Molecular and Histopathological detection of Cryptosporidiosis in calves in Chittagong

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Dedication

To my Family members
who always valued education
above everything else.

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(Md. Anwar Pavez)

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

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The Author
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS USED

Abbreviations and Symbol	Elaboration
CVASU	Chittagong Veterinary & Animal Sciences University
DPP	Department of Pathology and Parasitology
˚C	Degree Celsius
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide phosphates
e.g.	Example
et al.	And his associates
etc.	Et cetera
H & E	Hematoxylin and Eosin
HIV	Human immunodeficiency virus
hrs	Hours
µg	Microgram
mg	Milligram
ml	Milliliter
mAb	Monoclonal antibody
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
%	Percent
/	Per
rpm	Rotation per minute
rRNA	Ribosomal RNA
UV	Ultra-Violet
US	United States
UK	United Kingdom
www	World Wide Web
Z-N	Ziehl-Neelsen
Fig.	Figure

ABSTRACT

Cryptosporidium is an intestinal protozoan parasite of livestock and human and cause cryptosporidiosis which is also a common disease of cattle especially neonatal calves. Cryptosporidiosis is commonly asymptomatic but sometimes associated with persistent diarrhea in calves. The aim of the present study was to carry out pathological and molecular investigation of *cryptosporidium* infection in calves. The study was conducted during June, 2015 to January, 2016. During the study period fecal sample were collected from 160 calves with less than 45 days age from a total of 50 dairy and beef farms located in Chittagong Metropolitan Area and Patiya Upazilla of Chittagong when 5 dead calves with a history of diarrheal illness were also examined for gross and histopathological examination. The fecal samples were first screened by modified Ziehl-Neelsen (Z-N) staining technique for the detection of cryptosporidium oocysts under light microscope which is characterized by pink to bright red spherical bodies and later confirmed by polymerase chain reaction (PCR) using 18s rRNA gene. Z-N positive fecal samples were used for DNA extraction *Cryptosporidium* spp. was confirmed by amplifying 18srRNA gene primer. The positive samples yielded 580bp band with the use of 1% agarose gel electrophoresis for PCR amplified products. The overall prevalence of Cryptosporidiosis in crossbred calves was 9.37% (15/160) by Z-N techniques and 1.88% (3/160) by nested PCR amplification which is statistically significant ($p<0.05$). The prevalence of Cryptosporidium was also significantly higher in diarrhoeic calves (42.85%) than non diarrhoeic (7.9%) calves. During necropsy examination bad odors, bloody mucous membrane with fluid filled watery fecal contents were found in two dead calves. For histopathological examination tissue samples were collected from dead calves and preserved in 10% buffered formalin. After tissue processing and routine H&E staining of intestinal tissue section revealed atrophied villi and sloughing of mucosa with reactive cellular infiltration. It is evident that the prevalence of cryptosporidiosis in bovine calves in these areas is under diagnosed and the clinical status of infection was not potentially high. It can be concluded that *Cryptosporidium* spp. infection is prevalent in Bangladesh and adult cattle could serve as reservoirs for the zoonotic infection in humans.

Keywords: Cryptosporidium, Z-N techniques, PCR, Histopathology, Calves

CHAPTER I
INTRODUCTION

Bangladesh is growing in the field of livestock and facing a lot of infectious as well as non-infectious threats. Most of the livestock is not producing to the highest enough as in developed countries. Major involvement for this lowered performance is of different parasitic infections. These infections are causing huge economic losses to livestock industry in Bangladesh. As an agriculture-based developing country with approximately 54.35 million livestock that includes 23.37 million cattle in Bangladesh. In current fiscal year livestock sector contributes 1.66 % to the GDP (BER, 2016). The main target of livestock sector is to increase supply of animal protein through boosting of calf production. Calf is the vital sources of demanding animal protein of livestock sector. Calf diarrhea causes more financial loss to cow-calf producers than any other disease-related problem they encounter. Acute diarrhea has been recognized as one of the foremost causes of death in neonates of animal and human globally (Estes and Kapikian, 2007; Dhama et al., 2009). Recently, a report showed that the mortality rate (53.4%) for dairy calves due to calf diarrhea in Korea (Hur et al., 2013). A number of infectious (bacteria, viruses, parasites) and non-infectious factors cause diarrhoea in neonatal calves. Previous studies show that the most important infectious agents *Cryptosporidium* spp. either singly or in combination (Snodgrass et al., 1986; Waltner-Toews et al., 1986; Steiner et al., 1996; De La Fuente et al., 1998). In Bangladesh, calf diarrhoea (gastroenteritis syndrome) remains the most often reported clinical problem in calf management and rearing system. Cryptosporidiosis is considered as the third major cause of diarrheal disease worldwide (Janoff and Reller, 1987; Casemore et al., 1997; Fayer et al., 1997, 2000; Morgan et al., 1999; Spano and Crisanti, 2000). In diarrhoea, the intestine fails to absorb fluids and/or secretion into the intestine is increased. Bovine cryptosporidiosis is a common disease affecting newborn calves (Del Coco et al., 2009). *Cryptosporidium parvum* incriminated for causing intestinal cryptosporidiosis in newborn calves (Nolan et al., 2009). *Cryptosporidium* have emerged as important parasites of dairy cattle because of their proven pathogenicity and the potential public health significance of zoonotic transmission (Olson et al., 2004). *Cryptosporidium* is an apicomplexan intestinal protozoon, which infects animals and humans

gastrointestinal tract and causes cryptosporidiosis (Carey et al., 2004; Huang and White, 2006). This apicomplexan parasite infects humans and animals, globally. Up to now eight valid *Cryptosporidium* species have been reported to be capable of infecting humans (Rimhanen-Finne, 2006). They are dwelling in the stomach or in the small intestine of mammals, birds and reptiles. Clinical disease is characterized by mucous to hemorrhagic diarrhoea, lethargy, pyrexia and loss of condition (Santin et al., 2008). *Cryptosporidium* spp. also infects the proximal small intestine, as well as the cecum and colon (Angus et al., 1982, Heine et al., 1984). The pathological findings associated with *Cryptosporidium* are a mild to moderate villous atrophy, villous fusion, crypt hyperplasia, disruption of the intestinal microvillus, and the infiltration of inflammatory cells and changes in the surface epithelium (Angus, 1990). Further, infiltration of mononuclear cells and neutrophils was seen in the lamina propria (Sanford et al., 1982). Mortality by cryptosporidiosis may reach up to 35 percent in calves (Singh et al., 2006). Contaminated environment surrounding the infected animal herds is a constant threat to healthy animals and human beings (Fayer et al., 2012). Immunocompromised individuals residing in close association with infected animals are more prone to infection (Semenza and Nichols, 2007). Cryptosporidiosis is more severe in newborn animals and causes severe diarrhea that is sometimes accompanied with anorexia, reduced milk intake, dehydration, growth retardation, stiffness, hyperpnoea, slow gait and depression (Casemore et al., 1997; Fayer et al., 2004). Although the adult animals are generally refractory to infection, infected animals can act as asymptomatic carriers and shed large numbers of oocysts into the environment and remain a main source of infection to other domestic and wild animals (Xiao and Herd, 1994). Cattle cryptosporidiosis is widespread and studies have shown a wide range of oocyst shedding dynamics depending on the age, clinical situation and breeding system of the animals (Maldonado-Camargo et al., 1998). Bovine cryptosporidiosis causes significant neonatal morbidity, weight loss and delayed growth, which leads to large economic losses (McDonald, 2000). Two species of *Cryptosporidium* have been identified in cattle: (i) *C. parvum* (cattle genotype) in the intestine; and (ii) *Cryptosporidium andersoni* in the abomasum. In addition to their site of predilection, the two species have morphologically distinct oocysts and differ genetically (Lindsay et al., 2000). Infection rate by parasites in cattle differ with growing age. Contamination of food or water by cattle manure has been identified as a cause of several foodborne and waterborne outbreaks of

cryptosporidiosis (Glaberman et al., 2002; Blackburn and Soave., 1997). Moreover, several Bovine intestinal protozoan parasites are zoonotic and are considered important to public health. Young calves are considered as a reservoir for these parasites, and transmission of Giardia and Cryptosporidium from cattle handlers has been suggested in Bangladesh and India (Hasan et al., 2006; Rahman et al., 1985).A single oocyst is sufficient to produce infection and disease in susceptible hosts. Transmission can occur from animal to animal, animal to person and person to person, by ingestion of contaminated surfaces (Smith et al., 2007). Cryptosporidiosis in cattle has been reported from different parts of the world with prevalence ranging from 24.5% to 45% (Kumer et al., 2005). The calf mortality in Bangladesh up to 12 months of age was reported from 9% under rural (Debnath et al., 1990) to 13.4% under a farm (Debnath et al., 1995) conditions. Cryptosporidium hominis as well as C. parvum were identified in patient with diarrhoea in Bangladesh (Haque et al., 2009). Young calves are considered as a reservoir for these parasites, and transmission *Cryptosporidium* from cattle to cattle handlers has been suggested in Bangladesh and India (Rahman et al., 1985). In microbiological surveys of diarrheic calves, the detection rate of mixed infections with two or more of the main enteropathogens ranged between 5% and 20%, and in most of them *Cryptosporidium* was involved for complicated infection (Bulgin et al., 1982). Previous study reported that cryptosporidium mixed infections associated with more enteropathogens in calf diarrhea are very limited from Bangladesh (Samad et al., 1977, 2001). Traditionally, the detection of *Cryptosporidium* oocysts has primarily relied on examination by microscopy (O'Donoghue, 1995; Quintero-Betancourt et al., 2002). Various staining techniques have been employed to aid in the diagnosis of *Cryptosporidium* oocysts from calves feces. The simplest and least expensive stains include Dimethyl Sulphoxide (DMSO)-Carbol fuchsin (Pohjola et al., 1984), safranin–methylene blue (Baxby et al., 1984) and acid fast (modified Ziehl–Neelsen) (Henriksen and Pohlenz, 1981). Examination of fecal smears with acid-fast stains such as Ziehl-Neelsen techniques are commonly used by diagnostic facilities (Scott, 1988). In this technique, the oocysts are stained with carbol-fuchsin and the dye is retained in the decolorizing step with the acid alcohol. Oocysts of *Cryptosporidium* spp. appear as pink to bright red spheres of approximately 5 micrometer in diameter (Marks et al., 2004). Morphological characters for identifying *Cryptosporidium* are few, and identification based on light microscopy alone is unreliable and relatively time consuming (Fall et

al., 2003). Several molecular tests to detect parasites have been developed in the last decade. Their specificity and sensitivity have gradually increased, and parasites that were previously difficult to diagnose using conventional techniques began to be identified by molecular techniques. The more sensitive molecular-based methods, such as PCR are required for detection of low numbers of oocysts as observed in fecal specimens taken from patients during the early or late phases of Cryptosporidiosis, or in fecal-contaminated soils and waters. Another tremendous advantage of PCR in *Cryptosporidium* diagnosis is that the parasite's genotype can be determined (Ward and Wang, 2001). This study compared the PCR detection of *Cryptosporidium* with conventional microscopic detection of *Cryptosporidium* spp. in order to determine the usefulness and practicality of PCR-based detection methods for clinical diagnosis of *Cryptosporidium* as well as the histopathological changes in intestine of dead calves. The purpose of present study was also to determine the distribution of cryptosporidium in dairy calves on different dairy farm with associated risk factors like age sex and season in Chittagong district of Bangladesh. Considering the above facts the present study was undertaken to fulfill the following objectives:

- To investigate the prevalence of *Cryptosporidium* spp. in neonatal calves in different dairy farms in Chittagong metropolitan area and Patiya Upazilla of Bangladesh.
- Comparative study of the microscopic identification and PCR based diagnosis.
- To study histopathological slide of the dead calves in associated with diarrhoea.

CHAPTER II
REVIEW OF LITERATURE

Cryptosporidiosis due to infection with *Cryptosporidium* spp. is a well-recognized cause of diarrhea in neonatal ruminants and has been reviewed extensively by others (Graaf DC et al., 1999). In ruminants, three species of Cryptosporidium are now recognized as common causes of infection. *C. parvum* is the most extensively studied of these three species and is primarily a parasite of neonatal ruminants, colonizing the ileum and proximal portions of the large intestine. C. *andersoni*, which was referred to as *C. muris* in cattle before 2000 (Ryan et al., 2004) resides in the abomasums and has so far only been identified in mature cattle. Few years back, a third species, *C. bovis*, which is morphologically identical to *C. parvum* but infects calves at 1 to 2 years of age, has been proposed (Fayer et al., 2008).This species was previously referred to as the *Cryptosporidium bovine* B genotype. Of these three species, only *C. parvum* is considered zoonotic, having been identified as a cause of gastrointestinal disease in humans. Cryptosporidiosis has been classified by the World Health Organization (WHO) as a 'reference pathogen' indicating water quality globally (Medema et al., 2006).

2.1. Historical background

Cryptosporidium infection in cattle were first reported in the early 1970s (Graaf et al., 1999).After that association with other viral or bacterial enteropathogens, the role of *Cryptosporidium* spp. as primary enteropathogens was uncertain until 1980, when (Tzipori et al., 1983) an outbreak of neonatal diarrhoea to cryptosporidium infection alone. In the following years methods to free the infective oocysts from other contaminating pathogens became available, which permitted the experimental demonstration that Cryptosporidium was capable of causing clinical diarrhoea in calves (Heine et al., 1984). In cattle two species of the genus Cryptosporidium can be distinguished: *Cryptosporidium parvum* infecting the distal small intestine, and *Cryptosporidium muris* infecting the abomasums. Substantial differences in the size and shape of C*ryptosporidium parvum* (5.0 mm to 4.5 mm and spherical (Esteban and Andersonal, 1995) and *C. muris* (7.4 mm to 5.6 mm and ovoid oocysts (Ryan et al., 2003) enables the two species to be distinguished readily on microscopical

examination. Only *C. parvum* has been associated with neonatal diarrhoea. *Cryptosporidium muris* is much less prevalent and was only found in weaned Calves or adult cattle (Bukhari and Smith, 1996) and *C. muris* infection is considered to be clinically mild, affecting weight gain (de Pena et al., 1997) and milk production (Esteban and Anderson, 1995).

2.2. Taxonomy and nomenclature:

Cryptosporidium is a genus in the family Cryptosporidiidae, suborder Eimeriina, order Eucoccidiida, subclass Coccidia and Class Sporozoa (Tzipori, 1983). Cryptosporidium are intracellular parasites found in the digestive and respiratory tract in a variety of vertebrate hosts worldwide (Tzipori et al., 1983). Cryptosporidium consisting of multiple species. The taxonomy of currently recognized species of genera is summarized in Table1 and Table2. The genus Cryptosporidium consists of 18 species (Table 1) and over 40 genotypes (Xiao and Fayer, 2008). As biological and molecular data increase, many of the genotypes are expected to be named as valid species. More than 150 mammalian hosts were reported to be infected with *C. parvum, C. parvum*-like parasite or simply Cryptosporidium (Fayer et al., 2008) based on microscopic observations of oocysts in fecal samples. Most will have to be re-examined using molecular methods to validate the species or determine the genotype. Today 3 species are known to regularly infect cattle: *C. parvum, C. andersoni* and the recently described *C. bovis* (Fayer et al., 2005, Langkjaer et al., 2007, and Thompson et al. 2007). *C. andersoni* is found mainly in adult cattle but has also been found in young stock and *C. parvum* usually infects calves younger than one month (Olson et al., 2004). *C. bovis* tends to infect older calves and young stock (Langkjaer et al., 2007). *C. parvum, C. hominis* and *C. bovis* can not be separated based only by traditional diagnostic methods such as microscopy or ELISA test, but DNA analysis has to be applied in order to distinguish between the species. Other species of Cryptosporidium have been found to infect a predominant host species and to a much lesser extent other hosts including humans (Table 2).

