MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF GASTROINTESTINAL PARASITES OF WILD WATER BUFFALO OF CHITWAN NATIONAL PARK

M.Sc. Thesis

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Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

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For Partial Fulfillment of the Requirements for

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By

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LIST OF ABBREVIATIONS

Adv.	-	Advances
Ani.	-	Animal
BLAST	-	Basic Local Alignment Search Tool
Bp	-	Base pair
CITES	-	Convention on International Trade in Endangered Species of Wild Fauna and Flora
Chem	-	Chemical
Conerv.	-	Conservation
GoN	-	Government of Nepal
DNA	-	Deoxyribose nucleic acid
Dia	-	Diagnosis
IJAIEM	-	International Journal of Application or Innovation in Engineering and Management
Intl	-	International
IUCN	-	International Union for Conservation of Nature and Natural Resources
J.	-	Journal
Km^2	-	Square Kilometer
Ltr	-	Liter
MEGA X	-	Molecular Evolutionary Genetics Analysis version X
NCBI	-	National Center for Biotechnology Information
PCR	-	Polymerase Chain Reaction
Pharma	-	Pharmacology
rDNA	-	Ribosomal DNA
Res.	-	Research
Rpm	-	revolution per minute
Sci.	-	Science
Trop	-	Tropical

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ABSTRACT

The microscopy-based identification of gastrointestinal parasites can be further strengthened at species-level, which shows the importance of molecular based identification.The main aim of this study is the molecular characterization of the gastrointestinal parasites in Wild Water buffalo of Chitwan National Park. A wide range of helminth and protozoan species can infect or colonize the gastrointestinal tract of humans and animals. Intestinal parasitic infections caused mainly by protozoa and helminths are most prevalent in Wild Water buffalo, which are of relevance to critically endangered species. Twenty-five fecal samples were collected from groups of Wild Water buffalo. The fecal samples were examined microscopically by direct wet mount method, sedimentation method, floatation method, and molecularly by PCR, sequencing and, analysis. An integrative taxonomic approach is followed, where conspicuous morphological traits and phylogenetic trees based on DNA sequences are used to test the validity of the species.

Nine intestinal parasites were detected microscopically, where the infection rate was 96%, of which 88% and 84% were due to protozoa and helminths respectively. The identified protozoan parasites were *Entamoeba* (80%), *Eimeria* (60%), *Balantidium* (8%), and *Cryptosporidium* (4%) and helminths were *Oxyruid* (64%) *Strongyloides* (28%), *Strongyle* (36%), *Eurytrema* (8%), and *Paramphistomum* (4%). The PCR assays were conducted using *Entamoeba* spp. 18S specific primers. Ribosomal genome sequence (550 bp) was obtained and DNA sequence analysis enabled more precise identification of *E. bovis*. *Entamoeba* was successfully sequenced and it was separated from those of other *Entamoeba* species/genotypes in phylogenetic analysis by the Neighbor Joining method. Also the amplification of *Eimeria* was done by using genus-specific primers targeting 18S ribosomal region. This investigation gives benchmark data of GI parasites and their conveyance in wild buffalo. This is the primary report for the molecular discovery of *E. bovis* in translocated wild buffalo in Nepal.

Keywords: *Bubalus arnee*, Chitwan National Park, Gastrointestinal Parasite, Protozoa, Helminthes, 18S rDNA

CHAPTER 1. INTRODUCTION

1.1 Background

Wild Water Buffalo (*Bubalus arnee*), known as Arna in Nepali belongs to family Bovidae of order Aritodactyla. The global population of Wild Water buffalo is estimated to be less than 4000 individuals (DNPWC, 2020). The third most important driver of the population decline of wildlife is infectious diseases (Bengis *et* al., 2004) after hunting and habitat degradation. Wild animal under free-range condition is vulnerable to most diseases as other animals, particularly helminth infection. The gastrointestinal tract (GIT) of animals harbor a wide assortment of parasites such as helminths, coccidian, etc. which gives rise to clinical and sub-clinical parasitism (Dappawar *et* al., 2020). The word “parasite” is derived from the Greek word *parasitos*, which implies the one who eats at the table of others (Harper,2001; Lourenco, 2008). Parasitism is a sort of consumer-resource interaction (Getz, 2011). Gastrointestinal (GI) parasites are ubiquitous, taxonomically diverse (Larsson *et* al., 2006) and can affect their hosts straightforwardly by consuming host resources, through pathological effects (blood loss, tissue damage, death of the fetus, congenital deformity, and death) or in a round about by reducing the host immunity, affecting the physical condition damaging intestinal function (Gorsich et al., 2014; Sengar *et* al., 2017). Parasitic nematodes are highly diverse and common in animal species (Cole and Viney, 2019). Infection with intestinal parasites regards as the most important causative agent for diarrhea (Ibhrahim *et* al., 2017). Parasitic diseases constitute one of the major management problems causing mortality and morbidity in wild animals in captivity (Singh *et* al., 2006). Infectious diseases impact buffalo populations despite their adaptations to the local environment (Guccione *et* al., 2016). Parasites and parasitic diseases are considered to be important threats in wildlife conservation, as they can potentially impair the health of wildlife, decrease fitness, cause population declines and even contribute to local extinction (Cao *et* al., 2020). Regardless of the close pervasive and often high levels of GI infection among wild buffalo, there’s still little data about host-parasite interactions within this group. Information on parasitic infections of wild animals is meager due to the paucity of systematic investigation (Thawait *et* al., 2014). The parasitic diseases, gastro-intestinal helminthiasis, coccidiosis, fasciolosis, and mange are not less important in buffaloes than other infectious diseases

(Sreedevi and Hafeez, 2014). Research on the parasite fauna of the water buffaloes is of great practical importance because it allows determining the composition and influence of environmental factors on the course of the parasitic invasion.

1.2 *Bubalus arnee*

Large ruminants are important for livestock production systems. There are approximately 170 million water buffaloes worldwide (Wanapat and Chanthakhoun, 2015). The Wild Water buffalo (*Bubalus arnee*), a large ungulate, is a herd cherishing mega-herbivores and falls in the bovid family (DPNWC 2020). Linnaeus (1758), who generally used very broad genera, initially named it *Bos bubalis*. Kerr (1792) named it *Bos arnee*. Later, both were assigned to genus *Bubalus* by H. Smith, 1827 (Choudhary, 2014). In 2003, the name was again amended to *Bubalus arnee* by the International Commission on Zoological Nomenclature, a name that is currently accepted as valid. Wild Water buffalo (*Bubalus arnee*), the ancestor of domestic water buffalo and is also known as Indian water buffalo and wild Asiatic buffalo (Cockrill, 1974; Heinen 1993). Historically, Wild Water buffalo populations were distributed across south-east Asia from Mesopotamia to Indo-China (Fischer, 1976). Most of the water buffalo population is settled in the Asian continent and other regions of the world like Africa, South America, and Europe (Ojeda-Robertos, *et* al., 2017). Morphologically they are quite distinguished, with the swamp buffalo exhibiting white chevron, socks, a tip of the tail, and comparatively straight pale-colored horns, and river buffalo having a black body and usually curved horns (Flamand *et* al., 2003). The rusticity of water buffalos and their ability to be reared in regions where other species of ruminants are not adaptable make the water buffalo a crucial animal within the economy of few nations. (Martins *et* al., 2018; Dubey, 2018). The habitat of wild buffalo has been described intimately by Choudhury (2010). Their event is unequivocally connected to grasslands with water bodies, preferably in low-lying areas. The buffaloes dwell in tall grassy areas near swamps, streams, riverbanks, open woods and, dry thorn scrub, preferably in floodplains.

1.3 *Bubalus arnee* in Nepal

Bubalus arnee is a large bovine native to the Indian subcontinent and Southeast Asia with Nepal's only population in the flood plain of the Koshi River at Koshi Tappu Wildlife Reserve (KTWR). It has been listed as Endangered in the IUCN Red List since 1986 (Kaul *et* al., 2019) and Appendix III of Convention on International Trade in Endangered Species (CITES, 2017). An aggregate of 15 water buffaloes are translocated to Chitwan National Park (CNP) (Thapa *et* al., 2020). In this regard, this translocation of Wild Asian Buffalo has immensely played a vital role in conservation (Shah *et* al., 2017).

1.4 Threats

Over the last half-century, the Asian wild buffalo population is continuously waning, because of habitat fragmentation, competition and habitat overlap and cross breeding with local buffaloes. The utmost threat to Wild Water buffalo is loss of genetic diversity due to introgression with domestic and feral buffaloes, as well as danger from hunting, and diseases from domestic livestock (Kaul *et* al., 2019; Kandel *et* al., 2019; Flamand, *et* al., 2003). Thus the conservation of wild buffalo is a global concern (Aryal *et* al., 2011). One of the main threats to the wild buffalo population is the outbreak of various contagious diseases spread by domestic livestock, such as rinderpest, foot-and-mouth (FMD), and anthrax (Choudhury, A. 2014). Malady and parasites (transmitted by domestic livestock) and the contest for food and water between wild buffalo and domestic stock are likewise serious threats (Hedges *et* al., 2008). Diseases that are shared between species also depict a potential encumber to the entire ecosystem, affecting biodiversity, changing behavior or composition of animal populations, and even consigning species to the fringe of extinction (Sengar *et* al., 2017).

Among many constraints, parasitism is believed to be a serious cause that impediments the development of livestock populations including buffaloes (Roy *et* al., 2011). Infection by gastrointestinal (GI) parasites is one of the serious issues, which can restrict the livestock population and also adversely influence the health, fertility, and productivity of the animals (Al-Jubury *et* al., 2020). Physical management of herds like rotational grazing is the only strategy for the control of GIT parasitism in buffaloes (William *et* al., 2019). Therefore, it's necessary to seek out an alternate place where the habitat is suitable for

sustainable conservation in Nepal. In this regard, 15 Wild Asian buffaloes are translocated in 30 hector enclosures on the Old Padampur area of CNP, Nepal. Park Authority is maintaining the habitat almost like KTWR in alternative ways. CNP had a population of the species at least until the 1950s and is located closer to KTWR, making it the more feasible site for translocation (Shah *et* al., 2017).

1.5 Chitwan National Park

Chitwan National Park (CNP) is the first and foremost protected area of the country which was established in 1973. CNP and its Buffer Zone is situated in the southern part of Central Nepal and covers an area of 952.63 km^2 (GoN/MoFSC, 2014). Although the presence of Wild Water buffalo was recorded in Chitwan valley until 1950, they were lost from that area presumably because of diseases carried by domestic cattle and buffalo (Seidensticker, 1976). Chitwan has extensive grassland areas with ample adjacent riverine forests, and a larger buffer zone than KTWR constituting ideal habitat for buffalo, that are extensively studied for the past several decades (Heinen and Rayamajhi 2001). Overall fifteen Wild Water Buffaloes (twelve from KTWR and three from Central Zoo) were translocated in a 30-ha enclosed area on the old Padampur of CNP, Nepal in January-February, 2017 (Shah *et* al., 2017). There are 13 Wild Water Buffaloes (8 F and 5 M), including four calves (DNPWC, 2020).

1.6 Protozoan parasites

Wild, as well as domestic ruminants, have been reported to be highly infected with a protozoan parasite. Among the protozoan parasites, coccidiosis has been found in wild ruminants of different parts of the world such as Nepal (Gupta, 2017), India (Varadharajan and Kandasamy, 2000), Pakistan (Azam *et* al., 2002), Egypt (Wahed *et* al., 2004), Spain (Vazquez *et* al., 2009). In buffalo, coccidiosis causes severe diarrhea, dysentery, dehydration, anorexia, as well as a weakness (Ahmed and Soad, 2007). The major protozoan parasites that infect wild ruminants include *Entamoeba* sp, *Cryptosporidium* sp, *Eimeria* sp, *Balantidium* sp, etc. *Eimeria* sp. infects their hosts when water or foods contaminated with sporulated oocysts are ingested (Roberts and Janovy, 2005). Infection with the *Eimeria* sp. has been reported in several parts of Indian subcontinents like Tirupati (Sreedevi *et* al., 2014), and in buffalo calves of Punjab (Singh and Juyal, 2014).

Similarly, *Eimeria* infection has been reported from African countries like Tanzania (Senyael *et* al., 2013). Besides coccidian parasites, buffaloes are infected with *Balantidium* sp. in India (Sreedevi *et* al., 2014) and Bangladesh (Biswas, 2012).

1.7 Helminths

Helminthes parasites include trematode, cestode, and nematode parasites that are found in the bile duct, liver, and digestive system of both wild and domestic ruminants.

1.7.1 Trematodes

Trematodes are commonly known as flukes and are found in the liver, bile duct, or small intestine and sometimes present in the lungs of buffaloes. They are the internal parasites of Mollusca (snail) and vertebrates. Most of the trematode parasites have a complex life cycle as they complete their lifecycle within two or more hosts. The common trematode parasites represent *Paramphistomum, Fasciola, Schistosoma.*

Paramphistomum is an intestinal fluke that inhabits mainly in a villi region of the small intestine. *Paramphistomum* sp. has been reported from Asian countries like Nepal (Sah, 2015; Pun, 2018), India (Marskole *et* al., 2016; Sreedevi *et* al., 2014), Bangladesh (Alam *et* al., 2016; Fagiolini *et* al., 2010), Europe, Italy (Cringoli *et* al., 2009; Condoleol *et* al., 2007), and some countries of Africa, Tanzania (Senyael *et* al., 2013), Egypt (Haridy *et* al., 2006). As the *P. cervi* was recorded in Pakistan (Raza *et* al., 2010), Bangladesh (Biswas, 2012; Mamun *et* al., 2011), India (Patel *et* al., 2012), Poland (Kobak *et* al., 2012).

1.7.2 Cestodes

Cestodes are ribbon-like tapeworms such as *Moniezia, Taenia, et*c. that are found in the gut of ruminants. These parasites require an intermediate host for their transmission and some are transmitted through contaminated water and food. The earlier literature has shown that cestode infections in buffaloes are not as common as nematode and trematode infections. Some of the researchers from Bangladesh indicated its presents in buffaloes (Biswas, 2012) while the absence of cestode by others (Saha, 2013). Several studies have been shown on the *Moniezia* infection in buffaloes of India (Marskole *et* al., 2016; Sreedevi *et* al., 2014; Muraleedharan, 2005), Italy (Cringoli *et* al., 2009), while some

reports had shown the absence of the cestode parasites in water buffalo from Bangladesh (Mamun *et* al., 2011), and from samples collected from Pakistan (Bachal *et* al., 2002).

1.7.3 Nematodes

Nematodes are the most important and widely prevalent of wild and domestic ruminants. These parasites have a direct life cycle and do not involve any intermediate host and are transmitted by fecal contamination of food, water, and soil. There are numerous nematode parasites such as *Strongyloides* sp., *Strongyle* sp., and *Oxyurid* sp. These nematodes are most important and widely prevalent in buffalo. These nematodes are found in the small intestine and may cause severe damage to the intestinal mucous membrane. *Strongyloides* sp. is also known as the threadworm and also called the common hematophagous parasitic nematode of cattle worldwide (Theodoropoulos *et* al., 2010). Its diagnosis is based on presenting clinical signs and conformation of eggs in fecal samples of infected ones (Keyyu *et* al., 2005). *Strongyloides* sp. has been reported in Buffalo of Asian countries like Nepal (Pun, 2018; Thapa Shrestha *et* al., 2020), India (Marskole *et* al., 2016; Singh and Juyal, 2014), Bangladesh (Alam *et* al., 2016; Mamun *et* al., 2011), Pakistan (Afridi *et* al., 2007, Azam *et* al., 2002), European country like Italy (Condoleol *et* al., 2007). Similarly, *Strongyle* has been reported in Wild Water buffalo of Africa (Budischak *et* al., 2012). *Strongyle* (16.33%) and *Strongyloides* (8.16%) are prevalent in North Kerela (Nimisha *et* al., 2017).

