MOLECULAR STUDIES ON *CRYPTOSPORIDIUM* AND *GIARDIA* SPECIES IN CATTLE CALVES AND THERAPEUTIC EVALUATION OF *CURCUMA LONGA* AGAINST EXPERIMENTAL CRYPTOSPORIDIOSIS IN MICE

By

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(J-14-D-74-V)

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IN
VETERINARY PARASITOLOGY

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Certificate – I

This is to certify that the thesis entitled **"Molecular studies on *Cryptosporidium* and *Giardia* species in cattle calves and therapeutic evaluation of *Curcuma longa* against experimental cryptosporidiosis in mice"** submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Sciences in subject of **Veterinary Parasitology** is a record of bonafide research, carried out by **Alveena Ganai**, Registration No. **J-14-D-74-V** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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ABSTRACT

Title of Thesis	**:**	**Molecular studies on *Cryptosporidium* and *Giardia* species in cattle calves and therapeutic evaluation of *Curcuma longa* against experimental cryptosporidiosis in mice**
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ABSTRACT

The prevalence studies were based on identification of *Cryptosporidium* oocyst and *Giardia* cyst in cattle calves of Jammu region by examining 614 faecal samples subjected to Diethyl ether sedimentation technique (DEES) followed by modified Ziehl-Neelsen staining technique and lugols iodine wet mount technique for identification of *Cryptosporidium* spp. and *Giardia* spp. infection. The study revealed an overall positivity of 144 animals (23.45%) for cryptosporidiosis and of 43 animals (7.00%) for *Giardia* infection. Highest prevalence of *Cryptosporidium* spp. was recorded in Samba (28.57%) district, whereas highest prevalence of giardiosis was recorded in Kathua (7.78%) district of Jammu region. The highest prevalence of *Cryptosporidium* infection was recorded in < 1 month of age animals (43.24%) than other age group whereas highest prevalence of *Giardia* infection (11.2%) was recorded in 1-6 month age group. Diarrhoeic animals revealed significantly higher prevalence (29.51%) of *Cryptosporidium* infection and (9.02%) *Giardia* infection as compared to non diarrhoeic animals. Highest prevalence of *Cryptosporidium* infection was recorded (34.65%) in winter months from December to February but in case of *Giardia* infection it was recorded highest (10.34%) in monsoon months from July to September. Cattle calves having mucus in the faeces showed significantly higher prevalence (37.33%) of cryptosporidiosis whereas in case of giardiosis faecal samples with no mucus/blood showed significantly higher prevalence (7.62%). Male animals showed non significantly higher incidence of *Cryptosporidium* infection

(37.77%) and *Giardia* infection (12.77%) as compared to females. Genetic characterisation of 120 samples (40 samples per age group) found positive by mZN was carried using molecular techniques. Nested PCR of 18S small subunit (SSU) rRNA gene of *Cryptosporidium* spp. amplified a product of 830 bp band size whereas β-giardin gene of *Giardia* spp. amplified a product of 511 bp band size. RFLP analysis of nested PCR product of *Cryptosporidium* spp. by three restriction enzymes namely *Ssp*I, *Vsp*I and *Mbo*II was carried which resulted in higher prevalence of *C. parvum* (74.07%) in young animals whereas 16.66% were positive for *C. andersoni* and 9.25% were found to be positive for *C. bovis*. For subtyping of *C. parvum*, DNA of 17 positive samples GP60 gene was amplified and sequenced and yielded clear band at 850 bp. Molecular phylogenetic analysis of these sequences showed that these *C. parvum* isolates belonged to one subtype family, i.e., IIa. Fifteen of the *C. parvum* specimens belonged to the subtype IIaA15G2R1, whereas two specimens belonged to subtype IIaA14G2R1. The 511 bp nested product of *Giardia* spp. when digested by *Hae* III restriction enzymes, zoonotic *Giardia intestinalis* Assemblage B with 1.6% prevalence and Assemblage E with 14.16% prevalence in young cattle calves of Jammu region was recorded. Administration of curcumin, obtained from ethanolic extract of *Curcuma longa* as therapeutic agent at dose rate of 8 mg/ kg body weight for five days resulted in decreased oocyst production in experimentally infected mice. Although higher body weight gain was recorded in treated groups, but it was not significantly affected. The findings of the present study clearly suggest that molecular characterization and subtypes/assemblages of *Cryptosporidium* spp. and *Giardia* spp. established zoonotic potential of *C. parvum* and *G. intestinalis* infecting cattle of Jammu region. *Curcuma longa* showed promising anticryptosporidial effects in reduction of oocyst count in experimentally infected mice and highlights the significance of further trial in other susceptible host like cattle.

Key words: *Cryptosporidium*, *Giardia*, *Curcuma longa*, Cattle, Molecular characterization, Zoonotic.

Signature of Major Advisor

Signature of the Student

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ABBREVIATIONS

^{0}C	Degree Celsius
gm	Gram
ml	Milliliter
%	Per cent
>	Greater than
<	Less than
vs	Versus
spp	Species
P/E	Positive/Examined
mZN	modified Ziehl-Neelsen staining technique
ELISA	Enzyme Linked Immuno Sorbent Assay
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
HIV	Human Immuno Deficiency Virus
r RNA	Ribosomal Nucleic Acid
β giardin	Beta giardin gene
DMSO	Dimethyl Sulfoxide
DNA	Deoxy Ribose Nucleic Acid

Chapter-1

Introduction

CHAPTER–I

INTRODUCTION

Animal husbandry is an integral part of agriculture and cattle are the most important domesticated animal used for milk production, motive power for various farm operation, and village transport and production of organic manure. This animal species has significant contribution in the overall economic development of our nation. In India, there are about 199.1 million of cattle, ranking first in the world in terms of cattle population. Over 65% population of India is living in rural area wherein agriculture is the main sources of livelihood (Gautam *et al.,* 2007). More than 75% population of rural India is deprived of fertile soils and assured water for irrigation and are dependent on livestock for supplementary income. The livestock sector plays a vital role in country's agricultural economy, contributing to about 30 % of GDP of agriculture, allied sector and milk accounts for nearly 67% of total value of livestock products in the GDP.

The neonatal calves and adult cattle suffer from many of the parasitic infestations which cause heavy economic loss to the dairy industry. Parasitic infections are the natural state of life for mammals, including cattle and are transmitted through vector e.g, leishmanosis, trypanosomosis, babesiosis etc. or directly through contaminated food and water to man e.g. giardiosis, amoebiosis and recently cryptosporidiosis, an important disease of public health significance. In India the effective control of parasitic disease in cattle has been obstructed by paucity of information one epidemiology of the disease and their variability depending on agroclimatic conditions. Hence to control parasitic diseases a multidisciplinary approach involving the integration of chemotherapy, grazing, management, biological control and genetic resistance of hosts is implemented (Sharma *et al*., 2015).

Cryptosporidium and *Giardia* are significant causes of diseases and morbidity in humans and mammals. These parasites are significant causes of diarrhea and nutritional disorders in institutional and community settings, affecting growth and cognitive functions of infected individuals and may cause production losses in livestock. The main impact of these diseases is found in developing countries, including India. Transmission of giardiosis and cryptosporidiosis is typically associated with poor faecal-oral hygiene. Both parasites are included in the WHO Neglected Diseases Initiative because they impair the ability to achieve full potential, development and socio-economic improvements, and they have a common link with poverty (Savioli *et al*., 2006).

Cryptosporidiosis is a parasitic zoonotic disease affecting all terrestrial and most aquatic animals caused by 26 validated species (18 other species are not yet considered validated) of the genus *Cryptosporidium*, family Cryptosporidiidae, order Eucoccidiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa. Cryptosporidiosis ranks 5th among the 24 most important food borne parasites globally (Akiyoshi *et al*., 2006 and Abe *et al*., 2006) with 40 genotypes that have not yet been classified. But according to the International Commission on Zoological Nomenclature (ICZN) there are 18 valid species of *Cryptosporidium* (OIE, 2008) and among them the most important species infecting bovines are *Cryptosporidium parvum, C. andersoni, C. bovis* and *C. ryanae* (formerly known as deer-like genotype) (Santin *et al.,* 2004; Fayer *et al.,* 2006; Fayer and Xiao, 2008). *Cryptosporidium* occurs in different species of hosts and today the parasite is known to infect more than 150 species of animals belonging to mammalian, avian, reptiles, amphibian and fish (Fayer and Xiao, 2008). Bovine cryptosporidiosis is predominantly a disease of young calves of 0-2 years old which subsides in older cattle.

The zoonotic Apicomplexan *Cryptosporidium parvum* is considered the most common enteropathogen of neonatal calves (de la Fuente *et al.,* 1998; Santin *et al.,* 2008). Infected calves can exhibit clinical signs ranging from asymptomatic infection to profuse diarrhoea and dehydration (Thompson *et al*., 2007). These animals readily contaminate their immediate environment as total oocysts output per infected calf can be up to 10^{10} over a week (Fayer *et al.*, 2004).

Giardiosis is an endemic disease common in developed and developing countries causing gastroenteritis. *Giardia* occurs frequently, especially in developing countries (Hellard *et al*., 2000). About, 41 species of *Giardia* with uncertain validity is found in the literature and named *Giardia* species based on the host of origin (Campbell *et al.*, 1990; Van Keulen *et al.*, 1993).At present, only six species of the genus *Giardia* are considered valid. Filice (1952) classified *Giardia* using morphological characteristics and recognized three distinct species: *G. duodenlalis* infecting a wide range of mammals, including humans, livestock, and companion animals; *G. agilis* in amphibians; and *G. muris* in rodents. Amongst the six currently accepted species of *Giardia*, *G. duodenalis* (syn. *intestinalis/lamblia*) has the broadest host range and is the species with the greatest public and animal health significance in terms of gastrointestinal disease. Itis detected frequently in many mammals (Feng and Xiao, 2011) and is one of the most common intestinal parasites in livestock (Thompson *et al*., 2008). Clinical presentations of giardiosis range from an asymptomatic cyst passer to cases of diarrhea, which can be acute, chronic, or intermittent (Meyer and Jarroll, 1980). The course of severity of giardiosis depends on host factor (the immunity response) and the virulence of *Giardia* or a combination of both that is still unclear (Thompson, 2000).

According to geographic distribution of *Cryptosporidium* species in India and abroad, it is not a conclusive indication of a difference in the dynamic of transmission. One of the most popular subtyping tool is the DNA sequence analysis of the 60 kDa glycoprotein, gp60, also called gp40/15. The use of gp-60 subtyping has allowed for the identification of geographic and temporal differences in the transmission of *Cryptosporidium* species, and a better appreciation of the public health significance of other *Cryptosporidium* species/ genotypes and the frequency of infection with mixed genotypes or subtypes (Alves *et al*., 2003).Within each subtype group, there are several subgenotypes based primarily on the number of tri-nucleotide repeats coding for the amino acid serine, as suggested by Sulaiman *et al.* (2005). Out of 11 different subtype families of *C. parvum* (IIb, IIc, IIe – IIi) are not zoonotic and IIa and IId, are found in both humans and ruminants responsible for zoonotic cryptosporidiosis (Xiao, 2010). Among all subtype families of *C. parvum*, IIa (especially IIaA15G2R1) is considered the most prevalent in cattle (Imre *et al*., 2011).

Giardia duodenalis is a complex of seven distinct genotypic assemblages (A to G) that can only be distinguished by PCR amplification and sequencing of appropriate genes. Humans appear to be exclusively infected with the assemblages A and B, but these two groups are also widely reported in dogs, cats, livestock and also wild mammals (Feng and Xiao, 2011). Thus, giardiosis is considered as a zoonotic disease.

In humans both these organisms have faeco-oral route of transmission either by direct ingestion of cyst/oocyst or indirectly by ingestion of food or water contaminated by animals or humans. The relative importance of these transmission routes is not entirely clear, largely due to the inability of traditional diagnostic tools to differentiate parasite genotype/subtypes, important to understand transmission pathways and dynamics. Molecular techniques are sensitive and in particular allow identification of species, subtypes and assemblages.

In India, cryptosporidiosis was reported for the first time in faeces of cattle by Nooruddin and Sarma (1987). The report of cryptosporidiosis in cattle and buffalo calves from Northern India (U.P.) was given by Dubey *et al.* (1992) and first PCR-based detection of bovine cryptosporidiosis was reported by Das *et al.* (2004a). Also, Yadav (2010) studied the epidemiology of bovine cryptosporidiosis and its zoonotic potential in children and HIV positive patients of Jammu region and revealed an overall prevalence of 26.52 % *Cryptosporidium* spp. in animals. Recently, Joute *et al*. (2016) recorded overall prevalence of 26.15 per cent *Cryptosporidium* species by using modified Ziehl-Neelsen (mZn) staining technique. Prevalence rates of *Giardia* infection in patients with diarrhea range from 0.4% to 70%, and asymptomatic cyst passage has been found to be as high as 50% in rural southern India. Deshpande and Shashtri (1981) observed *Giardia* cysts in the faeces of 81 of 157 calves aged 1 month to 6 months from Maharashtra. Recently, Khan *et al*. (2011) reported the overall prevalence of *G. duodenalis* in cattle was 12.2% (22/180), the infection being more prevalent in younger calves than in adult cattle. Also, Sharma *et al*. (2013) used zinc sulphate floatation technique and copro antigen ELISA kit and recorded an overall prevalence of 40.83% *Giardia* infection in diarrhoeic dogs from Jammu

Microscopy coupled with differential staining techniques is considered as a conventional method for detection of oocysts of *Cryptosporidium* and *Giardia* cyst but they cannot be confirmed up to species level e.g modified Ziehl-Neelsen staining technique (mZN) (Henricksen and Pohlenz, 1981) for identification of *Cryptosporidium* and simple iodine wet mount method for *Giardia* identification (Soulsby, 1982). Apart from simple microscopic examination, other sensitive diagnostic techniques included immunofluorescence assay for detection of *Cryptosporidium* oocyst and *Giardia* cyst by Immunofluorescence examination by a direct immunofluorescent antibody test (IFAT) MeriFluor, test kit (Rieux *et al*., 2013) and molecular detection assay based on PCR and nested PCR-RFLP have the potential of addressing many of the limitations of traditional methods and are frequently introduced in many of previous studies for genus or species-specific detection of *Cryptosporidium* and *Giardia* and were found to be useful for large scale sample analysis. Polymerase chain reaction (PCR) is more sensitive and in particular allow species identification and subtyping of *Cryptosporidium* species (Xiao, 2010). PCR can be utilized to detect as low as one oocyst per sample (Gibbons *et al*., 1998; Xiao *et al*., 1999; Coupe *et al*., 2005). Subtyping of *C. parvum* isolates is performed by nested PCR amplification of the GP60 gene of *C. parvum* using primers and protocols by Alves (2006) and Xiao (2010). DNA-based genotyping studies have identified more subtypes from both humans and animals isolates, e.g., (Lalle *et al*., 2005) and (Sulaiman *et al*., 2003) have confirmed sub-structuring within major *G. duodenalis* assemblages.

A major problem concerning *C. parvum* is the lack of an effective means for controlling infection and decreasing environmental contamination with oocysts. Because oocysts are highly resistant to environmental stresses and many disinfectants, hygienic measures on their own are not sufficient to avoid infection and long term contamination of calf rearing facilities (O'Donoghue, 1995). Several studies have been performed, however, with limited success regarding efficient control measures. With increasing awareness of *Cryptosporidium*, effort is continuing to develop new potent drugs.

More than 200 substances have been tested against cryptosporidiosis. Some exhibit promising effects but none of them were able to consistently control clinical signs or completely eliminate the infection. Scientific interest on natural products with anti-cryptosporidial properties is increasing in recent years. Useful leads which are provided by practitioners of traditional system of medicine continue to help us in development of modern medicine and novel therapeutic targets. Many drugs are used for its treatment, but there is evidence of drug resistance, insufficient efficacy and unpleasant side effects. Therefore, natural products are good candidates for discovering more effective compounds.

Most experimental studies on cryptosporidiosis have been undertaken using rodent models because of their wide availability. Mice appear susceptible to inocula derived from other hosts, and in the absence of appropriate tissue culture systems provide a convenient model for testing therapeutic and prophylactic drugs (Current and Garcia, 1991). The use of a murine model may promote understanding of immunopathogenic mechanisms and the development of active and passive immunotherapy against *Cryptosporidium* infections (Arrowood and Sterling, 1989 and Perryman, 1990)

In Ayurveda (Indian traditional medicine), turmeric has been used for its medicinal properties for various indications and through different routes of administration, including topically, orally, and by inhalation. Turmeric is a spice that comes from the root *Curcuma longa*, a member of the ginger family, Zingaberaceae. Curcuminoids are components of turmeric, which include mainly curcumin (diferuloyl methane), demethoxycurcumin, and bisdemethoxycurcmin.

Curcuma longa commonly known as turmeric has been reported to have anti-inflammatory, antiproliferative and antioxidant properties. Curcumin is a yellow natural polyphenolic compound extracted from turmeric root (*Curcuma longa*). The active compound (coloring agent) diferuloylmethane [1,7-bis-(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione] is responsible for various pharmacological effects of curcumin. Curcumin is an effective antioxidant and scavenges superoxide radicals, hydrogen peroxide, and nitric oxide (NO) from activated macrophages (Joe and Lokesh, 1994).

Antiprotozoal activitites of curcumin (active ingredient of *Curcuma longa*) have been described for *Cryptosporidium*, *Plasmodium falciparum*, *Leishmania* spp. and *Giardia lamblia* trophozoites (Shahiduzzaman *et al*., 2009).

Curcumin acts against parasites through unique biomolecular mechanisms which would explain its activity on both drug-sensitive and drug resistant parasite strains. Various studies have shown that curcumin has antioxidant**,** antiparasitic and anti-inflammatory properties and that it modulates numerous targets and cell signaling pathways. These include growth factors, growth factor receptors, transcription factors, cytokines, enzymes, and genes regulating apoptosis.

Histone deacetylase regulates transcription and is one of the novel therapeutic targets for fungal derived antiprotozoal agents (like acipidin), thereby such drugs may alter proliferation of apicomplexan parasites such as *C. parvum* (Darkin-Rattray *et al*., 1996). A new member of the apicomlexan histone deacetylase family has been recently described in *C. parvum* (Rider and Zhu, 2009), and it seems to be possible that a respective mechanism is involved in inhibition of growth of *Cryptosporidium* by curcumin.

Objectives of Investigation:

In the light of the above facts the present study has been under taken with the following objectives viz;

1. Molecular characterization of *Cryptosporidium* and *Giardia* spp. in cattle calves.

2. To evaluate therapeutic activity of *Curcuma longa* against experimental cryptosporidiosis in mice.

Chapter-2

Review of Literature

CHAPTER–II

REVIEW OF LITERATURE

The purpose of this review is to provide a brief summary about the systematic investigations of conventional, immunological, molecular epidemiologic studies, clinical and genetic profiling and molecular diagnosis of *Cryptosporidium* and *Giardia* infection. This review discusses recent progresses in molecular characterization and subtyping of *Cryptosporidium* spp., *Giardia* spp. and chemopreventive drug against cryptosporidiosis.

2.1 CRYPTOSPORIDIOSIS

2.1.1 GENERAL

Cryptosporidium is an opportunistic coccidian protozoan parasite which is one of the most prevalent enteropathogens worldwide and the etiological agent of a diarrheal disease dependent largely on the immunological status of the affected individual. It can also infect farm animals, companion animals and wild animals (Fayer *et al*., 2004).

Cryptosporidium has attracted a great deal of attention since the late 1970s as an organism for epidemiological and molecular biological studies. The first individual to establish the genus *Cryptosporidium* and to recognize its multispecies nature was Ernest Edward Tyzzer, who described the type species, *C. muris*, from the gastric glands of laboratory mice **(**Tyzzer, 1907). He later published a more complete description of the life cycle (Tyzzer, 1910) and subsequently described a second species, also from laboratory mice (Tyzzer, 1912). *C. parvum* differed from the type species not only by infecting the small intestine rather than the stomach but also because the oocysts were smaller (Upton and Current, 1985).

Cryptosporidiosis is a parasitic zoonotic disease caused by 26 validated species affecting all terrestrial and most aquatic animals (18 other species are not yet considered validated) of the genus *Cryptosporidium*. Cryptosporidiosis ranks 5th among the 24 most important food borne parasites globally (Akiyoshi *et al*., 2006 and Abe *et al*., 2006) with 40 genotypes that have not yet been classified (Aloisio *et al*., 2006). The genus remained relatively obscure for about 5-6 decades and then *C. meleagridis* was reported in turkeys (Slavin, 1955). Panciera *et al*. (1971) for the first time associated *Cryptosporidium* with bovine diarrhoea. Subsequently, it was reported from wide range of mammals, birds, and reptiles from different parts of the world (Brownstein *et a1*., 1977, Iseki, 1979; Current *et al*., 1986; Lindsay *et al*., 2000; Fayer *et al*., 2001; Morgan *et al*., 2002). Several *Cryptosporidium* parasites named during or before the period, such as *C. meleagridis* in turkeys (Slavin, 1955). *C. wrairi* in guinea pigs (Vetterling *et al*., 1971) and *C. felis* in cats (Iseki, 1979). More recently, several other *Cryptosporidium* spp. were also named in a less haphazard fashion, such as *C. baileyi* in birds (Current *et al*., 1986) and *C. saurophilum* in lizards (Koudela and Modry, 1998).

The classical definition of species as groups of interbreeding natural populations reproductively isolated from other groups (Mayr, 1942) is difficult to apply to many organisms like *Cryptosporidium*, because it is very difficult to conduct genetic crossing studies with many *Cryptosporidium* spp. The taxonomic status of *Cryptosporidium* and the naming of species are undergoing rapid change as new information, based on molecular data is showing variation at genetic level among different susceptible host. The genus *Cryptosporidium* is classified under the family Cryptosporidiidae, order Eucoccidiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa (Levine, 1985; Fayer and Ungar, 1986). *Cryptosporidium* has strong morphological similarities throughout the life cycle and also possesses mitochondrion-specific genes like coccidia (Riordan *et al*., 1999), but *C. parvum* do not possesses a mitochondria-like organelle (Tetley *et al*., 1998) as found in classical coccidia. Molecular studies also suggest that *Cryptosporidium* may be more closely related to Gregarines (Carreno *et al*., 1999) and the life cycle stage similar to those of gregarines supports this suggestion (Hijjawi *et al*., 2002). Molecular data suggest an ancestral relationship of *Cryptosporidium* to *Helicobacter* bacteria (Striepen *et al*., 2002).

2.1.2 LIFE CYCLE

Tyzzer (1910) published detailed description of *Cryptosporidium* life cycle. *Cryptosporidium* are homoxenous parasite. These are intracellular but projects from lumanal side of cell and attached to cytoplasm of host cell by "feeder organelle." It has sexual and asexual developmental stages (Fayer and Ungar, 1986). Excystation of oocyst occurs after ingestion by a suitable host, with release of four motile sporozoites which invade and parasitize epithelial cells primarily of gastrointestinal tract and rarely in extraintestinal cells. Subsequent developmental stages are intracel1ular but extra-cytoplasmic, usually found at the microvillar surface of epithelial host cell. They finally produce micro and macro gametes. Absence of flagella on microgamete also differentiates from other coccidian parasites. Fertilization of female and male gametes produces sporulated oocysts which are discharged in the faeces of infected host in magnitude of billions. These oocysts are the thick walled oocysts excreted in faeces and are infective to other hosts, and the thin-walled oocysts, which burst while in intestine and release sporozoites which give rise to endogenous auto-infection (Soulsby, 1982; Levine, 1984; Mehlhorn, 1988).

Cryptosporidial infections can be transmitted directly in humans (Koch *et al.*, 1985 and McNabb *et al.*, 1985), and animals (Current *et al.*, 1983 and Tzipori *et al.*, 1983) from one individual to another or through the environment via contaminated water (Mc Kenzie *et al.*, 1994 and Atherton *et al.*, 1995). Calves eliminate a large number of oocysts in the faeces, contaminating fresh food, drinking and recreational water. This has originated several cryptosporidiosis water borne outbreaks, affecting humans and animals (Fayer *et al.*, 2000). The infection results from oral ingestion of very low number of oocysts (9-100) can cause an infection (Chen *et al*., 2002), sporozoites of which invade microvillous border of epithelial cells of intestines (Current *et al.*, 1983; Current *et al.*, 1986). However, the infectivity depends upon infecting isolate and the immune status of the host (Teunis *et al.*, 2002a; Teunis *et al.*, 2002b and Dupont *et al.*, 1995). The symptoms of infection with *C. parvum* are essentially acute diarrhoea, which occurs between the ages of 5 and 20 days (Paraud *et al*., 2010). There is concomitance between a high number of excreted oocysts and the severity of the diarrhoea. The prepatent period, the time from ingestion of infective oocysts to excretion of oocysts following completion of the life cycle, can be completed in as few as 3-5 days or can take as long as 2 weeks. The incubation period range from 3 to 22 days, with an average of 1 week (Jokipii *et al.*, 1985). The disease is self-limiting in the immune competent host but poses a significant threat in immune deficient individuals (Alves *et al*., 2001; Mohandas *et al*., 2002). This mainly leads to gastro-enteritis and diarrhoea as major symptoms.

2.1.3 BOVINE CRYPTOSPORIDIOSIS

Cryptosporidiosis is recognized worldwide, primarily in neonatal calves but also in lambs, kids, foals, and piglets. *Cryptosporidium* spp. infection causes varying degrees of naturally occurring diarrhea in neonatal farm animals. The parasites commonly act in concert with other enteropathogens to produce intestinal injury and diarrhoea. Association of cryptosporidiosis with diarrhoea in bovines was for first time recorded by Panciera *et al*. (1971). It was further documented as primary enteropathogen responsible for neonate diarrhoea in bovine (Tzipori *et al.,* 1980). The disease is manifested by diarrhea (varies from pale yellow with mucus to profuse watery diarrhea), depression, anorexia and abdominal pain. The disease mainly affected young bovine calves of 0-3 month's age. Santin *et al.* (2004) observed the first peak of prevalence of infection in calves at 2 weeks of age followed by a second peak at 6 months. The infection has been reported throughout the world including India.

2.1.4 Pathobiology

The developmental stages of *Cryptosporidium* can be identified at all levels of gastrointestinal tract. Although the jejunum is usually the most heavily infected site, the parasite can be found throughout the length of the colon, as well as the duodenum including the ampulla of vater, ileum, gall bladder, bile duct, and pancreatic duct. The complicated life cycle, variety of parasitic forms within the host, different *Cryptosporidium* species and predilection to different tissues in different host species leads to various pathobiological changes in the host. The pathogenesis of *Cryptosporidium* is associated with diarrhoea, weight loss, abdominal cramping, electrolyte imbalance etc. *Cryptosporidium* induced diarrhoea is believed to be a result of parasite invasion and epithelial destruction with the result of mild to moderate villus atrophy and microvillii shortening and destruction (de Graaf *et al.,* 1999). This also leads to impaired nutrient absorption and transport. The pathogenesis of cryptosporidiosis is frequently complicated by concurrent viral (rota virus, coronavirus), bacterial (*E. coli, Salmonella*) and parasitic (*Giardia*) infections (Fayer *et al.,* 1998; O'Handley *et al.,* 1999). Animals with sub clinical infection excrete oocysts which can be transmitted to other susceptible hosts. Calves can die from dehydration and cardiovascular collapse but cryptosporidiosis mortalities are highly variable. Colostrum antibodies and milk antibodies protect calves from developing severe clinical signs by blocking parasite invasion and immobilization of gut luminal parasitic forms. The parasite is located in the brush border of the epithelial cells of the small intestine, but are are mainly located in the jejunum. When the sporozoites attach the epithelial cells membrane envelops them. Thus, they are "intracellular but extracytoplasmic" and cause damage to the microvilli where it attaches. The immune system reduces the formation of Type 1 merozoites as well as the number of thin-walled oocysts. This helps prevent autoinfection and B cells do not help with the initial response or the fight to eliminate the parasite.