Table 1: Species of Cryptosporidium:

Species	Author	Types of host
Cryptosporidium andersoni	Lindsay et al. (2000)	*Bos taurus* (domestic cattle)
Cryptosporidium baileyi	Current et al. (1986)	*Gallus gallus* (chicken)
Cryptosporidium bovis	Fayer et al. (2005)	*Bos taurus* (domestic cattle)
Cryptosporidium Canis	Fayer et al. (2001)	*Canis familiaris* (domestic dog)
Cryptosporidium fayeri	Ryan et al. (2008)	*Macropus rufus* (red kangaroo)
Cryptosporidium felis	Iseki et al. (1979)	*Felis catis* (domestic cat)
Cryptosporidium galli	Pavlasek et al. (1999)	*Gallus gallus* (chicken)
Cryptosporidium hominis	Morgan-Ryan et al. (2002)	*Homo sa*piens (human)
Cryptosporidium macropodum	Power and Ryan et al.(2008)	*Macropus giganteus* (grey kangaroo)
Cryptosporidium meleagridis	Slavin et al. (1955)	*Meleagris gallopavo* (turkey)
Cryptosporidium molnari	Alvarez and Sitja-Bobadilla, 2002.	*Sparus aurata* (gilthead seabream) *Dicentrarchus labrax* (European seabass)
Cryptosporidium muris	Tyzzer, (1910)	*Mus musculus* (house mouse)
Cryptosporidium parvum	Tyzzer, (1912)	*Mus musculus* (house mouse)

Cryptosporidium scophthalmi	Alvarez-Pellitero and Sitja-Bobadilla, 2004	*Scophthalmi maximus* (turbot)
Cryptosporidium serpentis	Levine et al. (1980) Brownstein et al. (1977)	*Elaphe guttata* (corn snake) *Elaphe subocularis* (rat snake) *Sanzinia madagascarensus* (Madagascar boa)
Cryptosporidium suis	Ryan et al. (2004)	*Sus scrofa* (domestic pig)
Cryptosporidium varanii	Pavlasek et al. (1995)	*Varanus prasinus* (Emerald monitor)
Cryptosporidium wrairi	Vetterling et al. (1971)	*Cavia porcellus* (guinea pig)
Cryptosporidium parvum	Ehsan et al., (2015)	*Bos taurus* (domestic cattle)
Cryptosporidium hominis	Dey et al. (2016)	*Homo sapiens* (human)
Cryptosporidium xiaoi	Siddiki et al. (2015)	*Capra aegagrus hircus* (Goat)

Table 2: *Cryptosporidium spp*. and genotypes that infect humans and other hosts:

Species	Host	Author
Cryptosporidium andersoni	Cattle, sheep, Bactrian camel, gerbil, multi-mammate mouse, wood partridge	Lindsay et al. (2000)
Cryptosporidium baileyi	Chicken, duck, Bob-white quail	Current et al. (1986)
Cryptosporidium canis	Dog, fox, coyote	Fayer et al. (2001)

		Morgan et al. (2000)
Cryptosporidium felis	Cat, cattle	Iseki, (1979) Caccio et al. (2002)
Cryptosporidium hominis	Human	Morgan-Ryan et al. (2002)
Cryptosporidium meleagridis	Turkey, chicken, Bobwhite quail, dog, deer mouse	Pedraza-Diaz et al. (2001) Xiao et al. (2001)
Cryptosporidium muris	Mouse, hamster, squirrel, Siberian chipmunk, wood mouse, bank vole, Bactrian camel, mountain goat, bilby ringed seal, monkey, frogmouth	Fayer et al. (2008) Palmer et al. (2003)
Cryptosporidium parvum	Calf, lamb, horse, alpaca, dog, mouse, raccoon dog, eastern squirrel	Tyzer et al. (1910) Panciera et al. (1971) Kennedy et al. (1977) Snyder et al. (1978)
Cryptosporidium suis	Pig, cattle	Xiao et al.,(2002) Ryan et al. (2004)
Cervine genotype	Cattle, sheep, ibex grey squirrel, eastern chipmunk, beaver, red squirrel, Blesbok woodchuck, deer mouse, raccoon deer, mouflon sheep, nyala, lemur	Perz and Blancq, 2001 Ong et al. (2002) Ryan et al. (2003,2004) Blackburn et al. (2006) Feltus et al. (2006) Leoni et al. (2006) Nichols et al. (2006) Feng et al. (2007)
Skunk genotype	Skunk, raccoon, eastern squirrel, opossum, river otter	Blackburn et al. (2006) Soba et al. (2006).

Table 3: Location of *Cryptosporidium* spp. (Xiao and Ryan, 2008):

Cryptosporidium spp.	**Host**	**Location**
C. parvum	Ruminants, human	Intestine
C. andersoni	Bovines	Abomasum
C. baileyi	Birds	Cloaca, bursa, respiratory tract
C. canis	Canids, human	Small intestine
C. felis	Felids, human	Small intestine
C. galli	Birds	Proventriculus
C. hominis	Human	Small intestine
C. meleagridis	Birds, Human	Intestine
C. molnari	Fish	Stomach
C. muris	Rodents, human	Stomach
C. saurophilum	Lizards, snake	Intestinal and cloacal mucosa
C. serpentis	Lizards, snake	Stomach
C. suis	Pigs, human	Small intestine
C. wrairi	Guinea pigs	Small intestine
C. bovis	Ruminants	Small intestine
C. scophithalmi	Fish	Intestine

2.3. Host specificity of Cryptosporidium species and genotypes in animals:

Most Cryptosporidium species and genotypes are host-adapted in nature, having a narrow spectrum of natural hosts without few expectations. Thus, one species or genotype usually infects only a particular species or group of related animals. Recognizing the zoonotic potential of each species is based upon knowing the host range or host specificity of that species. This can be determined accurately identifying the species/genotype from the oocyst/cyst in the feces of naturally infected hosts using molecular methods and obtaining oocyst or cysts from one host species and feeding those to putative hosts of another species. When feeding these stages results in completion of the life cycle in the putative host and particular stages are excreted that are genetically identical to those that initiated the infection, the confirmed host range

is extended. However, the inability to obtain sufficient numbers of oocysts or cysts and difficulty in obtaining and/or housing wildlife, or scarce or expensive domesticated animals, limits testing. Which rely on molecular methods to clearly identify the species because morphological methods lack the specificity required to distinguish many species and genotypes. For example, oocysts of *C. parvum*, *C. hominis*, C. *meleagridis* and *C. bovis* have no apparent internal features that are unique among them and they overlap in size, differing only at the extremes by a few tenths of a micrometer. The oocyst size ranges for each species are 4.8–6.0 µm to 4.8–5.4 µm, 4.4–5.9 to 4.4–5.4 µm, 4.5–6.0 to 4.2–5.3 µm and 4.8–5.4 µm to 4.2–4.8 µm, respectively (Fayer et al, 2005 ; Fayer et al., 2008) . Confirmation of infection is complicated when only one or two fecal specimens serve as the source of oocysts or cysts because it can be argued that these stages were ingested and simply passed through the gut without actually infecting the host. This has been demonstrated experimentally (Graczyk et al., 1996). For humans, even when it appears conclusive that there is an infection with a potentially zoonotic species or genotype one might argue that the infection was acquired from a human source rather than an animal and therefore transmission is anthroponotic not zoonotic.

2.4. Morphology of the oocyst:

The oocysts of *Cryptosporidium* are sporulated where each contains four sporozoites and a residuum composed of numerous small granules with a spherical or ovoid membrane-bound globule. The dimension of oocyst for *C. parvum* 4.5 × 5.5 *µm*, *C. andersoni* 5.6 × 7.4 *µm* (5.0–6.5 × 8.1–6.0 *µm*), *C. bovis* 4.7–5.3 × 4.2–4.8 *µm* and *C. muris* 5.5 × 7.4 *µm* respectively (Upton and Current, 1985). The small sizes of Cryptosporidium oocysts for the various species are difficult to distinguish from each other during routine fecal examination based solely on morphology by light microscopy (Fall *et al*., 2003). The oocysts four naked parallel sporozoites surrounded by a smooth oocyst wall. At the wall, a faint suture can be seen through which the sporozoites exit during encystations (Morgan-Ryan et al., 2002). There is some variation from species to species. The length of the oocyst ranges from 4.5 to 7.5 µm and the width from 4.2 to 5.7 *µm* (Fig. 1) (Marquardt et al., 2000).

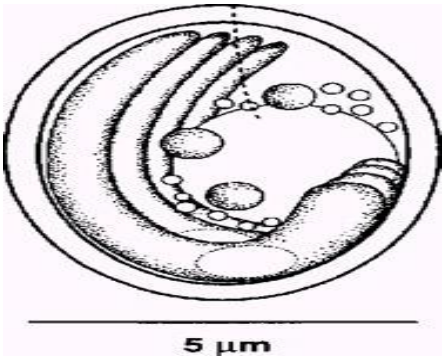

Fig 1: oocyst of Cryptosporidium (Marquardt et al., 2000).

2.5. Life cycle of Cryptosporidium:

Cryptosporidium spp. have monoxenous life cycles, where all stages of development (asexual and sexual) occur within one host. Thick-walled oocysts are excreted from the infected host in fecal material and represent the infective stage of the parasite. Infection of Cryptosporidiu*m* in a new host results from the ingestion of these oocysts. Once they are ingested, oocysts excyst in the gastrointestinal tract releasing the infective sporozoites. Encystation has been reported to be triggered by various factors including reducing conditions, carbon dioxide, temperature, pancreatic enzymes and bile salts (Robertson et al., 1993). The sporozoites escape through a slit-like opening created at one end of the oocyst by dissolution of a special suture in the oocyst wall (Fayer et al., 2000). The freed sporozoites attach to epithelial cells where they become enclosed within parasitophorus vacuoles and develop attachment organelles (stages generally referred as trophozoites). The trophozoites then undergo asexual proliferation by merogony (previously called schizogony). Cell division occurs by endopolygeny where multiple daughter cells are formed by internal budding within the mother meront (Fig. 2). Most studies performed on *Cryptosporidium* spp. have described sequential development involving two types of meronts (Fayer et al., 1997; Spano et al., 2000). Type I meronts form 8 merozoites which are liberated from the parasitophorus vacuole when mature. The merozoites then invade other epithelial cells where they undergo another cycle of type I merogony or develop into type II meronts. The type II meronts form 4 merozoites which do not undergo further merogony but produce sexual reproductive stages (called gamonts). Sexual reproduction occurs by gametogony and both microgamonts (male) and macrogamonts (female) are formed (O'Donoghue, 1995; Fayer et al., 1997; Collinet-

Adler and Ward, 2010; Huang et al., 2014). Microgamonts develop into microgametocytes which produce up to 16 non-flagellated microgametes. Macrogamonts develop into uninucleate macrogametocytes which are fertilized by mature microgametes. The resultant zygotes undergo further asexual development (sporogony) leading to the production of sporulated oocysts containing 4 sporozoites (Fig. 2). Most oocysts (80%) are thick-walled and are excreted from the host in fecal material. Some oocysts (20%), however, there are thin-walled and have been reported to excyst within the same host animal leading to a new cycle of development (autoinfection). The presence of these auto-infective oocysts and recycling merozoites of type I meronts are believed to be the means by which persistent chronic infections may develop in hosts without further exposure to exogenous oocysts (Current and Garcia, 1991; Ramirez et al., 2004).The entire life cycle of the parasite may be completed in as little as 2 days in many hosts and infections may be short-lived or may be persisting for several months. The prepatent period (time between infection and oocyst excretion) ranges from 1-3 weeks, whereas the patent period (duration of oocysts shedding) can range from several days to months or years, demonstrating the potential of this infection to persist. Many factors may influence the longevity of infections but the most important appear to involve the immunocompetency of the host and the parasite species involved (O'Donoghue et al., 1995). Consequently, in the absence of a protective immune response, Cryptosporidium may persist inside a single host even without further exposure to exogenous oocysts and the thick-walled oocysts are already fully sporulated when they leave the body with the feces and are therefore immediately infectious. Thus, Cryptosporidium seems to have an extraordinary reproductive ability. In addition, the oocysts can travel a considerable distance following runoff can survive for a relatively long time in an aqueous environment and are infections to a wide range of animals, thus having many potential excretors. As a result, this parasite undoubtedly has an exceptional capacity to dessiminat (Mawdsley et al., 1996; Tzipori and Griffiths, 1998).

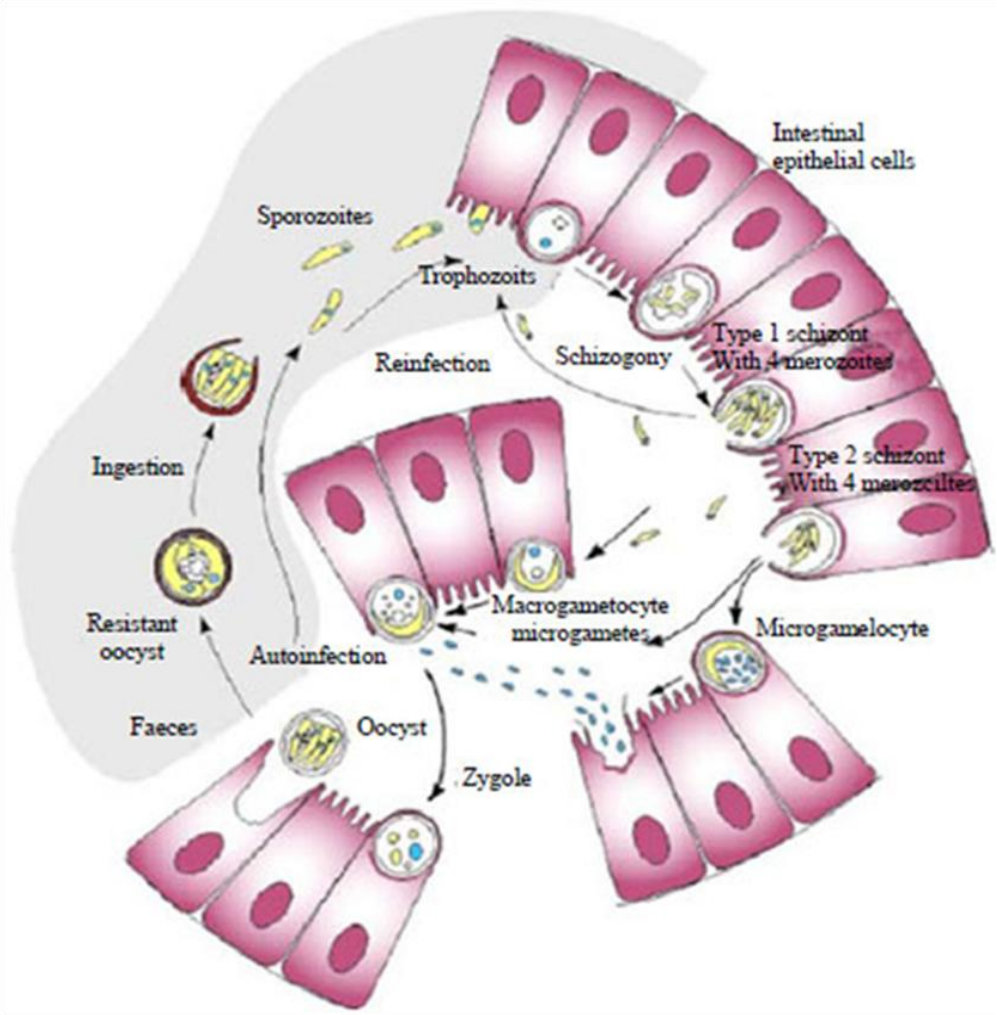

Fig.2: Life cycle of *Cryptosporidium* spp. in farm animals. (Smith et al., 2007)

2.6. Transmission of Cryptosporidium:

Transmission can occur via any route by which material contaminated with viable oocysts excreted by infected individuals can be ingested. Transmission of *C. parvum* is conducted by fecal contamination (Radostits et al., 2000). Practices likely to enhance the spread of cryptosporidiosis include indoor calving and lambing and the communal feeding and husbandry of neonates, where young susceptible animals are in close contact with each other and the feces of infected animals. Similarly, the disposal of feces, farmyard manure or other contaminated waste in land-based dumps, when followed by periods of heavy rainfall or melting snow can lead to *C. parvum* oocyst contamination of water courses (Robertson and Gierde, 2001). These courses

may be used as a source of drinking water for other animals and for potable water for human consumption. Contaminated waste includes both the liquid and solid by-products of animal husbandry. The parasite can be transmitted from animals to humans and transmission from human to human has also been shown. The latter transmission route seems to be more common than the first (Bowman et al., 2003).

