DEVELOPMENT OF SIMPLEX AND MULTIPLEX PCR ASSAY FOR RELIABLE IDENTIFICATION OF HAEMOPARASITIC DISEASES OF CATTLE, BANGLADESH

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Parasitology

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Session**:** 2020-2021

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DECEMBER-2022

Authorization

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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TO MY

BELOVED PARENTS

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List of abbreviations and symbols

Abbreviation and symbols	Elaboration
A. marginale	*Anaplasma marginale*
A. centrale	*Anaplasma central*
B. bigemina	*Babesia bigemina*
B. bovis	*Babesia bovis*
B. taurus	*Bos Taurus*
B. indicus	*Bos indicus*
T. annulata	*Theileria annulata*
%	Percentage
°C	Degree Celsius
°F	Degree Fahrenheit
>	Greater than
≥	Greater than equal
<	Less than
≤	Less than equal
bp	Base Pair
CVASU	Chattogram Veterinary and Animal Sciences University
DPP	Department of Pathology and Parasitology
DNA	De-oxy Ribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ID NO.	Identification Number

FY	Financial Year
GDP	Gross Domestic Product
gm	Gram
ml	Mililitre
mPCR	Multiplex Polymerase Chain Reaction
NCBI	National Centre for Biotechnology Information
NFW	Nuclease Free Water
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
RBC's	Red Blood Cells
rRNA	Ribosomal Ribonucleic Acid
rpm	Rotation Per Minute
sp.	Species
TAE	Tris Acetate EDTA
Tams1	Main Merozoite Surface Antigen Gene-1
TBDs	Tick Borne Diseases
µl	Microliter
µm	Micrometer
ng	Nanogram
Pmol	Picomole
UV	Ultra-violet
W/V	Weight / Volume
X	Magnification

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Abstract

Bangladesh is a tropical, agro-based developing country. Anaplasmosis, Babesiosis, and Theileriosis are the most common vector-borne haemoparasitic diseases in cattle. This study not only describes the development and evaluation of a multiplex PCR assay for simultaneous detection of haemoparasitic diseases (*Theileria annulata, Babesia bigemina,* and *Anaplasma marginale*), but also their prevalence ratio. In the multiplex PCR assay, three sets of pre-designed primers were used that targeted the genes *Tams1* for *T. annulata*, *18S rRNA* for *B. bigemina,* and *16S rRNA* for *A. marginale,* with desired amplicons sizes of 751 bp, 504 bp, and 270 bp, respectively, with the use of 2% agarose gel for electrophoresis of amplified PCR products. A total of 350 blood specimens were collected that were tentatively diagnosed as haemoparastic diseases on the basis of clinical signs from three districts of Bangladesh: Chattogram, Rangpur, and Sylhet. Blood samples were stained and preserved at -20°C for further molecular study. In this study, 52% (out of 350) cases were tentatively diagnosed as positive by microscopic examination, among the isolates *A. marginale* (30.3%), *Babesia sp.* (12.9%), and *Theileria sp.* (9%). (Whereas, 21.7% in Chattogram, 17.1% in Sylhet, and 13.1% in Rangpur.) However, in the simplex PCR assay, 35.7% of the cases (14.9% in Chattogram, 12% in Sylhet, and 8.9% in Rangpur) showed a positive band in electrophoresis. Mixed haemoparasitic infection is very common in cattle. Overall, 13.1% of infections were detected positive by multiplex PCR; among them, 2% were diagnosed as positive for all three haemoparasites and 3.71% were diagnosed as anaplasmosis+Babesiosis and so. The partial gene sequencing and phylogenetic analysis of the nucleotide sequences expressed the fidelity of the primer pairs that were used in multiplex PCR, which was found to be 100% sensitive and 85% specific for the detection of infections. In multiplex PCR, amplification of multiple target sequences in one assay is helpful for diagnosis of multiple organisms at a time. This is also time-saving and cost-effective compared to other two methods, but requires several trials for optimization of annealing temperature and shows false negative results in minor errors. Beyond all limitations, multiplex PCR assay is precise and could be used as a robust tool for easy, sensitive, specific, and simultaneous diagnosis of haemoparasitic diseases in cattle.

Keywords: Vector-borne haemoparasitic diseases, Anaplasmosis, Babesiosis, Theileriosis, simplex PCR and Multiplex PCR assay.

Chapter-1: Introduction

Bangladesh, officially known as "The People's Republic of Bangladesh," is the eighth-most populous agro-based developing country in South Asia's northeastern part. Bangladesh is a predominately fertile country with 148,460 square kilometers and more than 165 million people (www.britannica.com). Bangladesh has a tropical monsoon climate, distinguished by significant seasonal fluctuations in rainfall, temperatures, and humidity. This weather plays a vital role in the growth and reproduction of several arthropod vectors, such as ticks, flies, and mosquitoes, which are crucial in the spread of haemoparasitic infections (Al Mahmud et al., 2015; Ali et al., 2016; Alim et al., 2012).

The livestock sector is a significant subsector of the agricultural economy of Bangladesh. At constant prices, the contribution of the livestock sector to the GDP in FY 2021–22 was 1.90 percent, and the contribution of livestock to the overall agricultural sector was 16.52 percent (Bangladesh Economic Review, 2022). The role of this sub-sector is immense in meeting the demand for essential animal proteins in the human body's daily diet. The government has already taken a multi-pronged approach to livestock development. The number of cattle in the country in FY 2020–21 stood at 245.50 lakhs. The population of total livestock was about 563.30 lakhs in our country (Bangladesh Economic Review, 2022).

Cattle, mainly crossbred cattle, are vital components of the mixed farming systems in Bangladesh and are found everywhere in the country. Over 80% of rural people raise crossbred cattle. Most significantly, they offer meat and milk, which are abundant in nutrients necessary for human health. As a result, the per capita availability of milk and meat was 193.38 ml/day and 136.18 gm/day, respectively, in FY 2020–21 (Bangladesh Economic Review, 2022). We can use them for their draft power, which is required for plows, roads, and farm transportation in Bangladesh's rural areas. However, upgrading livestock is limited by several factors, including the damage caused by various hemoparasitic diseases transmitted by ticks. The cornerstone of controlling disease is limited due to the accurate identification of both infections and vectors. Since 1976, reports of the prevalence of several blood protozoa in Bangladesh have been made (Ahmed, 1976).

Haemoparasitic diseases, namely anaplasmosis, babesiosis, and theileriosis, are tick-borne diseases (TBDs) of cattle distributed throughout the world, particularly in tropical and subtropical countries, including Bangladesh (Ghosh et al., 2007). Bovine anaplasmosis (*Anaplasma marginale, A. centrale),* babesiosis (*Babesia bovis, B. bigemina,* and *B. divergens),* and theileriosis *(Theileria annulata*, *T. parvum*) are known to represent a severe threat to the health and output of cattle (Ananda et al., 2009; Rajput et al., 2005). These illnesses cause a high morbidity and mortality rate in the susceptible population, which has a significant negative economic impact on the dairy business (Shanawaz et al., 2011; Velusamy et al., 2014).

Haemoparasitic diseases are highly fatal. Those haemoparasitic diseases were defined as those that are visible through a light microscope and remain in the bloodstream to complete their life cycle (Demessie and Derso, 2015). Vector-borne protozoa and rickettsia cause most blood parasitic diseases. They are the most prevalent in endemic areas and cause devastating and deadly losses in developing countries, including Bangladesh. The diseases are transmitted through vectors, including ticks, flies, and mosquito species, which are common in farm areas. In addition, these are not only considered a severe economic challenge but also involve zoonoses in many countries around the world (Sparagano, 1999).

Tropical anaplasmosis, bovine babesiosis, and theileriosis cause health and management issues, reducing productivity and causing economic losses in domestic cattle and buffalo worldwide (Ananda et al., 2009). Important consideration in these three diseases is the presence of a carrier state after the animal recovers from the disease. Carrier animals serve as an important infection reservoir for ticks that transmit the infection to healthy susceptible animals. Importantly, outbreaks of these diseases occur when carrier cattle are transported to non-endemic areas. Hence, a laboratory test capable of detecting the carrier state in bovines can help in restricting and controlling the infection (Aubry and Geale, 2011).

However, anaplasmosis is a tick-borne haemoparasitic disease of cattle, caused by the rickettsial organism of the genus *Anaplasma* (Order Rickettsiales, Family Anaplasmataceae). *A. marginale* and *A. centrale* cause the disease in cattle. It occurs in tropical and subtropical climates all over the world and commonly affects ruminants like

cattle, sheep, goats, buffalo, and some wild ruminants. It is mechanically and physiologically transmitted in cattle by ticks. All cattle are susceptible to anaplasmosis; however, it only manifests clinically in young animals. Clinical symptoms of the aforementioned disorders can also include pyrexia, anemia, anorexia, haemoglobinuria, icterus (Kocan et al., 2004), weight loss, decreased milk production, abortion, hyperexcitability due to cerebral anoxia, and finally, the death of affected animals (Richey and Palmer, 1990; Stuen et al., 2003).

According to several studies (Abdullahi et al., 2014; Njiiri et al., 2015), co-infection of these parasites is frequently observed in animals worldwide. Therefore, it is crucial to accurately identify the cause before beginning any treatment and taking any necessary preventative measures (Reetha et al., 2012). Any study has not yet reported the actual economic cost associated with anaplasmosis in Bangladesh. However, considering the calculated loss in other countries, one would assume a substantial loss in small or large-scale dairy farming. Cattle that survive anaplasmosis become lifelong carriers and act as reservoir hosts for the infection of susceptible animals (Radostits et al., 2000; Aubry and Geale, 2011).

B. Bigemina, B. divergens, B. bovis, and *B. major* are all responsible for the tick-transmitted blood protozoa illness known as bovine babesiosis. In tropical and subtropical nations, *B. bigemina* and *B. bovis* are particularly detrimental to the health and production of cattle (Iseki et al., 2010). Infections in cattle are characterized by fever, anorexia, listlessness, dehydration, and progressive anemia, which may be followed by hemoglobinuria and hemoglobinemia leading to jaundice (Ellis et al., 2003; Zintl et al., 2005). Babesiosis in cattle results in significant economic loss due to death, loss of output, expense of control methods, and potential effects on the worldwide trade embargo for cattle (Bock et al., 2004).

Bangladesh frequently faces bovine babesiosis, brought on by *Boophilus microplus* and caused by *B. bigemina* (Samad et al., 1988). The parasite's infliction of hemolytic anemia results in the development of babesiosis, and the disease progresses due to the

overproduction of cytokines and other immune response-related substances (Bock et al., 2004).

Lastly, the most important blood protozoon is *Theileria. Theileria* is a genus of parasites that belongs to the Apicomplexa phylum. Many *Theileria sp.* are found in domestic and wild ungulates in tropical and subtropical regions of the world. The most important species affecting cattle are *T. parva* and *T. annulata*, which cause acute disease and result in high mortality levels. In the past twenty years, theileriosis, a lethal disease of crossbred animals brought on by *Theileria sp.* (*T. annulata, T. parva*) and spread by ticks named *Hyalomma sp.,* has become one of the most common diseases of cattle. Tropical theileriosis, recorded in numerous parts of the world, is caused by *Theileria sp.* (Oliveira et al., 1995; Durrani et al., 2010; Tavassoli et al., 2011).

They greatly impact livestock, affecting 80% of the world's cattle population and causing economic losses due to high morbidity and mortality (Kasozi et al., 2014). About 250 million cattle are at risk of tropical theileriasis worldwide (Erdemir et al., 2012). Clinical signs vary according to the challenge level, ranging from inapparent or mild to severe and fatal. Typically, fever occurs 7–10 days after parasites are introduced by feeding ticks, continues throughout infection, and may be >107°F (42°C). Lymph node swelling becomes pronounced and generalized as infected lymphoblasts increase. Anorexia develops, and the animal rapidly loses condition; lacrimation and nasal discharge may occur. Terminally, dyspnea is common. Just before death, a sharp decrease in body temperature is usual, and pulmonary exudates pour from the nostrils. Death usually occurs 18–24 days after infection (www.msdvetmanual.com).

Several conventional and modern methods are used to detect *Theileria sp.* in host animals. The conventional method involves a microscopic examination of Giemsa-stained blood smears. This method is typically sufficient for identifying acute infections but cannot be used to identify carriers because their parasitemia may be minor (Altay et al., 2008). The gold standard method for diagnosing acute anaplasmosis, babesiosis, and theileriosis has historically been the microscopic examination of thick and thin smears of peripheral blood for detecting *Anaplasma, Babesia,* and *Theileria* parasites (Böse et al., 1995).

Microscopic examination of the blood smear is helpful for diagnosis of haemoparasitic diseases but may not be so in sub-clinical and carrier infections (Salih et al., 2015). Although challenging, accurate diagnosis is essential for controlling bovine haemoparasites, which is usually uncertain (Fosgate et al., 2010). We commonly used peripheral blood for the detection of hemoparasites through the Giemsa staining technique, and other molecular methods such as simplex polymerase chain reaction (PCR) and multiplex polymerase chain reaction (multiplex PCR) were also included (Aubry and Geale, 2011; Molad et al., 2006).

However, nucleic acid-based techniques can detect infection, even in the latent phase, where the level of parasitemia is often below the detection limit of the conventional microscopic method (Gasser, 2006). However, even at low parasitemia stages, PCR-based diagnosis is a very accurate and sensitive molecular diagnostic technique that can distinguish between different species of haemoparasites (Almeria et al., 2001).

Simplex PCR assay has its limitations, as for the diagnosis of each organism species, a separate PCR assay is required, which is labor intensive and expensive. PCR assays with higher sensitivity and specificity can detect the parasite within six hours after blood collection (Charaya et al., 2016). However, these techniques are somehow time-consuming and labor-intensive for their use as epidemiological tools. Therefore, the need of the hour is the development of low-cost and technically less demanding methods that could detect multiple pathogens at once. Multiplex PCR is a modification of the conventional PCR assay that offers the significant advantage of detecting two or more target loci from one or more organisms in a single reaction (Heredia et al., 1996; Markoulatos et al., 2000).

In multiplex PCR, multiple pairs of primers specific for different DNA segments are included in the same reaction to enable the amplification of multiple target sequences in one assay. Multiplex PCR assays are valuable in field studies for the detection of viruses (Heredia et al., 1996; Markoulatos et al., 2000); bacteria (Charaya et al., 2015); and haemoparasites (Bilgiç et al., 2013). Considering the importance of timely and specific diagnosis for controlling haemoparasitic diseases, our present study planned to develop a multiplex PCR assay for simultaneous detection of the most common haemoparasites of cattle in Bangladesh, viz. *A. marginale*, *B. bigemina,* and *T. annulata.*

To verify the specificity of the PCR reactions, we evaluated three sets of primers to analyze samples containing different blood pathogens. A total of 350 blood DNA samples were used to evaluate the reproducibility of multiplex PCR compared with simplex PCR assay. The PCR primers used in this study were confirmed to be 100% species-specific using blood pathogens previously identified by other methods.

The optimized multiplex PCR assay specifically detected *A. marginale, T. annulata,* and *B. bigemina* from both single and mixed parasite DNA preparations. DNA of *A. marginale, T. annulata,* and *B. bigemina* can be detected using a multiplex PCR assay with equal sensitivity. A difference was observed in the sensitivity of the simplex PCR assay compared with the multiplex PCR assay.

Our neighboring country, India, also developed and evaluated a multiplex PCR assay for simultaneous detection of the same parasitic infections in bovines using different sets of primers and different species of parasites (Kundave et al., 2018), and a similar assay was studied by Ashuma et al. (2014) for the diagnosis of three parasites, viz., *T. annulata*, *Trypanosoma evansi,* and *B. bigemina*. Having the same environmental conditions in a neighboring country like India, they have done so much research from this perspective, but no one else has done this type of work in our country previously; we researched it for the first time.

In this study, we have tried to develop an accurate, specific, and sensitive multiplex PCR assay, using a combination of three sets of primers for the identification of haemoparasitic diseases such as *A. marginale*, *B. bigemina,* and *T. annulata* species from blood samples collected from different regions of Bangladesh.

Objectives

1. Determination of the prevalence of vector-borne haemoparasitic diseases (Anaplasmosis, Babesiosis and Theileriosis) through conventional blood smear.
2. Identification and molecular characterization of different haemoparasites through simplex PCR and multiplex PCR
3. Partial gene sequencing and phylogenetic analysis of the nucleotide sequences.

Chapter-2: Review of literature

The key motive of this section is to provide a selective review of the recent and past research works relevant to this study done by the previous researchers both at home and abroad. However, some important findings on different aspects directly or indirectly related to the present studies have been reviewed in this section.

2.1 Etiology, transmission, and epidemiology of haemoparasitic diseases

Bovine anaplasmosis, babesiosis and theileriosis are the most important haemoprotozoan diseases of cattle and small ruminants and are transmitted by various species of ticks (Jongejan and Uilenberg, 2004; Kocan et al., 2004; Kamani et al., 2010).

2.1.1 Bovine Anaplasmosis

Table 2.1 Etiology, clinical signs, and different types of host of anaplasmosis

Etiology	**Clinical signs**	**Others host**
Rickettsial organism of the genus *Anaplasma* (Order: Rickettsiales, Family:Anaplasmataceae). Anaplasmosis in cattle is mainly caused by *A. marginale* and *A. centrale* (De La Fuente et al., 2008) and *A. bovis* (Goethert and Telford, 2003).	Fever (4 to 10 days), weight loss, decreased milk production, severe anaemia, brownish urine, hyper-excitability, jaundice, abortion (Krishnamoorthy, 2018) and mortality without showing any sign of hemoglobinemia and hemoglobinuria during acute form of the disease (Chandratre et al. 2018; Richey and Palmer, 1990). Animals act become carriers for life and act as a reservoir of infection for susceptible animals (Aubry and Geale, 2011; Radostits et al., 2000).	**Cattle** : *A. marginale* and *A. centrale* (De La Fuente et al., 2008; (Kumar and Sangwan, 2010; Lucimar et al., 2014) Small mammals and cattle by *A. bovis* (Goethert and Telford, 2003). Ruminants (*A. phagocytophilum*) (Stuen, 2007).

