Prevalence of gastrointestinal parasitic infections and haemoprotozoan diseases of buffalo in coastal areas of Chattogram division, Bangladesh

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Roll No.: 0122/01

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A thesis submitted in the partial fulfilment of the requirements for the degree of Masters of Science in Parasitology

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JUNE 2023

Authorization

I hereby declare that I am the sole author of the thesis submitted in fulfillment of the requirements for the Degree of Masters of Science (MS) in the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, Chattogram Veterinary and Animal Sciences University (CVASU). I authorize CVASU to lend this thesis or to reproduce the thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

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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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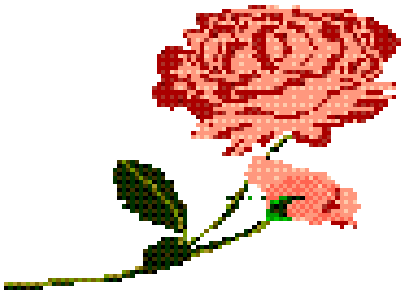
JUNE 2023

DEDICATION

To my parents, thank you for your constant support

&

To my teachers, my mentor. I am forever grateful to you as you took me straight to the top.



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List of Contents

List of Tables

List of Figures

Acronyms and Symbols Used

Acronyms and symbols	Elaboration
BBS	Bangladesh Bureau of Statistics
BER	Bureau of Economic Research
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CFT	Complement Fixation Test
CI	Confidence Interval
CVASU	Chattogram Veterinary and Animal Sciences University
d.f.	Degree of Freedom
DLS	Department of Livestock Services
DNA	Deoxyribonucleic Acid
DPP	Department of Pathology and Parasitology
EPG	Egg per gram
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked Immunosorbent Assay
et al.	et alia
etc.	Et cetera
FAO	Food and Agriculture Organization
FECs	Faecal Egg Counts
FY	Fiscal Year
GDP	Gross Domestic Product
GI	Gastrointestinal
GINs	Gastro Intestinal Nematodes
gm	Gram
ID	Identification
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
pmol	Picomole
RLB	Reverse Line Blot
rpm	Revolution per minute

Acronyms and symbols	Elaboration
sp.	Species
TAE	Tris acetate EDTA
TBD	Tick Born Disease
TBP	Tick Born Parasite
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
UV	Ultraviolet
℃	Degree Celsius
>	Greater than
<	Less than
X	Magnification
µ	Micro
W	Omega
%	Percent

Abstract

Buffalo rearing is an important component of livestock sector in Bangladesh. It is considered as the "Black Gold" next to cattle due to its significant contribution in milk and meat production. The growth, development, and productivity of buffaloes are adversely affected by various types of gastrointestinal (GI) parasitic infections (e.g., helminths, protozoa) and haemoprotozoan diseases (e.g., Anaplasmosis, Babesiosis, and Theileriosis) contributing a great economic loss. Therefore, the current cross-sectional study was designed to determine the prevalence of GI parasitic infections and haemoprotozoan diseases and their associated risk factors (e.g., location, age, sex, deworming status) in buffaloes from four upazillas of two coastal districts of Chattogram division. A total of 158 faecal and 145 blood samples were collected from Kabirhat and Companigonj upazilla of Noakhali district and Sandwip and Boalkhali upazilla of Chattogram district. The routine coproscopy (e.g. direct smear, floatation and sedimentation) were carried out to identify the eggs/oocysts/cysts of GI parasites. Blood smears were first examined by the Giemsa's stain and then, subsequent, polymerase chain reaction (PCR) was performed on the same blood samples to identify haemoprotozoan diseases of buffalo. Partial gene sequencing and phylogenetic analyses were performed on selected positive isolates of haemoprotozoa by using Sanger sequencing method and UPGMA method, respectively. Results demonstrated that overall prevalence of GI parasitic infections was 44.30% (95% CI: 36.41–52.41) in buffalo where, prevalence of nematode was the highest (20.25%, 95% CI: 14.28–27.37) in comparison to other helminths (trematode). Besides, among different nematodes, the prevalence of *Toxocara vitulorum* was the highest (17.72%, 95% CI: 12.11–24.58). Among the GI protozoa, the occurrence *Balantidium coli* was the highest (3.16%, 95% CI: 12.11- 24.58). Based on location, the buffaloes of Sandwip had the highest GI parasitic infections (61.54%) compared to other three upazillas. Moreover, adult buffaloes (46.05%) mostly infected with GI parasites in comparison to young (44.26%) and calf (38.10%). Male buffaloes (48.28%) were more prone to GI parasitic infection in comparison to female buffaloes (42.00%). It was also found that, infection caused by nematodes was higher in non-dewormed buffalo (31.58%) where, *T. vitulorum* infection was found almost two folds lower (p<0.05) in dewormed buffalo (12.87%) compared to non-dewormed buffalo (26.32%). On the other hand, the overall prevalence of haemoprotozoan infection was

14.48% (95% CI: 09.19–21.28), and 31.03% (95% CI: 23.62–39.24) in simple microscopy and PCR, respectively. Among the haemoprotozoa, *Anaplasma marginale* was the highest (30.34%) followed by *Babesia bigemina* and *Theileria annulata* in buffaloes using PCR technique. Geographically, the buffalo of Boalkhali region had the highest prevalence (48.89%) of all hemoprotozoan diseases in comparison to other locations. Among the three different age groups, calf (36.84%) showed more susceptibility to different haemoprotozoan diseases in comparison to young and adult buffalo. Female buffalo had significantly (p<0.05) higher prevalence of haemoprotozoan infections (36.84%) than male buffalo (20.00%). Sequencing and phylogenetic analyses revealed that *Babesia bigemina*, *Anaplasma marginale*, and *Theileria anulata* were circulating in buffaloes of aforementioned coastal areas of Chattogram division and these isolates were found to have close relation with those of China, India, Pakistan, and Japan. Therefore, the current investigation will help in epidemiological forecasting to the veterinarians and farmers in designing appropriate prevention and control measures against those infections in this region.

Keywords: buffalo, coastal areas, gastrointestinal parasitic infections, haemoprotozoan diseases, prevalence

Chapter-1: Introduction

Bangladesh is an agricultural based developing country. The area of the country is 147,570 square kilometres and its population is more than 165 million. (Britannica, 2023). The livestock sector is an important subsector of agriculture and contributes significantly to the national economy of the country. The majority of Bangladesh's livestock resources are made up of cattle, goats, sheep, buffalo, and poultry. However, among all the agricultural subsectors in Bangladesh, livestock production is growing at the second-highest rate (BER, 2012). Department of livestock services estimates that Bangladesh has 57.14 million ruminants in total. The entire livestock population includes 24.86 million cattle, 1.52 million buffaloes, 26.95 million goats, and 3.83 million sheep (DLS, 2023). According to the recent statistics, this subsector contributes 1.85% of national GDP, shares 16.52% in Agricultural GDP and provides 20% employment directly and 50% indirectly for the total population of the country (BBS, 2023). The contribution of this sector in supplying the daily diet of the human body with necessary animal proteins is significant. In fiscal year 2022–2023, livestock generated 140.68 metric tons of milk and 87.10 metric tons of meat, both of which were crucial in meeting the demand for animal protein (BBS, 2023).

Large ruminants particularly buffalo comprise an important component of the livestock sector. It is considered as the “Black Gold” next to cattle due to its contribution in milk and meat production (Ahmad et al., 2020). It is also a growing industry that creates jobs and improves the socioeconomic conditions of rural populations by raising family income, especially for small and marginal farmers. Buffaloes are concentrated in specific agro-ecological zones of Bangladesh due to their contribution and significance (Rahim et al., 2018). There are 194.29 million buffaloes worldwide, with 179.75 million (92.52%) of them living in Asia (Chakravarty, 2013; FAO, 2012). About 79.74% of the buffaloes in Asia are found in South Asian nations, with the remaining 20.26% found in other nations India and Pakistan are the two buffalo-rich countries contributing 58.11% and 16.83%, respectively of the total world buffalo population. The total number of buffalo in Bangladesh is estimated to be 1.52 million (DLS, 2023), with coastal areas having roughly 40% of that population (Hamid et al., 2016). With the exception of a few swamp types in the east, most of the buffalo in Bangladesh are of indigenous origin

and are low producers. Around the Indian border, there are also some cross breeds with Murrah, Nili-Ravi, Surti, and Jaffrabadi (Faruque et al., 1990; Huque and Borghese, 2012).

According to Chakravarty (2013), South Asian nations produce 93.19% of the world's total output of buffalo milk, making up 96.05% of all Asian production. In terms of global buffalo milk production, India and Pakistan were responsible for 67.99% and 23.96%, respectively. According to the FAO (2010), Asia accounted for 91.89% of the world's 3.08 metric tons of buffalo meat. Asia generated about 78.5% of the world's buffalo meat, with Pakistan and India contributing the lion's share of that production. According to Islam et al. (2018), it makes a sizeable contribution to Bangladesh's Gross Domestic Product (GDP) in the form of milk, meat, and skin, which together make up around 27.0%, 23.0%, and 28.0% of the country's total output from the livestock industry.

The growth, development, and productivity of these animals are adversely affected by many diseases including gastrointestinal and haemoportozoan diseases (Krishna et al., 2016; Mamun et al., 2020).

Gastrointestinal (GI) parasitism is a world-wide problem (Regassa et al., 2006). It is believed to be one of the main obstacles preventing the growth of the livestock population (Kakar et al., 2008) and it also adversely affects the health and productivity of animals (Irfan, 1984). The majority of the day is spent by the buffaloes eating in the river, grazing on pastureland, congregating in paddy fields and resting alongside the road to meet their physiological needs (Rahman et al., 2015). As the low-lying, muddy land and the stagnant water in coastal locations usually retain the intermediate hosts and infective stages of parasites during this time, they may become infected with numerous parasites by ingesting them. The digestive tract of a larger organism, or host, is where the GI parasites reside (Rahman et al., 2017). According to Roy et al. (2016), parasitic infections affect buffaloes more severely than other infectious disorders. *Neoascaris vitulorum* was shown to be the gastrointestinal parasite that had a more serious impact on buffalo calves based on the level of infection (Ara et al., 2021). The other species of parasites that are found in buffaloes are *Fasciola* sp., *Paramphistomum* sp., *Ascaris* sp., *Strongyloides* sp., *Bunostomum* sp., and *Oesophagostomum* sp. (Alam et al., 2016; Mamun et al., 2020).

GI parasitic infections causes lowered general health conditions, retarded growth rate, diminishing the working efficiency, decrease milk and meat production, abortion, cost associated with preventive measures and reduces the disease resistance capability, which may ultimately lead to higher mortality (Radostits et al., 1994; Chavhan et al., 2008). However, infections caused by GI parasites especially nematodes are one of the major causes of calf mortality and act as a big threat for dairy industry of this country. Earlier reports revealed that 26.47% calves up to 1 year of age died due to GI parasitism (Debnath et al., 1995; Ahmad et al., 2020).

Similar to GI parasitic infections, haemoprotozoan diseases are distributed world-wide. Bangladesh is usually hot and humid except in winter, and the geo-climatic condition of Bangladesh is highly favourable to a wide variety of parasites including ticks (Roy, 2018) which act as natural vectors of haemoprotozoa. Blood protozoa such as *Babesia bigemina, Theileria annulata, Theileria mutans,* and *Anaplasma marginale, Anaplasma centrale* have been reported in animals of Bangladesh (Kispotta et al., 2016). Haemoprotozoan diseases, namely Babesiosis, Anaplasmosis, and Theileriosis, are the tick borne diseases (TBDs) of ruminants, distributed throughout the world, particularly in tropical and subtropical countries including Bangladesh (Rahman et al., 2015). Tick-borne diseases cause substantial losses to the livestock industry throughout the world (Ananda et al., 2009) as these have got a significant economic impact due to obvious reason of death, decreased productivity, increased cost for control measures (Makala et al., 2003) and limited introduction of genetically improved animal in an area (Radostits et al., 2000). Ticks are obligatory blood sucking arachnid arthropods infecting mammals, birds, reptiles, and amphibians. They cause anaemia, dermatitis, paralysis, and toxaemia (Schmidt and Roberts, 1989).

The diversified topography of Bangladesh includes plane, hilly, and coastal areas. In coastal areas of Bangladesh, buffalo farming is growing in a noticeable way (Alim et al., 2012). But, investigations for GI and haemprotozoan diseases in buffalo especially in coastal areas of Chattogram region were less focused by the previous researchers. Occurrence of GI and haemoprotozoan diseases varies greatly upon the diverse intrinsic and extrinsic epidemiological and biological factors associated with them (Sardar et al., 2006). Epidemiological pattern of the parasitic diseases in the different agro-climatic zones of a country usually provides a basis for developing strategic and

tactical control systems against them (Kamal, 2020). In different regions of Bangladesh, several epidemiological studies were conducted on GI parasitic and haemoprotozoan diseases of buffalo (Mamun et al., 2010; Mamum et al., 2011; Biswas et al., 2014; Alam et al., 2016; Anwar et al., 2016; Roy et al., 2016; Ara et al., 2021; Zaman et al., 2022). Unfortunately, in Chattogram division, there are a few studies (Hossain et al., 2011; Mamun et al., 2020; Biswas et al., 2021) that were investigated only for GI parasitic infections in buffalo. To the best of our knowledge, there was no study conducted to address the prevalence of haemoprotozoan diseases of buffalo using simple microscopy or molecular techniques (e.g, PCR). Therefore, the current investigation has been proposed to fulfil the following objectives.

1.1. Objectives of the study

i. To determine the prevalence of gastrointestinal and haemoprotozoan diseases in buffalo of four upazillas (Companigonj, Kabirhat, Boalkhali, and Sandwip) of two districts (Noakhali and Chattogram) Chattogram division, Bangladesh
ii. To find out the associated risk factors in the occurrence of gastrointestinal and haemoprotozoan diseases of buffalo
iii. Molecular identification of haemoprotozoan diseases of buffalo
iv. Partial gene sequencing of haemoprotozoa to investigate the evolutionary relationship of haemoprotozoa available in buffalo of Chattogram

Chapter-2: Review of Literature

Gastrointestinal (GI) parasitism is a global issue (Regassa et al., 2006). It is critical for the veterinarian and producer to distinguish between different ruminant GI parasites since they can have negative clinical and economic effects on their hosts (Irfan, 1984; Kakar et al., 2008). Clinical diseases are prevalent in ruminants and present as aberrant symptoms in the cardiovascular, gastrointestinal, or cutaneous systems. Economic illness, as seen in buffalo, is the degree of parasitism that results in a rate of gain, feed conversion, development, reproduction, or less-than-optimal production of milk or meat that is below the genetic potential rate. In ruminants, economic losses predominate over morbidity and mortality. With rising stocking rates and insufficient nutrient intake, parasitism has the potential to become the most significant source of financial loss in the livestock business, according to Craig (2018).

This chapter includes the review of different literatures about prevalence of different GI parasitic and haemoprotozoan diseases of buffalo along with risks factors responsible for this diseases.

2.1. Gastrointestinal parasitic infection in buffalo

Form different literatures it is known that, GI parasitism occurs by nematodes, trematodes, cestodes, and protozoa. The nematodes include *Strongyloides papillosus Capillaria* sp. (*C. bilobata, C. bovis*) *Setaria digitate, Onchocerca armillata, Thelazia rhodesii, Gongylonema pulchrum, Oesophagostomum radiatum,* Hookworms (*Agriostomum vryburgi, Bunostomum phlebotomum, Trichostrongylus axei, Mecistocirrus digitatus, Haemonchus contortus,* and *Toxocara vitulorum.* The trematodes include *Fasciola gigantica,* Paramphistomes (*Gigantocotyl explanatum, Ceylonocotyl scoliocoelium, Cotylophoron cotylophorum,* and *Gastrothylax crumenifer*, *Schistosoma indicum*, *S. spindale*, and *S. nasalis*. Hydatid cyst, and *Cysticercus tenuicollis* are the most common cestodes (Soulsby, 1986; Islam et al., 1992; Urquhart et al., 1996).

This chapter reviews the admissible scientific literature on the prevalence and diagnosis of GI and haemoprotozoan diseases in buffaloes, as well as the risk factors

linked to these diseases. The most recent and pertinent information related to the research will be highlighted in this chapter.

2.1.1. Gastrointestinal nematodes of buffalo

The majority of parasitic illnesses in buffalo are caused by several parasite species, although a select handful are more economically significant than others. *Toxocara vitulorum* is the most frequent species found in calves, and from the perspective of egg counts, it frequently dominates the worm population. The parasite is pathogenic and can result in severe clinical illness. It can also induce enteritis, stunt calf growth, and even result in calf death when it is present in relatively low levels (Stromberg et al., 1997; Forbes et al., 2000). *Trichostrongylus*, *Bunostomum*, *Oesophagostomu*m, and *Trichuris* are further genera of significant local importance (Craig, 2018). Currently, programs to control *Toxocara vitulorum* are likely to help in the control of other species, but in order to prevent disease brought on by GI nematodes in young animals, it is important to take into account their surroundings, season, history, and source of mother or worms.

2.1.1.1. Population biology of nematodes

According to Hansen and Perry (1994); Urquhart et al., (1996); Taylor et al., (2007), and Craig (2018), all buffalo GINs have a direct life cycle. The sexes are distinct in the Nematode, and the females, which lay eggs or larvae, are typically smaller than the males. A nematode undergoes periodic moults throughout development, shedding its cuticle. There are four moults in the entire life cycle, with the succeeding larval stages being called L1, L2, L3, L4, and then L5, which is the immature adult. After hatching, the free living larvae go through two moults in the usual form of direct life cycle, and infection is caused by eating of the free L3.

There are several significant exceptions, though. Infections can occasionally occur when a larva penetrates the skin or when a larval egg is consumed. In indirect life cycles, the first two moults often occur in an intermediate host, and infection of the final host occurs either through ingestion of the intermediate host or through inoculation of the L3 during feeding by the intermediate host, which is typically a bloodsucking bug. After infection, the L5, or immature adult parasite, is created by two further moults. Immediately after copulation, a new life cycle begins. The

development of GI parasites may occur exclusively in the gut lumen or with some migration into the mucosa. However, many species have migratory life cycles in which the larvae traverse long distances through the body before settling in their preferred location. The hepatic-tracheal route is one of the more used ones. This involves developing stages traveling from the gut to the liver via the portal system, then from the liver to the heart via the hepatic vein and posterior vena cava, and finally from the heart to the lungs via the pulmonary artery. The larva then makes its way to the gut through the oesophagus, bronchi, and trachea (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007; Johannes et al.,2020). It should be emphasized that the nematode life cycle is complex and that the information above only provides a basic overview.

2.1.2. Gastrointestinal trematodes of buffalo

The Fasciolidae, Dicrocoeliidae, Paramphistomidae, and Schistosomatidae families are among the several in the class Trematoda that contain significant veterinary parasites. The Troglotrematidae and Opisthorchiidae are less significant (Taylor et al., 2007; Soulsby, 1986; Urquhart et al., 1996).

The Monogenea, which have a direct life cycle, and the Digenea, which need an intermediate host, are the two main subclasses of the class Trematoda. The bile ducts, digestive tract, and circulatory system are where adult digenetic trematodes, often known as "flukes," are most usually seen (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007).

2.1.2.1. Population biology of trematodes

The larval stages develop in a molluscan intermediate host after the eggs leave the final host, typically in the form of faeces or urine, depending on the preference location. The mollusk is crucial for all species in the group, with the exception of a few (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). The key aspect of the life cycle is that, as opposed to nematodes, which can produce only one adult from an egg, trematodes can produce hundreds of adults from one egg. This is a result of the molluscan intermediate host’s parthenogony phenomenon, which is the development of new individuals by single larval forms (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). Flukes that are adults always lay eggs with a lid or operculum on

one pole. The embryo inside the egg transforms into a miracidium, a pyriform (pear-shaped), ciliated larva (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). The proteinaceous cement holding the operculum in place is attacked by an enzyme that is released by the miracidium in response to light stimulation. The miracidium is driven through the water by its cilia and must find a compatible snail within a few hours in order to continue growing (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). About 30 minutes into the penetration process, the cilia are gone, and the miracidium transforms into an extended sac called a sporocyst that contains a number of germinal cells. Rediae, which are also larval forms with an oral sucker, some flame cells, and a primitive intestine, arise from these cells and move to the snail's hepato-pancreas (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). The last stages, the cercariae, develop from the rediae's germinal cells, though a second or daughter generation of rediae is frequently formed in its place if environmental conditions for the snail are unfavorable (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). The cercariae actively emerge from the snail, generally in large numbers. They are essentially immature flukes with lengthy tails. Cercariae continue to be created indefinitely once a snail contracts the infection, although the majority of afflicted snails pass away before their time due to severe hepato-pancreatic damage (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007). The cercariae often swim for a while, using even a thin coating of water, and then, after about an hour, they adhere to vegetation, lose their tails, and encyst. The name of this stage is metacercaria. Encysted metacercariae have a strong chance of living for months. The exterior cyst wall is mechanically removed during mastication after being swallowed. An enzymatic mechanism that is triggered by an appropriate oxidation-reduction potential and a carbon dioxide system provided by the intestinal environment causes the inner cyst to rupture in the gut. The emerging juvenile fluke then enters the intestine and travels to the place of preference, where it develops into an adult after a number of weeks (Soulsby, 1986; Urquhart et al., 1996; Taylor et al., 2007).