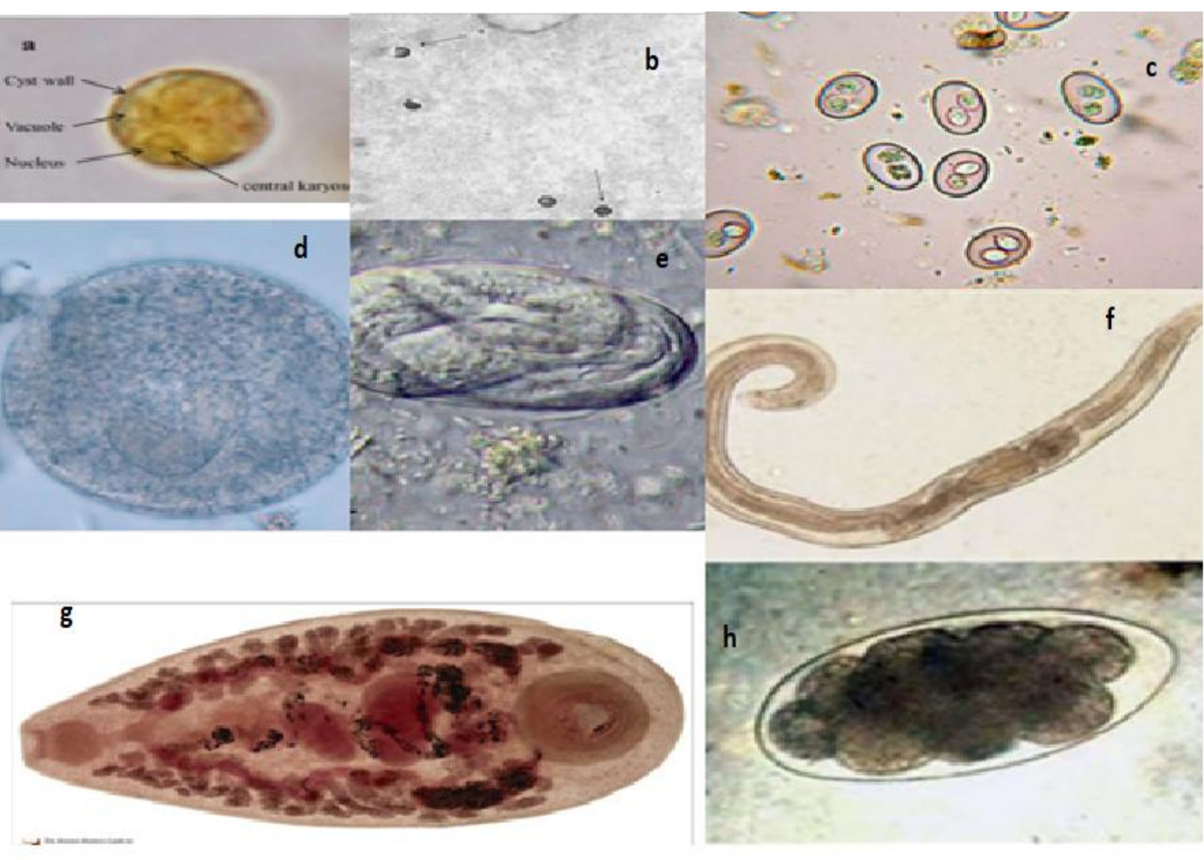


Figure 1: Gastrointestinal parasites in ruminants. a) *Entamoeba* cyst b) *Cryptosporidium* c) *Eimeria* d) *Balantidium* Cyst e) *Strongyloides* egg f) *Oxyurid* larva g) *Paramphistomum* sp. h) *Strongyle* egg (Veterinary Diagnostic parasitology)

1.8 Microscopic and Molecular Method

Classification of parasites is based on host specificities, parasite life cycles, and morphological features of sporulated oocysts. Clinical diagnostics is usually performed by light microscopy (Hamzah *et* al., 2006). However, it's often difficult to differentiate among species based on morphology, namely cyst size and the number and morphology of the nuclei because many similarities exist between the cysts of several species (Vlckova *et* al., 2018) so, molecular techniques have proven very useful to resolve the identification, taxonomy, epidemiology and clinical significance of *Entamoeba* species without relying on parasite cultures or experimental infections (Takeo *et* al., 2014; Stensvold *et* al., 2011; Jacob *et* al., 2015). The morphological comparisons are supplemented by a molecular phylogenetic analysis employing both mitochondrial protein-coding and nuclear

ribosomal genes, which offer more evidence for the relationships among other members of *Entamoeba* for which DNA sequences are available (Hamzah *et* al., 2006).

Customarily, species are arranged into those that produce either no cysts or cysts with one, four, or eight nuclei. Notwithstanding, while taxonomically useful, the phylogenetic significance of these groupings is ambiguous as is the validity of several described species. To better understand the relationships among and within species of the genus *Entamoeba*, an investigation was done on phylogeny at the molecular level by sequencing (Clark and Diamond, 1997).

1.9 Research questions

i. What is the infection level by intestinal parasites among the translocated Wild Water buffalo of CNP?
ii. Previously the translocated buffaloes were assumed to be extinct from CNP due to diseases so what is the present parasitic condition of recently translocated wild buffaloes?

1.10 Rationale

The study of GI parasites of Wild Water buffalo in Nepal is very rare. Thus, this study is the first of its kind to reveal the prevalence of GI parasites in wild buffalo of CNP, Nepal. Similarly, *Bubalus arnee,* being one of the endangered species and the burden due to parasitic infection cannot be left unnoticed; therefore, this study reveals the health condition of wild buffalo. This may provide a parasitic relationship with wild herbivores.

1.11 OBJECTIVES

1.11.1 General objective

i. Identification and estimation of the prevalent intestinal parasite infecting Wild Water buffaloes of CNP using morphological and molecular tools.

1.11.2 Specific objectives

i. To find and record species of parasites identified on Wild Water buffalo feces of CNP.
ii. To identify the parasites species at molecular level using Sanger sequencing.

1.12 Research Hypothesis

Null Hypothesis (H_0): The infections with protozoa and helminths are not prevalent among the translocated Wild Water buffalo of CNP.

Alternative Hypothesis (H_1): The infections with protozoa and helminths are prevalent among the translocated Wild Water buffalo of CNP.

CHAPTER 2. LITERATURE REVIEW

2.1 Parasites in wildlife

Parasites constitute significant detrimental jeopardy to the performance, health, and welfare of livestock grazing in all countries and impose a colossal loss in terms of productivity (Sutherland and Leathwick, 2011). Parasitic nematodes have significant impacts on their human, livestock, and wildlife hosts (Albon *et* al. 2002; Charlier *et* al. 2009; Lustigman *et* al. 2012). Gastrointestinal (GI) parasitic infections are common in buffaloes particularly calves (Singh and Juyal, 2014). Infection and dissemination of the parasites in herds are suffering from various factors that influence the development, survival, distribution, or migratory behaviors of the free-living larvae found on pasture are primarily dependent on weather. Different other environmental factors like temperature, moisture, rainfall, and the quality of the soil influence the development and survival of the larvae on pasture, also their distribution on the herbage (Shrestha *et* al., 2020; Thapa Shrestha *et* al., 2020). The predominant factors influencing the parasitic infections are grazing habits, climate, nutritional deficiency, pasture management, poor immunological status, presence of the vector/intermediate host, and the number of infective larvae and eggs in the environment (Sargison, 2016). The consequences of GI parasites may vary with age, sex of cattle, nutritional condition, and severity of infection (Gunathilka *et* al., 2018). Parasites are important pathogens of animals (Jex *et* al., 2019), and have evolved sophisticated mechanisms to communicate with their hosts to survive and successfully establish an infection (Quintana *et* al., 2017). The nematode is reported to cause severe micro- and macroscopic lesions (Gaur and Dutt, 1973) in the abomasum of infected buffalo.

However, techniques to detect, identify and quantify parasitic infection in wildlife lag far behind approaches used in humans and livestock (Budischak *et* al., 2012). Additionally, there is also a demand for simpler and better recognition of these infections, not only for diagnostic purposes and care management but also for a far better understanding of the epidemiology of these parasites within the population.

2.1.1 *Entamoeba*

Protozoans of *Entamoeba* spp. are globally distributed zoonotic parasites that infect diverse animal hosts and humans (Ai *et* al., 2021). *Entamoeba* is a pseudopod-forming, protozoan parasite in the phylum Amoebozoa, class Archamoebae and family Entamoebidae (Royer and Petri, 2014), Amoebas may cause a variety of clinical presentations, from asymptomatic commensal colonization to invasive amebic dysentery and extraintestinal infections. An infected individual may be initially asymptomatic and develop symptoms later in the course of the infection (Santos *et* al., 2013). *Entamoeba* species can be distinguished based on morphological characteristics into uninucleate (*E. bovis*-like group), quadrinucleate (*E. histolytica*-like group), octonucleate (*E. coli*-like group), and non-cyst-producing (*E. gingivalis*-like group) groups (Stensvold *et* al., 2010; Stensvold *et* al., 2012). Most of those parasites, except the non-cyst-forming parasite *Entamoeba gingivalis*, show two forms during their life cycles: cysts and trophozoites (Clark *et* al. 2006). In general, the parasites infect as trophozoites at an active stage in the hosts, since others form cysts that can survive under utmost environmental conditions, unlike trophozoites (Matsubayashi *et* al., 2014). The original depiction of *Entamoeba bovis* from cattle (*Bos taurus*) by Liebetanz (1905) was bound to the trophic stages. *Entamoeba bovis* inhabit the rumen of ruminant mammals; rarely, *Entamoeba* that is morphologically identical to *Entamoeba bovis* have been accounted for to cause genuine intrusive disease in some ruminants other than cattle. Diarrhea infection of neonatal and young calves is a common disease seen in cattle. The other most common parasitic agents responsible for diarrhea infection are parasites such as *Giardia, Cryptosporidium, Eimeria.* The dominant species obtained in ruminants are *E. bovis*, *E. ovis, E. histolytica, E. dilimani,* and *E. bubalus,* of which *E. bovis* was predominantly found in cattle (Noble and Noble 1952; Jacob *et* al., 2015).

Recently, high titers of specific serum antibodies, DNA, and antigen detection can be utilized to support the identity of *E. bovis* at the species level or to verify the diagnosis of amebiasis. However, such approaches are not available for the differentiation of the other *Entamoeba* spp (Santos *et* al.,2010). Recently, sensitive and specific molecular techniques that can distinguish *Entamoeba* spp. have been developed by use of the polymerase chain reaction (PCR) to amplify amoebic DNA (Ngui *et* al., 2012).

2.1.2 *Eimeria*

Eimeria spp. is an enteric coccidian parasite that infects a broad range of vertebrate hosts (McDonald and Shirley, 2009). *Eimeria* is a genus of apicomplexan parasites that has various species liable for coccidiosis (Vrba *et* al, 2011). Members of the genus *Eimeria* are obligatory intracellular parasites found in a broad variety of wild and domestic vertebrates (Zhao *et* al 2001). Several species cause significant mortality or morbidity in some hosts (Power *et* al 2009). Parasites within the *Eimeria* genus represent more than 1,100 described species isolated from vertebrates including livestock and birds, and invertebrates (Takeo *et* al., 2014). Members of Eimeriidae share distinct morphological features, like a Stieda body during a variable number of sporocysts that varies with the genus, and refractile bodies within the enclosed sporozoites (Ogedengbe *et* al 2011). The pathogenicity or life cycle of *Eimeria* species are known to vary even within one host genus, especially as seen with avian or bovine *Eimeria* spp (Daugschies *et* al., 2005; Sharman *et* al., 2010; Dkhil *et* al., 2014).

2.2 Molecular markers for parasite-species identification

In taxonomy, the necessity for species identification at the genetic level has been increasingly recognized (Gu *et* al., 2013). Several genetic markers have been used for GI parasites identification, the ribosomal DNA small subunit (SSU) (Blaxter *et* al,.1998), large subunit (LSU) (Subbotin *et* al., 2008), and internal transcribed spacer (ITS) region (Floyd *et* al., 2002; De Ley *et* al., 2005) and cytochrome c oxidase subunit I (COI) (Elsasser *et* al., 2009). Molecular assays utilizing the conserved 18S rDNA (small subunit: SSU) gene are developed as an alternative to studying the diversity of nematodes as a monitoring tool (Chen *et* al. 2010). The 18S ribosomal RNA (rRNA) gene was chosen as a target region because it is usually utilized in molecular characterization (Huggins *et* al., 2017).

2.2.1 Ribosomal Genome for marker selection

Ribosomes are large, multi-subunit ribonucleoprotein complexes, essential for protein synthesis (Salim and Gerton, 2019). Ribosomal DNA (rDNA) is a DNA sequence that codes for ribosomal RNA. These sequences regulate transcription initiation and amplification and comprise both transcribed and non-transcribed spacer segments. The rDNA gene sequences are easy to access due to the most evolutionarily conserved segment of the genome (Wang and Lemos, 2019) allowing the use of universal primers (Meyer *et* al.,

2010). This genetic locus has the added attraction of multiple genomic copies that made this region a good PCR target because the multiple copies increase sensitivity. For these reasons, the first large-scale phylogenetic studies depended on this gene (El-Sherry *et* al., 2013). Generally, rDNA sequences show less variation within species than between species variations and are, therefore considered as reliable markers for species identification (Khan *et* al., 2019). Ribosomal DNA (rDNA) has been widely used for molecular phylogenetic analyses of organisms (Suga and Hyakumachi, 2004).

2.2.1.1 SSU unit

The small subunit (SSU) rDNA is part of the nuclear ribosomal DNA (rDNA) locus (Wang *et* al., 2017). The small subunit (SSU) 18S rRNA gene is one of the most commonly used genes in phylogenetic studies and a crucial marker for random target polymerase chain reaction (PCR) in parasitic screening (Meyer *et* al., 2010). Their repetitive arrangement within the genome gives an excessive quantity of template DNA for PCR, even within the smallest organisms. In particular, the SSU rDNA is a universal marker for phylogenetic analyses, as well as identifications and classifications of microbes (Wang *et* al., 2017).

2.2.1.2 18S Subunit

The 18S molecule is part of the small subunit (SSU) of the ribosome (Martínez-Calvillo *et* al., 2001). The 18S gene is a component of the ribosomal functional core and is exposed to similar selective forces in all living beings. It is one of the essential components of all eukaryotic cells due to its being well conserved among species. 18S rRNA amplicon sequencing is showed as an effective and sensitive method to study the eukaryotic diversity (Tanaka *et* al., 2014, Kim *et* al., 2017). Thus, the first large-scale phylogenetic studies based on 18S sequences were published at Field *et al.*, 1988. The genes coding for 18S rRNA is stated as 18SrDNA. 18S rRNA gene has always been a highly conserved locus and is widely used for the molecular characterization of many parasites such as *Entamoeba, Cryptosporidium, Sarcocystis, Giardia*, and *Toxoplasma* (Feng *et* al., 2013; Yang *et* al., 2001; Moreno *et* al., 2018). The small subunit 18S rRNA gene is one of the most widely used genes in the study of phylogenetic relationships among species.

The 18S gene has been used extensively for the classification of apicomplexan parasites (Morrison *et* al., 2004). The *Entamoeba* spp. 18S rRNA gene exists as multicopy loci and exhibits genetic variation and therefore a good target for detection and differentiation of

members of this genus (Mulinge *et* al., 2021). From several potentially suitable markers (e.g. 18S rRNA, ITS, 5.8S rRNA) selection of the 18S rRNA gene, is the most widely used marker in previous studies addressing the phylogenetic relationships among *Entamoeba* spp. (Verweij *et* al., 2001; Stensvold *et* al., 2010, 2011; Jirků-Pomajbíková *et* al., 2016; Tuda *et* al., 2016). Sequences of 18S rDNA contain highly conserved flanking regions suitable for the design of the universal primers. This genetic locus has the added benefit of occurring in multiple copies which facilitates PCR amplification, especially in the case of a low infection level (Vlckova *et* al., 2018).

2.2.2 Mitochondrial COI

The gene encoding mitochondrial cytochrome c oxidase subunit I (COI) has been widely used for phylogenetic analysis of many organisms due to its near-universal presence in organisms that use oxidative phosphorylation as an energy source (El-Sherry *et* al., 2013). Partial COI sequences have proven to be effective species-specific markers for *Eimeria* spp. and related coccidia (Ogedengbe *et* al., 2011). The COI locus is thus far free of the gene duplication and divergence that has been demonstrated for nuclear rDNA. These observations confirm the utility of the mitochondrial COI locus as a species-level genetic marker for *Eimeria* spp. infecting turkeys as well as of other galliform birds (i.e. Ogedengbe *et* al., 2011; Vrba *et* al., 2011).

2.3 Review on techniques and instrumentations concerned

2.3.1 Fecal and sample Preparation

Feces are a complex matrix, and it is necessary to get rid of non-target fecal components. The fecal pre-purification steps like sedimentation, low-speed centrifugation steps, remove large particles present in the fecal matrix before any DNA extraction procedure to obtain DNA suitable for amplification.

2.3.2 DNA extraction

DNA extraction is the separation of DNA from any type of cell or sample for a particular purpose. DNA extraction follows a series of steps. In particular, DNA extraction begins with the lysis of the cell that contains the DNA of interest; this lysis is followed first by the removal of all proteins from the DNA and then by the final step of precipitating the DNA (Kuk *et* al., 2012). DNA extraction is a technique to purify DNA by using physical and/or

chemical methods from a sample differentiating DNA from cell membranes, proteins, and other cellular components (Leban *et* al., 2019). Friedrich Miescher in 1869 did DNA isolation for the first time (Dahm, 2008). Removal of fecal components requires effective modus operandi as an integral part of the DNA extraction process. The QIAamp DNA stool mini kit includes a commercial polysaccharide mixture for removing PCR inhibitors of fecal origin. The overall efficiency of this kit in removing PCR inhibitors was demonstrated by the purity of the extracted nucleic acids, assessed by using the A260 ⁄A280 ratio (Ruiz and Rubio 2009). Effective removal of fecal components has got to reach an equilibrium between obtaining a concentration of inhibitors at a level that doesn't affect PCR amplification, and a concentration of target DNA ample for PCR amplification (Rapp, D. 2010).