2.1.2: Bovine babesiosis

Babesiosis is a vector borne disease caused by blood protozoan of the genus *Babesia* which had worldwide distribution and affecting many species of mammals with a major impact on cattle (Bock et al., 2004). The disease also call at different name in different area of the world that are piroplasmosis, tick fever, red water, Texas fever, splenic fever, or tris teza, which is transmitted only by *Ixodid* ticks (Ristic, 1988).

Table 2.2 Etiology, clinical signs, and different types of host of babesiosis

Etiology	**Clinical signs**	**Other Hosts**
B. bovis and *B. bigemina* (Dumler et al., 2001; Lucimar et al., 2014) Babesiosis is the second most common blood parasite (Yabsley and Shock, 2013), *B. bigemina, B. divergens, B. major, B. bovis, B. ovata* (Japan) and *B. occultans* (South Africa) (Bock et al., 2004; Iseki et al., 2010; Uilenberg, 2006)	High fever, haemolytic anaemia (coffee colored urine) (Kocan et al., 2004), jaundice, haemoglobinuria (Kumar and Sangwan, 2010; Soulsby, 1982; Krishnamoorthy, 2018), in appetence, depression, increased respiratory rate, weakness, reluctant move, muscle wasting, tremors and abortion (Ellis et al., 2003; Babes, 1888). In severe cases animals die within 24 hours (De Vos and Potgieter, 1994) general circulatory shock (Sengupta and Ligi, 2018).	Host specific disease, mammals with a major impact on cattle (Bock et al., 2004) *B. bovis* and *B. bigemina* mainly infect cattle, water buffalo and some of wild ruminants (Kistner and Hayes, 1970). white-tailed deer (Holman et al., 2011) Captive woodland caribou in America (Petrini et al., 1995).

2.1.3: Bovine theileriosis

Theileria sp., responsible for tropical theileriosis, is a common protozoan parasite of cattle transmitted by *Hyalomma sp.* Ticks (Oliveira et al., 1995; Durrani et al., 2010; Tavassoli et al., 2011)

Table 2.3 Etiology, clinical signs, and different types of host of theileriosis

Etiology	**Clinical signs**	**Other Hosts**
T. annulata and *T. orientalis* (Vector-*Haemaphysalis sp*.) (Purnel, 1978; Uilenberg, 1981). *T. buffeli*, and *T. sergenti* in Australia and New Zealand (Watts et al., 2016) *T. mutans* and *T. velifera* are found in Africa (Vector-*Amblyomma sp*.) (Abanda et al., 2019)	Anorexia, pyrexia, enlarged prescapular and prefemoral lymph nodes, trembling while standing, pale conjuctival mucus membrane (Singh et al., 2017), corneal opacity, haemoglobinuria, nasal discharge, coughing, grinding of teeth, cachexia, diarrhea and enlargement of superficial lymph nodes is characteristic feature. . Anemia is not a major diagnostic sign of theileriosis (as it is in babesiosis) because there is minimal division of the parasites in RBC and thus no massive destruction of them (Kumar and Sangwan, 2010 ; Krishnamoorthy, 2018).	Cattle (*T. parva* and *T. annulata*) (Chisu et al., 2019) Buffalo (*T. parva*) (Pienaar et al., 2020) Sheep and goats (Hornok et al., 2017; Awad et al., 2020) Horse (*T. equi* and *T. haneyi*) (Bishop et al., 2020)

2.1.4 Transmission

In experiments, anaplasmosis transmission by over 20 tick species has been demonstrated (Kocan et al., 2004). Ixodid ticks, specifically *Boophilus microplus*, are the primary carriers of bovine anaplasmosis, while other ticks from the genera *Rhipicephalus, Dermacentor, Haemaphysalis, Hyalomma*, and *Ixodes* can also spread *Anaplasma sp.* (Kumar and Sangwan, 2010). The infectious agent can also be spread mechanically by contaminated objects, such as needles, castrating knives, ear taggers, and other surgical instruments, or by the mouthparts of biting insects (Ewing et al., 1997). *A. marginale* can be transmitted mechanically, biologically, and transplacentally from a cow to a calf during the gestation phase (Kocan et al., 2004).

Ticks that ingest infected blood can get infected and spread the *Babesia* species when conditions are natural. The main vector for the spread of babesiosis was the tick, *Boophilus* (Singh et al., 2017). These vectors are frequently found in tropical and subtropical nations, including Bangladesh. However, other tick species were also able to spread the disease (Uilenberg, 1981). All around the world, *B. microplus* is the most important vector, with the possible exception of southern Africa, where *B. decoloratus* is the significant vector (Sahibi et al., 1998).

T. annulata, which is widely distributed in North Africa, Southern Europe, India, the Middle East, and Asia, is the cause for theileriosis (Purnel, 1978). All continents are habitats for *T. orientalis*, a parasite belonging to the same genus that was once thought to be benign or very weakly harmful. It is mostly transmitted by *Amblyomma, Rhipicephalus*, and *Haemaphysalis* ticks and causes benign theileriosis (Uilenberg, 1981).

Theileria sp., the protozoan parasite of cattle that causes tropical theileriosis, is frequently reported to be spread by *Hyalomma sp.* ticks (Oliveira et al., 1995; Durrani et al., 2010; Tavassoli et al., 2011).

The vector population, vector transmission capacity, and host vulnerability are just a few examples of the variables that affect the dynamics of these parasite infections (Kocan et al., 2010). Animals that survive anaplasmosis develop a lifelong infection-carrying capacity and

serve as a reservoir for susceptible animals (Aubry and Geale, 2011; Radostits et al., 2000). Compared to zebu and buffaloes, which primarily serve as carriers, the crossbred cattle displayed a higher rate of susceptibility (Jithendran, 1997).

2.1.5 Epidemiology

Anaplasmosis is very common tick-borne disease in worldwide and frequently reported in Asia, Russia, South Africa, Australia, South America and USA (Woldehiwet and Ristic, 1993). Most frequently, *A. bovis* has been found in cattle and buffalo from Africa, the Middle East, and South America (Ooshiro et al., 2008). Animal cases of *A. marginale* and *A. centrale* have been documented in Bangladesh (Ahmed, 1976).

The prevalence of tick-borne babesiosis in cattle varies greatly around the world and is mostly dependent on the environment (AbouLaila et al., 2010). Africa, Asia, Central and South America, southern Europe, and the United States are where they are most frequently reported (Zulfiqar et al., 2012). Several tropical and subtropical regions of the world, including Africa, Asia, Australia, Central and South America, and the West Indies, are home to the significant blood parasites *B. bovis* and *B. bigemina* that affect cattle (Bock et al., 2004).

B. divergens usually affect the cattle of north-western Europe and France (Telford et al., 1993). *B. major* cases have primarily been recorded in European nations such the UK, Netherlands, France, Germany, and Spain (Hornok et al., 2014). *B. ovata* was discovered in eastern Asia (Uilenberg, 1995). *B. bigemina*, which has been identified by various researchers, is the most common *Babesia sp.* in cattle in Bangladesh (Banerjee et al., 1983; Chowdhury et al., 2006; Samad et al., 1989).

2.2 Pathology of haemoparasitic diseases

Table 2.4 Pathology found in anaplasmosis, babesiosis, and theileriosis

Disease name	Pathology
Anaplasmosis	Pathology associated with bile pigments may accumulate in various tissues and gall bladder may be distended due to accumulation of bile. It is not known whether or not *Anaplasma sp* produce toxins (Radostits et al., 2000).
Babesiosis	Those most often associated with an intravascular haemolytic condition, Pale or icteric mucous membranes, thin and watery blood, icteric subcutaneous tissues and abdominal fat, swollen liver with an orange-brown or paler coloration, enlarged gall bladder containing thick granular bile, enlarged, darker kidneys with possible petechial haemorrhages, dark, friable spleen, petechiae or ecchymoses on surface of heart and brain (Krishnamoorthy, 2018).
Theileriosis	These frequently associated with oedematous lymph nodes, hepatic lesions (enlargement, focal necrosis, icteric and fibrosed). The kidneys often were enlarged and congested. Frequently the spleen was enlarged, congested and atrophic shrinkage. Abomasum in many instances was ecchymotic and showed punched out ulcers in mucosa. It also found haemorrhages in subcutis and on most of the serous and mucous membranes on endocardium, pericardium and epicardium, and ulcers in abomasum which rarely extended to intestine, oesophagus, tongue and gums (Krishnamoorthy, 2018; Panda et al., 2011).

2.3 Factors influencing occurance of haemoprotozoan diseases

2.3.1 Effect of Age

All age groups of cattle can develop infections from *Anaplasma spp* and the severity and mortality rate increase with animal age (Alim et al., 2012; Ananda et al., 2009). Contrary to calves, which are more sensitive, animals over two years of age typically acquire clinical illness linked to a high mortality rate (Aubry and Geale, 2011). When calves are older than 6 months, they often suffer modest clinical illness, and clinical indications infrequently appear in calves under 6 months of age (Kocan et al., 2010). One to two year old cattle may show more severe symptoms of the disease, which is usually devastating (Urquhart et al., 1996 and Annetta et al., 2005). In cattle older than 3 years old, anaplasmosis was more common than in young animals (Chakraborti, 2002; Ananda et al., 2009; Nath et al., 2013).

One of the most significant factors affecting the occurrence of bovine babesiosis is the age of the animals (Alim et al., 2012; Ananda et al., 2009). Colostrums provide innate immunity, which helps calves fight against acute infections. This colostral antibody provides calves protection, which may last for up to 6 months in the case of *B. bovis* and 3 to 4 months in the case of *B. bigemina* (Wright, 1990). The proportion of infected animals in enzootic environments rises after 6 months of age. Animals aged 6 to 12 months experienced the highest infection rates. Infection is rare in animals older than five years. Animals under 1 year old are primarily infected with *B. bigemina*, while those over 2 years old are typically infected with *B. bovis* (Radostits et al., 2006). However, there have been numerous reports of babesiosis in calves under a month old (Vairamuthu et al., 2012; Venu et al., 2015). Babesiosis is more common in young cattle than in adults (Kamani et al., 2010; Atif et al., 2012; Rahman et al., 2015).

Both *Babesia* and *Anaplasma* organism are more prevalent in young cattle (>1-2.5 years) than those of adult cattle (>2.5 years). The prevalence of *Anaplasma* infection was 3.8% and 2.3% in young and adult cattle respectively while it was 2.2% and 1.4% in case of *Babesia* infection (Rahman et al., 2015).

Theileriosis and babesiosis are most common in animal over the age of 3 (7.25% and 3.10%, respectively), followed by those between the ages of 2 and 3 (5.12% and 1.70%,

respectively) and those between the ages of 6 months and 2 years (3.52% and 1.17%, respectively) (Al Mahmud et al., 2015).

Whereas newborn calves were protected by colostral immunity, the endemic instability of the research locations may be responsible for frequent infections in adult cattle. Maternal antibodies are responsible for the reduced number of clinical outbreaks in young animals (Cynthia et al., 2011).

The increased infection rate was found in animals between the ages of 6 and 12 months. Infection in animals older than five years old is not prevalent, according to the authors (Chakraborti, 2002; Chowdhury et al., 2006).

2.3.2 Effect of Season

Higher incidence of hemoprotozoan diseases was found soon after pecking of the tick population depending on temperature, humidity, rainfall, etc., which might be the reason for the higher prevalence of such infections in the rainy season (Radostits et al., 2000).

Table 2.5 Prevalence of haemoparasitic diseases in different seasons

Disease name	Summer	Rainy season	Winter	References
Theileriosis	5.83%	6.25%	5.05%	Mahmud et al., 2015
Babesiosis	2.50%	2.27%	2.02%	Mahmud et al., 2015
Anaplasmosis	22%	18%	2%	Nath et al., 2013
Babesiosis		10%	6%	Nath et al., 2013
Tick infestation	21.58%	24.33%	4.03%	Sanjay et al., 2007
Tick infestation		High (Salih et al., 2008)	Low (Mohammad et al., 2017; Zahid et al., 2005)	

2.3.4 Effect of Breed

Numerous investigations were carried out to compare the susceptibilities for *A. marginale* infection between European breeds (*Bos taurus*), local breeds (*Bos indicus*), and their crosses (Chowdhury et al., 2006). Compared to crossbred Zebu cattle, *B. taurus* cattle have a higher risk of developing acute anaplasmosis (Jonsson et al., 2008). However, in an experimental infection, both local and cross breed animals had identical clinical symptoms and were equally susceptible to *A. marginale* (Bock et al., 1997).

Babesiosis is a vector-borne disease that was previously known to have greater levels of intrinsic resistance in pure zebu cattle compared to *B. taurus* and their crosses to *B. bovis* and *B. bigemina* infections (Bock et al., 1997). This resistance is shown by the decreased parasitemia in *B. indicus* compared to *B. taurus* fed in same grazing conditions (Aguirre et al., 1987). Since zebu cattle have a strong resistance to both ticks and babesiosis, farm owners in high-tick-infestation areas of Australia decided to replace pure *B. taurus* cattle with *B. indicus* cattle and their hybrids (Jonsson, 2008). In Bangladesh, it has also been shown that cross-bred cattle are more susceptible to *B. bigemina* than native/local cattle. They reported that the prevalence of babesiosis is higher in crossbred cattle in compare to the local cattle (Chowdhury et al., 2006; Al Mahmud et al., 2015; Alim et al., 2012).

In crossbred cattle as opposed to locally native cattle, hemoprotozoan infections are substantially more common. Babesiosis was found in 2% and 14% of these same calves whereas anaplasmosis was found in 4% and 38% of indigenous and crossbred cattle, respectively. Crossbred cattle are more susceptible to *Babesia* and *Anaplasma* infections than native cattle (Chowdhury et al., 2004; Nath et al., 2013; Sajid et al., 2014; Rahman et al., 2015; Rahman et al., 2015). In various areas of the Chittagong region, red Chittagong cattle have a lower prevalence (1%) of haemoprotozoan infections (Siddiki et al., 2010).

However, paying more attention to the management of crossbred cattle lessens the possibility of pre-exposure to vectors and resulting in little or little immunity development, which causes the occurrence of such diseases to occur more frequently (Ananda et al., 2009; Chowdhury et al., 2006; Siddiki et al., 2010).

2.3.5 Effect of Sex

Male animals were typically less affected by clinical anaplasmosis than female animals. Numerous published studies backed up this assertion (Alim et al., 2012; Atif et al., 2012; Rajput et al., 2005). Female calves may have a higher incidence of anaplasmosis due to physiological changes that reduce immunity during advanced pregnancy or to significant energy loss during nursing in high-yielding animals (Kocan et al., 2010). The extensive use of contaminated needles to provide drugs for milk letdown may potentially contribute to the increased occurrence in female animals (Atif et al., 2012).

Theileriosis was more common in females than in males. Theileriosis and babesiosis were shown to be more common in females (6.66% and 2.59%, respectively) than in males (4.0% and 1.60%, respectively) (Al Mahmud et al., 2015; Singh et al., 2012; Altay et al., 2008).

Male cattle did not show any Babesia infections, but female cattle had a slightly higher anaplasmosis infection rate (3.8%) than male cattle (2.3%) (Rahman et al., 2015). According to the study, Babesia infections are only detected in female cattle. According to the study's data on anaplasmosis susceptibility in relation to sex, which is similar to previous findings, anaplasma infection was more common in female cattle (Alim et al., 2012; Atif et al., 2012; Chowdhury et al., 2006; Kamani et al., 2010; Sajid et al., 2014).

The prevalence of *Babesia* and *Anaplasma* infections is higher in female cattle, possibly due to the fact that they were kept longer for breeding and milk production purposes and were supplied with insufficient feed against their high demand (Kamani et al., 2010).

2.3.6 Effect of Tick infestation

The prevalence of *Babesia* and *Anaplasma* infections was significantly higher in tick-infested cattle than in apparently tick-free cattle. Tick-infested cattle were about six times more at risk of *Babesia* infection than the non-infested cattle. Similarly, tick-infested cattle were found to be seven times more at risk of *Anaplasma* infection than tick-free cattle (Rahman et al., 2015).

Blood-sucking ticks are the vectors of both *Babesia* and *Anaplasma* organisms; the presence of these ticks might influence the occurrence of infections with these organisms. The role of hematophagous flies may not be excluded in the occurrence of and Anaplasma infections (Costa et al., 2013; Francisco de et al., 2013).

2.4 Current trends in the diagnosis of haemoprotozoan diseases

The diagnosis of these diseases in cattle has relied mostly on clinical signs (Kanyari and Kagira, 2000), microscopic examination of blood smears (Sharma et al., 2013; Rahman et al., 2015; and Nath et al., 2013), and antibody detection (Banerjee et al., 1983; and Mahmoud et al., 2015). Several studies employ molecular methods, namely PCR (Karim et al., 2012; Njiiri, 2015), real-time PCR (Njiiri, 2015), multiplex PCR (Bilgiç et al., 2013; Kundave et al., 2018), and genome sequencing (Githaka et al., 2014).

The classical microscopic examination of haemoprotozoan parasites in a Giemsa-stained thin blood smear is a gold standard test (Bisht and Yadav, 2020) that is a relatively cheap and quick method (Salih et al., 2007); however, in chronic infection, it has low sensitivity and usually fails to detect carrier animals (Criado-Fornelio et al., 2009). Traditional diagnostic methods rely on the microscopical demonstration of infective stages in blood or tissue fluids, which is laborious, less sensitive, and cannot differentiate between morphologically similar organisms (Maharana et al., 2016).