Micronutrients also play a significant role in the clinical outcome of cryptosporidiosis as severe clinical signs have been observed in calves with selenium deficiency (McAllister *et al.,* 2005*)*. Calves that recover from *C. parvum* do not have recurrent cryptosporidiosis-associated diarrhoea and do not appear to shed oocysts for the rest of their life.

The epithelium damage also leads to villus shortening, fusion and crypt hyperplasia. Colonization of the epithelial surface leads to decreased intestinal surface area, loss of membrane-bound digestive enzymes and impaired nutrient and electrolyte transport (Buret *et al.,* 2003). A putative enterotoxin has been proposed to lead to chloride secretion resulting in secretory diarrhoea (Guarino *et al.,* 1995). *Cryptosporidium* disrupts epithelial tight junctions that can lead to increased epithelial permeability (Buret *et al.,* 2003) and so, malabsorptive and secretary diarrhoea are noticed in cryptosporidiosis. The mechanism by which *Cryptosporidium* causes diarrhoea includes a combination of increased intestinal permeability, chloride secretion, and malabsorption, which are all thought to be caused by the host response to infection. Therefore, calves can die from dehydration and cardiovascular collapse but the mortalities are highly variable. In endemic herds, morbidity rates are usually 100% but mortalities are infrequently observed.

2.1.5 Clinical signs

Clinical signs can persist for 4-14 days and the severity and duration is highly variable. The recovered animals become carriers, thereby serving as a potential source of infection to susceptible population. Cryptosporidiosis in calves has economic significance due to its high prevalence and losses associated with mortalities, body weight loss, impaired body weight gain and costs of treatment.

Calves begin shedding *C. parvum* oocysts as early as 2 days of age and peak occurs at approximately 14 days of age (Becher *et al*., 2004). Typically, shedding is observed between 1 to 4 weeks of age with infection lasting for about 2 weeks. The disease is manifested by diarrhoea which varies from pale yellow with mucus to profuse watery diarrhoea, depression, anorexia and abdominal pain.

Bovine cryptosporidiosis is predominantly a disease of young calves of 0-2 years of age and with the attainment of immunological maturity, the infection subsides in older cattle. Occasionally dehydration, collapse and mortality rate of up to 35% was recorded due to *C. parvum* in calves less than 1 month of age from dairy animals of Punjab (Singh *et al.*, 2006). Shobhamani and Singari (2006) reported profuse watery faeces with some blood clots, mucus and undigested milk clots, accompanied by mild-fever, depression, lethargy and varying degree of emaciation in diarrhoeic calves affected with *C. parvum*, whereas non-diarrhoeic calves showed undigested food materials in faeces, reduced weight gain and progressive emaciation. *C. andersoni* infects abomasum of juvenile, post-weaned and mature cattle (Olson *et al.*, 2004; Enemark *et al.*, 2002). Its endogenous stages destroy microvilli of peptic glands and increase the concentrations of plasma pepsinogen in infected animals (Ralston *et al.*, 2003). There is also inhibition of protein digestion by decreased gastric proteolytic function and increased gastric pH. Thus, *C. andersoni* does not cause diarrhoea but reduces milk production, body weight gain, and feed efficiency (Ralston *et al.*, 2003). Using technique *C. andersoni* was reported in 12.85 % of the bovine calves of 6-24 months of age (Paul *et al.*, 2009a).*Cryptosporidium* *bovis* (former bovine genotype B) is found to be the predominant species in 2-11 months old dairy calves but was not associated with any overt clinical signs. It is highly prevalent species that infects primarily post-weaned calves (Fayer *et al.,* 2005).

Sporadic infections in cattle with other *Cryptosporidium* spp. have also been reported. Bornay-Llinares *et al.* (1999) confirmed *C. felis* infection in cattle by morphological and molecular methods. Fayer *et al.* (2006) reported *C. suis* in cattle. Infection in calves with *C. canis* (Fayer *et al.,* 2001) and *C. hominis* (Feng *et al.,* 2007) is also on record. A fourth *Cryptosporidium* species i.e. *Cryptosporidium ryanae* (formerly known as the deer-like genotype) has been reported from both pre-weaned and 2-11 month old calves, without any signs of disease (Santin *et al.*, 2004).

An economic analysis of *Cryptosporidium* in calves is necessary to clearly demonstrate the high cost of this parasitic infection in beef and dairy calf production. Adult cattle shedding *C. parvum* –like oocysts appear to have a unique genotype of *C. parvum* that is not associated with clinical signs (Santin *et al*., 2004).

2.1.6 Detection of *Cryptosporidium* spp. in the faecal samples

Numerous techniques have been used to detect *Cryptosporidium* infection in animals. These include histology and ultrastructural examination of biopsy material for life-cycle stages, examination of faeces for the presence of oocysts and detection of *Cryptosporidium* antigens or DNA (Smith, 2008). The diagnosis of cryptosporidiosis rests on the identification of the 5 µm spherical oocysts of *Cryptosporidium* (or oocyst components) in the faecal sample. A variety of diagnostic options are available for the detection of *Cryptosporidium* in clinical faecal samples. Methods such as (DFSS) direct faecal smear staining, (DESS) diethyl ether sedimentation staining, (SFSS) Sheather’s floatation sedimentation staining, (DFAT) direct fluorescent antibody test, (PCR) polymerase chain reaction for detection of *Cryptosporidium* spp. oocysts in faeces. (Smith *et al*., 2008, Plutzer and Karanis, 2009, Paul *et al*., 2009 and Mirhashemia *et al*., 2015).

2.1.6.1 Microscopic staining method

Cryptosporidium is conventionally diagnosed by microscopic staining method. This methodology includes the concentration of oocysts and staining of faecal smears (Casemore *et al.,* 1985). The other supportive methods are the differential staining methods that include the safranin-methylene blue stain (Baxby *et al.,* 1984 and Soave, 1983), the Ziehl-Neelsen (Henricksen and Pohlenz, 1981) and the DMSO-carbol fuchsin (Pohjola *et al.,* 1984). However, the differential staining method is time-consuming and its result depends on the sensitivity and specificity (Baxby *et al.,* 1984; Smith *et al.,* 1989). The fluorochrome staining method (Campbell *et al.,* 1992) has a high sensitivity but is complicated. Modified Ziehl–Neelsen and Kinyoun staining with diagnostic sensitivity and specificity of 83.8% and 98.9%, respectively (Morgan *et al*., 1998). These staining methods are labour-intensive and require experienced microscopist for slide examination. The "gold standard" and most widely used staining technique for the detection of *Cryptosporidium* oocyst in stool was the modified acid-fast or Kinyoun stain (Fayer *et al.,* 2000).

The detection limit of acid fast staining has been reported to be 50,000 oocyst per gram of faeces, whereas the detection limit was 5, 00,000 oocysts per gram faeces with flurochrome dye (Balatbat *et al.,* 1996). Detection limit of modified Kinyoun technique was reported to be 1-5 x 10^4 oocysts per gram of faeces (Weber *et al.,* 1991).

2.1.6.2 Concentration techniques

The coprodiagnostic methods may fail to detect *Cryptosporidium* oocysts in stool specimens of infected patients. The concentration procedures provide excellent separation of parasites from stool debris and enhanced detection of *Cryptosporidium* oocysts in stool samples.

Various procedures for concentration of *Cryptosporidium* oocyst from faeces namely formol-ether concentration (Allen and Ridley, 1970), salt floatation (Weber *et al.,* 1992), sucrose centrifugal floatation (Anderson, 1981), zinc sulphate floatation were discussed in literature in which Sheather's sugar floatation was the most widely used and sensitive technique (Fayer *et al.,* 1997). Webster *et al.* (1996) reported detection limit of 4000 oocyst per gram of faeces by sugar floatation coupled with modified Ziehl Neelsen staining / Auramine-phenol staining. Current *et al.* (1983) also reported modified Sheather's floatation technique to be highly sensitive for selective purification and recovery of oocysts from faeces.

Arrowood and Sterling (1987) reported up to 72% recovery of oocysts from crude faeces by discontinuous sucrose step-gradient centrifugation technique. This method was comparable to isopycnic percoll gradient centrifugation which yielded 79% recovery of oocysts from crude faeces. Caesium chloride (CsCl) step gradient centrifugation technique for oocyst recovery was reported highly efficient by Kilani and Sekla (1987). A further modification of the technique was done by Arrowood and Donaldsen (1996) to obtain higher concentration of oocysts. A modification of discontinuous sucrose step-gradient centrifugation with high sensitivity and threshold of detection upto 10 oocysts was reported by Ramirez and Sreevatsan (2006). Barwick *et al.* (2000) reported 93% sensitivity and 100% specificity of sucrose floatation method when a sample containing 1.5×10^3 oocysts was tested. Sodium chloride floatation method exhibited a detection limit of less than 40 oocysts per gm soil/faeces (Walker *et al.,* 1998; Kuczynska and Shelton, 1999) and efficiency of 61.6% for 1 gram faecal samples (Mawdsley *et al.,* 1996).

Among different density gradient methods, CsCl density gradient centrifugation was reported to be the most sensitive technique (Fayer *et al.,* 1997). A sophisticated procedure for concentration and purification of oocysts was immuno-magnetic separation (IMS) using magnetisible particles coated with antibodies. This method was used to obtain highly purified oocysts (Parker and Smith, 1994).

2.1.6.3 Immunological detection methods

Immunological-based techniques include polyclonal fluorescent antibody tests (Stibbs and Ongerth, 1986), latex agglutination reactions (Pohjola *et al.,* 1986), immunofluorescence with monoclonal antibodies (Rusnak *et al.,* 1989; Xiao *et al.,* 1993; Chan *et al.,* 2000), enzyme linked immunosorbent assays (Anusz *et al.,* 1990; Ungar, 1990; Siddons *et al.,* 1992; Rosenblatt and Sloan, 1993; Dagan *et al.,* 1995; Kehl *et al.,* 1995), reverse passive haemagglutination (Farrington *et al.,* 1994), immunoserology using immunofluorescence detection (Fayer *et al.,* 2000) and solid-phase qualitative immunochromatographic assays (Garcia and Shimizu, 2000). The drawback of these methods is the non-specificity due to cross-reactivity with other genera and species.

The sensitivity and specificity of direct fluorescent antibody (DFA) test were reported to be 96-100% and 99.8-100% respectively, and was equal to traditional examination of permanent smears prepared from concentrated stool specimens (Johnston *et al.,* 2003). A number of antigen capture ELISAs were reported with detection limit in the range of 3 x 10^5-10^6 oocysts per gram of faeces, which indicated that the assays did not appear to have superior sensitivity over microscopical methods (Anusz *et al.,* 1990; Robert *et al.,* 1990). The antigen-capture ELISA exhibited 66.3% sensitivity and 99.8% specificity (Newman *et al.,* 1993) but both false positive and false-negative cases were recorded in the assay (Rosenblatt and Sloan, 1993). The monoclonal antibody based immunofluorescence test was found to be more efficient than modified Kinyoun technique (Alles *et al.,* 1995), whereas equal sensitivity and specificity of direct fluorescent antibody (DFA) test and modified Ziehl Neelsen staining (mZN) technique was reported by Kehl *et al.* (1995).

2.1.6.4 Molecular techniques

More than two decades have passed since the first report of describing the detection of *Cryptosporidium parvum* by PCR (Laxer *et al*., 1991). These techniques have been developed to detect and differentiate *Cryptosporidium* species at species/genotype and subtype level (Sulaiman *et al*., 1999 and Morgan *et al*., 1995). PCR based detection of *Cryptosporidium* in clinical samples is attractive due to its extreme accuracy. A variety of PCR tests have been developed (Smith, 1998) which generate rapid, highly sensitive and accurate result. However, these techniques have several limitations in discriminating the wide range of different species or genotypes. False positives can result from naked nucleic acids, from non-viable microorganisms, laboratory contamination and also from the interference of some environmental contaminants (Toze, 1999).

Carraway *et al.* (1996) used internal transcribed spacer-1(ITS-1) based PCR protocol and the assay reported to detect upto 100 oocysts per gm of faeces. Bonnin *et al.* (1996) and Morgan *et al.* (1997) used primers of random DNA sequences and reported threshold of detection of 20-100 oocysts in PCR. Spano *et al.* (1997) recorded a threshold limit of up to 10 oocysts with a PCR assay using gene primer of *Cryptosporidium* oocyst wall protein (COWP) but the threshold level was decresed to 20 oocysts when a gene primer of thrombospondin related adhesive protein 1 (TRAP-C1) was used (Spano *et al.*, 1998).

A threshold of detection of 50 oocysts per gm of faeces using gene primers of thrombospondin related adhesive protein 2 (TRAP-C2) was reported by Sulaiman *et al.* (1998.) Both dihydrofolate reductase (DHFR) based assay (Gibbons *et al.*, 1998) and the small sub-unit ribosomal RNA (18S ssu rRNA) based protocol (Xiao *et al.*, 1999) yielded threshold limit of upto 1 oocyst per gm of sample. Coupe *et al.* (2005) described another SSU rRNA based PCR protocol capable of amplifying DNA from a single oocyst. Balatbat *et al.* (1996) reported a *C. parvum* specific PCR assay which involved amplification of 194 bp DNA fragment and internal probing with enzyme linked chemiluminience system. A Nested PCR-RFLP protocol based on a 844 bp (Primary PCR) and 593 bp (secondary PCR) was described with 100% sensitivity for 10 oocysts per sample, 94% for 7 oocysts per sample, 92% for 5 oocysts per sample, 88% detection limit for sample containing 4 oocysts and 76% and 38% detection limit for samples containing 2 oocysts and 1 oocyst, respectively (Sturbaum *et al.*, 2001).

Sulaiman *et al.* (2000) reported heat shock protein-70 (HSP-70) based PCR which was both sensitive and specific for detection of *Cryptosporidium* spp. Another HSP-70 based Nested PCR was reported having detection limits of 10-100 oocysts per sample (Lindergard *et al.,* 2003). Reverse-transcriptase PCR (RT-PCR) for detection of *C. parvum* oocysts after immunomagnetic separation was reported by Hallier and Guillot (2003). They used the HSP-70 heat shock induced mRNA and the threshold of detection was upto 10 oocysts per sample.

Real-time PCR using Taq man probe was reported to be highly specific and sensitive for detection of *C. parvum* in faecal samples (Verweji *et al.*, 2004; Fontaine and Guillot, 2003; Ramirez and Sreevatsan, 2006; Godiwala *et al.*, 2006). A PCR hybridization assay for detection of *C. parvum* oocysts in faecal samples was reported by Ramirez and Sreevatsan (2006).

Paul *et al.* (2009_b) compared four conventional coprological techniques, *viz.*, direct faecal smear staining (DFSS), normal saline sedimentation staining (NSSS), Sheather's floatation (SF) and Sheather's floatation sedimentation staining (SFSS) with PCR directed against the 18S SSU rRNA gene as standard reference test for the diagnosis of cryptosporidiosis in bovines. Sheather's floatation sedimentation staining was found to be the most sensitive (82.6%) and specific (98.76%) among the coprological techniques. As sample processing based cost analysis, DFSS was found to be the most economical method (15 cents) followed by NSSS (19.6 cents), SF (23.6 cents) and SFSS (33.9 cents). The time taken for complete processing and diagnosis varied between 70 and 100 min. PCR based diagnosis of a sample took about 7.5–8 h for completion and cost of diagnosis was estimated at approximately 7.604 US$ per sample. The study concluded that among the conventional coprological methods, SFSS provided the required sensitivity and specificity along with nominal cost for diagnosis on per sample basis, and may be considered as a viable diagnostic alternative when PCR is not an option for a particular laboratory setting, especially in developing countries.

Mirhashemi *et al*. (2015) compared three molecular tests to identify *Cryptosporidium* species, viz., DFAT which showed the best specificity (93%) for the detection of oocysts followed by PCR (84%), ELISA (82%) and microscopy of Kinyoun's stained slides (78%); PCR was the most sensitive test (78%), followed by examination of slides stained with Kinyoun's (76%) which in turn appeared to be more sensitive than DFAT (58%), or ELISA (22%) in cattle. Also, almost perfect agreement between PCR vs DFAT with kappa values 0.72 as compared to PCR vs Kinyoun's with kappa value 0.68 as substantial agreement and PCR vs ELISA with kappa value 0.61 as moderate agreement.

2.1.7 PHYLOGENETIC RELATIONSHIPS AND TAXONOMY

According to ICZN four criteria are to be fulfilled for characterization of *Cryptosporidium*: (i) Morphometric studies of oocysts (ii) Genetic characterization (iii) Demonstration of natural and whenever feasible, at least some experimental host specificity (iv) Compliance with ICZN (Xiao *et al*., 2004).

2.1.8 Morphometric characterization

Although morphometry serves as the cornerstone of Apicomplexan taxonomy but in case of *Cryptosporidium* spp. it cannot differentiate the species of *Cryptosporidium* present if the oocysts fall within the range of 4-6 µm and it varies from species to species. The length of oocyst ranges from 4.5 to 7.5 µm and the width from 4.2 to 5.7 µm (Marquardt and Speer, 2000). The small size of *Cryptosporidium* oocysts makes them indistinguishable at the species level based on morphology by light microscope (Fall *et al*., 2003). The oocysts are spherical or ovoid in appearance and contain 4 naked parallel sporozoites surrounded by a smooth oocyst wall. At the wall, a faint suture can be seen through which the sporozoites exist during excystation (Morgan *et al*., 2002). At the electron microscope level, the zoites of *Cryptosporidium* show some of the elements of the apical complex; such as electron dense collar similar to a conoid, micronemes and electron-dense bodies that may be similar to rhoptries (Marquardt and Speer, 2000). The two apical rings can also be seen at the tip of the zoite. The pellicle is similar to that of other apicomplexans and subpellicular tubules are also present. They lack a true conoid, perhaps rhoptries, and mitochondria (Marquardt and Speer, 2000). *Cryptosporidium* have a few life cycle stages; oocyst, trophozoite, schizont, merozoit and sexual stages including micro- and macrogamonts.

Therefore genetic and biological criteria were much important for proper characterization of *Cryptosporidium* spp. (OIE, 2008).

2.1.9 Genetic characterization

A host can be naturally infected with multiple *Cryptosporidium* spp. which frequently cannot be differentiated from each other on the basis of morphology and development. So, the genetic differences between species can play pivotal role in differentiating *Cryptosporidium* spp. (Xiao *et al.,* 2004). Various genetic loci like ssu rRNA (Awad-El-Kariem *et al.,* 1994; Leng *et al.,* 1996; Xiao *et al.,* 1999), *Cryptosporidium* oocyst wall protein (COWP) (Spano *et al.,* 1997; Xiao *et al.*, 2001$_b$), thrombospondin related adhesive protein (TRAP-C1) (Spano *et al.,* 1998), TRAP-C2 (Sulaiman *et al.,* 1998); dihydrofolate reductase (DHFR) (Gibbons *et al.,* 1998), Poly-T (Carraway *et al.,* 1997; Barnes *et al.,* 1998), internal transcribed spacer-1 (ITS-1) (Carraway *et al.,* 1996; Morgan *et al.,* 2001), microsatellites (Feng *et al.,* 2000; Caccio *et al.,* 2000), heat shock protein-70 (HSP-70) (Sulaiman *et al.,* 2000) acetyl CoA, β-tubulin and glycoprotein 60 (gp60) (Caccio *et al.,* 2005) were used for molecular characterization of *Cryptosporidium* isolates from bovines.

A genotyping technique based on the SSU rRNA was developed by Xiao *et al.* (1999) which provided valuable information about sequence variation of different species of *Cryptosporidium* affecting bovines. The 18S SSU rRNA based nested PCR protocol was very sensitive as it amplified DNA from a single oocyst (Xiao *et al.,* 1999, 2004). The 834 bp nested product when digested by restriction enzymes *Ssp*I and *Vsp*I separately, provided specific diagnosis of the species of *Cryptosporidium* involved. *Cryptosporidium parvum* yielded 3 visible bands at 449, 267 and 108 bp with *Ssp*I and 2 visible bands of 628 and 105 bp with *Vsp*I.

Coupe *et al.* (2005) detected up to 95% of infection by *Cryptosporidium* spp. by amplifying the polymorphous region between nucleotides 179-271 of SSU rRNA gene. However this was not able to differentiate between *C. andersoni* and some *C. parvum* genotypes and found *C. parvum* as the predominant species among bovines in India.

Feng *et al.* (2007) reported that single digestion of 834 bp nested product with restriction enzyme *Mbo*II also differentiated the species of *Cryptosporidium*. *Cryptosporidium parvum* yielded 2 bands at 771 and 70 bp.

2.1.10 Biological characterization

Small animal models such as neonatal mice have been successfully infected with *Cryptosporidium* (Meloni and Thompson, 1996). Extensive infection could be induced in 1-4 days old neonatal mice or by suppressing the immunity of adult mice with immunosuppressive drugs (Reese *et al.,* 1982; Fayer *et al.,* 1997; Bednarska *et al.*, 1998). Various routes of experimental infection namely oral, intra-uterine, intra-cervical, rectal and intra-gastric injection of oocysts were followed to infect mice (Fayer *et al.,* 1997; Bednarska *et al.,* 1998). The dosage of oocysts varied with age of the animal and isolate of *Cryptosporidium parvum*. A technique for isolating purified oocysts from the gut of mice for further *in vitro* cultivation, molecular and biochemical studies has been successfully established (Current, 1990; Meloni and Thompson, 1996). Differences in the infectivity for laboratory animals also exist between *C. hominis* and *C. parvum* cattle genotype with the cattle genotype readily infecting mice and cattle, whereas *C. hominis* does not (Peng *et al.*, 1997 and Fayer *et al.,* 2006).

In experimentally infected mice, colony of *C. parvum* was detected in small intestine, caceum, colon, liver, lung and heart (Sherwood *et al*., 1982; Ernest *et al.,* 1986; Fayer *et al*., 1989). Fayer *et al.* (1997) reported transient villous atrophy, crypt hyperplasia, inflammatory cell accumulations in distal small intestine and endogenous stages were found on brush border epithelium.

2.1.11 Prevalence and molecular characterization of *Cryptosporidium* spp.

There are enormous biological and genetic diversity in mammalian *Cryptosporidium* spp. and because of a plethora of molecular studies, many new species have been discovered and described (Xiao *et al*., 2004). *Cryptosporidium* spp. infection can be detected as early as 5 days of age, with the greatest proportion of calves excreting organisms between days 9 and 14. Cryptosporidiosis is considered to be an emerging disease in developing countries (Tzipori and Ward, 2002), which has greatest prevalence, although the actual cases have been under reported (Hunter and Nicholas, 2002).

2.1.11.1 Work done in India

Bovine cryptosporidiosis was first reported by Nooruddin and Sarma (1987). The report of cryptosporidiosis in cattle and buffalo calves from Northern India (U.P.) was given by Dubey *et al.* (1992). In West Bengal Das *et al.* (2004_b) recorded highest prevalence of cryptosporidiosis in cattle in winter (45.16%) followed by summer (27.2%) and rainy season (19.2%) in the state of West Bengal. Kumar *et al.* (2004) reported 25% prevalence of cryptosporidiosis in both diarrhoeic and non-diarrhoeic calves in Puducherry. Jeyabal and Ray (2005) reported 35.7% prevalence of *Cryptosporidium* spp. in calves less than 1 month old and 50% prevalence among 1-3 month old buffalo calves in Izatnagar (U.P state).

In India first PCR-based detection of bovine cryptosporidiosis was reported by Das *et al.* (2004a). Roy *et al.* (2006) characterized the isolates of *Cryptosporidium* spp. from cattle from various parts of West Bengal and found *C. parvum* as the species responsible for bovine cryptosporidiosis. The prevalence of cryptosporidiosis, both in diarrhoeic (61.64%) and non-diarrhoeic (47.22%) cases was highest in 0–1-month age group. The highest prevalence was recorded in rainy season (27.55%) followed by summer (16.99%) and winter (8.71%). A total of 166 positive cases were genotyped by PCR-RFLP analysis of SSU rRNA gene and found *C. parvum* as the species responsible for bovine cryptosporidiosis.

Singh *et al*. (2006) reported 50 per cent prevalence of *C. parvum* in diarrhoeic and 25.68 per cent prevalence in non-diarrhoeic calves from Punjab. Both shedding and intensity of shedding were significant in calves with diarrhoea. The prevalence of the infection peaked in young calves between 0 and 30 days in both the diarrhoeic and non-diarrhoeic groups (86.4% and 66.6%, respectively). High mortality rate and case fatality rate of 35.2% and 44.40% respectively were observed in young calves between 0 and 30 days of age.

Shobamani *et al.* (2006) screened 651 faecal samples by acid fast staining method from non diarrhoeic (374) and diarrhoeic (277) calves from Andhra Pradesh and revealed 19.52 % and 48.38 % infection of *Cryptosporidium* spp. Prakash *et al*. (2009) used modified Ziehel Neelsen staining technique and recorded an overall prevalence of 9.05 per cent cryptosporidiosis in young calves in Chennai. The incidence was highest (14.66%) in young calves in the age group of 5-30 days followed by 31-60 days age group (9.38%). In older calves (above 3 months) it was 6.06% and the lowest (3.23%) in adult calves (3 months to1 year). Crossbred and female animals were more susceptible.

Paul *et al*. (2008) examined 457 faecal samples based on PCR assay of 18S SSU rRNA gene and revealed a 30.2% infection with *Cryptosporidium* spp. The PCR-RFLP pattern of the gene in all the positive cases established the species as *Cryptosporidium parvum.* Further as per season maximum infection was recorded during the monsoon season followed by pre monsoon and minimum during the post monsoon season. As per age highest incidence of infection was found in the 0-15 day's age group and the lowest incidence was recorded in the 75-90 days age group. Similarly, Paul *et al*. (2009a) by using PCR-RFLP technique reported 12.85% incidence of *C. andersoni* in bovine calves of 6-24 months of age.

Paul *et al.* (2009) examined 457 faecal samples collected from neonatal bovine calves, out of which specific PCR amplification was achieved in 138 samples, whereas, 65 samples turned positive by DFSS. This is the first comparative study describing the sensitivity and specificities of four conventional coprological techniques altogether with respect to PCR compared four conventional coprological techniques, viz., direct faecal smear staining (DFSS), normal saline sedimentation staining (NSSS), Sheather's flotation (SF) and Sheather's flotation sedimentation staining (SFSS) with PCR directed against the 18S SSU rRNA gene as standard reference test for the diagnosis of cryptosporidiosis in bovines. The study concluded that Sheather's flotation sedimentation staining was found to be the most sensitive (82.6%) and specific (98.76%) among the coprological techniques. The sensitivity and specificity of different coprological procedures were calculated on the basis that PCR which was 100% sensitive and specific for the diagnosis of bovine cryptosporidiosis.

In West Bengal, Khan *et al.* (2010) examined the genetic diversity and zoonotic potential of *Cryptosporidium* from 180 calves, heifers and adults and 51 farm workers on two dairy farms. Phylogenetic analysis was carried out on the DNA sequences obtained in the study and those available in GenBank. The overall prevalence of *Cryptosporidium* in cattle was 11.7% though the infection was more prevalent in younger calves than in adult cattle. The PCR-RFLP analysis of the 18S rRNA gene of *Cryptosporidium* followed by DNA sequencing of the PCR products revealed the occurrence of *Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium ryanae* and *Cryptosporidium andersoni*. A *Cryptosporidium suis*-like genotype was also detected in a calf. Farm workers were infected with *Cryptosporidium hominis, C. parvum* and a novel *C. bovis* genotype. The findings clearly suggested that there is a potential risk of zoonotic transmission of *Cryptosporidium* infections between cattle and humans on the studied dairy farms.

Das *et al*. (2011) examined 149 samples microscopically and it was observed that 32.9% from diarrhoeic faecal samples (72) and 7.1% from healthy faecal samples (70) revealed the presence of oocysts. *Cryptosporidium* genus was confirmed by DNA typing with nested PCR. The nested PCR positive samples were 100% identified to be *Cryptosporidium* species positive by *Ssp*I RFLP. The PCR-RFLP analysis was carried out for genotype identification. For detection upto species level of *Cryptosporidium*, RFLP analysis using restriction enzymes *Ssp*I of nested PCR products were performed. The positive *Cryptosporidium* bovine species yields three distinct bands at 444 bp, 247 bp and 106 bp after the digestion of second PCR products with *Ssp*I. In course of PCR-RFLP, unique band patterns were obtained in two samples. The unusual RFLP products were characterized by DNA sequencing and homology analysis with other reported variants.