2.3.2.1 QIAamp DNA Stool Mini Kit

The QIAamp DNA Stool Mini Kit provides fast and easy purification of total DNA from stool samples. QIAamp purified DNA is of high quality and well suited for use in PCR and other downstream enzymatic reactions. Stool samples typically contain many compounds that can deteriorate DNA and inhibit downstream enzymatic reactions. To confirm the removal of these substances, the QIAamp DNA Stool Mini Kit contains InhibitEX Tablets, a unique reagent provided in an easy tablet form. InhibitEX Tablets efficiently adsorb these substances early in the purification process so that they can easily be removed by a quick centrifugation step. Additionally, the kit has Buffer ASL, which causes pathogens in the stool to be effectively lysed by incubating the stool homogenate at 70°C. The entire DNA concentration in the lysate will rise 3- to 5-fold by lysis at 70°C. The QIAamp DNA purification procedure involves digestion of proteins, binding DNA to the QIAamp silica membrane, washing away impurities, and elution of pure DNA from the spin column. (QIAamp® DNA Stool Handbook, 2012).

2.3.3 DNA Concentration and Purity

DNA yields are determined from the concentration of DNA within the eluate, measured by absorbance at 260 nm. Purity is decided by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7–1.9. Additionally, the concentration of the sample report in ng/µl (QIAamp® DNA Stool Handbook, 2012).

2.3.4 PCR Principle and DNA Sequencing

Polymerase chain reaction (PCR) is a tremendously important tool for molecular diagnosis (Erlich, H. A. 1989; Sarkar *et* al., 1990). It is a primer-mediated in-vitro method (Lo and Chan, 2006) that can generate an ample supply of a specific segment of DNA (i.e., an amplicon) from only a small amount of starting material (i.e., DNA template or target sequence) (Joshi and Deshpande, 2010; Lorenz T.C., 2012). Each PCR assay needs the presence of template DNA, primers, nucleotides, and DNA polymerase. DNA polymerase is the essential enzyme that links individual nucleotides together to make the PCR product. The nucleotides comprise the four bases—adenine, thymine, cytosine, and guanine (A, T, C, G)—that are found in DNA. These act as the building blocks that are utilized by the DNA polymerase to make the PCR product. The primers in the reaction specify the precise DNA product to be amplified. The primers are short DNA fragments with an outlined sequence complementary to the target DNA that is to be detected and amplified. These serve as an extension point for the DNA polymerase to build on (Joshi and Deshpande, 2010; Garibyan and Avashia, 2013).

PCR consists of exponential amplification of a DNA fragment, and its principle is predicated on the mechanism of DNA replication in vivo: dsDNA is denatured to ssDNA, duplicated, and this process is repeated along with the reaction (Rodriguez and Hernandez, 2013). PCR is done in a reaction mixture that consists of the DNA extract (template DNA), Taq polymerase, the primers, and the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution. The tubes comprising the mixture reaction are subjected to repetitive temperature cycles several tens of times in the heating block of a thermal cycler. The apparatus permits the programming of the duration and the succession of the cycles of temperature steps. Each cycle incorporates three periods of a few tens of seconds. The process of the PCR is subdivided into three stages as follows: a denaturation of the template at 90˚C to 95˚C followed by annealing with specific primers (45 ˚C to 60 ˚C), and an elongation of the hybrid by the action of DNA Polymerase at 72˚C. The products of each synthesis step serve as a template for the subsequent steps, thus exponential amplification is achieved (Kadri, 2019; Bhat and Rao, 2020).

Nucleic acid sequencing is a process of determining the exact order of nucleotides present in a given DNA or RNA molecule (Grada and Weinbrecht, 2013). In the enzymatic method of sequencing, DNA fragments are synthesized by DNA polymerase, which includes deoxynucleotide monomers into a polymeric complementary copy of a template DNA fragment (Noolandi, 1992). DNA sequencing is the process of determining the arrangement of nucleotides bases, adenine, guanine, cytosine, and thymine in a molecule of DNA. Cycle sequencing is a method used to elevate the sensitivity of the DNA sequencing process and permits the utilization of tiny amounts of DNA starting material. This is achieved by using a temperature cycling process similar to that employed in the PCR (Craxton, 1991).

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during in vitro DNA replication (Sanger and Coulson, 1975; Sanger *et* al., 1977).

In chain-termination DNA sequencing, a single-stranded DNA molecule with the difference in length by a single nucleotide can be separated. For this sequencing, the preparation of identical single-stranded DNA molecules is a prerequisite. The first step in this method is to anneal a short oligonucleotide at the same position on each molecule. This oligonucleotide subsequently acts as the primer for the synthesis of a new DNA strand, complementary to the original template. DNA polymerase enzyme along with the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP, one or more of which is radioactively labeled) and a small amount of a dideoxynucleotide (e.g. ddNTP) are added. The polymerase enzyme does not discriminate between dNTPs and ddNTPs, hence dideoxynucleotide can be incorporated into the growing chain, but blocks further elongation because of the lack of the 3' hydroxyl group required to form a connection with the next nucleotide. The entire sequencing reaction can now be carried out using the PCR Thermocycler. For automated sequencing, the primer or the ddNTPs are labeled with a fluorescent dye. Thus, rather than running the gel, the machine uses a laser to read the fluorescence of the dye as the bands pass a fixed point. A DNA sequencer consists of capillary electrophoresis facility for size separation of DNA fragments, detection and recording of the dye fluorescence, and data output as fluorescent peak based chromatograms. Amplification of the template DNA by PCR, purification of PCR product,

and re-suspension in a buffer solution is done before loading the samples onto the sequencer. Samples are subjected to capillary electrophoresis. The laser beam causes the dyes on the fragments to fluoresce. Each base is labeled with a different color. The color of the fluorescent bases is detected by a camera and the presence or absence of a DNA strand is then recorded in a detector. The shorter strands of DNA move faster through the gel matrix and hence are detected sooner and there is a direct correlation between the length of the DNA strand and time at the detector. This relationship is used to determine the actual length of the DNA sequence. (Heather and Chain,2016; Khandpur, 2019; Bhat and Rao, 2020).

2.3.5 Sequencing analysis

The epoch of DNA sequencing began about 30 years ago (Heather and Chain, 2016). Sequence analysis is the procedure of subjecting a DNA, RNA , or peptide sequence to any wide range of analytical methods to understand its features, function, structure, or evolution. Methodologies used include sequence alignment, searches against biological databases, and others (Durbin *et* al., 1998). Information about the primary DNA sequence is a vital piece of data utilized in areas of research as medical studies, gene therapy, drug development, evolutionary studies, biotechnology, forensic, and numerous others (Prjibelski *et* al., 2018). Software packages are available which can trim low-quality DNA traces automatically and score only the quality peaks (Bhat and Rao, 2020).

2.3.6 Sequence editing and alignment

Multiple sequence alignment aims to align multiple related sequences to achieve their optimal matching. Typically, such related sequences are identified through database closeness searching. As the process generates multiple matching sequence pairs, it is often mandatory to somehow transform separate pairwise alignments into a single alignment, which aligns sequences in such a way that evolutionarily equivalent positions across all sequences are matched (Prjibelski *et* al., 2018). Sequence data is directly imported into MEGA by an alignment explorer that retrieves sequences from databases through an in-built web browser that directly downloads the selected sequence files in FASTA or other formats (Khan, 2017).

2.3.6.1 Bioedit and Sequencer

Bioedit is perhaps the most widely recognized program used in molecular biology studies. It comprises numerous features for sequence alignments modes of easy hand alignment, user-defined color, information-based shading, and auto integration with other programs such as ClustalW and Blast. Bioedit can accept a wide variety of formats that are commonly used with other bioinformatics applications (Hall *et* al., 2011).

Sequencer vs 4.1.4 has a facility to let import sequences in many formats, show chromatogram, and is usually exploited to generate a consensus sequence. It displays chromatogram in multiple columns and allows easy visualization and editing (Nishmura and Rating, 2000).

2.3.6.2 BLAST

The Basic Local Alignment Search Tool (BLAST) algorithm is the most prevalent sequence alignment tool in bioinformatics (Altschul *et* al., 1990). BLAST discovers areas of local similarity between biological sequences. The program juxtaposes nucleotide or protein sequences to sequence databases and computes the statistical significance of matches. BLAST can be utilized to derive functional and evolutionary relationships between sequences as well as aid identify members of gene families.

2.3.6.3 MEGA and Neighbor-Joining method

MEGA (Molecular Evolutionary Genetics Analysis) has been developed for estimating evolutionary distances, reconstructing phylogenetic trees, and computing basic statistical quantities from molecular data (Kumar *et* al., 1994). This software is user-friendly and capable of analyzing both distance-based and character-based tree methodologies. The latest version of MEGA X software contains many sophisticated methods and tools for phylogenomics and phytomedicine (Kumar *et* al., 2016). MEGA runs a statistical re-sampling process called bootstrapping to check trees reliability by measuring the probability of branch recovery if the taxa were sampled again. Its values are typically from 1000 repeated calculations and values >70% are acceptable (Khan, 2017).

NJ Methods initially sum individual distance to calculate the difference of an organism from all other organisms and then based on this sum corrected distance method is calculated (Kaur and Bawa, 2016). The neighbor-joining method is put forward for

reconstructing phylogenetic trees from evolutionary distance data. The principle of this method is to discover sets of operational taxonomic units (OTUs) that reduce the total branch length at each stage of clustering of OTUs starting with a starlike tree. The branch lengths as well as the topology of a parsimonious tree can quickly be obtained by using this method (Saitou and Nei, 1987).

2.3.6.4 Phylogenetic Tree construction

A phylogenetic tree is a tree showing the evolutionary interrelationships among various species or other entities that are believed to have a common ancestor (Qin *et* al., 2006). Phylogenetic studies use various multiple sequence alignment methods to detect the level of sequence dissimilarity. The distance between compared sequences is used to construct phylogenetic trees, in which the length of the branches typically corresponds to the distance between analyzed sequences. To construct a biologically meaningful and realistic tree, various clustering methods can be used as well as different sequences may be provided as input (Prjibelski *et* al., 2018). Robust phylogenies give the basis for interpreting biological variation of evolution (Littlewood *et* al., 1999).

CHAPTER 3. MATERIALS AND METHODS

3.1 Research Methodology and Data Analysis

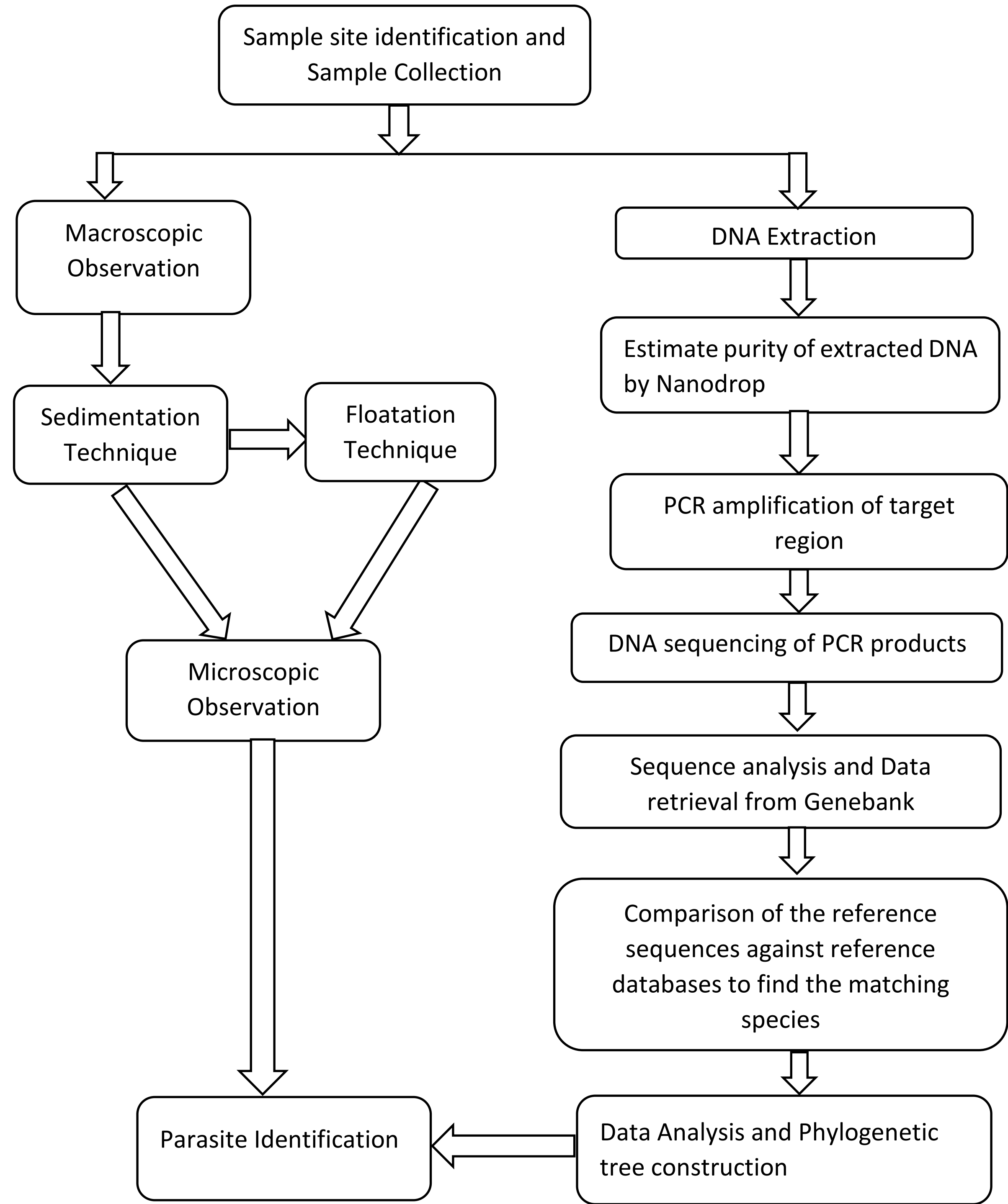


Figure 2: Overall study design in Flowchart

3.2 Sample collection

The fecal samples were collected from the enclosure of Padampur, CNP. The team collected 25 samples from CNP. Non-invasive sampling techniques were employed. Before collecting the samples, the fecal sample was carefully examined for blood and mucus. About 10 gm of fecal samples were collected in two separate sterile vials. Collected samples were mixed with 2.5% potassium dichromate that helps in preserve the morphology of cysts and eggs and then stored at -20°C when brought to the laboratory.

3.3 Morphological Identification and species description

The fecal samples were macroscopically examined for the presence of blood, mucus, segments of cestodes, as well as whole adult nematodes and microscopically examined for the ova/cyst/oocyst and larva of different parasites according to shape, size, and color by the techniques based on the literature (Rijal 2008, Ghimire and Bhattarai, 2019).

3.3.1 Direct Wet Mount Technique

One to two drops of carefully stirred fecal samples were put on a clean glass slide with the help of a plastic dropper. Samples were emulsified in a drop of Lugol's Iodine solution and then covered with a clean coverslip. The smear was examined under the electric microscope at 10X and 40X.

3.3.2 Concentration procedure

Eggs, cysts, and trophozoite were often in such low number, that they are difficult to be detected in direct smears or mounts so to separate parasites from fecal debris and increase the chances of detecting parasitic organisms this procedure is conducted. They are divided into flotation techniques and sedimentation techniques.

3.3.2.1 Sedimentation Technique

About two gms of the fecal samples were thoroughly mixed in 13mL normal saline (0.9% w/v), and filtered with the help of a tea strainer into a 15mL centrifuge tube, and then proceeded to centrifuge (1200rpm for 5 minutes). The supernatant thus obtained was discarded, and one to two drops of the sediment was put on a glass slide. Gram's iodine was used in the deposits for the microscopic examinations at 40X and 100X magnification.

This technique is used for the detection of trematode eggs as the eggs of trematode are a bit heavier than the other.

3.3.2.2 Saturated Salt Floatation Technique

In the previous sediment, 12mL of salt solution (45% w/v) was added and proceeded to centrifuge (1200rpm for 5 minutes). Then, few drops of salt solution (45% w/v) was added to the tube to fill it, and a coverslip was placed on the mouth of the tube. After 10 minutes, the coverslip was carefully removed and put on the glass slide with or without Lugol's iodine for microscopic observation at 40X and 100X magnification. This technique ensures the eggs float in the floatation liquid, which helps to identify the nematode and cestode eggs present in *Bubalus arnee* dung.