2.1.3. Gastrointestinal cestodes of buffalo

The order Cyclophyllidea contains almost all of the important tapeworms for veterinarians; the two exceptions are in the order Pseudophyllidea. The two most prevalent cestodes are hydatid cysts and *Cysticercus tenuicollis* (Soulsby, 1986; Islam et al., 1992; Urquhart et al., 1996).

2.1.3.1. Population biology of cestodes

The buffalo acts as intermediate host of hydatid cyst. Infected final hosts specially dogs expel proglottids and eggs in their faeces. The eggs may contaminate the herbage in the environment. By consuming the contaminated herbage, ruminants become affected. Metacestode i.e. hydatid cyst forms in the liver, lung, and some other organs of infected buffalo. After ingestion of these contaminated organs with cyst, final host become infected (Soulsby, 1986).

2.1.4. Development of immunity and its influences on epidemiology of gastrointestinal parasitic infections

Ruminant variables, parasite factors, and factors influencing host-parasite contact, such as grazing management and anthelmintic treatments, all have a role in the development of immunity to GI parasites. The genetic make-up, age, sex, and nutritional condition of the animals are all considered hosts. In animal, worm load frequency distributions are often too distributed (Johannes et al., 2020). This large variety in worm burdens is a result of animal sensitivity variations due to individual genetics.

Reduction in faecal egg production is the most significant phenotypic characteristic linked to genetic resistance to GI parasites (Gasbarre et al., 1990). Calves can be classified into one of three phenotypes based on their egg output, according to Gasbarre et al. (2001): type I, which never exhibits high faecal egg counts (FECs); type II, which exhibits increases in FECs during the first two months on pasture, which then decline and stay at levels associated with type I calves; and type III calves, which maintain high FECs. According to Herd et al. (1992), bulls are typically more vulnerable to GI parasite infections than females. According to Hammerberg and Michel et al. (1979) and Lamm (1980), cows are more prone to infection during early lactation and around the time of parturition. For *C. oncophora* but not for *O. ostertagi*, there is an age impact, with older animal gaining resistance more quickly than calves (Kloosterman et al., 1991). The resilience and resistance of the host to GI parasites are also influenced by nutrition. Increased immune development and resistance to reinfection are outcomes of protein supplementation (Holmes and Cooper, 1996). The site of infection has a significant impact on the immune response to GI parasites in addition to host-related variables. As opposed to immunization

against intestinal-dwelling genera such *Cooperia* and *Nematodirus*, protective immunity against the abomasal parasites *O. ostertagi*, *Trichostrongylus axei*, and *H placei* develops more slowly in ruminant (Kloosterman et al., 1991; Hilderson et al., 1995). In the end, the length and severity of the infection determine whether immunity develops (Claerebout et al., 1998; Ploeger et al., 1995). As a result, actions or situations like mowing, reducing grazing time, (preventive) anthelmintic treatments, and drought and housing conditions lower host immunity (Ploeger et al., 1990; Claerebout et al., 1998). Immunity growth significantly influences GI parasites' epidemiology. Host immunity controls the establishment, growth, fertility, and survival of worms. Immunity is also a factor in the hypobiosis (arrested development) of L4 (Johannes et al., 2020). The total impact of all immune response manifestations is a decrease in parasite transmission among the herd of animal (Gasbarre et al., 2001). Nematode vaccines are regarded as a promising control method if several research bottlenecks regarding understanding of immune effector mechanisms and production of recombinant protective antigens can be overcome (Charlier et al., 2018). In experimental settings, these reductions can reach 50% to 90% (Ravinet et al., 2017). For these reasons, nematode vaccines are considered a promising control method. More research is required in this area because there have been reports that nematode infection in buffalo can change the way vaccinations react to viral diseases. However, the evidence supporting these claims is not strong enough at this time (Charlier et al., 2013). Thus, host immunity affects GI parasites' epidemiology, which in turn affects the productivity of animals and the profitability of farms.

2.1.5. Diagnosis of gastrointestinal parasitic infections

The diagnosis of ruminant parasites is based on the clinical history, clinical signs, and presence of eggs, oocysts, and larvae in faecal samples or the presence of parasites that have been removed from the animals' digestive tracts or other viscera. Sometimes, imaging strategy, serological test, and molecular test (PCR) are also followed for confirmatory diagnosis of parasites. In actual practice, the majority of diagnostic laboratories as well as academic and research institutions use their own set of test procedures (Kamal, 2020).

Faecal egg counts in small ruminants can be quite telling; in the spring, counts larger than 1000 eggs per gram (EPG) and in the late summer or fall, counts greater than

2000 EPG, are indicative of disease or are about to be. We might observe clinical symptoms in calves with such high numbers. Adult cows with counts above 20 EPG are presumably not getting enough pasture to meet all of their demands, so they give less milk, which prevents their calves from developing as effectively as those nursing cows with lower egg counts can (Craig, 2018). However, the diagnostic techniques outlined by Soulsby and Thienpont et al. in 1986 are typically used.2.1.6. Prevalence of gastrointestinal parasitic infections

2.1.6.1. In local (Bangladesh) perspective

A study was conducted on total of 480 live buffaloes and 180 visceral samples from Dhaka, Mymensingh, Bogura and Rajshahi of Bangladesh in late 1980s revealed that there were eight trematodes, two cestodes, fourteen nematodes, two protozoa and two arthropods parasites present in water buffaloes. The trematodes included *Fasciola gigantica* (18.9%-46.4%), Paramphistomes (*Gigantocotyl explanatum, Ceylonocotyl scoliocoelium, Cotylophoron cotylophorum,* and *Gastrothylax crumenifer* (29.5%-48.3%), *Schistosoma indicum* (1.6%-31.6%), *S. spindale* (13.9%-27.7%), and *S. nasalis* (4.6%-8.3%). Hydatid cyst (24.4%) and *Cysticercus tenuicollis* (11.1%) were the cestodes. The nematodes included *Strongyloides papillosus* (14.8%-21.6%), *Capillaria* sp. (*C. bilobata, C. bovis*) (8.5%-20.0%), *Setaria digitata* (7.2%), *Onchocerca armillata* (27.2%), *Thelazia rhodesii* (2.3%), *Gongylonema pulchrum* (3.9%), *Oesophagostomum radiatum* (6.6%-41.6%), Hookworms (*Agriostomum vryburgi*, *Bunostomum phlebotomum*) (8.1%-17.2%), *Trichostrongylus axei* (11.2%-21.6%), *Mecistocirrus digitatus* and *Haemonchus contortus* (15.2%-25.5%) and *Toxocara vitulorum* (1.1%-9.8%) (Islam et al., 1992).

In Kurigram, the prevalence GI parasites through coprological examination in buffaloes was 61.02% whereas nine species of GI parasites were identified. Of them three species were nematodes, including *Toxocara vitulorum* (2.54%), Strongyles (0.85%) and *Strongyloides* sp. (0.42%); two species were protozoa, including *Eimeria* sp. (3.39%) and *Balantidium coli* (37.29%); four species were trematodes, including *Paramphistomum cervi* (29.24%), *Fasciola gigantica* (22.46%), *Schistosoma indicum* (1.27%), *S. spindale* (0.85%). No cestodes were found. Mixed infections were frequent among GI parasites. GI parasite infection was about equally common in

males and females (odd ratio: 1.08). In the age groups, young (<2 to 5 years) were more likely (p<0.01) to be sensitive to GI parasites (Mamun et el., 2011).

In Bhola, 84.90% of buffaloes were found infected with thirteen species of GI parasites. Among them six species were nematodes including *Haemonchus contortus* (9.70%), *Trichuris ovis* (3.0%), *Toxocara vitulorum* (3.0%), Strongyles (0.80%), *Strongyloides papillosus* (2.0%), and *Capillaria* sp. (0.40%); four species were trematodes including *Paramphistomum cervi* (41.40%), *Fasciola gigantica* (25.40%), *Schistosoma indicum* (5.80%) and *Schistosoma spindale* (2.40%); one species was cestode namely *Moniezia expansa* (0.60%); two species were protozoa including *Eimeria zuernii* (7.0%) and *Buxtonella sulcata* (37.40%). Females (87.53%) were 1.20 times more likely sensitive to GI parasitic infections than males (84.37%). Calves (68.05%) had a much lower prevalence than young people (83.45%) and adults (88.81%) in their age group. Poorly conditioned buffalo in the nutritional status group had a considerably higher infection rate (96.61%) than did medium body conditioned buffalo (75.51%) (Biswas et el., 2014).

In Sylhet, the prevalence of GI helminths infection in buffalo was 85.01% whereas six species of GI parasites were identified including *Fasciola* sp. (34.9%), *Paramphistomum* sp. (26.98%), *Bunostomum* sp. (7.49%), *Ascaris* sp. (7.28%), *Oesophagostomum* sp. (4.5%), *Strongyloides* sp. (3.85%). This study examined the prevalence of GI parasites in relation to seasonal dynamics, sex, and age. Females (86.9%) were more susceptible to GI parasitic infection than males (81.92%). According to age category, adult (89.18%) had the highest prevalence rates followed by young (83.46%) and buffalo calves (71.21%) (Alam et el., 2016).

Another study on buffalo calves conducted in Sylhet found that 36.47% of the animals had GI parasites. Two species of GI parasites were found, of them 26.47% was *Neoascaris vitulorum*, 5.88% was *Strongyloides* sp. and 4.12% was Strongyle type. Male calves had a lower frequency of GI parasites (44.12%) than female calves (55.88%). Compared to calves aged 6 to 12 months (32.35%) and calves aged 13 to 18 months (23.53%), the prevalence of GI parasitic infections was significantly higher (p≤0.05) in calves aged 6 months (44.12%) (Ahmad et el., 2020).

A study in Mymensingh revealed that 68.7% of buffaloes were infected with one or more endoparasite species while GI parasites (ova/cyst/oocyst) were also identified.

Among them, two species were trematodes including *Paramphistomum cervi* (28.7%), *Fasciola gigantica* (16.0%); two species were nematodes including strongyles (1.3%), *Trichuris* sp. (2.0%); one species of cestode namely *Moniezia* sp. (0.7%) and two species of protozoa including *Balantidium coli* (44.0%), *Eimeria* sp. (4.7%). Adult buffaloes aged >5 years (80.0%) had a substantially greater (p<0.05) prevalence of GI parasites than young buffaloes aged 2-5 years (42.2%) (Al Numan et al., 2022).

A study at Mongla in Bagerhat to investigate the prevalence of GI parasites in buffaloes by using faecal ova counting technique revealed that all animals were afflicted with one or more gastrointestinal parasites. Among the five species of GI parasites identified, two species were trematodes including *Fasciola gigantica* (24.41%), Amphistomes (78.40%); two species were nematodes including *Haemonchus contortus* (29.58), *Toxocara vitulorum* (18.78%); one species was protozoa namely *Balantidium coli* (80.28%). There were no cestodes found. Infection with several parasites in the GI tract was prevalent. Buffaloes of all ages were prone to infection. The GI parasites affected both males and females equally. It was also revealed that the dietary status of buffaloes had no significant (p>0.05) effect on GI parasite infection (Roy et al., 2016).

A study in Barishal revealed that 39.6% of buffaloes found positive for GI helminths. The direct smear method and the simple sedimentation method of faecal sample examination technique were used. Five species of helminths were identified including Amphistomes (60.75%), *Fasciola gigantica* (26.17%), *Neoascaris vitulorum* (1%), *Trichostrongylus axei* (2%), *Schistosoma bovis* (1%). The prevalence of combined infection with *Fasciola gigantica* and Amphistomes was 9.34%. No cestode was found. In the age groups 0-6 months, 7 months-2 years, 3 years- 6 years, and 7 years and over, the prevalence was 12.15%, 14.02%, 40.19%, and 33.65%, respectively. The study concluded that the application of an anthelmintic (Levamisole hydrochloride) on the calf may have resulted in the lowest prevalence of parasite infection at an early age in the research area (Saha et al., 2013).

2.1.6.2. In global perspective

In India, a study in Nimar region of Madhya Pradesh revealed 39.88% prevalence of GI parasites in buffaloes. Different GI parasites including strongyles (15.37%), amphistomes (10.19%), *Eimeria* sp. (9.17%), *Fasciola* sp. (5.97%), *Toxocara* sp.

(5.24%), *Moniezia* sp. (2.91%), *Trichuris* sp. (1.60), and *Strongyloide*s sp. (1.31%) were identified in this region. Other parasites, with the exception of Strongyle and *Toxocara* sp., did not demonstrate significant variation ($p<0.05$) between the males and females. The prevalence of strongyles, amphistomes, *Fasciola* sp., and *Moniezia* sp. was significantly higher in adult buffaloes ($p<0.01$; $p<0.05$) whereas the prevalence of *Toxocara* sp. was significantly high in buffaloes less than one-year-old ($p<0.01$) (Jamra et al., 2017). Another study conducted in Tirupati, Andra Pradesh, discovered that 40.20% of buffaloes were afflicted with one or more species of GI parasites. Ten species of GI parasites were found, including seven helminths (Amphistome, *Fasciola*, Strongyles, *Strongyloides*, *Toxocara*, *Trichuris*, *Moniezia* sp.) and two protozoa (*Buxtonella*, *Eimeria*, and *Entamoeba* sp.). Amphistomes were the most common (15.42%), followed by Strongyles (6.19%). Mixed infection was prevalent among GI parasites (3.17%). Adults aged above one year were the most sensitive to GI parasites ($p<0.05$) (Sreedevi and Hafeez, 2014). Furthermore, a study on buffalo calves up to 6 months old from 13 districts in Punjab state, India, indicated a prevalence of GI parasitic infections of 73.58% calves, with *Eimeria* sp. (54.55%) being the most prevalent GI parasite. *Toxocara vitulorum*, strongyles, and *Strongyloides papillosus* were identified in buffalo calves from all four major agro-climatic zones of Punjab, with significant variations ($p<0.01$) (Jyoti et al., 2013).

In Pakistan, a study was carried out in various tehsils of the district Khushab to assess the epidemiology of various GI helminths in buffaloes in relation to the host's age, sex, and bodily condition. And the prevalence of various parasites was recorded as 40.92%. The parasites included *Toxocara vitulorum* (18.54%), *Fasciola*, (17.21%), *Haemonchus contortus* (15.26%), *Paramphistomum cervi* (6.62%), *Oesophagostomum radiatum* (12.44%), *Ostertagia* (17.88%), *Trichuris* (1.98%), and *Bunostomum* (5.96%). The prevalence of parasites in calf (<1 year) and adult buffaloes (>2 years) was 32.03% and 46.34%, respectively, while the prevalence of parasites in male and female buffaloes was 42.5% and 39.71%, respectively. According to different body condition (good, normal, and poor), the prevalence of parasites was 23.63%, 36.34% and 58.99% respectively (Deeba et al., 2019). Another study in Lower Dir, Khyber Pakhtunkhwa, Pakistan found that 58.59% of 314 buffaloes and cattle tested positive for eggs, cyst/oocyst of one or more species of GI parasites. The prevalence of parasitic infection was higher in buffaloes (63.55%) than

in cows (55.61%), although the difference was not significant (p>0.05) *Entamoeba* sp., *Moniezia* sp., *Haemonchus* sp., and *Coccidian* sp. were found in this study. The percentage of infection in non-treated animals was highest, in cows it was 57.71% and in buffaloes it was 68.13%. Female cows had a 62.58% parasite prevalence and female buffalo had a 77.33% parasite prevalence more than their male counterparts, although the difference is non-significant (p>0.05). Calves, on the other hand, showed a lower rate of GI parasitic infection than adults (Khan et al., 2022).

A study on GI parasites in water buffalo reared under Mexican humid tropical circumstances revealed that the prevalence of GI parasites in buffalo was 42%, regardless of their age, with 60% of calves parasitized. The presence of GI parasites was strongly associated with age. (Xi^2 = 77.4014, d.f. = 1, p = 0.001). The Trichostrongylidae family was found in both age groups. The genera found were including *Strongyloides* sp. (47.2%), *Cooperia* sp. (33.9%), and *Haemonchus* sp. (10.4%), as well as *Eimeria* sp., *Moniezia* sp., *Trichuris* sp., and *Strongyloides* sp. (Ojeda-Robertos et al., 2017).

A study on GI parasitic infections of buffaloes in Sarawak Borneo of Malaysia found that the prevalence of *Paramphistomum* sp., strongyles, and coccidia were 75.2% (95% CI±7.5), 52.7% (95% CI±8.6) and 48.1% (95% CI±8.6), respectively. Farms with a grazing area of less than 50 acres had significantly higher prevalence of strongyles (70.5%, χ^2 = 8.34, p = 0.004) and paramphistomes (88.6%, χ^2 = 6.46, p = 0.01) compared to farms with a larger grazing area (43.5% and 68.2%, respectively). Deworming was practiced by the majority of farmers, and ivermectin was the most widely used anthelminthic (60.4%); only 1.9% of farmers used albendazole. Overall, this study found a significant prevalence of GI parasites in Sarawak's buffalo. (Harizt et al., 2021).

2.2. Haemoprotozoan diseases of buffalo

Heamoprotozoan infections, which are caused by vector-borne blood parasites, inflict devastating losses in the livestock business and thereby pose major restraints to the global dairy industry (Palmerand and Wright, 1989). *Babesia, Theileria, Anaplasma*, and *Trypanosoma* are examples of haemoparasites, although the most important are *Babesia, Theileria*, and *Anaplasma*. These protozoa are transmitted through ticks and the hot and humid climate is ideal for tick development and survival, and is a

persistent source of damage. Among the many haemoprotozoan illnesses, bovine Babesiosis and Theileriosis are known to be diseases of great economic importance, causing heavy losses due to mortality, decreased output, and impaired working efficiency of infected animals throughout the world's tropics and subtropics (Suryanarayan, 1990; Rajput et al., 2005; Zahid et al., 2005).

2.2.1. Anaplasmosis

Anaplasmosis is an emerging disease caused by intraerythrocytic rickettsia of the genus *Anaplasma* that is gaining attention globally because it affects animal body weight, causes fever, miscarriage, and progressive haemolytic anemia, and eventually leads to death, resulting in significant losses in meat and milk production. Clinical disease is most common in cattle, but other ruminants such as water buffalo, bison, African antelopes, and several deer species can become chronically infected (Kuttler, 1984; Kocan et al., 2010; Mubashir et al., 2022).

2.2.1.1. Etiology

Anaplasma marginale infection causes bovine Anaplasmosis, formerly known as gall sickness. The organism belongs to the genus *Anaplasma*, which is in the family Anaplasmataceae of the order Rickettsiales. *Anaplasma centrale*, a less harmful but closely related organism, is used as a live vaccine for cattle in Israel, South Africa, South America, and Australia. However, *Anaplasma centrale* has never been reported in North American countries. Furthermore, *Anaplasma ovis*, the agent of ovine Anaplasmosis, can cause mild to severe disease in sheep, deer, and goats but is not infectious to cattle (Aubry and Geale, 2011). *Anaplasma marginale* was the most common tick-borne infection in buffaloes, and was considered the most widespread TBD globally in bovines (El-Alfy et al., 2023).

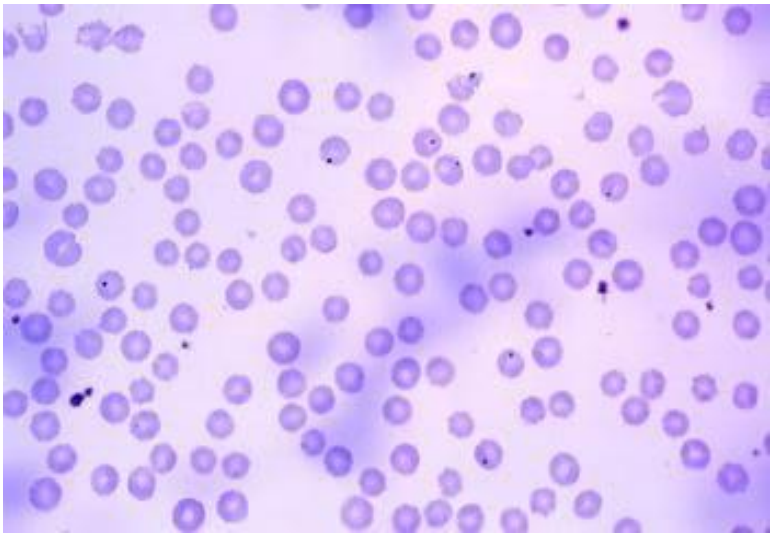


Figure 2.1 Intraerathrocytic stages of *Anaplasma marginale* (Taylor et al., 2007).

