economic loss associated with each specific vector-borne hemoparasitic diseases need to be ascertained before planning any control and prevention initiatives.

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Annex-1: Questionnaire used for collection of farm data (Blood parasite)

1a. Farm level data

1. Name of the farm:……. Mobile No.
2. Address of the farm:……..Ward……Thana…….Dist.
3. Economic condition of farm owner: 1=ultra poor, 2=poor, 3=Moderate
4. Type of Farm: 1= cattle, 2= sheep, 3= Goat, 4 = mixed
5. Govt. Registration: 1=Yes 2=No 3=Applied If yes Reg No:
6. Receive training on Farming**:** 1=Yes; 2=No
7. Record of farm data? 1= Yes; 2= No
8. Current population of cattle at farm ……
9. Farm composition: Total adult female……Milking ……
Pregnant ……Total Heifer……Prepubertal……..Pubertal…….
Total calf: Male ……Female ……Total male animal……
10. Last year farm dynamics/changes: animals bought……..animal sold…….
11. Floor type, 1=Paka (Cemented/semi-semented), 2= Mati (uncemented)
12. Drainage system of the farm: 1= Good 2= Moderate 3= Poor 4= Nothing
13. Position of drain: 1= Back of the stall/house 2=between animal rows (middle) 3= Other
14. Passage of animal waste: 1= Fast, well slopped and connected to central outlet
2=Slow passage, not well connected to outlet
15. Frequency of the cleaning of the floor: 1= Once daily 2=every alternate day 3=more than once daily 4=No schedule
16. Do you use disinfectant for cleaning? 1= Yes (Frequent) 2= Yes (infrequent/sometimes); 3= not at all
17. Visual hygienic score 1= Most cows hind parts appear clean; 2=Most cows buttock and tail areas painted with excreta; 3= most of the cows back and hind quarter paint extended up to lower abdomen.
18. Do you practice routine vaccination? 1= Yes; 2= No
19. Do you practice routine deworming? 1= Yes; 2= No

1b. Individual animal data

Sl. no.	Farm ID	Study ID	Age	Sex	Breed	Parity	Current status

Annex-2: Questionnaire used for collection of farm data (Toxoplasma)

2a. Farm level data

1. Name of the farm:……. Mobile No.
2. Address of the farm:……..Ward……Thana…….Dist.
4. Type of Farm: 1= cattle, 2= sheep, 3= Goat, 4 = mixed
5. Govt. Registration: 1=Yes 2=No 3=Applied If yes Reg No:
6. Receive training on Farming**:** 1=Yes; 2=No
7. Record of farm data? 1= Yes; 2= No
8. Current population of farm ……
9. Farm composition: Total adult female……Kid/Lamb ……
Pregnant ……Total male animal……
10. Last year farm dynamics/changes: animals bought……..animal sold…….
11. Sources of drinking water: 1= Tubewell; 2= Tap water (wasa); 3=Pond water; 4= other sources
12. Do you practice chlorination of water? **:** 1= Yes; 2= No
13. How frequently do you clean feeder and waterer: 1= daily; 2=every alternate day; 3= weekly; 4= No schedule.
14. Do you practice routine vaccination? 1= Yes; 2= No
15. Do you practice routine deworming? 1= Yes; 2= No
16. Presence of cat in the farm area: 1= yes 2= no
17. Disposal methods of carcass/placenta: 1 = Burial, 2 = throwing to nearby field, 3= left on ground, 4 = offer to dog/cat
18. History of abortion? 1= Yes, 2=No

2b. Individual animal data

Sl. no.	Farm ID	Study ID	Age	Sex	BCS	Parity	Current status	history of abortion

2c. Aborted animal data

Sl. no.	Study ID	Species	Age	Body condition	Clinical history	Presence of cat	Abortion age

Annex-3:

Submitted sequence to NCBI gene bank

3a. *Anaplasma marginale* **isolate CVASU/DPP/cattle/1 *major surface protein 4* (*msp4*) genes, partial cds**