The serological tests, including the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA), are capable of detecting antibodies in carrier animals; therefore, they are often used for monitoring surveillance and export certification (Maharana et al., 2016), as well as for large-scale epidemiological studies, but they fail to detect early infection (Sharma et al., 2016).

Different molecular techniques, especially DNA hybridization assays and polymerase chain reaction and its modifications, ensure the detection of infection in the latent phase of the disease. Nucleic acid-based assays are highly sensitive, free from immunocompetence, and can differentiate between morphologically similar parasites (Maharana et al., 2016).

PCR has been widely used for the detection of *Babesia* parasites owing to its high specificity and sensitivity. PCR is now becoming a common tool to detect fragments of genes in any microbe in the shortest possible period of time. Uniplex PCR (uPCR) and multiplex PCR (mPCR) have been used to detect hemoprotozoan diseases with the highest sensitivity and species specificity and can be us ed to detect co-infection in a reaction (Sharma et al., 2016; Markoulatos et al., 2002).

2.5 Parasitological and Molecular identification of Haemoparasites

The dynamics of infection of these parasites depends on factors such as vector population, transmission capability of the vector, and host susceptibility (Kocan et al., 2010). Babesiosis, theileriosis and anaplasmosis are widely distributed throughout the world, particularly in tropical and subtropical countries including India, Pakistan and Bangladesh (Ghosh et al., 2007).

2.5.1 Prevalence of anaplasmosis

Numerous sero-epidemiological and molecular investigations have tried to find out how common anaplasmosis is around the world (Table 2.5). When compared to serological tests, where the researchers detected a higher number of cases, it was discovered that molecular examinations were less sensitive. According to traditional detection methods like microscopy and staining, the prevalence of anaplasmosis ranges among different countries from 0.20 to 70.0%. However, the variation in the serological prevalence of anaplasmosis from 16.57% to 79.40% could be interpreted as demonstrating greater sensitivity of the serological tests. A prevalence rate ranging from 19.05% to 98.6% was observed in certain regions of the world in research employing molecular analysis. In Bangladesh, a number of studies using a parasitological methodology were carried out in the northern, southern, and north-eastern regions of the country. The prevalence ranged from 0.20% to 70.0%, with varying sample sizes. While the incidence ranged from 4.07% to 17.26% in Pakistan, it ranged from 2.64% to 12.08% in neighboring India.

Table 2.6 The prevalence of anaplasmosis in cattle had been reported from different countries. (Different authors used different tools, and sample size were variable)

Country	Regions	Sample size (n)	Methods applied	Prevalence (%)	References
Bangladesh	Sirajgonj	60	Parasitological	70.00	Chowdhury et al., 2006
Bangladesh	Sylhet	100	Parasitological	42.00	Nath and Bhuiyan, 2013
Bangladesh	Chittagong	166	Parasitological	1.00	Siddiki et al., 2010
Bangladesh	Chittagong	648	Parasitological	4.05	Alim et al., 2012
Bangladesh	Rangpur	400	Parasitological molecular	3.50	Rahman et al., 2015a
Bangladesh	Hilly areas	475	Parasitological	14.94	Mohanta and Mondal, 2013
Bangladesh	Sirajgonj	395	Parasitological	25.82	Belal et al., 2014
Bangladesh			Parasitological	5.93% (Subclinical)	Samad et al., 1989
Bangladesh	Baghabari milk shed		Parasitological	33%	Talukdar and Karim, 2001
Pakistan	Sargodha	350	Parasitological	9.71	Atif et al., 2012
Pakistan	Islamabad and Attock	307	Parasitological	17.26	Khan et al., 2004
Pakistan	Punjub	836	Parasitological	4.07	Sajid et al., 2014
India	Tamil Nadu	2637	Parasitological	2.64	Velusamy et al., 2014
India	Punjub	298	Parasitological PCR	12.08 51.01	Ashuma et al., 2013
India	Ludiana District	703	Parasitological	8.53	Singh et al., 2012

	Punjub				
Iran	Khorasan Province	160	Parasitological	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

2.5.2 Prevalence of Babesiosis

In Bangladesh, several studies were conducted in different parts of the country where the classical approach of examining Giemsa-stained blood smears was used to detect babesiosis. 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%.

Table 2.7 The prevalence of babesiosis in cattle had been reported in different countries:

Country	Regions	Sample size (n)	Methodology applied	Prevalence (%)	References
Bangladesh	Sirajgonj	395	Parasitological	2.27	Al-Mahmud et al., 2015
Bangladesh	Sirajgonj	60	Parasitological	3.30	Chowdhury et al., 2006
Bangladesh	Sylhet	100	Parasitological	16.00	Nath and Bhuiyan, 2013
Bangladesh	Chittagong	166	Parasitological	1.00	Siddiki et al., 2010
Bangladesh	Chittagong	648	Parasitological	4.05	Alim et al., 2012
	Noakhali	108	Parasitological	1.85%	
	Khagrachori	108	Parasitological	2.78%	
Bangladesh	Hilly areas	475	Parasitological	16.63	Mohanta and Mondal, 2013
Bangladesh	Rangpur	400	Parasitological and molecular	1.50	Rahman et al., 2015a
Bangladesh	Mymensingh and Dhaka districts	60	serological survey	14.53% (Subclinical)	Banerjee et al., 1983
Bangladesh	Milk vita project		Parasitological	3.28% (subclinical)	Samad et al., 1989
Bangladesh	Sirajgonj	42	Parasitological	24.85%	Shahidullah et al., 1983
Pakistan	Qadirabad	100	Parasitological PCR	18.00 29.00	Chaudhry et al., 2010

Pakistan	Islamabad and Attock	307	Parasitological	0.65	Khan et al., 2004
Pakistan	Sargodha	350	Parasitological	6.57	Atif et al., 2012
India	Tamil Nadu	2637	Parasitological	1.00	Velusamy et al., 2014
India	North-Eastern part	333	PCR	3.60	Laha et al., 2015
India	Punjub	703	Parasitological	1.56	Singh et al., 2012
Iran	Kurdistan	6469	Parasitological	2.10	Fakhar et al., 2012
Srilanka		316	PCR	30.1	Sivakumar et al., 2012
Myanmur		713	PCR	15.75	Bawm et al., 2016
Thailand	Northern Part	700	ELISA IFAT	71.45 72.3	Iseki et al., 2010
Malaysia		100	IFAT	14.00	Rahman et al., 2015
Italy	Southern part	117	ELISA	23.10	Cringoli et al., 2002
Phillipines	Five province	250	PCR	9.4	Yu et al., 2013
Portugal	Central and Southern part	406	ELISA PCR	65.5 62.5	Silva et al., 2009
Nigeria	-	168	Parasitological	9.50	Onoja et al., 2013

2.5.3 Prevalence of theileriosis

Table 2.8 The prevalence of theileriosis in cattle had been reported in different countries

Country	Regions	Methodology applied	Prevalence (%)	Reference
Bangladesh	Mymensing District	Reverse line hybridization assay	55.2%	Roy et al., 2018
Bangladesh	Rajshahi District	ELISA	34%	Ali et al., 2016
Bangladesh	Sirajgang District	Giemsa staining method	5.82%	Al Mahmud et al., 2015
Bangladesh	Dinajpur District	Giemsa staining method	0.29%	Mohammad et al., 2017
Bangladesh	Noakhali district	Giemsa staining method	4.62%	Alim et al., 2012
	Khagrachori district	Giemsa staining method	8.33%	
India	Gujrat	Giemsa staining method	82.94	Vahora et al., 2012
India	Odisha state	Giemsa staining method	74%	Acharya et al., 2017
India	Banglore north	Giemsa staining method	71%	Ananda et al., 2009
India	Uttarkhand	Giemsa staining method and PCR	27%	Afifi et al., 2014
India	Maharashtra state	PCR	15.8%	Kolte et al., 2017
India	Mizoram State	Giemsa staining method	10.75%	Gosh et al., 2018

Pakistan	Chakwal, Faisalabad, Jhang	PCR and recombinant PCR	7.66%	Hassan et al., 2018
Pakistan	Peshawar	Giemsa staining method	69.23%	Rashid et al., 2018a
Pakistan	Sahiwal	Giemsa staining method	38.3%	Qayyum et al., 2010
Pakistan	Kohat, Peshawar	Giemsa staining method and PCR	5.3% and 34%	Khattak et al., 2012
Pakistan	Vehari Muzaffargarh Bahawalnag	Giemsa staining method and PCR	3% and 19%	Shahnawaz et al., 2011
Pakistan	Kasur	Giemsa staining method and PCR	14% and 36%	Durrani et al., 2008
China	Qingyuan Moaming, Changehun, Jilin, Yanan, Linxia, Zhangye	Smear method, PCR, ELISA	43.5% to 67.5%	Zhao et al., 2017
China	Fuling, Tongnan, Khaizou, Rongchang	PCR	27%	Zhou et al., 2019
	Qianjiang	PCR	6.67%	
	Changshou	PCR	2%	
Iran	Ahvaz	Giemsa staining method and PCR	86%	Jalali et al., 2016
Iran	Sistan	Giemsa staining method and PCR	46.8%	Majidiani et al., 2016
Iran	Ardabil	Giemsa staining method and PCR	15.94%	Yamchi et al., 2016

2.6 Reviews of multiplex PCR of haemoparasitic disease

Table 2.9 Occurrence of multiplex PCR of haemoparasitic disease reported in different countries

Country	Infectious agents	Prevalence (%)	References
Bangladesh	*Babesia sp.* and *Theileria sp.*	1%	Hassan et al., 2019
India	*Theileria sp., B. bigemina, Trypanosoma evansi*	17.7%	Ashuma et al., 2014
India	*T. annulata, Trypanosoma evansi, B. bovis*	53.8%	Gaurav et al., 2020
India	*T. annulata* and *T. sergenti.*	2.91 %	Liu et al., 2015
India	*T. annulata, A. marginale ,B. bigemina*	17.8%	Kundave et al., 2018
Japan	*B. caballi* and *B. equi,*	17.9%	Alhassan et al., 2005
Turkey	*Anaplasma sp.* and *Babesia sp.*	41.99%,	Zhou et al., 2019

Chapter-3: Materials and Methods

3.1 Description of study areas

The reference population of this study was conducted in different dairy farms located in the western, northern and southeast parts of Bangladesh. These include Nahar dairy farm at Mirshorai in Chattogram, Rangpur dairy limited at Gangachara in Rangpur, and Sylhet dairy farm in Sylhet. Three Hundred Fifty (350) blood samples from different areas of Bangladesh were selected for this study.

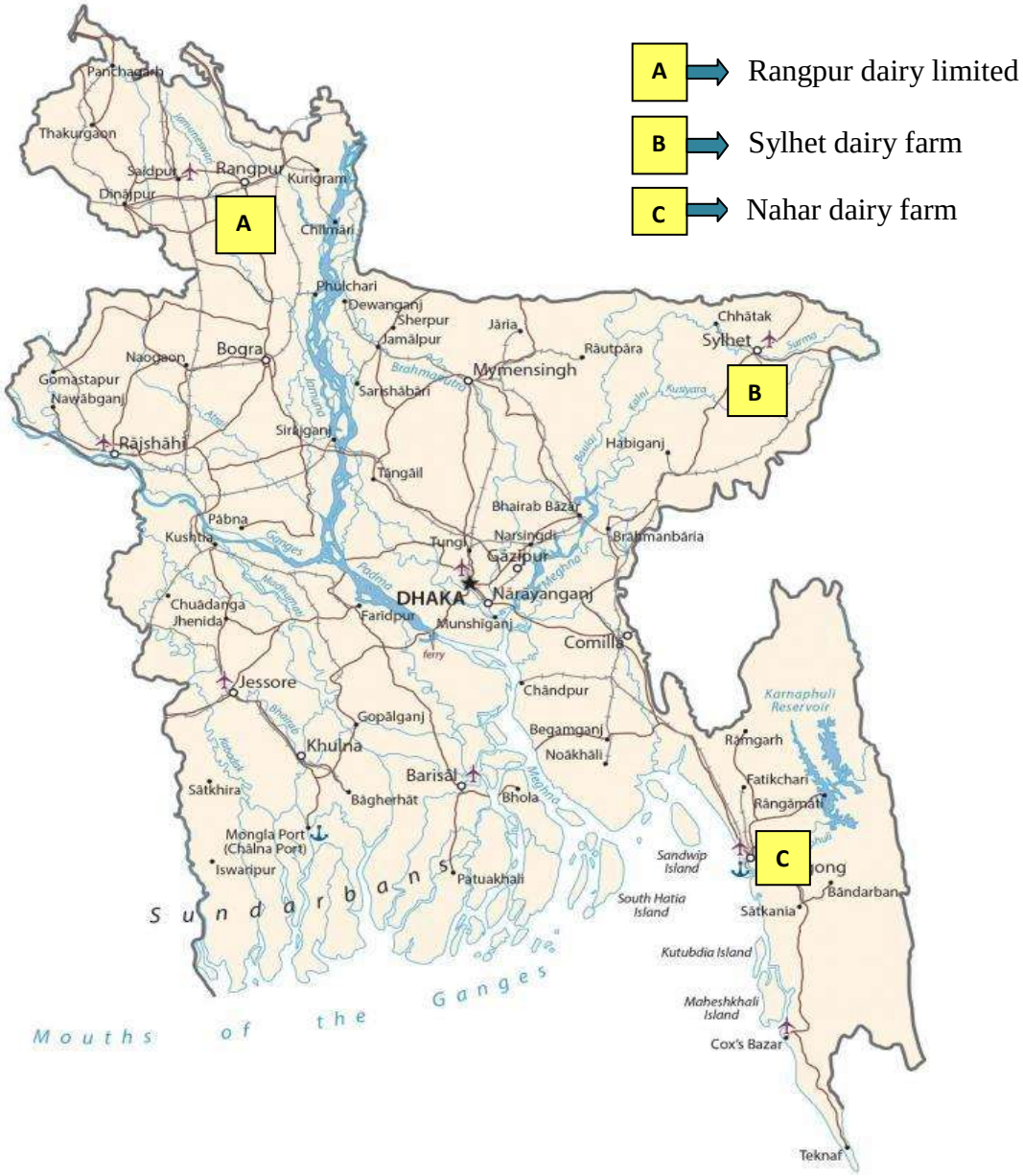


Figure 3.1: Different sample collection sites of Bangladesh

3.2 Study period

The study was conducted for 12 months. The field works started in September 2021 and ended in October 2022; the comprehensive study was conducted in winter (November to March).

3.3 Target animals

Holstein Friesian (HF) crossbred was selected as the target animal. To determine the age and sex susceptibility of different parasites, cattle were classified into three sub-groups such as calf (≤1 year), Young (>1 – < 2.5 years) and Adult (≥ 2.5 years).

3.4 Target sampling

Blood samples were taken from several chosen locations for the diagnosis of blood protozoa. In the subsequent season, samples were taken from the Holstein Friesian crossbreed from both sexes and various age groups. Different types of raw data were collected from dairy farm information such as the farm names, addresses, and animal identification number (ID NO) with their age. Above, all recorded were present on a questionnaire (Appendix A).

3.5 Sample collection

Blood samples were collected from live cattle. Blood samples were collected in an anticoagulant for direct thin blood smears. Approximately 3-5 ml of blood were collected from the jugular vein using a 5 ml disposable plastic syringe from each animal and then preserved in a BD Vacutainer® tube containing anticoagulant (Lithium Heparin). The blood sample in anticoagulant should be held and transferred at 4°C unless it can reach the laboratory within a few hours. The collected blood samples were carried out to the Parasitology Laboratory of Chattogram Veterinary and Animal Sciences University (CVASU). Further examination was done by preparing thin smears from each blood sample (Hendrix and Robinson, 2006) and subsequently staining them with Giemsa stain. Twenty fields from each stained slide were examined under a binocular microscope (100X) to identify blood protozoa at the genus level (Urquhart et al., 1996).

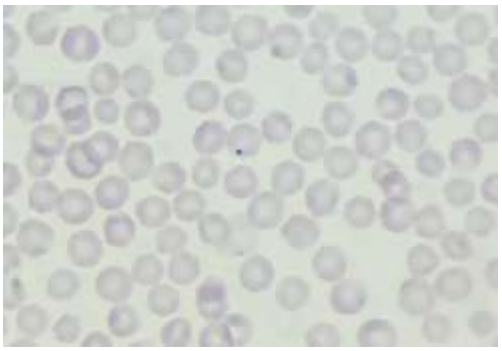
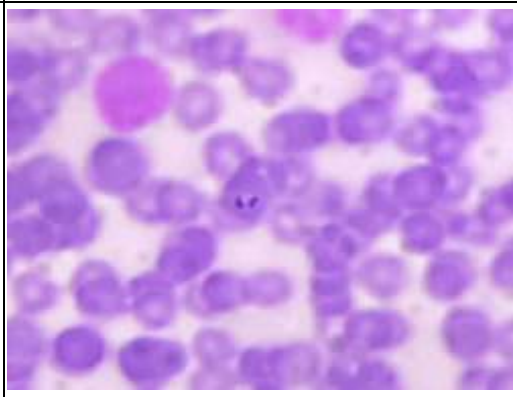
3.6 Staining techniques (Appendix B)

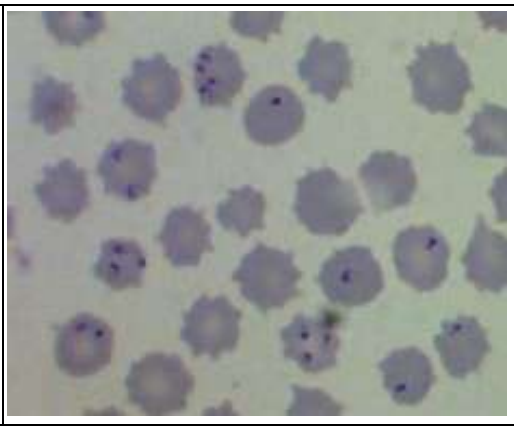
The Giemsa strain is the most popular and common method of identifying blood protozoa and rickettsial disease through microscopic examination of blood smears in clinically affected animals. Three hundred fifty whole blood samples are examined in this study.