3.4 DNA extraction

3.4.1 Modification in DNA extraction

The commercially available DNA extraction kit, QIAamp DNA Stool Mini kit was selected because Qiagen is recommended kit for stool samples. The kit was tested with a preceding bead beating step and freeze-thaw-boiling step (Ayana *et* al., 2019). This step aims to mechanically rupture the egg shells, but this is not included in commercial Qiagen protocols.

To withhold the large debris and to remove 2.5% potassium dichromate, about two gms of the fecal samples were thoroughly mixed in 13mL normal saline (0.9% w/v), were filtered with the help of a tea strainer into a 13mL centrifuge tube, and were proceeded to centrifuge (1500rpm for 5 minutes). The supernatant was discarded and the pellet was washed twice with PBS by centrifugation at 1500 rpm for 5 min and approximately 666µl of suspension with 200µl PBS was transferred in 2ml Lysing Matrix A tubes which contains garnet matrix and one 1/4" ceramic sphere (MP Biomedicals). It was then stored overnight at -20°C.

To enhance egg shell rupture and minimize inhibition, a freeze-thaw-boiling step was included in all extraction protocols (not included in the manufacturer's protocols). This step consisted of a frozen phase at -20˚C for overnight followed by a thaw-boiling phase in a preheated heating block at 100˚C for 10 min. Subsequently, for the bead beating extraction protocols, Lysing Matrix A tube containing samples were subjected to bead

beating (Fastprep-24 5G™ Sample Preparation Instrument, MP Biomedicals) at speed 6m/sec for 65sec . Thereafter, further, the manufacturer's protocol for extraction protocols was followed.

Another modification was done in step 4 of the Qiagen protocol by heating the lysate sample at 70 °C for 30 min for further cell lysis.

3.4.2 QIAamp DNA Stool Mini kit

Briefly, 2 ml of buffer ASL was added to each sample tube and thoroughly mixed by vortexing. Subsequently, 1.6 ml of this suspension was transferred to a 2 ml Eppendorf tube and heated at 70˚C for 30 min. After that centrifugation was done at 10,000 rpm (8,944 g) for 2 min, 1.2 ml of supernatant was transferred to a new 2 ml Eppendorf tube and 1 InhibitEX tablet was added, which adsorbs inhibitors from the suspension. After centrifugation at 10,000 rpm (8,944 g) for 3 min, 200µl of supernatant was added to an Eppendorf tube, to which 15µl of Proteinase K and 200µl AL lysis buffer was added. Tubes were then incubated at 70˚C for 10 min. 200 µl of ethanol (96–100%) was added to the lysate, and mix by vortexing. Centrifuge briefly. The complete lysate was added to the QIAamp spin column and centrifuged until the lysate completely passed through the spin column membrane enabling nucleic acids to attach to the column membrane. Subsequently, the column was washed with buffers AW1 and AW2. Finally, bound DNA was eluted in 200µl of AE buffer. All DNA extracts were stored at -20˚C for PCR analysis.

3.5 Determination of concentration, yield, and purity of DNA

Elution buffer was used as blank. DNA concentration and purity of the extracted DNA were measured by the absorbance at 260 nm and 280 nm in a microplate Nanodrop. (Multisky, Thermofisher Company).

3.6 Primer selection

The use of DNA primers to amplify the 18S region of specific *Entamoeba* spp. has been previously described (Verweij *et* al., 2003; Santosh *et* al., 2010; Santos *et* al., 2013; Ren *et* al., 2020). Universal, genus-specific DNA primers targeting two genomic regions from *Eimeria* spp. (COI and 18S rRNA) were selected based on previous studies by Schwarz *et* al., 2009; Vrba et al., 2011. The Primers were ordered from the Macrogen Company, Korea.

Table 1: Primers used in this study

Region	Direction	Primer sequence 5'-3'	Tm(˚C)	GC%	O.D.
18S *Ent.*	JVF_ Forward	GTTGATCCTGCCAGTATTATATG	59.3	39	7.5
	DSPR2_ Reverse	CACTATTGGAGCTGGAATTAC	57.4	43	7.1
18S *Eimeria*	ERIB1_ forward	ACCTGGTTGATCCTGCCAG	59.5	58	6.3
	ERIB10_ reverse	CTTCCGCAGGTTCACCTACGG	65.3	62	6.3
COI *Eimeria*	KM204_ forward	GTTTGGTTCAGGTGTTGGTTG	59.4	48	6.7
	KM205_ reverse	ATCCAATAACCGCACCAAGAG	59.4	48	7.3

3.7 Polymerase Chain Reaction (PCR) amplification and PCR success rate

After the selection of suitable primer and reviewing the PCR conditions, the PCRs targeting the 18S rDNA and COI were performed. All the necessary optimization was carried out according to the requirement such as alteration in the number of cycles, annealing temperature, and components for successful PCR. All the PCR reactions were carried out in PCR tubes, prepared in ice, and then incubated in a PCR machine for an average of 2 hours.

The reaction mixture for amplification of the 18S region of *Entamoeba* and *Eimeria* and COI region of *Eimeria* from the DNA templates with all the basic components were prepared. For amplification of the 18S region of *Entamoeba*, extracted DNA sample was taken as a template and PCR amplification was carried out as the primers (JVF and DSPR2) were *Entamoeba* specific. Similarly, for amplification of the 18S region of *Eimeria*, extracted DNA sample was taken as a template and PCR amplification was carried out as the primers (ERIB1 and ERIB10) were *Eimeria* specific. For amplification of mitochondrial region COI of *Eimeria*, extracted DNA sample was taken as a template and PCR

amplification was carried out as the primers (KM204 and KM205) were *Eimeria* specific. The two pairs of markers were used because joint analyses of nuclear and mitochondrial markers may be necessary to distinguish species.

A PCR reaction mixture was prepared by mixing GoTaq® Green Master Mix (PCR buffer, dNTPs, Taq polymerase, Magnesium chloride) to a final concentration of 1X, forward and reverse primers to that of 0.4 pmoles, Magnesium Chloride to that of 25mM (if required) and Taq polymerase to that of 0.25U/10µl (if required). The final volume of the reaction mixture (10µl) is fulfilled by nuclease-free water. The details of each reaction mixture and PCR conditions are shown in Table 5, Table 6, and Table 7.

After preparation of the reaction mixture, the PCR tubes were vortexed for 2 secs, short-spinned, and then incubated in PCR machines (Veriti 96-well, Applied Biosystems, USA) for target amplification. PCR incubation conditioned varied according to the marker and details for 18S and COI regions respectively are presented in respective tables in appendices. PCR amplifications were carried out in a thermal cycler.

3.8 Agarose gel electrophoresis

PCR products can be visualized by gel electrophoresis. DNA is loaded into an agarose gel where the DNA fragments are separated based on size. After electrophoresis, the target DNA-EtBr complex in the agarose gel is illuminated on the exposure to UV-Light and compared to a standard ladder, thereby confirming the presence of the target band.

Agarose gel (1.5%) was prepared in a freshly prepared 1X TAE in a conical flask. The flask was swirled to evenly distribute the agarose. The solution was heated in the microwave oven for 1-2 minutes. The gel was cooled until it was comfortable to touch the flask with a gloved hand. Just before pouring the gel, 0.5µg/ml ethidium bromide was added to the dissolved agarose and swirled to mix. As EtBr is carcinogenic it was handled with caution. The dissolved agarose was poured into the gel mold carefully to avoid air bubbles and the comb was adjusted perpendicularly to one side of the mold. The gel was allowed to solidified and then the comb was removed. The gel was placed in the electrophoresis rig with the wells closest to the negative (black) end and the tank was filled with 1x TAE buffer till it covered the gel. As the GoTaq® Green Master Mix contains dye so 3 µl PCR products were loaded directly into the well. To correspond the band size DNA marker (Dye Plus 250 bp ladder) was loaded in the first lane of the well. The electrophoresis was started to run

in 100 volts for 30 min and the gel was placed to visualize under the UV rays on the UV transilluminator and also in gel documentation instrument (Syngene Bio-imaging, UK).

3.9 DNA sequencing

3.9.1 Purification of the PCR products

For the further processing of the target amplicon, Amplified PCR products generated from the 18S rDNA gene of *Entamoeba* spp. were purified with EXOSap-IT^{TM} (Applied Biosystems, USA) which removes excess PCR primers and unincorporated dNTPs after PCR.

PCR products were purified following EXOSap kit protocol. To 5 µl PCR product, 2 µl of EXOSap mix was added and blend well by pipetting. The mixture was incubated at 37°C for 10 minutes followed by incubation at 80°C for 10 min (both in a PCR machine) to inactivate the enzymes. Purified PCR product was tested loading 1 µl of each product into 1% agarose gel and electrophoresis was performed. The corroboration of the presence of purified product was done and the difference in the purity before and after the purification was also analyzed. The rest of the purified product was preserved for down-streaming applications (DNA sequencing).

3.9.2 Cycle sequencing

DNA sequencing reaction incorporates the PCR incubation of amplified DNA (to be sequenced) with different reagents and using only a single primer at once; either forward or reverse. DNA sequencing was performed by using high-quality single band PCR purified product after gel visualization under UV light. The sequencing reactions were prepared on 96- well plate by following BigDye^{TM} Terminator v 3.1 Cycle Sequencing Kit.

Primers, Ready Reaction mix (fluorescent-labeled ddNTPs, dNTPs, DNA polymerase), PCR buffer, PCR products, and BSA were completely thawed and kept on ice after short-spinning. BSA was added to improve the adequacy of the reaction. Among the components used, the Ready Reaction mixture being light-sensitive was taken care of cautiously with the minimum exposure into the light as possible (covering with aluminum foil, turning off the chamber light, etc.). The sequencing reaction mixtures were prepared separately for each of the respective markers of respective direction (forward or reverse)

according to Table 2. After preparation of mixture on a 96-well plate, the plate was properly sealed with MicroAmp^{TM} Clear Adhesive film (Applied Biosystems, USA). The plate was vortexed for 2-3 secs and short- spinned in a swinging bucket centrifuge (ST8R, Thermo Scientific, USA). The plate was then immediately put in a PCR machine for incubation with appropriate sequencing reaction conditions (Table 3).

Table 2: DNA sequencing and reaction mixture

Components	Volume in µl	Final concentration
Ready Reaction mixture (2X)	0.5	
PCR buffer (5X)	1.75	1X
Primer (forward or reverse)	1.5	1.5 pm/ µl (µM)
PCR product	1	not quantified
BSA (1mg/ml)	0.025	2.5 µg/ml
NFW	5.225	
Total	10	

Table 3 : DNA sequencing and reaction condition

Steps	Initial Denaturation	Denaturation	Annealing	Extension	Final extension	Hold
Cycles	1	35			1	1
Temperature	94°C	94°C	52°C	72°C	72°C	4°C
Time	3 min	30 sec	30 sec	45 sec	10 min	∞

3.10 Purification of the synthesized DNA fragments

The presence of both unlabeled and dye-labeled reaction components can interfere with electrokinetic injection, electrophoretic separation, and data analysis that obscure the desired signal and interfere with base calling. To avoid the possible obstruction of used buffers, primers, and other utilized reagents, the DNA fragments synthesized after the PCR incubation was further purified before the sequence-reading electrophoresis. Purification was carried out by the following purification method.

3.11 Sequencing purification by EDTA precipitation:

A master mix I of 10 µl Mili-Q and 2 µl of 125mM EDTA per reaction was prepared. Twelve µl master mix I was added to each 10 µl of sequencing reaction in PCR strips with attached caps a master mix II of 2 µl of 3M Sodium acetate pH 5.7 and 50 µl of absolute ethanol per reaction was prepared and 52 µl of the master mix II was added to each reaction. The PCR strips were capped properly and the reagents were blended by vortexing. The capped -strips were incubated at room temperature for 15 minutes and centrifuged at 3800 rpm for 30 minutes at room temperature. The supernatant was decanted by inverting the plate on the tissue paper. The plate was invert-spinned at 300 rpm for 2 minutes to remove residual supernatant. After that 100 µl of freshly prepared 70% ethanol was added to each reaction of the plate and the capped strips were spinned at 4000 rpm for 15 minutes at room temperature. The ethanol was drained out (washed) by inverting the PCR strips immediately over layers of UV- sterile tissue paper after opening the caps. Invert spin was done at 300 rpm for 2-3 minutes. The washing was done with absolute care and was repeated for removal of residual ethanol and then left to dry for 20 minutes. Finally, 10 µl of Hi-Di formamide was added to each of the reactions in the plate and spinned. Denaturation at 95°C for 5 minutes was performed in a Thermo cycle machine and the plate was promptly chilled (snap-chilling) on ice.

3.12 Sequence: Capillary electrophoresis

The plate with the purified reaction was then forwarded for analysis on a Genetic Analyser (Sequencer). The plate was stacked on the deck of the sequencer, bolted, and connected to the 3500XL Genetic Analyser (Applied Biosystems, USA) for capillary electrophoresis using BigDye XTerminator run module. Applied Biosystems Gene Analyser ABC3500XL is based on Sanger's dideoxy chain termination method. The base allocation and chromatographic interference for *Entamoeba* were given by the analyzer which was further evaluated to generate information.

3.13 Sequence editing and Alignment

The Sequence file was imported to Bioedit vs 7.2.5 to check the sequence peaks to confirm the quality of the sequenced and the ambiguous peaks were examined carefully for a single strand. Whereas for the double strands (with both forward and reverse sequences) the consensus sequences (contigs) were prepared.

To prepare the contigs, sequences (forward and reverse primer based) obtained through the Genetic analyzer were imported to the Sequencer vs 4.1.4 and the contig of overlapping fragments was created by assembling the sequences keeping the assembly parameters: minimum match percentage 70-85. The sorted sequence was exported as Consensus as taken for further analysis. Based on the base call of a nitrogen base corresponding to forward and reverse sequence, the assignment of bases, deletion of bases, deletion of the gap, assignment of gaps, replacement of nitrogen bases was performed to obtain a reliable and valid sequence. Sequences were prepared for identification of species by removing discrepancies from both 5' and 3' ends, if necessary. Consensus sequences were used for multiple sequence alignment comparing with the primer sequences and the fasta file of alignment was saved for further analysis. The individual sequences based on the alignment were also saved for the marker for further analysis.

3.14 NCBI BLAST

Sequences were compared to sequences stored in GenBank. Similarity search was performed by using NCBI -nucleotide BLAST. The method takes the top hit and percentage identity to give the identity score from query-reference comparison. For this method, all the top hits within 10 points deviation down of the maximum score were considered: if the maximum score (-10points) included only a single species then a species-level identification was assigned. The consensus sequence thus obtained was entered in NCBI nucleotide-BLAST to observe the matches of samples(queries) with the available specimens (subjects). Considering the maximum score, identity, and the E - value, the samples were checked for the match of sequence-based species identification with the phenotypic-based sample labeling.

3.15 Nucleotide composition

The nucleotide composition of all the marker-sequences corresponding to the respective markers of the samples was obtained by 'Model' application on MEGA. The percentage composition of A, T, G, and C was known and further analyzed to find the average G+C content in the genes of *Entamoeba*.

3.16 Phylogenetic tree construction and distance estimation

A phylogenetic tree is a tree showing the evolutionary interrelationships among various species or other entities that are believed to have a common ancestor (Qin *et* al., 2006). The phylogenetic trees were constructed for *Entamoeba* using the Neighbour-joining method (NJ) in MEGA X software program using p-distance as the genetic measure. NJ Methods initially sum individual distance to calculate the difference of an organism from all other organisms and then based on this sum corrected distance method is calculated (Kaur and Bawa, 2016). Bootstrap testing of 1000 replicates was performed to estimate the confidence level of the topology of the consensus tree. Note support was estimated base on the following scale: 50-70% (weak bootstrap support) and 70-100 % (strong bootstrap support). The efficiency of the species resolution was considered successful only when all the single clades have at least more than ≥50% bootstrap value. In addition, the sampled species was considered successful when a discriminate individual of species formed a monophyletic group in the phylogenetic tree.

The aligned sequences in MEGA-acceptable format (fasta format) were imported to MEGA X and continued with the 'Analyze' option. The tree was constructed on a Phylogeny application with the Neighbor-Joining method with the number of bootstrap 1000 as the changed parameter. The tree was visualized in different formats as available in MEGA X and one of them is saved for interpretation and analysis.

The program BLASTn is used to identify samples in GenBank with similar sequences to those isolated in this study. Sequences were imported into the MEGA X and aligned using 'clustalW'. Sequences were trimmed at both the 5'and 3' to prepare a data set of sequences homologous to isolates from this study.