2.7. Clinical and pathological features:

Cryptosporidium is a well-known cause of diarrhea in neonatal ruminants. Numerous reports detail the occurrence of clinical disease due to cryptosporidiosis in calves, lambs, goat kids, and other ruminants (Fayer et al., 1997).The clinical manifestation of cryptosporidiosis are similar in calves, lamb, and kids and include diarrhea, which is often pale yellow with mucus, lethargy, anorexia, and dehydration (Fayer et al., 2005, Koudela and Giri, 1997) experimentally *C. parvum* infected neonatal calves, revealed a prepatent and a patent period ranging from 3-6 and 4-13 days, respectively (Fayer et al., 1998). Severe cryptosporidiosis can result in extreme dehydration, metabolic acidosis, and death, but these cases are rare. Although it is less frequently identified in beef calves than in dairy calves, outbreaks of cryptosporidiosis can be more severe in beef calves than in dairy calves and may result in mortality as high as 30% (Olson et al., 2004). In many of these cases, the introduction of dairy calves into the herd during calving season was a contributing factor. In addition, low levels of serum selenium in beef herds were associated with high mortality due to cryptosporidiosis (Olson et al., 2004).It is important to emphasize that *C. parvum* is commonly identified along with other enteropathogens during cases of neonatal diarrhea (Anderson et al., 1998), and the parasite may often be identified in calves that have no signs of clinical disease (O'Handley et al., 1999). Cryptosporidial diarrhoea is associated with the excretion of tremendous numbers of oocysts (Current et al., 1985). The severity of cryptosporidiosis is influenced by a number of factors, including the infectious doses, herd immunity, presence of concurrent infections, nutrition, and husbandry (Olson *et al*., 2004). Thus, in most well-managed herds, cryptosporidiosis due to *C. parvum* is typically a mild disease and not a serious health concern (Anderson, 1998). The clinical signs associated with intestinal cryptosporidiosis are the result of both malabsorption and an increase in fluid secretion. Although the exact mechanisms are still not fully understood, both parasite and host factors play a role. *C. parvum* primarily colonizes the distal small intestine

(figure below). However, the parasite may also infect the proximal small intestine, as well as the cecum and colon (Angus et al., 1982, Heine et al., 1984). The pathological findings associated with Cryptosporidium are a mild to moderate villous atrophy, villous fusion, crypt hyperplasia, disruption of the intestinal microvillus, and the infiltration of inflammatory cells and changes in the surface epithelium (Angus et al., 1990). Further, infiltration of mononuclear cells and neutrophils was seen in the lamina propria (Belchev et al., 1987). Recent studies suggest that Cryptosporidium can also induce apoptosis and disrupt the epithelial tight junction, resulting in the loss of barrier function (Buret et al., 2003).The culmination of these pathologic changes is the development of both a malabsorptive and a secretory diarrhea. Abomasal cryptosporidiosis, caused by C andersoni, does not result in the development of noticeable clinical signs Increased in the number of CD8 + T-cells was found due to accumulation of T-cells, both $CD4^+$ and $CD8^+$ in the intestinal villi (Abrahamsen et al., 1997). Respiratory cryptosporidiosis has been reported (Mascaro et al., 1994), but can be considered to be of less economic importance than the enteric form.

Fig 3: Cryptosporidium infected calf

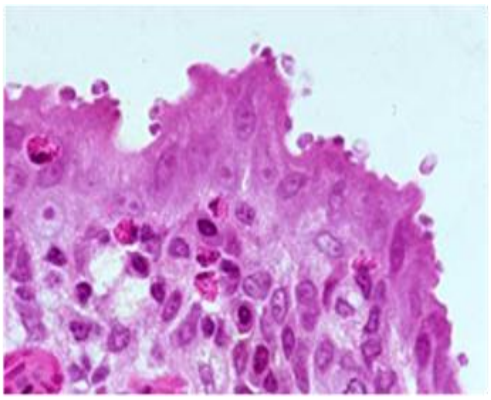

Fig 4 : Cryptosporidium ocyst in the surface of intestine

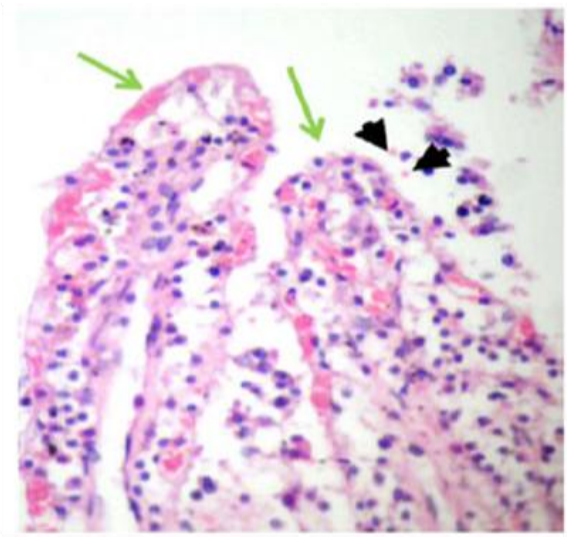

Fig 5: *Cryptosporidium* infected calf showing severe hemorrhagic enteritis with congestion of blood vessels (double arrow), blunting of villi (blue arrow) and sloughing and complete erosion of epithelial cells (dashed arrow) (H and E X100 (intestine)

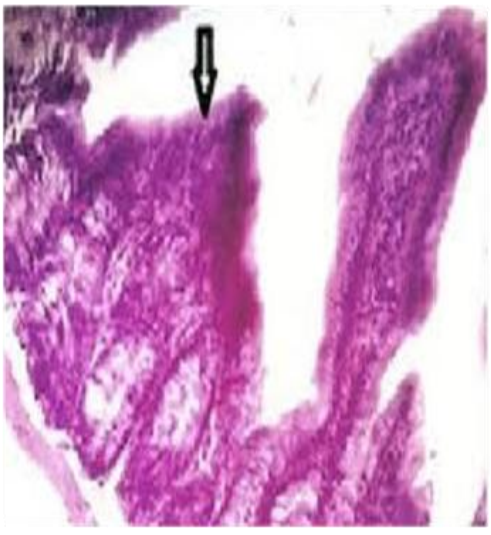

Fig 6: Altered mucosa of intestine infected with *Cryptosporidium spp.*

2.8. Interactions with other enteropathogens and prevalence:

In case of neo-natal diarrhoea, both in large and small ruminants, is a clear example of a multifactorial disease governed by a wide range of factors related to the animal, conditions of the environment and husbandry, and a variety of viruses, bacteria and protozoan parasites. A number of infectious (bacteria, viruses, parasites) and non-infectious factors cause diarrhoea in neonatal calves. Previous studies show that the most important infectious agents Cryptosporidium either singly or in combination (Snodgrass et al., 1986; Waltner-Toews et al., 1986; Steiner et al., 1997; De La Fuente et al., 1998). Numerous infectious pathogens capable of causing diarrhoea among food animals have been associated with food born disease and zoo-noses in humans (enterohaemorrhagic E. coli, Salmonella, Rotavirus and C. parvum) (Trevejo et al., 2005).Cryptosporidium have emerged as important parasites of dairy cattle because of their proven pathogenocity, and the potential public health significance of zoonotic transmission (Olson et al., 2004).In calves, enterotoxigenic Escherichia coli (ETEC), with thermo labile and/or thermo stable enterotoxins and with colonization factors are recognized as a common cause of diarrhoea in calves under 3 days old (Blanco et al., 1991). This age-dependence is caused by a diminished adherence of ETEC to enterocytes after the first few days of life (Tzipori et al., 2000). Between 4 days and 6 weeks of age digestive problems can mostly be attributed to C. parvum and/or a variety of viruses, with rotavirus, corona virus and bovine viral diarrhoea (BVD) virus being the most important.

2.9. Maintenance of infection:

A variety of wild mammals act as hosts to *C. parvum* (Fayer et al., 2008), particularly neonates, but little is known of the importance of their involvement in transmitting infection to, or maintaining infection, in domesticated species in farmyard environments. Their role in ‘on farm’ epidemiology in domesticated species is also uncertain. The methods used for diagnosing infection in small mammals and wildlife are the same as those described for farm animals. Oocysts are environmentally robust and can survive for long time periods (>6 months) in moist, cool microclimates. Evidence exists for transmission of cryptosporidiosis from clinically normal dams to suckling neonates, but, in general, the duration of the carrier state remains unknown.

2.10. Treatment and prophylaxis:

In the immunocompetent individual the signs and symptoms of cryptosporidiosis usually abate in less than 2 weeks. Supportive therapy may be required if diarrhea is excessive (Blagburn and Soave, 1997). In cases of very severe watery diarrhea, symptomatic treatment, such as liquid and elelctrolyte replacement and agents such as parenteral octreotide may be used to reduce secretory diarrhea in either immuncompetent or imunodeficient individuals (Moroni et al., 1993). Nutritional support may be required in the event of severe malabsorption. There are no consistently effective, approved products for either animals or humans Which had proven long time research. Nitazoxanide, a nitrothiazole benzamide with a wide spectrum of activity against bacteria, protozoa, and helminths, has shown efficacy in early trials with human subjects (Blagburn and Soave, 1997). Presently there is no effective treatment for Cryptosporidial infections. Three drugs; azithromycin, Immuno-C and DAP-092 are currently in clinical trials in the USA (Cryptosporidium Capsule, 1998). Studies evaluating the efficacy of azithromycin have been inconclusive, however a recent study by Smith et al. (1998) demonstrated that azithromycin combined with paromomycin is effective. The development of anti-Cryptosporidial drugs is tremendously difficult, as there are few patients with cryptosporidiosis who can be used in control trials. Since there is no drug that achieves the complete removal of Cryptosporidium from an infected host, and no compound is clearly recognized, widely accepted and immediately available as a prophylactic or therapeutic agent, the preferred treatment in both humans and domestic animals is supportive treatment. Generally, this consists of replacement of fluid and electrolytes, nutritional support and anti-diarrhoeal drugs. Broad-spectrum antibiotics, gastric protectants and anti-ulcer medication may also be beneficial. Until now numerous in vivo and in vitro drug trials have been con-ducted against this important opportunistic pathogen but none was found to be completely effective against cryptosporidiosis. Calves suffering from diarrhoea need fluids and electrolytes orally and parenterally. The calves should be fed milk continuously to prevent loss of body weight and digestion function. Parenteral nutrition could be considered in cases of valuable calves. Further transmission should be avoided through isolation of unhealthy calves and feeding calves adequate amounts of colostrums. It is useful to keep the farm clean and to use an all-in, all-out system so that all areas could be properly cleaned. Rodents and pets should not have access to the calves to avoid

transmission through vectors (Radostits et al., 2000). Fresh bedding in combination with strict sanitation is probably the best prophylaxis against calf diarrhoea (Krogh and Henrikse, 1985).

2.11. Cryptosporidiosis in cattle:

For *Cryptosporidium* infection in Cattle mostly infected with *C. parvum*, *C. andersoni, C. bovis* and *C. ryanae* (Xiao et al., 2010). In dairy cattle, *C. parvum* is mostly found in pre-weaned calves, *C. bovis* and *C. ryanae* in weaned calves and *C. andersoni* in yearlings and adult cattle (Fayer *et al.,* 2006; Santín et al., 2008). Most cases, outbreak of disease occurs at young age, in calves less than three weeks or in immunocompromised individuals (Ballweber et al., 2001). *Cryptosporidiosis* is characterized by high morbidity and the most common clinical sign is diarrhoea (Holland et al., 1990). Other symptoms that might show at the same time are depression, various degrees of apathy, inappetence and poor condition. Only at some occasions does severe dehydration and collapse occur (Radostits et al., 2000). The symptoms may appear 2-7 days after inoculation and the calf will recover within 6 -10 days after the appearance of diarrhoea. Oocysts are shed in feces for 3-12 days (Radostits et al., 2000). The illness is usually self-limiting in healthy animals but it enhances in severity in the presence of other pathogens such as rotavirus in calves (Holland et al., 1990). The protozoa cause tissue reactions in the intestines, which may lead to diarrhoea. Villous atrophy, a shortening of microvillus, villous fusion and cryptitis can be seen. The intestinal digestion and absorption of dietary nutrients is decreased due to the loss of microvilli and decreased activity in mucosal enzymes and villous function. It is mostly the lower part of the small intestine that is infected but cecum and colon can occasionally be affected (Radostits et al., 2000). Sometimes the parasite causes bacterial overgrowth by decreasing disaccharidase activity. Less disaccharidase reduces the breakdown of sugars and causes a good environment for bacterial growth (Ballweber, 2001). Once the calves have been exposed to *C. parvum,* it has been indicated that they will become resistant to further infections of the pathogen (Harp et al., 1990). Calves that have been isolated from the parasite and then experimentally challenged to an infection have acquired diarrhoea, oocyst shedding and a raised antibody titre. At a second challenge, no symptoms of infection, no oocyst shedding and no increase in antibody titres were observed (Harp et al., 1990). Age related resistance, unrelated to prior exposure, has been observed in lambs but

not in calves (Radostits et al., 2000). Separating the effects of non-immunological age-related resistance from immunity through natural exposure on older individuals is difficult since *C. parvum* is common in the environment and the animals could be assumed to continuously be exposed for an infection (Harp et al., 1990). *Cryptosporidium parvum* is often the only pathogen found in diarrhoeic calves (Singh et al. 2006). It has been reported from Norway that the prevalence of the parasite seems to increase as the number of calves in a herd increases, and that small farms tend to have fewer problems with cryptosporidiosis (Hamnes et al. 2006). However, the importance of *C. parvum* infection as a cause of calf diarrhoea is under debate since *C. parvum* is commonly found in healthy animals. In some studies, no association between infection with *C. parvum* and diarrhoea or other clinical signs were found (Huetink et al., 2001). However, results from Canada showed a three time higher risk for calves shedding oocysts to be diarrhoeic than non-infected calves (Trotz-Williams et al., 2005).