Rana *et al.* (2011) screened 142 fecal samples to establish the diversity of parasites in neonatal buffalo calves in Haryana. Samples were examined by specific techniques including morphometry of oocysts. An overall prevalence of 3.52% was found for *Cryptosporidium* spp. with the highest infection during peak winters.

Bhat *et al.* (2012) investigated *Cryptosporidium* infection in 152 faecal samples of neonatal buffalo calves by Zehl-Neeslen technique. Overall prevalence of disease was

38.3%. A gradual decline in the prevalence values was seen with the increase in age, highest in 0-30 days of age-groups (65.71%) and lowest in 4-5 months of age group (5.88%). This trend in decline was observed in both diarrhoeic and non-diarrhoeic calves. Higher prevalence (40.65%) was recorded during monsoon season followed by pre-monsoon season (30.35%) and lowest prevalence (34.04%) was recorded during post-monsoon season. Female calves showed higher prevalence (40.35%) than males calves (33.3%).

Venu *et al*. (2012) examined 459 faecal samples from south India and found 182 samples positive with a prevalence of 39.65%. Highest prevalence of *Cryptosporidium* was observed in Puducherry (86.67%) and lowest in Kerala (17.65%). Genotyping by PCR-restriction fragment length polymorphism (RFLP) revealed the presence of all the four major *Cryptosporidium* species of cattle viz. *Cryptosporidium andersoni*, *Cryptosporidium ryanae, Cryptosporidium parvum* and *Cryptosporidium bovis*. *C. andersoni* was widely distributed in calves of Tamil Nadu, Karnataka and Puducherry whereas in Andhra Pradesh *C. ryanae* was the major species out of the 64 samples subjected to PCR-RFLP, 39 (60.94%) could be classified as *C. andersoni*, 18 (28.13%) as *C. ryanae*, 4 (6.25%) as *C. parvum* and 3 (4.69%) were confirmed as *C. bovis*. The results were also confirmed by sequencing of 19 *Cryptosporidium* DNA samples.

Singla *et al.* (2013) examined 50 faecal samples from 5 organized dairy farms for cryptosporidiosis by employing simple and rapid chromatographic lateral flow immunoassay for antigenic detection of cryptosporidial infections in buffalo (34) and crossbred cattle (16) calves in Punjab state. The percentage of positivity from buffalo and crossbred cattle calves by lateral flow immunoassay was 23.52% and 12.50%, respectively, while by modified Ziehl-Neelsen (mZN) staining it was 17.65% and 6.25%, respectively. The sensitivity of mZN staining was reported to be 70% as compared to chromatographic lateral flow immunoassay.

Venu *et al.* (2013) studied the factors like age, sex, breed, dung consistency and rearing system on prevalence of *Cryptosporidium* spp. in south Indian cattle. Examination of 459 dung samples from the calves by nested PCR revealed 39.65%

positive for the *Cryptosporidium* spp. Age wise comparison showed a high prevalence of *Cryptosporidium* in the age group of one month old calves. Depending on the consistency of dung, the highest prevalence of *Cryptosporidium* was observed in semi-solid dung, followed by formed and the diarrhoeic group animals. Cow calves had an overall prevalence of 40.75% and the infection rate in buffalo calves was 36.28 %. In relation to rearing system, individual animals had 42.18 % infection and farm animals showed 38.46 % of *Cryptosporidium* infection.

Rakesh *et al.* (2014) examined 363 faecal samples of cattle calves, buffalo calves, kids and lambs under 3 months of age. Microscopically, 20 positive samples were genotyped by PCR amplification of partial 18S rRNA region and subsequent digestion by SspI, VspI and MboII restriction enzymes. Based on PCR-RLFP patterns of 18S rRNA, all 20 samples were positive for *Cryptosporidium parvum.* For further confirmation of spp. of *Cryptosporidium,* amplified 818 bp partial actin gene of 3 representive isolates of was cloned and sequenced. The partial actin gene sequences obtained in this study were available in the GenBank with the accession numbers as KC469977, KC469978 and KC469979. The sequence and phylogenetic analysis of PCR-positive samples confirmed presence of *Cryptosporidium parvum.*

Bhat *et al.* (2014) studied the comparative efficacies of different conventional parasitological methods and nested PCR for diagnosis of bovine cryptosporidiosis in 100 faecal samples collected from calves in and around Ludhiana. Direct faecal smear staining technique revealed 25.0 % positivity for the oocysts of *Cryptosporidium* spp. with sensitivity and specificity of 68.12 and 92.98 %, respectively. Zinc sulphate solution floatation and saturated sugar solution floatation staining techniques showed sensitivity and specificity of 83.92 and 96.36; 81.03 and 98.14 %, respectively. Nested PCR directed against small subunit (18S) ribosomal RNA revealed the positivity of *Cryptosporidium* spp. in 47.0 % of the samples. The study concluded that PCR assays are highly sensitive and specific techniques for the screening of the samples for *Cryptosporidium* spp.

Sivajothi *et al.* (2014) recorded an overall prevalence of 37.75% for bovine cryptosporidiosis on coprological examination of 98 faecal samples collected from neonatal diarrhoeic and non-diarrhoeic cattle calves from an organized dairy farm in Tirupati by modified Ziehl–Neelsen staining. Further, a gradual decline in the percent prevalence was seen with increase in the age of the host from <1 month (52.45 %) to 2–5 months (13.51 %). Further, prevalence of cryptosporidiosis was significantly higher ($p < 0.05$) in the diarrhoeic calves (78.57 %) as compared to the non diarrhoeic calves (21.42 %). It is clearly indicating a relatively higher risk of the disease in diarrhoeic than normal calves.

Joute *et al*. (2016) recorded overall prevalence of 26.15 per cent *Cryptosporidium* species in Punjab, India by using modified Ziehl-Neelsen (mZn) staining technique. The highest prevalence of 37.25 per cent was recorded in 0–30 days calves followed by 22.97 percent, 20.27 and 17.30 per cent in1 to 2 months, 2 to 3 months age groups and in3 to 4 months of animals. The prevalence values decreased with increase in age in both diarrhoeic as well as non-diarrhoeic calves. Prevalence rate was found to be highest during monsoon (42.85 %) followed by pre-monsoon (30.66 %). PCR analysis of all the samples showed expected bands at 1,325 and 835 bp in primary PCR and secondary PCR respectively.

Hingole *et al*. (2017) recorded overall prevalence of *Cryptosporidium* spp. in Mumbai on 141 samples (36.06 %) out of 391 samples with higher occurrence in buffaloes (36.99 %) than cattle (34.48 %). Diarrhoeic loose faeces showed higher prevalence (42.07 %) than apparently normal faeces (31.72 %) irrespective of the host species. The highest prevalence was noted in the youngest group (47.12 %) declining gradually with the advancing age with lowest (6.25 %) in adults. These differences were statistically significant in case of buffaloes. *Cryptosporidium andersoni* was tentatively identified by morphometric analysis. PCR, PCR–RFLP and sequence analysis of few samples showed good correlation in the identification of species of *Cryptosporidium* involved in the infection and demonstrated occurrence of *C. parvum*, *C. ryanae* and *C. bovis*.

Swain *et al*. (2018) examined total of 402 faecal samples of buffalo calves of below three months of age collected from various villages and organized buffalo farms located in Hisar, Bhiwani, Fatehabad and Sirsa districts of Haryana. The overall prevalence of *Cryptosporidium* in buffalo calves was 8.7% with the highest prevalence of 25% at the university buffalo farm. District wise, highest prevalence was observed in district Hisar (10.8%) followed by Bhiwani (8.6%), Fatehabad (5.0%) and Sirsa (2.5%).

2.1.11.2 Global scenario:

Infection by *Cryptosporidium* spp. in cattle was first reported in the early 1970's (Panciera *et al*., 1971). However, Tzipori *et al*. (1980) documented for the first time the role of *Cryptosporidium* spp. as primary enteropathogen associated with bovine neonatal diarrhoea. The disease mainly affected young bovine calves of 0-3 months old.

de la Fuente *et al.* (1999) examined faecal samples from 218 diarrhoeic dairy calves in 65 dairy herds of 1 to 30-day-old age in Spain, for the presence of *Cryptosporidium* and the infection was detected in 43.8%, 71.9%, 63.2% and 6.9% of the calves having 1–7, 8–14, 15–21 and 22–30 days of age respectively. *Cryptosporidium* was the only enteropathogen detected in 60 of the 114 (52.6%) diarrheic calves. Concurrent infections with other enteropathogens were detected in 64.3%, 46.3%, 39.5% and 0% of the *Cryptosporidium*-infected calves in the age groups 1–7, 8–14, 15–21 and 22–30 days, respectively.

Naciri *et al.* (1999) studied the importance of *Cryptosporidium parvum* in diarrhoea of neonatal calves and reported it as the major etiological agent responsible for neonatal diarrhoea as compared to *E. coli*, rotavirus, coronavirus, and *Salmonella.*

Lefay *et al.* (2000) using qualitative ELISA for detection of coproantigens in calves of seven Administrative Regions (Aquitaine, Bretagne, Franche-Comté, Lorraine, Normandie, Nord, Pays de Loire) in France observed prevalence to be 17.9% (4–12-day-old) of which only 5.3% had diarrhoea, whereas from ten Administrative Departments (Allier, Cantal, Creuse, Doubs, Ille-et-Vilaine, Maine-et-Loire, Manche, Pas-de-Calais, Saône-et-Loire, Vendée) 43.4% (4–21-day-old) showed positivity but diarrhoea was observed in 90.5% calves. They attributed monthly variation to seasonal peaks in calving with a lower infection rate during summer.

Elwin *et al*. (2001) recorded that PCR-restriction fragment length polymorphism (RFLP) techniques represent a more rapid and simple method of genotyping to support epidemiological and clinical investigations than conventional DNA analytical techniques. They described a nested PCR-RFLP technique that identifies polymorphisms in the *C. parvum* thrombospondin-related adhesive protein gene locus.

Santin *et al.* (2004) in USA observed the first peak of prevalence of infection in calves at 2 weeks of age followed by a second peak at 6 months. He observed 35.5% calves (345 of 971) to be positive irrespective of age for *Cryptosporidium* using PCR testin 15 dairy farms of seven states on east coast. Infection was 50.3% in pre-weaned calves of 5 days to 2 months (253 of 503) and 19.7% in post-weaned calves of 3–11 months (92 of 468). Genetic characterization of 278 positive specimens by gene sequencing revealed *C. parvum*, in 85% pre-weaned calves and 1% in post-weaned calves. The study recommended that persons handling or exposed to calves less than 2 months of age are at greater risk of zoonotic infection from *Cryptosporidium* as compared to person handling older calves.

Becher *et al.* (2004) conducted a longitudinal study in Western Australia on calves from birth to weaning. *Cryptosporidium* was detected in 48% of animals and no significant association was observed between *Cryptosporidium* occurrence, season or management practices (housing). Calf-to-calf contact was the most common source of transmission. Molecular characterization of isolates revealed presence of *C. parvum* which signifies its importance as a public health risk in terms of the potential for zoonotic transmission.

Coupe *et al.* (2005) detected up to 95% of infection by *Cryptosporidium* spp. by amplifying the polymorphous region between nucleotides 179-271 of SSU rRNA gene. However the technique was not able to differentiate between *C. andersoni* and some *C. parvum* genotypes.

Fayer *et al.* (2006) examined the prevalence of *Cryptosporidium* species in 571 heifers (aged 1–2 year) on 14 dairy farms in seven states on the East Coast of the United States by nested PCR of 18S rRNA gene of *Cryptosporidium*. The study revealed that

11.9% of the examined animals were infected with *Cryptosporidium*, whereas the zoonotic species, *Cryptosporidium parvum* was recorded in 0.7% heifers. Further it was reported that out of 68 PCR-positive specimens characterized by gene sequencing 1, 4, 10, 24, and 29 calves were infected with *Cryptosporidium suis, Cryptosporidium parvum, Cryptosporidium deer-like genotype, Cryptosporidium bovis, and Cryptosporidium andersoni*, respectively.

Kváč *et al.* (2006) examined 7021 faecal samples of calves by Sheather's floatation method and staining by aniline–carbol–methyl violet in South Bohemia (Czech Republic) and observed *Cryptosporidium* oocysts in 1814 (25.8%) samples. Based on oocysts morphology *C. parvum* was found in 561 samples (8%) and *C. andersoni* in 1253 (17.8%) calves. Pre-weaned dairy calves had higher infection levels of *C. parvum* than pre-weaned beef calves. The prevalence of *C. parvum* ranged from 1.4 to 56.5% on dairy farms.

Fayer *et al*. (2007) observed *Cryptosporidium* species infection by two-step nested PCR protocol of the SSU rRNA gene. Positive animals were recorded in 11 of the 14 examined farms sequencing revealed that *Cryptosporidium parvum, Cryptosporidium bovis and Cryptosporidium andersoni* were found on 2, 6, and 8 farms, and infected 0.4, 1.7, and 3.7% of the 541 cows, respectively.

Cocklin *et al.* (2007) revealed *Cryptosporidium* spp. in 27.3% faecal samples of 143 animals from adults, heifers and calves collected from two dairy cattle farms in eastern Ontario, Canada. Molecular characterisation using 18S rRNA gene and the heat-shock protein 70 (HSP-70) genes revealed *Cryptosporidium parvum* in 21.7%, and *C. bovis* in 1.4% animals.

Nagano *et al*. (2007) identified the species of the isolates by analyzing the partial sequences of the 18S rRNA. They analyzed both the 18S rRNA and the COWP gene sequences of a single oocyst passaged from mice using a modified multiplex PCR that was able to amplify both genes. The study observed that that two distinct genotypes (Types A and B) of a novel *C. andersoni* type existed in the 18S rRNA gene, whereas the COWP gene sequences of both oocysts were identical to *C. andersoni*. It was reported that although the sequence of the 18S rRNA gene of Type A was identical to that of *C. andersoni*, that of Type B had a thymine insertion and was not identical to any sequence registered with GenBank. The study reported that this is a new type of *C. andersoni*.

Feng *et al.* (2007) in USA reported that approximately 85% of pre-weaned dairy calves were infected with zoonotic *Cryptosporidium parvum*, whereas only 1-2% of post-weaned calves and 1-2-year-old heifers were infected with this species. *Cryptosporidium bovis and Cryptosporidium* deer-like genotype were much more prevalent in the post-weaned animals. It was also observed that in Georgia, the deer-like genotype was found frequently in pre-weaned and post-weaned calves and *Cryptosporidium andersoni* was found in one post-weaned calf. Both *C. bovis* and the deer-like genotype were found in the few milking cows examined in Georgia. There were no differences in the small subunit rRNA gene sequences obtained from *C. bovis* or deer-like genotype among the three areas. All four common bovine *Cryptosporidium* spp. were differentiated from each other by restriction fragment length polymorphism analysis of PCR products with enzymes *Ssp*I and *Mbo*II.

Rinaldi *et al.* (2007) examined 347 faecal samples of calves in Italy, using commercially available enzyme-linked immunosorbent assays and observed 63 (18.1%) buffalo calves (1-9 weeks age) positive for *C. parvum.* Among 90 farms screened 22 (24.4%) were found positive for *C. parvum*. Co-infection with *Giardia duodenalis* was observed only in ten (11.1%) farms.

Nguyen *et al.* (2007) examined 266 fecal samples from diarrhoeic and non-diarrhoeic cattle were by the modified Ziehl-Neelsen staining method and the prevalence of *Cryptosporidium* infections was 33.5% (89/266). Study also reported that *Cryptosporidium* spp. infection was significantly higher (44.3%) in less than 6 months old calves than animals (28.9%) having more than 6 months of age. The percentage of diarrhoeic and non-diarrhoeic cattle identified to be shedding *C. parvum* oocysts was 46.5% (74/159) and 14.0% (15/107), respectively. DNA sequences of 18S rRNA genes of *C. parvum* type indicated that they were *C. parvum* bovine genotype.

Santin *et al.* (2008) examined 30 calves from birth to 24 months of age in Maryland using immunofluorescence microscopy and PCR and reported shedding of oocysts of *Cryptosporidium* at some time by all calves during 24 months of the study. Highest prevalence was observed in calves of 2 weeks of age (29 out of 30). Pre-weaned calves (1–8 weeks of age) showed higher prevalence (45.8%) than post-weaned calves (3–12 months of age) 18.5% and lowest in heifers (12–24 months of age) 2.2%. Sequence data for 190 PCR-positive specimens revealed *C. parvum*, *C. bovis*, the *Cryptosporidium* deer-like genotype and *C. andersoni*, with cumulative prevalences of 100, 80, 60, and 3.3%, respectively. *C. parvum* constituted 97% of infections in pre-weaned calves but only 4% and 0% of infections in post-weaned calves and heifers, respectively.

El-Khodery and Osman (2008) examined 458 fecal samples of buffalo calves (<3 month age) in 55 small scale herds in Middle Egypt by modified Ziehl- Neelsen staining technique and revealed 14.19% positivity for *Cryptosporidium* in calves. Calves of 1–15 days age were at the highest risk, and a significant relationship between season and infection was recorded. Further the study demonstrated the strong relation between infections by *Cryptosporidium* spp. and diarrhoea in buffalo calves.

Szonyi *et al*. (2008) identified *Cryptosporidium* genotypes from feces collected from urban and peri-urban dairy cattle in Nairobi, Kenya. Two *Cryptosporidium* isolates examined at the 18S rRNA locus were identified as the deer-like genotype by DNA sequencing. Brook *et al.* (2008) conducted a cross-sectional study for *Cryptosporidium* in young calves of 41 farms of North West England. Molecular diagnosis of 215 faecal samples by PCR of the 18S rRNA gene confirmed *Cryptosporidium* infection in 60 animals. The study suggested that intervention strategies should be targeted at calves under 21 days old as they represent a significant reservoir of infection on the farm and also pose a risk to public health.

Del Coco *et al.* (2008) reported 48 calves positive for shedding of *Cryptosporidium* oocysts out of the 280 examined from dairy calves (≤ 30 days of age) of a rural area of Buenos Aires, Argentina using a modified Ziehl-Neelsen acid fast method. Prevalence was higher in ≤7 days calves (37.5%) as compared to ≥8 ≤ 14 days calves

(21.4%). Both normal and diarrhoeic faeces with blood were observed to be negative for *Cryptosporidium* spp. However 37.5% of diarrhoeic faeces without blood were found positive for *Cryptosporidium* oocysts. Faeces with mucus revealed 83.3% positivity. The relationship between intensity of infection and age group showed that 66.7% of positive samples from calves ≤7 days old, presented an average of >10 oocysts/field.

Fayer *et al.* (2008) identified and described new species, *Cryptosporidium ryanae*, from cattle. It was observed that oocysts of *C. ryanae*, previously identified as the *Cryptosporidium* deer-like genotype and recorded as such in GenBank (AY587166, EU203216, DQ182597, AY741309, and DQ871345), are similar to those of *Cryptosporidium parvum* and *Cryptosporidium bovis* but smaller. Fragments of the SSU-rDNA, HSP-70, and actin genes amplified by PCR were purified and PCR products were sequenced. Multi-locus analysis of the three unlinked loci demonstrated the new species to be distinct from all other species and also demonstrated a lack of recombination, providing further evidence of species status.

Santin *et al.* (2008) examined 30 calves from birth to 24 months of age in Maryland using immune fluorescence microscopy and PCR and reported shedding of oocysts of *Cryptosporidium* at some time by all calves during 24 months of the study. Highest prevalence was observed in calves of 2 weeks of age (29 out of 30). Pre-weaned calves showed higher prevalence (45.8%) than post-weaned calves (18.5%) and lowest in heifers, 2.2%. *C. parvum* constituted 97% of infections in pre-weaned calves but only 4% and 0% of infections in post-weaned calves and heifers, respectively.

Keshavarz *et al.* (2009) studied the prevalence, variability with host age, and the genotypes of species of *Cryptosporidium* in cattle from 15 dairy farms in Qazvin province, Iran. Oocysts from 51 positive samples were analyzed using PCR assay of 18S SSU rRNA, restriction fragment length polymorphism (RFLP) and sequencing. They identified 72.6% of the positive samples as *Cryptosporidium parvum,* 17.7% as *Cryptosporidium andersoni*, 7.8% as *Cryptosporidium bovis* and 1.9% as a novel genotype of *C. parvum* possessing a single mutation on *Mbo*II restriction. An infection rate of 19.5% of *C. parvum* among 174 pre-weaned calves was significantly higher than the 3.1% among 98 post-weaned calves.

Santin and Zarlenga (2009) in USA developed a multiplex PCR assay for simultaneously detecting the 4 species of *Cryptosporidium* that commonly infect cattle. It was concluded that the assay specifically identifies *Cryptosporidium* oocysts present in cattle feces, improves the detection of mixed infections, reduces the time and cost relative to current sequencing methods, and further demonstrates the shortcomings of sequencing as the definitive method for identification when analyzing samples containing mixed infections.

Ayinmode *et al.* (2010) examined 65 fecal samples from 12-24-week-old diarrheic calves in four white Fulani herds in southwestern Nigeria for *Cryptosporidium* spp. using PCR, RFLP and analysis of the small subunit rRNA gene. Thirty-four (52.3%) of the samples were positive for *Cryptosporidium*. RFLP analysis of PCR products showed that 18 (27.7%) and five (7.7%) of the positive samples had *Cryptosporidium bovis* and *Cryptosporidium ryanae*, respectively, and 11 (16.9%) had mixed infections of the two species. The absence of *C. parvum* suggests that the age group of calves studied is not likely to be source of zoonotic infection to humans.

Silverlas *et al.* (2010) examined 1202 faecal samples collected from 50 cattle herds in Sweden and revealed 176 positive for *Cryptosporidium* spp. by FITC-labelled anti *Cryptosporidium* monoclonal antibody and PCR method. Molecular characterisation of 18S rRNA gene of *Cryptosporidium* revealed *C. bovis, C. parvum, C. ryanae* and *C. andersoni* in 83, 15, 10 and 2 samples, respectively. An age related prevalence pattern in species distribution was seen and it was observed that *C. parvum* was detected only in calves younger than 6 weeks, and 11 of the 15 *C. parvum* positive calves were younger than 2 weeks.

Meireles *et al.* (2011) studied the molecular characterization of *Cryptosporidium* spp. isolated from cattle from the state of Sao Paul, Brazil, using nested polymerase chain reaction for amplification of fragments of the 18S rRNA gene and the glycoprotein GP60 gene, following sequencing of amplified fragments. Positivity for *Cryptosporidium* was found in 10.7% (21/196) of the samples. Four species of *Cryptosporidium* were identified viz. *C. andersoni*, *C. bovis*, *C. parvum* and *C. ryanae.*

Follet *et al.* (2011) examined the veal calf faeces from 142 animals on 15 farms in the region of Brittany, France. Samples were collected on 5, 15 and 22 weeks of age. DNA extraction, Nested PCR was performed to amplify 18S rDNA and 60 kDa glycoprotein genes of *Cryptosporidium.* The sequencing of amplified fragments of*Cryptosporidium* species revealed the presence of *C. parvum* (43.8%), *C. ryanae* (28.5%), and *C. bovis* (27%). *C. parvum* caused 86.7% of infection in 5 week old calves and only 1.7% in 15 week old animals.

Imre *et al.* (2011) from Romania examined diarrheic fecal samples from 258 pre-weaned calves (1–30 days old) from 9 dairy farms located in Banat region, Romania, for the presence of *Cryptosporidium* oocysts. Overall, 65 (25%) samples were found positive and a higher percent of infection was recorded in calves aged between 8 and 14 days compared with other age categories. Genetic characterization was carried out by a nested PCR of the small subunit rRNA gene (18S) followed by RFLP analysis with *Ssp*I, *Vsp*I and *Mbo*II restriction enzymes. The restriction patterns showed that animals were infected with *Cryptosporidium parvum*.

Nguyen *et al.* (2012) investigated the prevalence of *Cryptosporidium* in native beef calves of 2-6 months age in Dac Lac province, central Vietnam. The overall prevalence of *Cryptosporidium* spp. using mZN on the sample and herd levels were 18.9% (44/232) and 50% (20/40), respectively. Genotyping based on PCR and sequence analysis of the 18S rRNA gene revealed occurrence of the two non-zoonotic species *Cryptosporidium ryanae* and *Cryptosporidium bovis,* with the former as a dominant species in the animals.

Helmy *et al.* (2013) from Egypt examined 804 cattle and buffalo faecal samples and 165 human samples by copro-antigen RIDA QUICK followed by PCR targeting the 18S rDNA and gp60 genes. PCR identified, 65.7% *Cryptosporidium parvum*, 11.8% *Cryptosporidium ryanae*, 4.1% *Cryptosporidium bovis* and combination of *C. parvum* and *C. ryanae* was 11.2%. *C. parvum* and *C. bovis* was 5.3% and *C. parvum* and *C.*

andersoni 1.8%. Analysis of gp60 variants allocated *C. parvum* found in animals to be zoonotic subtype family IIa (18.9%subtype IIaA15G1R1 only) and to IId (8.1% mostly IIdA20G1).

Rieux *et al.* (2013) from France used a two-step nested PCR protocol to amplify an 830 bp segments of the 18S rRNA gene by using primers and 32 isolates were successfully identified, where in 27 as *Cryptosporidium bovis*, 4 as *Cryptosporidium ryanae* and 1 as *Cryptosporidium parvum*. *C. bovis* was isolated from samples of calves between 11 and 33 days old. *C. ryanae* was isolated from samples of calves between 17 and 34 days old. *C. parvum* was isolated from one calf aged 13 days. PCR products were sequenced and sequences were compared with known sequences by BLAST analysis against the NCBI database.

Murakoshi *et al.* (2013) examined 107 faecal samples from calves with percentage of each genotype of *Cryptosporidium* represented among 25 *Cryptosporidium* positive specimen in the two age categories (preweaned; 1-8 weeks and postweaned; 3-12 months). Microscopic examination and sugar flotation method was performed. *Cryptosporidium* species were detected and subtyped by nested PCR amplification targeting an 830 bp and 850 bp fragments of the small subunit (SSU) rRNA and 60 kDa glycoprotein (gp60) genes. All these secondary PCR products were sequenced in both directions with secondary primers and sequencing kit. Study revealed that 23% (n=25) animals were positive for Cryptosporidiosis. Genetic characterization by PCR technique reported that 8 samples (7%) having *C. parvum*, 10 (9%) having *C. bovis* and 7 (7%) having *C. ryanae* were positive for *Cryptosporidium*. The zoonotic species *C. parvum* was statistically correlated with diarrhea in calves.

Amer *et al.* (2013) examined the prevalence and molecular characteristics of *Cryptosporidium* in dairy cattle in four Nile River delta provinces in Egypt. Screening of faecal specimens from animals of different ages on 12 farms by mZN staining recorded an overall prevalence of 13.6%. As per age, the infection rate were 12.5% in pre-weaned calves, 10.4% in post-weaned calves, 22.1% in heifers, and 10.7% in adults. PCR-RFLP and DNA sequence analyses of microscopy-positive fecal specimens revealed the presence of four major *Cryptosporidium* species. In pre-weaned calves, *C. parvum* was most common (30/69 or 43.5%), but *Cryptosporidium ryanae* (13/69 or 18.8%), *Cryptosporidium bovis* (7/69 or 10.2%), and *Cryptosporidium andersoni* (7/69 or 10.2%) were also present at much higher frequencies seen in most industrialized nations. In contrast, *C. andersoni* was the dominant species (193/195 or 99.0%) in post-weaned calves and older animals.