Blood smears can be stained in a 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol (100% for 1 min). After staining, the smears are rinsed three or four times with tap water to remove excess stain and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory, but distilled water is not recommended to dilute Giemsa stock. Water should be pH 7.2–7.4 to attain the best resolution with Giemsa stain. Smears were examined under oil immersion at a magnification of 100X.

3.7 Microscopic Examination

Table 3.1 Microscopic identification of haemoparasitic diseases

Infectious agent	**Identifying characteristics**	**Figure 3.2 Microscopic observation**
Anaplasma marginale	Organisms in blood smears appeared as dense, spherical dot-like bodies (0.3-1.0µm) located in periphery of the infected RBCs.	
Babesia sp.	Typically pear-shaped bodies (3-3.5µm) are usually located in the periphery of the infected RBCs. It is frequently found in pairs that are practically parallel or at an acute angle to one another.	

Theileria sp.	Theilerial piroplasms were thin and thick rod-shaped or annular with light staining trailing cytoplasm.	

3.8 Extraction of DNA (Appendix B)

According to the manufacturer's recommendations, genomic DNA was extracted from 200 µl of each blood sample using a commercial DNA purification kit (Addprep Genomic DNA Extraction kit).

At a glance, the extraction procedures of DNA were present in below:

i) 20µl of Proteinase K buffer was transferred into 1.5ml micro-centrifugal tube.
ii) 200µl of whole blood was mixed with Proteinase K solution.
iii) 200µl of binding buffer was added to the sample and mixed well by pulse-vertexing for 15 seconds.
iv) Incubation was done at 56°C for 10 minutes
v) 200µl of absolute ethanol was added and mixed properly by pulse-vertexing for 15s.
vi) Carefully transferred the lysate into the upper reservoir of the spin column with 2.0 ml collection tube without wetting the rim.
vii) Thereafter, centrifugation was done at 13,000 rpm for 1 minute.
viii) 500µl of washing buffer 1 to the spin column with collection tube and centrifuge at 13,000 rpm for 1 minute.
ix) 500µl of washing buffer 2 was mixed to the spin column with collection tube and centrifuge at 13,000 rpm for 1 minute.
x) Additional centrifugation at 13,000 rpm for 1 minute for drying of spin column.
xi) Transferred the spin column to the new 1.5ml micro-centifugal tube.
xii) 70-80 µl of Elution buffer was added and stood for at least 1 min.

xiii) The genomic DNA was eluted by 13,000 rpm for 1 minute.

Next, concentration and purity of isolated DNA was measured using spectrophotometer and diluted in aliquots of 100 ng/µl as well as quality was checked in agarose gel electrophoresis (2.0% gel). Aliquots of extracted DNA were labelled properly and stored at –20°C until further use.

3.9 Optimization of single PCR assay

The custom synthesized PCR primer sets viz. Tamulti-F/R, Bb18S F/R and Amar16S F/R were initially used to optimize single PCR assay to amplify the template DNA of *T. annulata, B. bigemina* and *A. marginale*, respectively.

Table 3.2 Reagents used for PCR amplification of the Haemoparasitic diseases

Serial No	Name of the Component	Amount
1	2X master mix (One Taq^{R}, Quick load^{R})	10 µl
2	Forward primer (10 pmol/ µl)	1 µl
3	Reverse primer (10 pmol/ µl)	1 µl
4	Nuclease Free water	5 µl
5	Template DNA	3 µl
	Total	20 µl

Table 3.3 Primer and gene used for the identification of *Anaplasma marginale*, *Babesia bigemina* and *Theileria annulata* are given at below:

Target Organism	**Primer name & Gene name**	**Primer sequence (5'-3')**	**Amplicon size**	**Ref.**
Anaplasma marginale	Amar*16S rRNA*	Forward primer 5'- GGC GGT GAT CTG TAG CTG GTC TGA - 3'	270 bp	Kundave et al., 2018
		Reverse primer 5'- GCC CAA TAA TTC CGA ACA ACG CTT- 3'		
Babesia bigemina	Bb*18S rRNA*	Forward primer 5'-TCC ATT CAA GTT TCT GCC CCA TCA -3'	504bp	Kundave et al., 2018
		Reverse primer 5'- CCA TTA CCA AGG CTC AAA AGC AAC AA - 3'		
Theileria annulata	Tamulti (*Tams 1*)	Forward primer 5'- CCG TTA ATG CTG CAA ATG AGG AGG - 3'	751bp	Kundave et al., 2018
		Reverse primer 5'- GAG GCG AAG ACT GCA AGG GGA G - 3'		

PCR conditions were optimized for *Anaplasma marginale* (*16S rRNA* gene) listed as table 3.4. Reaction was performed in an automatic thermal cycler (Bio-Rad S1000TM, USA).

Table 3.4 Cycling conditions used during PCR for detection of *A. marginale*

SL NO	Steps	Temperature and time	Number of cycles
1	Initial denaturation	94°C for 5mins	
2	Cyclic denaturation	94°C for 45s	40 cycles
3	Annealing	57°C for 45s	
4	Cyclic extension	72°C for 45s	
5	Final extension	72°C for 15mins	
6	Final holding	4^{o}C	

PCR conditions were optimized for *Bebesia bigemina* (*18S rRNA* gene) listed as table 3.5.

Table 3.5 Cycling conditions used during PCR for detection of *B. bigemina*

SL NO	Steps	Temperature and time	Number of cycles
1	Initial denaturation	94°C for 5 mins	
2	Cyclic denaturation	94°C for 1min	35 cycles
3	Annealing	57°C for 1min	
4	Cyclic extension	72°C for 1min	
5	Final extension	72°C for 15mins	
6	Final holding	4^{o}C	

PCR conditions were optimized for *Theileria annulata* (*Tams 1* gene) listed as table 3.6.

Table 3.6 Cycling conditions used during PCR for detection of *T. annulata*

SL NO	Steps	Temperature and time	Number of cycles
1	Initial denaturation	95°C for 5 mins	
2	Cyclic denaturation	94°C for 30s	37 cycles
3	Annealing	55°C for 30s	
4	Cyclic extension	72°C for 30s	
5	Final extension	72°C for 15mins	
6	Final holding	4^{o}C	

Total 5µl PCR product of each sample was electrophorosed in 2% Agarose gel containing 4µl/ml Ethidium bromide.PCR amplification was visualized under UV light (Syngene, UK).

3.10 Visualization of PCR products by Agar Gel Electrophoresis (Appendix B)

2% agarose gel (W/V) was used to visualize the PCR product. Briefly, the procedure followed as follows:

i) 0.75 gm of agarose powder and 50 ml of 1X TAE buffer was mixed thoroughly in a conical flask and boiled in a microwave oven until agarose powder was dissolved.

ii) Then the agarose mixture was cooled at 50°C in a water bath, and one drop of ethidium bromide was added to the mixture.

iii) The gel casting tray was assembled by sealing the gel chamber's ends with tape and placing an appropriate number of combs in the gel tray.

iv) The agarose-TAE buffer mixture was poured into the gel tray and kept for 20 minutes at room temperature for solidification; then combs were removed

and the gel was shifted into an electrophoresis tank filled with 1X TAE buffer and kept until the gel is drowned completely.

v) An amount of 5 µl of PCR product for a gene was loaded into a gel hole.

vi) 3 µl of 100 bp plus DNA marker (Addbio INC, Korea) was used to compare the size of a gene product's amplicon, and the electrophoresis was run at 110 volts and 80 mA for 40 minutes.

vii) Finally, the gel was examined using a gel documentation system (UVP UVsolo touch - Analytik Jena AG).

3.11 Optimization of multiplex PCR assay

In this study, multiplex PCR reaction conditions were optimized based on the results of a single PCR assay. The final concentration of multiplex PCR reagents in 25 µl reaction volume were; 2X master mix 12.5 µl, Tamulti-F (10 pmol/µl) 0.75 µl, Tamulti-R (10 pmol/µl) 0.75 µl, Bb18S-F (10 pmol/µl) 0.75 µl, Bb18S-R (10 pmol/µl) 0.75 µl, Amar 16S-F (10 pmol/µl) 0.75 µl, Amar 16S-R (10 pmol/µl) 0.75 µl, template DNA 2.0 µl and nuclease free water (NFW) to make the final volume.

Table 3.7 Reagents used for Multiplex PCR amplification of the Haemoparasitic diseases

Serial No	Name of the component	Quantity
1	2X master mix	12.5 µl
2	Forward Primer (3 sets)	0.75 µl (each)
3	Reverse Primer (3 sets)	0.75 µl (each)
4	nuclease free water (NFW)	4 µl
5	Template DNA	4 µl
	Total	25µl

PCR conditions were optimized as: initial denaturation at 94°C for 5 mins followed by 37 cycles of denaturation (95°C for 45s), primer annealing (56.5°C for 45s) and extension (72°C for 45s) along with a final extension of 72°C for 15mins. Reaction was performed in automatic thermal cycler (Bio-Rad S1000TM, USA) and total 10µl PCR product of each sample was electrophorosed in 2% agarose gel containing 10µl/ml ethidium bromide. PCR amplification was visualized under UV light (Syngene, UK).

Table 3.8 Cycling conditions used during Multiplex PCR of *A. marginale, B. bigemina* and *T. annulata*

SL NO	Steps	Temperature and time	Number of cycles
1	Initial denaturation	94°C for 5mins	
2	Cyclic denaturation	95°C for 45s	37 cycles
3	Annealing	56.5°C for 45s	
4	Cyclic extension	72°C for 45s	
5	Final extension	72°C for 15mins	
6	Final holding	4^{o}C	

3.12 Selection of primers for multiplex PCR

Three sets of primers targeting the *Tams1* gene of *T. annulata*, *18S rRNA* gene of *B. bigemina* and *16S rRNA* gene of *A. marginale* were designed (Table 3.3) on the basis of sequence information in public domain (GenBank accession No. AF214840, KM046917, HM538192) with the help of Primer Select (DNASTAR-Lasergene) software. All the primers were checked for their properties like Tm value, length, and presence of self and cross dimer using oligonucleotide analyzer software 1.0.2 and were also checked for their specificity in BLAST tool of NCBI before being custom synthesized.

3.13 Phylogenetic analysis

3.13.1 Gene Sequencing

A total of nine samples were randomly selected from the positive blood samples: three *A. marginale* (*16S rRNA* gene), three *B. bigemina* (*18S rRNA* gene), and three *T. annulata* (*Tam1* gene). Selected all gene PCR amplicons were purified using a DNA purification kit (AddPrep Genomic DNA Extraction Kit). The purified PCR products were Sanger-sequenced with a BigDye terminator v3.1 sequencing kit and a 373XL automated sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined on both strands (forward and reverse) of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). The forward and reverse sequences of each of the strains were assembled by the Mega Software alignment program. All the sequences were submitted to Genbank at the National Center for Biotechnology Information (NCBI).

3.13.2 Evolutionary analysis by Neighbor-joining method

BLASTn was performed to compare the different gene sequences that are available in GenBank. By the BLASTn homology search, the nucleotide sequences were determined to correspond with *A. marginale*, *B. bigemina,* and *T. annulata* 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 the 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 11. A bootstrap analysis estimated the tree's stability for 1,000 replications (Felsenstein, 1985).

3.14 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.

Chapter-4: Results

4.1 Parasitological examination

The present study was designed to analyzed samples from different geographic locations in Bangladesh, including Chattogram, Sylhet and Rangpur divisions. A total of 350 whole blood samples (150 blood samples from Nahar dairy farm in Chattogram, 100 samples from Sylhet dairy farm, and 100 samples from Rangpur dairy Ltd.) (Figure 4.1) were collected from clinically suspected cattle with the presence of high fever (≥105°C), loss of appetite, anemia, jaundice, and coffee-colored micturation.

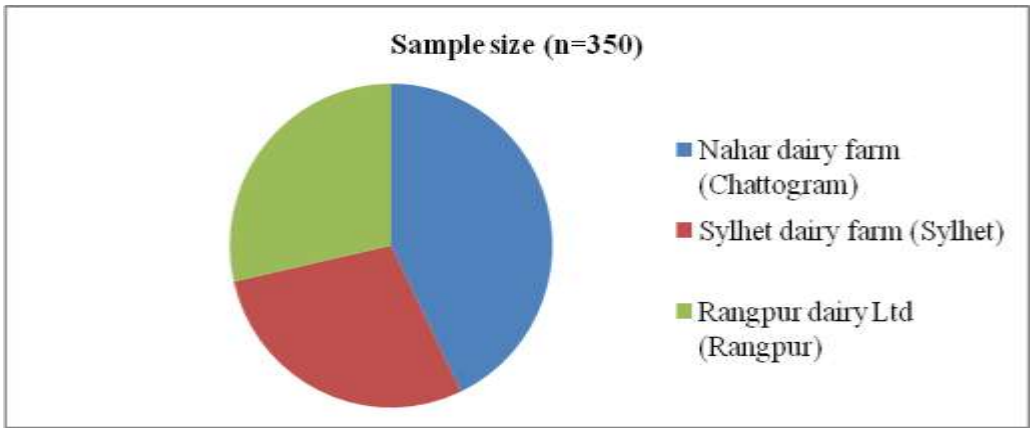


Figure 4.1: Location and sample size from different dairy farm

Using conventional blood smear examination, the organisms of *Anaplasma marginale* in blood smears appeared as spherical dot-like bodies in the periphery of the infected RBCs (Figure 4.2) that was stained with Giemsa solution for all hemoparasites from different areas of Bangladesh.

For *Babesia sp.* infection, the organisms in thin blood smears appeared as pear-shaped bodies, usually located in the periphery of the infected RBCs (Figure 4.3).

On the other hand, during the *Theileria sp.* infection, various morphological appearances of theilerial piroplasms were observed. They were thin and thick rod-shaped or annular, with light staining trailing cytoplasm (Figure 4.4).

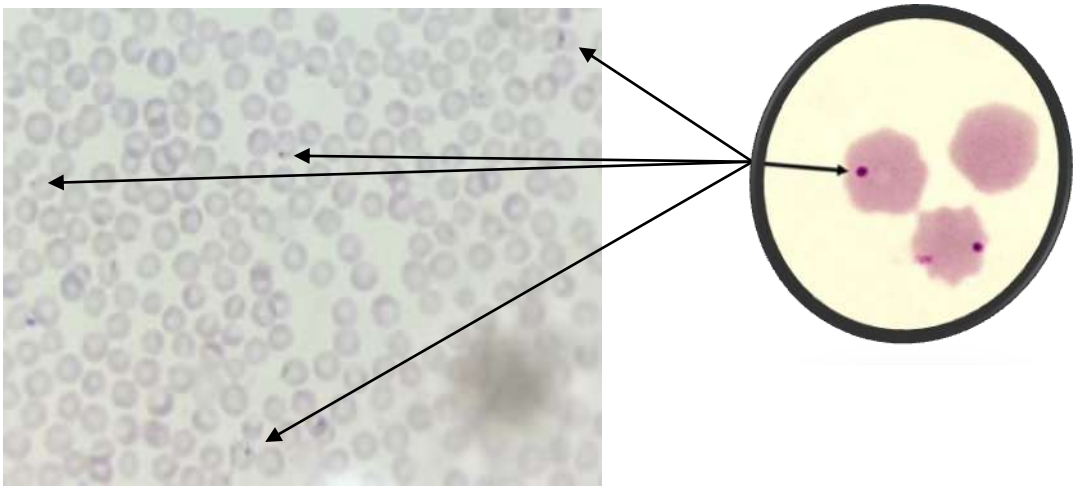


Figure 4.2: Spherical dot shaped bodies in the periphery of infected RBCs (*A. marginale*)

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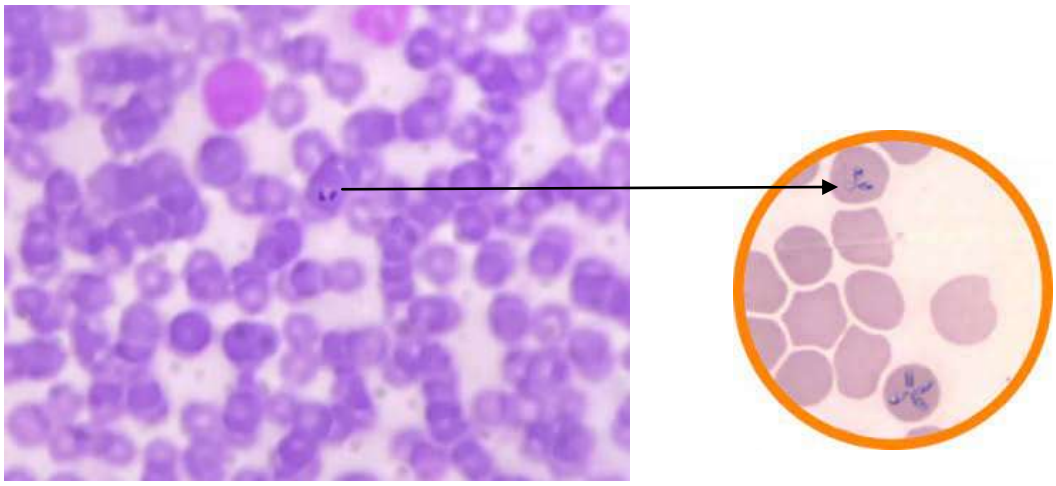