Table 4: Infection dynamics Cryptosporidium spp. in cattle:

Periods	*C. parvum*	*C. andersoni*	References
Age of oocyst and/or cyst shedding (weeks)	1–5 weeks	> 7 weeks	Huetink et al., 2001, Joachim et al., 2003
Duration of cyst shedding	1–2 weeks	5 months to years	Huetink et al., 2001, Joachim et al., 2003
Age of peak shedding (weeks)	1–2 weeks	N/A	Ralston et al., 2003, Joachim et al., 2003
Age of onset of diarrhoea (weeks)	1–2 weeks	N/A	Fayer et al., 1998, Huetink et al., 2001
Duration of diarrhoea (weeks)	1–3 weeks	N/A	Fayer et al., 1998, Huetink et al., 2001
Duration of peri-parturient shedding (weeks)	0–2	0– > 4 weeks	Casmir et al., 1997, Ralston et al., 2003

2.12. Prevalence of Cryptosporidiosis in cattle:

Parasitic infestation in cattle are common throughout the world but reported prevalence rates vary widely In dairy cattle, *C. parvum* is mostly found in pre-weaned

calves, *C. bovis* and *C. ryanae* in weaned calves and *C. andersoni* in yearlings and adult cattle (Fayer et al. , 2006; Santin et al., 2008). Parasite prevalence varies from 1% (Kvac et al., 2006) to 59% (Olson et al., 1997) in individual calves and up to 100% on farm level (Santín et al., 2004). The highest prevalence is observed in calves under the age of 5 weeks (Quílez et al., 1996). This difference in prevalence results can be explained by differences in age of the animals, management, and diagnostic methods applied (Robertson, 2009). *Cryptosporidium parvum* oocysts were detected in bovines ranging from 3 days old to adults, although the prevalence was significantly higher in suckling calves (Lorenzo-Lorenzo, 1993).In a Spanish epidemiological study on the prevalence and age distribution of *C. parvum* infections, as many as 44.4% of calves aged 3-4 days were infected, but infection rates peaked (76.7%) at 6-15 days of age. Prevalence was also high in weanling calves aged 1.5-4 months (14%), fattening calves and heifers 4-24 months old (7.7%) and adults (17.8%). However, Cryptosporidium infection was only statistically associated with diarrhoea in suckling calves (Quilez et al,. 1996). The same USDA: APHIS: VS study estimated that on any given day, 22 % of preweaned dairy calves, and as many as 50% of dairy calves in the 1 to 3 week age group are shedding Cryptosporidium. Prevalence is higher in the summer than in other months. Although oocysts are shed in greater numbers during the diarrheic phase, the organism has been found in normal feces. In stool surveys of patients with gastroenteritis, the reported prevalence of Cryptosporidium is 1–4% in Europe and North America and 3–20% in Africa, Asia, Australia, and south and Central America (Current et al., 1991). Peaks in the prevalence in developed countries are observed in spring (Casemore et al., 1990) and in the late summer (Van Asperen et al., 1996).

2.13. Cryptosporidiosis in Bangladesh:

Cryptosporidiosis is widespread in Southeast Asia in humans and animals (Lim et al., 2013).Recently *C. hominis*, *C. parvum,* and mixed infections were detected and this was associated with higher stool frequency and a watery stool. A high parasite loads, vomiting and nausea was more frequently associated with *C. hominis* than with *C. parvum* (Ehsan et al., 2015). Cryptosporidiosis in Asia appears to be associated with urbanization and rapid increase of population. Water sources are insufficient and not well maintained and open to contamination by animals and birds. In Bangladesh, *Cryptosporidium* spp. are a significant cause of diarrheal disease in young children

(Albert et al., 1999, Bhattacharya et al., 1999). Rahman et al., 1990, Shahid NS et al., 1987). In Bangladesh, the researchers at the International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B) in Dhaka, study the etiology, pathogenesis, treatment, and prevention of diarrheal diseases. Since 1979, there has been a surveillance system at the ICDDR, B in which every 25th patient (4% sample, 1979−1995) or 50th patient (2% sample, 1996−2003) is studied in detail. Retrospective case-control studies from this surveillance data as well as from other prospective studies from this institute have reported *Cryptosporidium spp*. infection in 1.4−3.5% of individuals, and have identified this parasite as one of the major enteropathogens significantly associated with diarrhea in young children. (Albert et al., 1999, Bhattacharya et al., 1999, Rahman et al., 1990, Shahid et al., 1987). Previous study reported the presence of this parasite in 8.4% of diarrheal stool samples from Bangladeshi children (Haque et al., 2003). However, it is possible that PCR-based methods may have increased detection rates, as reported in other studies (Ajjampur SSR et al., 2008), raising the possibility that infection with these parasites could be even more common in children with diarrhea in this region of the world than has been reported. A significant advance in the study by Haque et al., (Haque et al., 2009), compared with previous studies from Bangladesh, is the molecular characterization of parasites and the association or lack thereof of different species and genotypes with diarrheal symptoms. PCR-based genotyping identified the two major *Cryptosporidium* species, *Cryptosporidium parvum* and *Cryptosporidium hominis.* Cryptosporidium were detected in 14% in calves with diarrhoea and 1% calves without diarrhoea at a dairy farm in Bangladesh (Rahman ASMH et al., 1984).In recent study showed that the rural areas of Bangladesh, calf handlers showed a prevalence of 3.2% for *Cryptosporidium spp*. (Dey et al., 2016).

2.14. Zoonotic potential and public health significance:

Zoonotic parasitic infections are playing a significant role in human health. The zoonotic parasites circulating in South Asia are a significant burden on human health and well being *C. parvum* is a zoonotic parasite, and numerous reports of cryptosporidiosis outbreaks in humans are associated with farm visits and contact with calves (Smith et al., 2004; Miron et al., 1991). However, research into the epidemiology of *C. parvu*m infection, including recent research employing molecular techniques, indicates that only young ruminants, primarily neonates, pose a risk for

the zoonotic transmission of *Cryptosporidium*. *C. parv*um infections are rare in dairy calves older than 30 days, and infections in older calves and adult cattle are most commonly due to *C. andersoni* and the recently proposed *C. bovis*, which are not zoonotic (Fayer et al., 2006). Given that neonatal ruminants, and dairy calves in particular, pose the greatest risk for direct or waterborne zoonotic transmission of *C. parvum*, the risk may be readily managed. Surface and ground water should be protected from young ruminants and their feces, and such manure should be composted or spread on a field where runoff cannot occur. The manure of older ruminants, including cattle on range, is generally not a zoonotic concern with respect to *Cryptosporidium. Cryptosporidiosis* is increasingly recognized as an important agent of diarrhea in normal and immune compromised humans, in particular in infants and young children in developing countries in whom it is the major cause of persistent diarrhoea (Lima and Guerrant, 1992). *Cryptosporidium parvum* has been recognized as a human pathogen since 1976 with the first cases being identified in a previously healthy 3-year old child from a farming community in rural Tennessee (Nime et al., 1976). Farming families may be especially susceptible to infection as indicated by Rahaman et al, (1984), in Bangladesh that showed 9.1% (7 of 77) of family members and infected farm attendants shed oocysts. The primary mode of transmission for enteric pathogens is the fecal-oral route because animal fur, skin, and saliva (Keen et al., 2002) can become contaminated with fecal organisms. Transmission has also occurred from fecal contamination of food and water (Anonymous, 1998). The finding of and pig genotype 1 in humans suggests that farm animals, domestic pets, and some wildlife can be potential sources of human cryptosporidiosis (Current et al., 1983). Animals have been attributed to be the main source of *C.* and other zoonotic *spp.* found in human and environment (Stantic and Pavlenic, 2003).

2.15. Economic impact of *C. parvum* infections:

Economic impact of *C. parvum* infections in ruminants is limited to losses due to mortality and costs involved in treating animals for clinical signs. Nevertheless, these costs may be substantial in some outbreaks of cryptosporidiosis. No evidence indicates that intestinal cryptosporidiosis results in any long-term production effects in ruminants. Abomasal cryptosporidiosis may be an important production-limiting disease of cattle. In fact, *C. andersoni* infections were first discovered during an investigation of feedlot cattle that had a lower than expected daily weight gain

(Anderson et al., 1987). A decreased feed efficiency has been reported in feedlot cattle with C andersoni infections (Ralston et al., 2003). Dairy cows excreting *C. andersoni* oocysts in their feces were found to produce significantly less milk than cows in which oocysts could not be detected (Esteban and Anderson, 1995). No reports on the effect of C andersoni on range cattle exist, and further studies are required to confirm these previous findings. However, the observations made so far indicate that *C. andersoni* could be of economic importance to both the beef and dairy industries. *Cryptosporidium parvum* is considered as the most commonly found enteropathogens in calves during their first weeks of life (Moore and Zeman, 1991). The parasite frequently acts alone, but the losses are more severe when concurrent infections occurred with both viral and bacterial enteropathogens.

2.16. Control of cryptosporidiosis in calves:

As elimination is difficult and relapse can occur, the control of human and animal *cryptosporidiosis* must focus on prevention targeting mainly the destruction of the oocyst in the environment. The following measures are designed for the control ling of outbreaks of cryptosporidiosis on the farms as applied with other enteric infections:
- Calf rearing should be on an all in all out basis. Individual pens should be stringently disinfected between batches of calves (Blewett et al., 1988). Calves should be born and raised in a clean, dry environment. Ideally, newborn calves should be penned individually for 2- 3 weeks (Blewett et al., 1989). Sick calves should be removed immediately from the company of health calves. Healthy calves should have different attendant s from those of sick calves. Utensils should be heated and sterilized if possible daily. Attendants should keep boots, protective clothing as free of faces as possible. Vermin, farm dogs and cats should be controlled. Colostrums management and nutrition should be satisfactory (Fayer et al., 1989). Approximate prophylactic measures against other agents such as rotavirus or ETEC-K99t vaccines should be employed.

2.17. Control of cryptosporidiosis in Human:

Sites that might be endemic need to boil all tap water regardless of whether an outbreak is ongoing or not depending on the source of water supply for an individual and the risk. Patients should be advised to avoid swimming in the water that may be risk of contamination e.g. lake water, or rain water. Education programs are important

means of prevention of cryptosporidiosis targeting immunocompromised patients, the dairy farms, the water supplysectors, swimming pool and recreational water operators and general public. The programs should include information on risks of infection or contamination from various exposures, advised accordingly on minimizing the risks. There should be initiatives to promote the HACCP techniques in dairy farms.

2.18. Diagnostic techniques of Cryptosporidium:

2.18.1. Microscopic staining methods:

A microscopic staining method depends on the concentration and staining of fecal samples indicated the conventional detection method of oocyst. The small size of the oocysts makes them indistinguishable at the species level based on morphology by light microscope (Fall et al., 2003). Various staining techniques have been employed to aid in the differentiation (on glass slides) of Cryptosporidium oocysts from other protists and environmental or fecal debris. The simplest and least expensive stains include dimethylsulphoxide (DMSO)- carbolfuchsin (Pohjola et al., 1984), Kinyoun (Ma and Soave et al., 1983), safranin–methylene blue (Baxby et al., 1984) and acid fast (modified Ziehl-Neelsen) (Henriksen and Pohlenz, 1981). Although useful, approaches employing these stains can suffer from low specificity and/or sensitivity, particularly for samples containing small numbers of oocysts (Morgan et al., 1998; Clark et al., 1999). In addition to these direct staining techniques, there are negative staining methods, using chemicals such as light green merbromide (Chichino et al., 1991) or malachite green (Elliot et al., 1999), which stain “background” material on slides and leave Cryptosporidium oocysts unstained. However, these latter methods are also relatively time consuming and require considerable skill by the microscopist to achieve reliable detection of oocysts. Although useful for the detection of oocysts, none of these approaches allow the identification of Cryptosporidium to species. To perform this staining method assisted by experienced microscopist by definite method.

2.18.2 Immunological-based detection methods:

For the detection of Cryptosporidium oocysts and diagnosis of cryptosporidiosis Immunological-based techniques had given some advantages over light microscope. Polyclonal fluorescent antibody tests (Stibbs and Ongerth, 1986) latex agglutination reactions(Sterling and Arrowood, 1986), immune fluorescence (IF) with monoclonal

antibodies (mAbs) (Arrowood and Sterling, 1989), enzyme-linked immunosorbent assays (ELISA) reverse passive haemagglutination (RPH) (Farrington et al., 1994), immunoserology using IF detection (Hernandez et al., 1994) and ELISA (Priest et al., 1999) and solid-phase qualitative immunochromatographic assays (Garcia et al., 2000)have been developed for the detection of cryptosporidiosis. Non-specificity of antibody based methods due to cross-reactivity with other microorganisms can be problematic. For example, in the study of gill washings and haemo-lymph from oysters that harbored oocysts of Cryptosporidium a variety of organisms and particulate material of many sizes and shapes were observed that fluoresced as brightly as the oocysts (Fayer et al., 1999)

2.18.3. Fluorescence in-situ hybridization (FISH):

FISH has been suggested as a tool for the specific detection of *Cryptosporidium parvum* (Lindquist, 1997; Vesey et al., 1997). Vesey et al. (1997) also showed that the stain ability of oocysts with the FISH method correlated with encystations. The FISH method could be combined with the immunofluorescent assay (IFA) method. However, the FISH-fluorescence signal is relatively weak, which makes microscopic interpretation difficult.

2.18.4. Molecular techniques:

One of the most extensively tested procedures is the polymerase chain reaction (PCR) for detection of specific sequences of nucleic acids that may be species or genus-specific. Clearly, the ability to distinguish between *C. parvum* and other morphologically similar members of the genus is useful and nucleic-acid-based techniques may prove useful for this. PCR is sensitive to the concentration of many compounds within the reaction mixture; those of particular concern to researchers working with water concentrates are divalent cations and humic and fulvic acids, which are frequently found in water and can cause a high degree of inhibition. Protocols for the detection of Cryptosporidium oocysts by PCR and a wide variety of primers have been described by many researchers. These primers have been designed from various regions of the genome. Primers with apparent specificity include those from regions coding for the 18S rRNA (Johnson, 1995), or mRNA coding for the Cryptosporidium heat shock protein Hsp70 (Stinear et al., 1996; Kaucner and Stinear, 1998), in combination with cell culture (Rochelle et al., 1996; Rochelle et al, 1997).