Mirzai *et al*. (2014) studied the prevalence and associated risk factors of *Cryptosporidium* infection in cattle herds of northwestern Iran. A total number of 246 fecal samples from 138 diarrhoeic and 108 non-diarrhoeic cattle were randomly collected and examined by fecal smears stained with Ziehl-Neelsen. The overall prevalence of *Cryptosporidium* infection was 22.3% (55/246). The prevalence of *Cryptosporidium* infection in examined calves less than 6 month-old was significantly higher than adult cattle. Molecular characterization using 18 SSU rRNA gene revealed that *C. parvum* and *C. andersoni* were identified in 20.3% (50/246) and 2.03% (5/246) of examined cattle, respectively. .

Adamu *et al.* (2015) studied the prevalence of *Cryptosporidium* infection in cattle in Northeastern Nigeria. A total of four hundred (400) fecal samples were examined for the presence of *Cryptosporidium* spp. oocysts using the modified Ziehl-Neelsen (mZN) staining method. The results showed that the overall prevalence of infection was 22.3%, with an infection rate of 23.4% in adult cattle and 19.1% in young cattle, respectively. There was no statistical significant difference between the age groups, with (OR: 1.298; 95%CI: 0.7507–2.245). Out of 89 positive samples, 21.2% were male and 25.0% were female, respectively. There was no statistical significant difference between the sex, with (OR: 0.8062; 95% CI: 0.4828–0.346).

Zhang *et al.* (2015) examined 2945 faecal samples of dairy cattle from Northwest China. Samples positive for *Cryptosporidium* oocysts were subjected to PCR amplification of SSU rRNA gene. A total of 150 samples were PCR positive for *Cryptosporidium.* Species identification showed *Cryptosporidium andersoni* in 36 samples, *Cryptosporidium ryanae* in 24 samples, *Cryptosporidium bovis* in 70 samples, *Cryptosporidium parvum* in 20 samples

Qi *et al*. (2015) examined a total of 258 faecal samples belonging to pre-weaned calves from 19 different farms using PCR and sequencing methodologies targeting the small subunit rRNA gene. *Cryptosporidium* infection was detected in 14 of 19 farms (73.7%), with a total prevalence of 20.2% (52/258).

Wegayehu *et al.* (2016) determined the prevalence and genotypes of *Cryptosporidium* species in dairy calves to assess the role of cattle in zoonotic transmission in central Ethiopia. Total of 449 fecal samples were collected and screened using modified Ziehl-Neelsen staining method and PCR targeting the small-subunit (SSU) rRNA gene. The prevalence of *Cryptosporidium* was 9.4% (42/449) and 15.8% (71/449) as detected by microscopy and nested PCR. Crossbred calves had significantly higher prevalence of *Cryptosporidium* than indigenous zebu. Genotyping results revealed the presence of *C. andersoni* (76.1%), *C. bovis* (19.7%) and *C. ryanae* (4.2%). The occurrence of these *Cryptosporidium* species appeared to be age-related. *C. andersoni* constituted 92.1% of the *Cryptosporidium* infection in calves older than 3 months. Sequence analysis also showed the existence of intra-species variation at SSU rRNA gene.

Wait *et al*. (2017) studied the prevalence of *Cryptosporidium* spp. on Tasmanian devil (*Sarcophilus harrisii*) is a carnivorous marsupial found only in the wild in Tasmania, Australia. *Cryptosporidium* was identified in 37.9% of wild devils but only 10.7% of captive devils. The novel *Cryptosporidium* genotype was 98.1% similar at the 18S rDNA to *Cryptosporidium varanii* (syn. *C. saurophilum)* with additional samples identified as *C. fayeri*, *C. muris*, and *C. galli*.

Wang *et al*. (2018) examined 344 faecal samples from yaks with diarrhoea in the Chenduo and Nangqian counties of Qinghai Province, China. *Cryptosporidium* spp. were detected by light and immunofluorescence microscopy and nested PCR (nPCR). Fifteen samples were found to be positive (4.5%) by Kinyoun staining, 40 (11.6%) samples were positive by immunofluorescence test (IFT), and 39 (11.3%) samples were positive by nPCR for *Cryptosporidium* spp. *Cryptosporidium* bovis (11/39, 28.2%) was the most prevalent species, followed by *C. ryanae* (6/39, 15.4%), *C. andersoni* (5/39, 12.8%), *C. struthionis* (5/39, 12.8%), *C. parvum* (5/39, 12.8%), *C. hominis* (4/39, 10.3%) and *C. canis* (3/39, 7.7%).

2.1.12 Subtyping of *Cryptosporidium parvum*

Subtyping tools have been used extensively in studies of the transmission of *C. hominis* in humans and *C. parvum* in humans and ruminants. One of the most popular subtyping tool is the DNA sequence analysis of the 60 kDa glycoprotein, gp60, also called gp40/15 (Xiao, 2010).The gp60 gene has tandem repeats of serine-coding trinucleotides TCA, TCG or TCT at the 5' (gp40) end of gene, in addition there are also several non-repeat regions which categorize *C. parvum* and *C. hominis* each to several subtype family. The name of gp60 starts with the subtype family designation (IIa, IIb, IIc, IId, etc), followed by the number of TCA (represented by letter T), TCG (represented by letter G) or TCT (represented by letter T) repeats (Sulaiman *et al*., 2005). In *C. parvum* IIa subtype family, a few subtypes also have two copies of the ACATCA sequence right after the trinucleotide repeats, which are represented by "R2" (R1 for most subtypes). Atleast 11 different *C. parvum* families with many subtypes have been described on the basis of sequence analysis of gp60 from humans and mammals including cattle (Xiao, 2010). Two subtype families of *C. parvum*, IIa and IId are considered zoonotic (Alves *et al*., 2006; Nazemalhosseini-Mojarad *et al.,* 2011). Among all subtype families of *C. parvum*, IIa (especially IIaA15G2R1) is considered the most prevalent in cattle (Imre *et al*., 2011 and Xiao, 2010).

2.1.12.1 Work done in India

The first report of sub-genotyping of *C. parvum* isolates of calves from India by Feng *et al*. (2007) reported occurrence of IIa subfamily and prevalence of zoonotic sub-types IIaA15G2R1, IIaA13G2R2, IIaA14G2R1a, IIaA14G2R1b.

In humans in India, Ajjampur *et al*. (2010) reported the sub-genotype IId of *Cryptosporidium parvum* from a child at St. Stephens Hospital, New Delhi, India

Maurya *et al*. (2013) revealed the presence of *C. parvum* sub-genotype IIdA15G1R1 in four cattle calves. Sequence analysis of all the 4 isolates examined in the present study revealed that each isolate had 15 copies of the TCA repeat and 1 copy of the TCG repeat, with ACATCA sequence present immediately after the trinucleotide repeats (TCA or TCG). The GP60 gene sequences of *C. parvum* IIdA15G1R1 generated in the present study are available in GenBank under accession numbers JN967649 to JN967652.

2.1.12.2 Work done in world

Alves *et al.* (2003) characterized *Cryptosporidium parvum* and *Cryptosporidium hominis* isolates from human immune-deficiency virus infected-patients, cattle and wild ruminants by PCR and 60 kDa glycoprotein gene. 7 alleles were identified, 3 corresponding to *C. hominis* and 4 corresponding to *C. parvum* and the study reported two subtypes IIa and IId.

Misic and Abe (2007) reported 60.2% of 103 examined dairy calves, from 10 farms in Belgrade area, positive for *Cryptosporidium* infection by PCR and all of them were identified as *Cryptosporidium parvum* by PCR-RFLP of SSUrRNA and COWP genes. 18 *C. parvum* isolates were selected randomly from 9 positive farms and were classified by molecular phylogenetic analysis of 60kDa glycoprotien gene into 3 different subtype allele, IIa and IId and a new family IIj. Furthermore, 10 isolates of IIa were classified into 3 subtypes, IIaA16G1R1b, IIaA18G1R1 and IIaA20G1R1; 2 in IId as IIdA18G1b; and 6 in IIj into IIjA16R2 and IIjA17R2. The last two subtypes in IIa and two subtypes in IIj were new subtypes.

Geurden *et al.* (2007) estimated prevalence of *Cryptosporidium* in calves younger than 10 weeks in a cross-sectional epidemiological study on 100 dairy and 50 beef farms in East Flanders (Belgium), using a previously evaluated immunoflouroscence assay. Subtyping of *Cryptosporidium parvum* positive isolates by sequence analysis of 60kDa glycoprotein gene indicated the presence of 4 allele IIa subtypes, along with one subtype IIdA22G1. The subtype IIaA15G2R1 was most prevalent, next to subtype IIaA13G2R1 and IIaA16G2R1, and a new subtype IIaA14G2R1.

Thompson *et al.* (2007) examined faecal samples of diarrhoeic calves less than 30 days of age, for *Cryptosporidium* spp. from farms across Northern Ireland, over a period of one year, by microscopic, genotyping and subtyping techniques to characterize transmission dynamics. *Cryptosporidium parvum* was identified in 213 (95.1%) specimens, *C. bovis* in 3 samples (3.6%) and *Cryptosporidium* deer-like genotype in three (1.3%). Sequence analysis of 60 kDa glycoprotein gene identified 16 IIa subtypes, with 120 out of 216 (55.6%) positive specimens having the subtype IIaA18G3R1.

Xiao *et al.* (2007) subtyped *Cryptosporidium parvum* DNA, from 175 neonatal calves on 16 farms in 8 eastern states in the United States, by sequence analysis of the 60-kDa glycoprotein gene. Six subtypes of the IIa subtype family were found. Subtype IIaA15G2R1, which is predominant *C. parvum* subtype in calves in many parts of the world, was identified in 77% of the *C. parvum* DNA from calves. Several farms had more than one *C. parvum* subtype and a few calves had infections with mixed subtypes.

Amer *et al.* (2009) detected *Cryptosporidium* oocysts in 5 out of 50 selected animals, around Osaki area, Miyagi prefecture, Japan. Out of these 5 positive samples, *Cryptosporidium parvum* was detected in 3 samples, *Cryptosporidium* deer-like genotype in one and *Cryptosporidium andersoni* in one sample. Sequence analysis of 60 kDa glycoprotein gene indicated all *C. parvum* samples are IIa subtype.

Plutzer *et al.* (2009) studied current status of taxonomy, genotyping and molecular phylogeny of *Cryptosporidium* species. To this date, 20 *Cryptosporidium* species have been recognized out of which two named species of *Cryptosporidium* have been found in fish, 1 in amphibians, 2 in reptiles, 3 in birds, and 12 in mammals. Nearly 61 *Cryptosporidium* genotypes with uncertain species status have been found based on SSUrRNA sequences. The gp-60 gene showed a high degree of sequence polymorphism among isolates of *Cryptosporidium* species and several subtype groups and subgenotypes have been identified, of which the *Cryptosporidium parvum* IIa and IId subtype groups were found to be zoonotic.

Brook *et al.* (2009) subtyped *Cryptosporidium parvum* DNA, from cross-sectional study of calves from 41 farms in Cheshire, UK. Out of 60 positive samples, 54 were sequenced at 18S rRNA locus and 51 were sequenced at gp60 locus. Six gp60 genotypes were identified. One subgenotype (IIaA15G2R1) was highly prevalent throughout the study area.

Amer *et al.* (2010) studied prevalence and molecular analysis *of Cryptosporidium* in dairy calves of less than 3 weeks of age at Kafr El Sheikh province, Egypt. Among 29 faecal samples positive for *Cryptosporidium* oocysts, 26 samples were sequenced for SSU rDNA and *Cryptosporidium* wall protein gene (COWP). Further sequence analysis of gp60 glycoprotein gene, 23 samples of *Cryptosporidium parvum* belonged to the allele IId (subtype IIdA20G1) and one sample belonged to allele IIa (subtype IIaA15G2R1).

Diaz *et al.* (2010) examined 61 faecal specimens from diarrhoeic pre-weaned calves collected over a 1-year period (2008–2009) at 27 cattle farms in Galicia (NW Spain), for the presence of *Cryptosporidium* oocysts. Overall, 30 calves (49·2%) tested positive for *Cryptosporidium* by microscopy. PCR products of the SSU rRNA locus were obtained for 27 *Cryptosporidium* positive calf isolates. Sequence analysis of the glycoprotein (gp60) gene revealed that one *C. parvum* isolate from calves was identified as the novel subtype IIaA13G1R1 and rest all isolates belonged to the subtype IIaA15G2R1.

Imre *et al*. (2010) carried out intraspecific characterization of 16 isolates of *Cryptosporidium parvum* from calves and lambs using gp60 as target gene. The analysis of 15 bovine *C. parvum* isolates from Western Romania revealed that they belong to two family subtypes: IIa (86.7%) and IId (13.3%). Two subtypes of IIa family were found: IIaA15G2R1 (53.4%) and IIaA16G1R1 (33.3%). The single ovine isolate of *C. parvum* belonged to subtype IIdA 22G2R1. The intraspecific characterization of these isolates showed the presence of zoonotic subtypes in calves and lambs.

Meireles *et al.* (2011) accomplished the molecular characterization of *Cryptosporidium* spp, isolated from cattle, from state of São Paulo, Brazil, using nested PCR for amplification of 18S rRNA gene and gp60 glycoprotein gene, following sequencing of amplified products. Four species were identified *Cryptosporidium parvum*, *C. andersoni, C. ryanae* and *C. bovis.* One subtype of *Cryptosporidium parvum* identified was IIaA15G2R1.

Wang *et al*. (2011) examined the prevalence and public health significance of cryptosporidiosis in pre-weaned calves in China, where 801 fecal samples from eight farms in seven areas in Henan Province were examined for *Cryptosporidium* oocysts. The overall infection rate of *Cryptosporidium* was 21.5%. The SSU rRNA-based PCR identified four *Cryptosporidium* species, including *Cryptosporidium parvum* (54/172), *C. bovis* (65/172), *C. ryanae* (19/172), and *C. andersoni* (12/172), and the occurrence of infections with mixed species (22/172). Sequencing analysis of the gp60 gene showed all 67 *C. parvum* samples belonged to subtype IIdA19G1.

Abeywardana *et al.* (2012), from Sri Lanka, carried out PCR-coupled sequencing and phylogenetic analysis of sequence data for regions in the 60kDa glycoprotein (go60) gene of *Cryptosporidium.* Three samples contained *Cryptosporidium parvum* subtypes IIaA15G3R1, IIaA19G3R1 and IIaA23G4. Twelve samples contained *Cryptosporidium hominis* subtype IbA10G2R2.

Feng *et al.* (2013) sequenced-characterized 26 IIaA15G2R subtype specimens and 26 non-IIaA15G2R subtype specimens from United States, Canada, United Kingdom and Spain, at seven other known polymorphic loci, including CP47, CP56, DZ-HRGP. MSC6-5, MSC6-7, RPGR and ZPT. Extensive heterogeneity within IIaA15G2R1 and discordance in typing results between gp60 and other genetic markers were observed. Results concluded were that the IIaA15G2R1 subtype at gp60 is likely a fitness marker for *C. parvum* and the widespread of IIaA15G2R1 subtype around the world is probably independent of the sequence characteristics at other genetic loci.

Abeywardana *et al*. (2014) examined 340 samples from pre-weaned cattle calves and buffalo calves from seven different farms in Sri Lanka. Based on SSU sequence data, *C. bovis*, *C. ryanae* and six new genotypes that were genetically similar but not identical to *C. andersoni* (n=1), *C. bovis* (n=1), *C. ryanae* (n=3) and *C. suis* (n=1) were recorded in cattle. No species or genotypes of *Cryptosporidium* with zoonotic potential were detected using sequence analysis of gp60 gene.

Couto *et al*. (2014) examined 143 fecal samples of calves from three dairy farms located in the state of Rio de Janeiro, Brazil. Among these samples, 19.6% (28) were positive by microscopy, and 82.1% (23) of these 28 samples had their diagnosis confirmed by PCR using 18S as gene target. In pre-weaning phase (< 2 months), 10% (3/30) of the calves were infected with *C. parvum*, whereas 14.2% (16/113) of post-weaning calves (≥ 2 months) were observed to be infected with *C. andersoni* and 1.8% (2/113) by *C. ryanae*. Samples positive for *C. parvum* were further characterized at the GP60 locus, and PCR products were cloned. Eight different subtypes (IIaA20G2R1, IIaA20G2R2, IIaA19G2R1, IIaA19G2R2, IIaA18G1R1, IIaA18G2R2, IIaA16G3R2 and IIaA14G2R2) of *C. parvum* were identified, all belonging to the IIa family subtype, which is considered of high zoonotic potential. .

Rahmouni *et al.* (2014) studied the zoonotic potential of *Cryptosporidium parvum* in an extensive cattle farming region of northern Tunisia. Seventy fecal samples from pre-weaning calves and 403 fecal samples from children were examined by microscopy after modified Ziehl–Neelsen (MZN) staining. Positive *Cryptosporidium* specimens were identified at a species level using an 18S rRNA RFLP analysis. *C. parvum* isolates were subgenotyped by sequence analysis of the glycoprotein 60 (gp60) gene. Among calf samples, 14 samples were positive by MZN method. *C. parvum* was identified in all cases. Twelve *C. parvum* isolates (85.7%) belonged to family subtype IIa. Subtype IIaA15G2R1 was more prevalent (50%). Two *C. parvum* isolates corresponded to the IIdA16G1 subtype. Seven human samples were positive by MZN method. *C. parvum* and *C. meleagridis* were identified in four and three cases, respectively. Intraspecific characterization of *C. parvum* identified two subtypes, the IIaA15G2R1 and the IIdA16G1, also found in calves.

Rieux *et al.* (2014) studied *Cryptosporidium* infection in pre-weaned calves over three successive years (2010-2012) in one beef cattle herd in western France. About 201 faecal samples were found to be microscopically positive for *Cryptosporidium* species. DNA was extracted from positive samples and a PCR-RFLP protocol, with restriction enzymes *Ssp*I and *Mbo*II, to amplify the partial SSU rRNA gene, was performed. For the subtyping of *C. parvum*, a gp60 PCR was carried out. The IIaA15G2R1 zoonotic subtype of *C. parvum* was the only subtype identified

Bjorkman *et al.* (2015) conducted a study in two regions in Sweden and included 30 herds. Faecal samples were collected from calves younger than 3 months of age. *Cryptosporidium* positive samples were analysed at the 18SS rRNA and gp60 gene. Two *C.parvum* subtypes were identified as IIaA16G1R1 and IIdA27G1.

Julius *et al*. (2016) showed that 6 (85.7%) of the 7 *Cryptosporidium parvum* that belong to the subtype family IIa had the subgenotype IIaA15G2R1 while 14.3% (1/7) belong to IIaA16G2R1 subgenotype. Only 1 (100.0%) belong to the *Cryptosporidium parvum* subtype family IIc and its genotype was IIcA5G3a. There were 4 specimens that belong to the subtype family IId and all of them were subgenotyped as IIdA15G1R1 (100.0%).

Benhouda *et al*. (2017) first reported about genotyping and subtyping of *Cryptosporidium* in calves in Algeria and examined 132 fecal samples from young calves (< 8 weeks old). Amplification of a fragment of the small subunit ribosomal RNA gene were found positive for 24 out of the 61 samples (40%), and sequence analysis identified three species, namely *Cryptosporidium bovis* (n = 14), *C. ryanae* (n = 6), and *C. parvum* (n = 4). *C. parvum* IIaA13G2R1 subtype, an uncommon zoonotic subtype, was identified in two isolates from a single farm by sequencing a fragment of the GP60 gene.

Gong *et al*. (2017) reported 10 *Cryptosporidium* species in cattle in China, with an overall infection rate of 11.9%. The highest rate of infection (19.5%) was observed in preweaned calves, followed by that in juveniles (10.69%), postweaned juveniles (9.0%), and adult cattle (4.94%). The dominant species were *C. parvum* in preweaned calves and *C. andersoni* in postweaned, juvenile, and adult cattle. Zoonotic *Cryptosporidium* species (*C. parvum* and *C. hominis*) were found in cattle, indicating the possibility of transmission between humans and cattle. Different cattle breeds had significant differences in the prevalence rate and species of *Cryptosporidium*, including IIa subtypes (IIaA14G1R1, IIaA14G2R1, IIaA15G2R1, IIaA16G2R1, and IIaA16G3R1) and IId subtypes (IIdA14G1, IIdA15G1, IIdA18G1, and IIdA19G1) for *C. parvum*.

Tao *et al.* (2018) investigated dairy cattles of all ages in north east china for the prevalence and genetic traits of *Cryptosporidium*. The parasites were detected in 130 animals out of 537 with an overall prevalence of 24.2%. *Cryptosporidium parvum* (5/130) was identified as the dominant species by sequence analysis followed by *C. bovis* (28/130), *C. ryanae* (5/130), *C. andersoni* (2/130), *Cryptosporidium suis* like genotype (2/130). Subtypes of *C. parvum* isolates was based on DNA polymorphisms of the 60 KDa glycoprotein gene. Subtyping of the *C. parvum* isolates recognized subtypes IIdA15G1 (24/87) in Harbin and IIdA20G1 (48/87) in Qiqihar.

2.2 GIARDIOSIS

2.2.1 GENERAL

Antoine van Leeuwenhoek first discovered *Giardia*, a protozoan parasite, in 1681(Erlandsen and Meyer, 1984). Previously the systematic nomenclature was based on host specificity and light microscopy characteristics. *Giardia* is very unusual, seemingly ancient, eukaryotic single cell organism as it shares many characteristics with anaerobic prokaryotes (Cavalier-Smith, 1987). The characteristic and distinctive morphological features of *Giardia* are well known and were described initially in the latter part of the 1800s. The newest systematic, however, is based on genetic, structural and biochemical data, and places *Giardia* in the phylum Protozoa, subphylum Sarcomastigophora, superclass Mastigophora, class Zoomastigophora, order Diplomonadida and family Hexamitidae (Thompson, 2004). On the basis of light and electro-microscopic characteristics (e.g., trophozoite shape and marginal grove) six species of *Giardia* has been identified (Adam, 2001, Plutzer *et al*., 2010). Giardiosis affects a wide range of host like man, dog, cat, cattle, buffalo, llama, mice, beavers and other domestic animals (kang *et al*., 1998: Barigye *et al*., 2008: Epe *et al*., 2010). Historically, 41 species of *Giardia* are found based on the host of origin (Campbell *et al*., 1990). At present, only six species of the genus *Giardia* are considered valid. *Giardia intestinalis*, includes from a large variety of mammalian hosts, including humans and animals. Other five species are host specific, *G. agilis* (amphibians), *G. ardeae* and *G. psittaci* (birds), *G. muris* (mice) and *G. microti* (voles) (McRoberts *et al*., 1996, Caccio *et al*., 2005).

Inclusion of isolates from a variety of mammalian host into the *G. duodenalis* has recently been supported by ribosomal RNA gene sequence and has been classified into seven assemblages from A to G, based on substantial sequence differences identified, such as glutamate dehydrogenase(gdh), triosephosphate isomerase(tpi) and b-giardin (bg) (Plutzer *et al*., 2010).Assemblages A and B include human isolates, however, the remaining assemblages have not been detected in humans appear to be a host specific but these two groups are also reported in dogs, cats, livestock and also wild animals (Feng, 2011). Humans, cats, dogs, cattle, sheep, swine, horse, as well as wildlife such as coyotes, birds, fishes and deer, have been describes as natural host of *G. intestinalis* assemblage A (Geurden *et al*., 2008, Armson *et al*., 2009, Yang *et al*., 2010b). Similarly, humans, livestock, beavers, coyotes and dogs have been identified as hosts of *G. intestinalis* assemblage B (Trout *et al*., 2006, Sprong *et al*., 2009a).

2.2.2 LIFE CYCLE

Giardia is a single celled protozoan parasite which has a simple and direct life cycle that involves two structural forms: pear shaped trophozoite (replicative stage) and the cystic (environmentally resistant and infective stage).

A. Trophozoite

The trophozoite is pear-shaped with a broad rounded anterior end and a tapering posterior end. The trophozoite measures about 12 to 15 µm long, 5 to 9 µm wide and 2 to 4 µm thick. The dorsal surface is convex while the ventral surface is concave. The organism is bilaterally symmetrical with a large sucking or adhesive disc on the ventral side (Carmena, 2010 and Robertson, 2014). It occupies most of the anterior region of the ventral surface. There are two large anterior vesicular nuclei. Four pairs of flagella are represented. The first pair of flagella emerges anteriorly between the cell edge and dorsal surface, the second or lateral pair is located in the posterio-laterally from a groove between the margin and the dorsal surface, the third or the ventral pair from a groove posterior to the adhesive disc and the fourth pair is caudally from the tapered end. The flagella which run backwards through the middle of the body have been referred to as the median rods or axonemes. Two median bodies composed of bundles of microtubules arranged irregularly in the shape of the claws of a claw- hammer are present. These disappear in the course of division and it has been suggested to have something to do with the formation of a new adhesive disc. The shape of the median body is used to divide *Giardia* into groups. The trophozoite has also two nuclei without nucleoli which are located interiorly and symmetrically to the long axis. The nuclei contain the same number of gene and the same amount of DNA (Kabnick and Peattie, 1990).

Trophozoites inhibit the small intestine of their host using the ventral adhesive disc to attach to the wall of the small intestine. The trophozoites usually encyst before they leave the jejunum. It is not known whether the factors that initiate the encystation are intrinsic or environmently dependent such as changes in the intestinal pH. Most probably, it is a combination of both. The living trophozoite moves in a very characteristic way, like a falling leaf, and this feature is used as a diagnostic criterion to discriminate *Giardia* trophozoites from other protozoan flagellates in fresh stool samples. Encystation into new cysts takes place in the lower part of the small intestine, and the excreted cysts, which are immediately infectious, are also resistant to environmental factors and can survive for a long time under favorable conditions (Ankarklev *et al*., 2010).

B. Cyst

The cysts are ovoid to ellipsoidal and slightly asymmetric, 8-14µ in length and 5-10µ in width, has 4 nuclei and 4 pairs of bristle like axoneme (Danciger and Lopez, 1975). The cysts from the same host often show variations in size and shape. The cysts have four nuclei when mature and a variable number of fibrillar remnants of the organelles of the trophozoite. The cyst wall is smooth without any characteristic markings. The wall composed of an outer filamentous layer and an inner membranous layer with two membranes. Cysts from different hosts appear to be morphologically indistinguishable (Tombes *et al*., 1979).

Infection with *Giardia* occurs after a susceptible host ingests an infective dose of viable cyst. Once the cysts are ingested, trophozoites are ingested, trophozoites are released from the cysts via excystation, which is triggered by exposure to the alkaline environment in the upper small intestine, as well as gastric acid and pepsin (Robertson, 2014).one cyst will produce four trophozoites as two rounds of cytokinesis occurs without immediate DNA replication (Robertson, 2014). The trophozoites then attach to the enterocytes of the mucosal surface in the lumen of small intestine by using a suction disc located on their ventral surface (Erlandsen *et al*., 2004, Robertson, 2014). Negative pressure at the host-parasite interface created by flaggelar movement is believed to facilitate this attachment (Erlandsen *et al*., 2004). Trophozoites are non-invasive and remain in the lumen of the small intestine either attached to the intestinal wall or freely motile, and multiply asexually via longitudinal binary fission (Robertson, 2014). Although trophozoites mainly reproduce asexually, there is evidence for sexual recombination among *Giardia* (Frazen *et al.,* 2009 and Robertson, 2014). It has been suggested that there are infrequent recombinatory events between different isolates and allelic sequence heterozygosity, particularly associated with *G. duodenalis* Assemblages B (Frazen *et al.*, 2009). To ensure survival inside the hosts intestine, *Giardia* trophozoites are capable of antigenic variation which involves continuosly switching specific antigens which is likely important for evading the hosts immune response (Nash, 2002).

2.2.3 Bovine Giardiasis

Giardia duodenalis infects bovines of almost all age groups (Olson *et al*., 1997; Goz *et al*., 2006). The pathogen is prevalent at 2-24 week age so it should be considered in animals with diarrhoea, or failure to thrive (Olson *et al*., 1997). Even in older calves prevalence and chronic infection has been reported (O'Handley *et al*., 1999) and so they may act as a carrier of infection. At high rate of infection, *Giardia* may lead to outbreak of diarrhoea in calves (Xiao *et al*., 1993). It has been observed that giardiosis has negative effect on production, performance, weight and hence may causes economic losses and thus have clinical importance (Olson *et al*., 2004). Throughout the world, *Giardia* has been reported at different prevalence rate which may reach up to 100 percent in bovines (Oviedo *et al*., 1987 and Himonas *et al*., 1998).