Figure 4.3: Pear-shaped bodies located in the periphery of infected RBCs (*Babesia sp*)

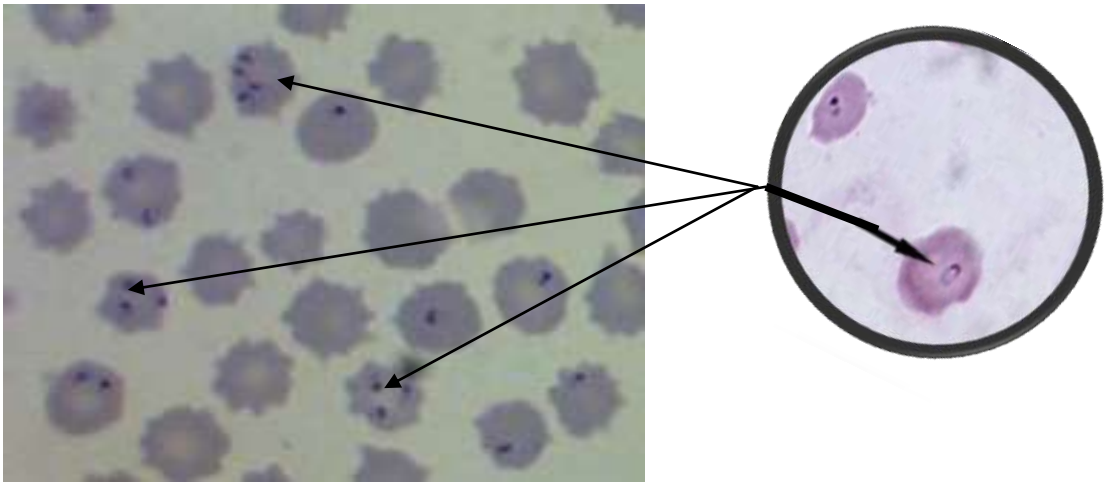


Figure 4.4: Theilerial piroplasms were observed on the infected RBCs (*Theileria sp.*)

Table 4.1 Prevalence and positive cases in microscopic haemoparasites from different areas of Bangladesh

Infectious agent	Chattogram (%)	Sylhet (%)	Rangpur (%)	Overall prevalence (%)
Anaplasma marginale	31.3% (47)	32% (32)	27%(27)	30.3% (106)
Babesia sp.	12.7% (19)	15% (15)	11% (11)	12.9% (45)
Theileria sp.	6.7% (10)	13% (13)	8% (8)	9% (31)
Total	21.7% (76)	17.1% (60)	13.1% (46)	52% (182)

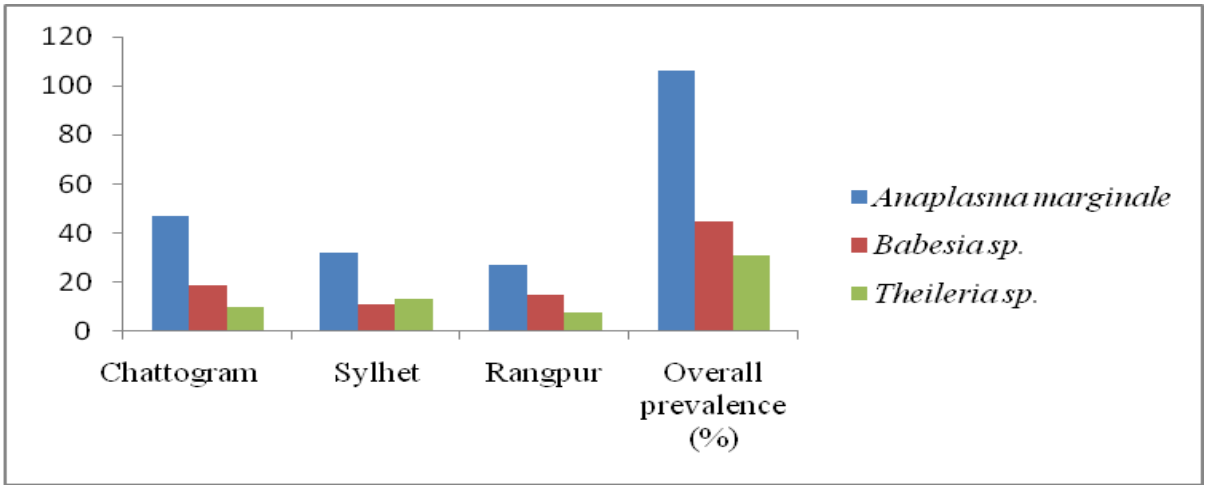


Figure 4.5 Number of microscopically positive haemoparasitic cases from different areas of Bangladesh

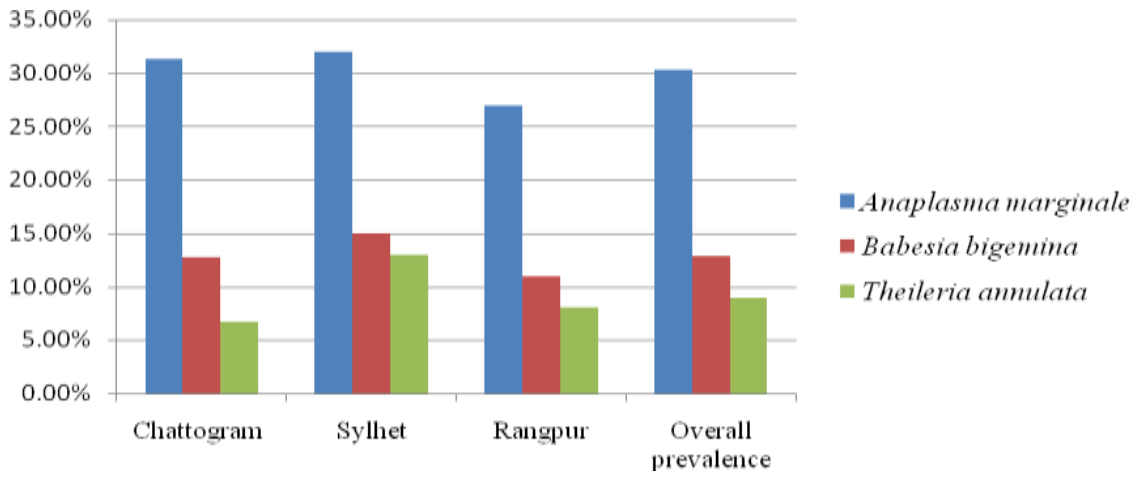


Figure 4.6**:** The prevalence of microscopically positive hemoparasites from different parts of Bangladesh

4.2 Molecular examination

Further molecular investigation using PCR based analyses were applied to all 106 (30.3%) positive samples (verified by microscopic) examination based on the amplification of different genes. However, we used different specific primer like Amar-*16S rRNA* which is specific for *A. marginale*, Bab-*18S rRNA* that is specific for *B. bigemina* and Tamulti-*Tams1* for *T. annulata*. All genes were amplified as a PCR product of expected size when the respective positive control DNA was used as template while no amplification was seen with negative control. All these amplicons produced characteristic bands of 270bp, 504 bp and 751bp on agar gel electrophoresis (Fig-4.10) of *A. marginale* (Fig-4.7), *B. bigemina* (Fig-4.8) and *T. annulata* (Fig-4.9) respectively.

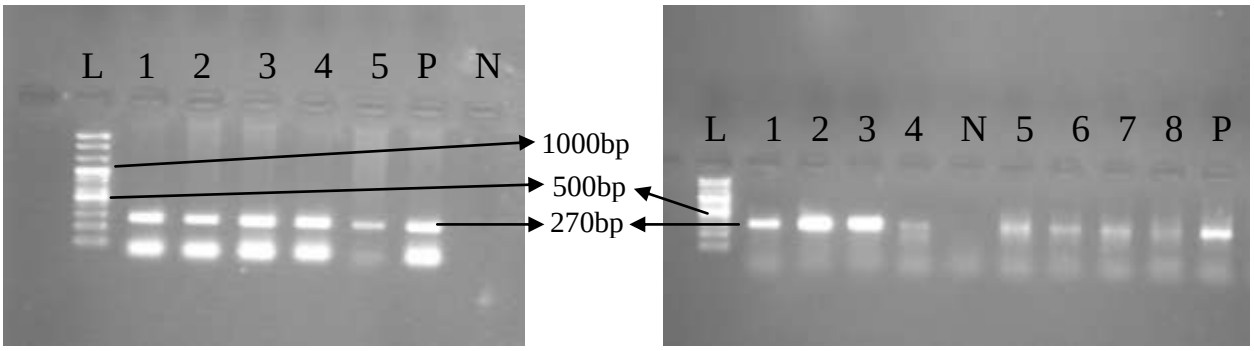
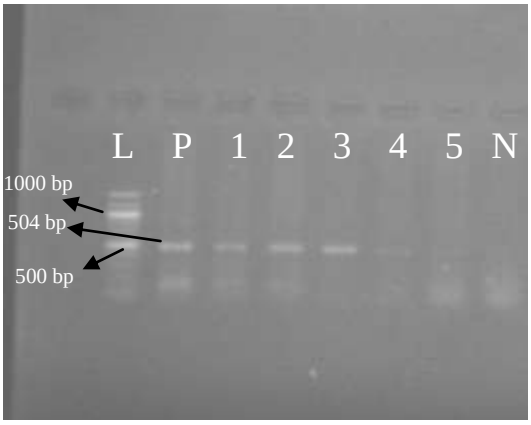


Figure 4.7: Results of PCR assay for *16S rRNA* gene of *A. marginale* isolates; Lane L: 1kb plus Ladder; Lane 1,2,3,4,5: Samples (270 bp); Lane P: Positive control and Lane N: Negative control



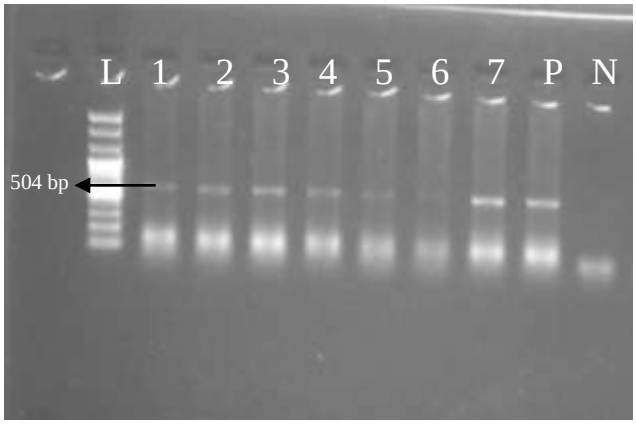


Figure 4.8: Results of PCR assay for *18S rRNA* gene of *B. bigemina* isolates; Lane L: 1kb plus Ladder; Lane 1, 2, 3, 4, 5, 6, 7: Samples (504 bp); Lane P: Positive control and Lane N: Negative control

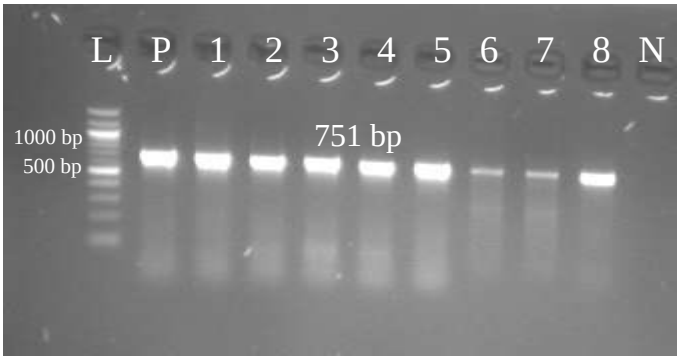


Figure 4.9: Results of PCR assay for *Tams1* gene of *T. annulata* isolates; Lane L: 1kb plus Ladder; Lane 1, 2, 3, 4, 5, 6, 7, 8: Samples (751 bp); Lane P: Positive control and Lane N: Negative control

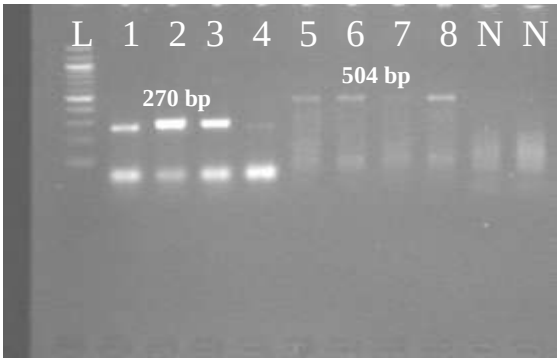


Figure 4.10: Agarose gel electrophoresis of the simplex PCR products with *16S rRNA* gene of *A. marginale* isolates (270 bp) and *18S rRNA* gene of *B. bigemina* isolates (504 bp); Lane L: 1kb plus Ladder; Lane 1,2,3,4,5,6,7,8: Samples ; Lane P: Positive control and Lane N: Negative control

On PCR, we observed overall 20.9%, 9.14% and 5.71% of prevalence on *A. marginale*, *B. bigemina* and *T. annulata*, by turns (table 4.4) on different geographical area.

Table 4.2 PCR positive cases with their percentage in different haemoparasites

Infectious agent	Chattogram N (%)	Sylhet N (%)	Rangpur N (%)	Overall prevalence N (%)
Anaplasma marginale	31 (20.7%)	23 (23%)	19 (19%)	73 (20.9%)
Babesia bigemina	14 (9.3%)	11 (11%)	7 (7.23%)	32 (9.14%)
Theileria annulata	7 (4.7%)	8 (8%)	5 (5%)	20 (5.71%)
Total	52 (14.9%)	42 (12%)	31 (8.9%)	125 (35.7%)

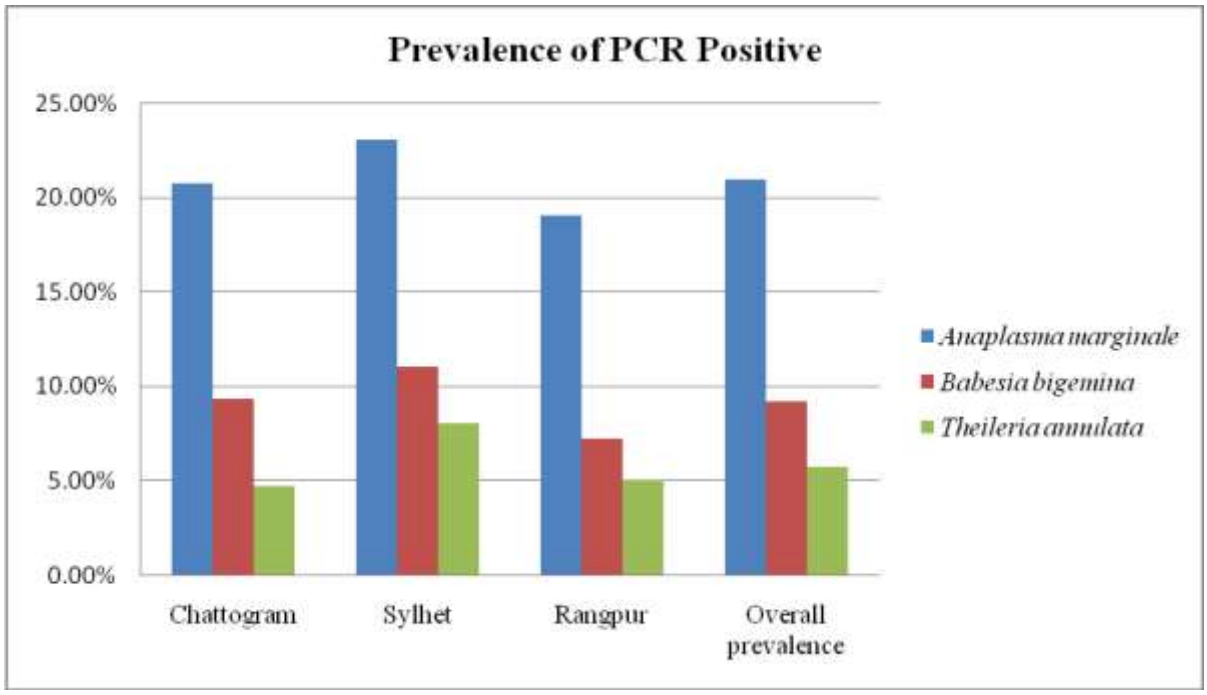


Figure 4.11: PCR positive cases with their percentage in different haemoparasites

4.3 Multiplex PCR

Besides this, mixed infection with *Anaplasma marginale* and *Babesia bigemina* in 19 (5.42%) on conventional blood staining method and 13 (3.71%) on multiplex PCR Method. We also found *T. annulata* and *B. bigemina* in 17 (4.85% on conventionally) and 11 (3.14% on multiplex PCR) as well as *T. annulata* and *A. marginale* in 23 (15) animals were also recorded (table 4.3). The incidence of all haemoparasitic infection in cattle were 16 (4.57%) by microscopy and 7 (2.00%) from multiplex PCR.