GenBank: KX110079.1

FASTA Graphics

Go to:

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  TITLE     Molecular identification and characterization Anaplasma spp in
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 546)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S.I., Ahaduzzaman,M., Akter,Y.,
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  TITLE     Direct Submission
  JOURNAL   Submitted (21-APR-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
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3b. *Babesia ovata* isolate DPP/CVASU-1 *18S ribosomal RNA* gene, partial sequence

GenBank: KU837251.1

FASTA Graphics

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            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular identification and characterization Babesia spp in
cattle
            in Chittagong division, Bangladesh
  JOURNAL   Unpublished
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  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-FEB-2016) Pathology and Parasitology, Chittagong
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3c. *Babesia ovata* isolate DPP/CVASU-2 *18S ribosomal RNA* gene, partial sequence

GenBank: KU877881.1

FASTA Graphics

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            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular identification and characterization Babesia spp in
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            in Chittagong division, Bangladesh
  JOURNAL   Unpublished
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3d. *Babesia ovata* isolate DPP/CVASU-3 *18S ribosomal RNA* gene, partial sequence

GenBank: KU947081.1

FASTA Graphics

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  TITLE     Molecular identification and characterization of Babesia spp. in
            cattle in Chittagong division, Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 370)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-MAR-2016) Pathology and Parasitology, Chittagong
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GenBank: KU947082.1

FASTA Graphics

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            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular identification and characterization of Babesia spp. in
            cattle in Chittagong division, Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 357)
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  TITLE     Direct Submission
  JOURNAL   Submitted (17-MAR-2016) Pathology and Parasitology, Chittagong
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3f. *Babesia bigemina* isolate DPP/CVASU-5 *18S ribosomal RNA* gene, partial sequence

GenBank: KX228228.1

FASTAGraphics

Go to:

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2016
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VERSION     KX228228.1  GI:1072839799
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            Eukaryota; Alveolata; Apicomplexa; Aconoidasida; Piroplasmida;
            Babesiidae; Babesia.
REFERENCE   1  (bases 1 to 284)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular identification and characterization of Babesia spp. in
            cattle in Chittagong division, Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 284)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAY-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
            Chittagong 4225, Bangladesh
COMMENT     ##Assembly-Data-START##
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                     /country="Bangladesh"
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                     /identified_by="Abdul Mannan"
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      121 gccgcggtaa ttccagctcc aatagcgtat attaaacttg ttgcagttaa aaagctcgta
      181 gttgtatttc agcctcgcga cttgtccatt tttttggttc acttcgctgg cttttgtttt
      241 actttgagaa aattagagtg tttcaagcag acttttttgtc ttga
```

3g. *Babesia ovata* isolate DPP/CVASU-6 *18S ribosomal RNA* gene, partial sequence

GenBank: KX228229.1

FASTAGraphics

Go to:

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2016
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partial
            sequence.
ACCESSION   KX228229
VERSION     KX228229.1  GI:1072839800
KEYWORDS    .
SOURCE      Babesia ovata
  ORGANISM  Babesia ovata
            Eukaryota; Alveolata; Apicomplexa; Aconoidasida; Piroplasmida;
            Babesiidae; Babesia.
REFERENCE   1  (bases 1 to 370)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular identification and characterization Babesia spp in
cattle
            in Chittagong division, Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 370)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAY-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
            Chittagong 4225, Bangladesh
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
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      121 atacagggct ttcgtcttgg aattggaatg atggtgacgt aaaagctccc cagaatacca
      181 attggagggc aagtctgggg ccagcagccg cggtaattcc agctccaata acgtatatta
      241 aacttgttgc agttaaaaag ctcgtaattg tatttcagcc cgtcgtattt tcccgttttg
      301 ggtttttgct cgggcctatt tttactttga taaaaataaa gtgtttgtag cagacttttg
      361 tctttgttga
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3h. *Trypanosoma evansi* isolate CVASU/Bangladesh/01 *18S ribosomal RNA* gene, partial sequence

GenBank: KC675213.1

FASTA Graphics

Go to:

```
LOCUS       KC675213                467 bp    DNA     linear   INV 26-MAY-
2013
DEFINITION  Trypanosoma evansi isolate CVASU/Bangladesh/01 18S ribosomal RNA
            gene, partial sequence.
ACCESSION   KC675213
VERSION     KC675213.1  GI:506458608
KEYWORDS    .
SOURCE      Trypanosoma evansi
  ORGANISM  Trypanosoma evansi
            Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;
            Trypanosoma.
REFERENCE   1  (bases 1 to 467)
  AUTHORS   Das,S., Alim,M.A., Das,A., Islam,M.S., Siddiki,A.Z.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular detection of Trypanosome from cattle in Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 467)
  AUTHORS   Das,S., Alim,M.A., Das,A., Islam,M.S., Siddiki,A.Z.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-FEB-2013) Molecular Biology and Genetics, Aarhus
            University, Blichers Alle 20, Tjele 8830, Denmark
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
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                     /mol_type="genomic DNA"
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                     /country="Bangladesh: Chittagong"
                     /collection_date="03-Jul-2012"
                     /note="db_xref=Taxon:5697"
rRNA<1..>467
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      121 aatatcctca gcacgtttct tacttcttca cgcaaaagct tggagggtac ctcaccgggg
      181 ggattattct ctctcaagtg aacacctcca aaactgcata aacaaccttg tgtcgacata
      241 tcgcttactt tacatttttc gcgtttagta tggtaactaa ccataaatta caaattgggg
      301 ttaaatcaac acttgtgtat atccgaatga taaagcctgc taatcagata aagctgctaa
      361 tggactctat ccgataatag attgatatcc aaatttaaat cctgcaaatt ggaaagacct
      421 gcaatggaag tcacatcggt aataccatta tgttaacatg cacattt
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3i. *Toxoplasma gondii* isolate CVASU/DPP/sheep/2015 *antigen protein* gene, partial cds

GenBank: KU877882.1

FASTA Graphics

Go to:

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LOCUS       KU877882                151 bp    DNA     linear   INV 13-MAR-
2016
DEFINITION  Toxoplasma gondii isolate CVASU/DPP/sheep/2015 antigen protein
            gene, partial cds.
ACCESSION   KU877882
VERSION     KU877882.1  GI:1003342650
KEYWORDS    .
SOURCE      Toxoplasma gondii
  ORGANISM  Toxoplasma gondii
            Eukaryota; Alveolata; Apicomplexa; Conoidasida; Coccidia;
            Eucoccidiorida; Eimeriorina; Sarcocystidae; Toxoplasma.
REFERENCE   1  (bases 1 to 151)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular characterization of Toxoplasma gondii in small
ruminant
            in Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 151)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAR-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
            Chittagong 4225, Bangladesh
COMMENT     ##Assembly-Data-START##
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      121 atcgatagtt gaccacgaac gctttaaaga a
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3j. *Toxoplasma gondii* isolate CVASU/DPP/Goat-1/2015 *antigen protein* gene, partial cds

GenBank: KU900746.1

FASTA Graphics

Go to:

```
LOCUS       KU900746                150 bp    DNA     linear   INV 26-APR-
2016
DEFINITION  Toxoplasma gondii isolate CVASU/DPP/Goat-1/2015 antigen protein
            gene, partial cds.
ACCESSION   KU900746
VERSION     KU900746.1  GI:1021207553
KEYWORDS    .
SOURCE      Toxoplasma gondii
  ORGANISM  Toxoplasma gondii
            Eukaryota; Alveolata; Apicomplexa; Conoidasida; Coccidia;
            Eucoccidiorida; Eimeriorina; Sarcocystidae; Toxoplasma.
REFERENCE   1  (bases 1 to 150)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular characterization of Toxoplasma gondii in small
ruminant
            in Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 150)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-MAR-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
            Chittagong, Chittagong 4225, Bangladesh
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      121 tcgatagttg accacgaacg ctttaaagaa
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3k. *Toxoplasma gondii* isolate CVASU/DPP/Goat-2/2015 *antigen protein* gene, partial cds

GenBank: KU900747.1

FASTA Graphics

Go to:

```
LOCUS       KU900747                144 bp    DNA     linear   INV 26-APR-
2016
DEFINITION  Toxoplasma gondii isolate CVASU/DPP/Goat-2/2015 antigen protein
            gene, partial cds.
ACCESSION   KU900747
VERSION     KU900747.1  GI:1021207566
KEYWORDS    .
SOURCE      Toxoplasma gondii
  ORGANISM  Toxoplasma gondii
            Eukaryota; Alveolata; Apicomplexa; Conoidasida; Coccidia;
            Eucoccidiorida; Eimeriorina; Sarcocystidae; Toxoplasma.
REFERENCE   1  (bases 1 to 144)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular characterization of Toxoplasma gondii in small
ruminant
            in Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 144)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-MAR-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
            Chittagong, Chittagong 4225, Bangladesh
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      121 agttgaccac gaacgcttta aaga
```

3l. *Toxoplasma gondii* isolate CVASU/DPP/Goat-3/2015 *antigen protein* gene, partial cds

GenBank: KU900748.1

FASTA Graphics

Go to:

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2016
DEFINITION  Toxoplasma gondii isolate CVASU/DPP/Goat-3/2015 antigen protein
            gene, partial cds.
ACCESSION   KU900748
VERSION     KU900748.1  GI:1021207587
KEYWORDS    .
SOURCE      Toxoplasma gondii
  ORGANISM  Toxoplasma gondii
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            Eucoccidiorida; Eimeriorina; Sarcocystidae; Toxoplasma.
REFERENCE   1  (bases 1 to 137)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Molecular characterization of Toxoplasma gondii in small
ruminant
            in Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 137)
  AUTHORS   Mannan,A., Siddiki,A.Z., Islam,M.S., Akter,Y., Ahaduzzaman,M.,
            Masuduzzaman,M. and Hossain,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-MAR-2016) Pathology and Parasitology, Chittagong
            Veterinary and Animal Sciences University, Zakir Hossain Road,
            Chittagong, Chittagong 4225, Bangladesh
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                     /country="Bangladesh"
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                     /collected_by="Abdul Mannan"
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                     /translation="MSICATLVYSQIGRLQSIVDHE"
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       61 agtgaaattc atgagtatct gtgcaacttt ggtgtattcg cagattggtc gcctgcaatc
      121 gatagttgac cacgaac
```

Annex-4: Presentation of research results in different workshops and conferences

Poster presentation:

1. Mannan, A., Hossain, M. A., Islam, M. S., Siddiki, A. M. A. M. Z., "Study on the prevalence of blood parasities of economic importance in cattle in selected areas of Bangladesh". 12th Annual Scientific Conference (Ascon-XII), CVASU, Chittagong, Bangladesh 21-22 march, 2015.
2. Mannan, A., Hossain, M. A., Islam, M. S., Siddiki, A. M. A. M. Z.,"A study on prevalence, molecular identification and characterization of *Babesia* infection in selected hilly, coastal and plain area of Bangladesh". 13th Annual Scientific Conference (Ascon-XIII), CVASU, Chittagong, Bangladesh 02-03 April, 2016.
3. Mannan, A., Hossain, M. A., Islam, M. S., Siddiki, A. M. A. M. Z., "Molecular identification and characterization of *Toxoplasma gondii* in sheep and goats in Chittagong Metropolitan area of Bangladesh". 12th Bangladesh Society for Parasitology Biennial Conference, Bangladesh Agricultural University, Mymensingh, Bangladesh 02 January, 2016.