2.2.4 Pathobiology

Giardia trophozoites has been reported to cause villous atrophy, diffuse shortening of microvilli, reduce disaccharidase activity, loss of epithelial barrier function, increase permeability and enterocyte apoptosis (Buret, 2007). Diarrheal symptoms mostly occur during the acute phase of the infection. The majority of *Giardia* infections are self-limiting, although reinfection and chronic infection can occur. Research over the past two decades has established that the pathophysiology of giardiasis is multifactorial and involves parasitic, host, dietary, and environmental factors, as well as immunological and non-immunological processes (Ankarlev, 2010)

The pathophysiology of acute diarrhea in giardiosis implicates increased rates of enterocyte apoptosis, a disruption of the intestinal barrier function, activation of host lymphocytes,CD8+ lymphocyte-mediated shortening of brush border microvilli with or without coinciding villous atrophy, disaccharidase deficiencies, small intestinal malabsorption, anion hypersecretion, and increased intestinal transit rates (Scott *et al.*, 2010 and Panaro *et al.*, 2007). Impaired enterocyte cell cycle and proliferation, via the consumption of host arginine by the parasite, have also been suggested to contribute to pathogenesis (Stadelmann, 2013). The reduction in intestinal barrier function induced by giardiosis implicates disruptions of F-actin, zonula occludens 1 (ZO-1), claudin-1 and claudin-4, occludin, and α-actinin, the latter being a component of the actomyosin ring that regulates paracellular flow (Halliez, 2014). Loss of epithelial barrier integrity is mediated by activation of epithelial myosin light chain kinase (Chin, 2006). Disruptions of epithelial tight junctional proteins by *Giardia* are caspase-3-dependent, similarly to other enteric disorder (Buret and Bhargava, 2013). However, recent findings also point to a direct role for cysteine proteases released by the parasite in the proteolytic disruption of epithelial villi, an important constituent of brush border microvilli (Bhargava *et al*., 2013). *Giardia*-induced diffuse shortening of brush border microvilli causes small intestinal malabsorption due to impaired absorption of water, glucose, and electrolytes. Anion hypersecretion may further contribute to the production of the resulting diarrhea (Baldi, 2009). Recent findings indicate that the pathogenic effects of *Giardia* may be further compounded by degradation of local mucin by the parasite (Amat, 2015), which may in turn contribute to the translocation of commensal bacteria through the epithelium (Beatty, 2013). An isolated report indicates that giardiasis may be associated with increased mucus secretion, but the biological significance of this observation has yet to be determined. Recent findings indicate that *G. intestinalis* may cause mucin depletion in goblet cells of the small intestine, as well as in the colon (Amat, 2015). While at least some of the pathogenic effects of *Giardia* are isolate-dependent, the identification of a *Giardia* “enterotoxin” has remained elusive.

In giardiosis and other enteric infections, severe diarrhea may lead to poor cognitive function caused by zinc and iron micronutrient deficiencies, as well as defects in the antioxidant system, which may all affect neuroplasticity (Koruk *et al.,* 2010 and Ajjampur *et al.,* 2011).The mechanisms responsible for pathophysiology in giardiasis include both parasite and host immune factors, as well as *Giardia* induced microbiota disruptions that may directly contribute to the post-infectious gastrointestinal disorder associated with the infection. *Giardia* induces enterocyte apoptosis and disrupts the epithelial barrier in myosin-light chain kinase dependent manner. In turn, CD8+ lymphocyte-dependent microvillus shortening causes maldigestion and malabsorption. Compounding these effects, *Giardia* may also increase chloride secretion and intestinal transit, all of which further contribute to diarrhea.

2.2.5 Clinical signs

Giardia infection in cattle are clinically important and may be of economic significance by impairing performance. Clinical manifestations of giardiasis usually appear 1 to 2 weeks after infection and may range from asymptomatic to acute or chronic diarrhea, abdominal pain, vomiting, and weight loss (Cotton *et al*., 2011). Recent evidence indicates that *G. intestinalis*, like other enteropathogens, may cause chronic postinfectious complications, including irritable bowel syndrome, via mechanisms that remain obscure (Buret, 2014). Similar to most infectious diseases, a variety of host and environmental factors modulate disease outcome in giardiasis. They include diet, immune factors, age, and concurrent infections (Ankarlev *et al*., 2010; Halliez and Buret, 2013 and Bhargava *et al*., 2013). In calves, diarrhoea, mucoid and fatty stool, weight loss and growth retardation are the main disease symptoms (Dwight, 1999 and Lebwohl *et al*., 2003). These symptoms may persist for few weeks, in the case of acute giardiosis or evolve into chronic reoccurring disease (Farthing, 1996). Eighty five percent at the age of 5 weeks which decline up to 25 percent at the age of 25 weeks (Olson *et al*., 1997). According to Singh *et al*. (2008) the prevalence of giardiosis peaks in diarrhoeic calves at the age of 1-6 months whereas in non-diarrhoeic calves peak reaches at the age of 6 months to 1 year. *Giardia* infection in ruminants cause intestinal malabsorption thereby reducing rate of body weight gain and affecting feed efficiency in growing animals by as much as 10%. Although mortality is not common, fatal *Giardia* infections have been reported in chinchillas and birds (Shelton, 1954 and Upcroft *et al.*, 1997).

2.2.6 Detection of *Giardia* spp. in the faecal samples

The diagnosis of giardiasis in asymptomatic cases plays an important role for controlling the disease. Thus, obtaining reliable results in the diagnosis is quite significant. There are different methods used to detect *G. intestinalis* cysts or trophozoites. Currently, microscopic techniques like direct (iodine wet mount) on fresh and concentrated faecal samples are still commonly utilized (Johnston *et al*., 2003 and Garcia, 2007). In addition, concentration method (FEST- Formal ether sedimentation technique, ZNSFST- Zinc sulphate floatation Sedimentation technique), immunoenzymatic (DFAT - Direct fluorescent antibody test) and molecular techniques are also available for routine diagnosis and research studies (de waal *et al*., 2012, Faust *et al*., 1938 and Bruijnesteijn van Coppenraet and Wallinga *et al*., 2009)

2.2.6.1 Microscopic staining method

Microscopy of direct fecal smears or smears prepared following formol-ether concentration and iodine staining has been reported to reach 97% sensitivity (Wolfe, 1979). Zajac and Conboy (2012) reported that *Giardia* spp. were identified on the basis of cysts/trophozoites color shape and size (8-14µ in length and 5-10µ in width)**.**The utility of sodium acetate-acetic acid-formalin (SAF) preservation (Yang and Scholten *et al*., 1977) in diagnosing intestinal protozoans and found both an increased yield with the concentration of preserved samples and a further increase of about 20% with permanent staining. Calves have been reported to the infected with *G. intestinalis* as early as four days of age and have the highest intensity of cysts excretion (10^5 to 10^6 cysts/ gram) which could be diagnosed by iodine wet mount technique. (Ahmad *et al*., 2016).

Kamath and Murugasu (1974) reported that microscopy of direct smears without preservation have found to be 50% sensitive. However, string test, duodenal aspirate, intestinal impression smear and intestinal biopsy have been proposed as techniques to improve microscopic diagnosis (Isaac, 1991 and Koneman *et al*., 1992).

2.2.6.2 Concentration techniques

Traditional approaches to diagnose the *Giardia* cyst by concentration techniques with standard protocols have been followed with more sensitivity than microscopy.

Formol-ether (FE) or formol-ethylacetate (FEA) concentration is a standard procedure for investigating cattle calves with diarrhea where *Giardia* spp. infection was suspected (de Waal, 2012 and Fletcher *et al*., 2012).

Zinc sulfate flotation for concentration of intestinal protozoa was first described in 1938 (Faust *et al*., 1938) and soon modified (Sawitz and Faust, 1941). The FE (formol-ether) concentration technique was described (Ritchie, 1948). The FE and the zinc sulfate flotation methods were compared with microscopy technique which found them equally sensitive (Bartlett *et al*., 1978; Ritchie *et al*., 1952). The use of ether has been substituted with ethylacetate, without any change in sensitivity (Erdman, 1981), to become the FEA concentration technique.

Additionally, the salt–sugar flotation (SSF) technique is also used as an alternative to FEA but this technique is rather laborious and may therefore not fit well in a diagnostic laboratory (Maddox-Hyttel *et al*., 2006).

Rasmussen *et al*., 2016 demonstrated that concentration using FEA or SSF followed by iodine staining and microscopy had a detection level at least 1500-fold lower than immunofluorescence- and PCR-based techniques.

2.2.6.3 Immunological detection methods

Many studies have demonstrated that direct immunofluorescence microscopy of faeces (fluorescent antibody [FAB] coproscopy), using fluorescent antibodies directed against *Giardia*, provides greater sensitivity and specificity for diagnosis of *Giardia* than flotation methods. Geurden *et al*. (2008) studied direct immunofluorescent coproscopy which has been adopted as the gold standard for identifying fecal *Giardia* cysts.

Immunofluorescence assays (IFAs) can be used to stain fecal smears without initial concentration and this method has an increased sensitivity compared to FEA (Garcia *et al*., 1992 and Rose *et al*., 1989). El-Nahas *et al*. (2013) studied about direct fluorescent antibody test, enzyme immunoassays (EIAs) and reported that it has high sensitivity and specificity similar to the most widely used immunological techniques than the conventional methods.

2.2.6.4 Molecular techniques

The introduction of PCR allows semi automation of the diagnostic procedure, and multiplex PCR permits the detection of several different parasite species (Bruijnesteijn van Coppenraet and Wallinga, 2009, Mary *et al*., 2013 and Stensvold and Nielsen, 2012).

A variety of PCR assays (nested PCR, PCR-RFLP, RT-PCR etc.) were developed for diagnosis of giardiosis. They have excellent sensitivity and specificity but require more specialized technical skills and high-cost equipment. RT-PCR is the most powerful method and it has the following advantages: targeting the small specific gene regions of the parasite, rapid cycling time (approximately 1 h), low contamination risk, and ability to measure the DNA amount during the assay without post-PCR analysis. The infection could be detected in patients with a low parasite count by ELISA or by PCR when only when two cysts are present (Calderaro *et al*., 2010). These methods capture the infection using the parasite antigen or DNA molecule, so even when the live parasite is absent, they produce accurate results (Jelinek and Neifer, 2013). RT-PCR was accepted as the gold molecular technique and the use of different diagnostic techniques together would increase the chances of obtaining true positives (Caccio and Sprong, 2011).

Rasmussen *et al*., 2016 reported qPCR had a sensitivity of 91%, specificity of 95.1%, a false-positive rate of 50%, a false-negative rate of 0.48%, a positive predictive value of 50%, and a negative predictive value of 99.5%. This showed that qPCR and IFA were significantly more sensitive than microscopy of iodine-stained concentrates using either FEA or SSF.

2.2.7 Prevalence and molecular characterization of *Giardia* spp.

2.2.7.1 Work done in India

In India diarrhoea occur in bovines because of various pathogens which has been widely reported with the global distribution of giardial assemblage, zoonotic transmission and the association of assemblages with disease and epidemiology of giardiasis in India.

Sulaiman *et al*. (2003) identified *Giardia duodenalis* parasites from animals and nucleotide sequences of the triosephosphate isomerase (TPI) gene were generated for 37 human isolates, 15 dog isolates, 8 muskrat isolates, 7 isolates each from cattle and beavers, and 1 isolate each from a rat and a rabbit. Phylogenetic analysis on the TPI sequences confirmed the formation of distinct groups and a major group (assemblage B) contained most of the human and animal isolate which confirmed that *G. duodenalis* from certain animals can potentially infect humans.

Traub *et al.* (2004) studied *Giardia* infection in humans and dogs in a tea growing community and the genotyping using three markers (*18S-rDNA, ef1-alpha, tpi*), revealed presence of genetically identical genotypes (AII-type) in a dog and two people in the same household.

Khan *et al*. (2011) examined fecal samples from 180 calves, heifers and adults and 51 dairy farm workers on two dairy farms in West Bengal by PCR-RFLP analysis of the β-giardin gene of *G. duodenalis* followed by DNA sequencing of the nested PCR products. The overall prevalence of *G. duodenalis* in cattle was 12.2% (22/180), the infection being more prevalent in younger calves than in adult cattle. Zoonotic *G. duodenalis,* Assemblage A1 was identified in both calves and workers although the most prevalent genotype detected in cattle was a novel Assemblage E subgenotype.

Sharma *et al*. (2013) used zinc sulphate floatation technique and copro antigen ELISA kit and recorded an overall prevalence of 40.83% *Giardia* infection in diarrhoeic dogs from Jammu.

2.2.7.2 Work done abroad

In cattle, a prevalence of between 6.6% in New Zealand (Learmonth *et al*., 2003) and up to 57.8% in Canada and Australia (O'Handley *et al*., 2000) has been reported. A cumulative parasite prevalence of 73% to 100% in both dairy and beef calves has been observed in North America (Ralston *et al*., 2003), and also a farm prevalence as high as 96% has been recently reported in Canada (Dixon *et al*., 2011). Several studies have demonstrated that *Giardia* infections in cattle tend to occur more commonly towards the end of the neonatal period, and they are often chronic in nature (O'Handley and Olson, 2006). A higher prevalence of *Giardia* in pre-weaned (40%) and post-weaned (52%) calves compared with adult cattle (27%) has been reported (Trout *et al*., 2007).

Xiao *et al.* (1993) examined faecal samples of diarrhoeic calves from Ohio farms in USA and found *Giardia* in 100 percent of diarrhoeic cases of both farms and conducted that the *Giardia* infection contribute to the outbreak of diarrhoea in calves.

Xiao and Herd (1994) using quantitative direct immunofluorescence assay in calves (0-20 week old) of an Ohio dairy farm observed cumulative infection rate of *Giardia* and *Cryptosporidium parvum* to be 100 percent with peak shedding of *Giardia* cyst at 2 week to 7 week of age.

Olson *et al*. (1997) conducted a study, using 386 calves (new born to 24 week ages) from 20 dairies located in the lower Frazer river valley area of British Columbia. They reported an overall prevalence of 73 percent (50-100 percent) with an average geometric mean cyst count of 1180 cyst per gram of faeces. They also observed that *Giardia* is not age dependent. Forty seven percent of calves with diarrhoea were found to have high number of *Giardia* cyst in their faeces.

Wade *et al*. (2000) conducted an observational analytic study in south eastern New York State and reported that maternity, management practices, age of the animals and the season of sampling were significantly associated with the risk of infection with *Giardia* spp. It was also observed that summer housing of herd heifers on pasture or in the stall, was associated with increased risk of infection and it decreased immediate weaning after birth and with age of animals.

Degerli and Ozcelik (2005) had reported prevalence rate of *Giardia* to be 14 percent in calves in Turkey. Goz *et al*. (2006) examined 231 calves ages range between 1 day to 8 months in Van, Turkey and found that 80 percent of the calves were infected with one or more parasitic load with 14.7 percent of prevalence of *Giardia* in diarrhoeic calves.

Santin and Zarlenga (2009) using immunofluorescence microscopy and PCR examined faecal samples of 30 calves (1 day to 24 month age) and observed that 31.5 percent of the samples (312/990) were *Giardia* positive. Higher prevalence (60.8 percent) in preweaned calves (<8 weeks) followed by 32.1 percent in age groups of 8-12 weeks and 11.4 percent in heifer (12-24 month). Zoonotic assemblages were reported in post weaned (6.9 percent) and heifer (4.7 percent) calves only.

Mark-Carew *et al*. (2010) using Zinc Sulphate floatation examined 10,672 faecal samples in New York water shed region and observed that *Giardia* infection occurred in calves within first 180 days of age. The incidence of cysts shedding ranged from 0.0004 per animal day for cattle at high risk cohort. In addition it was observed that the intensity of shedding *Giardia* cysts varied significantly with age ($p<0.0001$) and season of collection.

Dixon *et al*. (2011) reported high prevalence of *Giardia* in dairy calves (96 percent) and beef calves (97 percent) in Ontario Canada. Further it was found that zoonotic genotype were more in dairy cattle than in beef cattle and parasitic infection was higher in calves than in adult. Study also supported the zooanthroponotic transmission of *Giardia*.

Fan *et al*. (2017) examined total of 339 faecal samples from pre- and post-weaned calves from four distinct locations in Hubei Province using markers in the large (LSU) or small (SSU) subunits of nuclear ribosomal RNA genes from which *Giardia duodenalis* assemblage E were detected in 0.6%, 10.9%, 4.1% and 22.6% of calves, respectively.

2.2.8 Assemblages of *Giardia intestinalis*:

DNA-based genotyping studies have identified more subtypes from both humans and animals isolates, e.g., (Lalle *et al*., 2005) and (Sulaiman *et al*., 2003), and have confirmed sub-structuring within major *G. duodenalis* assemblages. In these studies, a different terminology was used to describe subtypes, such as A3, A4, A5, or B1, B2, B3 at the *bg* locus (Lalle *et al*., 2005), B S1, B S2, B S3, or C S1, C S2, C S3 at the *tpi* locus (Sulaiman *et al*., 2003), or even B0, B1, B2 at the *gdh* locus. This has generated a certain degree of confusion, particularly when different subtypes were found by sequencing different genetic markers, i.e., when there was a lack of concurrence between the results for these genes (Robertson *et al*., 2006).

Lalle *et al*. (2005) analysed beta-giardin nested PCR assay applied to 24 isolates collected from calves. Sequencing of the amplification products revealed that eight isolates displayed subgenotype A1, one subgenotype A2, two subgenotype A3, and one subgenotype A4. The Assemblage B was found in five isolates, one isolate displayed a subgenotype B3 sequence, whereas two new subgenotypes (B5 and B6) were each found in two isolates. Two mixed A and B infections were also detected. The host-specific Assemblages E was detected in two isolates, as well as in three mixed infections with Assemblages A and E, or subgenotype E1 and E2. The comparison of the beta-giardin sequence of the reference strain P15.

Uehlinger *et al*. (2006) performed Polymerase chain reaction (PCR) on 74 of 248 faecal samples in Atlantic Veterinary College, University of Prince Edward Island, Canada which were found to be positive for *G. duodenalis* under the epifluorescence microscope, genetic sequencing was successful on only 14 of these 28 samples. *G. duodenalis* genotypes were identified by comparison to GenBank sequences as genotypes A and E.

Geurden *et al.* (2008) and Santin and Zarlenga (2009) reported high prevalence of zoonotic assemblages A in dairy calves than beef calves where the host specific assemblages were more common suggesting that the calves may be an important source of human infection. Genetic similarity of some isolates from human and animals also indicates possible infection of human from livestock (Ey *et al.*, 1997). Hunt *et al.* (2000) and Learmonth (2003) reported that human assemblages were common in cattle and assemblages E was largely absent in New Zealand.

Barigye *et al.* (2008) reported *Giardia duodenalis* in 189 scouring calves in North Dakota USA and tested by different assays during a 1-year-study period. *Giardia* antigens were detected in 22/189 scouring calves by a fecal-based enzyme-linked immunosorbent assay and 10 of these were positive for assemblage E, by polymerase chain reaction.

Minvielle *et al*. (2008) investigated the genotypes of *Giardia lamblia* from human and animal feces and their epidemiological and clinical characteristics in Argentina, South America.

They examined seventy isolates out of which 60 were humans (adults and children), eight from dogs and two from cows were processed by polymerase chain reaction-restriction fragment length polymorphism. The triosephosphate isomerase gene was amplified from 43 (71.66%) of the 60 human fecal samples and among these, 3/43 (6.98%) were genotype AII and 40/43 (93.02%) were genotype B. Assemblage AII was detected in three children and this genotype was not found in animals.

Oates (2012) analyzed *Giardia* isolated from beef cattle revealed that all faecal samples comprised of assemblage E. All beef cattle isolates shared 99% homology with each other and the reference genotype E sequence (GenBank AY178740) from bp 59 to 482. Isolates Cattle 236 and Cattle 240 differed by one base pair (C to T at 447 and A to Gat bp 382, respectively). Isolate Cattle 032 differed by two base pairs (A to G at bp 382 and C to T at bp 447).

Minetti *et al*. (2012) examined 370 faecal samples tested by PCR out of which 127 (34.3%) gave a positive result for *Giardia* spp. and 91 (71.6%) were successfully sequenced, confirming infection with *G. duodenalis* assemblages occurring in domestic livestock species in the UK. *G. duodenalis* assemblage E and mixed infections with assemblages A and E was found to be predominant in cattle and sheep.

Huang *et al*. (2014) Twenty-nine *G. duodenalis* isolates were analyzed by DNA sequencing of the triosephosphate isomerase (tpi) and glutamate dehydrogenase (gdh) genes. Two *G. duodenalis* assemblages were identified, assemblages E (n = 15) and B (n = 4, one subtype B1 and three subtype B2) in preweaned calves, and assemblage E (n = 10) in 3–11-month-old calves.

Fan *et al*. (2017) sequenced the amplicons from *Giardia*-positive samples (n = 77) faecal samples from pre and post-weaned calves from other regions in China which revealed the assemblage E in the present study and it had not been considered zoonotic.

Malekifard and Ahmadpour (2018) identified subspecies of *G. duodenalis* by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method from fecal samples of naturally infected cattle in the Urmia, West Azerbaijan province, Iran. The PCR-RFLP analysis of glutamate dehydrogenase *(gdh)* locus was used to identify the genotypes found in cattle. In this method, 432 bp expected size was amplified and then specific restriction *Nla*IV enzyme was used for subspecies detection. The PCR-RFLP analysis revealed that 19 samples (82.60%) have the genotype E and 4 samples (17.39%) belong to the subgroup AI.

Jian *et al.* (2018) examined 389 faecal samples from cattle in the Qinghai-Tibetan Plateau Area, northwestern China out of which 39 specimens from cattle were *G. duodenalis*-positive by PCR amplification of the SSUrRNA gene with a prevalence of 10% (39/389). Sequencing and phylogenetic analysis of the positive samples identified *G. duodenalis* assemblage E.

2.3 Therapy of cryptosporidiosis

One of the most biologically intriguing, and clinically frustrating features of cryptosporidiosis is its resistance to antimicrobial drugs. Unlike many other parasites (*Toxoplasma, Eimeria* and *Plasmodium*), there is no curative therapy for cryptosporidiosis, despite the in vitro and in vivo testing of many compounds (obiad *et al.,* 2012).Woods *et al*. (1996) analyzed that an experiment on more than 100 drugs *in vitro* only 40 drugs showed some affection on the parasite, no one of the aniparasitic drugs used (Guinive, Chloroquine, Pyrimethamine, Difluromethyl orinthine, Trimethoprim- sulfamethoxazol, Diclazuril). Although large number of chemotherapeutic agents have been screened against cryptosporidiosis, but there is no reliable curative treatment for the infection (Armson *et al.*, 2003). The poor response to many chemotherapeutic agents has been explained by the parasite's unique intracellular location (Sterling, 2000), which may serve as "escape mechanism" to protect the parasite from anticryptosporidial drugs. A limited number of chemotherapeutic agents (nitazoxanide, paromomycin, macrolide, spiramycin, azithromycine and refaxmin) have demonstrated efficacy in animal models and under clinical trials. The current optimal therapy for cryptosporidiosis is nitazoxanide and is the most common drug for treating cryptosporidiosis in humans and animals (Certad, 2010). NTZ, a nitrothiazole benzamide compound, has proved to be effective against a wide variety of protozoa and helminthes (Hussien, 2013). Nitazoxanide exhibits antiprotozoal activity by interfering with the pyruvate ferredoxin/flavodoxin oxidoreductase dependent electron transfer reaction, an essential reaction need for anaerobic energy metabolism of various microorganism. Sporozoites of *Cryptosporidium parvum* are therefore inhibited, relieving symptoms of diahrroea (Shakya *et al*., 2017).

Blagburn *et al.* (1998) published first report on evaluation of nitazoxanide against *C. parvum* in a mouse model which was studied in Atlanta, Georgia. In this study, two formulations were considered, i.e., powder formulation with moderate effect against *C. parvum* when tested at 100 mg/kg. This was further supported by substantial improvement in efficacy (4.3% of the oocyst output in the controls) when the dose was increased to 150 mg/kg. The drug yielded moderate efficacy as powder and injectable formulations administered at 100 mg/kg orally (reduction of oocyst output to 42 and 26% of that in controls, respectively). But, oral administration of the injectable formulation of nitazoxanide at a dose of 150 mg/kg resulted in improved efficacy (oocyst output, <5% of that in controls) as compared to 100 mg/kg dose rate.

Theodos *et al*. (1998) evaluated the study against chronic cryptosporidiosis in cell culture in two animal models (piglets and mice) at Bethesda, Maryland. A concentration of 10 mg of nitazoxanide/ml (32 mM) consistently reduced parasite growth in cell culture by more than90% with little evidence of drug-associated cytotoxicity, in contrast to an 80% reduction produced by paromomycin at 2,000 mg/ml (3.2 mM). In contrast to its efficacy in vitro, nitazoxanide at either 100 or 200 mg/kg of body weight/day for 10 days was less effective at reducing the parasite burden in *C. parvum*-infected, anti-gamma interferon conditioned SCID mice. NTZ was partially effective at reducing the parasite burden in a gnotobiotic piglet diarrhea model when given orally for 11 days at 250 mg/kg/day but not at 125 mg/kg/day.

Abdou *et al.* (2013) carried out the study in Egypt that nitazoxanide was tested as a treatment for cryptosporidiosis in both the experimentally infected immunocompetent and dexamethasone-immunosuppressed groups. The study demonstrated the effectiveness of nitazoxanide in both groups with significant differences regarding levels of oocyst excretion in the faeces and the number of endogenous developmental stages of the parasite in both groups, being lower in group with immunocompetent mice than in immunosuppressed groups ($p < 0.05$). Histopathological findings in experimental animals confirmed the gastrointestinal changes, including parasitological and pathological changes, induced by *C. parvum* infection in both immunocompetent and in chemically immunosuppressed mice, together with immunohistochemical assessment of cyclin D1 expression in infected tissues. In addition, the effectiveness of nitazoxanide in the treatment of cryptosporidiosis was evaluated and revealed that the drug was not effective without an appropriate immune response, in which a competent immune system is needed to reject the parasite.

Taha *et al.* (2017) carried out the study in Egypt about the combination of Atorvastatin and nitazoxanide which showed a synergistic effect through reduction of the number of *Cryptosporidium* oocysts shed and improvement of the histopathological changes in the small intestine, colon, stomach and lungs of infected immunosuppressed mice in comparison to that induced by either nitazoxanide or Atorvastatin alone. In the study, it was concluded that Atorvastatin was used alone at low dose (20 mg/kg), high dose (40 mg/kg), and combined with nitazoxanide (1000 mg/kg) with either the low dose or high dose for five consecutive days. Parasitological assessment of the drug effect was done using Modified Z-N staining of stool samples collected from mice. Therefore, results revealed a reduction of the number of oocysts shed with percentage of reduction on the 21st day post infection by 53.7%, 67.2%, 70.1% & 77.5%, respectively, compared to the infected untreated group and nitazoxanide treated alone group showed 52.7% reduction.

Mostafa *et al.* (2018) reported in Egypt the combined therapeutic study on artesunate and nitazoxide which resulted in highest reduction percentages in oocyst shedding among the immunosuppressed group (68.5%, 75.9% and 99%) of experimental animals when measured on days 7, 14 and 21 consequently. According to percent efficacy of used drugs, nitazoxide had 12.2%, 10.8% and 8.2% efficacy and artesunate with35.4%, 54% and 60.8%efficacy. Finally, the study concluded that the combined therapy of artesunate and nitazoxide gave the highest efficacy in oocyst reduction among the immunosuppressed groups (68.5 %, 75.9% and 99%).