CHAPTER 4. RESULTS

4.1 Distribution of Wild Water buffalo in Chitwan National Park

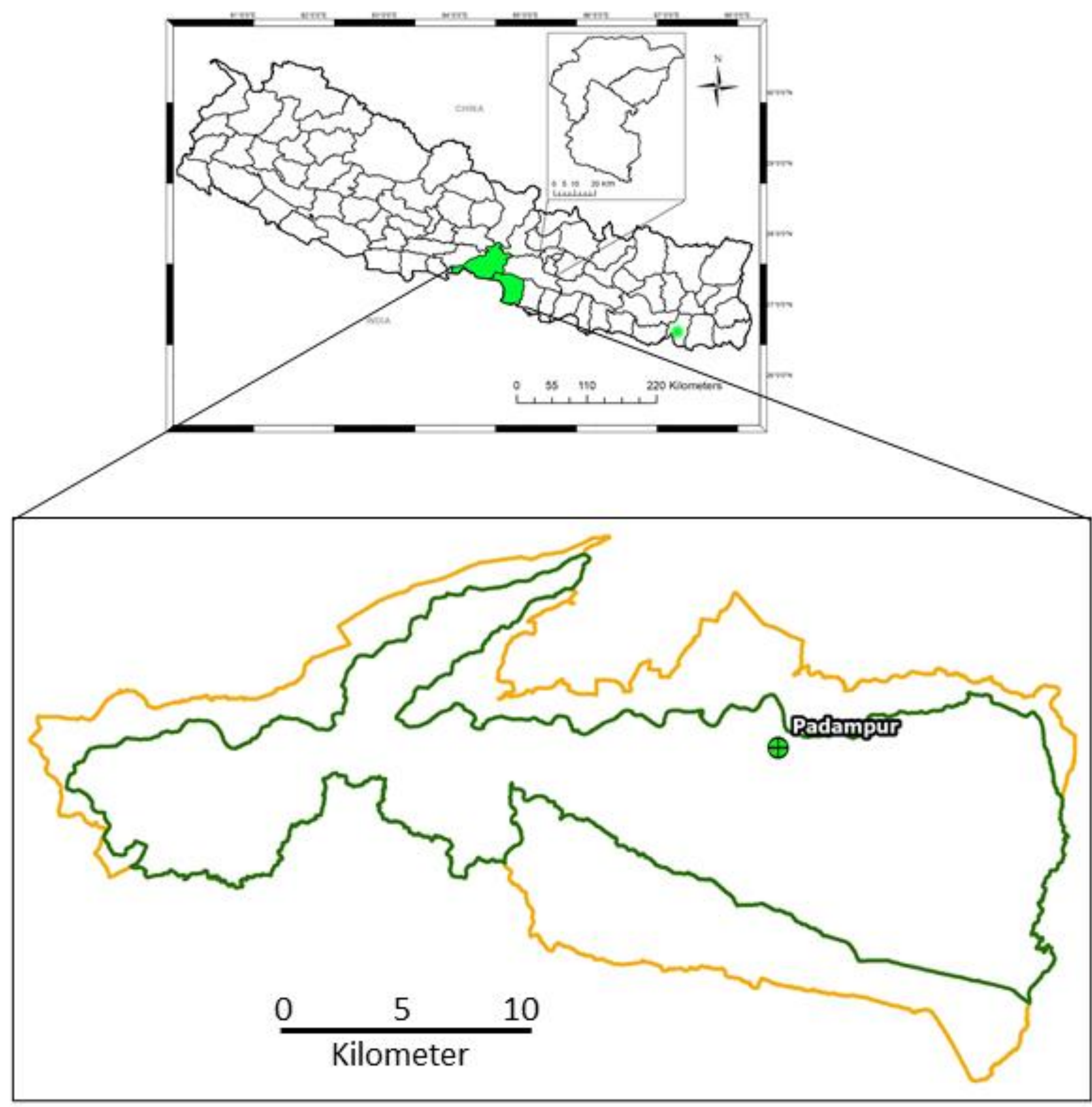


Figure: 3 Distribution of Wild Water buffalo in Chitwan National Park

4.2 Distribution of intestinal parasites

In the present study, out of 25 faecal samples 22 (88%) faecal sample were positive for protozoan parasites which is followed by nematode 21 (84%) and trematode 3 (12%).

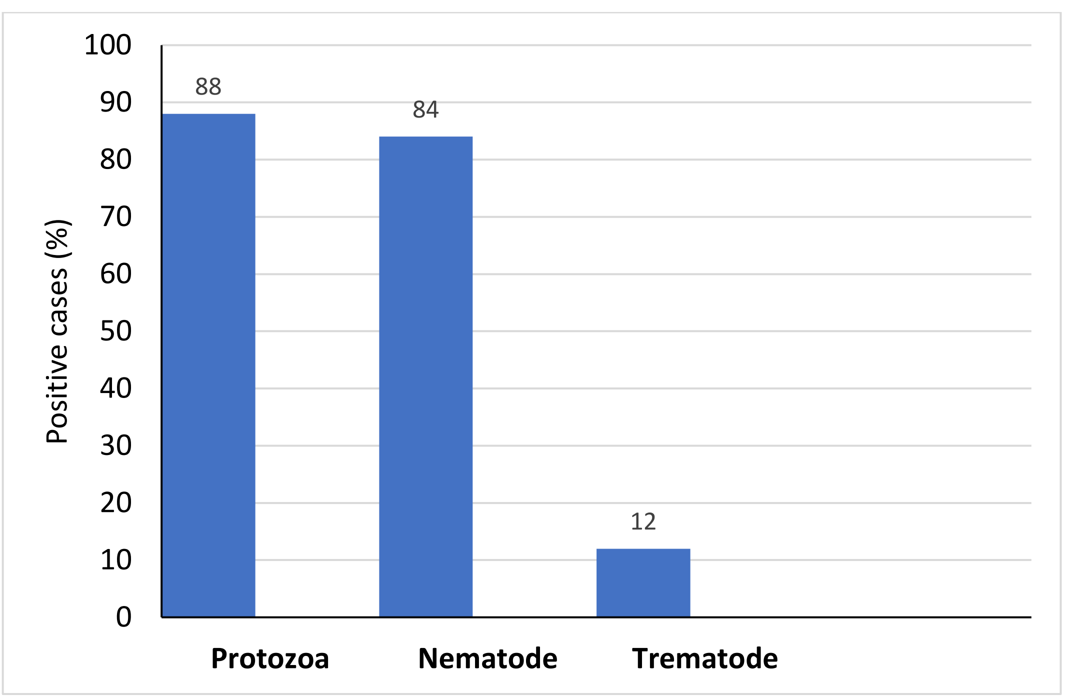


Figure 4: Group wise parasitic prevalence in Wild Water buffalo of CNP

4.3 General prevalence of intestinal parasites.

Out of 25 fecal samples collected from Wild Water buffaloes protected at Padampur enclosure of the CNP, 22 (88%) were positive for protozoan parasites, and 21 (84%) were positive for helminth parasites. In total, nine intestinal parasites were detected under microscopic examination and they included four protozoa (88%), two trematodes (12%), and three nematodes (84%) (Figure 4).

The prevalence rates of five helminth parasites were as follows: *Oxyruid* (68%) *Strongyloides* (28%), *Strongyle* (36%), *Eurytrema* (8%), and *Paramphistomum* (4%). Similarly, the prevalence rates of four genera of protozoan parasites were assessed as *Entamoeba* (80%), *Eimeria* (8%), *Balantidium coli* (4%), and *Cryptosporidium* (60%) (Figure 3). In this study, *Entamoeba* was one of the common intestinal parasites found in the present study.

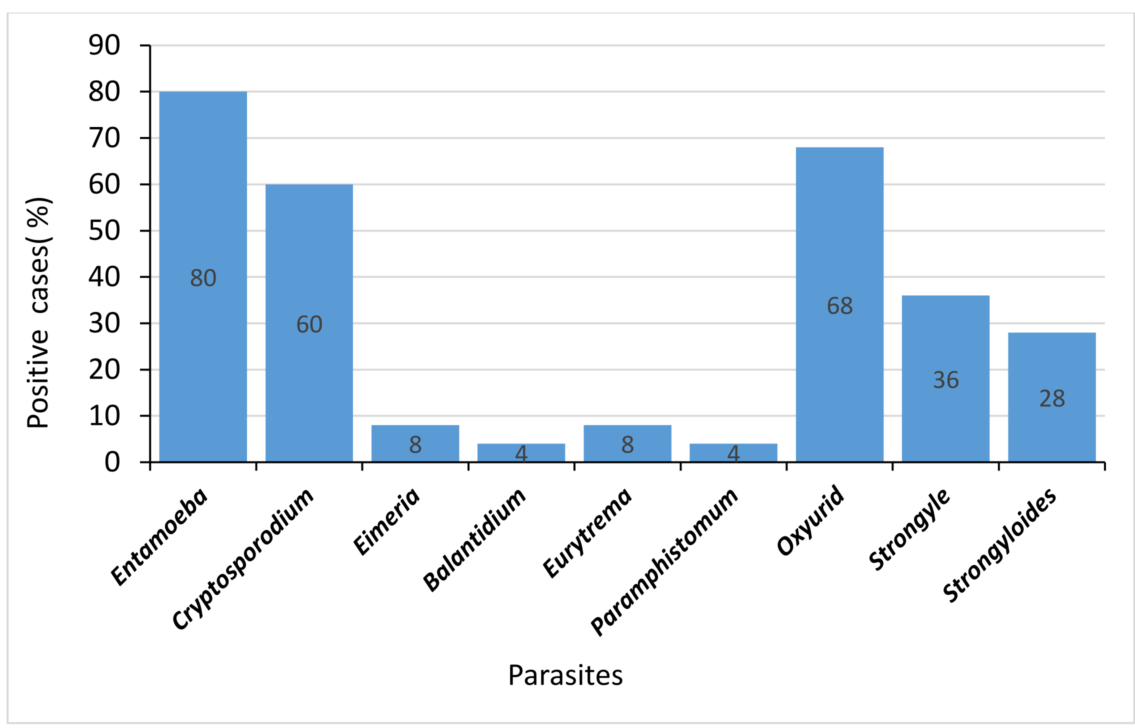


Figure 5: Overall parasitic prevalence in Wild Water buffalo of CNP

4.4 Mixed infection:

The study showed that only one fecal sample did not contain any parasite, one fecal sample had single infection, and the leftover 23 samples were infected with multiple parasite infections (Figure 5). The double infection was the highest, present in eight samples (32%) followed by triple in six samples (24%), in quadruple in five samples (20%), quintuple in two samples (8%), and sextuple in two samples (8%).

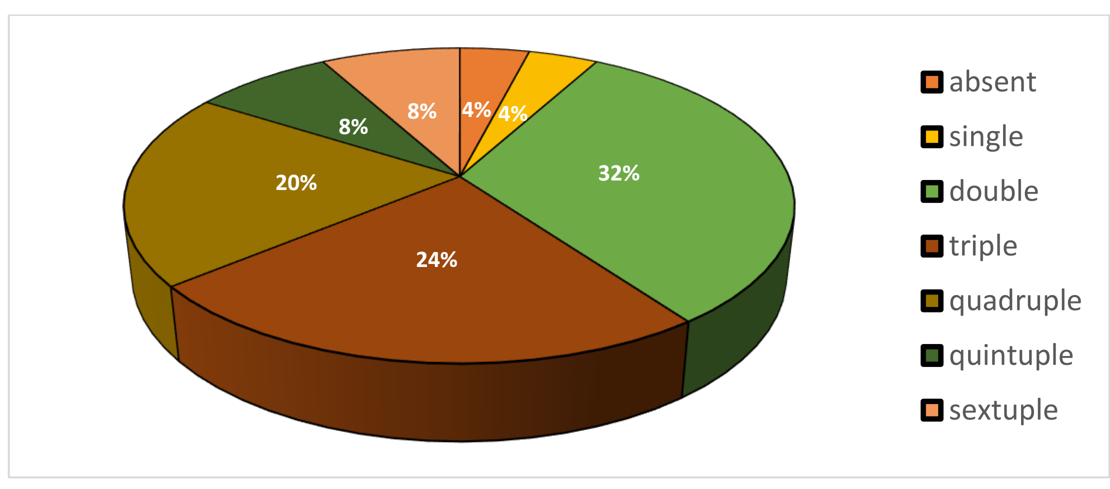
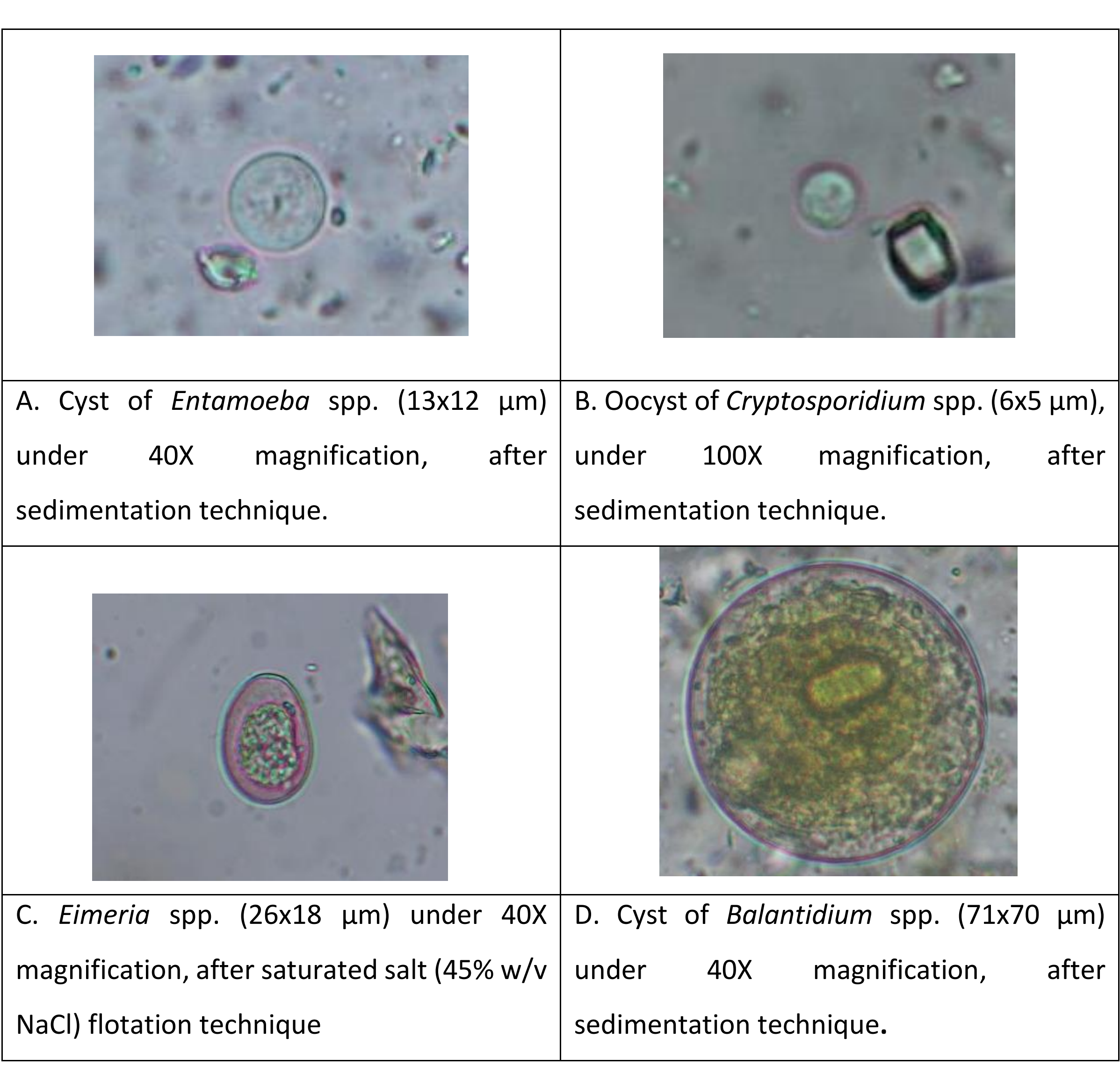