PCR and The direct sequencing of an amplified gene or gene portion remains the gold standard approach for detecting genetic variation or polymorphisms and, consecutively, accurate specie assignment. It is the most common technique used throughout the world, regarding the genotype of *Cryptosporidium and Giardia*. PCR-single strand conformation polymorphism (PCR-SSCP) is a particularly useful approach based on the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturating gel and its dependence on the conformation and size of the molecule. This technique may detect a single point mutation in amplicons till a size of 500bp (Gasser, 2006). PCR-SSCP has been particularly used for display sequence variation in SSU and hsp70 genes *of* Cryptosporidium and is useful for the screen of genetic variability and unknown mutations (Chalmers et al., 2005; Gasser, 2006; Jex et al., 2008). Real Time PCR was developed in the early 1990s (Higuchi et al., 1993). It allows the amplification in PCR to be monitored in real time. The Real Time principle consists in the incorporation of a specific intercalating dye in the PCR and measuring the changes in the fluorescence via a digital camera (Higuchi et al., 1993). Although it is technically very similar to a standard PCR, RT-PCR has several advantages: it does not requires handling after the amplification since it allows high throughput analysis in a ―closed-tube‖ format, and it can be used to differentiate amplicons of varying sequences by melting curve analysis. The most recent improvements inside this technique, introduced better dyes and capillary thermal cycler, enabling the quantification (Ct or cycle threshold) of the sample by comparison with DNA standards. Also, the use of the dyes allows the determination of the melting temperature or denaturation of the amplicons: since this temperature depends on the sequence composition, the melting curve is used to characterize the variation among samples. Furthermore, the range of Real Time application became larger by the introduction of probes sequence-specific (Monis et al., 2005). On the otherhand, there are Real Time PCR approaches using TaqMan probes described targeting Cryptosporidium and Giardia genes. In 2001 and 2003 some author developed probes targeting the Cp11 and 18S rRNA gene of Cryptosporidium (Higgins et al., 2001; Keegan et al., 2003), and in 2002 the β-tubulin gene of Cryptosporidium (Tanriverdi et al., 2002). TaqMan probes are one of the most widely used Real Time PCR chemistries mainly because the assay design is easy and the assays are robust. TaqMan assays can be multiplexed by using probes with different colored fluorophores (Monis et al., 2005).

2.18.5. PCR-RFLP:

PCR-restriction fragment length polymorphism (PCR-RFLP) is a technique widely used in *Cryptosporidium* and *Giardia* genotyping. Different PCR-based techniques employing specific primer pairs for the selective amplification of different genetic loci, followed by enzymatic cleavage or sequencing, have been used to characterize and classify Cryptosporidium species or "genotypes" (Quintero-Betancourt et al., 2002; Xiao et al., 2004).specific primers under a specific temperature cycles is the base of the PCR for amplification of a chosen target sequence. During translation of RNA to proteins two scenarios had seen in DNA sequence (a) the mutation does not cause a change in the amino acid (synonymous mutation), or (b) the mutation may change the codon changing the amino acid (non-synonymous mutation). If these mutations or polymorphisms exist in different species and strains, the PCR-RFLP takes advance of this fact and shows different restriction patterns in agarose gel for particular species. For C*ryptosporidium* species assignment, the most common genes used are the *Cryptosporidium* oocyst wall protein (cowp) (Spano et al., 1997), the 70 kDa heat shock protein (hsp70) (Gobet et al, 2001), the thrombospondin-related adhesive protein (trap) genes (Spano et al., 1998) and the 60 kDa glycoprotein (gp60) gene (Wu et al., 2003). 18S rRNA gene was first cloned and the sequence determined in *C. parvum* in 1992 (Cai et al., 1992). Latter, some authors determine the ribosomal RNA gene organization in the same organism, based on the hypothesis that the genes encoding the cytoplasmic ribosomal RNAs (rRNA) in most eukaryotes are organized into transcriptional units with a small subunit rRNA gene, a 18S rRNA gene, and a large subunit rRNA gene in a 5-3'orientation separated by internal transcribed spacers, what they called the rDNA unit (Le Blancq et al., 1997). In this work, the authors calculated the size of the large subunit rRNA in 3.6 kb, and concluded that the rDNA unit in *C. parvum* has the standard arrangement of 5' small subunit rRNA - internal transcribed spacer 1-5.8S rRNA - internal transcribed spacer 2- large subunit rRNA 3', the minimum size would be 6.5 kb, there are five copies of the rDNA unit per haploid genome in *C. parvum*, there are two types of rDNA unit in *C. parvum* and rDNA units are dispersed through the genome of *C. parvum.* From the CryptoDB database, part of the EuPathDB, these rDNA units are localized in chromosome 1, 2, 7 and 8 of *C. parvum*. Fixed differences on the sequence of this gene among Cryptosporidium species turn it useful in genotyping Cryptosporidium species. PCR

amplification from small amounts of genomic DNA from a single Cryptosporidium oocyst is not practical; amplicons are almost always produced from an isolate representing a population of oocysts.

2.19. Recent developments in molecular epidemiologic tools

Different types of molecular diagnostic tools have been used in the differentiation of Cryptosporidium species/genotypes and *C. parvum* and *C. hominis* subtypes. Small subunit (SSU) rRNA based tools are now generally used in genotyping .Cryptosporidium in humans, animals and water samples. A review of original studies on *Cryptosporidium* genotyping during the last three years revealed the use of SSU rRNA tools in 100 (86%) of 116 publications. In particular, a PCR-RFLP tool that targets an 830-bp fragment of the gene and uses SspI and VspI restrictions for genotyping (Xiao et al., 2001) is commonly used, being reported in 70 (60%) publications. The widespread use of the SSR rRNA gene in Cryptosporidium genotyping is largely due to the multi-copy nature of the gene and presence of semi conserved and hyper-variable regions, which facilitate the design of genus-specific primers. Other molecular diagnostic tools based on other genes were popular previously, but their use in Cryptosporidium genotyping has decreased in recent years.

CHAPTER-III
MATERIALS AND METHODS

3.1. Sources of samples:

Fresh fecal samples were collected from 160 calves, located on different dairy farms in Chittagong Metropolitan Area and Patiya Upazilla. In 2015 fecal specimens were obtained from calves aged less than 45 days.

3.2. Study area and period:

In the present study was carried out in selected areas in Chittagong Metropolitan Area and Patiya Upazilla. The study period was June, 2015 to January, 2016.

3.3. Collection and Preservation of the samples:

All fecal samples were collected from 200 calves and kept in labeled sterilized plastic containers that were immediately capped, labeled and placed on ice box. Then collected fecal samples were taken into two different containers as one part was collected in a empty sterilized plastic container for deep freezing and another part of fecal sample were collected in a vial containing 10% formalin with labeling. Then the sample was transported to the clinical pathology laboratory, CVASU and kept refrigerated at -20^0 C without preservatives until processed.

3.4. Oocyst Examination:

Microscopic diagnosis of Cryptosporidium Oocyst was performed by Ziehl-Neelsen (Z-N) technique in the clinical pathology laboratory, Department of pathology and parasitology, CVASU.

3.4.1. Ziehl-Neelsen staining technique:

This technique was used for the demonstration of oocysts of Cryptosporidium species in feces. Alternatively, the modified auramine-phenol stain may be used (www.evaluationsstandards.org.uk 2007)

3.4.1.1. Materials and reagents:

Materials required:

- Fecal sample
- Tooth pick
- Slides
- Coplin jar
- Compound microscope
- Immersion oil
- Plastic tray
- Electric drier

Reagents required

- Methanol
- 3% Carbol fuchsin
- 1% Acid alcohol
- 0.4% brilliant green

3.4.1.2. Procedure of staining:

1. Prepared a medium to thick smear by using the tooth pick and air dried.
2. Fixed in methanol for 3 min and air dried
3. Flooded the slide with 3% Carbol fuchsin and left for approximately 15 minutes.
4. The slide was than rinsed with running tap water.
5. Flooded the slide with 1% acid methanol to decolorized and left for 15 to 20 sec.
6. Again rinsed with running tap water
7. 0.4% brilliant green was then poured to counter stain for 30 seconds.
8. Finally Rinsed with tap water and air dried.
9. Then examined under compound light microscope using x10, x 40 and x 100 x 50 objective.

3.4.1.3. Microscopic examination:

Air dried slides were examined under compound light microscope. In case of ×100 microscopy oil of emulsion was used for increasing resulation. The slides were examined in zik jark method. Several fields were examined to detect oocyst of

Cryptosporidium. Oocysts of Cryptosporidium are found under microscope as pink color round and spherical bodies as it take carbol fuchin stain. The back ground of slide takes blue color of brilliant green.

3.5. DNA extraction from selected fecal samples:

Total genomic deoxyribonucleic acid (DNA) was directly extracted from Z-N staining positive fecal samples, using available stool DNA isolation kit (FavorPrepTMstool DNA Isolation Mini Kit .Cat. No.:FASTI001). The experiment was carried out at Molecular pathology laboratory, CVASU. DNA was extracted following the manufacturers procedures.

Table 5: Composition of FavorPrep^{TM}stool DNA Isolation Mini Kit. (Cat. No.: FASTI001)

Materials	**Amount**
Glass beads	12 g
SDE1 Buffer	20 ml
SDE2 Buffer	7 ml
SDE3 Buffer	15 ml
SDE4 Buffer	20 ml
Wash Buffer	20 ml
Elution Buffer	15 ml
Proteinase K+	11 mcg
SDE mini column	50 pcs
2.0 ml Collection tube	100 pcs
1.5 ml Elution tube	50 pcs
Bead tube	50 pcs

3.5.1. Protocol for isolation of DNA from stool samples:

(FavorPrep^{TM} stool DNA Isolation Mini Kit.)

1. At first 200 mg of Glass beads was added into a 2.0 ml bead tube and 50-100mg of stool sample was transferred into bead tube then placed on ice.

2. 300µl of SDE1Buffer and 20 µl of proteinase K was added to the sample and vortex was performed at maximum speed for 5 minutes.

3. Sample was incubated at 700c for 10 min and vortexes twice during incubation. 4. Brief spinning of the tube was done to remove drops from the inside of the lid.

4. Sample was allowed to cool and 100 µl of SDE2 buffer was added to the sample then then mixed by vortexing.

5. Incubated the sample on ice for 5 min.

6. Sample was centrifuged at 1400rpm for 5 min.

7. Supernatant was transferred carefully to a 1.5ml microcentrifuge tube and discarded the stool pellet. 8. 200 µl of SDE3 buffer was added to the sample and mixed well by vortexing. Then incubated at room temperature for 2 minutes.

9. Sample was centrifuged at 1400rpm for 2 min.

10. 250 µl of supernatant was transferred to a clean 1.5 ml micro centrifuge tube and the pellet was discarded.

11. 250 µl of SDE4 buffer and 250 ml of 99%ethanol was added to the sample and mixed thoroughly by vortexing.

12. A SDE column was placed into a collection tube and transferred all the sample mixture to the SDE column. And then centrifuged at 1400rpm for 1 min and discarded the flow-through.

13. SDE column was placed into a new collection tube.

14. 750 µl of wash buffer was added to the SDE column. Sample was centrifuged at1400rpm for 1 min and discarded the flow–through. This step was done repeatedly one more time.

15. SDE column was centrifuged at 1400 rpm for 3 min to dry the column.

16. SDE column was placed into an elution tube and 50-200 µl of preheated elution buffer was added to the membrane of SDE column.

17. The spin column was centrifuged for 1 min to elute DNA.

Fig. 7. Stool DNA extraction kit

Fig. 8. PCR primers: 18srRNA gene primer

3.6. PCR analysis of 18s rRNA gene:

In case of identifying the *Cryptosporidium* infection the oocysts/cysts DNA was tested by nested polymerase chain reaction (PCR) of 18S rRNA. PCR of the extracted DNA was conducted using the commercially available Intron ® PCR 2x Master mix and primer (provided in the table).

Table 6: Primer for 18s rRNA gene amplification

Name of primer	Sequence of primer	Sequence length	Start from	Stop in
C18S F1	GACATATCATTCAAGTTTCTGACC	24bp	271	294
C18S R1	CTGAAGGAGTAAGGAACAACC	21bp	1028	1008
C18S NF1	CCTATCAGCTTTAGACGGTAGG	22bp	293	314
C18S NR1	CTAAGAATTTCACCTCTGACTG	22bp	873	852

Length of primary PCR: 758bp (Ryan et al., 2003)

Length of nested PCR: 581bp

3.6.1. *Cryptosporidium* spp. CSP06 18S ribosomal RNA gene, complete sequence: Gene Bank accession no: AF112573.1

1 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC AT GCATGTCT

61 AAGTATAAAC TTTTATACGG TTAAACTGCG AATGGCTCAT TATAACAGTT ATAGTTTACT

121TGATAATCTT TTTACTACAT GGATAACCGT GGTAATTCTA GAGCTAATAC ATGCGAAAAG

181 GCCTGACTTT ATGGAAAGGT TGTATTTATT AGATAAAGAA CCAATATTAT TTGGTGATTC

241 ATAATAACTT TACGGATCAC AATTATTTGT GACATATCAT TCAAGTTTCT **GACCTATCAG**

301 CTTTAGACGG TAGGGTATTG GCCTACCGTG GCAATGACGG GTAACGGGGA ATTAGGGTTC

361 GATTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACATCTA AGGAAGGCAG CAGGCGCGCA

421 AATTACCCAA TCCTAATACA GGGAGGTAGT GACAAGAAAT AACAATACAG GACCTTACGG

481 TTTTGTAATT GGAATGAGTT AAGTATAAAC CCCTTTACAA GTATCAATTG GAGGGCAAGT

541 CTGGTGCCAG CAGCCGCGGT AATTCCAGCT CCAATAGCGT ATATTAAAGT TGTTGCAGTT

601 AAAAAGCTCG TAGTTGGATT TCTGTTAATA ATTTATATAT AATATTACGG TATTTATATA

661 ATATTAACAT AATTCATATT ACTTTATTTT TAGAGTATAT GAAATTTTAC TTTGAGAAAA

721 TTAGAGTGCT TAAAGCAGGC ATATGCCTTG AATACTCCAG CATGGAATAA TATTAAAGAT

781 TTTTATCTTT CTTATTGGTT CTAAGATAAA AATAATGATT AATAGGGACA GTTGGGGGCA

841 TTTGTATTTA ACAGTCAGAG GTGAAATTCT TAGATTTGTT AAAGACAAAC TAGTGCGAAA

901 GCATTTGCCA AGGATGTTTT CATTAATCAA GAACGAAAGT TAGGGGATCG AAGACGATCA

961 GATACCGTCG TAGTCTTAAC CATAAACTAT GCCAACTAGA GATTGGAGGT TGTTCCTTAC

1021 TCCTTCAGCA CCTTATGAGA AATCAAAGTC TTTGGGTTCT GGGGGGAGTA TGGTCGCAAG

1081 GCTGAAACTT AAAGGAATTG ACGGAAGGGC ACCACCAGGA GTGGAGCCTG CGGCTTAATT

1141 TGACTCAACA CGGGAAAACT CACCAGGTCC AGACATAGGA AGGATTGACA GATTGATAGC

1201 TCTTTCTTGA TTCTATGGGT GGTGGTGCAT GGCCGTTCTT AGTTGGTGGA GTGATTTGTC

1261 TGGTTAATTC CGTTAACGAA CGAGACCTTA ACCTGCTAAA TAGACATAAA AAATTCTTTT

1321 TTTATTTGTC TTCTTAGAGG GACTTTGTAT GTTTAATACA GGGAAGTTTG AGGCAATAAC

1381 AGGTCTGTGA TGCCCTTAGA TGTCCTGGGC CGCGCGCGCG CTACACTGAT GCATCCATCA

1441 AGTTAATTAT CCTGTTTCGA AGGAAATGGG TAATCTTTTG AATATGCATC GTGATGGGGA

1501 TAGATCATTG CAATTATTGA TCTTTAACGA GGAATTCCTA GTAAGCGCAA GTCATCAGCT

1561 TGCGCTGATT ACGTCCCTGC CCTTTGTACA CACCGCCCGT CGCTCCTACC GATTGAATGA

1621 TCCGGTGAAT TATTCGGACC ATACTTTGTA GCAATACATG TAAGGAAAGT TTCGTAAACC

1681 TTATCTCTTA GAGGAAGGAG AAGTCGTAAC AAGGTTTCCG TAGGTGAACC TGCAGAAGGA

1741 TCA

Forward – Green color

Reversed – Red color

*CC-common for both primers

Table 7: PCR Master Mix solution [Catalog no. 25027 (1ml). Intron Biotechnology] contained the following reagents:

2X PCR master mix solution(i-TaqTM)	0.5 ml x 2
TaqTM DNA polymerase (5U/µl)	2.5 U
dNTPs	2.5 mm each
PCR reaction buffer	1X
Gel loading buffer	1X

3.6.2. Other instrument and chemicals used for PCR

- Thermo cycler (Q-cycler)
- Thin walled PCR tubes (0.2ml capacity)
- RNase free water
- Agarose gel (1%)
- Ethidium bromide
- Micropipettes
- Tips for micropipettes
- Ice
- Gloves

Table 8: Composition of reaction mixture for 1st round nested PCR or normal PCR

Components	Quantity	Total amount	Final Concentration (20 µl)
2X PCR master mix	10 µl	10 µl	-------
Forward Primer (10pmole/µl)	1 µl	1 µl	10 pmole
Reverse Primer (10pmole/µl)	1µl	1 µl	10 pmole
Water	6 µl	6 µl	----
Total volume	18 µl	18 µl	----
Extracted DNA Template	2 µl	2 µl	----
Grand Total volume	20 µl	20 µl	----

Table 9: Composition of reaction mixture for nested PCR

Components	Quantity	Total amount	Final Concentration (20 µl)
2X PCR master mix	10 µl	10 µl	-------
Forward Primer (10 pmole/µl)	1 µl	1 µl	10 pmole
Reverse Primer (10 pmole/µl)	1µl	1 µl	10 pmole
Water	7µl	7 µl	----
Total volume	19µl	19 µl	----
Extracted DNA Template	1µl	1 µl	----
Grand Total volume	20 µl	20 µl	----

3.7. Nested PCR analysis of 18s rRNA gene amplification

A region of the *Cryptosporidium* 18s ribosomal RNA (18SrRNA) gene was amplified using the primers C18S F1, C18SR1, C18SNF1and C18SNR1 (Table 6). A PCR product of 758bp was amplified by C18S F1, C18SR1 in primary amplification and in the secondary PCR a fragment of 581 bp was amplified by nested primer C18SNF1and C18SNR1. The thermal cycler was programmed according to Ryan et al. (2003) to run the PCR (Table 10)

Table 10: Steps and conditions of thermal cycling for 18s rRNA gene PCR, according to Ryan et al. (2003)

SL No.	Steps	Temperature	Time
Step 1	Initial Denaturation	94°C	2 min
Step 2	Denaturation	94°C	30sec
Step 3	Annealing	54˚C	30sec
Step 4	Extension	72 °C	30sec
Step 5	45 cycles from step 2 to step 4		
Step 6	Final Extension	72 °C	10 minutes

3.8. Agar gel electrophoresis

1% agarose gel was made by using 0.5 g agarose powder and 50 ml TAE buffer with ethidium bromide. The DNA amplicons were visualized using 4 µl of the final PCR product and 2 µl standard 100bp DNA markers (Invitrogen) at 120 V/100mA for 30 min. Gels were photographed using a gel documentation system Positive or negative amplifications were evaluated as presence or absence of visible orange colour bands on agarose gels under UV light (Ryan et al., 2003).

Procedure of Agar gel electrophoresis was as follows:

3.8.1. Materials and Reagents

- Conical flask
- 50 ml test tube
- Aluminum foil
- Electronic balance
- Microwave oven
- Gel electrophoresis tank
- Agarose
- Micropipette
- UV transilluminator
- 50x TAE buffer.
- Distilled water.
- Ethidium bromide

3.8.2. Procedure of agar gel electrophoresis

1. For 1 agarose, 500 mg of agarose and 50 ml of 1 X TAE buffer was mixed thoroughly in a conical flask.
2. The mixture was heated in a microwave oven until agarose was completely dissolved.
3. The agarose-TAE buffer solution was then allowed to cool in room temperature.
4. Gel casting tray was prepared by sealing ends of gel chamber with tape or appropriate casting system and placed appropriate number of combs in gel tray.
5. 10 microlitre of ethidium bromide was added to agarose-TAE buffer mixture, shaked well and poured into gel tray.

6. The gel was then allowed to be cool (left for 15-30 minutes at room temperature).
7. The comb(s) were removed and the electrophoresis chamber was filled with 1x TAE buffer until the casted gel is drowned completely.
8. 4 µl of DNA and 2 µl of 100bp marker (ladder) were loaded into gel.
9. The electrophoresis was run at 120 volt and 100 mA for 40 minutes.
10. Then the gel was taken to the UV transilluminator for image acquisition and analysis.

3.9. Precautions followed in the PCR laboratory

All procedures were carried out under strict aseptic conditions at the laboratory. Maximum precautions were taken to avoid contamination. Hand gloves and musk were used all the time, nothing were touched in bare hands to avoid contamination with RNase from the skin. All the samples were processed under the Bio-safety cabinet Class II. Isolation of DNA, preparation of PCR reaction mix, thermo cycling and analysis were performed in three separate rooms to avoid carry-over contamination. Eye protector was used while working with transilluminator (produces UV radiation) for documentation of PCR products.

3.10. Histopathological Examination:

Finally total numbers of 5 dead calves were post-mortem for the hispathological investigation to find out the lesion in intestinal part. For histopathological study formalin fixed tissue samples were washed and dehydrated in graded ethanol and embedded in paraffin wax. Fixed tissues were sectioned at 5 µm thickness and stained with hematoxylin and eosin as per standard method (Lee Luna, 1968).

3.10.1. Collection of tissue and tissue processing

During tissue collection the following point were taken into consideration; the tissues were collected in conditions as fresh as possible. Normal and diseased tissues were collected side by side. The thickness of the tissues were as less as possible (5mm approximately). Formalin fixed tissues were processed by following protocol.

3.10.1.1. Fixation: 10% neutral buffered formalin was added in the plastic container. (10 folds of the tissue size and weight) and fixed for 3-5 days.

3.10.1.2. Washing: The tissues were trimmed into a thin section and washed over night in running tape water to remove formalin.

3.10.1.3. Dehydration: The tissues were dehydrated by ascending ethanol series to prevent shrinkage of cells as per following schedule. The tissues were dehydrated in 50%, 70%, 80%, 95%, 100%, 100%, 100% ethanol one hour in each.

3.10.1.4. Cleaning: The tissues were cleaned in chloroform for 3 hours to remove ethanol (two changes; one and half hour in each).

3.10.1.5. Impregnation: Impregnation was done in melted paraffin (56- 60°c) for 3 hours.

3.10.1.6. Sectioning: Then the tissues were sectioned with a microtome at 5-µm thickness. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The sections were allowed to spread on warm water bath at 40-42°C. Then the sections were taken on grease free clear slides.

3.10.1.7. Drying: The slides containing section were air dried and kept in cool place until staining**.**

3.10. 2. Routine hematoxylin and eosin staining procedure:

The sectioned tissues were deparaffinized in three changes of xylene (three minutes in each). Then the sectioned tissues were rehydrated through descending grades of alcohol (three changes in absolute alcohol, three minutes in each; 95% alcohol for two minutes; 80% alcohol for two minutes; 70% alcohol for two minutes) followed by distilled water for five minutes. The tissues were stained with Harris hematoxylin for fifteen minutes and then washed in running tap water for 10-15 minutes. The tissues were differentiated in acid alcohol by 2 to 4 dips (1 part HCL and 99 parts 70% alcohol) and washed in tap water for five minutes followed by 2-4 dips in ammonia water until sections were bright blue. Stained with eosin for one minute and differentiated and dehydrated in alcohol (95% alcohol: three changes, 2-4 dips each; absolute alcohol: three changes 2-3 minutes for each). Cleaned by xylene and three changes was made in every five minutes in each. Tissues were mounted with cover

slip by using DPX. The slides were dried at room temperature and examined under a low (10X) and high (40X, 100X) power objectives.

3.10.3. Acid Fast (Ziehl–Neelsen) staining for detection of Cryptosporidium spp.

The paraffin embedded tissue samples from nodular lesions were subjected to acid fast (Ziehl–Neelsen) staining. First, tissue sections were deparaffinized and after rehydrated in distilled water. Then the slides were dipped in heated Carbol-fuchsine solution (heated in microwave for 45 seconds) for 5 minutes followed by a washing step in running tap water to remove the excess stains. Further destaining was performed in 1% Acid alcohol until the tissue appearance changes into light pink color. The slides were then washed again in running tap water for 5 minutes, rinsed in distilled water followed by dipping into working methylene blue for 30 seconds. Finally the slides were rinsed in water, dehydrated, cleared and mounted with cover-slip using DPX.

3.11. Statistical analysis

Data obtained were stored and cleaned in the Microsoft Excel programme 2007 and then exported to STATA-13(College Station, Texas 77845, USA) for statistical analysis where significant was determined $P<0.05$. Descriptive analysis was performed on the data of collected sample history according to different variables. Descriptive analysis was also done on status of Age, sex, diarrhoeic and months. Results were presented as frequency number and percentage.

CHAPTER IV

RESULTS

In this study a total of 160 fecal samples were tested for oocyst examination by Z-N techniques and 15 samples (9.37%) were found positive under light microscopy (Fig. 9) which was then further examined by PCR for more confirmation. Among the 15 samples 3 samples (1.88%) showed positive reaction in PCR (Fig.10).

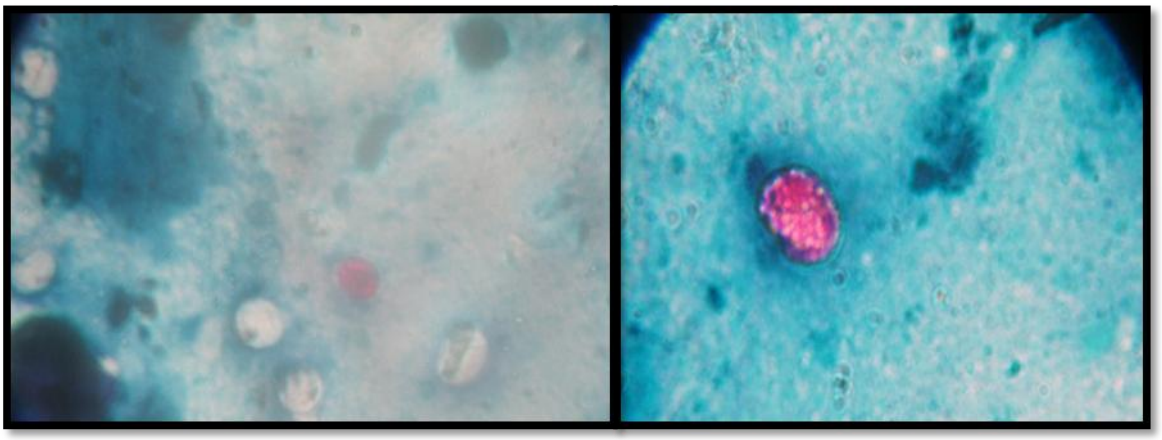

Fig. 9: Bright pink coloured oocysts of *Cryptosporidium spp*. modified Z-N staining under 100 x objectives.

PCR amplification of the *Cryptosporidium* DNA:

A nested PCR was done by using 18s rRNA gene primer and after running through agar gel electrophoresis the samples were found positive based on 580bp band visualization (Fig.10).

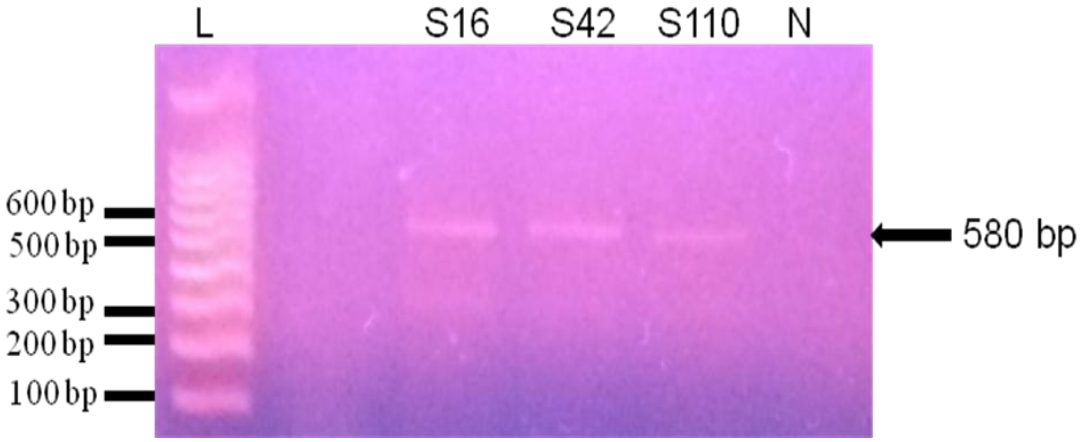

Fig. 10: positive PCR amplification by nested PCR of 18srRNA gene of three positive samples. Lane (L) consists of DNA (100bp plus Intron®) marker, calves fecal sample was marked as S16, S42, and S110. Negative control (N) was determined by using water instead of DNA template.

Overall prevalence of *Cryptosporidium* infection includes microscopic examinations are given below with chart:

Table 11: Diarrhoea related prevalence of Cryptosporidium in neonatal dairy calves.

Diarrhoea	No. of animals examined	No. of positive cases	% positive	p-value
Yes	7	3	42.85	0.002
No	153	12	7.9	

In this study we represent the prevalence of positivity of cryptosporidiosis in calves based on age, sex, diarrhoeic condition and seasonal status. Out of 160 collected samples, only 7 samples were diarrhoeic (4.37%) and 153 were non diarrhoeic (95.63%) in consistency. Among these samples three (42.85%) were detected positive in diarrhoeic and twelve (7.9%) were positive in non diarrhoeic samples (Table 11).

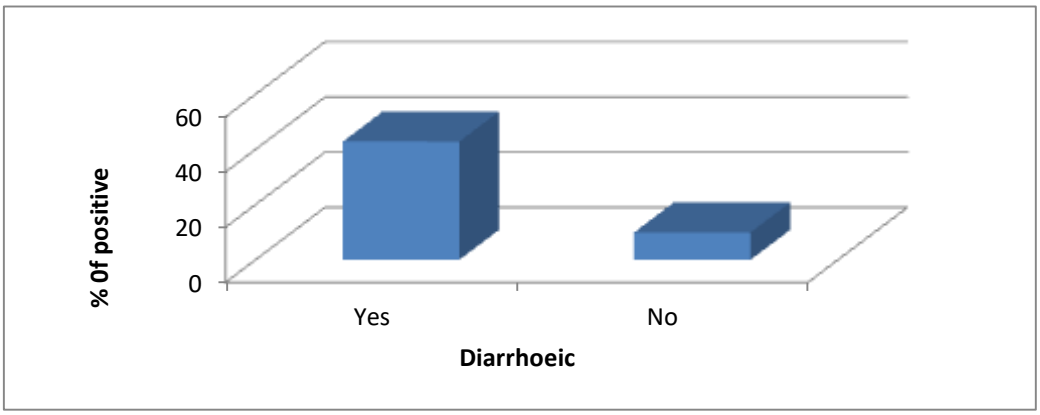

Fig. 11: Diarrhoea related prevalence of Cryptosporidium in neonatal dairy calves.