2.2.1.2. Epidemiology

2.2.1.2.1. Geographical distribution

Anaplasma marginale is distributed in tropical and subtropical regions all over the world (~40^0N–32^0S), including South and Central America, the United States, southern Europe, Africa, Asia, and Australia. Bovine Anaplasmosis is said to be endemic in cattle throughout Mexico, Central and South America, and the Caribbean. This widespread and expanding distribution was most likely caused by increased animal transportation, with subsequent mechanical or biological transmission from asymptomatic continuously infected animals to vulnerable ones (Aubry and Geale, 2011; Mubashir et al., 2022).

2.2.1.2.2. Transmission

Anaplasma marginale can be spread in three ways: biologically, mechanically, and transplacentally (Aubry and Geale, 2010). Firstly, by biological mode-infected erythrocytes are ingested by ticks after which *A. marginale* replicates within the tick's gut and salivary glands and is subsequently transmitted into uninfected ruminants via tick saliva. Because of ticks' replication and persistence powers, biological transmission via ticks is the most efficient mode of spreading *A. marginale*. At least 20 tick species have been found to transmit *A. marginale* globally, with *Rhipicephalus*

(Boophilus) microplus being the predominant transmission agent (Aubry and Geale, 2011; Mubashir et al., 2022). *A. marginale's* tick vectors include *Boophilus* sp., selected *Dermacentor* sp., *Ixodes ricinus*, and *Rhipicephalus* sp., although *Amblyomma* sp. do not appear to transmit *A. marginale* (Aubry and Geale, 2011). Secondly, by mechanical mode-without amplification of *A. marginale*, infected erythrocytes are spread from infected to susceptible animals by biting insects or blood-contaminated fomites such as needles, dehorning saws, nose tongs, tattooing instruments, ear tagging devices and castration instruments (Kocan et al., 2010; Aubry and Geale, 2011; Mubashir et al., 2022). And the last one by transplacental mode-infected erythrocytes pass from infected animals to their offspring via the placenta in the uterus, without *A. marginale* amplification (Aubry and Geale, 2011).

2.2.1.2.3. Host occurrence

Anaplasma sp. only infects ruminants. Buffalo are naturally vulnerable to *A. marginale* and *A. centrale*, while sheep are susceptible to *A. ovis* (Aubry and Geale, 2011). Although Anaplasmosis is most common in cattle, *A. marginale* can infect other ruminants such as water buffalo, American bison, and numerous species of deer such as white-tailed deer, mule deer, black-tailed deer, and Rocky Mountain elk. In some areas, wild ruminants, particularly mule deer and elk, may be involved in the epizootiology of bovine Anaplasmosis (Kuttler, 1984; Kocan et al., 2010).

2.2.1.2.4. Factors influencing occurrence of Anaplasmosis

Maternal immunity in calves are covered partially against colostral antibodies (Corrier and Guzman, 1977). This protection lasts about 3 months and is usually accompanied by an age range of 9 to 12 months for animals (Jones et al., 1968; Paul et al., 1980). When mother or age resistance is high, calves exposed to Anaplasmosis rarely display clinical signs but develop a strong, long-lasting immunity. It is thus conceivable to have both *Anaplasma* sp. and vectors present in the same area without suffering from animal losses or clinical illnesses. This is referred to as endemic stability. In contrast to bovine Anaplasmosis, age does not appear to be a factor in ovine and goat infection susceptibility. Young and adult animals normally develop only a minor illness, though varied stressors in particular cases can exacerbate this (Stoltsz, 1994).

Anaplasmosis prevalence varies from animal to animal and is also affected by seasonal dynamics. Anaplasma infections are more common in cattle than in buffaloes (Mubashir et al., 2022). Because of the abundance of ticks during the hot, humid, and rainy seasons, Anaplasmosis is more frequent in these circumstances (El-Metenawy 2000). The occurrence of bovine Anaplasmosis may be expected to change in some extent as a result of climatic change, which may influence the tick population mobility as well as the survival of *A. marginale* in overwintering ticks. However, predicting the influence of climate change on vector-borne disease epidemiology is not a straightforward task (Aubry and Geale, 2011).

Immunity of the animals also affects susceptibility to Anaplasmosis. Immune-compromised animals have demonstrated vulnerability to heterologous threats, either through splenectomy or treatment with immunosuppressants such as cyclophosphamide and corticosteroids (Kuttler et al., 1984). It was also claimed that environmental stress or other stressors could impair cattle immunity (Kuttler et al., 1984).

2.2.2 Babesiosis

Babesiosis is a major zoonotic disease caused by tick-borne intra-erythrocytic protozoan of the genus *Babesia*. Domestic and wild mammals are both affected by the disease. Over 100 species of *Babesia* have been described, many of which are found in domesticated animals (Nyindo, 1992; Homer et al., 2000). *Babesia* infections, in general, progress with variable degrees of severity, which can often be linked to the host's age, immune condition, concomitant infections with other pathogens, and/or genetic factors (Schnittger et al., 2012). Clinical manifestation of babesiosis are numerous that includes anemia, fever (41^0C), depression, splenomegaly, jaundice, malaise, lethargy, cessation of rumination leads to anorexia, circulatory disturbances (shock), cerebral complications, mild hepatitis, hemoglobinuria, and multiorgan failure can lead to death if left untreated (Schnittger et al., 2012; El-Ashker et al., 2015; Suarez et al., 2019).

2.2.2.1 Etiology

The most common species of *Babesia* that cause babesiosis in cattle and buffalo are namely *Babesia bigemina*, *Babesia bovis*, and *Babesia divergens*. *B. bigemina* and *B.*

bovis affected cattle, water buffalo and African buffalo (*Syncerus caffer*) whereas *B. divergens* frequently affected cattle and reindeer (Rangifer tarandus) (Zahid et al., 2005). Water buffalo is the only natural host and *Rhipicephalus haemaphysaloides* is the only vector for *B. orientalis* and which is a new species distinct from *B. bigemina* and *B. bovis* (He et al., 2017).

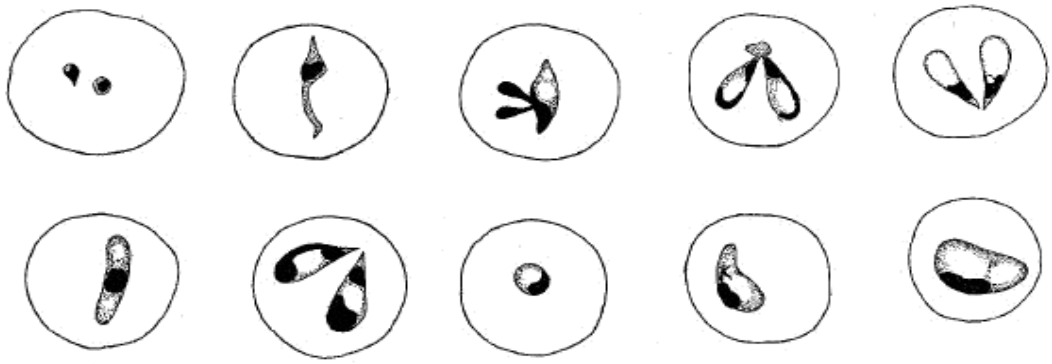


Figure 2.2 Diverse forms of *B. bigemina* in bovine erythrocytes (Riek, 1964)

2.2.2.2. Epidemiology

2.2.2.2.1. Geographical distribution

The most economically important bovine *Babesia* spp. are *B. bovis*, *B. bigemina* and *B. divergens. Babesia bovis*, and *B. bigemina* are mostly found in tropical and subtropical regions of the world, such as Australia, Africa, Asia, and the Americas, and are transmitted by the tick vectors *Rhipicephalus* (*Boophilus*) *microplus* and *R. annulatus*, and *R. decoloratus* for *B. bigemina* alone (El-Alfy et al., 2023). However, *B. divergens* primarily affects cattle in Europe, ranging from Scandinavia to the Mediterranean and Northern Africa. Its widespread distribution is linked to the wide temperature range tolerated by its tick vector, *Ixodes ricinus* (Schnittger et al., 2012).

2.2.2.2.2. Transmission

Hyalomma within the Ixodidae four Genera, *Rhipicephalus, Ixodes, Haemophysalis*, and *Dermacentor* have been identified as vectors of *Babesia* species. *B. ovis, B. motasi*, and *B. crassa.* are predominantly transmitted by *Haemaphysalis qinghaiensis* and *H. longicornis* (Niu et al., 2016). *Babesia* species multiply in the erythrocytes of vertebrate host via binary fission, endodyogeny, endopolygeny (budding), or

merogony to generate merozoites. These break out from the erythrocytes and infiltrate other cells. The asexual cycle continues indefinitely, and the animals may be infected for the rest of their lives. When ingested by a tick, these forms transform into vermiform and enter the body cavity, then the ovary to penetrate the eggs, where they round up and divide to form small round organisms. The parasites enter the salivary gland and undergo a series of binary fissions before entering the cells of the salivary gland acini when the larval tick moults into the nymph stage. They continue to multiply until the host cells are infested with thousands of minute parasites. When the tick consumes blood, they become vermiform, burst out of the host cell, and lie in the lumen of the gland before being injected into the mammalian host (Taylor et al., 2007).

2.2.2.2.3. Host occurrence

Due to the wide range of species reported to serve as *Babesia* hosts mostly in three host groups: domestic animals, humans, and some wildlife species, all vertebrates might be potential carriers, as long as they are adequate hosts for Babesia-vector ticks. Mammal species that belong to the same or related genera are frequently susceptible to the same *Babesia* species. For instances, *B. bigemina*, *B. divergens*, and *B. bovis* can infect buffalo and cattle; *B. motasi* and *B. ovis* infect sheep and goats. Babesiosis in dogs is also caused by a variety of *Babesia* sp. including *B. vogeli*, *B. canis*, *B. rossi*, *B. conradae*, *B. gibsoni*. And, clinical cases of cat babesiosis in Africa have largely been linked to *B. felis* infections. Finally, *Babesia* parasites have been found in a wide range of wildlife species, including lion, panther, elephant, giraffe, several deer species, wolf, hyaena, rhinoceros, and some birds such as seagull and kiwi. *Babesia* seen in wildlife animals are classified into two types: parasites that appear to be primarily unique to a single host, for instances, *B. leo* in lions or *B. bicornis* in rhinoceros, and *Babesia*, which are commonly found in closely similar domestic animals, for example, *B. bigemina* in gazelles or *B. canis* in wolves (Schnittger et al., 2012).

2.2.2.2.4. Factors influencing occurrence of Babesiosis

It is commonly stated that *Babesia* infection has an inverse age resistance in that young animals are less vulnerable to babesiosis than older animals (Urquhart et al., 1996; Taylor et al., 2007). In contrast to other infectious diseases that have a greater

impact on young animals, inverse age resistance is unique. This phenomenon is hypothesized to be caused by innate resistance that is independent of the mother's immunological condition (Christensson, 1987).

The prevalence of babesiosis is mostly determined by the vector's distribution. Babesiosis vector distribution is influenced by factors such as latitude, altitude, and its consequences (sunlight, temperature, rainfall, wind) (Morel, 1989). Seasonal changes in a bioclimatic environment may assist or hinder tick production or behavior during certain periods (Rabo et al., 1995). If the number of ticks suddenly increases under these situations due to favorable climatic conditions or a drop in dipping frequency, the incidence of clinical illnesses may skyrocket. This is referred to as enzootic instability (Taylor et al., 2007).

There are differences in virulence of the certain species of *Babesia*. For instances, *B. bigemina* and *B. bovis* are extremely pathogenic in tropical and subtropical regions, *B. divergens* is reasonably pathogenic in northern Europe, whereas *B. major* causes only mild and transient anaemia (Taylor et al., 2007). Moreover, the physiological state is frequently influenced by the normal or learnt defence system. Any deterioration of bodily status due to fatigue, dietary issues (lactation, mistletoe, gestation) or anabolism makes the animal more susceptible to infection or recurrence (Morel, 1989; Urquhart et al., 1996). The level of tick challenge also affects the Babesiosis occurrence. In endemic areas with a high number of infected ticks, the host's immunity is maintained at a high level through recurrent challenge and overt disease is uncommon. In contrast, if ticks are scarce or restricted to specific places, the population's immunological state is low, and young animals acquire little, if any, colostral protection (Taylor et al., 2007).

2.2.3. Theileriosis

Theileriosis, caused by the apicomplexan haemoparasite *Theileria* sp., is a significant constraint to livestock productivity in many parts of the world, particularly in South Asia (Zeb et al., 2022). *T. annulata* and *T. parva*, two *Theileria* species, are major buffalo parasites. Tropical Theileriosis is caused by *T. annulata*, whereas East Coast fever is caused by *T. parva*. Ticks are the vectors of *Theileria* (Brown, 2008). The clinical manifestations include anemia, lymphatic proliferation, weakness, dyspnea,

leucopenia, petechial haemorrhages on the conjunctiva, nasal discharge, and neurological complications (Farooq et al., 2019; Sharma et al., 2019).

2.2.3.1. Etiology

Theileria parasites infect a wide range of hosts, including domestic and wild ruminants, and frequently cause clinical diseases in the animals affected. The tick-borne parasites undergo repeated merogonony in lymphocytes, eventually releasing microscopic merozoites that infiltrate red cells to produce piroplasms (Taylor et al., 2007). Ticks from the genus *Hyalomma*, which includes *H. anatolicum*, *H. detritum*, *H. dromedarii*, and *H. lusitanicum*, play a role in transmission. (Jabbar et al., 2015). *Theileria* parasites are widely classified into two groups based on their ability to alter host leukocytes in such a way that the infected cells can multiply infinitely with the parasites occupying them. These two groups are host-cell transforming and non-transforming species. Host-cell transforming *Theileria* are *Theileria parva*, *Theileria annulata*, *Theileria lestoquardi*, and *Theileria taurotragi*. Non-transforming parasites include *Theileria orientalis*, *Theileria mutans*, *Theileria velifera*, and *Theileria cervi* (Sivakumar et al., 2014).

2.2.3.2 Epidemiology

2.2.3.2.1. Geographical distribution

Theileriosis is caused by *Theileria* species extensively present in South Asia, Middle East, Central Africa, South Europe (South), America, and Australia (Taylor et al., 2007). According to a rigorous study of current data (2000–2019) from five Asian nations, China had the greatest prevalence rate of bovine Theileriosis (39%), followed by Iran (33%), India (31.7%), Pakistan (21.2%), and Bangladesh (2.69%). The distinction can be ascribed to ecological and graphical causes, and it may differ in housing systems (Zaman et al., 2020).

2.2.3.2.2. Transmission

The *Theileria* species infect a wide range of both domestic and wild animals and are transmitted by ixodid ticks of the genera *Amblyomma, Haemaphysalis*, *Hyalomma,* and *Rhipicephalus* (Mans et al., 2015). *Theileria*'s lifecycle is divided into two stages: vertebrate host (asexual reproduction) and tick vector (sexual reproduction). The

lifecycle starts when an infected tick bites and transmits sporozoites into vertebrate hosts while blood-feeding, where they can transform into schizonts. Merozoites may then infect host erythrocytes (RBCs) and evolve into piroplasms after being released from infected leukocytes. The piroplasms multiply further in the red blood cells (merogony). In case of non-transforming *Theileria*, merogony has been seen in RBCs. Finally, when a tick feeds on an infected host, it picks up blood-stage *Theileria* parasites, including gametes. Sexual reproduction of the gametes occurs in the midgut of the competent vector tick species, where genetic recombination occurs during meiosis. As a result, *Theileria* parasites are transmitted trans-stadially by tick vectors, and the recognized transmission vectors may be two- or three-host tick species (Zeb et al., 2022). **Figure 2.4** depicts the modified life cycle of *Theileria* species.

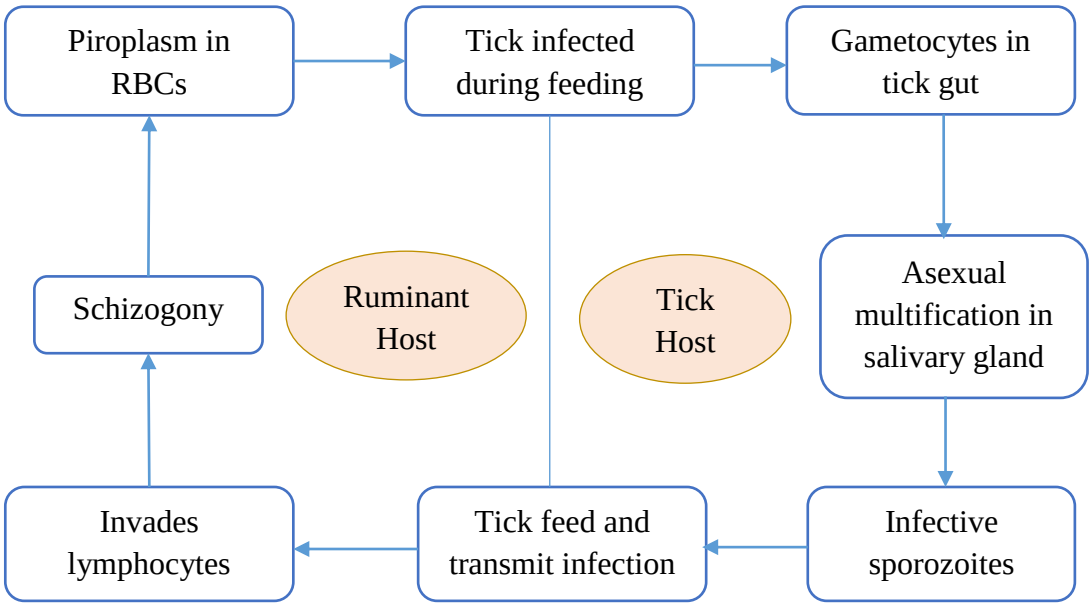


Figure 2.3 Life cycle of *Theileria* with distinct phases in ruminants and tick hosts (Zeb et al., 2022)

2.2.3.2.3. Host occurrence

Buffaloes and cattle have been found infected with different *Theileria* species, including *T. parva* and *T. annulata*, *T. orientalis*, on the other hand, infects yaks, cattle, and buffaloes. Furthermore, numerous *Theileria* species (*T. lestoquardi*, *T. separata*, *T. uilenbergi*, *T. luwenshuni*, *T. capreoli*, and *T. ovis*) have been observed to infect small ruminants. Wild ruminants such as deer, antelope, and giraffe are

afflicted with various unclassified *Theileria* parasites, some of which are highly deadly (Sivakumar et al., 2014; Zeb et al., 2022).

2.2.4. Diagnosis of haemoprotozoan diseases

Diagnosis of haemoprotozoan infection is based largely on clinical signs and parasitological approaches to detect the causative agent. Although classic Giemsa's-stained blood smears have several limitations, they remain the gold standard for diagnosing haemoparasite infections worldwide (Marcondes, 2017). A microscopic examination, however, does not detect all haemoprotozoans, particularly in subclinical cases. In addition to the direct microscopic approach, antigens or antibodies against TBPs may be detected with indirect diagnostic techniques such as multiple serological assays (e.g., ELISA, indirect fluorescence assays and CFT) (Lew-Tabor, 2016; Lempereur et al., 2017; Shabana et al., 2018).

The indirect fluorescent antibody approach has long been used to diagnose parasites such as *Babesia* sp. (Morzaria et al., 1977; Anderson et al., 1980). ELISA is used to evaluate the immunization program (Guglielmone et al., 1997) as well as epidemiological surveys (Passos et al., 1998). Microscopic and serological approaches are of limited value due to numerous limitations such as reduced sensitivity and precision, cross reactivity, inability to detect carriers, and the need for time and skill (Igarashi et al., 2014; Mans et al., 2015; Lew-Tabor, 2016). These constraints have been overcome through the use of highly sensible molecular approaches such as conventional PCR (cPCR), nested PCR (nPCR), quantitative PCR (qPCR), Loop Medium Isothermic Amplification (LAMP), reverse line blotting (RLB), high-throughput microfluidics-based real-time PCR, high-resolution melting (HRM) assays, and next-generation sequencing (NGS) (Schnittger et al., 2004; Criado-Fornelio et al., 2009; Johnsen et al., 2013; Michel et al., 2014; Wang et al., 2019).