Figure 6: Mixed parasitic infection in Wild Water buffalo of CNP

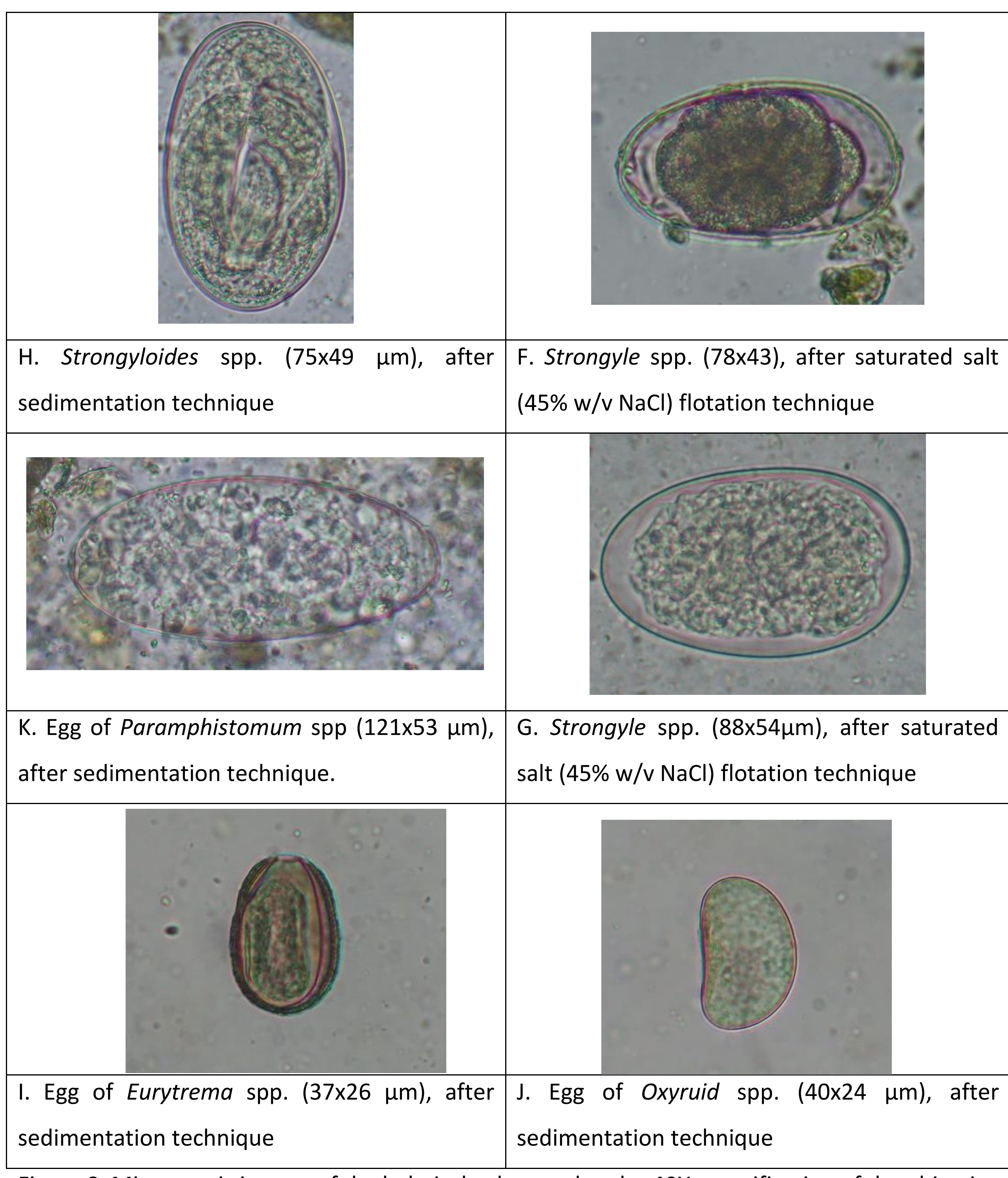
4.5 Morphological Identification

The diameter (length by width) of eggs/oocyst of different gastrointestinal parasites of Wild Water buffalo which were observed under a light microscope (Optika Microscopes Italy, B-383PLi) at a total magnification of 100x, 400x, and1000x. Photographs were taken by the camera (SXView2.2.0.172 Beta (Nov 6, 2014) Copyright (C) 2013-2014) accompanied by the microscope. The size of the parasites assessed by using ImageJ 1.51k (National Institute of Health, USA) in this study are given below:



A. Cyst of *Entamoeba* spp. (13x12 µm) under 40X magnification, after sedimentation technique.	B. Oocyst of *Cryptosporidium* spp. (6x5 µm), under 100X magnification, after sedimentation technique.
C. *Eimeria* spp. (26x18 µm) under 40X magnification, after saturated salt (45% w/v NaCl) flotation technique	D. Cyst of *Balantidium* spp. (71x70 µm) under 40X magnification, after sedimentation technique.

Figure 7: Microscopic images of the protozoa detected under objective lens of a compound microscope at Gram's iodine stain.



H. *Strongyloides* spp. (75x49 µm), after sedimentation technique	F. *Strongyle* spp. (78x43), after saturated salt (45% w/v NaCl) flotation technique
K. Egg of *Paramphistomum* spp (121x53 µm), after sedimentation technique.	G. *Strongyle* spp. (88x54µm), after saturated salt (45% w/v NaCl) flotation technique
I. Egg of *Eurytrema* spp. (37x26 µm), after sedimentation technique	J. Egg of *Oxyruid* spp. (40x24 µm), after sedimentation technique

Figure 8: Microscopic images of the helminths detected under 40X magnification of the objective lens of a compound microscope at Gram's iodine stain.

4.6 Extracted DNA quality

DNA isolation from stool samples is hampered by the inhibitory compounds that are present in feces, which include the degradation products of hemoglobin, bilirubin, bile

acids, and mineral ions. Frequently, stool samples can produce failed attempts to isolate DNA and false-negative amplification.

In this study, DNA from the 25 stools was extracted by using Qiagen Kit. PCR amplification was done for *Entamoeba* and *Eimeria* using specific primer and visualized under UV illumination. The purity and concentration of the extracted DNA from the sample was evaluated by Nanodrop and the result is shown in Table 4. The purity of the extracted DNA was recorded from the samples (with values of absorbance A260/A280 ratio, close and higher to 1.7-1.9).

4.7 Amplification of target gene

4.7.1. 18S region of *Entamoeba*

Of the 20 microscopy *Entamoeba* positive samples, all samples were successfully amplified and characterized the *Entamoeba* species based on its amplicons size using conventional PCR. The preliminary experiments evaluated the effectiveness of the primers JVF/DSPR2 targeted to amplify a conserved region of 18S rDNA of *Entamoeba* species, which produced amplified products of 550bp.

The success of the PCR amplification reaction was observed performing agarose gel electrophoresis. The amplified bands of DNA fragments in the gel were observed under UV illumination. The visible bands corresponded to the positive results. Negative control (reaction mixture with no template) and DNA ladder were used to compare size and validate the presence of target amplicons. A representative diagram of agarose gel electrophoresis of PCR amplification is shown in (Figure 8) showing confirmation of *Entamoeba* spp. For *Entamoeba* spp., the overall amplification efficiency was 100% among the 20 positive specimens.

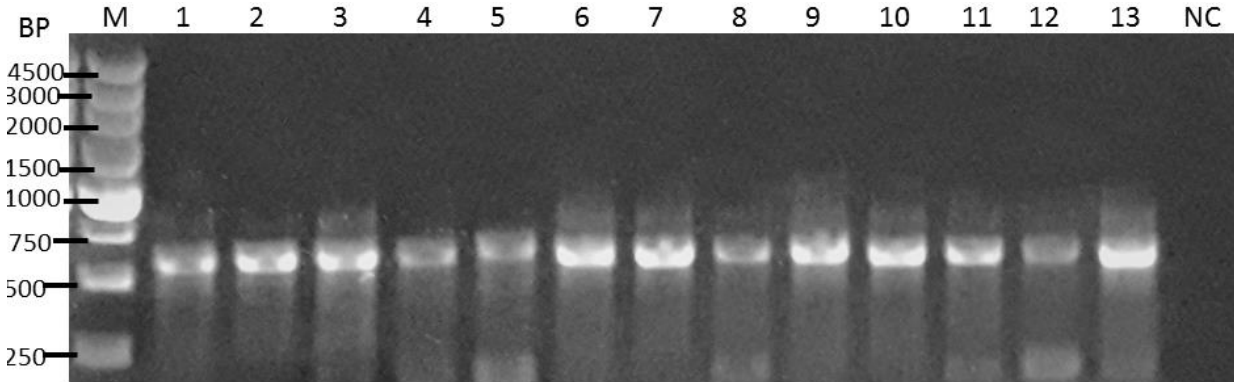


Figure 9: Visualization of PCR amplified DNA bands on agarose gel electrophoresis. PCR amplification of 18S region from ribosomal DNA of *Entamoeba* spp on 1.5% agarose gel. M: DNA ladder Takara Dye Plus of 250 bp; lane: 1-13 samples from wild buffaloes and NC: negative control.

4.7.2 18S and COI region of *Eimeria*

Every sample (n=2) that was *Eimeria* positive by microscopy showed successful PCR amplification of 18S region. The amplified bands of DNA fragments were observed under UV-illumination after performing agarose gel electrophoresis. Figure 10 shows the confirmations of amplification of 18S regions of ribosomal DNA around 1790 Bp by using the *Eimeria* specific primers ERIB1 and ERIB10.

The PCR amplifications for COI regions of *Eimeria* wasn't successful. For negative results, it was retested by optimization at different concentrations and different PCR conditions, and each was again found to be PCR negative. Amplification of 18S ribosomal regions of both the samples were successful but COI region of *Eimeria* was not successful.

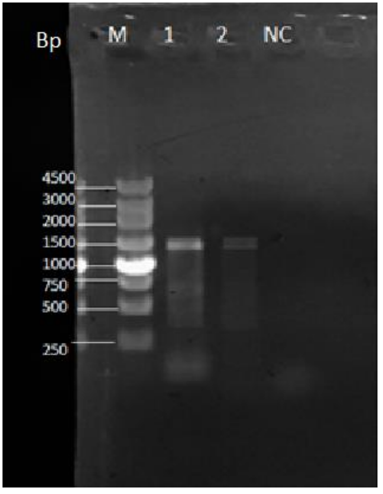


Figure 10: Visualization of PCR amplified DNA bands on agarose gel electrophoresis showing PCR products of 18S region from ribosomal DNA of *Eimeria* spp. M: DNA ladder Takara Dye Plus of 250 bp; lane 1 and 2 samples from wild buffaloes and NC: negative control.

4.8 Purified PCR amplicons after clean up

Successfully amplified PCR products were purified to avoid the chemical interference in sequencing by the EXOSap purification kit. After the reaction, the samples were loaded in 1% agarose gel to visualize the improved quality of DNA bands and also to compare the tentative intensity of DNA band per microliter of the sample.

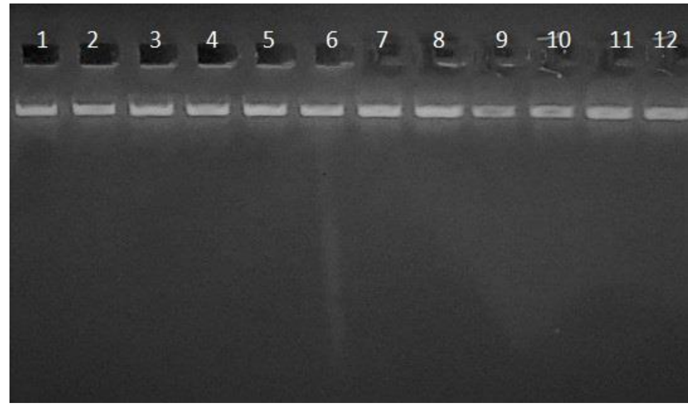


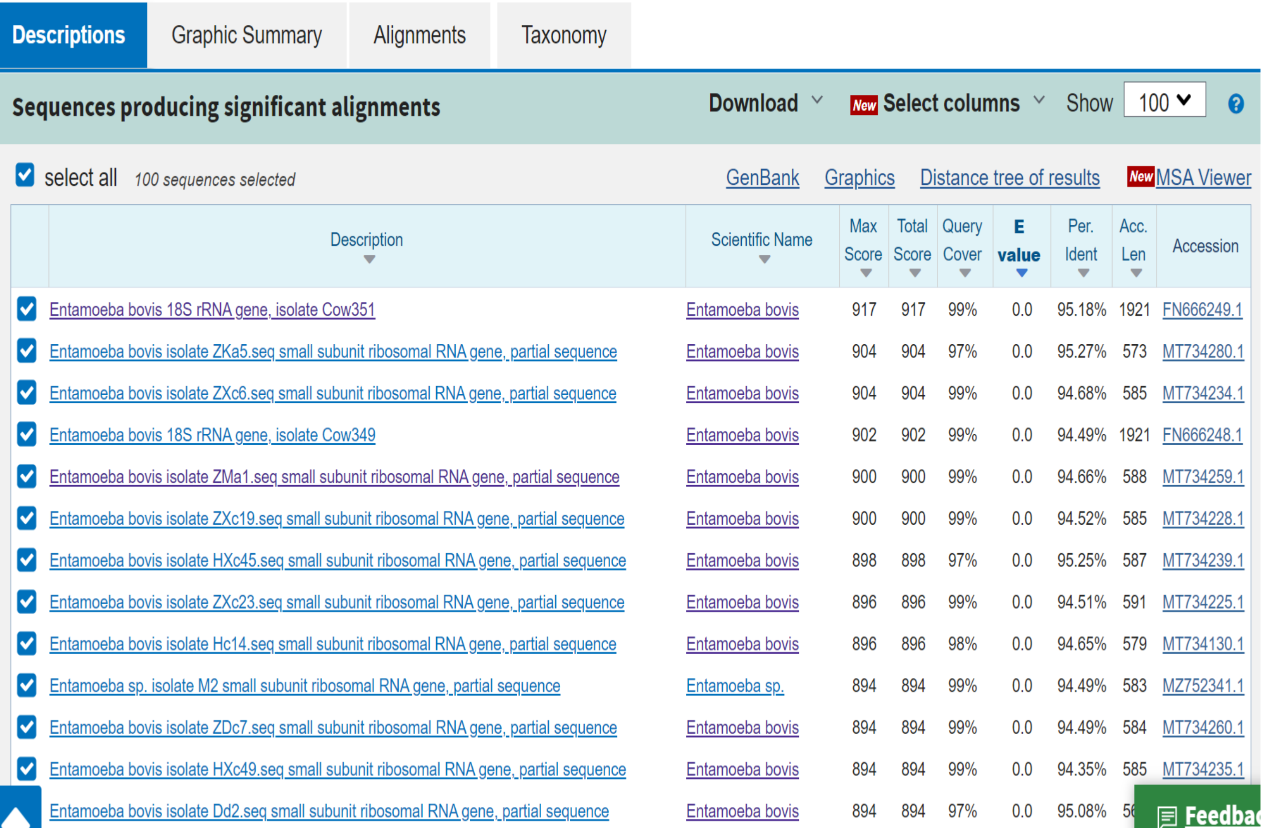
Figure 11: Visualization of bands of purified PCR amplicons after clean up in 1% agarose gel electrophoresis showing PCR products of 18S region from ribosomal DNA of *Entamoeba* spp. lane; 1 to 12 purified PCR products

4.9 Sequence Identification

After sequences comparison to sequences stored in GenBank, the program BLASTn revealed sequence similarities. The species of *Entamoeba bovis* was inferred from this comparison.

4.10 PCR and sequencing efficiency

18S region sequences (550 bp) were obtained from nine PCR positives. Phylogenetic analyses using NJ analyses produced similar results (Fig. 9) and grouped the 18S *Entamoeba* sequences -from Rangeland goats in a clade with *Entamoeba bovis* and were most closely related to an *E. bovis* isolate accession no. FN666249 from a Cow isolate from Sweden. Amongst all *E. bovis* isolates, the genetic similarity ranged from 93% to 95%.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments

select all 100 sequences selected

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Entamoeba bovis 18S rRNA gene, isolate Cow351	Entamoeba bovis	917	917	99%	0.0	95.18%	1921	FN666249.1
Entamoeba bovis isolate ZKa5.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	904	904	97%	0.0	95.27%	573	MT734280.1
Entamoeba bovis isolate ZXc6.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	904	904	99%	0.0	94.68%	585	MT734234.1
Entamoeba bovis 18S rRNA gene, isolate Cow349	Entamoeba bovis	902	902	99%	0.0	94.49%	1921	FN666248.1
Entamoeba bovis isolate ZMa1.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	900	900	99%	0.0	94.66%	588	MT734259.1
Entamoeba bovis isolate ZXc19.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	900	900	99%	0.0	94.52%	585	MT734228.1
Entamoeba bovis isolate HXc45.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	898	898	97%	0.0	95.25%	587	MT734239.1
Entamoeba bovis isolate ZXc23.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	896	896	99%	0.0	94.51%	591	MT734225.1
Entamoeba bovis isolate Hc14.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	896	896	98%	0.0	94.65%	579	MT734130.1
Entamoeba sp. isolate M2 small subunit ribosomal RNA gene, partial sequence	Entamoeba sp.	894	894	99%	0.0	94.49%	583	MZ752341.1
Entamoeba bovis isolate ZDc7.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	894	894	99%	0.0	94.49%	584	MT734260.1
Entamoeba bovis isolate HXc49.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	894	894	99%	0.0	94.35%	585	MT734235.1
Entamoeba bovis isolate Dd2.seq small subunit ribosomal RNA gene, partial sequence	Entamoeba bovis	894	894	97%	0.0	95.08%	5[illegible]	[illegible]

Fig 12: Sequences producing significant alignments showing genetic similarity ranged from 93% to 95%

4.11 Nucleotide composition of *Entamoeba*

Nucleotide composition provided the idea about the composition of A, T, G, and C in terms of their percentage of total length. The computation by using MEGA X showed average composition of A was 31%, T was 25.5%, G was 25% and C was 18.5%.