Table 4.3 Prevalence on mixed infection through simplex PCR and multiplex PCR

Mixed infection	**Simplex PCR**	**Multiplex PCR**
A. marginale + *B. bigemina*	19 (5.42%)	13 (3.71%)
A. marginale + *T. annulata*	23 (6.6%)	15 (4.3%)
B. bigemina + *T. annulata*	17 (4.85%)	11 (3.14%)
A.marginale + *B.bigemina* + *T.annulata*	16 (4.57%)	7 (2.00%)
Total	75 (21.4%)	46 (13.1%)

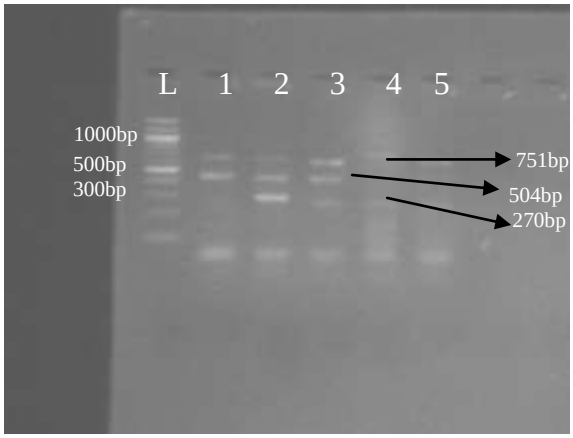


Figure 4.12: Field application of multiplex PCR: Agarose gel (2%) electrophoresis showing amplified 751bp product for *T. annulata*, 504 bp product for *B,bigemina* and 270 bp product for *A. marginale* (L= l00 bp plus DNA molecular marker, 1 = *B. bigemina* and *T. annulata* infection, 2, 3 = *A. marginale, B. bigemina* and *T. annulata* infection, 4, 5=*A. marginale* and *T. annulata* infection)

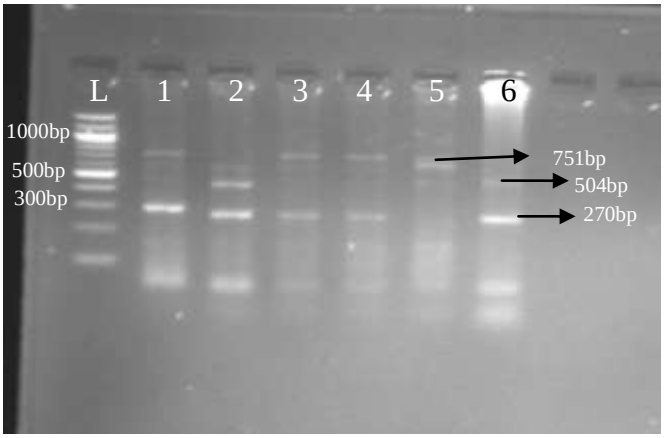


Figure 4.13: Field application of multiplex PCR: Agarose gel (2%) electrophoresis showing amplified 751 bp product for *T. annulata*, 504 bp product for *B,bigemina* and 270 bp product for *A. marginale* (L= l00bp plus DNA molecular marker; 1, 2= *A. marginale B. bigemina* and *T. annulata* infection; 3,4 = *A. marginale and T. annulata* infection, 5= *T. annulata* 6= *A. marginale* and *B. bigemina*

4.4 Phylogenetic Analysis, Genetic Divergence and Multiple Sequence Alignment

We have selected *A. marginale, B. bigemina,* and *T. annulata* for phylogenetic and genetic divergence analysis. Those species may infect all ruminants, especially cattle, and could be of great economic concern. The evolutionary history was inferred using different specific regions like *16S rRNA* for *A. marginale*, *18S rRNA* for *B. bigemina,* and merozoite-piroplasm surface antigen (*Tams1*) for *T. annulata* as a taxonomic marker. Homology searches of the selected isolates revealed 90–100% similarity with local and global isolates deposited in the NCBI GenBank.

The neighbor-joining algorithm was used to establish a phylogenetic relationship among different selected isolates. The selected isolates were clustered into different clades (Figures

4.15, 4.17, and 4.19). However, no single isolate was selected from Bangladesh as different sequences (16S rRNA, 18S rRNA, and Tams1) were unavailable for different hemoparasites (*A. marginale, B. bigemina,* and *T. annulata*) from Bangladesh. We select all isolates as genetic markers, as several previously published studies used this genetic marker in Pakistan, India, and other parts of the world to identify and establish the phylogenetic profile of hemoparasites circulating in ruminants (George et al., 2015; Zeb et al., 2019; Parveen et al., 2021).

The different specific genes such as 16S rRNA of *A. marginale,* 18S rRNA of *B. bigemina,* and Tams 1 gene of *T. annulata* play an important role in genetic variability due to the presence of conserved sequences and some hypervariable regions, which are crucial in determining the evolutionary patterns and discriminating the various haemoparastic species (Ica et al., 2007; Bilgiç et al., 2013). Furthermore, targeting the amplification of different genes is preferably used for accurate identification, classification, and exploration of the population structures of the blood parasites (Nair et al., 2011).

The multiple alignment analysis also showed changes in the nucleotide sequences of different isolates, as seen in Figures 4.14, 4.16, and 4.18. These genetic variations may be due to the genetic variability contributed by the deletions, insertions, and substitutions in the nucleotide sequences of different isolates, which may result in the various degrees of pathogenecity and treatment measures in the field. Thus, on the basis of this analysis and previous findings, we may suggest that using a single diagnostic or immunogenic molecule may not be sufficient for achieving the required goals.

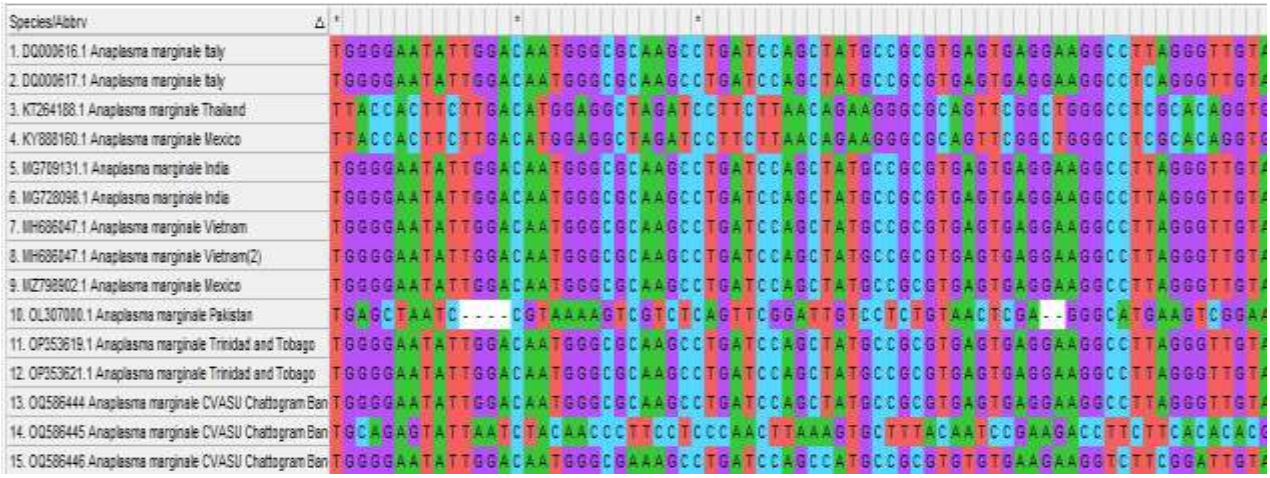


Figure 4.14: CLUSTAL W multiple sequence alignment analysis for the different *A. marginale* isolates from India, Pakistan, Vietnam, Italy, Mexico and Trinidad and Tobago. This figure shows the nucleotide sequence changes between A. marginale isolates from different countries. * means nucleotide similarities.

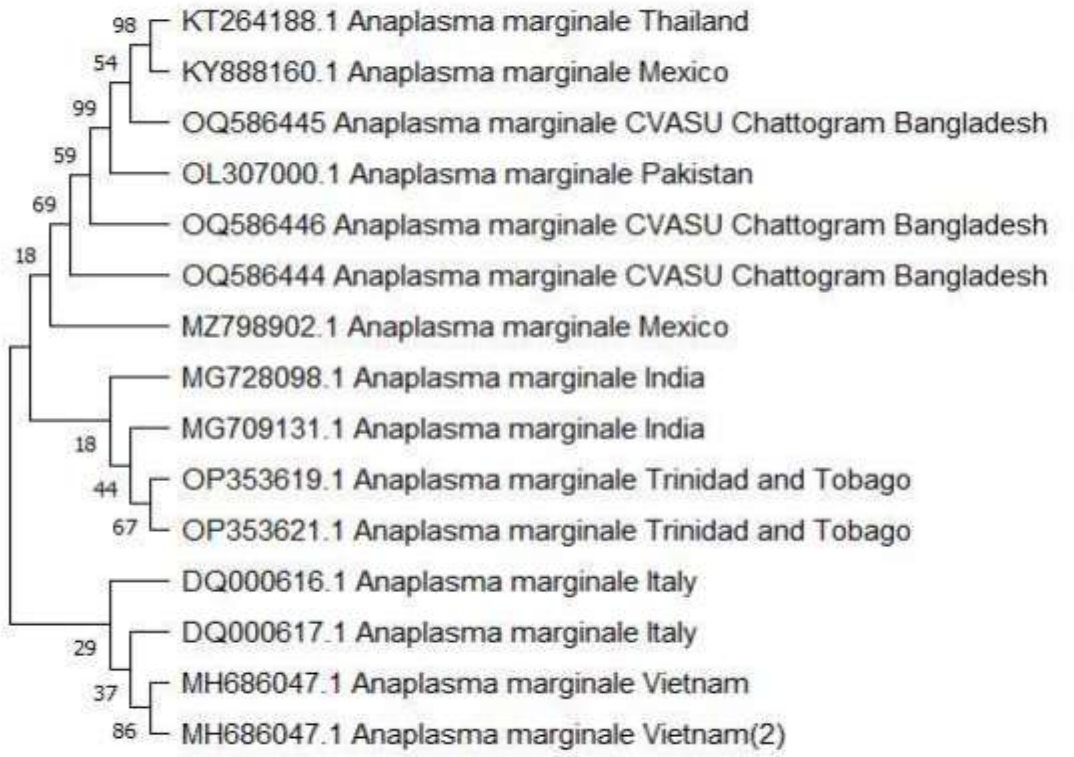


Figure 4.15: Phylogenetic analysis of selected *A. marginale* isolates (16S rRNA gene).

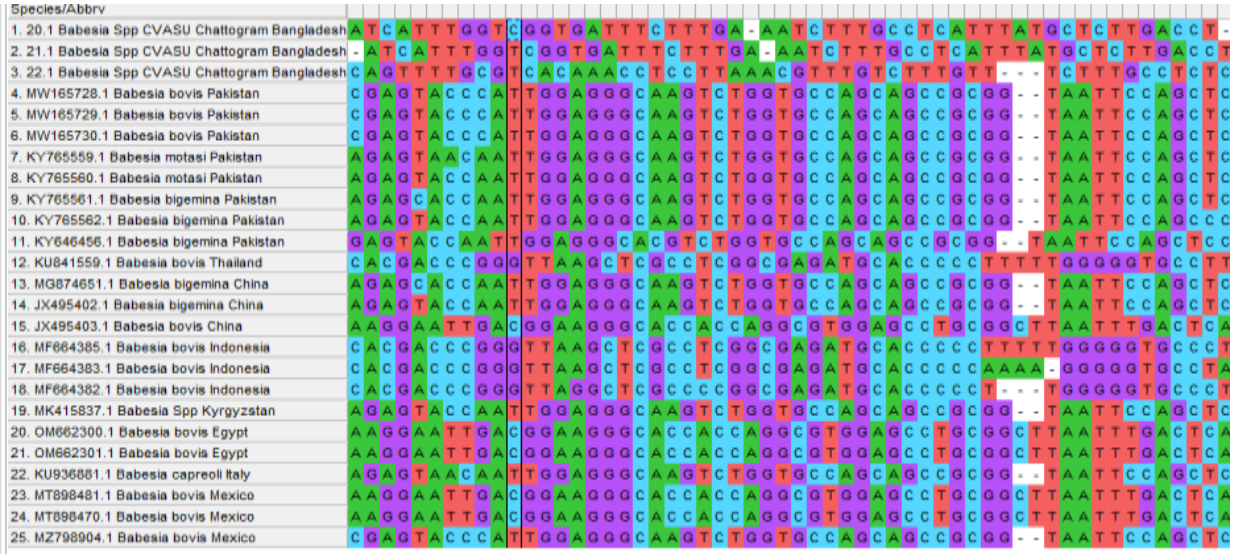


Figure 4.16: CLUSTAL W multiple sequence alignment analysis for the different *B. bigemina* isolates from Pakistan, Thailand, China, Indonesia, Kyrgzstan, Egypt, Italy and Mexico. This figure showing the changes in nucleotide sequences between A. marginale isolates from different country.* means nucleotide similarities.

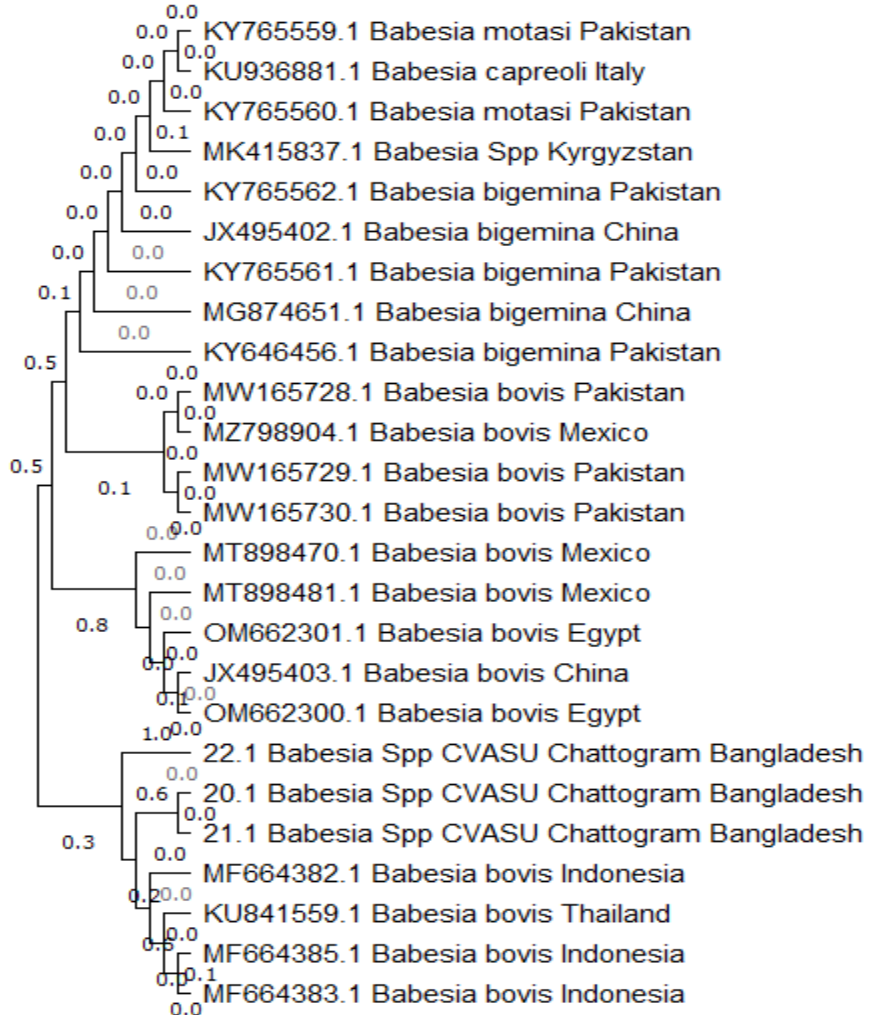


Figure 4.17: Phylogenetic analysis of selected *B. bigemina* isolates (18S rRNA gene).

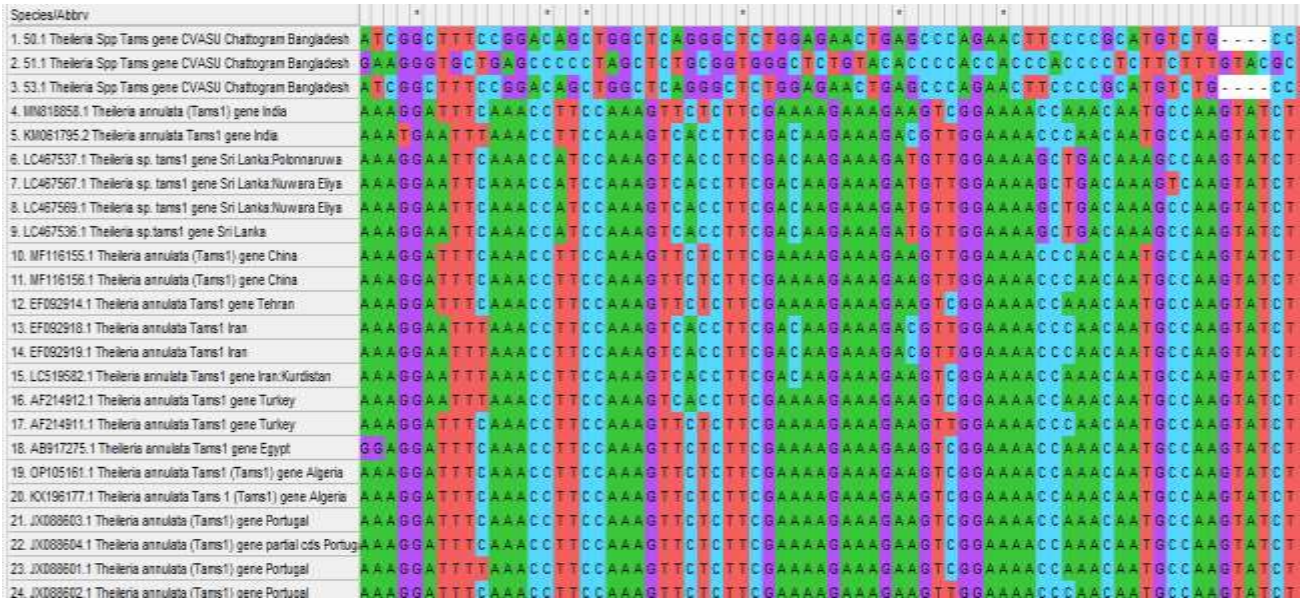


Figure 4.18: CLUSTAL W multiple sequence alignment analysis for the different *T. annulata* isolates from India, Sri Lanka, China, Iran, Turkey, Algeria and Portugal. This figure shows the nucleotide sequence changes between A. marginale isolates from different countries.* means nucleotide similarities.