2.2.5. Prevalence of haemoprotozoan diseases

2.2.5.1. In local (Bangladesh) perspective

Epidemiologic research on common blood parasites in buffaloes has been limited in Bangladesh. In Kurigram district, a study on the prevalence of haemoprotozoan infections in buffaloes indicated that 12.27% of the animals investigated were infected with three species of haemoprotozoa, namely *Anaplasma marginale* (8.89%),

Theileria sp. (2.12%), and *Babesia* sp. (1.69%). There were no mixed infections found. The prevalence of parasites in connection to age, sex and seasonal dynamics revealed that there had considerably higher prevalence of haemoprotozoa in rainy season (16.98%) than summer (12.33%) and winter seasons (10.91%). Female animals had a much higher prevalence (23.81%) than males (10.31%). In case of age groups, young aged (>2 to 5 years) buffaloes were the most susceptible to haemoprotozoa (17.07%) followed by older (>5 year) buffaloes (12.50%) and calves of 0.5–2 years (5.26%). These findings indicate that haemoprotozoa are the most common parasites of buffaloes, regardless of the host's age, sex, or season of the year (Mamun et al., 2010).

A study conducted in Sylhet, the prevalence of haemoprotozoan diseases in crossbred and indigenous cattle was 52%. Three types of haemoprotozoan diseases have been identified namely Anaplasmosis (28%), Babesiosis (08%) and Mixed infection (15%) Sex-wise prevalence was not significant (p>0.05) in any of the diseases, with males having the highest prevalence (31.48%) in the case of Anaplasmosis. Only mixed infected cattle differed substantially (p<0.05) in terms of age, with Anaplasmosis having the highest prevalence (30.43%). Summer (36.11%) was the most predominant season for hemoprotozoan infections, followed by rainy (29.41%) and winter (16.67 %) season. Adult cattle exhibited a much greater prevalence of mixed infection, which was statistically significant (p<0.05) (Hosen et al., 2020).

2.2.5.2. In global perspective

In India, a study was conducted to record the prevalence of haemoprotozoan infections in bovines of Shimoga region where a total of 300 blood samples (including 215 from cattle and 85 from buffaloes) were examined using Giemsa's staining technique. Among 300 blood samples examined, 43.3 % were found positive for Haemoprotozoan infections. Out of 85 buffalo samples examined, 12.9 % were showed *Theileria* sp., 4.7 % found positive for *Babesia bigemina*, 3.5 % were found positive for *T. evansi* and 2.3 % were positive for *Anaplasma marginale* (Krishna Murthy et al., 2014). Another study was conducted in three districts of Madhya Pradesh, namely Indore, Dhar, and Alirajpur, to study the epidemiology of haemoprotozoan diseases in buffaloes using Giemsa's staining technique revealed that the prevalance of haemoprotozoan diseases was 58.325 in Indore district, followed by

29.75% and 28.56% in Alirajpur and Dhar districts, respectively. In all three districts, the highest prevalence was recorded in adults, followed by heifers, and the lowest in calves (Asha et al. 2022). Furthermore, the seasonal prevalence of haemoprotozoan diseases in crossbred cattle and buffalo was studied in Gujarat, which revealed a higher prevalence of haemoprotozoan diseases in crossbred cattle and buffalo from June to September and June to August, respectively. In crossbred cattle, 375 of 3152 blood smears tested positive for haemoprotozoan infection, while 17% of 1129 blood smears tested positive for haemoprotozoan infection in buffalo. Theileriosis was shown to be more common in both species during the monsoon season than in other protozoan infections (Vahora et al., 2012).

A study was conducted in Al-Najaf, Iraq, to identify *Babesia* species in buffalo by microscopic identification, molecular analysis, and phylogenetic analysis whereas the direct microscopic prevalence data demonstrate that *Babesia* sp. has the highest prevalence of haemoprotozoa at 45.74%. The prevalence of *Babesia* sp. was found to be 43.48% in males and 52% in females, with no significant differences. *Babesia* sp. prevalence was found to be 12.50%, 92.86%, and 30% in young, adult, and old age groups, with significant differences ($p<0.05$). The results of the molecular study, which used PCR and DNA sequencing identified two *Babesia* species; the high prevalence of *Babesia bovis* (38.30%) was closely related to NCBI-Blast *Babesia bovis* (HQ264126.1) with homology sequence identity 97–100%, and *Babesia bigemina* 7.45% were closed related to NCBI-Blast *Babesia bigemina* (KU206291.1) with homology sequence identity 95-99% (Ateaa et al., 2019).

In Pakistan, a study revealed the prevalence of Theileriosis in buffaloes from 21 villages of Lahore. Microscopic examination revealed a 39.9% prevalence, compared to 53.3% with the polymerase chain reaction (PCR) test (Durrani et al., 2008). Furthermore, a study in peri-urban and urban areas of Hyderabad, Pakistan revealed that out of 2400 buffaloes evaluated during the study, 76.87% were found infested with ticks and among the tick infested bovine samples 91.05% were found positive for *Theileria* species using the Giemsa's-stained method. *Theleiria annulata* was found in 70% of the samples examined under the microscopic haematological examination. The prevalence of parasite infection in peri-urban settings was significantly higher ($p<0.05$) than in urban areas (Memon et al., 2016). Another study conducted at Landhi Dairy Colony Karachi identified the prevalence of haemoprotozoan diseases

in buffaloes was 14%. Females had a higher prevalence of blood parasites (15%) than males (10%). The highest percentage of infection found was that of *Anaplasma marginale* (64.28%) followed by *Babesia bovis* (21.42 %) and *Theileria* (14.285%) (Bhutto et al., 2012).

A study conducted in Hubei province, south China to determine the prevalence of tick-borne haemoparasites in water buffalo by the reverse line blot (RLB) hybridization assay and phylogenetic analysis of the parasite 18S rRNA gene revealed that *Theileria buffeli* (19.1%) was the most commonly found species in all of the locations investigated, followed by *Babesia orientalis* (8.9%), *Babesia bovis* (1.0%) and *Babesia bigemina* (0.7%). Only 3.9% of the samples exhibited mixed infection (He et al., 2012).

Chapter-3: Materials and Methods

3.1. Study areas and study period

The study was conducted in two coastal districts of the country namely Chattogram and Noakhali of Chattogram division of Bangladesh. Boalkhali and Sandwip upazilla of Chattogram district and Companigonj and Kabirhat upazilla of Noakhali district were considered. The study upazillas were selected as they are located in the coastal belt of the country and availability of the study population (e.g., buffalo). The investigation was conducted for a year starting from June, 2022 to June, 2023.

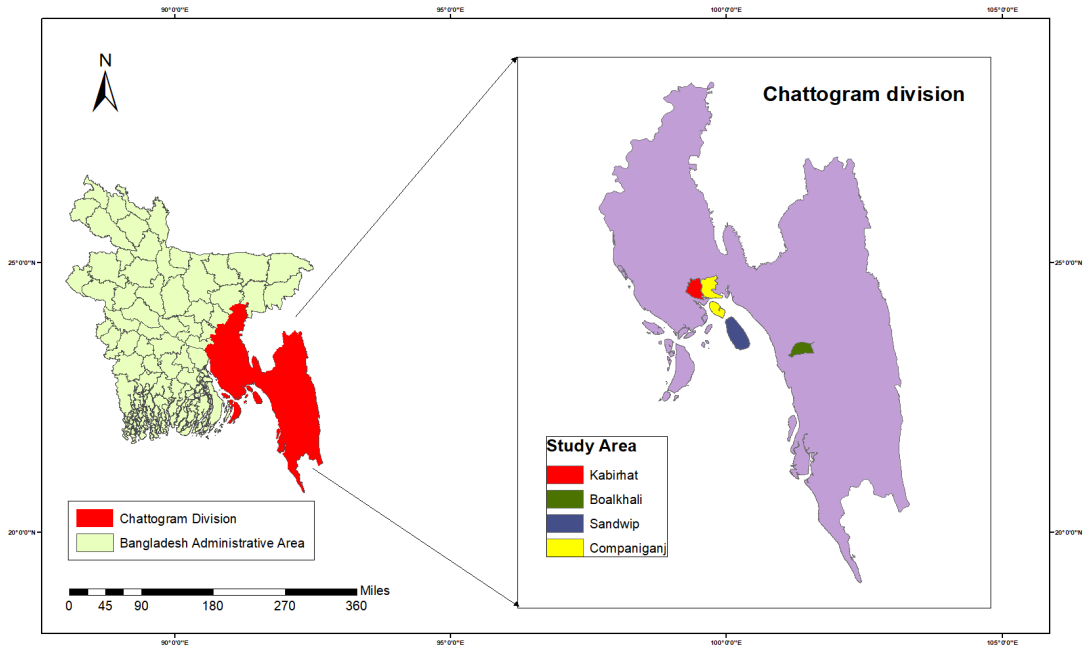


Figure 3.1 Map of Bangladesh (inset: Chattogram division) showing the study upazillas of Chattogram and Noakhali district

3.2. Selection of animals and study design

3.2.1. Target animals and age groups

Indigenous buffaloes (river type) were selected for this study as target animals. Age of the buffalo was determined according the previously published literature (Samad, 2008). To determine the age susceptibility for different parasites, the target animals were categorized into three sub groups as calf (<1 year), young (1–3 year) and adult (>3 year) (Samad, 2008).

3.2.2. Study design, sample collection, and preservation

The current investigation was a cross sectional study and samples were collected purposively. A prototype questionnaire was used to record the information (e.g., owner's name and address, animal identification number, breed, age, sex, deworming history etc.) (Appendix A). In the present study, the minimum age of the buffalo was 6 months, median 1.5 year, and the maximum age was 5 years. We are also unknown to the type of anthelmintic that were utilized in these cases.

An individual animal was considered as a sampling unit. Two different types of biological samples (faeces and blood) were collected during this study. Individual faecal sample (approximately 5–10 gm) was collected directly from rectum and stored in a plastic specimen container. Then, the containers were filled with 10% formalin and refrigerated at 4^0C until further examination. Blood was collected from ear vein by puncturing with a sterile needle. Initially, a drop of blood was taken on a FTA card (CAT. No. 1102110) and kept it in a sterile zip lock bag. Another drop of blood was taken to make thin blood smear (Alim and Rana, 2023). The blood smears were air dried and fixed by 100% methyl alcohol for 3–5 minutes. However, labelling of the samples were strictly maintained to prevent the misinterpretation during sample collection. All the faecal and blood samples were then carried to designated laboratories (Parasitology, Molecular pathology lab.) of Chattogram Veterinary and Animal Sciences University, Chattogram for examination.

A total of 158 faecal samples and 145 blood samples were collected from Noakhali and Chattogram district of Chattogram division. In case of Chattogram district, only 13 faecal samples were collected from Sandwip upzilla, and 45 faecal and blood samples from Boalkhali upazilla. Whereas, in case of Noakhali district, 50 faecal and 50 blood samples were taken from each upazilla (Companigong and Kabirhat). However, we are unable to collect equal number of the samples for each upazilla of each district due to difficulty in sample collection which was a limitation of this study.

3.3. Examination of samples

3.3.1. Faecal samples examination

A total of 158 faecal samples were examined using three different types of qualitative tests, namely direct smear, floatation, and sedimentation techniques (Alim and Rana, 2023). Briefly, the individual faecal sample was first homogenized and strained to remove the undigested materials. Next, a drop of faecal suspension was placed on a glass slide to perform a direct smear. Floatation technique was carried out by taking 5 mL of faecal suspension in a 20 mL test tube and rest of the volume was filled with the sugar-salt floatation fluid. A coverslip was then placed on the e convex meniscus and kept it aside for 15 minutes. The coverslip was taken off and placed on the glass slide for microscopic examination. Sedimentation test was conducted by allowing the faecal suspension in a test tube for 15 minutes. After discarding the supernatant, a drop of the sediment was examined. Duplicate smears were made for every specimen examined. A sample was considered "positive" if at least one egg or oocyst/oocyst or trophozoite was found in the same examined smears. Gastrointestinal parasitic infections were identified upto genus level based on the morphological features of eggs, cyst, oocysts, trophozoite etc. (Hendrix, 2006 and Soulsby, 1982). Due to time constraint, we were unable to perform quantitative technique (e.g., McMaster) which was a limitation of the study.

3.3.2. Blood smears examination

A total of 145 blood samples were tested. The prepared thin blood smears were stained with the 10% Giemsa's stain ((Hendrix, 2006) for 25–30 minutes. After rinsing with tap water, the stained blood smears were air dried and examined under a compound microscope (X 100) with immersion oil for the identification of blood protozoa (Krishna et al., 2016; Urquhart et al., 1996; Soulsby, 1982).

3.3.3. Examination for molecular identification of haemoprotozoa

3.3.3.1. Extraction of DNA

A total of 145 blood samples were considered for molecular confirmation of haemoprotozoan diseases. Total genomic DNA was extracted from all samples using the commercially available kit following manufacturer's instruction (AddPrep

Genomic DNA Extraction Kit®). Briefly, the samples (20 mg blood containing FTA card) were taken in Eppendorf tubes and labelled accordingly and added 200µl lysis buffer. Then 20µl of proteinase-k was added into the Eppendorf tube and incubated at 56°C until the tissue was completely lysed. Again, the mixture was properly mixed by pulse vortexing and after mixing 200µl binding buffer was added. Following incubation of the mixture at 56°C for 10 minutes, 200µl absolute ethanol (96–100%) was added into it and vortexed. Mixture was then transferred to a spin column and centrifuged at 13000 rpm for 1 minute. After centrifuging, we discarded the flow through and assembled the column. Then, 500µl wash Buffer-I was added into the tube and centrifuged at 13000 rpm for 1 minute. This process was repeated twice where 500µl wash Buffer-II was added into the tube and centrifuged at 13000 rpm for 1 minute. Then empty spin column was centrifuged at maximum speed at 13000 rpm for 1 minutes to dry the column. Then, 100 to 200µl elution buffer was added and incubated at room temperature for 1 minute. Later, mixture was centrifuged at 13000 rpm for 1 minutes and DNA was collected into new Eppendorf tube. Finally, the DNA product was preserved at -20°C for PCR

3.3.3.2. 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 in order to amplify the template DNA of *T. annulata, B. bigemina,* and *A. marginale*, respectively (**Table 3.1**). The PCR reaction volume was made 20µl containing 5µl of extracted DNA, 1µl (10 pmol/ µl) of each primer, 10µl master mix (2X) and 3µl nuclease free water.

The conditions used for amplification of *Anaplasma marginale* includes the initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 45 seconds followed by final extension at 72°C for 15 minutes.

For *Babesia bigemina*, PCR was performed for 5 minutes at 94°C to initial denaturation, and then the reaction was repeated for 35 cycles under the following conditions: 1 minute of denaturation at 94°C, 1 minute of annealing at 57°C and 1 minute of extension at 72°C followed by a 15 minutes extension at 72°C.

Theileria annulata PCR's thermal profile consisted of 95°C for 5 minutes, then 37 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, and 30 second extension at 72°C, with a final extension step of 72°C for 15 minutes. After the PCR reaction was finished, it was stored at 4°C.

Table 3.1 Primer and Oligonucleotide sequences used for the identification of *Anaplasma marginale*, *Babesia bigemina*, and *Theileria annulata*

Target Organism	**Primer name**	**Primer sequence (5'-3')**	**Amplicon size**	**Reference**
Anaplasma marginale	Amar16S-F	5'- GGCGGTGATCTGTAGCTGGTCTGA-3'	270bp	Kundave et al., 2018
	Amar16S-R	5'- GCCCAATAATTCCGAACAACGCTT-3'		
Babesia bigemina	Bb18S-F (18S rRNA)	5'-TCCATTCAAGTTTCTGCCCCATCA-3'	504bp	Kundave et al., 2018
	Bb18S-R (18S rRNA)	5'-CCATTACCAAGGCTCAAAAGCAACAA-3'		
Theileria annulata	Tamulti-F (Tams 1)	5'- CCGTTAATGCTGCAAATGAGGAGG-3'	751bp	Kundave et al., 2018
	Tamulti-R (Tams 1)	5'- GAGGCGAAGACTGCAAGGGGAG-3'		

For visualization of PCR products, an aliquot of 5µl of each PCR product was subjected to electrophoresis on a 2% agarose gel. 1gm agarose powder was added to 50ml 1X TAE buffer (Tris, Acetic acid and EDTA) and was mixed thoroughly. The mixture was then heated in the oven for 2 minutes. Then 4µl ethidium bromide was added into the mixture. Finally, the mixture/gel was poured on a gel tray. Then 5µl PCR product was put into each well of the gel tray run for the gel electrophoresis for 30 minutes. The bands were visualized using in a gel documentation system (UV-illuminator). A 100bp DNA ladder was used as a molecular-weight size marker and previously confirmed positive sample was used as positive control whereas nuclease free water was as negative control.

3.4. Nucleotide sequencing and phylogenetic analyses

Sanger dideoxy sequencing using both the forward and reverse primers was done on twelleve (12) purified PCR amplicons from the positive samples. Purification was performed by addprep genomic DNA extraction kit® according to the manufacturer's

instructions. The Sanger sequencing was carried out using the dideoxy chain termination method at Genecreate Biotech, China. (Sanger et al., 1977). The acquired sequences were cleared and combined with Chromas, BioEdit, and the chromatogram peaks were used to confirm the results. Consensus sequences were then produced by performing multiple sequence alignments of the cleared sequences using the ClustalW technique and Neighbor Joining cluster method on MEGA 11. The consensus sequences were compared with the right A*naplasma, Babesia*, and *Theileria* species identities using BLASTn for nucleotide analyses, which was accessed through GenBank of the NCBI database. The closest BLASTn match to those homologues discovered in the GenBank, with an identity of between 90%–100%, was determined to be the species confirmation. Using the UPGMA method and Maximum Composite Likelihood model, three phylogenetic trees *(Anaplasma*, *Babesia*, and *Theileria*) of evolutionary history were constructed (Sneath and Sokal, 1973). The percentage of duplicate trees in which the connected taxa clustered together in the bootstrap test (1000 repetitions) is indicated next to the branches (Felsenstein, 1985). The evolutionary distances were calculated using the Maximum Composite Likelihood approach (Tamura K et al., 2004) and were measured in base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). 18 nucleotide sequences from *Anaplasma,* 12 nucleotide sequences from *Babesia, and* 15 nucleotide sequences from *Theileria* were analyzed. The final dataset had a total of 1462, 1589, and 145 positions for *Anaplasma*, *Babesia,* and *Theileria,* respectively. Evolutionary analyses were conducted in MEGA 11 (Tamura et al., 2021).

3.5. Statistical analyses

All data were inserted and coded in Microsoft office Excel 365 spreadsheet and analysed using The STATA/SE-13.0 (Stata Corporation College Station). Descriptive statistics were expressed as the proportion with 95% Confidence Interval. The chi-square test was performed and results were expressed in percentage with *p*-value. Significance was determined when $p^{*} \leq 0.05$, $p^{**} \leq 0.01$, and $p^{***} \leq 0.001$. In this research, two types of infections (single and mixed) were taken into consideration. In the case of GI parasitic infection, mixed infection was defined as helminths + helminths; helminths + GI protozoa; GI protozoa + GI protozoa; and in the case of hemoprotozoan diseases, as mixed infection was defined as Anaplasmosis +

Babesiosis; Anaplasmosis + Theileriosis; Anaplasmosis + Babesiosis + Theileriosis. Single infections did not include mixed infections.

Chapter-4: Results

4.1. Prevalence of gastrointestinal parasitic infection

4.1.1. Overall prevalence of gastrointestinal parasitic infection

The overall prevalence of gastrointestinal (GI) parasitic infections was 44.30% (95% CI: 36.41–52.41, N= 70) in buffalo **(Table 4.1**). Among helminths, prevalence of nematodes was higher (20.25%, 95% CI: 14.28–27.37, N= 32) than other. Besides, among different nematodes, the prevalence of *Toxocara vitulorum* was the highest (17.72%, 95% CI: 12.11–24.58, N= 28) and the lowest GI parasitic infections were recorded for *Trichostrongylus* sp. and *Trichuris* sp. infections **(Table 4.1)**. However, among protozoa, *B. coli* cyst was highly prevalent (4.43%, 95% CI=01.79–08.91, N=7). Furthermore, more than one GI parasitic infections were found in 12.03% of buffalo **(Table 4.1, Figure 4.1)**.