Among all included factor age is the most potential factor of susceptibility of cryptosporidiosis in calves. The presented result indicated that June-July to august calves is more positive with cryptosporidiosis. Infection rate was varied in different age group. Highest percentage (11.59%) is found in 16-30 days age group followed by 31-45 days age (8.33%) and 0-15 days age (7.27%). (Table 12).

Table 12: Age related prevalence of Cryptosporidium in neonatal dairy calves.

Age (Days)	No. of animals examined	No. of positive cases	% positive
0-15	55	4	7.27
16-30	69	8	11.59
31-45	36	3	8.33
Total	160	15	9.37

The result estimated that, 16-30 days old calves are more susceptible than other given aged.

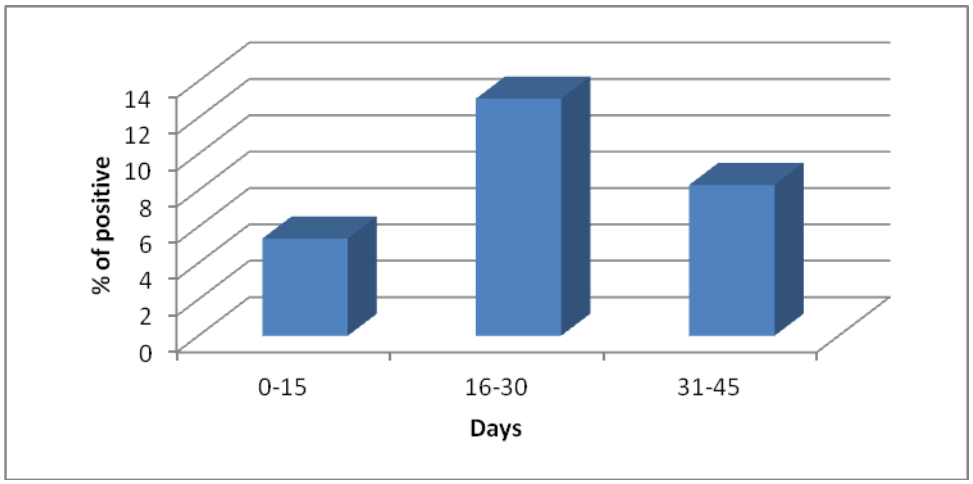

Fig. 12: Age related prevalence of Cryptosporidium in neonatal dairy calves.

The majority of the dairy farming younger than 45 days olds were headed by males (53.75%; 86/160) while the majority of the respondents were female (46.26%; 74/160).

Table 13: Sex related prevalence of Cryptosporidium in neonatal dairy calves.

sex	No. of animals examined	No. of positive cases	% positive
Male	86	11	12.79
Female	74	4	5.4

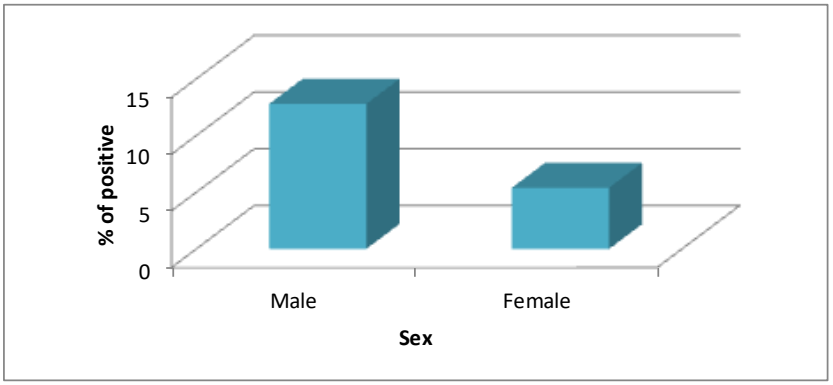

Fig 13: Sex related prevalence of Cryptosporidium in neonatal dairy calves.

Overall the highest percent prevalence of Cryptosporidiosis in cattle recorded was during summer (June-July) (12.35%), followed by August to September (summer) (6.6%) and the lowest in autumn (October-November) (3.84%) (Table14).

Table 14: season related prevalence of cryptosporidiosis in neonatal dairy calves.

Season	No. of animals examined	No. of positive cases	% positive
June-July	89	11	12.35
August-September	45	3	6.6
October-November	26	1	3.84

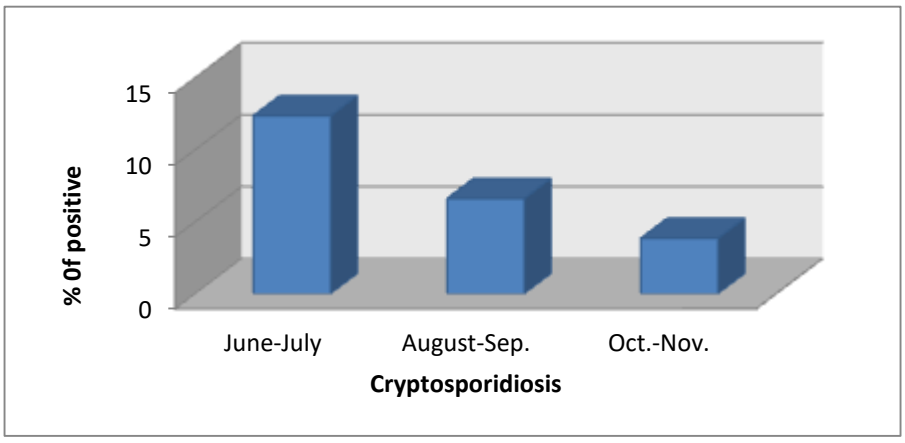

Fig 14: Season related prevalence of cryptosporidiosis in neonatal dairy calves.

Histopathological Features:

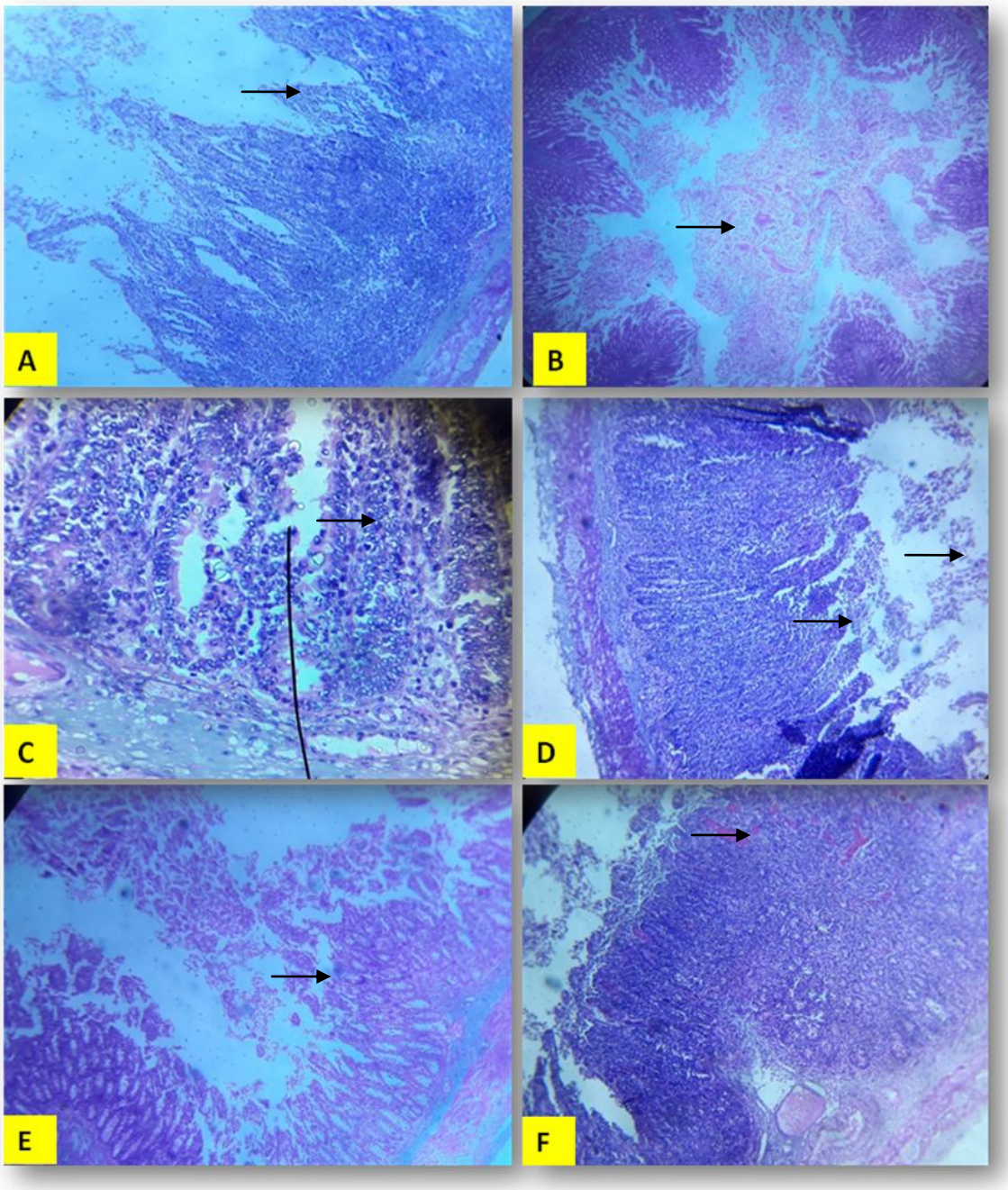

Fig 15: Histopathological findings in diarrhoeic calves. (Arrow indicated the different changes observed under microscope (4x, 10x and 100x) for each represented slide.

Histologic sections of intestine from collected dead calves in the study were examined to confirm that *Cryptosporidium* spp. infection of intestinal epithelium induced severe enteritis includes sloughing of epithelium, reactive cell infiltration, villous atrophy etc that compared with from disease. The above picture shows atrophied villi (A, D), sloughing of mucosa (B, D). Histological section of intestine from less than two month old dead calves showed following changes under microscope :Atrophied villi with lymphocytic infiltration and the hyperplasia of the crypts (A), Sloughing off mucosa with huge reactive cell infiltration in lamina propia (B), oocyst like structure on the tip of villi (C), Atrophied villi and sloughing of mucosa (D), presence of neutrophilic infiltration in lamina propia ; (E) and slightly congested mucosal layer with cellular infiltration.

CHAPTER V
DISCUSSION

Results revealed that the prevalence of Cryptosporidiosis was significantly higher ($p<0.05$) in diarrhoeic than the non diarrhoeic individuals indicating the importance of cryptosporidium in the causation of diarrhoea. In Bangladesh, previous study also recorded higher incidence of infection in diarrhoeic (14%) calves than non diarrhoeic (1%) calves (Shahid et al., 1987; Ehsan et al., 2015). Paul et al. (2008) reported the infection is greater among diarrhoeic (32.33%) than the non diarrhoea (22.64%) calves in India which also supports the present study. Small sample size in the study of singh et al. (2006) may be the cause of the cryptosporidiosis at higher in diarrhoeic calves than the present study. The study was also done to assess the effect of age, sex, diarrhoea and status on the occurrence of the *cryptosporidium* spp. It should be noted that the estimated prevalence in the cross-sectional study could have been affected by intermittent shedding of oocyst. From this result, it was observed that the infection was highest in the age of 16-30 days old calves in comparison to the other recorded aged calves. High degree of prevalence was detected in diarrhoetic calves than non diarrhoetic calves. Susceptibility of different age groups of calves to cryptosporidium spp. Infection has been reported in different countries (Sturdee et al., 2003; Santin et al., 2008; Paul et al., 2008) and in Bangladesh (khair et al., 2014; Ehsan et al., 2015). Higher incidence of the infection among cattle calves of 14- 28 days old was recorded by Olson et al. (1997) in British Columbia. Higher incidence of the infection was also recorded in male calves than in female calves by Khair et al. (2014) in Mymensingh and Sirajgonj district of Bangladesh. In contrast Nouri and Torogi (1991) also reported higher rate of infection in male diarrhoeic calves than female calves in Iran. As the complexes of dairy farm used to cull the male calves soon after birth therefore, the number of fecal samples collected male comparatively less than female calves. This may be significant reason for the difference of *cryptosporidium* spp. Infection between male and female calves in present study (Table 13). Dairy calves shedded crypto oocyst apparently higher in the summer and autumn season than the winter season due to high temperature and humidity with sudden heavy rainfall for transmission of faster oocyst. These results of present study (Table 14) are in congruent with Masood et al. (2013) who recorded higher prevalence in summer

(15%) than winter (6.6%).The findings of present study also corroborates the results obtained in the earlier studies done in Bangladesh (Shahid et al., 1987; Ehsan et al., 2015 and Khair et al., 2014) who observed the infection highest in summer and autumn season in bovine calves. Higher infection rates in calves were previously observed in the rainy season and in autumn in Bangladesh, India and Pakistan (Rahman et al., 1985; Saha et al., 2006; and Ayaz et al., 2012). H&E staining of the histological tissue sections revealed the presence of altered mucosal architecture with congestion of blood vessels, infiltration, sloughing and complete erosion of epithelial cells and blunting and atrophy of the intestinal villi in the present study which indicates acute enteritis. Those lesions are also supported by Angus, (1990); Carol et al. (1997) followed by Cryptosporidium are a mild to moderate villous atrophy, villous fusion, crypt hyperplasia, disruption of the intestinal microvillus, and the infiltration of inflammatory cells and changes in the surface epithelium. However, worldwide the prevalence of cryptosporidiosis has been reported with considerable variation due to differences in study design, age of the animals under study, management system, geographical and seasonal differences. Due to intensive and Semi intensive housing method having the little opportunity for the young animals to be infected by the contaminated cyst. It was also described that the samples were collected from both healthy and infected calves included diarrheic or non diarrhoeic. It may be have the chance of other enteropathogens mixed infection which was the main reason for declined prevalence of cryptosporidium in calves. In microbiological surveys of diarrheic calves, the detection rate of mixed infections with two or more of the main enteropathogens ranged between 5% and 20%, and in most of them *Cryptosporidium* was involved for complicated infection (Bulgin et al., 1982) with The result of this study thus implies that *Cryptosporidium* infection in cattle, as well as the possible risk of transmission to humans by cattle, is important in the study area as it is elsewhere in the world. Therefore, considerable attention should be paid to preventing the spread of the infection. Cryptosporidium infection is prevalent in neonatal calves, especially in young age groups and it is suggested that veterinarian should consider this protozoan while investigating the etiology of diarrhoea in young calves.