El Shafei *et al.* (2018) carried out the study in Egypt that the combined therapy of phenyl vinyl sulfone (PVS) as cysteine protease inhibitors @ 35 mg /day and nitazoxanide (NTZ) @ 100mg/day, in immunocompetent and immunosuppressed groups showed highest reduction in the mean number of oocyst shedding per milligram of faeces than treatment with NTZ and with PVS individually. The mean oocyst shedding in immucompetent group PI was less (0.778 ±1.20), whereas in immunosuppressed group, the mean oocyst count was more (4.43± 0.49).

In Ayurveda (Indian traditional medicine), tumeric has been used for various purposes and through different routes of administration. Hundreds of *in vitro* and animal studies have been published describing the antioxidant, anti-inflammatory, antiviral, and antifungal (Roth *et al*., 1998) properties of curcuminoids (White and Foster, 2000; Young-Joon, 1999) which gives tumeric its bright yellow color.

Turmeric is prepared by grinding of dried rhizomes mainly from *Curcuma longa* of the Zingiberaceae family. These powdered dried rhizomes have at least 76 synonyms listed in the 1999 WHO monograph. Among them some important names are *Haldi* (in Hindi), *Haridra* or *Gauri* (in Sanskrit), *Kurkum* (in Arabic), *Besar* (in Nepali), *etc*. *Curcuma longa* grows naturally throughout the Indian subcontinent and in tropical climates. It is a short-stemmed perennial, which grows to up to 100 cm in height. It has curved, oblong, and ovate leaves with beautiful white to colourful flower and cylindrical rhizomes. More recently, it has been used by the food industry as additive, flavouring, preservative, and colouring agent.

The first recorded scientific article referring to *Curcuma* spp. was published in 1748, and the first pharmacologic review of turmeric appeared 67 years later (Vogel and Pelletier, 1815). The yellow colour of turmeric is mainly due to the presence of polyphenolic curcuminoids, which constitute approximately 3% to 5% of most turmeric preparations. The alcoholic extract of turmeric mainly contains three curcuminoids, namely curcumin (also referred as curcumin I or diferuloylmethane), desmethoxycurcumin (curcumin II), and bisdesmethoxycurcumin (curcumin III). Its chemical structure was confirmed in 1973 by Roughley and Whiting (Roughley and Whiting, 1973) and the solution structure was only confirmed by Payton *et al.* (2007).

The most commercially available “curcumin” is a mixture of curcumin (approx. 77%), desmethoxycurcumin (approx. 18%) and bisdesmethoxycurcumin (approx. 5%) (Basnet *et al*., 2010 Curcumin [chemical name: (*E,E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5 dione) is a bis-α,β-unsaturated β-diketone. It has a molecular weight of 368.38, a melting point of 179–183 °C, and chemical formula of C21H20O6.

Curcumin is readily soluble in dimethylsulfoxide (DMSO), ethanol or acetone, but it is sparingly soluble in water. In acidic and neutral solutions as well as in the solid state, the keto form predominates, and curcumin acts as a potent donor of H-atoms. In contrast, under alkaline conditions (≥pH 8), the ethanolic form predominates, and the phenolic part of the molecule plays the principal role. Curcumin is similarly unstable at basic pH, but in presence of calf serum or human blood less than 20% of curcumin was found to be decomposed in 1 h (Wang *et al*., 1997)

The absorption, distribution, metabolism and excretion studies of curcumin in recent years suggest that curcumin undergoes a rapid metabolism which has been suggested as the root cause of low bioavailability in systemic circulation. Curcumin administered orally at a dose of 1,000 mg/kg to rats resulted in approx. 75% of the dose being excreted in faeces and negligible amounts were detected in the urine (Wahlstrom and Blennow, 1978).

In addition to inhibiting the growth of a variety of pathogens, curcumin has been shown to have anthelmintic and antiprotozoal activities (Araujo *et al*., 1998 and Koide *et al*., 2002). The current experience regarding curcumin effects in various scenarios rewards more research into the drug targets that might be suitable for therapeutic invention.

Antiprotozoal activities of curcumin have been reported extensively over the last decade. The spice rhizome of turmeric (1% crude extract), as well as its main medicinal component, curcumin (0.05%), appear effective in reducing upper- and mid-small-intestinal infections of certain protozoans like *Eimeria acervulina* and *Eimeria maxima* (Allen *et al*., 1998). Curcumin was found to be effective against *Cryptosporidium parvum* in cell culture. *C. parvum* appears to be more sensitive to curcumin than *Plasmodium, Giardia* and *Leishmania* (Shahiduzzaman *et al*., 2009). Synergistic antiprotozoal effects were shown when curcumin was applied in combination with other drugs. Leitch and Qing (1999) reported that both reactive nitrogen species (RNS) and ROS play protective roles in experimental cryptosporidiosis in mice. *Cryptosporidium* has a poor capacity to scavenge ROS (Entrala *et al*., 1997), making it potentially more susceptible to killing by such oxygenic compounds. Certain ROS, especially hydroxyl radicals and hydrogen peroxide, produced as a result of parasite exposure to ultraviolet irradiation, resulted in inactivation (photo-toxicity) of *C. parvum* oocysts (Gerrity *et al*., 2008 and Ryu *et al*., 2008).

Cui *et al.* (2007) carried out the study in Pennsylvania that curcumin's prooxidant activity promote the production of reactive oxygen species (ROS) in *P. falciparum*, whose cytotoxic effect could be antagonized by coincubation with antioxidants and ROS scavengers. Curcumin treatment also resulted in damage of both mitochondrial and nuclear DNA, probably due to the elevation of intracellular ROS. Results showed that brief treatment of the parasite culture for 8 h with 25 to 100 M curcumin significantly reduced the HAT activity in the parasite nuclear extracts, whereas treatment with 50 M curcumin nearly completely blocked parasite maturation.

Shahiduzzaman *et al*. (2009) reported that curcumin was found to be effective (>95% inhibition of parasite growth) at 50 μM for 24 h when infected cultures were exposed for more than 12 h, the infection level was reduced to 36%. Withdrawal of curcumin after 24 h of exposure did not result in a significant resumption of *C. parvum* growth. The invasion of host cells by sporozoites (infectivity) was found to be inhibited at least 65% in the presence of 200μM curcumin.

Abbas *et al.* (2010) studied the efficacy of *Curcuma longa* crude powder and salinomycin sodium on the occurrence of coccidiosis and growth performance of broiler. Maximum coccidiostatic effect was observed with 3% turmeric as compared to other infected groups with turmeric containing rations. Therefore, weight gain in the group treated with salinomycin sodium (2280g) and 3% turmeric (2293g) were significantly higher ($p \leq 0.05$) than the infected control group (1955g).

El khtam *et al.* (2014) studied in Egypt the in vitro analysis of sporulated oocysts of mixed *Eimeria* species isolated from naturally infected chickens were randomly assigned to 10, 5, 2.5, 1.25, 0.6, 0.3, 0.2 and 0.08 g turmeric and garlic powders /liter distilled water (g/L). The efficacy turmeric (up to 66.6%) at different concentrations. In the in vivo study, one-day old chicks were divided into 7 equal groups and were infected with 10^3 viable sporulated oocysts of mixed *Eimeria* spp. orally except negative control. Therefore turmeric powders can inhibit or impairs invasion and/ or replication and development of *Eimeria* parasites species in the gut tissues of chickens before the relatively inert oocysts are formed and finally released (i.e. they effect on the intracellular stages of the infected *Eimeria* specially at the 2nd schizogony stage (late stages of asexual cycle) as well as those of the sexual stage of *Eimeria* at the lumen of the intestine).

Dyab *et al*. (2016) evaluated the study in Berlin that curcumin @ 10 mg kg−1 day−1 was reduced *Giardia* cyst count significantly (P≤0.001). The percentages of reductions were 91.2% and 72.3% respectively. The reduction rate with curcumin at 20 mg kg−1 day−1 was 84.7 % (P≤0.001).

Gutiérreza *et al.* (2017) reported in Mexicot that curcumin inhibited *Giardia* proliferation and adhesion in a time concentration- dependent mode. The higher inhibitory concentrations of curcumin (3 and 15 μM) disrupted the cytoskeletal structures of trophozoites and the damage was evident on the ventral disk, flagella and in the caudal region, also the membrane was affected. Also, curcumin caused a clear reduction of tubulin expression and by docking analysis and molecular dynamics we showed that curcumin has a high probability to bind at the interface of the tubulin dimer close to the vinblastine binding site.

Asadpour *et al*. (2018) carried out the study in Iran undertaken to examine the anti-cryptosporidial efficacy of curcumin in experimentally infected mice compared with that of paromomycin. Administration of curcumin stopped oocyst shedding at dose of 4.33 mg/kg/day dissolved in 50 μl of corn oil (Nonose *et al*., 2014) and dose of 50 mg/kg/day of paromomycin without recurrence for 10 days after drug withdrawal. Histopathological findings confirmed that treatment with curcumin could improve the lesions and decrease the number of oocyst on the villi.

2.3.1 Cryptosporidiosis in experimental animal

Cryptosporidium parvum isolated from the faeces of an infected, diarrhoeic calf colonized intestinal tissues of neonatal mice in a significantly heavier manner as compared to germ free or conventional adult mice. By 7^{th} DPI, 4 out of 7 infant mice had *C. parvum* infection as determined by examining their ileal or colonic contents (Harp *et al*., 1988).

Fayer *et al*. (1990) studied the immunotherapeutic efficacy of bovine colostral immunoglobulins from a hyperimmunized cow against cryptosporidiosis in neonatal BALB/c mice. After infection with 250,000 *C. parvum* oocysts and treatment with immmunoglobulins, it was seen that histologically at 72 h post infection, the most significant reduction in the endogenous developmental stages of *C. parvum* occured in the intestines of treatment group as compared to untreated controls.

Bjorneby *et al*. (1991) evaluated the effects of monoclonal antibody immunotherapy in nude mice persistently infected with *C. parvum*. Oral infection with 2 x 10^7*Cryptosporidium parvum* oocysts and subsequently treatment with monoclonal antibody (MAb) for 10 days significantly decreased the infection load in the intestines.

A murine model of biliary tract cryptosporidiosis was developed by direct intra-gall bladder injection of *C. parvum* oocysts in BALB/C and C57BL/6 mice. Intracellular parasitic stages were detected in the epithelium of the common bile duct in all animals on day **7** PI and was associated with a strong inflammatory reaction. Infection cleared between days 14 and 21 PI. However, dexamethasone treated animals were unable to eradicate infection and an establishment of ileal infection was seen in the animals of both strains (Verdon *et al*., 1998**)**.

Using a neonatal mouse model Downey *et al.* (2008) determined that pyrvinium pamoate is a potential drug candidate for the treatment of cryptosporidiosis in both immunocompetent and immunocompromised individuals. Beginning 3 days after infection with oocysts of *C. parvum*, pyrvinium @ 5 or 12.5 mg/kg of body weight/day for 4 or 6 consecutive days reduced the numbers of oocysts in the fecal smears and also decreased the intensitiy of trophozoite infection in the ileocecal intestinal regions in hematoxylin-and-eosin-stained histological slides.

Rasmussen *et al*. (1992) studied the pathology of *Cryptosporodium parvum* infection in mice. After immunosuppression by dexamethasone given orally @ 0.25 µg/gm/days for 14 days, inoculation with *Cryptosporodium parvum* @ 10^6 /os was done. Microscopical examination revealed that *Cryptosporodium parvum* was localized predominantly in the small and large intestine and on the contrary no parasites were observed in non- immunosupressed mice.

Tarazona *et al.* (1998) studied the dynamics and effect of immunosupression after infection with *Cryptosporodium parvum* oocysts @ 10^4, 10^5, 10^6/os infection in BALB/c and Porton mice. Immunosupression was induced by oral administration of cyclophosphamide @ 50 mg/kg/day for 10 days before intragastric inoculation of *Cryptosporodium parvum* oocyst. They observed cyclophosphamide induced marked depletion of IgA in gut secretion & a more severe clinical course of cryptosporidiosis.

Stephens *et al*. (1999) studied liver and bile duct pathology after experimental *Cryptosporodium parvum* infection @ 10^5 oocyst in SCID mice or mice with disrupted genes for CD154, CD40, or interferon gamma (IFN-gamma) .The histological lesions observed in mice included lobular hepatitis in CD154 deficient mice, triaditis in CD40 deficient mice & portal fibrosis, biliary sclerosis & dysplasia in IFN-gamma mice respectively.

Kim and Healey (2001) administered the protective pycnogenol (pine bark abstract) to immunosuppressed adult C57BL/6N mice infected with *Cryptosporodium parvum* oocysts @ 10^6/os. The treatment with pycnogenol @30mg/kg/day was found to be therapeutically effective against *Cryptosporodium parvum* and significantly reduced the faecal oocyst shedding. However, there was no effect on parasitic colonization of intestinal tissue.

Complex interactions between *Cryptosporidium parvum* infection & nutritional status in a weaned murine model were studied by Costa *et al*. (2011). C57BL/6 mice on a protein-deficient diet infected with 5 × 10^7 excysted *C. parvum* oocyts suffered from weight loss after *C. parvum* challenge upto 20%. Further, a significantly higher fecal *C. parvum* shedding was detected in malnourished infected mice compared to the nourished infected mice. Also, higher oocyst counts were found in ileum and colon tissue samples from malnourished infected mice, as well as a significant reduction in the villous height–crypt depth ratio in the ileum as compared to control infected mice.

Castro *et al*. (2012) investigated the role of L-arginine supplementation to undernourished and *Cryptosporidium parvum*-infected C57BL6J suckling mice. Mice infected with *C. parvum* oocysts @ 10^6/os. Peak infection was seen at 14^{th} day PI. Cryptosporidiosis was associated with ileal crypt hyperplasia, villus blunting, and inflammation. L-arginine improved mucosal histology following infection.

Certad *et al.* (2012) infected SCID mice, which were also administered dexamethasone @ 4mg/l with *Cryptosporodium parvum* oocysts isolated from human stool @ 10^5 oocyst by oral gastric lavage. The infection resulted in well differentiated adenocarcinomas which spread to all layers of the digestive tract upto the subserosa and from there spread via blood vessels. Moreover, microscopic studies also demonstrated intense cryptosporidial colonization along with infilammatory cells in the ileoocecal region and also moderate and intense presence in the duodenum, colon.

Benamrouz *et al.* (2014) studied the involvement of ileo-caecal adenocarcenoma that developed in a mouse model immunosuppressed with dexamethasone @ 4mg/l orally and after infection with *Cryptosporodium parvum* @ 10^5 oocyst. Utrastructurally lesions were found at ileo-caecal region of mice and at the antropyloric region of mice and included alterations in intercellular junction of GIT epithelial cells.

Yousof *et al*. (2017) evaluated the study in Egypt on *Cryptosporidium* infection using coproscopy, copro-antigen and copro-DNA for early detection of infection in mice. Three infectious doses (2500, 5000 and 10^4 oocysts/mouse) of *Cryptosporidium* infection were taken in the experiment. *Cryptosporidium* coproantigen and copro-DNA were detected 4 and 8 h P-I in infected neonatal and adult mice, respectively, and intestinal mucosal DNA was detected after 12 h in both. Microscopy was able to detect oocysts 48 h P-I. Inoculated *C. parvum* oocysts were recovered in feces of infected mice without genotypic changes. The study concluded that all the doses of oocysts were effective in producing cryptosporidial infection in mice and neonate mice showed higher susceptibility for cryptosporidial infection than adults without statistical differences for the given infectious doses with no detectable statistical differences (P value<0.05) for the given infectious doses in either the oocyst or antigen and DNA shedding.

Chapter-3

Materials and Methods

CHAPTER–III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Faecal Samples

Faecal samples were collected from cattle calves from three age groups viz.<1 month, 1-6 months and > 6 months of age from private dairy farms of Jammu region and screened for presence of *Cryptosporidium* and *Giardia* spp.

3.1.2 Staining solutions and Chemicals

The staining solutions and chemicals were purchased from Merck's chemicals Ltd, Ziehl-Neelsen stain (Carbol fuchsin, Methanol, 10% sulphuric acid, 5% Malachite green), N/10 Iodine solution, Zinc sulphate.

3.1.3 KITS

The following kits were used: Ziehl-Neelsen stain kit, DNA extraction (Qiagen) kit, Merifluor (*Cryptosporidium/Giardia*) immunoflourescent kit, Gel Purification kit (Promega).

3.1.4 DNA studies

Primers were custom synthesized from Sigma (Aldrich, USA), Ladder 1 Kb MBI Fermentas, Chloroform (Amresco); Saturated phenol and EDTA (SRL, India); Ethidium bromide (Hi Media). Taq polymerase, RNase A and Agarose (Promega). All the restriction enzymes were purchased from MBI Fermentas.

3.1.5 Other Chemicals

Sodium dodecyl sulphate and Tris base (Sigma), Sulphuric acid, Polyvinyl pyrollidone, Potassium dichromate, Hydrochloric acid, Sodium chloride, Sucrose, Methanol and Sodium hydroxide, (SD Fine chemicals); Sodium dihydrogen phosphate, Glycerol, Potassium chloride, Disodium hydrogen phosphate and Potassium dihydrogen phosphate (Merck); Sodium carbonate, Sodium bicarbonate (HiMedia).

3.1.6 Glass wares and Plastic wares

All glasswares and plasticwares used for the present study were purchased from Borosil, Polylab, Tarson, and Eppendorff. Microscopic glass slides and coverslips were purchased from Blue Star.

3.1.7 Equipments

The instruments used are as follows:-

Olympus CH20i compound microscope, Sartorius BP 2215 weighing balance; SPINWIN microfuge spin apparatus, Master cycler gradient Peqlab, Bio Rad Gel documentation system; Lambda 35 UV/Vis spectrometer from Perkin Elmer; 3µ pore size Millipore membrane filters, micropipette (Eppendorf), Deep freezer (Vest frost). Horizontal gel electrophoresis apparatus and Vertical gel electrophoresis apparatus from BioRad; pH meter and BOD incubator (Scientronic), Liquid Nitrogen (LN2) container (MSA, USA).

3.1.8 Mice

Swiss albino mice of either sex of 2weeks of age were procured from the Indian Institute of Integrated Medicine, Jammu (CSIR). The mice were maintained on concentrates containing feed and water *ad libidum*. They were used for evaluation of therapeutic efficacy of *Curcuma longa* against cryptosporidiosis.

3.2 METHODS

3.2.1 Collection of faecal samples and data recorded

Faecal samples of cattle calves of three age groups (< 1 month, 1-6 months, > 6 months) were collected from private dairy farms of four districts of Jammu region (Rajouri, Samba, Kathua and Udhampur) and minimum of fifty samples from each age group per district were collected. After collection, the samples were immediately put in 2.5% potassium dichromate solution and at the time of faecal sample collection, data related to the age, sex of the animals and consistency of faeces were recorded. The samples were further screened for presence of *Cryptosporidium* and *Giardia* spp. oocyst/cysts.

To study the prevalence of *Cryptosporidium* and *Giardia* spp. in cattle calves of Jammu region, a total of 614 faecal samples were collected and subjected to Diethyl ether sedimentation technique (DEES) followed by modified Ziehl-Neelsen staining technique (Henricksen and Pohlenz, 1981) for screening of *Cryptosporidium* spp. infection and lugols iodine wet mount technique for identification of *Giardia* spp. infection. Intensity of infection was measured by counting the number of *Cryptosporidium* oocysts and *Giardia* cysts in the faeces.

3.2.2 Quantitative examination of positive faecal samples

The intensity of *Cryptosporidium* infection among positive samples (by DESS followed by mZN staining) was measured based on the number of oocysts observed under x 40 objective lens (OIE, 2008) i.e. 1+; less than 5 oocysts per slide, 2 +; 1 to 10 oocysts per field of view and 3 +; 11 or more oocysts per field of view. Samples positive for *Giardia* spp. were subjected to modified Mc Masters technique (Soulsby, 1982) for determination of cysts per gram (OPG) of the faeces. About 3 gm of faecal samples were taken in a graduated container upto 45 ml mark. Water was added upto 45ml mark. Sample was allowed to soak water for about one hour. The samples were collected in a beaker and triturated with a pestle and mortar. After sieving, 15 ml of solution was taken in a centrifuge tube and centrifuged it at 1500 r.p.m for 2 minutes. The supernatant was discarded and zinc sulphate solution was added upto 15 ml mark in the centrifuge tube. The solution was mixed well with glass rod. Mc Master Chamber was filled with this solution by using a pipette. Number of eggs were counted in each chamber. The average number was taken and multiplied it by 50 to get e.p.g.

3.2.3 Sensitivity and specificity of diagnostic techniques:

To study the sensitivity and specificity of various diagnostic tests for identification of *Cryptosporidium* spp. and *Giardia* spp. infection, a total of 75 faecal samples (25 samples from each age group viz. <1 month, 1-6 months and > 6 months) were randomly selected from total 614 faecal samples collected from Jammu region. All the 75 samples were subjected to five techniques for identification of *Cryptosporidium* oocysts/*Giardia* Cyst. Among five techniques, three conventional microscopy techniques were DFSS; direct faecal smear staining, DESS; Diethyl ether sedimentation staining, SFSS; Sheather's floatation sedimentation staining (sheathers solution for floatation of *Cryptosporidium* oocysts and zinc sulphate solution for floatation of *Giardia* cyst). Smear was formed after performing conventional technique and then stained with Modified Ziehl-Neelsen staining (mZN) for examination of *Cryptosporidium* and lugols iodine for identification of *Giardia* cyst. The other two techniques used for diagnosis of *Crptosporidium* and *Giardia* spp. infection in the faecal samples were DFAT; direct fluorescent antibody test (MeriFluor *Cryptosporidium/Giardia*, Meridian Bioscience Europe, Nice, France)and nucleic acid based assay (polymerase chain reaction, PCR) using standard primers.

3.2.4 Direct faecal smear examination (DFSS)

A faecal smear was made directly from the collected samples on a clean grease free microscopic slide. The smear was air dried and stained by modified Ziehl-Neelsen technique (mZN) (Henricksen and Pohlenz, 1981). The air dried smear was fixed with methanol for 5 minutes, air dried and then the smear was transiently fixed over flame and kept on staining rack. Concentrated carbol fuchsin was poured over the smear and kept for 30-40 minutes. The slide was washed thoroughly under running tap water for 5 minutes, decolorized using 10% sulphuric acid for 15-30 seconds and then washed in water. The smear was counterstained with 5% malachite green for 5 minutes. The slide was again washed in running tap water for 5 minutes. After drying, examined using the high-power, dry objective (40 fold magnification) and confirmed the morphology using oil immersion.

For examination of *Giardia* cysts by iodine wet mount staining technique a very small amount of freshly collected faecal samples was taken on a clean grease free microscopic glass slide. The sample was mixed with a drop of water and evenly spread on slide with the help of a tooth pick. A drop of lugols iodine solution was poured on the slide and finally smear was covered with clean glass cover slip and examined using high power, dry objectives (40 fold magnification).

3.2.5 Examination of stained faecal smear after Diethyl ether sedimentation technique (DESS)

About 5gms of faeces was mixed thoroughly with an equal volume of phosphate buffer saline (PBS) solution (w/v) and strained first through a wire mesh of 400µ pore size and then of 200µ pore size. The faecal sample was then mixed with an equal volume of diethyl ether in a 15 ml centrifuge tube and vigorously shaken. The sample was centrifuged at 500 x g for 10 minutes. The fat plug and other fat soluble materials in the upper organic/aromatic layer were discarded. The lower aqueous phase was taken in another 15 ml centrifuge tube, mixed with equal volume of PBS and centrifuged at 1000 x g for 15 minutes. The sediment was taken and stained by mZN technique or by lugols iodine technique.

3.2.6 Examination of stained sediment of floated material following sheather's floatation sedimentation staining (SFSS) or zinc sulphate floatation sedimentation technique (ZNSFST)

About 5 gm of faecal sample was thoroughly homogenized with phosphate buffered saline (PBS) and strained through a 400 µ pore size wire mesh. The suspension was transferred to a 15 ml centrifuge tube and centrifuged at 1000 x g for 5 minutes. The supernatant was discarded and the sediment was mixed with 10 ml of Sheather's sugar solution/33 percent zinc solution (specific gravity= 1.18). The suspension was centrifuged at 500 x g for 10 minutes (Anderson, 1981). About 2-3 ml suspension of faecal sample in Sheather's solution or zinc sulphate solution was aspirated from the top after centrifugation. The suspension was diluted 3 times with PBS and centrifuged at 1500 x g for 10 minutes. The supernatant was discarded and smear was made on clean microscopic glass slide from the sediment, air dried and stained by mZN technique or by lugols iodine. The stained smear was examined under the high power and oil immersion microscopy.

3.2.7 Examination by direct fluorescent antibody test (DFAT):

Cryptosporidium oocysts and *Giardia* cysts were detected by a direct fluorescent antibody test (DFAT) as per manufacturer's protocol (MeriFluor *Cryptosporidium/*

Giardia, Meridian Bioscience Europe, Nice, France). Using a transfer loop (provided in kit) the faecal sample was spread over the entire well marked on the glass slide provided in the kit. A drop of positive control and negative control sample were spread over the separate treated slide wells. The slides were allowed to get air dry completely at room temperature for 30 minutes. Then, a drop of detection reagent was placed in each well followed by counter stain. The contents were mixed and spread over the entire well, without scratching the treated surface of the slide. The slide was incubated in humidified chamber for 30 minutes at room temperature. By using a wash bottle, slides were rinsed until excess detection reagent and counterstain was removed. The specimen was kept undisturbed to avoid cross contamination of the specimens. The excess stain was removed by buffer by tapping the edge of the slide on a clean paper or towel and the slide was allowed to dry. One drop mounting medium was added to each well and cover slip was placed to observe under fluorescent microscope. Each well was scanned thoroughly using 100X magnification. The presence of *Cryptosporidium* oocysts and *Giardia* cysts was confirmed at higher magnification.

3.2.8 Examination by polymerase chain reaction

Polymerase chain reaction was carried on faecal suspension obtained after performing diethyl ether concentration technique on the faecal samples of cattle calves.

3.2.9 Extraction and purification of genomic DNA from the purified oocysts and Cysts

The DNA was extracted from faecal samples using the protocol of the commercial kit (QIAamp genomic DNA kit-Qiagen) procured with minor modification.

About 200µl of faecal suspension was taken in a 2 ml microcentrifuge tube and 1.4 ml ASL buffer provided with the kit was added. Stool samples was vortexed for 1 minute or until the stool samples were thoroughly homogenized. The oocysts were lysed by five freeze thaw cycles by freezing in liquid nitrogen (-196ºC) for 30 seconds and thawing at 90 ºC in a water bath for 30 seconds. Finally the suspension was heated for 5 minutes at 70 ºC and centrifuged at 20,000g for 2 minutes to pellet stool particles. Discarding the pellet, 1.2 ml of the supernatant was pipetted into a new 2 ml microcentrifuge tube. One inhibit EX tablet was added to the sample and vortexed immediately and continuously for 2 minutes or till the tablet was completely suspended. The suspension was incubated for 2 minutes at room temperature to allow inhibitors to adsorb to the inhibit EX matrix. Samples centrifuged at 20,000g for 3 minutes and 200 µl supernatant was transferred into another 1.5 ml microcentrifuge tube already pipetted with 15 µl proteinase K. 200 µl of AL buffer was added, vortexed for 30 seconds followed by incubation at 70 ºC for 10 minutes. This complete lysate was poured into a QIAamp spin column (placed in a 2 ml collection tube) in such a way that no moistening of the rim of spin column took place. The column was centrifuged at 20000 rpm for 2 minutes and tube containing filtrate was discarded. QIAamp spin column was placed on a new 2 ml collection tube and 500 µl of buffer AW1 was added, followed by centrifugation at 20000g for 2 minutes. The filtrate so obtained was discarded and QIAamp spin column was again placed on a new 2 ml collection tube. The QIAamp spin column was added with 500 µl and centrifuged at 20,000g for 4 minutes. After discarding the filtrate, the spin column was placed on a new 1.5 ml microcentrifuge tube. On the spin column 200 µl buffer AE was added, incubated at room temperature for 2 minutes, centrifuged at 20,000g for 2 minutes. Finally, the DNA was collected and stored at -20 ºC for further use.