4.12 Sequence alignment and interface

The raw forward and reverse sequences (chromatogram) were carefully checked and aligned in Sequencer 4.1.4. Following the inspection, all sequences were aligned and sorted in MEGA X. In a second control step, all unique positions in the alignment were rechecked in the corresponding chromatograms. For the phylogenetic analysis, GenBank sequences for *Entamoeba* species for which the whole sets of 18S rDNAs were available (Additional) were included. Sequences of raw genomes were retrieved by performing BLAST searches against the “whole-genome shotgun contigs” database of NCBI GenBank.

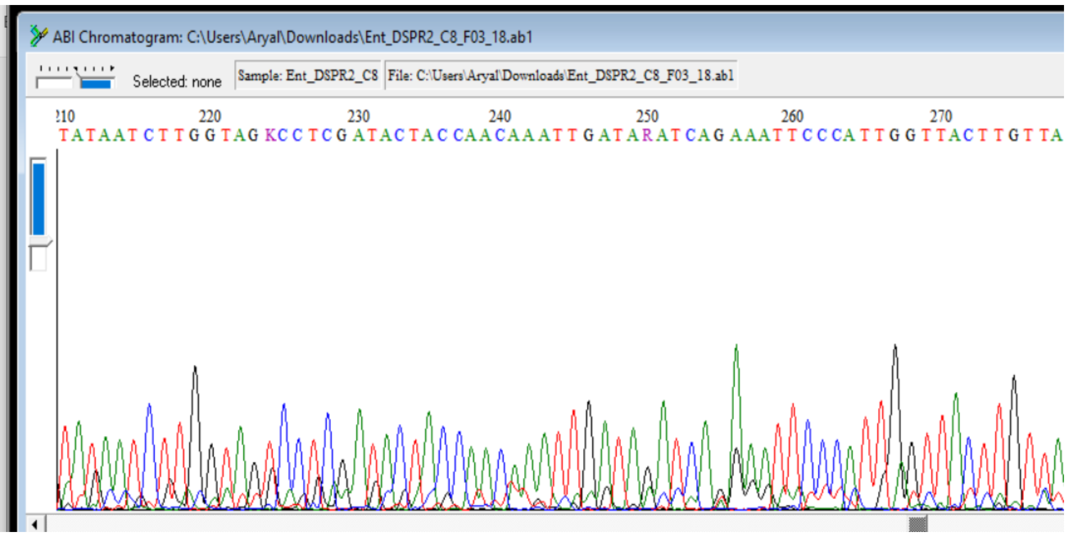


Figure 13: The raw reverse sequence of sample 8 chromatogram of 18S rRNA gene from the isolate in Wild Water buffalo of CNP.

4.13 Phylogenetic interface

18S sequences (550 bp) were obtained from all nine PCR positives samples. Phylogenetic analyses of three contig sequence using NJ analyses produced similar results (Fig.9) and grouped the 18S *Entamoeba* sequences from wild buffalo in a clade with *Entamoeba bovis* and were most closely related to *Entamoeba bovis* accession no. FN666252 from Reindeer of Iceland and *Entamoeba bovis* accession no. MT734128 from free-ranging Yak of China . Amongst all *E. bovis* isolates, the genetic similarity ranged from 93% to 95%.

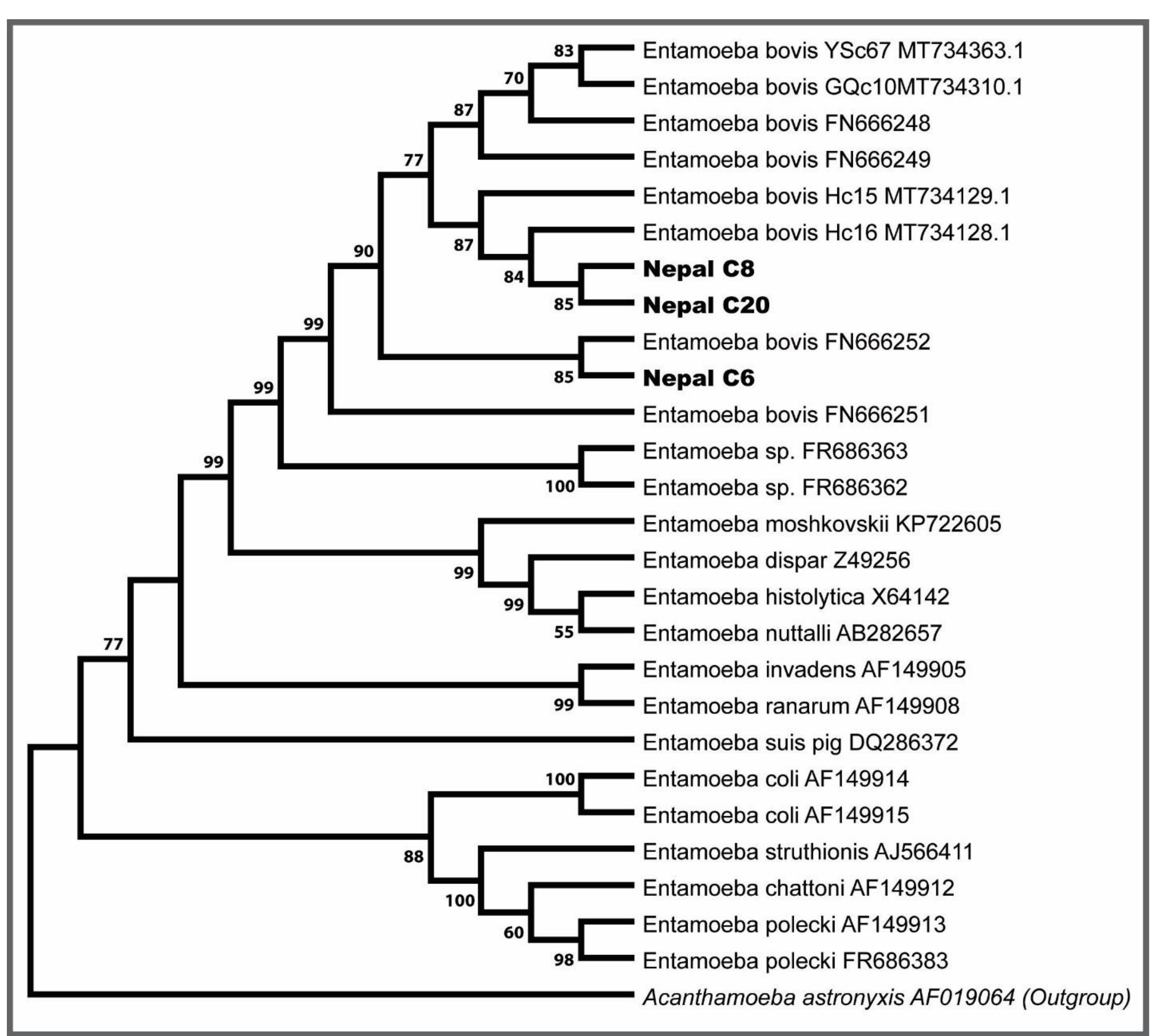


Figure 14: A Neighbor-Joining of 18S region of *Entamoeba* showing the relationship between individuals *Entamoeba* genus

Phylogram of *Entamoeba* spp., including isolates by using MEGA X is shown in the present study. Isolates from the present study were found to be closely related to *E. bovis*, as inferred using the neighbor-joining method generated from partial 18S rDNA gene sequences. The evolutionary distances were computed using the p-distance method. All ambiguous positions were removed for each sequence pair (pairwise deletion option).Accession numbers of samples are shown in parentheses following the species name. Scale bar represents substitutions per nucleotide, and numbers below the branches indicate bootstrap values (> 1000).

Phylogenetic relationships between the *Entamoeba* spp. identified in this study and reference strains were analyzed by sequence alignment. The results showed that *E. bovis* has the closest evolutionary relationship with *Entamoeba bovis* accession no. FN666252 with bootstrap value 85 and *Entamoeba bovis* accession no. MT734128 bootstrap value 84, which together form a monophyletic branch.

CHAPTER 5. DISCUSSION

GI infections in water buffalo

In the present study, a high proportion of animals were recorded positive (96%) for GI infections, showing the susceptibility of buffaloes to the different GI parasites infections. The high prevalence of *Entamoeba* infection may be due to the transmission via a cyst stage through contact with infected feces and pathogenesis. In this study, cestode was not detected which is supported by Roy *et* al. 2016; Saha *et* al. 2013 and Mamun *et* al., 2011 who reported no cestode in their studies. This is not startling because cestodes of buffaloes are rare in recent years (Liu *et* al; 2009). Nutrition and the condition of an animal can have a major impact on the resistance and resilience of ruminant species to GIT nematodes (Coop and Kyriazakis, 2001). The high liability of water buffaloes to be infected with GI parasites could also be attributed to factors like the level of host immunity, and the stage of parasite infection. Other inherent Marsh-specific ecological factors include moisture, humidity, vegetation, soil, and animal interactions (Gunathilaka *et* al., 2018). Wild buffalo naturally are nomadic, but the wild buffaloes of CNP are enclosed in a fence. So, the constant stress of captivity makes animals more susceptible to parasitic infection as the immune system of these captive animals becomes weak (Gracenea *et* al. 2002; Cordon *et* al. 2008). Some of the translocated Wild Water buffaloes died due to parasitic diseases, consequences of a severe flood, and tiger attacks. A previous study detected ungulate malaria parasites in Wild water buffaloes for the first time from Nepal (Kandel *et* al., 2019). In the wild, animals might have a natural resistance against parasitic infections or board a balanced system with their parasites. But the change in environment and living conditions influences the animal's ecology and might enhance the sensitivity for parasitic infections (Goossensa *et* al., 2005).

There were no particular criteria associated with the Wild Water buffalo selected for the fecal collection. Fresh feces were collected off the ground in a yard where a herd of buffalo was grazing. Although we did not see them defecating, it is not difficult to suspect that the samples were derived from the wild buffalo but not from other animals. Therefore, it is thought that most of the samples collected were derived from different individuals.

DNA extraction and Molecular characterization

Stool encompass serious PCR inhibitor materials, a number are known to us and a few are unknown; for reducing this risk, a DNA extraction kit was used where the specific tablets (InhibitEX) were used for removing PCR inhibitors. The degrees of restraint of the PCR by fecal components showing various physico-chemical properties can introduce bias in the DNA extraction process, affecting the quality and the quantity of the DNA obtained, thereby curbing the sensitivity of molecular studies. The DNA extraction protocols used for stool usually include from one to each of the three of the subsequent basic elements: physical disruption, chemical lysis, and enzymatic lysis (Sambrook *et* al. 1989). In our experiment, DNA extraction of parasites from stool samples with lysis mixtures yielded quantifiable DNA. The Qiagen kit protocol succeeded to obtain a parasite DNA extract that could be amplified by PCR.

The purity of DNA of extracts from the Qiagen kit method ranged from about -0.187 nm to 2.17 nm (Table 4). This could probably be related to both, the variable abundance of parasites in a sample and to the different contributions of DNA associated with the size of parasites in each condition of sample collection. This wide range of DNA yield confirms the efficiency of the Qiagen kit extraction method for a variable abundance of DNA in stool samples. In some samples A260/A280 ratio for purified nucleic acids was low which may be due to inefficient elimination of inhibitory substances due to insufficient mixing with InhibitEX matrix or decreased proteinase activity or insufficient mixing with Buffer AL.

Sequence interpretation

Some of our chromatogram was noisy which could be due to multiple strains of parasites in the sample and cloning could be helpful to seperate them.

Sequence comparison by BLAST for 18S locus disclosed that the *Entamoeba* isolated from Wild Water buffalo in the current study shared 95% similarity with *E. bovis* accession no. FN666249 from a Cow315 in Sweden. Among the distinguished *E. bovis* 18S sequences available in GenBank, the genetic similarity ranged between 93% to 95% .

Phylogeny of *Entamoeba*

Phylogenetic analysis was conducted to assess the relationships of the sequences from the current study to those available from public databases for species or genotypes of *Entamoeba*. Phylogenetic analysis was used to provide unequivocal support for the classification of species and genotypes of *Entamoeba*.

We did not comprise the species-specific PCR for *E. bovis* into our experiment. Nonetheless, using genus-specific PCR followed by sequencing, we recognized numerous samples that were positive for *E. bovis*. Our sequences branched within the well-bolstered lineage of *Entamoeba bovis*, which included isolates from Reindeer in Iceland (FN666252).

Multiple intestinal protozoa and helminthic infections

Buffaloes are raised on meadow with high humidity which favors the growth and multiplication of parasites as well as their vectors.

Protozoan infectivity to water buffalo

The main objective of this investigation was to identify a region in the *Entamoeba* genome that could be used as a target for a PCR to amplify all species found in Water buffalo feces. To address this goal, we amplified and sequenced a fragment of approximately 550 bp, corresponding to the 18S rDNA gene of *Entamoeba* species from 24 DNA samples. The findings of this study confirmed a trend of high risk of infection with *Entamoeba* species among wild buffalo of CNP. The source of infection could also be from the consumption of water contaminated with *Entamoeba* cysts from feces animals as they come to the river bank to drink and at the same time defecate in or near the river. Many investigations have reported the successful application of PCR to the diagnosis of amebiasis, a tool for confirmatory identification (Kebede *et* al., 2004; Santos *et* al., 2010). The PCR assay provided discriminatory power in detecting and differentiating *Entamoeba* species, even when a considerable number of mixed infections were observed. In this study, we used stool samples positive by microscopy, PCR followed by DNA sequencing analysis. There was 100% agreement between the results of these tests and the identification derived by the comparative DNA sequencing analysis, microscopy, and PCR combined, thus yielding an assay specificity of 100%. Our molecular technique showed that *E. bovis* (80%) was

found to be the most common species detected in this study. The reason is due to the use of contaminated water or using the same areas for grazing which facilitates the transmission of parasites themselves. The first study by Refaii, 1993 recorded *Entamoeba bovis* infection in large ruminants in Egypt with a percentage of about 85% in cattle, and 80% among buffaloes. Another study recording the presence of *Entamoeba bovis* in calf samples and sheep by (28.5%; 14.2%) (Ghaidaa and Musafer 2015). Goz *et* al., 2006 reported *Entamoeba* spp was the least common parasite causing diarrhea in young calves, only 6.4% of calves had *Entamoeba* cyst and trophozoites. Newton *et* al., (1990) first reported *E. bovis* like organisms intruding and causing pathological changes in the tissues of their host. So far, *E. bovis* has been reported in several countries, namely, Egypt from large ruminants (El-Refaii A.H.1993), 2.5% and 1.1% of dairy and beef cattle from Costa Rica (Jiménez *et* al., 2007), 4.8% (alluded to as *Entamoeba* spp.) of beef cattle from Korea, only based on morphology (Jiménez *et* al. 2007; Ismail *et* al. 2010), Iraq (Al-shabbani, 2016), Australia (Al-Habsi *et* al., 2017), Japan (Matsubayashi *et* al., 2017), Uganda (Nolan *et* al., 2017), Iran (Tavassoli *et* al., 2018) and China (Ren *et* al., 2020). *E. bovis* infection was detected in all (100 %) fecal samples of cattle, 97.2 % of yak, 96.8 % of goats, 96.7 % of Tibetan sheep, 87.5 % of camels, 87.2 % of Mongolian sheep, and 37.5 % of the horse, indicating that ruminants would be more susceptible to *E. bovis* than monogastric herbivores (Ai *et* al., 2021).

Entamoeba with a prevalence (80%) among buffaloes that considered same to our ratios was reported in Mohammed (2017). Several uninucleate cyst-producing *Entamoeba* species are reported from ruminants, like cattle and sheep, and identified based on morphological findings and the host within which the parasites were detected, e.g., *E. bovis* from cattle and *E. ovis* from sheep (Noble and Noble 1952; Jacob *et* al., 1990; Hampton *et* al., 2006; Stensvold *et* al., 2010; Stensvold *et* al., 2011). In the present study, all successfully amplified and sequenced isolates were recognized as *E. bovis*, which encompassed some previously reported sequence types in the *E. bovis* cluster (Nolan *et* al. 2017; Matsyubashi *et* al., 2017). A uninucleate *E. bovis*-like *Entamoeba* sp. was identified from rangeland goat (Al-Habsi *et* al., 2017) and yaks in Qinghai province, China (Ren *et* al., 2020). More recently, *E. bovis* isolated from cattle and goat included subtypes containing nucleotide substitutions compared to reference strains in the GenBank database (Nolan *et* al. 2017). Several other *E. bovis* hosts have been found, including Bay

duiker (Bray, 1964, white-tailed deer (Kingston and Stabler 1978), reindeer (Stensvold *et* al., 2011), and kangaroo (Jacob *et* al., 2015).