CHAPTER VI
CONCLUSIONS

Cryptosporidiosis is an emerging global zoonosis that is still poorly understood and largely neglected. Cryptosporidiosis is important calf health problems in the study area. This study indicates that the occurrence of Cryptosporidium in calves, with higher prevalence among calves 16-30 days old when compared with other age groups. Mostly the disease affects inadequate development of the immune system in new born calves and less immunity in poor body condition and due to stress as result of poor adaptation to climatic condition concerning other risk factors, higher occurrence was observed in intensive management system than in extensive and semi intensive management system. Since large number of extensive management system was present in the study area considerable contamination to the communal pasture grazing system could be the other factor which favors the transmission of the oocyst.

The present study demonstrated the presence of *Cryptosporidium* spp. in calves in selected area of Chittagong, Bangladesh. Results of this study indicate that the prevalence of cryptosporidiosis in crossbred calves in these areas is under diagnosed and the clinical status of infection is not higher value. The prevalence of the *Cryptosporidium* species/genotypes appeared to be age, sex, season related. Because calves less than 45 days old are the predominant population infected with *C. parvum* (zoonotic species), any effort designed to control this infection must be directed primarily at this age group. A further prospective study, capturing seasonal variations to elucidate the magnitude of the disease (mortalities and reduced production), is desirable. From these findings efforts should be directed towards the diagnosis and prevention of Cryptosporidiosis in Bangladesh so as to prevent the transmission of the disease to human beings.

CHAPTER VII
RECOMMENDATION

Therefore, based on the above conclusions, the following points were recommended: The owner of calves should be aware to improve the management system and health care of calves. The molecular technique of nested PCR was found more effective but time consuming which can be replaced by Real-time PCR which is also highly specific and allow rapid and sensitive diagnosis of cryptosporidiosis. Further sequencing and phylogenetic analysis with necessary bioinformatics tools can be quite interesting to understand the specific detection of cryptosporidium through genomic data analysis can be done in future for identification of genetic variation within and among species of *Cryptosporidium.*

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ANNEX-I

Questionnaire for factors associated to Cryptosporidium agent in calf diarrhea

**Serial no. : ……. Date…/…/…

1. Name of the farm and owner: ……
2. Educational Status……..
3. Upazilla/Thana: ……...District: ……..…
4. Location: ……..Latitude: ……Longitude: ……..
5. Region of location: ☐ Plain ☐ Hilly ☐ Coastal
6. Herd size: ☐ 5-10 ☐ 11-20 ☐ 21-50 ☐ >50 (Numbers……..)
7. Number of Calves: ……
8. Population density: No. of animals..../sqft
9. Age:
10. Sex: ☐ Male ☐ Female
11. Breed of dam: ☐ Local ☐ cross
12. Type of barn: ☐ Closed ☐ Partialy open ☐ Open barn
13. Flooring type in calving area: ☐ Concrete ☐ Slatted ☐ Brick ☐ Grass
14. Type of litter in calf pen: ☐ Straw ☐ Rubber pad ☐ Litter less
15. Source of drinking water: ☐ Ponds ☐ River ☐ Deep tube well ☐ Supply ☐ More than one type
16. Bedding cleaning method: ☐Water cleaning ☐water cleaning with disinfectant
17. Calving month:☐ J ☐ F ☐ M ☐ A ☐ M ☐ J ☐ J ☐ A ☐ S ☐ O ☐ N ☐ D
18. Separation of calf from dam: ☐ Immediately ☐ <24 hr ☐ >24hr ☐ No information
19. First feeding of colostrum after birth: ☐ Within 30 min ☐ within 2 hr ☐ within 2-6hr ☐ no information
20. Feeding calf with waste milk:☐ Yes ☐ No (☐ from mastitis ☐ Contain antibiotics)
21. Sucking as feeding regimen: ☐ Yes ☐ No (☐ Restricted ☐ No restricted)
22. Confinement from birth: ☐ Single ☐ Group
23. Maximum age difference between youngest and adult calf housed in same pen: ☐ <4wk ☐ 4-8wk ☐ >8wk
24. Diarrhoeic calves in farm: ☐ Yes ☐ No
25. History of calf scour: ☐ Yes and dead ☐ Still Diarrhoeic ☐ Recovered ☐ No

26. Therapy: ☐ Antibiotics ☐ Antiparasitics ☐ Others ☐ None
27. Feces consistency: ☐ Liquid ☐ Semi-liquid or semi-solid ☐ Solid or formed
28. Dehydration (Skin fold test): ☐ Within 2 second ☐ Within 2-6 second ☐ >6sec
29. Body weight(kg):
30. Newly introduced calves from other farms within----days/months: ☐ Yes ☐ No
31. Dystocia during delivery of this calf: ☐Yes ☐ No
32. Parity: ☐1st ☐ 2nd ☐ 3rd ☐ 4th ☐ 5th/ ☐ More
33. Hygiene of calf feeding utensils:

☐ Not shared ☐ Shared & rinsed with water ☐ Shared and disinfected

34. Navel treatment: ☐ Yes ☐No
35. Physical contact with other:

☐ None ☐ unweaned ☐ weaned ☐> 6 month ☐ adult

36. Surrounding environment of the farm: ☐ Good ☐ Moderate ☐ Bad
37. Drainaige system: ☐ Good ☐ Moderate ☐ Bad
38. Number of other animals on the farm and if any have diarrhoea: Yes/No

Bull: Sheep: Goat: Poultry:

39. History of calf death within -----days/months: Yes ☐ No; If yes, mention number:

Clinical signs: ☐ Respiratory ☐ Digestive ☐ Still birth

40. Floor disinfection system: ☐ Yes ☐ No .If yes, Frequency: ------/month; Name of agent using now:
41. Grazing system: ☐ Zero ☐ Community grazing ☐ Tethering; If zero grazing, Practice of washing before offering: ☐ Yes ☐ No
42. Storage system of feed: ☐ Good ☐ Fair ☐ Poor
43. Is calf experiencing concurrent condition: ☐ Respiratory ☐ Umbilical ☐ Others…
44. If diarrhea calves present, any of your family members has loose motion?

☐ Yes ☐ No

Signature of Interviewer

Table 15: Vital information with results of research working.

SL NO.	Farm Id.	Breed	Thana/Upazila	sex	Age(Days)	Z-N results	PCR results
1	1	Cross	Chandgaon	female	25	- ve	-ve
2	2	Cross	Chandgaon	male	30	-	
3	3	Cross	Chandgaon	male	12	-	
4	4	Cross	Chandgaon	male	26	-	
5	5	Cross	Chandgaon	female	41	+ve	-ve
6	6	Cross	Chandgaon	male	26	-	
7	7	Cross	Chandgaon	female	25	-	
8	8	Cross	Chandgaon	female	15	-	
9	9	Cross	Chandgaon	male	15	-	
10	10	Cross	Chandgaon	female	20	-	
11	11	Cross	Chandgaon	female	16	-	
12	12	Cross	Chandgaon	female	26	+ve	-ve
13	13	Cross	Chandgaon	female	30	-	
14	14	Cross	Chandgaon	female	30	-	
15	15	Cross	Chandgaon	female	5	-	
16	16	Cross	Chandgaon	female	15	+ve	+ve
17	17	Cross	Chandgaon	female	16	-	
18	18	Cross	Chandgaon	male	20	-	
19	19	Cross	Chandgaon	female	21	-	
20	20	Cross	Chandgaon	male	40	-	
21	21	Cross	Chandgaon	female	42	+ve	-ve
22	22	Cross	Bakalia	female	18	-	
23	23	Cross	Bakalia	male	15	-	
24	24	Cross	Bakalia	male	21	-	
25	25	Cross	Bakalia	female	11	-	
26	26	Cross	Chandgaon	female	20	-	
27	27	Cross	Chandgaon	female	28	-	
28	28	Cross	Chandgaon	female	40	-	
29	29	Cross	Chandgaon	female	22	-	
30	30	Cross	Chandgaon	female	30	-	
31	31	Cross	Chandgaon	male	36	-	
32	32	Cross	Chandgaon	male	43	-	

33	33	Cross	Chandgaon	female	33	-	
34	34	Cross	Bakalia	female	30	+ve	-ve
35	35	Cross	Bakalia	male	24	-	
36	36	Cross	Bakalia	female	17	-	
37	37	Cross	Bakalia	female	45	-	
38	38	Cross	Bakalia	female	16	-	
39	39	Cross	Bakalia	male	16	-	
40	40	Cross	Bakalia	female	6	-	
41	41	Cross	Bakalia	male	18	-	
42	42	Cross	Bakalia	male	44	+ve	+ve
43	43	Cross	Bakalia	female	17	-	
44	44	Cross	Bakalia	female	15	+ve	-ve
45	45	Cross	Bakalia	male	16	-	
46	46	Cross	Bakalia	male	20	-	
47	47	Cross	Bakalia	female	31	-	
48	48	Cross	Chandgaon	male	9	-	
49	49	Cross	Chandgaon	male	12	+ve	-ve
50	50	Cross	Chandgaon	female	15	-	
51	51	Cross	Chandgaon	male	31	-	
52	52	Cross	Potenga	female	16	-	
53	53	Cross	Potenga	male	14	-	
54	54	Cross	Potenga	male	18	-	
55	55	Cross	Potenga	female	2	+ve	-ve
56	56	Cross	Potenga	female	42	-	
57	57	Cross	Potenga	female	40	-	
58	58	Cross	Potenga	female	32	-	
59	59	Cross	Potenga	female	20	-	
60	60	Cross	Potenga	female	31	-	
61	61	Cross	Potenga	male	38	-	
62	62	Cross	Potenga	female	42	-	
63	63	Cross	Potenga	male	45	-	
64	64	Cross	Potenga	female	35	-	
65	65	Cross	Potenga	female	15	-	
66	66	Cross	Potenga	female	41	-	
67	67	Cross	Potenga	male	14	-	

68	68	Cross	Potenga	female	42	-	
69	69	Cross	Potenga	female	33	-	
70	70	Cross	Khulshi	female	35	-	
71	71	Cross	Khulshi	male	45	-	
72	72	Cross	Khulshi	female	18	-	
73	73	Cross	Khulshi	female	5	+ve	-ve
74	74	Cross	Khulshi	male	14	-	
75	75	Cross	Khulshi	male	41	-	
76	76	Cross	Chandgaon	female	30	-	
77	77	Cross	Chandgaon	male	45	-	
78	78	Cross	Chandgaon	male	30	-	
79	79	Cross	Chandgaon	male	35	-	
80	80	Cross	Chandgaon	male	31	-	
81	81	Cross	Chandgaon	female	11	+ve	-ve
82	82	Cross	Chandgaon	female	17	-	
83	83	Cross	Chandgaon	male	14	-	
84	84	Cross	Chandgaon	female	45	+ve	-ve
85	85	Cross	Chandgaon	female	29	-	
86	86	Cross	Chandgaon	male	40	-	
87	87	Cross	Chandgaon	male	45	-	
88	88	Cross	Chandgaon	male	11	-	
89	89	Cross	Chandgaon	male	42	-	
90	90	Cross	Chandgaon	male	45	-	
91	91	Cross	Chandgaon	female	12	-	
92	92	Cross	Chandgaon	female	16	-	
93	93	Cross	Bayezid	male	22	+ve	-ve
94	94	Cross	Bayezid	female	17	-	
95	95	Cross	Bayezid	female	20	-	
96	96	Cross	Bayezid	male	14	-	
97	97	Cross	Bayezid	female	28	-	
98	98	Cross	Bayezid	female	15	-	
99	99	Cross	Bakalia	male	20	-	
100	100	Cross	Bakalia	male	15	-	
101	101	Cross	Chawkbazar	male	12	-	
102	102	Cross	Chawkbazar	female	15	-	

103	103	Cross	Chawkbazar	female	13	-	
104	104	Cross	Chawkbazar	female	42	-	
105	105	Cross	Chawkbazar	female	15	-	
106	106	Cross	Chawkbazar	female	15	-	
107	107	Cross	Chawkbazar	male	14	-	
108	108	Cross	Chawkbazar	male	20	-	
109	109	Cross	Chawkbazar	female	5	-	
110	110	Cross	Chawkbazar	male	22	+ve	+ve
111	111	Cross	Chawkbazar	male	6	-	
112	112	Cross	Kotoali	female	22	-	
113	113	Cross	Kotoali	male	21	-	
114	114	Cross	Kotoali	male	20	-	
115	115	Cross	Kotoali	male	22	-	
116	116	Cross	Kotoali	male	21	-	
117	117	Cross	Kotoali	male	26	-	
118	118	Cross	Kotoali	male	20	-	
119	119	Cross	Kotoali	female	22	-	
120	120	Cross	Kotoali	female	11	-	
121	121	Cross	Kotoali	male	11	-	
122	122	Cross	Kotoali	male	23	-	
123	123	Cross	Kotoali	female	22	-	
124	124	Cross	Kotoali	female	11	-	
125	125	Cross	Potiya	male	15	-	
126	126	Cross	Potiya	male	10	-	
127	127	Cross	Potiya	male	20	-	
128	128	Cross	Potiya	male	22	-	
129	129	Cross	Potiya	male	14	-	
130	130	Cross	Potiya	male	21	-	
131	131	Cross	Potiya	female	7	-	
132	132	Cross	Potiya	female	13	-	
133	133	Cross	Potiya	male	7	-	
134	134	Cross	Potiya	male	22	-	
135	135	Cross	Potiya	male	20	-	
136	136	Cross	Potiya	female	21	-	
137	137	Cross	Potiya	male	21	-	

138	138	Cross	Potiya	male	21	-	
139	139	Cross	Potiya	male	2	-	
140	140	Cross	Potiya	male	35	-	
141	141	Cross	Potiya	male	14	-	
142	142	Cross	Potiya	male	12	-	
143	143	Cross	Potiya	female	24	-	
144	144	Cross	Potiya	male	21	-	
145	145	Cross	Potiya	male	4	-	
146	146	Cross	Potiya	female	22	-	
147	147	Cross	Potiya	female	24	-	
148	148	Cross	Kornafully	male	25	-	
149	149	Cross	Kornafully	female	23	-	
150	150	Cross	Kornafully	male	35	-	
151	151	Cross	Kornafully	male	12	-	
152	152	Cross	Kornafully	female	15	-	
153	153	Cross	Kornafully	female	12	-	
154	154	Cross	Kornafully	female	10	-	
155	155	Cross	Kornafully	female	12	-	
156	156	Cross	Kornafully	female	20	-	
157	157	Cross	Kornafully	female	15	+ve	-ve
158	158	Cross	Kornafully	female	12	-	
159	159	Cross	Chandgaon	male	13	-	
160	160	Cross	Chandgaon	female	12	-	

ANNEX-II

Activities during the research work

Fig: A. Fecal sample collection, B. Using Z-N techniques, C. oocyst identification under microscope, D. Sample preparation for DNA extraction.

Fig: E. Centrifugation during DNA extraction, F. Placing PCR mixture in thermal cycler G. Gel electrophoresis of PCR products, H. Observing of PCR products under UV-illuminator.

BRIEF BIOGRAPHY OF THE STUDENT

This is Md. Anwar Parvez; son of Md. Shafir Uddin and Ambia Akter from Ishwarganj Upazilla under Mymensingh district of Bangladesh. He passed the Secondary School Certificate Examination in 2005 followed by Higher Secondary Certificate Examination in 2007. He obtained his Doctor of Veterinary Medicine (DVM) Degree in 2013 (held in 2015) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a candidate for the degree of MS in Pathology under the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, CVASU. He enjoys providing diagnostic consultation to private veterinarians, non-governmental organizations. He has Keen interest to work on laboratory animal and comparative pathology.