3.2.10 Purity and concentration of genomic DNA

3.2.10.1 Agarose gel electrophoresis

The purity of DNA was checked by agarose gel elecctrophoresis in a submarine horizontal electrophoresis apparatus (Biorad, Germany). Agarose gel (0.7%) was prepared by boiling agarose (Bangalore Genei) in a measured volume of 10X TAE and poured on a gel casting tray fitted with comb, which was removed after solidification of the gel. Ten µl of DNA suspension was mixed with 2 µl of 6x loading dye and loaded into the well. Electrophoresis was performed at 2 - 3 volts / cm (or at 40 V) and the mobility was monitored by the migration of dye. After sufficient migration (3 – 4 h), the gel was visualized in a gel documentation system and thermal print was obtained.

3.2.10.2 Quantification of DNA

Two microliters of the DNA solution in TE buffer (pH 8.0) was added to 2 ml of distilled water. The blank was prepared by adding 2 µl of TE in distilled water. The optical density (OD) of the sample was then read at 260 nm and 280 nm in a UV Spectrophotometer after adjusting the absorbance to zero with the blank. The purity of the DNA samples was estimated by calculating the ratio of A260 and A280. The concentration of DNA was calculated from the A260 value using the formula: concentration of DNA (ng/µl) = A260 x 50

After quantification, working solutions were prepared by adjusting the concentration of DNA to10 ng/µl. Two µl of the working solution was used as template in all PCR reactions.

3.2.11 Amplification of 18S SSU rRNA gene of *Cryptosporidium* spp.

The oligonucleotide primers described by Xiao *et al.* (1999) were used in this study. The primers were obtained in lyophilized form and were resuspended in autoclaved milliQ water and a working solution of 20 p mole/µl was made. One µl of the working solution was used in 25 µl PCR mixture. The nested PCR protocol described by Xiao *et al.* (1999) was followed with minor modifications.

Primers for primary PCR

CRP-DIAG1 Forward Primer : 5' TTCTAGAGCTAATACATGCG 3'

CRP-DIAG1 Reverse Primer : 5' CATTTCCTTCGAAACAGGA 3'

Primers for secondary/nested PCR

CRP-DIAG2 Forward Primer : 5'GGAAGGGTTGTATTTATTAGATAAAG 3'

CRP-DIAG2 Reverse Primer : 5' AAGGAGTAAGGAACAACCTCCA 3'

3.2.11.1 Standardization of primary PCR

In order to amplify the 18S small sub-unit (SSU) rRNA gene of *Cryptosporidium* spp. by PCR, the following protocol was standardized. The 25 µl PCR mixture composed of:

Genomic DNA	2.0 µl (10 ng/ml)
CRP-DIAG 1 F1	1.0 µl (20 p mole/µl)
CRP-DIAG 1 R1	1.0 µl (20 p mole/µl)
Taq polymerase buffer (10X)	2.5 µl
MgCl2 (50 mM)	1.0 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.2 µl
Water up to	25 µl

The cycling conditions used for amplifying the 18S ssu rRNA gene of *Cryptosporidium* spp. were as follows:

Step 1	:	Denaturation	94^0C for 5 minutes	
Step 2	:	Denaturation	94^0C for 1 minute	× 35 cycles (Steps 2–4)
Step 3	:	Annealing	56^0C for 1 minute	
Step 4	:	Elongation	72^0C for 1 minute	
Step 5	:	Final elongation	72^0C for 10 minutes	

The amplification of specific PCR product was checked by gel electrophoresis of the PCR product in 1% agarose and viewed in UV transilluminator system.

3.2.11.2 Standardization of Secondary/nested PCR

The amplified fragment from the primary PCR was diluted in the ratio of 1:10 with autoclaved milliQ water. One µl of the diluted amplicon was used as template in secondary PCR. The 25 µl PCR mixture composed of:

Template	1.0 µl
CRP-DIAG 2 F2	1.0 µl (20 p mole/µl)
CRP-DIAG 2 R2	1.0 µl (20 p mole/µl)
Taq polymerase buffer (10X)	2.5 µl
MgCl2 (50 mM)	0.5 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.2 µl
Water up to	25 µl

The cycling conditions used for amplifying the 18S SSU rRNA gene of *Cryptosporidium* spp. were as follows:

Step 1	:	Denaturation	94^0C for 5 minutes	
Step 2	:	Denaturation	94^0C for 1 minute	× 35 cycles
Step 3	:	Annealing	57^0C for 1 minute	
Step 4	:	Elongation	72^0C for 1 minute	
Step 5	:	Final elongation	72^0C for 10 minutes	

The amplification of specific PCR product was checked by electrophoresis of the PCR product in 1% agarose gel and viewed in UV transilluminator system.

3.2.12 Amplification of the β-giardin gene of *Giardia intestinalis*

Amplification of β-giardin gene was carried using the primers given by Lalle *et al.* (2005) and Caccio *et al*. (2005). The primers (forward and reverse) used for β-giardin gene amplification are as

Primers for primary PCR:

Forward primers G7: 5’AAG CCC GAC GAC CTC ACC CGC AGT GC-3’

Reverse primers G 759: 5’GAG GCC GCC CTG GAT CTT CGA GACGAC3’

Primers for secondary PCR:

Forward primers Giar F: 5’GAA CGA ACG AGA TCG AGG TCCG -3’

Reverse primers Giar R: 5’CTC GAC GAG CTT CGT GTT-3’

3.2.12.1 Standardization of primary PCR

In order to amplify the β-giardin gene of *Giardia intestinalis* by PCR, the following protocol was standardized. The 25 µl PCR mixture composed of:

Genomic DNA	2.0 µl (10 ng/ml)
G7F1	0.5 µl (20 p mole/µl)
G759 R1	0.5 µl (20 p mole/µl)
Taq polymerase buffer (5X)	5.0 µl
MgCl (25 mM)	1.5 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.5 µl
Water up to	25 µl

The cycling conditions used for amplifying the β-giardin gene of *Giardia intestinalis* were as follows:

Step 1	:	Denaturation	95^0C for 3 minutes	
Step 2	:	Denaturation	95^0C for 30 seconds	× 35 cycles
Step 3	:	Annealing	65^0C for 30 seconds	
Step 4	:	Elongation	72^0C for 60 seconds	
Step 5	:	Final elongation	72^0C for 7 minutes	

The amplification of specific PCR product was checked by gel electrophoresis of the PCR product in 1% agarose and viewed in UV transilluminator system.

3.2.12.2 Standardization of Secondary/nested PCR

The amplified fragment from the primary PCR was used as template in secondary PCR. The 25 µl PCR mixture composed of

Template	3.0 µl
Giar F2	0.5 µl (20 p mole/µl)
Giar R2	0.5 µl (20 p mole/µl)
Taq polymerase buffer (10X)	5.0 µl
MgCl2 (50 mM)	1.5 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.5 µl
Water up to	25 µl

The cycling conditions used for amplifying the β-giardin gene of *Giardia intestinalis* were as follows:

Step 1	:	Denaturation	95^0C for 15 minutes	
Step 2	:	Denaturation	95^0C for 30 seconds	× 35 cycles
Step 3	:	Annealing	55^0C for 30 seconds	
Step 4	:	Elongation	72^0C for 1 minute	
Step 5	:	Final elongation	72^0C for 7 minutes	

The amplification of specific PCR product was checked by electrophoresis of the PCR product in 1% agarose gel and viewed in UV transilluminator system

3.2.13 Detection of PCR product by gel electrophoresis

3.2.13.1 Preparation and casting of gel

One percent agarose in 1X TAE buffer was prepared. The boiled agarose was allowed to cool down to about 50 ^{0}C and ethidium bromide 0.5 µg/ml was added. The molten agarose was poured in casting tray of the electrophoresis unit and allowed to set. After solidification of agarose, the comb was removed and the gel tray was immersed in the main electrophoresis unit containing 500 ml of 1X TAE buffer.

3.2.13.2 Electrophoresis of PCR/digested product

Two µl of 6X gel loading dye was added to 10 µl of PCR/digested product and mixed thoroughly by pipetting several times. This mixture of 12 µl was loaded in the wells and in one well 100 bp DNA Ladder plus was loaded. Electrophoresis was performed at 2 - 3 volts / cm (or at 40 V) and the mobility was monitored by the migration of dye.

3.2.13.3 Visualisation of PCR/digested product

After sufficient migration (3–4 h), the gel was examined in a gel documentation system and thermal print was obtained.

3.2.14 Purification of the nested PCR product

The nested PCR product was purified using Qiagen gel extraction kit (Qiagen, Gmbh, Germany) following manufacturer's protocol.

3.2.15 Molecular characterization of *Cryptosporidium* and *Giardia* spp.

Molecular characterization of *Cryptosporidium* and *Giardia* spp. was performed on 120 faecal samples of cattle calves collected from Jammu region. The study samples consisted of 75 faecal samples which served as the part of the study used for evaluating sensitivity and specificity of various diagnostic tests. In addition, 45 samples (15 per age group) were randomly selected to form the part of the objective of the study. DNA using QIAamp kit was extracted from all the 120 faecal samples and then nested PCR using 18S SSU rRNA gene of *Cryptosporidium* and β-giardin gene of *Giardia intestinalis* were performed as discussed previously (under head 3.2.9). For detection of *Cryptosporidium* species prevalent, Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was carried on the samples found positive for *Cryptosporidium* using *Ssp*I, *Vsp*I and *Mbo*II restriction endonucleases enzymes. The samples found as *Cryptosporidium parvum* by PCR-RFLP were further confirmed positive by nested PCR of glycoprotein 60 (gp60) gene of *C. parvum*.

Assemblage of *Giardia intestinalis* prevalent in field samples of Jammu region was determined by PCR-RFLP of β-giardin gene of *Giardia intestinalis* using BsuRI (*Hae* III) and BspLI (*NIa*IV) restriction endonucleases enzymes.

3.2.16 Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) of the 18S SSU rRNA gene of *Cryptosporidium* spp

Five µl of the eluted PCR product (834 bp) was separately subjected to the restriction endonucleases enzymes *Ssp*I, *Vsp*I and *Mbo*II in 10 µl reaction mixture, in 0.2 ml thin walled tubes for 3 hrs in a humid chamber.

The 10 ml reaction mixture composed of:

Eluted amplicon (DNA)	5.0 µl
10 X RE buffer	1.0 µl

Enzyme (*Ssp*I/*Vsp*I/*Mbo*II)	0.5 µl (5 IU)
Water up to	10 µl

The confirmation of the digested product was made by electrophoresis of the product in 3% agarose gel and viewed in UV transilluminator system.

3.2.17 Amplification of gp60/glycoprotein 60 gene of *Cryptosporidium parvum*

Amplification of gp60 gene was carried using the primers given by Alves *et al.* (2003) and Peng *et al*. (1997). Subtyping of *C. parvum* was achieved by sequence analysis of the gp60 gene product in both directions. The sequence of primers (forward and reverse) for gp60 amplification are as:

Primers for primary PCR

CRP-DIAG1 Forward Primer (AL3531) : 5' ATA GTC TCC GCT GTA TTC 3'

CRP-DIAG1 Reverse Primer (AL3535) : 5' GGA AGG AAC GAT GTA TCT 3'

Primers for secondary PCR

CRP-DIAG2 Forward Primer (AL3532) : 5' TCC GCT GTA TTC TCA GCC 3'

CRP-DIAG2 Reverse Primer (AL3534) : 5' GCA GAG GAA CCA GCA TC 3'

3.2.17.1 Standardization of primary PCR

In order to amplify the gp60 gene of *Cryptosporidium parvum* by PCR, the following protocol was standardized. The 25 µl PCR mixture composed of:

Genomic DNA	2.0 µl (10 ng/ml)
CRP-DIAG 1 F1	0.5 µl (20 p mole/µl)
CRP-DIAG 1 R1	0.5 µl (20 p mole/µl)
Taq polymerase buffer (5X)	5.0 µl
MgCl (25 mM)	3.5 µl
dNTP (10mM)	0.5 µl

Taq polymerase	0.3 µl
Water up to	25 µl

The cycling conditions used for amplifying the gp60 gene of *Cryptosporidium parvum* were as follows:

Step 1	:	Denaturation	95^0C for 3 minutes	
Step 2	:	Denaturation	95^0C for 45 seconds	× 35 cycles
Step 3	:	Annealing	49.2^0C for 45 seconds	
Step 4	:	Elongation	72^0C for 60 seconds	
Step 5	:	Final elongation	72^0C for 10 minutes	

The amplification of specific PCR product was checked by gel electrophoresis of the PCR product in 1% agarose and viewed in UV transilluminator system.

3.2.17.2 Standardization of Secondary/nested PCR

The amplified fragment from the primary PCR was used as template in secondary PCR. The 25 µl PCR mixture composed of

Template	1.0 µl
CRP-DIAG 2 F2	0.5 µl (20 p mole/µl)
CRP-DIAG 2 R2	0.5 µl (20 p mole/µl)
Taq polymerase buffer (5X)	5.0 µl
MgCl2 (50 mM)	3.0 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.3 µl
Water up to	25 µl

The cycling conditions used for amplifying the gp60 gene of *Cryptosporidium* spp. were as follows:

Step 1	:	Denaturation	95^0C for 5 minutes	
Step 2	:	Denaturation	95^0C for 1 minute	× 35 cycles
Step 3	:	Annealing	52^0C for 45 seconds	× 35 cycles
Step 4	:	Elongation	72^0C for 1 minute	× 35 cycles
Step 5	:	Final elongation	72^0C for 10 minutes	

The amplification of specific PCR product was checked by electrophoresis of the PCR product in 1% agarose gel and viewed in UV transilluminator system.

3.2.18 Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) of the β-giardin gene of *Giardia intestinalis*

Five µl of the eluted PCR product (511 bp) was separately subjected to the restriction endonucleases enzymes, BsuRI (*Hae* III) and BspLI (*NIa*IV) in 20 µl reaction mixture, in 0.2 ml thin walled tubes for 3 hrs in a humid chamber.

The 20 µl reaction mixture composed of:

Eluted amplicon (DNA)	1.0 µl
10 X RE buffer	2.0 µl
Enzyme (*Hae* III/ *NIa*IV)	2.0 µl (3000U/200U)
Water up to	15 µl

The confirmation of the digested product was made by electrophoresis of the product in 3% agarose gel and viewed in UV transilluminator system

3.2.19 Sequencing of nested PCR product:

Nested PCR products of 18S SSU rRNA gene of *Cryptosporidium* spp., gp60 gene of *Cryptosporidium parvum* and β-giardin gene of *Giardia intestinalis* were purified using gel extraction kit (Qiagen, Gmbh, Germany) and the eluted product was commercially sequenced in an automated DNA sequencer at Agrigenome pvt. Ltd., Kochi, Kerela. The sequence information received was analysed using DNA Star, Laser gene software and Basic local allignment search tool (BLAST, NCBI).

3.2.20 Therapeutic activity of *Curcuma longa* against experimental cryptosporidiosis in mice

The study was undertaken to assess the therapeutic efficacy of *Curcuma longa* against *Cryptosporidium parvum* infection in experimentally infected mice. According to the previous study (Asadpour *et al*., 2018) therapeutic effect of *C. longa* against *Eimeria* spp. (another coccidian parasite) at the dose of 4.33 mg/kg/day has been reported. Keeping in view this information in the backdrop, initially a pilot study was performed, wherein *Curcuma longa*@2 mg, 4mg and 8 mg/kg/day was assessed against *C. parvum* infection in 3 weeks old mice. As per the method of OIE (2008) oocyst count was assessed in the faecal samples and the dose which resulted in maximum reduction in oocyst count was selected. The selected dose further varied in narrow range and evaluated for the final therapeutic trial.

3.2.21Collection and purification of parasite

Cryptosporidium oocysts were collected from a young, 18 day old cow calf showing clinical form of cryptosporidiosis and belonging to an organized cattle farm of Jammu region. Infection was confirmed by modified Ziehl Neelsen staining technique (Henricksen and Pohlenz, 1981).

Cryptosporidium oocysts were purified from the faeces by primary and secondary purification methods. Primary purification of the oocysts from faeces was done by modified Sheather’s sucrose floatation (Current *et al*., 1983) technique. About 5gms of faeces was mixed thoroughly with an equal volume of 2.5% potassium dichromate solution (w/v) and strained first through a wire mesh of 400µ pore size and then of 200µ pore size. The faecal sample was then mixed with an equal volume of diethyl ether in a 15 ml centrifuge tube and vigorously shaken. The sample was centrifuged at 500 x g for 10 minutes. The fat plug and other fat soluble materials in the upper organic/aromatic layer were discarded. The lower aqueous phase was taken in another 15 ml centrifuge tube, mixed with equal volume of 2.5% Potassium dichromate solution (w/v) and centrifuged at 1000 x g for 15 minutes. The sediment was taken and resuspended in 1 ml of 2.5% potassium dichromate solution (w/v) mixed with 0.2% Tween-20. The 1 ml faecal suspension was then mixed with 9 ml of modified Sheather's sugar solution in a 15 ml centrifuge tube and centrifuged at 1000 x g for 10 minutes. One ml of 0.2% aqueous solution of Tween- 20 was layered over the floatation medium. The top layer of the mixture was stirred carefully with a pipette and about 1 ml was aspirated with it. The remaining material was again centrifuged. This step was repeated several times and oocysts harvested each time were monitored under phase contrast microscopy. Equal volume of aqueous solution of 0.2% Tween-20 was added to the harvested oocyst suspension and centrifuged at 1000 x g for 15 minutes. The pelleted oocysts were resuspended in antibiotic solution and stored at 40^0C.

Secondary/final purification of the oocysts was done by discontinuous step-gradient centrifugation technique (Arrowood and Sterling, 1987). Sheather's sucrose solution was diluted with 0.025M phosphate buffered saline (PBS) supplemented with 1% (v/v) Tween-80, in the ratio of 1:2 and 1:4. Five ml of the 1:4 solution was layered carefully over 5 ml of the 1:2 solution in a 15 ml centrifuge tube. One ml of the oocyst suspension from the primary purification procedure was layered over the 1:4 solution and centrifuged at 1500 x g for 30 minutes. The fluid from interface of the two sucrose solutions was aspirated carefully, centrifuged and the pellet was examined for presence of oocysts.

Confirmation of *Cryptosporidium* species oocyst was done by nested PCR of GP60 gene of *C. parvum* as discussed previously (under head 3.2.17).

3.2.22 Preparation of ethanolic extract of *Curcuma longa*

Turmeric (*Curcuma longa*) tuber was purchased from the local market, completely dried, powdered and then ethanolic extract was prepared.

3.2.22.1 Preparation of Extract

The ethanolic extract of turmeric powder (*Curcuma longa)* was prepared by using ethyl alcohol in extract container of soxhlet apparatus according to Harborne (1998). In brief, 30 gm of powered material was extracted with 500 ml of ethyl alcohol extract container. All extractions were done by maintaining plate temperature (70 - 80°C) on soxhlet apparatus. The extract was further concentrated using vacuum rotary evaporator maintained at 45-50°C (Linskens and Jackson, 1985; Harborne, 1998).

The per cent extractability of ethanolic extract was determined by the following formula.

% Extractability = Weight of the extract obtained / Weight of plant material (powder) taken for extraction×100

3.2.22.2 Preparation of the stock solution of *Curcuma longa* extract

Different concentrations of *Curcuma longa* extract (sigma-Aldrich, USA) were prepared in HPLC grade methanol and stored in a refrigerator at 4°C.

3.2.22.3 Preparation of the stock solutions and calibration of standard compound curcumin

Stock solution of the pure reference compound, curcumin (sigma-Aldrich, USA) @ 1.000 mg/ml was prepared in HPLC grade methanol and stored in a refrigerator at 4°C. From the stock solutions, working solutions for each reference compound were prepared by dilution with HPLC grade methanol. 2 µL of these diluted solutions was injected for the preparation of calibration curve (Figure 2).

3.2.22.4 HPLC analysis

The curcumin was separated and quantified by a Shimadzu HPLC system (Kyoto, Japan) consisting of an LC-10 ATvp pump, SIL-10 ADvp automatic sampling unit (auto sampler), CTO-10 and SCL-10 Avp as the system controller. Class VP software (version 6.10) (Company, City and Country) was used for data analysis and data processing. The samples were analyzed at 30°C on RP-18.5 μm, 250x 4 mm i.d. Merck (Darmstadt, Germany) column. Photo Diode Array (PDA) detection was performed at 423 nm. The analysis was carried out using a mobile phase of methanol: water (2:3) which was delivered at a flow rate of 0.7 ml/min. The curcumin was quantified by using the external standard method.

3.2.23 Therapeutic activity of *Curcuma longa* ethanolic extract w.r.t. curcumin pure salt

The main constituent of *Curcuma longa* (turmeric) is curcumin. Curcumin is a polyphenol that gives turmeric its colour and is lipophillic in nature, hence insoluble in water and ether but soluble in ethanol, dimethylsulphoxide, and other organic solvents (Aggarwal *et al*., 2003). The other constituents present in *Curcuma longa* are volatile oils including tumerone, atlantone, and zingiberone and sugars, proteins, and resins (Leung, 1980). Due to the presence of curcumin as an active constituent and the volatile oils turmeric is widely used as a medicine for the treatment of many ailments. Anticryptosporidial activity of curcumin has been documented in literature, but to assess that whether the other chemicals constituents available in *Curcuma longa* (turmeric powder) has synergestic/inhibitory action against *Cryptosporidium parvum*, the efficacy of curcumin available naturally was compared with synthetic pure salt of curcumin (sigma-Aldrich, USA).

3.2.24 Preparation of Infection for mice

Purified oocysts of *C. parvum* were counted by Neubuaer Chamber. Final concentration of purified oocysts was adjusted @ 10^8/ml and these were fortified with streptopencillin where streptomycin @ 100 µg/ml and pencillin @ 100 IU/ ml and stored at 4 ºC for further use.

3.2.25 Experimental animals

The study was conducted on 200 female Swiss albino mice. These mice aged two weeks, were procured from Indian Institute of Integrative Medicine (IIIM), Jammu.

3.2.25.1 Housing and maintenance

On their arrival, all the animals were examined for any abnormality and overt ill health and were weighed. The animals were maintained under strict hygiene condition and were given standard laboratory diet and clean drinking water *ad libitum*. To confirm the absence of infection, all the mice were tested microscopically by taking direct faecal smear followed by modified Ziehl Neelsen acid fast staining method (Henricksen and Pohlenz, 1981). Mice were housed in polypropylene cages. Rice husk was provided as the bedding material. All the animals were checked thrice daily for any signs of ill health.

3.2.25.2 Experimental design

All the experimental mice were acclimatized to the experimental conditions for one week. The mice were randomly divided into ten groups i.e., Group I to Group X, each group comprising of 20 animals and housed in separate cages during the experiment. Group I mice served as healthy control and received distilled water. Group II mice served as control for immunosuppression and individual mice in this group were administered dexamethasone. Group III (infected control) mice were given dexamethasone and also were infected with *C. parvum* oocysts @ 1x 10^4 per os. Group IV animals were infected with 10^4 oocysts/animal, immunosupressed with dexamethasone and treated orally with Nitazoxanide @ 150 mg/kg/day (Blagburn, 1998). Group V to Group VII animals were infected with 10^4 oocysts/animal, immunosupressed with dexamethasone and treated orally with ethanolic extract of *Curcuma longa* @ 4, 6 and 8 mg/kg/day, respectively. Group VIII to Group X animals were infected with 10^4 oocysts/animal, immunosupressed with dexamethasone and treated orally with pure salt of curcumin (Sigma-Aldrich, USA) @ 4,6 and 8 mg/kg/day, respectively. The detailed experimental design is depicted in Table 1.

Table 1: Experimental design on therapeutic evaluation of *Curcuma longa* extract w.r.t. curcumin

Groups	No.of mice	Timeline of infection and drug administered in various groups
Group I	20	Uninfected (Healthy control)and received distilled water
Group II	20	Uninfected and immunosupressed with dexamethasone @ 30µg/ml in drinking water
Group III	20	Infected with 10^4 oocysts/ animal and immunosupressed with dexamethasone @ 30 µg/ml in drinking water
Group IV	20	Infected with 10^4 oocysts/ animal, immunosupressed and received standard drug nitazoxanide @ 150mg/kg/day
Group V	20	Infected with 10^4 oocysts/ animal, immunosupressed and received ethanolic extract of *Curcuma longa* @ 4mg/kg/day
Group VI	20	Infected with 10^4 oocysts/ animal, immunosupressed and received ethanolic extract of *Curcuma longa* @ 6mg/kg/day
Group VII	20	Infected with 10^4 oocysts/ animal, immunosupressed and received ethanolic extract of *Curcuma longa* @ 8mg/kg/day
Group VIII	20	Infected with 10^4 oocysts/ animal, immunosupressed and received pure salt curcumin (Sigma-Aldrich, USA) @ 4mg/kg/day
Group IX	20	Infected with 10^4 oocysts/ animal, immunosupressed and received pure salt curcumin (Sigma-Aldrich, USA) @ 6mg/kg/day
Group X	20	Infected with 10^4 oocysts/ animal, immunosupressed and received pure salt curcumin (Sigma-Aldrich, USA) @ 8mg/kg/day

3.2.25.3 Immunosupression

Dexamethasone @ 30 µg/ml in drinking water was administered in Group II to Group X animals seven days prior to start of the experiment (i.e. at 3 weeks of age) and continued till the end of experiment.

3.2.25.4 Infection

Mice of Group III to Group X were infected orally with 10^4 oocysts/ 100 microlitre/animal as suggested by Tarazona (1998). The prepared infection inoculums was given at 4 weeks of age i.e. after one week of commencement of immunosupression.

3.2.25.5 Treatment

Treatment in experimental Groups IV to X started simultaneously at the beginning of oocyst shedding (i.e. 5th day post infection) and continued for 5 successive days i.e upto 9th day post infection. The therapeutic dose was prepared based on pilot trial conducted initially. Group IV animals were treated with nitazoxanide @ 150 mg/kg/day and was dissolved in 1 % DMSO and administered orally to the mice (Blagburn *et al*., 1998). Ethanolic extract of *Curcuma longa* @ 4, 6 and 8 mg/kg/animal/day was dissolved in DMSO and then administered orally in each mice belonging to Group V to Group VII, respectively. Similarly, pure salt of curcumin (Sigma Aldrich, USA) was dissolved in DMSO and then orally administered to mice of Group VIII to X @ 4, 6 and 8 mg/ kg/animal, respectively.

3.2.26 Parameters for assessment of therapeutic activity of *Curcuma longa* against *C. parvum*

Two parameters viz. oocysts per gram of faeces and average body weight was assessed for therapeutic evaluation

1. Oocysts per gram of faeces:-

In order to monitor oocyst count throughout the experiment, pooled faecal sample of the animals of each group were collected. Initially the oocyst count was measured on day zero and third day post infection, followed by alternate day till 11^{th} day PI (7^{th} day post treatment) to find percent mean oocyst reduction. The number of oocysts per gram of faeces was calculated using the method of Grinberg *et al*. (2002). In this method, 1 gram of faecal sample was mixed with 10 ml of tap water, passed through a 100-mesh sieve, the suspension centrifuged and the sediment resuspended in 4 ml of normal saline. Afterwards, 10 microlitre of this suspension was poured as a drop on a slide, air-dried and stained with modified Ziehl-Neelsen. The entire area of smear was examined with 40x objective lens and oocysts counted. The number of oocysts was counted in all microscopic fields (×1000) (Ortolani, 2000).

% Mean oocyst reduction =Mean OPG (0 day) - Mean OPG (Post treatment) / Mean OPG (0 day) × 100

2. Average body weight:-

Mean body weight of the experimental animals was recorded on day 0, 5, 7, 9 and 11 post infection.