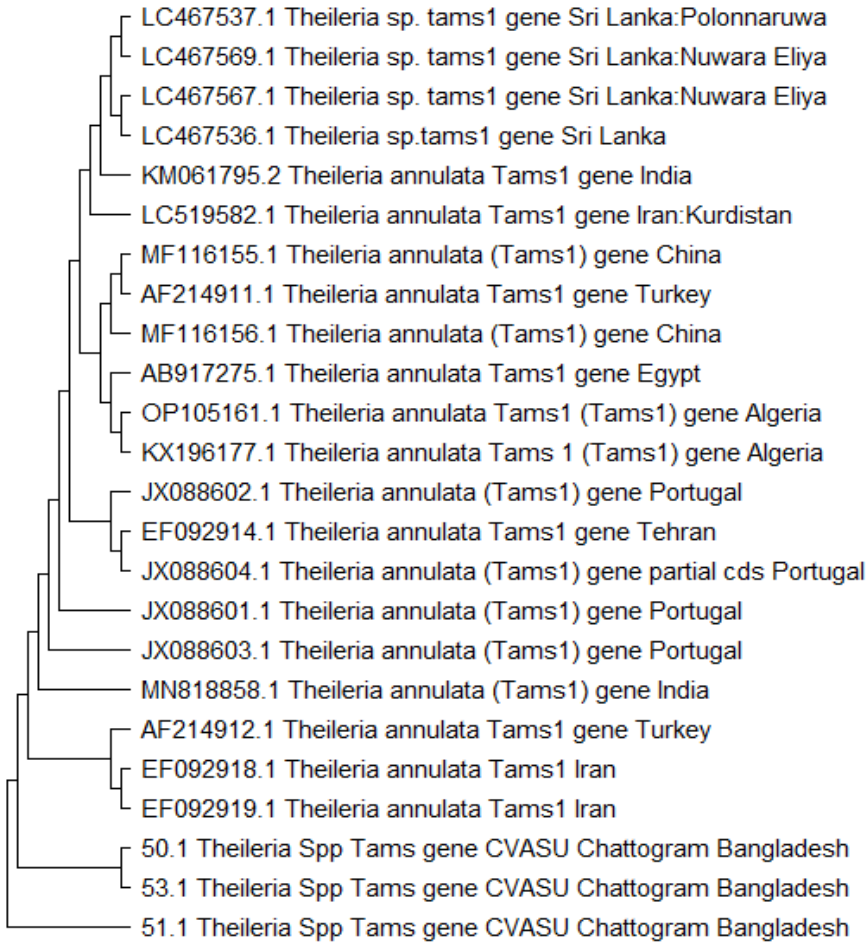


Figure 4.19: Phylogenetic analysis of selected *T. annulata* isolates (Tams1 gene).

Chapter -5: Discussion

Anaplasmosis, babesiosis, and theileriosis are the hemoparasitic illnesses most frequently found in Bangladesh. *T. annulata*, *B. bigemina*, *T. mutans*, and blood rickettsia such as *A. marginale* and *A. centrale* have been previously reported in animals from Bangladesh (Samad and Goutam, 1984; Siddiki et al., 2010; Talukdar and Karim, 2001). The most prevalent cases were associated with babesiosis and anaplasmosis compared to other blood protozoa (Ahmed, 1976; Alim et al., 2012; Banerjee et al., 1983; Rahman et al., 2015).

Several epidemiological studies in Bangladesh have been conducted on vector-borne hemoprotozoan diseases (Belal et al., 2015; Chowdhury et al., 2006; Siddiki et al., 2010; Talukdar and Karim, 2001). Only a few studies were conducted on molecular analysis; the majority were based on microscopic examination of blood smears taken from peripheral blood circulation (Alim et al., 2012; Banerjee et al., 1983; Rahman et al., 2015). As far as we know, Bangladesh has not published any research on the molecular characterization of all blood parasites in cattle.

The overall prevalence of anaplasmosis in this study was 24.3% (n = 350). In this present study, we recorded 31.3%, 27%, and 32% infection rates of anaplasmosis in Chattogram (Nahar and Nolkho dairy farms), Sylhet (Sylhet Dairy Farm), and Rangpur (Rangpur Dairy Ltd). This finding greatly differs with the findings of some other investigators who used samples from other different geographic areas of Bangladesh, such as 70% of *anaplasma* infection being stated in Sirajgonj district (the northern district of Bangladesh) by Chowdhury et al. in 2006, which supports the earlier report of *anaplasma* infection in Bangladesh (Talukdar and Karim, 2001), who recorded 33% anaplasmosis present at Baghabari Milk Shed Area.

The occurrence of subclinical Anaplasma infection in cattle (5.93%) has been reported since 1989 (Samad et al., 1989), and in 2013, Nath and Bhuyian reported that they found a 42% prevalence in Bangladesh. The findings of the current study were a little lower than the findings of some investigators who had different sample sizes and locations (Siddiki et al., 2010; Rahman et al., 2015; Sajid et al., 2014). A somewhat similar observation (9.71% prevalence) was reported by other researchers in neighboring Pakistan (Atif et al., 2012).

The variations in the results could be due to the random selection of clinically suspected animals rather than healthy animals. Moreover, geographical variation plays a vital role in the prevalence of anaplasmosis inside Bangladesh. However, a far lower prevalence (3.7%) was recorded in indigenous cattle in Khagrachori district in Chattogram by a separate report (Alim et al., 2012). But our study just focused on different dairy farms that were located in different areas of Bangladesh. In this research, we were unable to show any differences between seasons, sex (male or female), and types of floor that have the capability to influence the rate of infection with Anaplasmosis.

In this investigation, two molecular methods were used: simplex PCR and multiplex PCR, in addition to the traditional Giemsa staining method. All the positive samples (from microscopy) were successfully amplified by PCR using specific primers for the Amar *16S rRNA* gene (*A. marginale*).

In this study, we found overall 20.9% Anaplasma infection through simplex PCR. However, 19% positive cases were found in Rangpur, which is a little higher than the previous report followed by Rahman et al. (2015), which stated that 3.5% positive cases were recorded in Rangpur Division in 2015. In this study, we also found 23% and 20.7% positive cases in Sylhet and Chattogram, respectively. On the other hand, 51.01% of infections were reported in India (Ashuma et al., 2013), 19.05% in Thailand (Saetiew et al., 2013), 21.90% in Morocco (Hamou et al., 2012), and 98.6% in Brazil (Brito et al., 2010).

Bovine babesiosis caused by *B. bovis* and *B. bigemina* is known to be endemic in Bangladesh (Al Mahmud et al., 2015; Chowdhury et al., 2006; Rahman et al., 2015). While few reports are published on these important protozoa, a variable prevalence rate (1–16.63%) has been reported in cattle in selected areas of the country (Siddiki et al., 2010; Mohanta and Mondal, 2013). The use of conventional detection tools like microscopy is also insufficient for reliable diagnosis of babesiosis, and therefore modern molecular tools were used to unravel the actual scenario of these important protozoa.

The study was therefore complementary to other previous reports and highlighted the need for further molecular investigation to formulate effective control strategies. On the contrary,

B. bigemina infection was at 12.85% positive out of 350 samples recorded in this study, which was similar to Banerjee et al. (1983), who detected a higher (14.53%) prevalence of *B. bigemina*, and also similar to Lima (2017), who recorded the overall 12.7% prevalence of babesiosis in cattle in Bangladesh.

On the other hand, Samad et al. (1989) recorded a 3.28% subclinical prevalence of *B. bigemina* infection in cattle of the selected Milk-Vita Project areas of Bangladesh. This observation was higher than that of the other investigator, who has reported 1.52%–2.29% prevalence in different parts of the country (Al Mahmud et al., 2015; Rahman et al., 2015; Siddiki et al., 2010). Shahidullah (1983) recorded a comparatively lower (2.29%) prevalence rate of such infections on microscopic peripheral blood smear examinations. However, some reports involving Bangladeshi cattle have indicated a higher prevalence, ranging from 3.28% (in subclinical cases) to 7.17% in some areas (Alim et al., 2012; Samad et al., 1989).

Furthermore, we stated that a higher prevalence of babesiosis up to 15% was found in Sylhet, compared with 12.7% and 11% in Chattogram and Rangpur, respectively. This similar type of investigation was also reported in other reports where 16% positive samples were collected from northern districts of Bangladesh (such as Mymensingh and Sylhet) (Banerjee et al., 1983; Nath and Bhuyian, 2013) and 16.67% prevalence was recorded in the hilly area of Chattogram (Alim et al., 2012; Mohanta and Mondal, 2013). Again, the prevalence of babesiosis in Noakhali district was reported as low as 4.62% by previous investigators, which is inconsistent with this present study (Alim et al., 2012).

In our neighboring countries (India and Pakistan), this prevalence was recorded as 1%–6.56% for *B. bigemina* in cattle, which is the most common species in their country (Velusamy et al., 2014; Atif et al., 2012; Singh et al., 2012). They found 17.23% infection of babesiosis through optical microscopy and 26.86% through PCR, and the highest prevalence was recorded at 29% at Qadirabad, Pakistan (Chaudhry et al., 2010), and 9.50% was recorded in Nigeria (Onoja et al., 2013).

In this investigation, we found 9.14% (*18S rRNA* gene) *B. bigemina* infection followed by simplex PCR, which is higher than the previous studies. In 2015, this infection was recorded at 1.50% in Rangpur (Rahman et al., 2015), 1% in Mymensingh (Roy et al., 2017), and 1.33% in Chattogram (Bary et al., 2018).

In India, they recorded the lowest prevalence at 3.60% in the north-eastern part (Laha et al., 2015) than other countries like Pakistan, where recorded at 29% (Chaudhry et al., 2010), Sri Lanka at 30.1% (Sivakumar et al., 2012), 15% in Myanmar (Bawm et al., 2016), and lastly in Portugal, where they stated the highest prevalence at 62.5% (Silva et al., 2009) of babesiosis in cattle

There are seven species of Theileria known to infect cattle; *T. parva* and *T. annulata* are two of the most significant. These parasites have been identified using a variety of criteria and techniques (Dumanli et al., 2005; Durrani et al., 2010). It is challenging to distinguish between *Theileria* species solely on the basis of the morphology of the piroplasm and schizont phases, and confusion may result if mixed infections take place. Although mostly round and circular, *T. mutans* piroplasms resemble those of *T. annulata* (Durrani et al., 2010).

Interestingly, 8.9% of the studied animals were positive for *T. annulata* through Giemsa staining analysis. We found a higher prevalence of 13% in Sylhet, 10% and 8% in Chattogram and Rangpur, respectively. Some researchers reported the same types of observations, like 5.82% of infections (Al Mahmud et al., 2015) found in Sirajganj district and 0.29% of infections recorded (Mohammad et al., 2017) in Dinajpur district. In this study, we found a higher prevalence in Sylhet, which may be due to Bangladesh's hot, humid environmental conditions, which favor the survival of tabanid flies.

Some researchers in India conducted similar research and reported a 10.75–82.94% prevalence (Gosh et al., 2018; Kala et al., 2018; Kumar et al., 2018; Vahora et al., 2012). The highest prevalence was 82.94% (Vahora et al., 2012) in Gujarat, India. These similar types of studies conducted by Shahnawaz et al. (2011) stated that 3% of positive cases,

9.2% (Saleem et al., 2014), 5.3% (Khattak et al., 2012), and 14% (Durrani et al., 2008) were recorded in Pakistan through the conventional blood smear technique.

The current study amplified the parasite's main merozoite surface antigen gene (*Tams 1*) by PCR. The *Tams 1* gene, which has a molecular mass of about 30 kDa, is the most common and immunodominant antigen on the surface of *T. annulata* merozoites that are present in the asexual blood stage. The molecular characterization of *T. annulata* in cattle and buffaloes has previously been the focus of this gene-focused study (Oliveira et al., 1995; Dumanli et al., 2005; Durrani et al., 2010).

According to several investigations, PCR is more sensitive and specific than conventional procedures for detecting carrier ruminants with *Theileria sp.* in their blood but no external symptoms of theileriosis (Oliveira et al., 1995; Kolte et al., 2017; Hassan et al., 2018). We had a similar experience, as the prevalence of *T. annulata* detected through PCR was 5.71% (20 positives out of 350) parasitic detection by microscopic examination of Giemsa-stained blood smears. Additionally, PCR testing revealed that 20 blood samples were parasite-positive. This can be due to the low sensitivity of this PCR because of the large size of the amplicon at 751 bp (Kolte et al., 2017).

In a similar study, the prevalence of *T. annulata* was recorded at 7.66% (Hassan et al., 2018), 19% (Shahnawaz et al., 2011), 23% (Durrani et al., 2010), and 34–36% (Khattak et al., 2012; Durrani et al., 2008) through PCR in cattle.

Further sequencing confirmed the protozoa's genus and species, which were analyzed for phylogenetics. The results from the present study (partial sequencing of the *16S rRNA* gene, the *18S rRNA* gene, and the *Tams1* gene) indicate that *A. marginale*, *B. bigemina*, and *T. annulata* are present in Bangladeshi cattle sporadically. Further comprehensive study with a wide sample number and geographic areas is essential to understand the molecular epidemiology of this important hemoparasite.

In many clinical laboratories, the PCR assay has been used for sensitively identifying hemoparasites and is recognized as an alternative to microscopy. There are numerous publications on establishing PCR assays for identifying specific haemoparasitic species that

infect ruminants (Eriks et al., 1989; Figueroa et al., 1992; Lew et al., 2002; Singh et al., 2012). Some researchers have also developed duplex PCR techniques to identify the co-infection of two hemoparasitic infections in cattle (Kaur et al., 2012; Sharma et al., 2013; Liu et al., 2014). However, field diagnosis will benefit more from multiple pathogen detection in a single tube than from single or duplex PCR experiments (Figueroa et al., 1993; Bilgic et al., 2013; Ashuma et al., 2014; Rahman et al., 2015). The multiplex PCR assay was optimized in the study because specific diagnosis of these hemoparasites has become an obstacle in the implementation of proper control measures for livestock.

The necessary amplicons for *A. marginale* (270 bp), *B. bigemina* (504 bp), and *T. annulata* (751 bp) could be simultaneously produced by multiplex PCR standardized for the simultaneous detection of *A. marginale, B. bigemina,* and *T. annulata* in a single reaction without any non-specific amplification. When different parasites or bovine DNA were employed individually, no amplification of non-target DNA was seen, establishing that each pair of primers is specific for the associated parasite DNA. One parasite species-specific primer failed to generate PCR results from other species. To identify the presence of mixed infections in field conditions, the multiplex PCR procedure standard for the simultaneous detection of hemoparasitic infection in bovine blood has been used (Lew et al., 2002; Bilgic et al., 2013; Ashuma et al., 2014).

Specific diagnosis of these hemoparasites has become a bottleneck in the institution of appropriate control measures; hence, the study optimized the multiplex PCR assay. The target genes, Amar-*16S rRNA*, Bb-*18S rRNA*, and Tamulti (*Tams1*) of *A. marginale, B. bigemina,* and *T. annulata,* were amplified using parasite-specific primers identified in previous studies (Kundave et al., 2018) employed in the investigation. Additionally, any two fragments could be distinguished by more than 200 bp, and the size of amplicons varied from 270 to 751 bp, making it simple to distinguish between bands on gel electrophoresis. The target parasite DNA from the field cases, whether they were single-species infections or infections involving multiple species, could be found using the optimized multiplex PCR conditions (Figueroa et al., 1993; Bilgic et al., 2013; Ashuma et al., 2014).

The test was also proven to be very specific, with no instances of non-specific amplification being noted and the specific amplification being validated by the sequencing of the PCR products. According to the study, multiplex PCR assays (2–4.5%) revealed a greater overall frequency of hemoparasitic infection in cattle than microscopic inspection (4.5–7%). One crucial aspect of bovine hemoparasitic infection is that survivors serve as carriers for that specific infection (Brown et al., 2006; Kocan and de la Fuente, 2003). The results of this study do support the idea that anaplasmosis (73%) in cattle is the most common tick-borne disease in Bangladesh. Another significant tick-borne parasitic disease of cattle, bovine theileriosis, has a low incidence rate (20%), suggesting that either the disease is less common in the targeted areas or that there may be seasonal variations in the amount of parasitaemia. *A. marginale* infection rates may be higher than *B. bigemina* infection rates due to mechanical and biological transmission via biting flies and a variety of tick species.

The overall prevalence of TBDs was 50.17% in cattle, of which *Anaplasma sp.* were 43%, *Babesia sp. were* 19%, *Anaplasma sp.* and *Babesia sp. were* 33%, *Theileria sp.* were 4%, and *Anaplasma sp.* with *Babesia* and *Theileria sp. were* 1% of blood protozoa at 68°C for 30 sec (annealing temperature) stated by Hass.

Similar types of research were also conducted in India, where they found that 20% of samples were positive for multiple (17.7%) and single (2.2%) haemoparasitic infections like *Theileria sp.*, *B. bigemina* at 57°C for 1 min (annealing temperature), and *T. evansi*, as compared to 8.8% (single- 6.6% and multiple- 2.2% positive) by conventional microscopy (Ashuma et al., 2014).