Oral presentation:

1. "Study on the prevalence of parasites of economic importance in cattle inBangladesh". Annual Research Review Workshop, Chittagong Veterinary and animal Sciences University 11 September, 2013.
2. "A serological and molecular study on *Toxoplasma gondii* in sheep and goats in Chittagong Metropolitan Area". 13th Annual Scientific Conference of CVASU (Ascon-XIII), CVASU, Chittagong, Bangladesh 02-03 April, 2016.
3. "A Study on prevalence, molecular identification and characterization of blood and tissue protozoan diseases in domestic ruminants". PhD progress seminar, CVASU 29th August, 2015.

Annex-5: Brief biodata of Dr. Abdul Mannan

Dr. Abdul Mannan is a doctoral student in Pathology and Parasitology at the Chittagong Veterinary and Animal Sciences University (CVASU). Before engaging as PhD research fellow he completed the undergradute degree and MS degree from the same institutes in the year of 2006 and 2011 respectively. Dr. Abdul Mannan is a veterinarian and field practitioner who is now working as a Veterinary Surgeon-1 at Sahedul Alam Quaderi Teaching Veterinay Hospital (SAQTVH) under Chittagong Veterinary and Animal Sciences University (CVASU) in Bangladesh. After graduating from Chittagong Veterinary and Animal Sciences University (CVASU), he joined as a Market development officer in the project “Chars Livelihoods Programme” (CLP) of Sirajgonj Sadar upazil under Sirajgonj district which was implemented by National Development programme (NDP) funded by DFID. During his working periods he has performed duties entitle on motivation and awareness raising, conducting and facilitation training and field monitoring and evaluation. After that he joined as Veterinary surgeon-2 in Chittagong Veterinary and Animal Sciences University (CVASU) and in the year 2014 he promoted as Veterinary surgeon-1. As a veterinary surgeon he gets scope to undertake the practical classes along with his regular hospital duties. He completed his master degree in Medicine and Surgery in 2011 under the supervision of Professor M. A. Matin Prodhan. His MS thesis was entitled as "Determination of maternally derived antibody (MDA) in day old broiler chicks and its effect on disease (IBD) prevention". During his tenure as an employee of CVASU, he participated on different scientific seminar, symposium and work to gather clinical knowledge and research methodology. He published six scientific papers on some national and international peer reviewed journal. He is interested to doing research on molecular identification and characterization of blood and tissue protozoa of large and small ruminants. He is now engaged in doing research entitled on " a study on prevalence, molecular identification and characterization of blood and tissue protozoa of domestic animals in Chittagong division, Bangladesh. After completion of his PhD degree he will provide services to SAQTVH, CVASU as a Veterinary surgeon-1.

Annex-6: Manuscript prepared from this research work

1. Mannan, A., Siddiki, A. M. A. M. Z., Masuduzzaman, M., Islam, M. S., Hossain, M. A., 2016. “A Study on prevalence, molecular identification and characterization of *Babesia* infection in cattle in selected Hilly, Coastal and Plain areas of Chittagong division Bangladesh”. Under review inVeterinary Parasitology: Regional Studies and Reports.
2. Mannan, A., Siddiki, A. M. A. M. Z., Masuduzzaman, M., Islam, M. S., Hossain, M. A., 2016. “A Study on prevalence, molecular identification and characterization of *Anaplasma* infection in cattle in selected Hilly, Coastal and Plain areas of Chittagong division, Bangladesh”.
3. Mannan, A., Siddiki, A. M. A. M. Z., Masuduzzaman, M., Islam, M. S., Hossain, M. A., 2016. “Study on prevalence, molecular identification and characterization of *Trypanosoma* infection in cattle in Chittagong Metropolitan area, Bangladesh”.
4. Mannan, A., Siddiki, A. M. A. M. Z., Masuduzzaman, M., Islam, M. S., Hossain, M. A., 2016. “Sero-epidemiological study of toxoplasmosis in small ruminants in Chittagong Metropolitan area of Bangladesh”.
5. Mannan, A., Siddiki, A. M. A. M. Z., Masuduzzaman, M., Islam, M. S., Hossain, M. A., 2016. “Molecular identification and characterization of *Toxoplasma gondii* in small ruminants in Chittagong Metropolitan Area”.