Table 4.1 Overall prevalence of gastrointestinal parasitic infection in buffalo

Gastrointestinal parasites		**Frequency**	**Percentage (n=158)**	**95% CI**
Parasite groups	**Parasites**			
Nematode	*Toxocara vitulorum*	28	**17.72**	12.11- 24.58
	Oesophagostomum sp.	2	1.27	00.15-04.49
	Trichostrongylus sp.	1	0.63	00.01-03.47
	Trichuris sp.	1	0.63	00.01- 03.47
	Total	**32**	**20.25**	**14.28- 27.37**
Trematode	*Paramphistomum* sp.	5	**3.16**	01.03-07.23
Protozoa	*Eimeria* cyst	2	1.27	00.15- 04.49
	Trophozoite of *B. coli*	5	3.16	01.03- 07.23
	Balantidium coli cyst	7	**4.43**	01.79-08.91
	Total	**14**	**8.86**	**04.92- 14.41**
Mixed		19	12.03	07.39- 18.14
Overall		**70**	**44.30**	**36.41- 52.41**

n= Total number of animals, CI= Confidence Interval

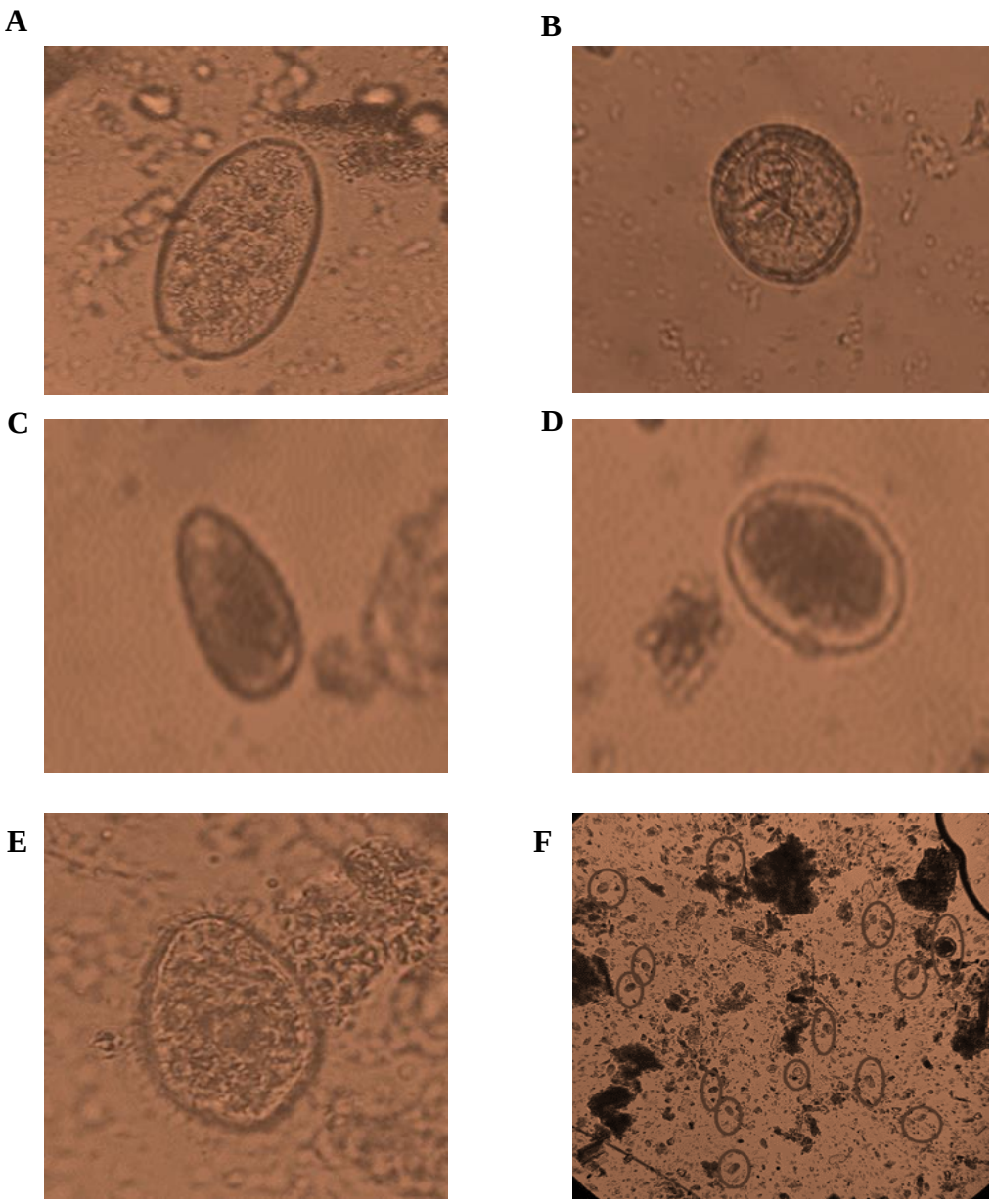
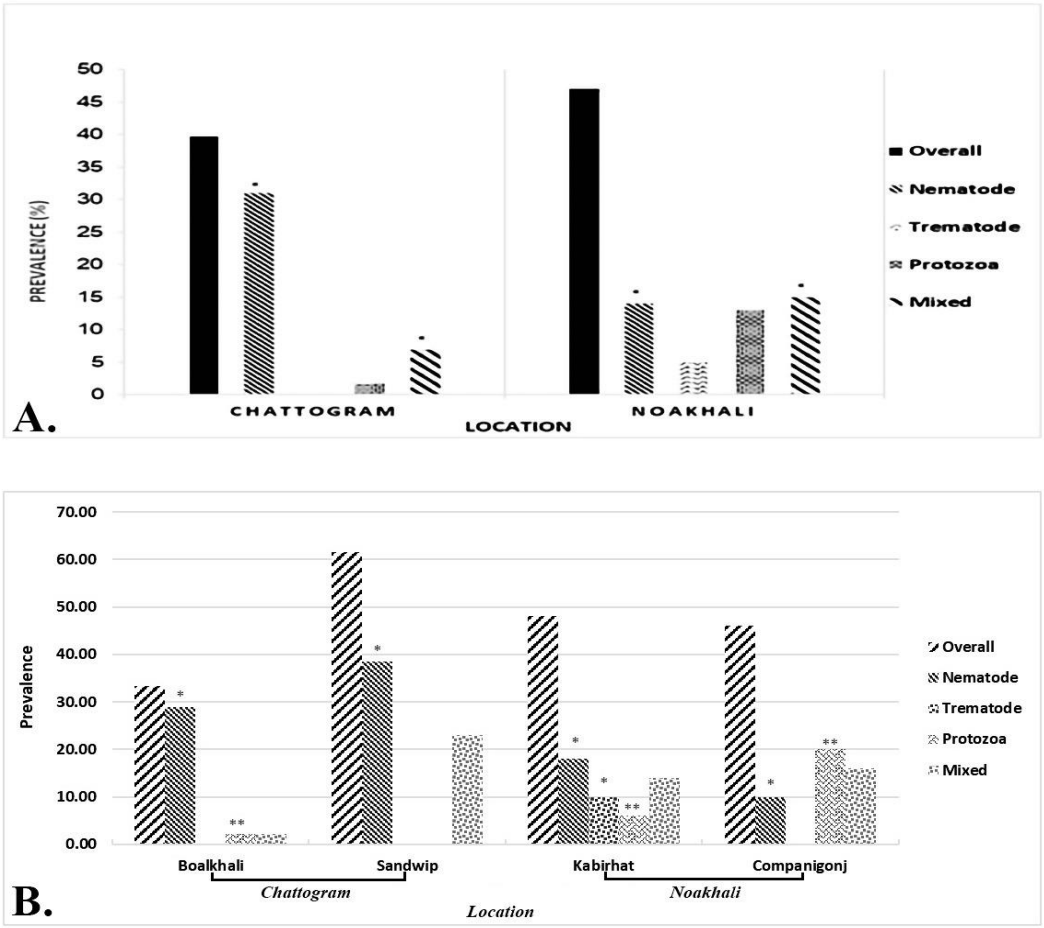


Figure 4.1 Diagnostic stages of gastrointestinal parasites*:* Figures showed the egg of *Paramphistomum* sp. (A); the Egg of *Toxocara vitulorum* (B); Egg of *Trichostrongylus* sp. (C); Egg of *Oesophagostomum* sp. (D); Trophozoite of *Balantidium coli* (E); Mixed infection of nematode (F)

4.1.2. Prevalence of gastrointestinal parasitic infection based on location

In terms of location, buffalo of Noakhali district had higher prevalence (47%) of GI parasites than buffalo of Chattogram district (39.65%) **(Figure 4.2)**. Again, buffalo of Sandwip had the highest (61.54%) prevalence of GI parasites in relation to buffalo of Kabirhat (48.00%), Companigonj (46.00%), and Boalkhali (33.33%). In terms of different parasitic groups, nematode was found most in relation to other parasite groups in all upazilla. Besides, among different parasites *Toxocara vitulorum* had the highest prevalence in most of the sampling location except for Companigonj. But no cestode was found in these study areas **(Figure 4.2)**.



Level of significance was indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

Figure 4.2 Prevalence of gastrointestinal parasite in buffalo in different areas; district wise (A); upazilla wise (B)

4.1.3. Age specific prevalence of gastrointestinal parasitic infection

Age specific prevalence of GI parasitic infections in buffalo was presented in **Table 4.2**. It was observed that adult buffalo showed greater susceptibility (46.05%) to GI parasitic infection than calf (38.10%) and young (44.46%). In case of nematode, young buffalo had the highest prevalence (27.86%) than calf and young, whereas, the highest prevalence of trematode was found in calf (9.52%) and the highest prevalence of protozoa was found in adult buffalo (13.16%). Highest prevalence of *Toxocara vitulorum* was 22.95% in young buffalo and it was 15.79% in adult buffalo. Occurrence of *Oesophagostomum* sp. and *Trichostrongylus* sp. was also highest in young buffalo. Furthermore, more than one GI parasitic infections were found highest in calf (14.29%) as shown in **Table 4.2**

Table 4.2 Age specific prevalence of gastrointestinal parasitic infection in buffalo

Gastrointestinal parasites		**Age**			***p*-Value**
Parasite groups	**Parasites**	**Calf (n=21) %**	**Young (n=61) %**	**Adult (n=76) %**	
Nematode	*Toxocara vitulorum*	9.52 (2)	22.95 (14)	15.79 (12)	0.316
	Oesophagostomum sp.	0.00 (0)	3.28 (2)	0.00 (0)	0.200
	Trichostrongylus sp.	0.00 (0)	1.64 (1)	0.00 (0)	0.449
	Trichuris sp.	0.00 (0)	0.00 (0)	1.32 (1)	0.581
	Total	**9.52 (2)**	**27.86 (17)**	**17.11 (13)**	
Trematode	*Paramphistomum* sp.	**9.52 (2)**	0.00 (0)	3.95 (3)	0.086
Protozoa	*Eimeria* cyst	0.00 (0)	0.00 (0)	2.63 (2)	0.335
	Trophozoite of *B. coli*	0.00 (0)	3.28 (2)	3.95 (3)	0.657
	Balantidium coli cyst	4.76 (1)	1.64 (1)	6.58 (5)	0.376
	Total	**4.76 (1)**	**4.92 (3)**	**13.16 (10)**	
Mixed		14.29 (3)	11.48 (7)	11.84 (9)	0.941
Overall		**38.10 (8)**	**44.26 (27)**	**46.05 (35)**	**0.810**

Calf (<1 year), young (1–3 year) and adult (>3 year) where the minimum age of the buffalo was 6 months, median 1.5 year, and the maximum age was 5 years.

4.1.4. Sex specific prevalence of gastrointestinal parasitic infection in buffalo

As presented in **Table 4.3**, male buffalo showed more susceptibility (48.28%) to different GI parasites than female (42.00%). However, prevalence of *Paramphistomum* sp., *Trichuris* sp. and *Eimeria* cyst was higher in female buffalo

than male buffalo. *Balantidium coli*, *Toxocara vitulorum*, *Oesophagostomum* sp., and *Trichostrongylus* sp. infection were more prevalent in male buffalo **(Table 4.3)**.

Table 4.3 Sex specific prevalence of gastrointestinal parasitic infection in buffalo

Gastrointestinal parasites		**Sex**		***p*-Value**
Parasite groups	**Parasites**	**Male (n=58) %**	**Female (n=100) %**	
Nematode	*Toxocara vitulorum*	18.97 (11)	17.00 (17)	0.755
	Oesophagostomum sp.	1.72 (1)	1.00 (1)	0.695
	Trichostrongylus sp.	1.72 (1)	0.00 (0)	0.188
	Trichuris sp.	0.00 (0)	1.00 (1)	0.445
	Total	**22.41 (13)**	**19.00 (19)**	
Trematode	*Paramphistomum* sp.	1.72 (1)	4.00 (4)	0.431
Protozoa	*Eimeria* cyst	0.00 (0)	2.00 (2)	0.278
	Trophozoite of *B. coli*	5.17 (3)	2.00 (2)	0.272
	Balantidium coli cyst	6.90 (4)	3.00 (3)	0.251
	Total	**12.07 (7)**	**7.00 (7)**	
Mixed		12.07 (7)	12.00 (12)	0.990
Overall		**48.28 (28)**	**42.00 (42)**	**0.444**

4.1.5. Prevalence of gastrointestinal parasites in buffalo based on deworming status

As shown in **Table 4.4**, overall no significant difference was found in case of prevalence of GI parasites in terms of deworming status. Overall GI parasitic infection was highest in dewormed buffalo (47.52%) than non-dewormed buffalo (38.60%). However, Nematode was higher in non-dewormed buffalo (31.58%) and among nematodes, *Toxocara vitulorum* infection was found almost two folds less in dewormed buffalo (12.87%) than the buffalo that were not dewormed (26.32%) during this investigation which was statistically significant ($p<0.05$). In case of *Balantidium coli* cyst statistically significant ($p<0.05$) difference was also found between dewormed (6.93%) and non–dewormed (0.00%) buffalo **(Table 4.4)**.

Table 4.4 Prevalence of gastrointestinal parasites in buffalo based on deworming status

Gastrointestinal parasites		**Deworming status**		***p*-Value**
Parasite groups	**Parasites**	**Dewormed (n=101) %**	**Non-dewormed (n=57) %**	
Nematode	*Toxocara vitulorum*	12.87(13)	26.32 (15)	**0.034***
	Oesophagostomum sp.	0.00 (0)	3.51 (2)	0.058
	Trichostrongylus sp.	0.00 (0)	1.75 (1)	0.182
	Trichuris sp.	0.99 (1)	0.00 (0)	0.451
	Total	**13.86 (14)**	**31.58 (18)**	
Trematode	*Paramphistomum* sp.	4.95 (5)	0.00 (0)	0.088
Protozoa	*Eimeria* cyst	1.98 (2)	0.00 (0)	0.285
	Trophozoite of *B. coli*	3.96 (4)	1.75 (1)	0.447
	Balantidium coli cyst	6.93 (7)	0.00 (0)	**0.042***
	Total	**12.87 (13)**	**1.75 (1)**	
Mixed		15.84 (16)	5.26 (3)	0.050
Overall		**47.52 (48)**	**38.60 (22)**	**0.278**

Level of significance was indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

4.2. Prevalence of haemoprotozoan diseases on the basis of Giemsa's staining and microscopic identification

The microscopic examination of thin blood smears (N=145) revealed only *Anaplasma* species in buffalo. The overall prevalence of haemoprozoan diseases (Anaplasmosis) was 14.48**%** (95% CI: 09.19–21.28, N= 21) **(Figure 4.3**). However, such findings under different variables such as age, sex, deworming status, and location was presented in **Table 4.5**.

Table 4.5 Prevalence of haemoprotozoan diseases (Anaplasmosis) in buffalo (in thin blood smear)

Traits		Anaplasmosis		95% CI
		Frequency	Percentage	
Overall prevalence (n=145)		21	14.48	09.19-21.28
Traits	**Categories**	**Frequency**	**Percentage**	***p*- Value**
Age	Calf (n=19)	4	21.05	0.639
	Young (n=54)	8	14.81	
	Adult (n=72)	9	12.50	
Sex	Male (n=50)	8	16.00	0.706
	Female (n=95)	13	13.68	
Deworming status	Dewormed (n=100)	13	13.00	0.449
	Non-dewormed (n=45)	8	17.78	
Location (District)	Chattogram (n=45)	8	17.78	0.449
	Noakhali (n=100)	13	13.00	
Location (Upazilla)	Boalkhali (n=45)	8	17.78	0.522
	Kabirhat (n=50)	8	16.00	
	Companigonj (n=50)	5	10.00	

Calf (<1 year), young (1–3 year) and adult (>3 year) where the minimum age of the buffalo was 6 months, median 1.5 year, and the maximum age was 5 years.

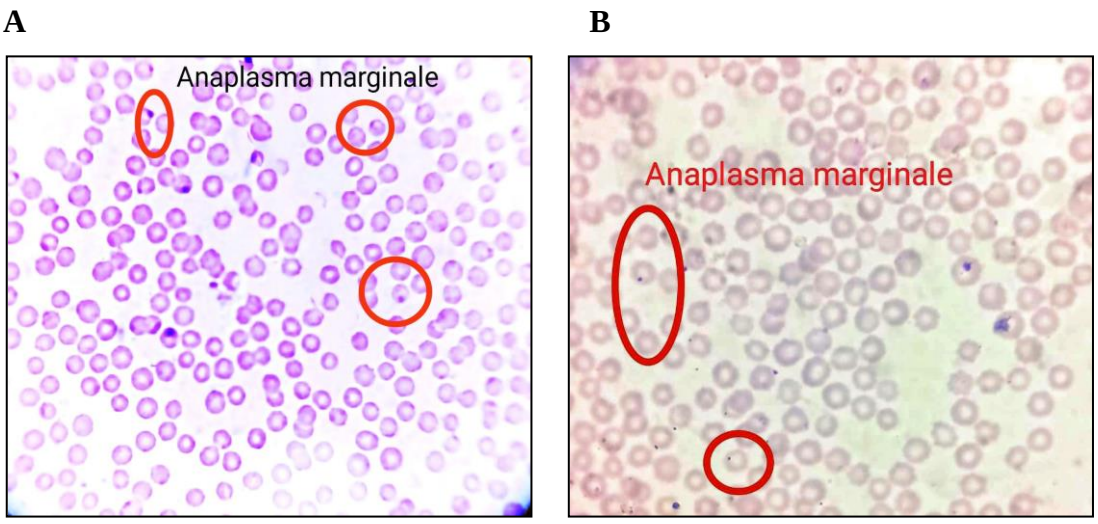


Figure 4.3 *Anaplasma marginale* (A, B) in microscopic examination by Giemsa's stain

4.3. Prevalence of haemoprotozoan diseases on the basis of molecular identification

4.3.1. Overall molecular prevalence of haemoprotozoan diseases

The overall molecular prevalence of haemoprotozoan diseases was 31.03% in buffalo. The highest prevalence was recorded in Anaplasmosis which was 30.34%. Occurrence of Babesiosis was 2.07%. However, mixed infection (Babesiosis, Anaplasmosis and Theileriosis) was found relatively higher than Babesiosis and Theileriosis alone **(Table 4.6)**.