The prevalence of *Entamoeba* noted in our study was higher than that observed in cattle from Japan (72 %) and equal to prevalence in Uganda (80 %). The samples used in these two studies were collected from farms where animals thrived in a small area (Nolan *et* al., 2017; Matsubayashi *et* al., 2017) and those collected in our study were from Wild buffalo raised in free-range, explaining the differences in results. In free-ranging yak, the prevalence was (76.14 %) in China (Ren *et* al., 2020). The percentages contrast depends on several of causes, like climatic conditions, geographic determination, nature of the places, the degree of the contamination by the causative agent, the parasites spread irregularly spreading in most countries. *Entamoeba bovis* is an important cause of enteric infection in 38% of calves in Iraq (Al-shabbani, 2016). Our result showed a discrepancy with Jacob *et* al (2015), which said there was low or no *Entamoeba* positivity in wild animals, even in wild ungulates.

According to Ai *et* al., (2021) the farm animals that were identified positive for *E. bovis* infection remained asymptomatic and sound. There are no sufficient studies remember for *Entamoeba bovis* infection in the calf. In general, *Entamoeba* infections are asymptomatic, and some calves develop diarrhea or dysentery (Al-shabbani, 2016). The high prevalence of *Entamoeba* infection might be because of the transmission and pathogenesis as well as other risk factors which favor the persistence of this infection. Given its fecal-oral route, habits related to eating, defecation may have an impact on the prevalence rates.

The prevalence of *Eimeria* was detected 8% microscopically in our study. The other studies reported higher infection rate by *Eimeria* spp than our study. The total infection rate was 35% in wild buffalo of KTWR (Gupta, 2017); 28% in water buffalo of Egypt (El-Alfy *et* al 2019); 53.8% in buffalo of Iraq (Sabbar *et* al., 2020); 81.5% in water buffalo farms (Morgoglione *et* al., 2020).

The preliminary experiments evaluated the effectiveness of the primers ERIB1/ERIB10 targeted to amplify a conserved region of 18S rDNA of *Eimeria* species, which produced amplified products around 1790bp bands which was visualized in UV trans illuminator. The comparatively conserved nature of the nuclear 18S rDNA locus has permitted

evolutionary relationships among many taxonomic groups in the Apicomplexa, including *Eimeria* spp., to be inferred successfully using 18S rDNA sequences (Barta *et* al. 1997). *Eimeria* genomes most commonly have been sampled by sequencing the 18S rDNA (Schwarz *et* al., 2009). Complete 18S ribosomal RNA gene sequences were determined for for *Eimeria bovis* of cattle (Barta *et* al., 1997).

But *Eimeria* positive samples weren't amplified by PCR for mtCOI regions. Our results showed discrepancy with El-Sherry *et* al.,2013; Ogedengbe *et* al., 2011 which stated Cytochrome c oxidase I (COI) partial sequences are more reliable species-level molecular markers than 18S rDNA for *Eimeria*. The sample was retested by PCR, each was again found to be negative. Some pitfalls complicate the reaction producing spurious results. Careful troubleshooting was employed to sort out and solve the problem. However, the amplification of most target sequences, and strategies for optimizing a reaction failed to produce result in *Eimeria*. Further study is required to increase PCR sensitivity so that the samples which were detected by microscopy but not PCR may belong to other *Eimeria* species. Therefore, a more sensitive method such as Real-Time PCR are to be considered in a future study.

Cases of 60 % cryptosporidiosis have been detected in water buffalos. However, the parasite was not characterized at the molecular level in this study. Since water buffalo belongs to the bovine family, the *Cryptosporidium* species identified may be expected to be *C. parvum, C. andersoni,* or another genotype closely related to these. However, the molecular pattern of the *Cryptosporidium* isolates detected was distinct from the patterns generated by these species. According to Abeywardena *et* al., 2014, *Cryptosporidium* is present in water buffalo in Sri Lanka. The zoonotic *Cryptosporidium* spp. are usually associated with intestinal pathology, resulting in diarrhea in both humans and animals (Ryan and Hijjawi, 2015). Chalise, (2013) reported *Cryptosporidium* in wild buffaloes of KTWR.

The ciliates of the genus *Balantidium* (Vestibuliferida: Balantidiidae) are intestinal parasites of a broad range of animals. *Balantidium* is also transmitted through fecal-oral route infection via contaminated pasture (Schuster and Ramirez-avila 2008). In this study, 4% prevalence of *Balantidium* was observed microscopically. Among the protozoan diseases balantidiasis caused by *Balantidium coli*, is a common disease of ruminants

(cattle, buffaloes, sheep and goats), guinea pig, man (Roy et al., 1970), and in ostrich (Ponce-Gordo *et* al., 2008). Bilal *et* al., (2009) reported 25% prevalence of *B. coli* in cattle in Pakistan. Palanivel et al., (2005) reported 45.45% and 51.43% prevalence of *B. coli* in cattle and buffaloes respectively.

Trematode infectivity

Wild buffalo may acquire the infections through contaminated food or water as most of these GI parasite eggs, cysts, and larvae are related to pasture. Digenetic trematodes like *Paramphistomum* have indirect life cycles where a snail play as an intermediate host of parasite who associates with water bodies. Snails usually play a dominant role in the ecology of freshwaters by providing food for many other animals and by grazing on vast amounts of algae and detritus (debris)(Johnson, 2005). Cercariae of these trematodes encyst on vegetation where herbivores feed (Sepalage and Rajakaruna, 2020). The mean prevalence of *Paramphistomum* infection in water buffaloes in this study is 4% (Fig. 3) and it was almost two times lower than that found by Kobak and Pilarczyk, 2012 and in the Wielkopolska Province in Poland (11%).

Nematode infectivity

Strongyloides sp., though is not considered an important parasite in adult animals, is commonly found in young animals, chiefly those animals younger than 6 months of age (Barbosa *et* al. 1992) generally causes minor health problems but it much affects the growth and productivity of the animals (Deepak et al., 2020). *Strongyloides* prevalence was 28% in this study which could be due to a more conducive environment for the development of the pre parasites stages in the hot and humid environmental condition of this region.

Strongyle parasites are ubiquitous nematodes of grazing livestock. A single egg encompasses different developmental stages and, hence, different amounts of DNA. (Anderson et al., 2013). The anticipation of water buffalo would harbour the *Strongyle*-type nematode species that are common in other livestock ruminant livestock systems, and at similar levels (William et al 2019). It is the second most prevalent (36%) helminthes found in our study. *Strongyle* eggs have been previously reported in wild Buffalo of Africa by Budischak *et* al. (2012).

Oxyurid has the highest prevalence (68%) among the helminths in this study. The prevalence of *Oxyurid* is higher than found in Koshi Tappu Wildlife Reserve in Wild Water buffalo (Gupta, 2017). According to Mohammed *et* al., (2020) prevalence of *Oxyurid* was 36% in horses and 22.4% in the donkey. Altogether five helminths in Wild buffalo *Oxyurid* sp. showed a 68 % prevalence rate which was higher than 58.14% recorded by Shrestha et al. (2015) in Red Panda. The highest prevalence rate of *Oxyurid* may be due to the infection intensity, environmental contamination with infective eggs . (Koehler et al., 2014) and cool climate of the area which engender suitable environment for the development of *Oxyurid* larva.

Multiple infectivity

Nine species of gastro-intestinal parasites were identified in our study. Similar to our study, the presence of nematodes and trematodes but absence of cestode was found in buffalo of Bangladesh (Mamun *et* al., 2011; Roy *et* al., 2016). Sreedevi and Hafeez, (2014) reported ten GIT parasites among which seven species were helminthes in buffalos from India. Consistent to the other studies in wild buffalo, mixed infection involving more than one protozoa and helminths genera was common in this survey and is in agreement with the results of Senyael *et* al., (2013) from Africa, Gupta, (2017) from Nepal. Mixed infection by gastrointestinal parasites were found in buffalo calf in Nepal (Adhikari and Ghimire, 2021), in Pakistan (Hussain *et* al., 1996; Bilal *et* al., 2009). Our study showed discrepancy with Marskole *et* al., (2016) which had more common single parasitic infections than mixed infections. Multiple infections in buffalo by helminths and protozoan were studied in buffalo previously by Gorsich *et* al., 2014 and Kalkal *et* al., 2020. Coccidia prevalence, coccidia intensity, and nematode prevalence were sensitive to factors that influence host immunity (Gorsich *et* al., 2014).

CHAPTER 6. CONCLUSION

This study revealed the prevalence of multiple GIT parasites infection in wild buffalo at CNP. The intensity of infestation by GIT coccidiosis and helminths also varies from no to heavy infestation. The high prevalence encountered may be explained by the existence of favorable climate condition which supports prolonged survival of infective helminths on pasture. For the detection of parasites in this research, the basic microscopy technique has been used, and molecular methods have constituted the tool for further identification. In this study, we have discussed the extraction of DNA from fecal samples and some modified steps.

In the present study, an uninucleated *E. bovis* was identified from Wild Water buffalo feces. In addition, for the first time, an *E. bovis* sequence was produced in wild buffalo of CNP, which will facilitate future phylogenetic analysis of *Entamoeba* in ruminants and other ungulates. The current survey and phylogenetic reconstructions have further expanded our knowledge of the diversity and host range of *Entamoeba* species. The clinical impact of the *E. bovis* isolates in wild buffalo is unknown. Therefore, *E. bovis* may be less pathogenic, and without being cured, the infection remains for a long time. The success was achieved for amplification of 18S region of *Eimeria.* But the amplification of mtCOI region wasn't successful. The presented conventional PCR showed an acceptable sensitivity and specificity for the detection of *Entamoeba* in fecal samples and encouraged us to believe molecular identification as a practical approach in the routine diagnostic laboratory stings for parasitic infections. However, whether the pathogenicity of *Entamoeba* depends on the severity of the infection, the presence of a mixed infection with other pathogens, and the health status of the host, remains unclear. Explanation of these issues will need further detailed genetic research and epidemiologic surveys. However, little is understood about the aggravation of gastrointestinal side effects by blended infection of ruminant parasites with different pathogens, like viruses or bacteria.

As the cysts can survive for a long time after being shed from the hosts, in contrast to trophozoites, the transmission of parasites may happen through cyst-contaminated environments. Thus, we must include the environment as well as soil and water for the survey to evaluate the distribution of parasites in the conservation areas. Therefore,

serious and unwavering preventive measures should be taken into consideration for the control of gastrointestinal parasites to augment the health of buffaloes.

The presence of parasites infection, in this study, should be an indication for devising an appropriate parasite control program. This baseline parasitological data provides important information for the elimination of such parasites. Based on the prevalence of gastrointestinal parasites and administration of desired anti helminths to the captive wild animals periodically coupled with better sanitary measures, we would be able to reduce the parasitic infection in the National Park. Wild animals are also vulnerable to deadly or debilitating pathogens, and coinfections can exacerbate clinical malady. Wild buffalo at CNP were exposed to parasites including some that are known to be pathogenic; however, most of the animals were in good physical condition. Parasitic prevalence is an important parameter to monitor the health of free-ranging Wild Water buffalo. Our findings may give a basis for assessing the effectiveness of future control strategies against parasitic infections in water buffalo populations.

RECOMMENDATION

Based on the outcome of the present study, the following recommendations have been made to reduce the risk of intestinal parasitic threat in the conservation of wild buffalo (*Bubalus arnee*). This study reports gastrointestinal infection in Wild Water buffalo in Nepal. Because of this, the correct management of wildlife parasitism requires this information to develop control and management strategies. Therefore,

- Periodical monitoring of parasitic species among wild animals would be beneficial to control and manage diseases at the early stages of infections in wildlife conservation practice.
- Proper pasture management programs should be conducted on CNP for the parasites' control.
- A detailed study on seasonal, age and, the sex-wise prevalence of intestinal parasites of Wild Water buffaloes should be carried out.
- The wildlife deworming programs together with screening and treatment of intestinal infections should be conducted.

Further research

Further study is needed to determine the virulence of parasites in wild buffalos of CNP. As nothing is known about the zoonotic/zooanthroponotic capabilities of the corresponding parasites, future work should emphasize on wider scale. This study recommends further studies on;

i. Intestinal parasitic infection rates assessing the seasonality of transmission.
ii. Analysis of water for cysts and/or helminths eggs to assess its safety
iii. Epidemiological investigations along with continued surveillance of parasites in humans, other mammals, the environment, and water in this mainly impoverished area.

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APPENDICES

Table 4: Nanodrop Reading of DNA concentration of the samples studied

Sample ID	Sample Type	Purity 260/280	Conc. (ng/µl)
1	dsDNA	1.71	4.95
2	dsDNA	2.00	24.0
3	dsDNA	2.17	15.0
4	dsDNA	0.722	-0.65
5	dsDNA	1.48	-6.35
6	dsDNA	1.69	-4.05
7	dsDNA	2.05	-1.35
8	dsDNA	1.80	5.05
9	dsDNA	-0.187	0.15
10	dsDNA	1.32	-4.75
11	dsDNA	1.14	-3.65
12	dsDNA	1.71	-5.65
13	dsDNA	1.68	-5.55
14	dsDNA	1.66	-2.65
15	dsDNA	1.76	5.10
16	dsDNA	1.57	6.90
17	dsDNA	0.50	-0.20
18	dsDNA	1.45	4.50
19	dsDNA	2.42	8.00
20	dsDNA	1.47	10.9
21	dsDNA	1.26	-12
22	dsDNA	1.22	3.30
23	dsDNA	1.57	3.60
24	dsDNA	-0.50	0.10
25	dsDNA	1.44	-1.30

PCR and Sequencing details of DNA samples

PCR reaction mixture

Table 5: PCR reaction mixture for 18S locus amplification from ribosomal DNA for *Entamoeba*

Components	Volume in µl	Final concentration
Master Mix (2X)	5	1X
Forward primer (JVF) (10 pmoles)	0.4	0.4 pmoles
Reverse Primer (DSPR2) (10 pmoles)	0.4	0.4 pmoles
Template	1	
NFW	3.2	
Total	10	

Table 6: PCR reaction mixture for 18S locus amplification from ribosomal DNA for *Eimeria*

Components	Volume in µl	Final concentration
Master Mix (2X)	5	1X
Taq polymerase (5U/ µl)	0.05	0.25U/ µl *
Magnesium chloride (25mM)	0.2	1Mm *
Forward primer (ERIB1) (10 pmoles)	0.4	0.4 pmoles
Reverse Primer (ERIB10) (10 pmoles)	0.4	0.4 pmoles
Template	1.5	
NFW	2.45	
Total	10	

Table 7: PCR reaction mixture for COI region amplification from mitochondrial DNA for *Eimeria*

Components	Volume in µl	Final concentration
Master Mix (2X)	5	1X
Taq polymerase (5U/ µl)	0.05	0.25U/ µl *
Magnesium chloride (25mM)	0.2	1Mm *
Forward primer (KM204) (10 pmoles)	0.4	0.4 pmoles
Reverse Primer (KM205) (10 pmoles)	0.4	0.4 pmoles
Template	1.5	
NFW	2.45	
Total	10	

Note: * added for those samples not amplified by basic component

Table 8: PCR condition for amplification of 18S locus of *Entamoeba*

Steps	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**	**Hold**
Cycles	1	40			1	1
Temperature	95 °C	95 °C	57 °C	72 °C	72 °C	4 °C
Time	5mins	30sec	30sec	30sec	2mins	∞

Table 9: PCR condition for amplification of 18S locus of *Eimeria*

Steps	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**	**Hold**
Cycles	1	35			1	1
Temperature	0	94°C	55°C	72°C	0	4°C
Time	0	1min	1min 30sec	2min	0	∞

Table 10: PCR condition for amplification of COI locus of *Eimeria*

Steps	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**	**Hold**
Cycles	1	40			1	1
Temperature	95°C	95°C	59°C	72°C	72°C	4°C
Time	7mins	30sec	30sec	1min	10mins	∞

Scientific Classification of *Bubalus arnee*

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Mammalia
Order	-	Artiodactyla
Family	-	Bovidae
Genus	-	*Bubalus*
Species	-	*B. arnee*

Scientific Classification of *Entamoeba bovis (Liebetanz, 1905)*

Kingdom	-	Protista
Phylum	-	Amoebozoa
Class	-	Archamoebae
Family	-	Entamoebidae
Genus	-	*Entamoeba*
Species	-	*bovis*