This study was quite similar to ours; here, results of a simplex PCR assay for the detection of individual parasites revealed 48 (41.02%), 27 (23.77%), and 5 (4.27%) samples positive for *T. annulata, T. evansi,* and *B. bovis*, respectively. Sixty-three (53.8%) samples were found positive at 55°C annealing temperature through a multiplex PCR assay, with 15 samples (23.8%) showing mixed infection (Gaurav et al., 2020).

In India, co-infection of *T. annulata* and *A. marginale* in cattle is very common, and *B. bigemina* may also be associated with any one of the two. 12.8% of animals suspected of

being haemoprotozoan were found to be co-infected with two parasites, while 41.02% were infected with only one species. Only a minor divergence was noted in the results of the multiplex PCR compared to the single species PCR.

Additionally, in India, similar types of work had also been done, where microscopic examination of blood samples revealed the presence of single and multiple species of hemoparasites in 25.8% and 2.4% of the samples, respectively. Results of multiplex PCR revealed the presence of single haemoparasitic species infection in 159 cases (34.5%), whereas mixed infection was recorded in 82 (17.8%) samples. The occurrence of individual species infection detected by mPCR at 55°C for 45 sec was used as the annealing temperature in this study. They found infections at 26.03% for *T. annulata*, 3.25% for *B. bigemina*, and 5.20% for *A. marginale* (Kundave et al., 2018).

These types of research were almost identical to that stated that in Turkey, the overall prevalence was 74.78%, *Anaplasma sp.* and *Babesia sp.* were 41.99%, and slightly higher than where the overall prevalence was 38%; there was some variation due to tropical and subtropical regions variation (Zhou et al., 2019).

Additionally, it is known that both the tick and cattle populations are carriers of *A. marginale*, serving as reservoirs for the susceptible and healthy populations (Kocan et al. 2010). As a result, the incidence would continue to be high.

B. bovis, *B. bigemina*, *Theileria sp.*, *A. marginale*, and *T. evansi* were used to detect five hemoprotozoans using multiplex PCR. It was reported that this method was easier and faster than blood film and that it was suitable for large-scale epidemiological studies and the monitoring of drug treatments. They described this technique as a quick and accurate test with good specificity and sensitivity for the identification of hemoparasitic infection in cattle. Multiplex PCR is a very desirable diagnostic method to simultaneously detect several drugs that induce related or identical clinical symptoms and share related epidemiological aspects (Markoulatos et al. 2002).

Moreover, they developed multiplex PCR for amplification of the genomic DNA of *T. annulata*, *A. marginale*, and *B. bovis* using three different primer sets (cytob1, MAR 1bB2,

and bovar2A), and they compared multiplex PCR to single PCR. When parasite DNA from either a single species or a combination of DNA from all three species was utilized in the assay, there was no difference in the detection limit of the multiplex PCR (Bilgic et al., 2013).

The sensitive hypothesis of the single PCR using the DNA template of each species separately was greater than that obtained for the multiplex PCR. The quantity of each product produced throughout the reaction is influenced by the use of template DNA in the reaction mixture as well as competition among primers for a finite number of reagents. Therefore, a high percentage of template DNA combined with a lack of competition between the primers would anticipate that these reactions would occur in the case of a single PCR experiment using single species DNA preparations (Edwards and Gibbs, 1994).

The multiplex PCR assay established in this study was found to be extremely sensitive and capable of detecting the presence of very little parasite DNA. The assay may aid in accurate assessment of tick-borne haemoparasitic illnesses prevalent in a certain geographic area due to its excellent sensitivity and throughput. The poor farming community, whose primary source of income is the animal population, will suffer losses that are reduced with timely diagnosis and the implementation of control measures.

Chapter-6: Conclusion

Bangladesh is a developing country where the livestock sector plays an important role in the rural economy. Various diseases, like parasitic infections, are responsible for substantial morbidity and mortality, leading to unexpected economic losses and decreased productivity for marginal farmers. Tick-borne hemoparasitic diseases (Babesiosis, Anaplasmosis, and Theileriosis) are now a crucial factor for livestock production in Bangladesh. In our present study, we wanted to determine the prevalence of hemoparasitic disease in crossbred cattle along with their conventional (microscopic) examination at 52% and, subsequently, the prevalence of 35.7% at PCR and 13.1% at multiplex PCR from different geographic areas of Bangladesh. The results of the multiplex PCR revealed that the *A. marginale 16S rRNA* gene, the *B. bigemina 18S rRNA* gene, and the *T. annulata tams1* gene are reliable target genes for species identification in clinical isolates, as well as being specific for each of the three target hemoparasitic species.

The developed multiplex PCR method in the present study provides an efficient, less time-consuming, cost-effective, and higher-sensitivity assay for detecting *A. marginale*, *B. bigemina*, and *T. annulata*, the carrier cattle. The method should be useful for addressing the prevalence of both parasites in more areas of Bangladesh and will be helpful in developing control strategies for the co-infection by these pathogens.

Chapter -7: Recommendations

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

a) Different variable factor such as sex, breed, progeny, health status those are the important factor towards the prevalence of hemoparasitic diseases in small and large ruminants that are not discussed in this study.

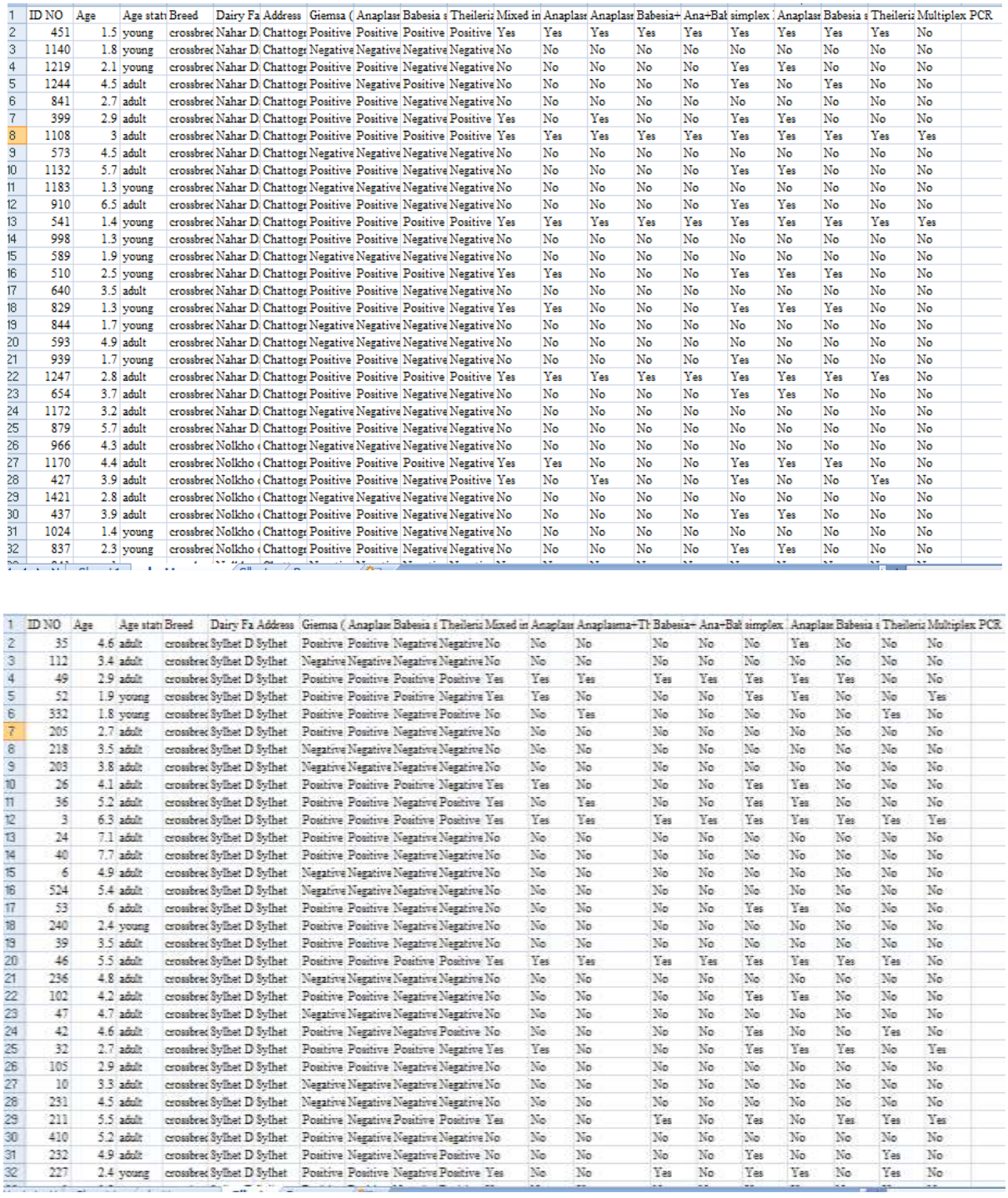
b) Design of primer

b) Identification of differentially expressed gene for selection of therapeutic target.

e) Development of effective drugs or vaccine.

Chapter-8: Appendix

Appendix A



ID NO	Age	Age stati	Breed	Dairy Fa	Address	Giemsa (	Anaplasr	Babesia s	Theileriz	Mixed in	Anaplasr	Anaplasr	Babesia+	Ana+Bal	simplex	Anaplasr	Babesia s	Theileriz	Multiplex	PCR
451	1.5	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	
1140	1.8	young	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
1219	2.1	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
1244	4.5	adult	crossbrec	Nahar D	Chattogr	Positive	Negative	Positive	Negative	No	No	No	No	No	Yes	No	Yes	No	No	
841	2.7	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
399	2.9	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Positive	Yes	No	Yes	No	No	Yes	Yes	No	No	No	
1108	3	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
573	4.5	adult	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
1132	5.7	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
1183	1.3	young	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
910	6.5	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
541	1.4	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
998	1.3	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
589	1.9	young	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
510	2.5	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Positive	Negative	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No	
640	3.5	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
829	1.3	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Positive	Negative	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No	
844	1.7	young	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
593	4.9	adult	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
939	1.7	young	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	No	No	No	No	
1247	2.8	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	
654	3.7	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
1172	3.2	adult	crossbrec	Nahar D	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
879	5.7	adult	crossbrec	Nahar D	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
966	4.3	adult	crossbrec	Nolkho (	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
1170	4.4	adult	crossbrec	Nolkho (	Chattogr	Positive	Positive	Positive	Negative	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No	
427	3.9	adult	crossbrec	Nolkho (	Chattogr	Positive	Positive	Negative	Positive	Yes	No	Yes	No	No	Yes	No	No	Yes	No	
1421	2.8	adult	crossbrec	Nolkho (	Chattogr	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
437	3.9	adult	crossbrec	Nolkho (	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
1024	1.4	young	crossbrec	Nolkho (	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
837	2.3	young	crossbrec	Nolkho (	Chattogr	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	

ID NO	Age	Age stati	Breed	Dairy Fa	Address	Giemsa (	Anaplasr	Babesia s	Theileriz	Mixed in	Anaplasr	Anaplasma+Th	Babesia+	Ana+Bal	simplex	Anaplasr	Babesia s	Theileriz	Multiplex	PCR
35	4.6	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	Yes	No	No	No	
112	3.4	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
49	2.9	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	
52	1.9	young	crossbrec	Sylhet D	Sylhet	Positive	Positive	Positive	Negative	Yes	Yes	No	No	No	Yes	Yes	No	No	Yes	
332	1.8	young	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Positive	No	No	Yes	No	No	No	No	No	Yes	No	
205	2.7	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
218	3.5	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
203	3.8	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
26	4.1	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Positive	Negative	Yes	Yes	No	No	No	Yes	Yes	No	No	No	
36	5.2	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Positive	Yes	No	Yes	No	No	Yes	Yes	No	No	No	
3	6.3	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
24	7.1	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
40	7.7	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
6	4.9	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
524	5.4	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
53	6	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
240	2.4	young	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
39	3.5	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
46	5.5	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Positive	Positive	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	
236	4.8	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
102	4.2	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	Yes	Yes	No	No	No	
47	4.7	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
42	4.6	adult	crossbrec	Sylhet D	Sylhet	Positive	Negative	Negative	Positive	No	No	No	No	No	Yes	No	No	Yes	No	
32	2.7	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Positive	Negative	Yes	Yes	No	No	No	Yes	Yes	Yes	No	Yes	
105	2.9	adult	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
10	3.3	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
231	4.5	adult	crossbrec	Sylhet D	Sylhet	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
211	5.5	adult	crossbrec	Sylhet D	Sylhet	Positive	Negative	Positive	Positive	Yes	No	No	Yes	No	Yes	No	Yes	Yes	Yes	
410	5.2	adult	crossbrec	Sylhet D	Sylhet	Positive	Negative	Negative	Negative	No	No	No	No	No	No	No	No	No	No	
232	4.9	adult	crossbrec	Sylhet D	Sylhet	Positive	Negative	Negative	Positive	No	No	No	No	No	Yes	No	No	Yes	No	
227	2.4	young	crossbrec	Sylhet D	Sylhet	Positive	Positive	Negative	Positive	Yes	No	No	Yes	No	Yes	Yes	No	Yes	No	

Figure 8.1: Appendix 1 (different types of data collected from different dairy farm)

Appendix B

Giemsa staining procedure

Figure 8.2: Collected of sample from cattle

Figure 8.3: Preparation of thin blood smear

Fig 8.4: Drying the smear

Fig 8.5: Administration of Giemsa stain

Fig 8.6: Washing the smear

Fig 8.7: After preparing of smear

Fig 8.8: Microscopic observation

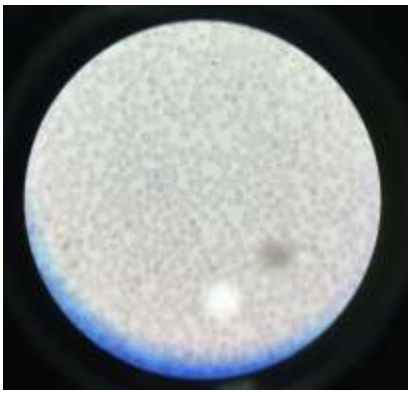


Fig 8.9: Parasites containing slide

DNA Extraction Procedure

Fig 8.10: 20µl of Proteinase K buffer was transferred

Fig 8.11: 200µl of whole blood was mixed

Fig 8.12: Pulse-vertexing for 15s

Fig 8.13: Incubation at 56°C for 10 minutes

Fig 8.14: 200µl of absolute ethanol was added

Fig 8.15: Transferred the lysate within spin column

Fig 8.16: Addition of Elution buffer

Fig 8.17: Centrifugation was done

Fig 8.18: Extracted DNA

Whole Process of PCR/Multiplex PCR

Fig 8.19: Preparation of PCR products

Fig 8.20: Placing PCR tubes in the PCR machine

Fig 8.21: Optimizing of running conditions

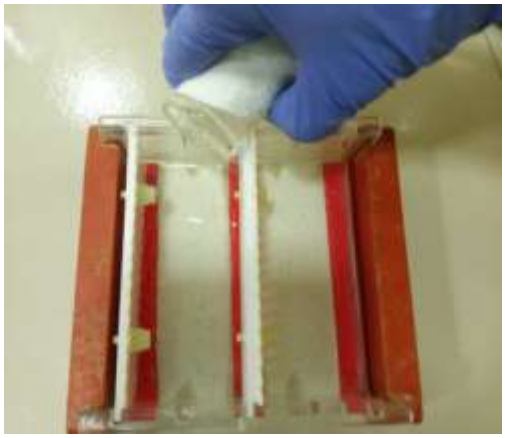


Fig 8.22: Preparation of gel

Fig 8.23: Gel documentation system

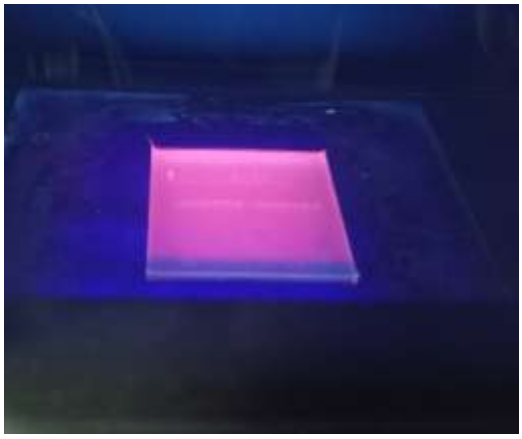


Fig 8.24: Examination of Gel

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Chapter -10:Biography

This is Ayesha Sultana who was born in 1995 at Kapasia Upazilla in Gazipur, Bangladesh. She is a registered veterinarian and daughter of Md. Lutfor Rahman and Morium Sultana. She completed her Secondary School Certificate (SSC) examination in 2011 with GPA-5.00 and Higher Secondary Certificate (HSC) examination in 2013 with GPA-5.00 from Shahid Asad Collegiate Girls' High School and College at Shibpur, Narsingdi. She also completed her graduation in Doctor of Veterinary Medicine (DVM) with CGPA- 3.78 from Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh in 2018. After completion her graduation, she involved in research. She has been author of two research articles published in renowned international journals. Through this research work, she gained hand on expertize and training in numerous analytical and biochemical tools. Her research interests lie in the field of clinical & molecular parasitology, entomology, protozoology, especially vector borne and zoonotic diseases. She is also a research assistant in the Department of Pathology and Parasitology, CVASU under the project "Development of Anaplasma vaccine in dairy cattle, Bangladesh" funded by University Grants Commission (UGC). She has been studying Masters of Science at the Department of Pathology and Parasitology, CVASU. She received fellowship from the National Science & Technology and University Grants Commission (NST) and University Grants Commission (UGC). Her favourite hobby is reading and exploring the unexplored issues. At present, she is doing her thesis project which is essential for awarding her degree of Master of Science (MS) in Parasitology.