Table 4.6 Overall molecular prevalence of haemoprotozoan diseases in buffalo

Haemoprotozoan diseases	Frequency	Percentage (n=145)	95% CI
Anaplasmosis	44	30.34	22.99-38.52
Babesiosis	3	2.07	00.42-05.92
Theileriosis	2	1.38	00.16-04.89
Mixed infection	4	2.76	00.75-06.91
Overall infection	**45**	**31.03**	**23.62-39.24**

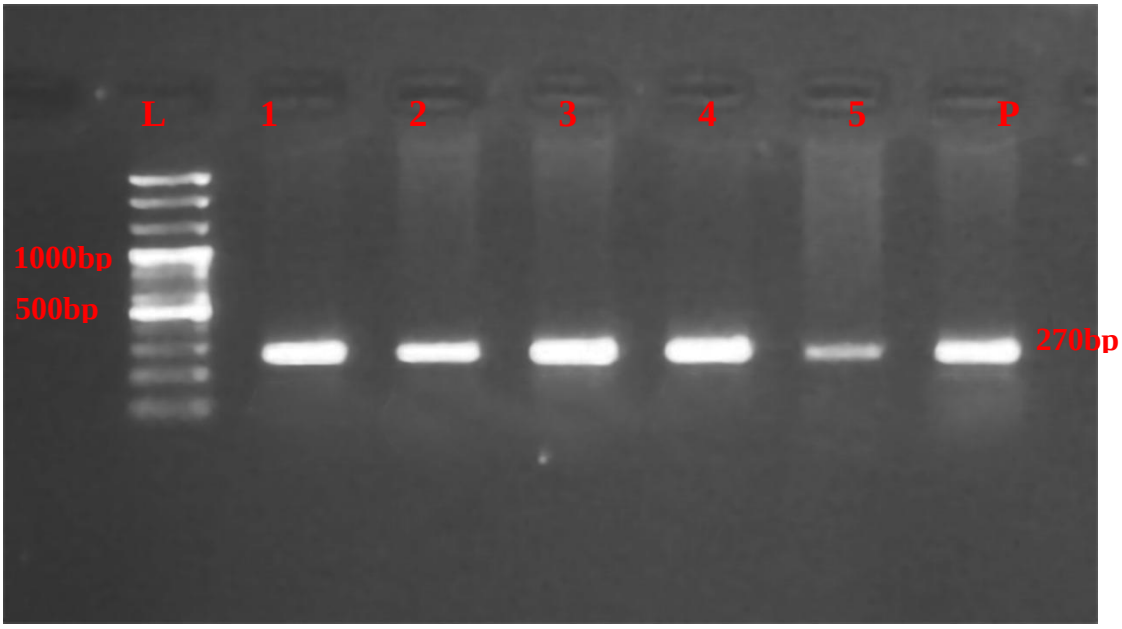


Figure 4. 4 PCR assay for 16S rRNA gene of *Anaplasma marginale* isolates; Lane L: 100bp Ladder; Lane 1,2,3,4,5: Samples (270bp) of *A. marginale* isolates and Lane P: Positive control

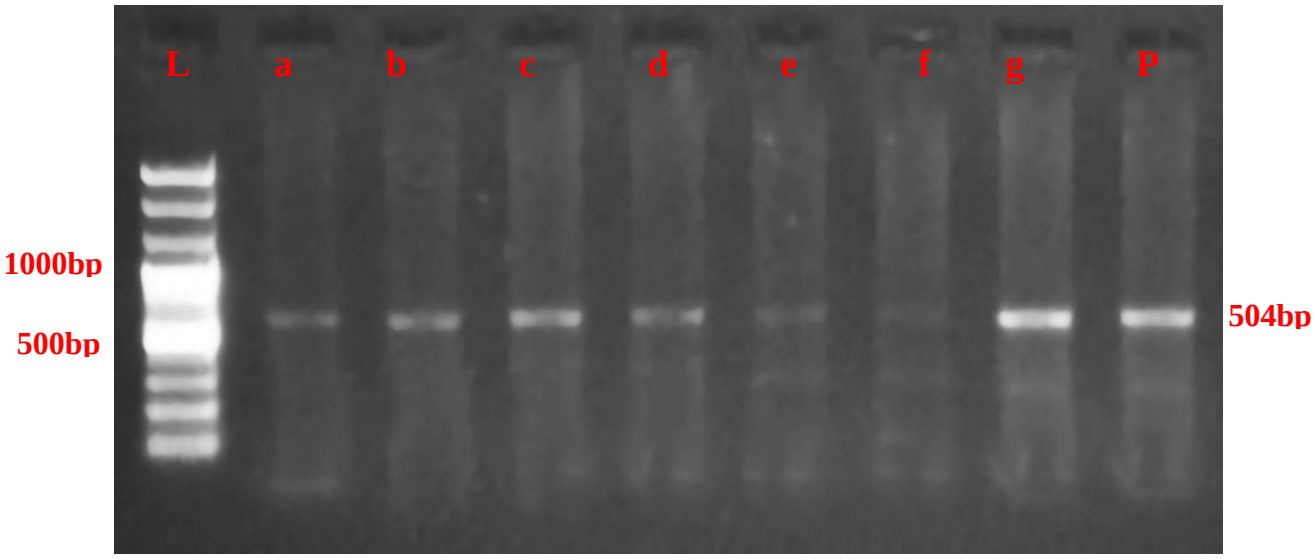


Figure 4.5 PCR assay for 18S rRNA gene of *B. bigemina* isolates Lane L: 100bp Ladder; Lane a, b, c, d, e, f, g: Samples (504bp) of *B. bovis* isolates; Lane P: Positive control

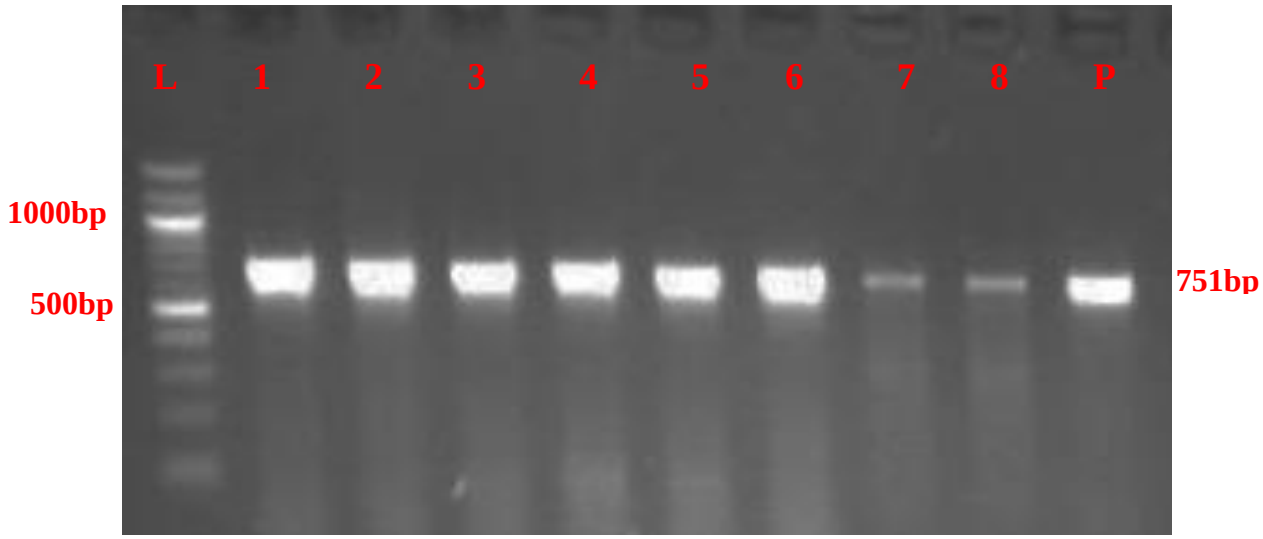


Figure 4.6 PCR assay for Tams1 gene of *T. annulata* isolates; Lane L: 100bp Ladder; Lane 1, 2, 3, 4, 5, 6, 7, 8: Samples (751bp); Lane P: Positive control

4.3.3. Age specific molecular prevalence of haemoprotozoan diseases

Among three different age groups, calf (36.84%) showed more susceptibility to different haemoprotozoan diseases in comparison to young and adult buffalo **(Table 4.7)**. It was also observed that prevalence of Babesiosis was the highest in young buffalo. Occurrence of Anaplasmosis was highest in calf. Theileriosis and mixed infections were also varied according to age of animals during this study **(Table 4.7)**.

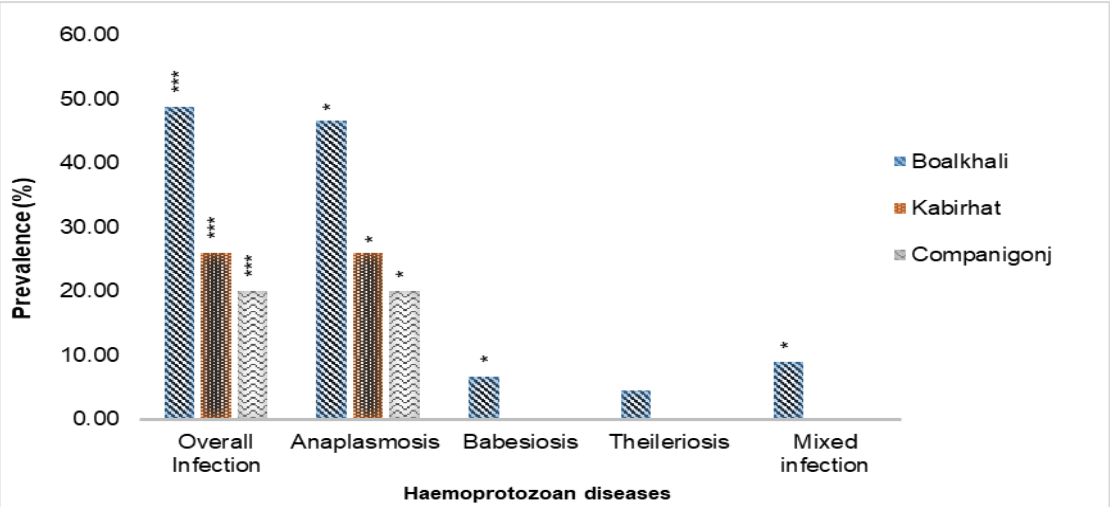
Table 4.7 Age specific molecular prevalence of haemoprotozoan diseases in buffalo

Haemoprotozoan diseases	**Age**			***p*- Value**
	Calf (n=19) %	**Young (n=54) %**	**Adult (n=72) %**	
Anaplasmosis	36.84 (7)	31.48 (17)	27.79 (20)	0.727
Babesiosis	0.00 (0)	5.56 (3)	0.00 (0)	0.076
Theileriosis	0.00 (0)	0.00 (0)	2.78 (2)	0.358
Mixed infection	0.00 (0)	5.56 (3)	1.39 (1)	0.270
Overall infection	**36.84 (7)**	**31.48 (17)**	**29.17 (21)**	**0.810**

Calf (<1 year), young (1–3 year) and adult (>3 year) where the minimum age of the buffalo was 6 months, median 1.5 year, and the maximum age was 5 years.

4.3.2. Molecular prevalence of haemoprotozoan diseases based on location

Geographically, the buffalo of Boalkhali region had the highest prevalence (48.89%) of all the hemoprotozoan disorders that were studied. However, it was revealed that, buffalo of Kabirhat had higher (26.00%) prevalence of Anaplasmosis in relation to Companigonj (20.00%) and it was 46.67% for buffalo of Boalkhali area **(Figure 4.7)**.



Level of significance was indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

Figure 4.7 Molecular prevalence of haemoprotozoan infection in buffalo in different areas

4.3.4. Sex specific molecular prevalence of haemoprotozoan diseases

Overall higher prevalence (molecular) of haemoprotozoan diseases was found in female which was 36.84% whereas it was 20.00% for male buffalo. However, Anaplasmosis infection was more prevalent in both groups and Babesiosis infection showed slightly variations in their occurrence **(Table 4.8)**.

Table 4.8 Sex specific molecular prevalence of haemoprotozoan diseases in buffalo

Haemoprotozoan diseases	Sex		*p*- Value
	Male (n=50) %	Female (n=95) %	
Anaplasmosis	20.00 (10)	35.79 (34)	**0.049***
Babesiosis	2.00 (1)	2.11 (2)	0.966
Theileriosis	0.00 (0)	2.11 (2)	0.302
Mixed infection	2.00 (1)	3.16 (3)	0.686
Overall infection	**20.00 (10)**	**36.84 (35)**	**0.037***

Level of significance was indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.0001$

4.3.5. Molecular prevalence of haemoprotozoan diseases based on deworming status of buffalo

As shown in **Table 4.9**, overall substantial difference was found in case of prevalence of haemoprotozoan diseases in terms of deworming status of the buffalo. The overall prevalence of haemoprotozoan diseases was 48.89% in non-dewormed and 23.00% in dewormed buffalo. However, buffalo that were not dewormed suffered from mixed infections than the dewormed buffalo.

Table 4.9 Molecular prevalence of haemoprotozoan diseases in buffalo based on deworming status

Haemoprotozoan diseases	Deworming status		*p*-value
	Dewormed (n=100) %	Non-dewormed (n=45) %	
Anaplasmosis	23.00 (23)	46.67 (21)	**0.004***
Babesiosis	0.00 (0)	6.67 (3)	**0.009***
Theileriosis	0.00 (0)	4.44 (2)	**0.034***
Mixed infection	0.00 (0)	8.89 (4)	**0.002***
Overall infection	**23.00 (23)**	**48.89 (22)**	**0.002***

Level of significance was indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

4.4. Partial gene sequencing of blood protozoa (*Anaplasma marginale, Babesia bigemina and Theileria annulata*)

Twelleve amplicons in all were chosen at random for sequencing, six of which came from *Anaplasma* positive samples, four from *Babesia* positive samples, and the other two from *Theileria* positive samples. All of the samples that produced nucleotide sequences were good candidates for additional examination. Six sequences from *Anaplasma*- 16s rRNA were subjected to bioinformatics analyses using the BLASTn method, which revealed homologues that were the same as those from *A. marginale.* Four *Babesi*a 18S rRNA gene sequence was found to be identical to *B. bigemina.* Next two *Theileria* tams1 gene sequences were found to be identical to *T. annulata.*

4.5. Results of nucleotides sequence alignment

4.5.1. Multiple sequence alignment of *Anaplasma* isolates with those of other regions

Multiple sequence alignments showed that, the sequences of the *Anaplasma marginale* isolates from India, China, Pakistan, and the USA were the identical and partially similar with those from Chattogram, Bangladesh. Anaplasma isolates from Chattogram, Bangladesh had diverse sequences from those of Egypt and Iraq **(Figure 4.8)**.

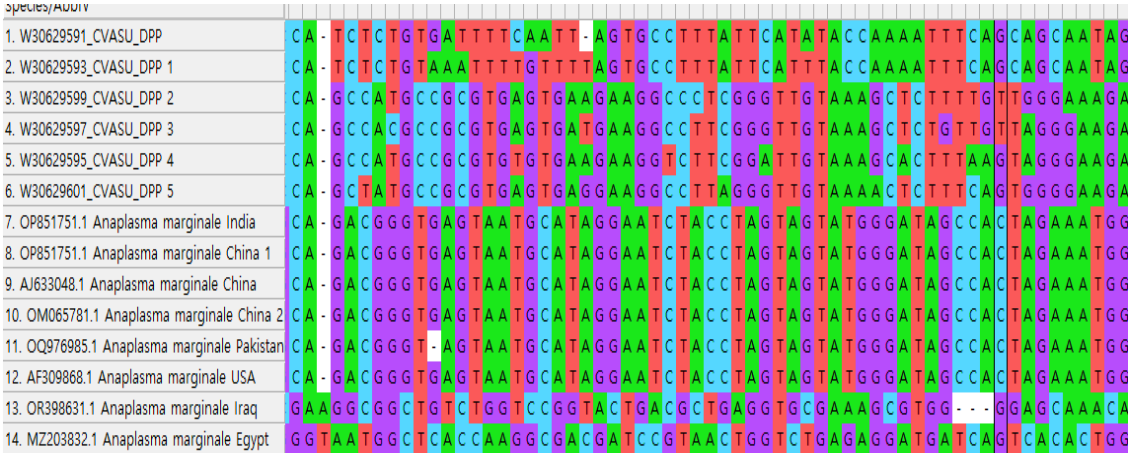


Figure 4.8 For the various *A. marginale* isolates from India, Pakistan, China, USA, Egypt, and Iraq, ClustalW multiple sequence alignment analysis was conducted. The

nucleotide sequence variations among *A. marginale* isolates from several countries are depicted in this figure.

4.5.2. Multiple sequence alignment of *Babesia* isolates with those of other regions

Multiple sequence alignments showed that, the sequences from buffalo samples were conserved. The sequences of the *Babesia bigemina* isolates from Switzerland, Columbia, Brazil, and India were the partialy similar as those from Chattogram, Bangladesh. *Babesia* isolates from Chattogram, Bangladesh and other location had distinct sequences from those from Japan **(Figure 4.9)**.

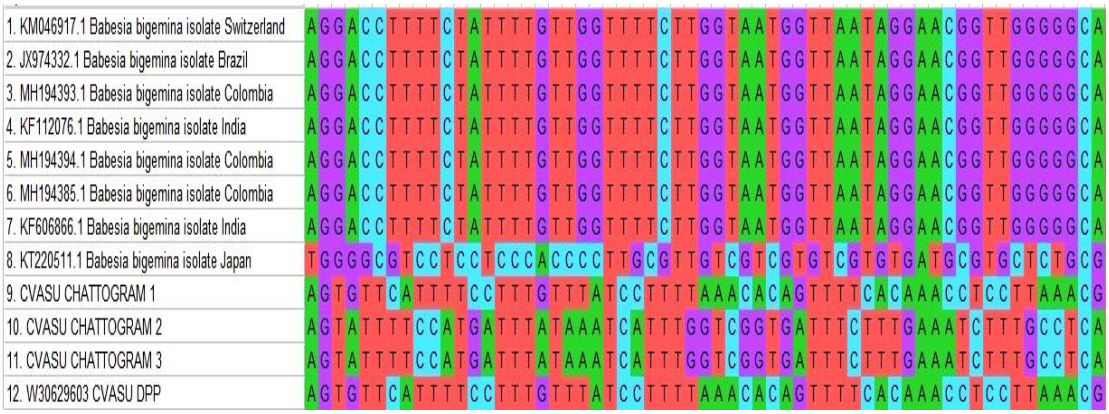


Figure 4.9 For the various *B. bigemina* isolates from India, Columbia, Switzerland, Brazil, and Iapan, ClustalW multiple sequence alignment analysis was conducted. The nucleotide sequence variations among *B. bigemina* isolates from several countries are depicted in this figure.

4.5.3. Multiple sequence alignment of *Theileria* isolates with those of other regions

Multiple sequence alignments showed that, the sequences of the *T. annulata* isolates from buffalo samples from Chattogram, Bangladesh showed smiliarity with the isolates of India, China, Pakistan, Egypt, and Iraq. **(Figure 4.10)**.

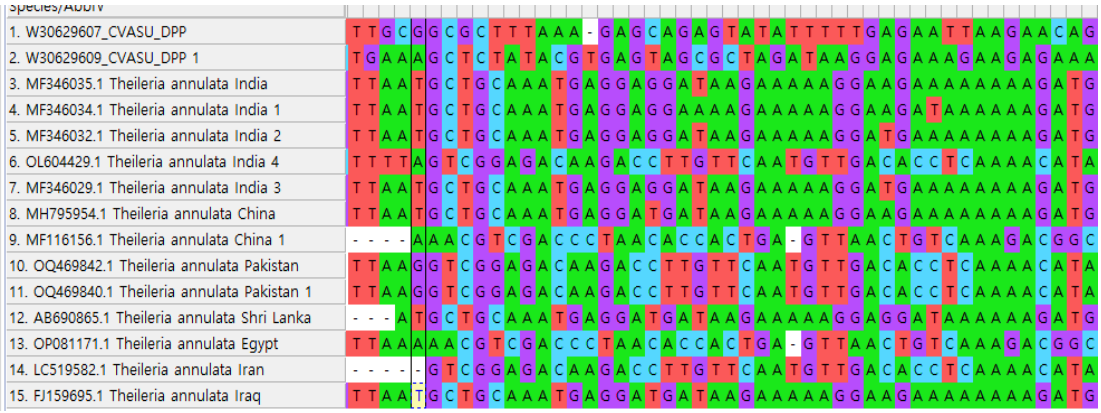


Figure 4.10 For the various *T. annulata* isolates from India, China, Pakistan, Egypt, Iran, and Iraq, ClustalW multiple sequence alignment analysis was conducted. Nucleotide sequence variations among *T. annulata* isolates from several countries are depicted in this figure.

4.6. Results of phylogenetic analyses

4.6.1. Phylogenetic analysis of *Anaplasma* isolates

To comprehend the evolutionary relationship between the *Anaplasma* isolates from Chattogram, Bangladesh, and *Anaplasma marginale* isolates from other countries that are available in Genbank, a phylogenetic tree was constructed. A close cluster was formed by three isolates from Chattogram, Bangladesh, (CVASU DPP 1, CVASU DPP 3, CVASU DPP 2) while other three isolates (CVASU DPP, CVASU DPP 5, CVASU DPP 4) belonged another cluster. Once more, three isolates had an ancestral relationship with those from Egypt and the other three had distal evolutionary relationship with the isolates from the USA. However, isolates from Egypt had slightly descendant from three isolates of Chattogram, Bangladesh and all the isolates of Chattogram, Bangladesh had distand evolutionary relationship with the isolates from India, Pakistan, China, and Iraq **(Figure 4.11)**.

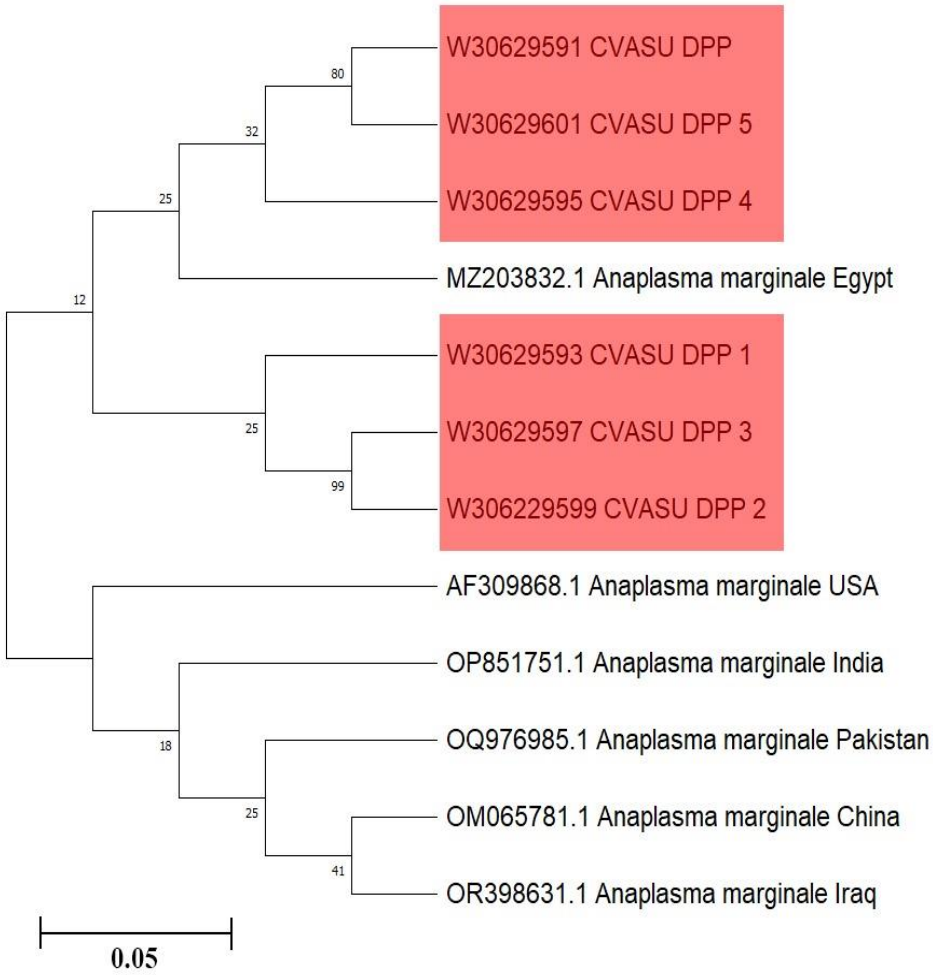


Figure 4.11 Phylogenetic tree inferred from partial *tams1* nucleotide gene sequences and showing the relationship between *A. marginale* isolates investigated in this study (indicated by red marking) and other *A. marginale* strains published in GenBank (accession numbers in starting). The tree was constructed using the UPGMA method of MEGA 11 software. The numbers at the nodes are bootstrap values expressed as percentages of 1000 replicates. The scale bar (0.05) represents the number of mutations per site.

4.6.2. Phylogenetic analysis of *Babesia* isolates

A phylogenetic tree was created to understand the evolutionary relationship between the *Babesia bigemina* isolates from Chattogram, Bangladesh, and other *Babesia bigemina* isolates from different countries that are available in Genbank. According to this tree, which also showed that two of the isolates from Chattogram, Bangladesh (CVASU CHATTOGRAM 1, CVASU DPP) formed a clade while the other two

(CVAU CHATTOGRAM 2, CVASU CHATTOGRAM 3) formed another clade, all four isolates from that city belonged to a single cluster. But each of these isolates had a close relationship with the Japan isolates, indicating that they are descendant from the isolates of Japan. Once more, it was discovered that the isolates from Chattogram, Bangladesh were distantly related to those from Brazil, Switzerland, India, and Columbia (**Figure 4.12)**.

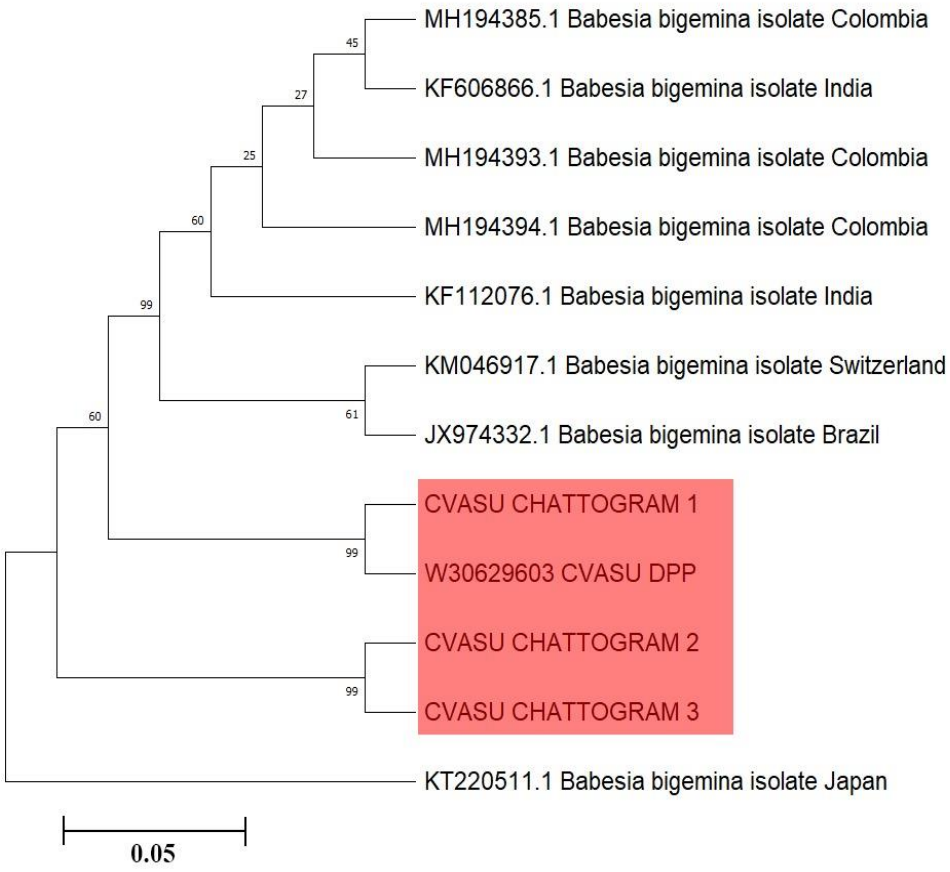


Figure 4.12 Phylogenetic tree inferred from partial *tams1* nucleotide gene sequences and showing the relationship between *B. bigemina* isolates investigated in this study (indicated by red marking) and other *B. bigemina* strains published in GenBank (accession numbers in starting). The tree was constructed using the UPGMA method of MEGA 11 software. The numbers at the nodes are bootstrap values expressed as percentages of 1000 replicates. The scale bar (0.05) represents the number of mutations per site.

4.6.3. Phylogenetic analysis of *Theileria* isolates

A phylogenetic tree was created in order to understand the evolutionary relationship between the *Theileria annulata* isolates from Chattogram, Bangladesh, and other *Theileria annulata* isolates from different countries that are found in Genbank. Two isolates from Chattogram, Bangladesh, were found to be members of the same clade, as evidenced by this tree. Again, isolates from Egypt, Pakistan, Sri Lanka, China, or India revealed distand evolutionary relationship with those from Chattogram, Bangladesh **(Figure 4.13)**.

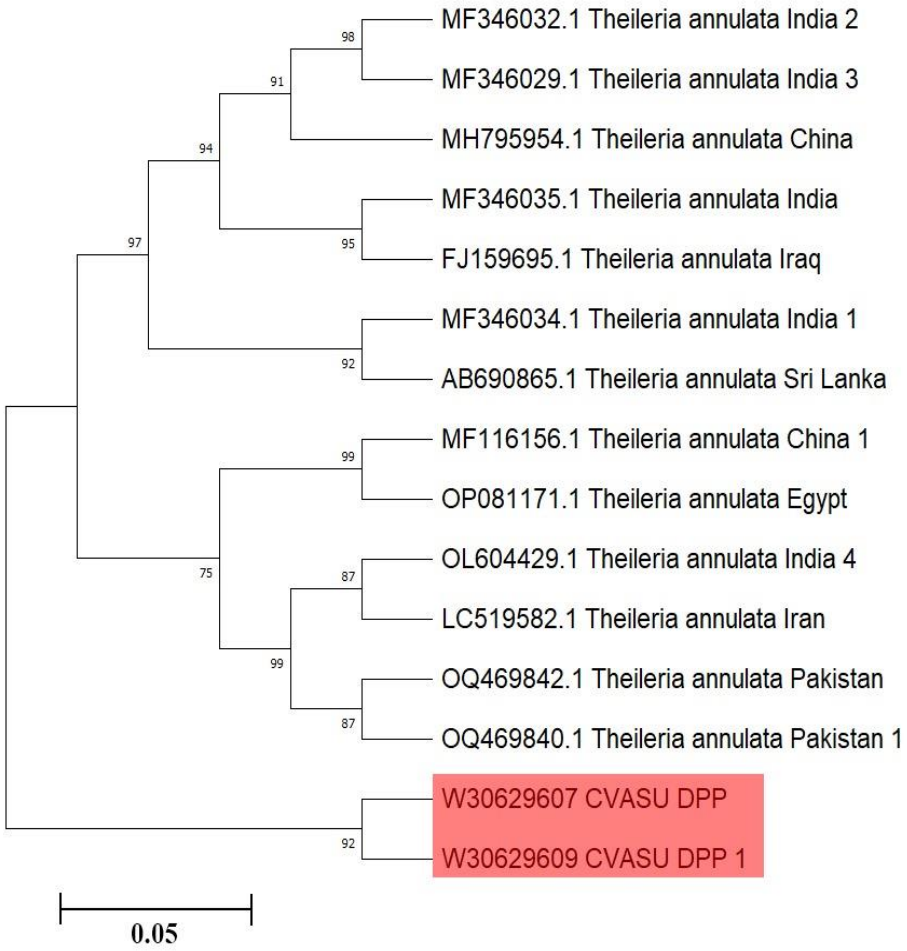


Figure 4.13 Phylogenetic tree inferred from partial *tams1* nucleotide gene sequences and showing the relationship between *T. annulata* isolates investigated in this study (indicated by red marking) and other *T. annulata* strains published in GenBank (accession numbers in starting). The tree was constructed using the UPGMA method of MEGA 11 software. The numbers at the nodes are bootstrap values expressed as

percentages of 1000 replicates. The scale bar (0.05) represents the number of mutations per site.

Chapter-5: Discussion

Many disorders, including as GI and haemoprotozoan diseases, have a negative impact on the growth, development, and production of these animals (Krishna et al., 2016; Mamun et al., 2020). This research was carried out to find out how common gastrointestinal (GI) parasites and blood protozoa were in buffalo, as well as what risk factors led to these disorders. Based on the degree of infection, it was determined that *Toxocara vitulorum* is the intestinal parasite that has a more detrimental effect on buffalo calves (Ara et al., 2002). According to Alam et al. (2016), additional parasite species that are present in buffaloes include *Fasciola* sp., *Paramphistomum* sp., *Ascaris* sp., *Strongyloides* sp., *Bunostomum* sp., and *Oesophagostomum* sp.

5.1. Prevalence of gastrointestinal parasitic infection on the basis of microscopic identification

The overall prevalence of gastrointestinal (GI) parasites (helminths and protozoa) in buffalo was 44.30% where 35.44%. were contributed by helminth infections. This finding of this study is slightly lower to the previous findings of Kashyap et al., (1997), who reported 40.30% GI helminth infections in buffaloes of the Malwa region of Madhya Prades, India. Besides, Mir et al., (2013) showed 38.70% prevalence of GI parasitic infection in buffaloes in the Jammu region, India which is slightly lower than the present study. While Gupta et al., (2012), Biswas et al., (2014), and Marskole et al., (2016), had reported 73%, 70.75%, and 84.30% GI parasitic infection in buffaloes, respectively which is much higher than the findingds of the current study. However, Mamun et al., (2011) and Azam et al., (2002), reported 60-65% prevalence of GI helminths in water buffalo in the Kurigram district of Bangladesh and Pakistan which is also higher than the findings of this current investigation. In addition, GI helminthiosis has also been linked to more than 50% incidence of parasite infections in cattle and buffaloes in Gujrat (Pethkar and Hiregaudar, 1972), Haryana (Chhabra et al., 1978), and Rajasthan (Godara and Manohar, 2004). This variation may be due to the variation in sampling strategies and sample size, season, diet, stocking density, geo-climatic conditions, grazing and housing, deworming and overall husbandry practices (Gunathilaka et al., 2018; Marskole et al., 2016)

This study also revealed that, *Toxocara vitulorum* had the greatest prevalence, which was 17.72%, among the several nematodes. This findingis in line with the prevalence (17% –35%) recorded in Bangladesh and Pakistan (Bhutto et al., 2002; Zaman et al., 2014; Ara et al., 2021). Akhter et al., (2001) also claimed that it was the parasite that affected buffaloes the most frequently. In case of *Trichuris*, its occurrence found to be lower (0.63%) than the findings of the Bhutto et al., (2002) who reported 2% prevalence in study animals. In case of *Oesophagostomum* sp., prevalence is much lower (1.27%) than the findings of Guzel and Kozan, (2013) who recorded 41.6% prevalence in buffalo.

Prevalence of *Paramphistomum* sp. is much lower (3.16%) as compared to the findings of Mamun et al., (2011) who recorded 29.24% prevalence. This lower prevalence may be dute to lower availability of intermediate host in study area. Furthermore, there was found no *Fasciola* in this study. This may be due to absence of intermediate host in the study area. But, Saha et al., (2013), Biswas et al., (2014), and Mamun et al., (2011) reported that Fasciolosis in buffaloes was 26.17%, 25.40%, and 22.46%, respectively at Barisal, Bhola, and Kurigram in Bangladesh.

Cestode was not found in this investigation, which is consistent with Saha et al., (2013), and Mamun et al., (2011)’s findings. As buffaloes are typically maintained in animal houses and are rarely available to intermediate hosts of cestodes, it is not surprising that cestodes of buffaloes have become less common in recent years (Liu et al., 2009).

Overall prevalence of protozoa was found 8.86% in this study which is much lower than Azam et al., (2002) and Biswas et al., (2014) who found that 72% and 37.40%, respectively of buffalo calves in Pakistan had intestinal protozoan infections. Moreover, found that the prevalence of intestinal protozoa was that is higher than the current findings. In the present study the prevalence of *Balantidium coli* (3.16%) is much lower than the findings reported by Bilal et al., (2009); Roy et al., (2011) and Mamun et al., (2011) who recorded 25%, 45.03%, and 37.2% prevalence, respectively.

The differences in the results could be a result of the choice of animal, sample collection methods, animal breed, numbers of faecal samples examined, the study period and geo-climatic conditions (temperature, humidity, etc.), which favor the

survival of intermediate hosts and the infective stage of the parasites, as well as management conditions and deworming practices. Cockrill, (1974) stated that the buffalo is exposed to a higher risk of infection with snail borne helminthes due to its wallowing behavior.

Adult buffalo showed greater susceptibility (46.05%) to overall gastrointestinal (GI) parasitic infection than calf (38.10%) and young (44.26%) in this study. The current study is parallel with an earlier publication by Biswas et al. (2014) that noted that adult buffaloes in Bhola, Bangladesh had greater infection rates than young buffaloes. Besides, this finding is also in agreement with Marskole et al., (2016), Quershi and Tanveer (2009); Cheru et al., (2014). On the other hand, among different nematodes, the highest prevalence of *Toxocara vitulorum* in this study was 22.95% in young buffalo which is dissimilar to Roy et al., (2016) where *T. vitulorum* (82.85%) was the highest in buffalo calves compared to young and adult. Moreover, the current conclusion does not concur with earlier reports of Regassa et al., (2006), and Deeba et al., (2019) stated that the younger animals are more susceptible than adult animals. This difference may be related to the immune system, grazing circumstances, and differences in the grazing region. In this study, adult animals were highly susceptible and it may be due to keeping them in breeding pupose, lactation, or drought purpose for a longer period of time as well as ideadequate feed sppuly against their higher demand. Younger animals may have less resistance to infection because they have had less exposure to various parasites than older animals (Bilal et al., 2009; Raza et al., 2012; Zaman et al., 2014). Baily (1971) claimed that helminthiosis in animals is not simply self-limiting, rather it is an immunological phenomenon. The variance in pasture and management variety of the animals may potentially be the reason of this discrepancy.

Though, it was detected that, there was no significant relationship of gastrointestinal (GI) parasites ($p>0.05$) in sex related prevalence, GI parasites were present in all of the buffaloes, both male and female. In this study, it was shown that males had a somewhat greater overall prevalence of GI parasites (48.28%) than females (42.00%). Similarly, according to a study by Marskole et al., (2016), the prevalence was in male buffaloes (85.71%) compared to female buffaloes (63.33%). According to Mamum et al., (2011) male buffaloes (61.34%) were also found to have slightly more GI parasites than female buffaloes (59.52%). Furthermore, Asif et al., (2007) also found

that, male buffaloes in Pakistan had more GI parasites than female counterpart and in Maulvibazar area, a slightly greater frequency was detected in males (66.6%) than in females (65.6%) by Ara et al., (2021) and all are in line with the findings of our study. This finding is also supported by findings from different parts of the world Fikru et al., (2006); Bilal et al., (2009), and Awraris et al., (2012), Raza et al. (2007) who indicated that male buffaloes in had a higher prevalence of GI parasites than female counterpart. Uncertainty surrounds the cause of the greater rates of infection in male buffaloes, however it is possible that farmers' careless handling of male animals is to blame. Additionally, male buffaloes are frequently employed for drought-related tasks, which causes stress and increases the risk of infection.

This result, however, contradicts with those of Deeba et al., (2019), Bhutto et al., (2002), Ara et al., (2021) who found that female buffaloes had a higher prevalence of GI parasitic infections than males in their study area. Furthermore, according to Biswash et al., (2014), there was a significant ($p<0.05$) association between the prevalence of GI parasites in male and female buffaloes, with females (87.53%) being 1.2 times more susceptible to GI parasitic infection than males (84.37%). However, it was known from a prior study by Azhar et al., (2002) found that buffaloes of either sex are equally impacted. There is no clear explanation for this inconsistency in the results. The increased incidence of infection in females may be related to changes in the animals' physiological state during pregnancy and lactation (production activity). Higher levels of the hormones, prolactin and progesterone make the female more vulnerable (Lioyd, 1983). According to Raza et al., (2012), insufficient/unbalanced nutrition versus increased demands and impaired resistance of female animals exacerbated by their reproductive events may be the causes of the higher incidence of parasites in female as compared to male.

Similar to Roy et al., (2016), who identified *Balantidium coli* (91.78%) and *Toxocara vitulorum* (19.18%) in male, in the present study the highest prevalence was also found in case of *Toxocara vitulorum* (18.97%), *Trichostrongylus* sp. (1.72%), *Oesophagostomum* sp. (1.72%), and *B. coli* (5.17%) in male. Although this discrepancy in the results cannot be explained precisely, it is possible to presume that it is related to the difference in the sample sizes between male and female.

In terms of deworming status, there was no significant difference in the occurrence of gastrointestinal (GI) parasites. Overall prevalence of GI parasites, trematode, protozoa and mixed infection was higher in dewormed buffalo (47.52%, 4.95%, 12.87% and 15.84%, respectively) than non-dewormed buffalo (38.60%, 0.00%, 1.75%, and 5.26%, respectively). In the instance of *Balantidium coli* cysts, the difference between buffalo that had been dewormed (6.93%) and those who hadn't (0.00%) was statistically significant ($p<0.05$) in this study. It may be due to imbalance in sample size collected from different study areas as well as anthelmintic (whose data was not known unfortunately) might not effective enough for lower dose or improper anthelmintic given.

However, it was revealed that *T. vitulorum* infection was nearly two times less in dewormed buffalo (12.87%) than in non-dewormed buffalo (26.32%) during this investigation which was statistically significant ($p<0.05$). In a similar vein, according to Gunathilaka et al., (2018), non-treated buffalo had the highest percentage of parasitic diseases. The incidence of GI parasites is reduced by deworming and management measures, claimed by Rajakaruna and Warnakulasooriya (2011).

5.2. Prevalence of haemoprotozoan diseases on the basis of microscopic and molecular examination

Blood protozoa, including *Anaplasma marginale*, *Anaplasma centrale, Babesia bigemina, Theileria annulata, Theileria mutans* have been discovered in animals in Bangladesh (Kispotta et al., 2016). Babesiosis, Anaplasmosis, and Theileriosis are three hemoprotozoan diseases that are transmitted to ruminants by ticks and are common in tropical and subtropical regions of the world, including Bangladesh (Rahman et al., 2015). In Bangladesh, several epidemiological research on vector-borne hemoprotozoan diseases have been carried out (Talukdar and Karim, 2001; Chowdhury et al., 2006; Siddiki et al., 2010; Belal et al., 2015) in cattle. But only a few regional studies (Mamun et al., 2010) along with numerous international studies (Durrani et. al., 2008; Bhutto et. al., 2012; Vahora et. al., 2012; Krishna et. al., 2016; Memon et. al., 2016; and Mehta et. al., 2022) had been conducted on blood protozoa, particularly on *Anaplasma, Babesia*, and *Theileria* of buffalo.

Only Anaplasmosis was found in buffalo based on microscopic identification (which is a limitation of this study) and overall prevalence of Anaplasmosis was 14.48%.

This finding supports those of Vahora et al., (2012) and Butto et al., (2012), who discovered a prevalence of haemoprotozoa in buffalo of 17% and 14%, respectively. But it is considerably lower than the prevalence (32.91%) in the Makwanpur district (Mishra, 2003) and the prevalence (41%) in Pakistan (Rajput et al., 2005).

By microscopic examination, no Babesiosis or Theileriosis were detected in the current study, but Krishna et al., (2016) reported prevalence of *Theileria* sp., *Babesia* sp., *Trypanosoma* sp., and *Anaplasma marginale* of 12.9%, 4.7%, 3.5%, and 2.4%, respectively. According to Lalchandani (2001), 58.82% of buffaloes had Theileriosis, suggesting that this region may have a somewhat higher frequency of Theileriosis as a result of its different geography or climatic conditions.

In this study, species specific primers i.e. only for *Anaplasma marginale*, *Babesia bigemina*, and *Theileria annulata* were used. These species were chosen for higher prevalent of them in ruminant than others (Atif et al., 2012). But we have a plan to do further research on other circulating haemoprotozoa of buffalo in this study area.

In this study, the overall prevalence of haemoprotozoan diseases was 31.03% in buffalo where, the highest prevalence (30.34%) was recorded in Anaplasmosis compared to Babesiosis (2.07%.) and Theileriosis (1.38%) by PCR examination. Similarly, Mamun et al., (2010) found that *Anaplasma marginale* had the highest incidence (8.89%), followed by *Theileria* sp. (2.12%) and *Babesia* sp. (1.69%), with a substantial lower prevalence in Anaplasmosis, and Babesiosis than the present study. This may be because the area is hilly, where the activity and density of the vectors are greatly reduced. While another study by Ajayta et al., (2013) found a higher frequency of Anaplasmosis (33.52%), Babesiosis (7.64%), and Theileriosis (1.76%) in Uttarakhand than the present study. Jithendran (1997) also noted that *Theileria annulata* (29.5%), *Babesia bigemina* (18.5%), and *Anaplasma marginale* (4.5%) were the three pathogens with the highest prevalence. Additionally, report of the prevalence of blood protozoa by Soundararajan and Rajavelu (2006) revealed that, the different rate of infections may be caused by difference in geographic location that encourages the proliferation of vector ticks.

In this study, young and adult buffalo demonstrated greater resistance to various haemoprotozoan diseases than calf, where calf had an overall infection rate of 36.84%. But Mehta et al., (2022) and Rani et al., (2015), discovered a contrary situation, where the prevalence of haemoprotozoan diseases was highest in adults followed by heifers and calves. It might be due to adult are kept long time in milk production. Mamun et al., (2010) observed that, in comparison to adults (12.50%) and calves (0.5–2%), young buffaloes (17.07%) were more sensitive to blood protozoan infections. Due to passive immunity, calves are less susceptible to diseases. However, in the current investigation, calf was found more sensitive. Less passive immunity or sampling error could be the reason.

Comparatively, overall higher prevalence of haemoprotozoan diseases was found in female which was 36.84% whereas it was 20.00% for male buffalo. This result agrees with the studies of Bhutto et al., (2012); Rajput et al., (2005), and Mamun et al., (2010). In the study of Bhutto et al. (2012), the prevalence was recorded as 15% and 10% in female and male buffaloes, respectively. Rajput et al., (2005) also reported that, the higher prevalence of Anaplasmosis was in female (30.28%) than male (29.33%). From Mamun et al., (2010), the higher prevalence of haemoprotozoan diseases was in female (23.81%) than male (10.31%). Though there is no clear explanation for this difference in the results. The increased incidence of infection in females may be related to changes in the animals' physiological state during pregnancy and lactation. Higher levels of the hormones, prolactin and progesterone, make the female more vulnerable (Lioyd, 1983). According to Raza et al., (2012), insufficient/imbalanced nutrition versus increased demands and impaired resistance of female buffaloes aggravated by their reproductive events may be the causes of the higher incidence of parasites in females as compared to male.

In all, 48.89% of buffalo that had not been dewormed had haemoprotozoan diseases, compared to only 23% of dewormed buffalo. Unfortunately, no research has been done yet to determine how deworming affects blood protozoa. However, Hassan et al., (2012) conducted a study to determine the effectiveness of an anthelmintic on blood protozoa in black Bengal goats and discovered a notable decrease in blood protozoa following deworming. As per my knowledge, anthelmintic has no direct impact on blood parasites. In this study, highest prevalence of blood parasites was

found in Boalkhali upazilla where deworming was not done. That's why, higher prevalence of blood parasites was found in non-dewormed buffalo.

5.3. Molecular characterization of haemoprotozoa by partial gene sequencing

The positive rates from amplification of 16S rRNA, 18S rRNA, and tams1 genes in buffalo were 2.07%, 30.34%, and 1.38%, respectively. During PCR, the amplified DNA fragment of 270bp, 504bp, and 751bp for, *Anaplasma* sp., *Babesia* sp., and *Theileria* sp., respectively considered as positive. Actually, it can be difficult to identify between these species based on the morphology of the schizont and piroplasm stages, especially in mixed infections. Because of this, PCR and gene sequencing may be effective tools for identifying different blood protozoa species. This gene-focused work has previously concentrated on the molecular characterisation of *T. annulata* in cattle and buffaloes (Oliveira et al., 1995; Dumanli et al., 2005; Durrani et al., 2010). The genus and species of the protozoa were identified through further sequencing, and phylogenetic analyses. According to the findings of partial gene sequencing of the current investigation, of the 16S rRNA gene, the 18S rRNA gene, and the tams1 gene, *A. marginale*, *B. bigemina*, and *T. annulata* are sporadic occurrences in Bangladesh. Using parasite-specific primers discovered in earlier work (Kundave et al., 2018), the target genes of *A. marginale*, *B. bigemina*, and *T. annulata*, Amar-16S rRNA, Bb-18S rRNA, and Tamulti (tams1), were amplified. to fully comprehend the molecular epidemiology of this significant haemoprotozoa. However, additional thorough research with a large sample size and geographically diverse locations is essential.

5.4. Phylogenetic analyses of *Anaplasma, Babesia,* and *Theileria* isolates

A great degree of genetic diversity, host tropisms, and variety in pathogenicity are displayed by *Anaplasma marginale*, a member of the genus *Anaplasma* (Barakova et al., 2014). Phylogenetic tree revealed that, three isolates from Chattogram, Bangladesh, had ancestral relationship with the isolates from Egypt whereas another three had descendant relationship. However, all the isolates had distal evolutionary realtionship with those from the USA, India, Pakistan, China, and Iraq. The most common and diversified blood protozoa that infects both humans and animals is *Anaplasma* sp. Despite greater diversity, the 16S rRNA gene sequences of various *Anaplasma* sp. showed a high degree of similarity, and a number of 16S rRNA gene variations have been discovered (Kawahara et al., 2006; Katargina et al., 2012).

The 18S rRNA gene sequence, which has been shown effective for phylogenetic investigations and genetic characterization of *Babesia bigemina*, was used to confirm the infections with *Babesia bigemina*. 18S rRNA sequences from Chattogram and previously acquired 18S rRNA sequences from other countries underwent phylogenetic analyses. It revealed that, each of these isolates of Chattogram had a close relationship with the isolates of Japan, indicating that they were descendant isolates. On the other hand, isolates from Chattogram, Bangladesh had distand evolutionary relation with those from Colombia, India, Switzerland, and Brazil. It may be due to genetic mutation of aforementioned isolates with time.

Previous researches have looked into the frequency of Theileriosis in buffalo in Bangladesh, but this is, as far as I can tell, the country's first investigation into the molecular and genetic variety of *Theileria* sp. The phylogenetic tree also showed that all of the tams1 sequences in our investigation belonged to the same clade and found as ancestor of other isolates from different countries. Again, isolates from Egypt, Pakistan, Sri Lanka, China, or India revealed distand evolutionary relationship with those from Chattogram, Bangladesh. This may be due to mutation of *Theileria sp.* found in this study area.

Chapter-6: Conclusion

As far my knowledge, about haemoprotozoan infection in buffalo this study is for the first time in Chattogram and in case of molecular identification of blood protozoa, it is first time in Bangladesh. It was revealed that, from 158 faecal samples GI parasitic infection occurred in 44.43% buffalo. Among different helminths, *Toxocara vitulorum* infection had the highest prevalence rate of all of them followed by mixed infection, and *Oesophagostomum* sp. Among different GI protozoa, *B. coli* cyst, *B. coli* trophozoite, and *Eimeria* cyst were found. 145 blood samples were randomly selected for microscopy and PCR to identify blood parasites like *Anaplasma marginale., Babesia bigemina*, and *Theileria annulata* at species level. In microscopic and PCR tests, the prevalence of haemoprotozoan infection was 14.48% and 31.03%, respectively, where Anaplasmosis occurred in 30.34% of them, Babesiosis in 2.07%, and Theileriosis in 1.38%. Buffalo of any age could be affected by GI parasites and haemoprotozoa but adults were found highly susceptible to GI parasites and calves were more susceptible to haemoprotozoan diseases. Besides, both male and female were affected by GI parasites and haemoprotozoa where female showed more susceptibility to both GI parasites and haemoprotozoa. From partial gene sequencing and phylogenetic analysis it is known that, *Anaplasma marginale, Babesia bigemina*, and *Theileria annulata* are circulating in buffalo of Chattogram, Bangladesh which showed evolutionary relationship with isolates from other contries like China, India, Japan, and Pakistan etc. As the current study's findings will help to better understand the epidemiology of parasitic diseases by providing epidemiological forecasts for the incidence of such diseases as well as more accurate identification of haemoprotozoan diseases and associated risk factors in buffaloes of two coastal districts (Chattogram and Noakhali), future vaccination and medication delivery schedules will be organized with the aid of the study's findings based on molecular research in an effort to control and stop the spread of parasitic infections in buffalo.

Chapter-7: Recommendations

In this study, the prevalence of gastrointestinal (GI) parasitic infections and haemoprotozoan diseases, as well as the risk factors for these diseases (such as location, age, sex, and deworming status), were examined in the districts of Chattogram and Noakhali. Future techniques may take the following forms depending on certain constraints (such as time and resource constraints):

1. Molecular identification of other circulationg haemoprotozoan species of buffalo
2. Determination of anthelmintic resistance as well as AMR pattern in buffaloes which will help to face the current AMR challenges in Bangladesh in the long run
3. Whole genome sequencing of haemoprotozoan diseases that have been found
4. Developing multiplex PCR protocol protocol to identify haemoprotozoan diseases in buffalo in short time
5. Development of a vaccine against ruminant haemoprotozoa using a cell culture methodology

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Appendix A

Questionnaire used in this research during sample collection

Questionnaire

Date:

Sample number:

Name of the farm/owner : ………………………………Location/Area:……………………………..

Cell phone number (optional):………………………………………Animal types: Sheep/goat/cattle/buffalo

Age of the animal:……………………month/year, Sex: Male/Female, Type: River/Swam type

BCS:……………………………………..Anthelmintic use (within last 3 months): Yes/no

Ectoparasites : Yes/No Water source: Pond/Tube well/ other

……………………………………………………………………………………………………

Sample number:

Name of the farm/owner : ………………………………Location/Area:……………………………..

Cell phone number (optional):………………………………………Animal types: Sheep/goat/cattle/buffalo

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Age of the animal:……………………month/year, Sex: Male/Female, Type: River/Swam type

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Ectoparasites : Yes/No Water source: Pond/Tube well/ other

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Cell phone number (optional):………………………………………Animal types: Sheep/goat/cattle/buffalo

Age of the animal:……………………month/year, Sex: Male/Female, Type: River/Swam type

BCS:……………………………………..Anthelmintic use (within last 3 months): Yes/no

Ectoparasites : Yes/No Water source: Pond/Tube well/ other

Appendix B

STATA-13 commands used in this study

1. tab Age Trophozoite of *B. coli*, row chi
2. tab Age *Toxocara vitulorum*, row chi
3. tab Age *Oesophagostomum* sp., row chi
4. tab Age *Trichostrongylus* sp., row chi
5. tab Age *Paramphistomum* sp., row chi
6. tab Age *Trichuris* sp., row chi
7. tab Age *Eimeria* cyst, row chi
8. tab Age *B. coli* cyst, row chi
9. tab Age Mixed, row chi
10. tab Sex Trophozoite of *B. coli*, row chi
11. tab Sex *Toxocara vitulorum*, row chi
12. tab Sex *Oesophagostomum* sp., row chi
13. tab Sex *Trichostrongylus* sp., row chi
14. tab Sex *Paramphistomum* sp., row chi
15. tab Sex *Trichuris* sp., row chi
16. tab Sex *Eimeria* cyst, row chi
17. tab Sex *B. coli* cyst, row chi
18. tab Sex Mixed, row chi
19. tab Deworming status Trophozoite of *B. coli*, row chi
20. tab Deworming status *Toxocara vitulorum*, row chi
21. tab Deworming status *Oesophagostomum* sp., row chi
22. tab Deworming status *Trichostrongylus* sp., row chi
23. tab Deworming status *Paramphistomum* sp., row chi
24. tab Deworming status *Trichuris* sp., row chi
25. tab Deworming status *Eimeria* cyst, row chi
26. tab Deworming status *B. coli* cyst, row chi
27. tab Deworming status Mixed, row chi
28. tab Water source Trophozoite of *B. coli*, row chi
29. tab Water source *Toxocara vitulorum*, row chi
30. tab Water source *Oesophagostomum* sp., row chi
31. tab Water source *Trichostrongylus* sp., row chi

32. tab Water source *Paramphistomum* sp., row chi
33. tab Water source *Trichuris* sp., row chi
34. tab Water source *Eimeria* cyst, row chi
35. tab Water source *B. coli* cyst, row chi
36. tab Water source Mixed, row chi
37. tab Age *Anaplasma* sp., row chi
38. tab Age *Babesia* sp., row chi
39. tab Age *Theileria* sp., row chi
40. tab Age *Mixed* sp., row chi
41. tab Sex *Anaplasma* sp., row chi
42. tab Sex *Babesia* sp., row chi
43. tab Sex *Theileria* sp., row chi
44. tab Sex Mixed sp., row chi
45. tab Deworming status *Anaplasma* sp., row chi
46. tab Deworming status *Babesia* sp., row chi
47. tab Deworming status *Theileria* sp., row chi
48. tab Deworming status *Mixed* sp., row chi
49. tab Water source *Anaplasma* sp., row chi
50. tab Water source *Babesia* sp., row chi
51. tab Water source *Theileria* sp., row chi
52. tab Water source Mixed sp., row chi
53. cii 158 5
54. cii 158 28
55. cii 158 2
56. cii 158 5
57. cii 158 1
58. cii 158 2
59. cii 158 7
60. cii 158 19
61. cii 145 21

Appendix C

Collection of faecal sample directly from rectum of buffalo

Ear vein puncturing for blood sample collection

Examination of faecal sample under microscope

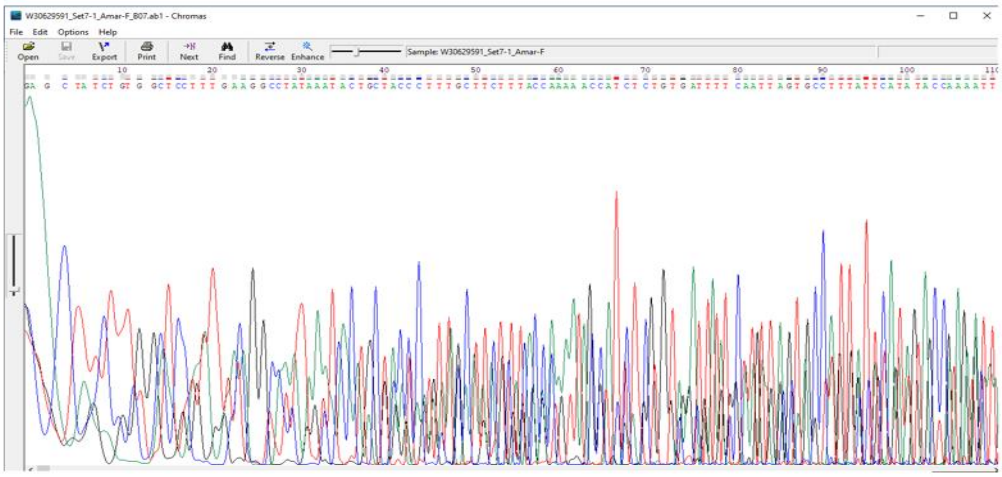
Examination of blood smear under microscope

Preparation of PCR product

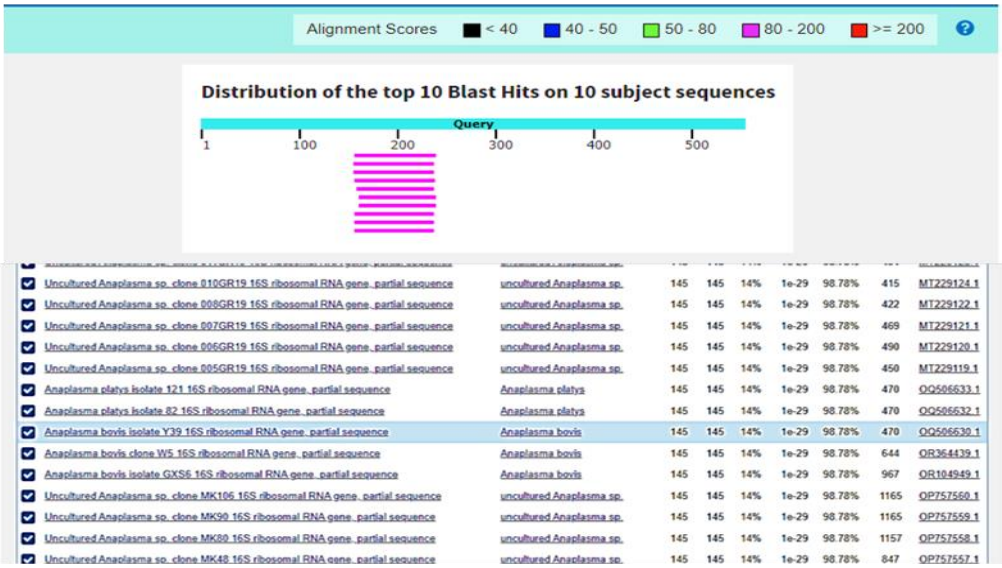
Placing of PCR product in thermal cycler

Setting up the run condition in thermal cycler

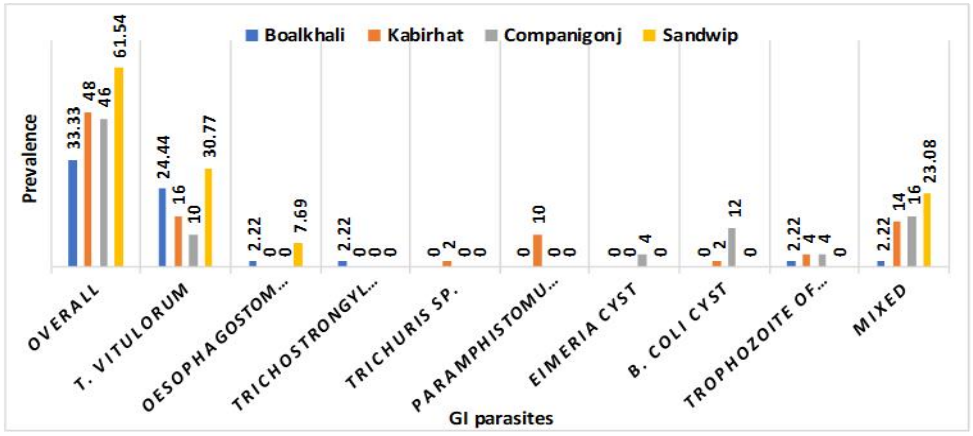
Loading of PCR product in gel



Checking chromatogram's peak to confirm the result



The graphic summary of 10 blast hits on the query sequence (*Anaplasma* sample 1)



Prevalence of different GI parasites in buffalo in different areas

Brief Biography

The author, passed Secondary School Certificate (SSC) examination from Kutubdia Model High School in 2012 and then Higher Secondary Certificate (HSC) examination from Chittagong College in 2014. She obtained her Doctor of Veterinary Medicine (DVM) Degree in 2022 from Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, she is a Candidate for the degree of MS in Parasitology under the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, CVASU. She has published two scientific articles in international journals. She has immense interest to work in new vaccine design against haemoprotozoa of animals in veterinary field.