3.2.27 Histopathological examination

Twelve mice from each group were euthanized humanely by ether on 11th day of post infection for histopathologiacal observation. The remaining 8 mice were sacrificed on 20th day post infection for histopathological examination. The stomach and duodenum were removed and the remaining parts of intestine were tied at both ends by thread and injected by 10% neutral buffered formalin for better fixation. The tissue samples from each mouse were fixed in 10% neutral buffered formalin, separately, and embedded in paraffin. The paraffin embedded sections were then passed through sequential steps of deparaffinisation in xylene, rehydration by passing through descending grades of ethyl alcohol to running tap water. All jejunum and ileum tissue blocks were sectioned serially at 5 μm in thick, and stained with hematoxylin and eosin stain (Luna, 1968). Then, the tissue sections were mounted on a separate slide and examined microscopically with a × 1000 objective on a compound microscope.

3.3 Statistical analysis

The association of *Cryptosporidium* and *Giardia* infection in cattle calves with relation to the prevalence study was analyzed through Chi-square test (http://statpages.org/ctab2x2.html). The sensitivity, specificity and over all agreement between two tests was analyzed by statistical method used by Samad *et al.* (1994).

Particulars	Test result	Gold standard test (PCR)		
		Positive	Negative	Total
The test to be compared	Positive	a	b	a+b
	Negative	c	d	c+d
Total		a+c	b+d	a+b+c+d=N

a = Number of samples positive to both conventional and the gold standard tests
b = Number of samples positive to conventional but negative to the gold standard test
c = Number of samples negative to conventional but positive to the gold standard test
d = Number of samples negative to both conventional and the gold standard tests
a + b + c + d = Total number of samples (N)

Data generated from various parameters of experimental work on mice were presented as Mean ± SE. The growth performance and oocyst count parameters were subjected to one way ANOVA employing duncan descriptive statistical analysis as per method described by Snedecor and Cochran (1994).

3.4 Permission from ethical committees

To carry out the experimental work on mice, the permission was taken from institutional Animal Ethical Committee of Sher-e- Kashmir University of Agricultural Sciences & Technology, Jammu, Vide letter number-3/IAEC-17/2017, Dated: 28-9-2017.

Chapter-4

Results

CHAPTER-IV

RESULTS

4.1 Prevalence studies

Examination of 614 faecal samples of cattle by diethyl ether sedimentation technique followed by modified Ziehl-Neelsen staining and iodine wet mount technique revealed an overall positivity of 144 (23.45%) and 43 animals (7.00%) for *Cryptosporidium* and *Giardia* spp. infection, respectively. The *Cryptosporidium* oocysts stain dark pinkish (modified Ziehl-Neelsen staining) and 4-7µm in diameter and have the crescenteric forms of the sporozoite (Plate1and 2).Iodine wet mount technique revealed that *Giardia* cysts measures about 12 to 15 µm long and 5 to 9 µm wide (Plate3 and 4).

4.1.1 District wise prevalence

The prevalence of *Cryptosporidium* spp. and *Giardia* spp. infection in cattle calves among four different districts of Jammu region was studied. The highest prevalence of *Cryptosporidium* was recorded in Samba (28.57%) followed by Udhampur (23.25%), Kathua (22.75%) and Rajouri (18.18%) districts whereas highest prevalence of *Giardia* spp. was recorded in Kathua (7.78%) followed by Rajouri (7.43%), Samba (6.49%) and Udhampur (6.39%) districts. The same has been shown in Table 2 and Fig.1.

Statistical analysis suggested that prevalence of *Cryptosporidium* infection in Udhampur varied significantly (p<0.05) than other three districts. On the other hand prevalence of *Giardia* infection varied non significantly (p<0.05) among all the four districts (Table 3).

4.1.2 Age related prevalence

The prevalence of *Cryptosporidium* spp. and *Giardia* spp. in different age groups in cattle calves of the Jammu region is shown in the Table 4 and Fig 2. The highest incidence of *Cryptosporidium* infection was found in< 1 month of age animals (43.24%) followed by 1-6 months (23.58%) and lowest prevalence in >6 months of age animals (12.54%). Further, it was observed that very young (< 1 month age) diarrhoeic and non-diarrhoeic calves showed highest prevalence of cryptosporidiosis

Table2: District wise prevalence of *Cryptosporidium* spp. and *Giardia* spp. in cattle calves of Jammu districts

District of jammu	Number of diarrhoeic faecal samples						Number of non-diarrhoeic faecal samples						Number of total faecal samples					
	Cryptosporidium spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp		
	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)
Rajouri	59	14	23.72%	59	5	8.47%	62	8	12.90%	62	4	6.45%	121	22	18.18%	121	9	7.43%
Samba	79	26	32.91%	79	6	7.59%	75	18	24.00%	75	4	5.33%	154	44	28.57%	154	10	6.49%
Kathua	81	23	28.39%	81	9	11.11%	86	15	17.44%	86	4	4.65%	167	38	22.75%	167	13	7.78%
Udhampur	69	22	31.88%	69	6	8.69%	103	18	17.47%	103	5	4.85%	172	40	23.25%	172	11	6.39%
Total	288	85	29.51%	288	26	9.02%	326	59	18.09%	326	17	5.21%	614	144	23.45%	614	43	7.00%

Where E= Examined P= Positive P (%) = Percent prevalence

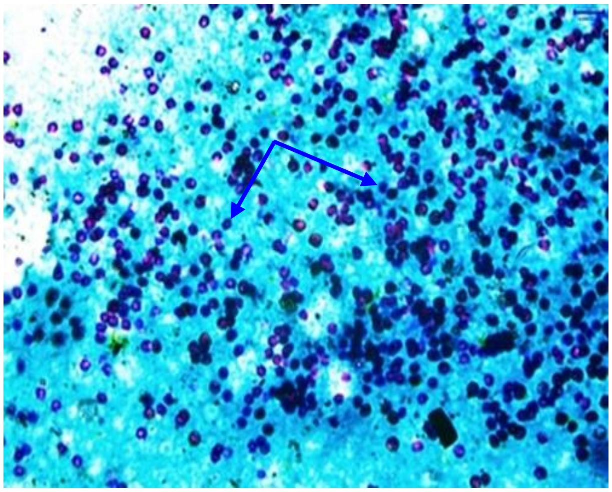


Plate 1: ***Cryptosporidium*** **oocyst (arrow marked) in faeces using modified Ziehl – Neelsen staining x 400**

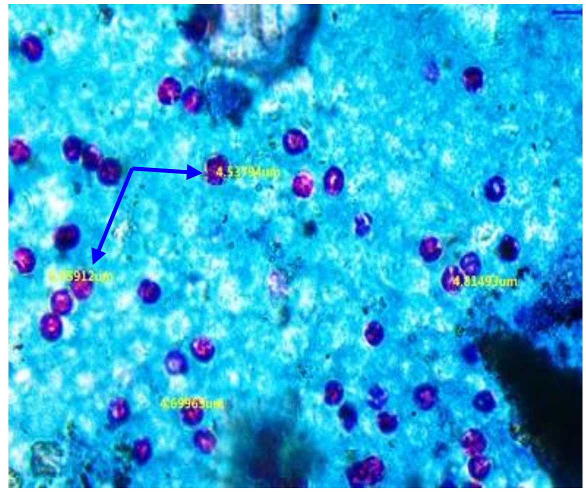


Plate 2: ***Cryptosporidium*** **oocyst (arrow marked) in faeces using modified Ziehl – Neelsen staining x 1000**

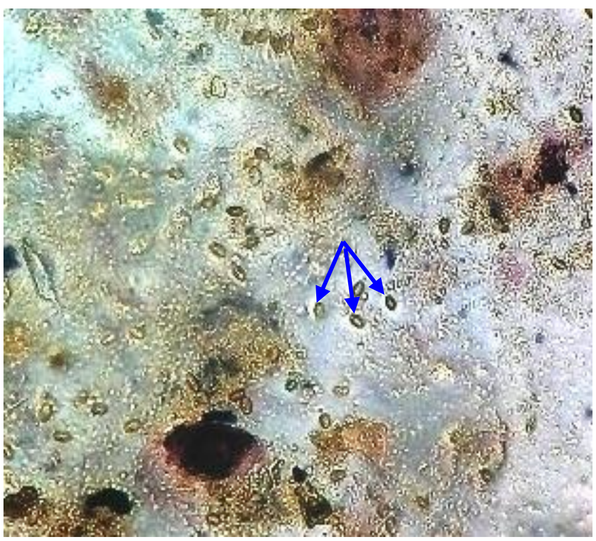


Plate 3: ***Giardia*** **cyst (arrow marked) in faeces using lugols iodine staining x 100**

Plate 4: ***Giardia*** **cyst (arrow marked) in faeces using lugols iodine staining x 400**

Table 3: Statistical analysis (χ^2- value) of *Cryptosporidium* spp. and *Giardia* infection in districts of cattle calves of Jammu region

Districts	Risk Factor	*Cryptosporidium* spp infection			*Giardia* spp infection		
		Odd ratio	95% Confidence Interval	χ^2- value	Odd ratio	95% Confidence Interval	χ^2- value
Rajouri	Diarrhoeic / Non diarrhoeic	2.100	0.740-6.076	1.709 [NS]	1.343	0.292-6.355	0.006 [NS]
Samba	Diarrhoeic / Non diarrhoeic	1.553	0.723-3.353	1.092 [NS]	1.459	0.345-6.475	0.059 [NS]
Kathua	Diarrhoeic / Non diarrhoeic	1.877	0.846-4.190	2.258 [NS]	2.563	0.681-10.387	1.608 [NS]
Udhampur	Diarrhoeic / Non diarrhoeic	2.210	1.017-4.821	4.033 **	1.867	0.478-7.426	0.478 [NS]

**Significant at $P<0.05$; NS=Non-significant at $P<0.05$

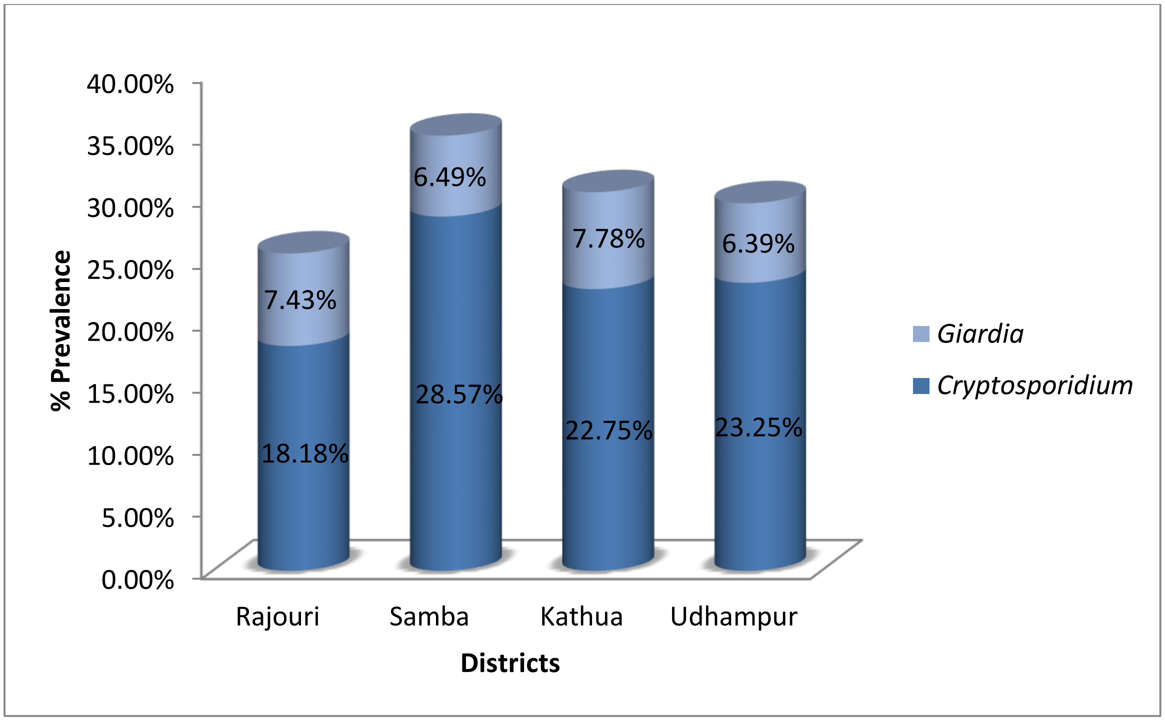


Fig 1: District wise prevalence of *Cryptosporidium* and *Giardia* spp. in cattle calves of Jammu region

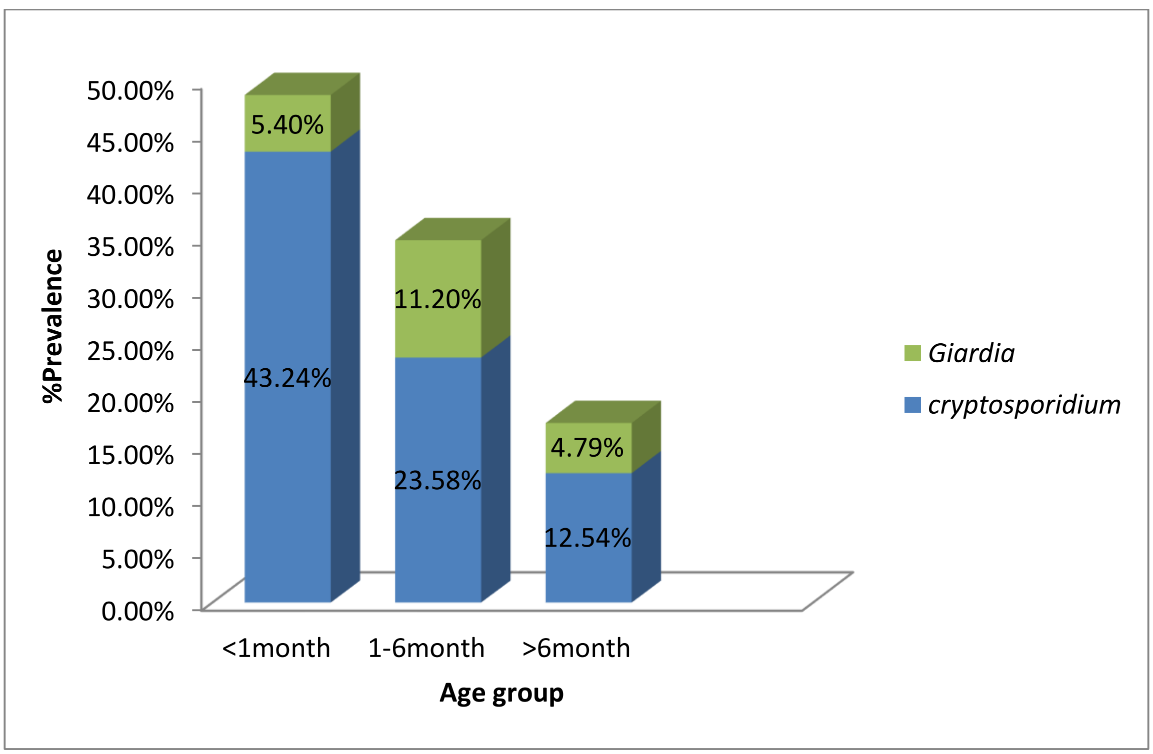


Fig 2: Age wise prevalence of *Cryptosporidium* spp. and *Giardia* spp. in cattle calves of Jammu districts

Table 4: Age wise prevalence of *Cryptosporidium* spp. and *Giardia* spp. in cattle calves of Jammu region

Age groups	Number of diarrhoeic faecal samples						Number of non-diarrhoeic faecal samples						Number of total faecal samples					
	Cryptosporidium spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp		
	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)
<1 month	64	38	59.37%	64	5	7.81%	84	26	30.95%	84	3	3.57%	148	64	43.24%	148	8	5.40%
1-6months	95	28	29.47%	95	13	13.68%	100	18	18.00%	100	9	9.00%	195	46	23.58%	195	22	11.2%
>6 months	129	19	14.72%	129	8	6.20%	142	15	10.56%	142	5	3.52%	271	34	12.54%	271	13	4.79%
Total	288	85	29.51%	288	26	9.02%	326	59	18.09%	326	17	5.21%	614	144	23.45%	614	43	7.00%

Where E= Examined P= Positive P (%) = Percent prevalence

than other age animals (59.37%; diarrhoeic and 30.95%; non-diarrhoeic). Prevalence of *Giardia* infection was found to be highest in 1-6 month age animals (11.2%) followed by <1 month (5.40%) and lowest was recorded in > 6month age group (4.79%).

Statistical analysis by chi square revealed that the prevalence of *Cryptosporidium* spp. infection in <1 month age animals varied significantly ($p<0.05$) with other age groups (<1month, 1-6 months and >6 months), whereas it was non-significant ($p<0.05$) between 1-6 months and > 6 months age group. On the other hand the prevalence of *Giardia* infection in animals varied non significantly among all the age groups (Table 5).

4.1.3 Prevalence among diarrhoeic and non diarrhoeiccattle calves

The prevalence of *Cryptosporidium* spp. infection was significantly (χ^2- value, 10.47, $p<0.05$) higher in diarrhoeic animals (29.51%) than non diarrhoeic animals (18.09%) and prevalence of *Giardia* infection was non significantly (χ^2- value, 2.85, $p<0.05$) higher in diarrhoeic animals (9.02%) than non diarrhoeic animals (5.21%). Further it was observed that irrespective of age, diarrhoeic animals showed higher prevalence than non diarrhoeic animals and the same has been shown in Table 6 and Fig.3.Different risk factors of *Cryptosporidium* and *Giardia* spp. infection in cattle calves of different age groups is shown in Table 7 and 8.

4.1.4 Sex related prevalence

The overall prevalence of *Cryptosporidium* and *Giardia* infection was recorded higher in male animals as compared to female animals and same has been shown in Table 9 and Fig.4

Male animals showed significantly ($p<0.05$) higher incidence of *Cryptosporidium* infection (46.34%; diarrhoeic, 30.61%; non-diarrhoeic) as compared to females (22.81%; diarrhoeic, 12.71%; non-diarrhoeic) (Table 10). Similarly, significantly ($p<0.05$) higher incidence of *Giardia* infection (15.85%; diarrhoeic, 10.20%; non-diarrhoeic) was recorded in males as compared to females (6.31%; diarrhoeic, 3.07 %; non-diarrhoeic) (Table 11).

Table 5: Statistical analysis (χ^2 - value) of *Cryptosporidium* spp. and *Giardia* infections between different age groups of diarrhoeic and non-diarrhoeic cattle calves of Jammu region

Diarrhoeic	*Cryptosporidium* spp		*Giardia* spp	
Age groups	<1 month	1-6month	<1 month	1-6month
1-6 month	12.877 **	-	0.793 NS	-
>6 months	38.855 **	6.313 NS	0.013 NS	2.779 NS
Non-diarrhoeic				
1-6 month	3.528 NS	-	1.406 NS	-
>6 months	13.433 **	2.160 NS	0.000 NS	2.305 NS
Diarrhoeic+Non-diarrhoeic				
1-6 Month	14.030 **	-	2.942 NS	-
>6 months	48.641 **	8.965 **	0.001 NS	5.964 **

** Significant at P<0.05; NS=Non-significant at P <0.05

Table 6: Risk of *Cryptosporidium* and *Giardia* infection in cattle calves with diarrhoeic and non diarrhoeic samples in Jammu region

Species	Risk Factor	Odd ratio	95% Confidence Interval	χ^2- value
Cryptosporidium	Diarrhoeic	1.895	1.275-2.818	10.473**
	non diarrhoeic			
Giardia	Diarrhoeic	1.804	0.919-3.560	2.853NS
	non diarrhoeic			

** Significant at P<0.05; NS=Non-significant at P <0.05

Table 7: Risk of *Cryptosporidium* spp. between different age groups of diarrhoeic and non- diarrhoeic cattle calves in Jammu region

Diarrhoeic			
Age groups	<1 month	1-6month	>6 month
<1 month	-	3.497 (1.707-7.209)	8.462(3.992-18.120)
1-6 months	0.286 (0.139-0.586)	-	2.419 (1.195-4.920)
>6 months	0.118 (0.055-0.250)	0.413 (0.203-0.837)	-
Non-diarrhoeic			
<1 month	-	2.042 (0.972-4.308)	3.795 (1.774-8.188)
1-6 months	0.490 (0.232-1.028)	-	1.859 (0.836-4.144)
>6 months	1.239 (0.526-2.909)	0.538 (0.241-1.195)	-
Diarrhoeic+Non-diarrhoeic			
<1 month	-	2.468 (1.512-4.033)	5.311 (3.183-8.887)
1-6 months	0.40 (0.248-0.661)	-	2.181 (1.302-3.661)
>6 months	0.188 (0.113-0.314)	0.465 (0.277-0.778)	-

Values in parenthesis indicates 95% confidential interval for the odd ratio value

Table 8: Risk of *Giardia* infection between different age groups of diarrhoeic and non diarrhoeic cattle calves in Jammu region

Diarrhoeic			
Age groups	<1 month	1-6month	>6 month
<1 month	-	0.535 (0.156-1.730)	1.282 (0.346-4.572)
1-6 months	1.871(0.578-6.399)	-	2.398 (0.879-6.665)
>6 months	0.780(0.219-2.888)	0.417(0.150-1.137)	-
Non-diarrhoeic			
<1 month	-	0.374(0.077-1.581)	1.015(0.187-5.038)
1-6 months	2.670(0.633-12.928)	-	2.710(0.797-9.647)
>6 months	0.985(0.198-5.361)	0.369(0.104-1.255)	-
Diarrhoeic+Non-diarrhoeic			
<1 month	-	0.449(0.178-1.102)	1.134(0.418-3.013)
1-6 months	2.225(0.907-5.626)	-	2.524(1.178-5.461)
>6 months	0.882(0.332-2.390)	0.396(0.183-0.849)	-

Values in parenthesis indicates 95% confidential interval for the odd ratio value

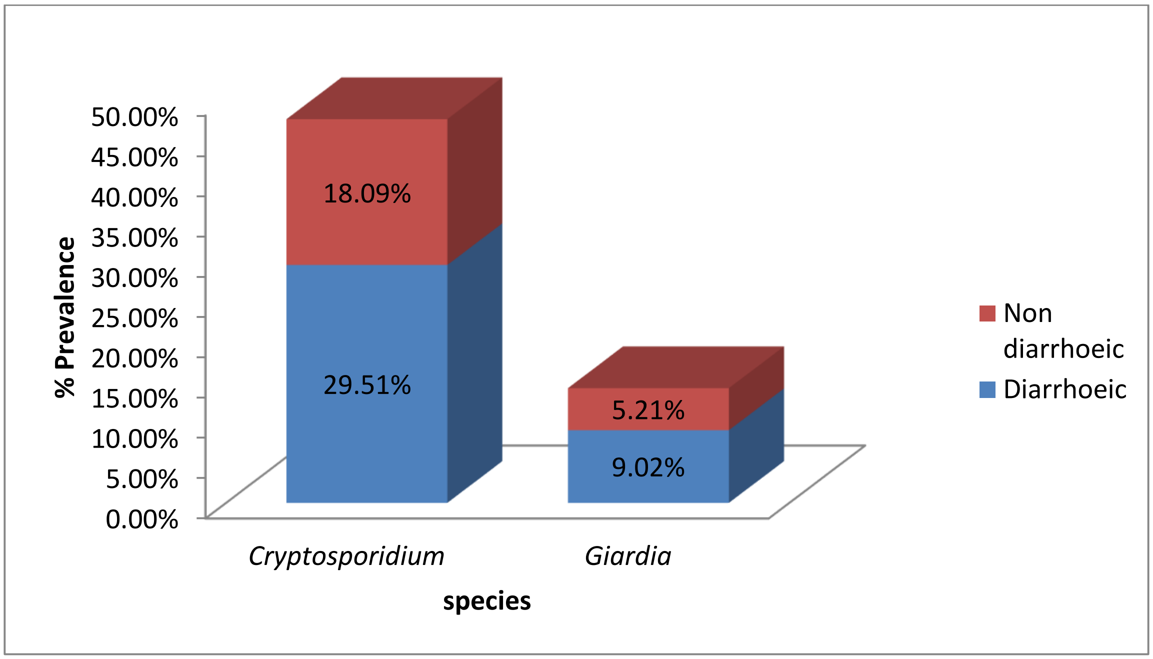


Fig 3: Prevalence of *Cryptosporidium* and *Giardia* spp among diarrhoeic and non diarrhoeic cattle calves of Jammu region

Table 9: Sex wise prevalence of *Cryptosporidium* spp. and *Giardia* spp. in cattle calves of Jammu region

Sex	Number of diarrhoeic faecal samples						Number of non-diarrhoeic faecal samples						Number of total faecal samples					
	Cryptosporidium spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp		
	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)
Male	82	38	46.34%	82	13	15.85%	98	30	30.61%	98	10	10.20%	180	68	37.77%	180	23	12.77%
Female	206	47	22.81%	206	13	6.31%	228	29	12.71%	228	7	3.07%	434	76	17.51%	434	20	4.60%
Total	288	85	29.51%	288	26	9.02%	326	59	18.09%	326	17	5.21%	614	144	23.45%	614	43	7.00%

Where E= Examined P= Positive P (%) = Percent prevalence

Table 10: Risk of *Cryptosporidium* spp. infection between male and female cattle calves of Jammu region

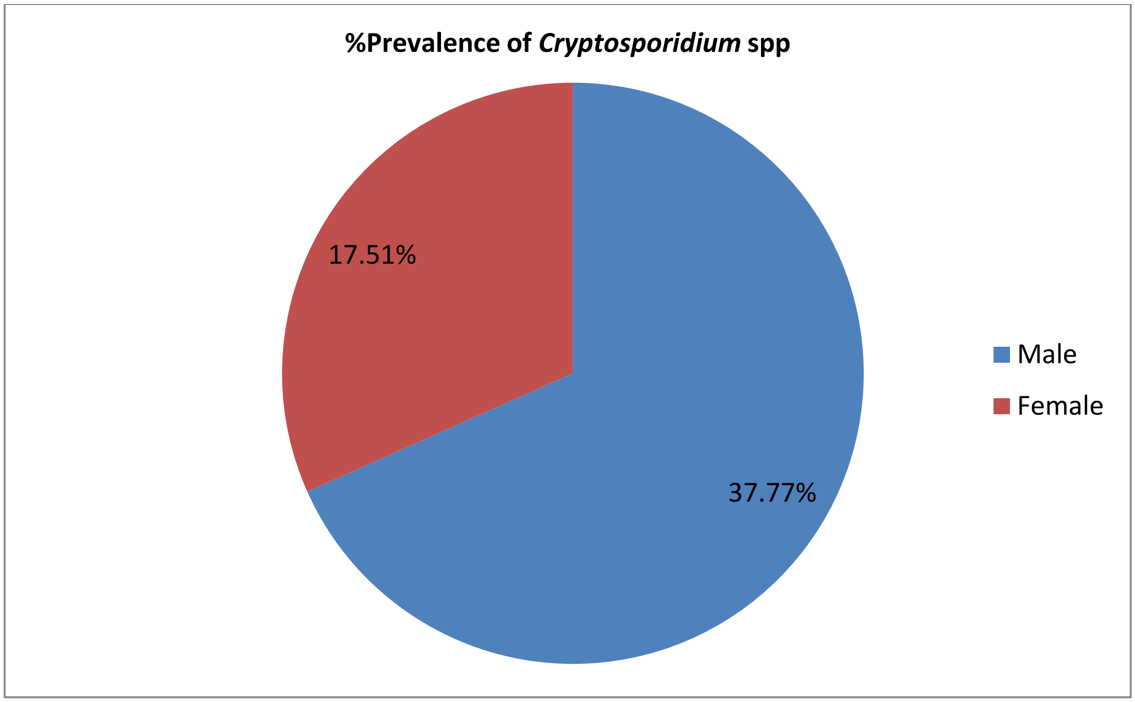
Risk Factor ↓	Male	Female	χ^2-value
Male	-	2.860(1.901-4.305)	27.990**
Female	0.350(0.232-0.526)	-	

95% Confidence Interval, ** Significant at P<0.05

Table 11: Risk of *Giardia* infection between male and female cattle calves of Jammu region

Risk Factor ↓	Male	Female	χ^2-value
Male	-	3.032(1.553-5.934)	11.814**
Female	0.330(0.169-0.644)	-	

95% Confidence Interval, ** Significant at P<0.05



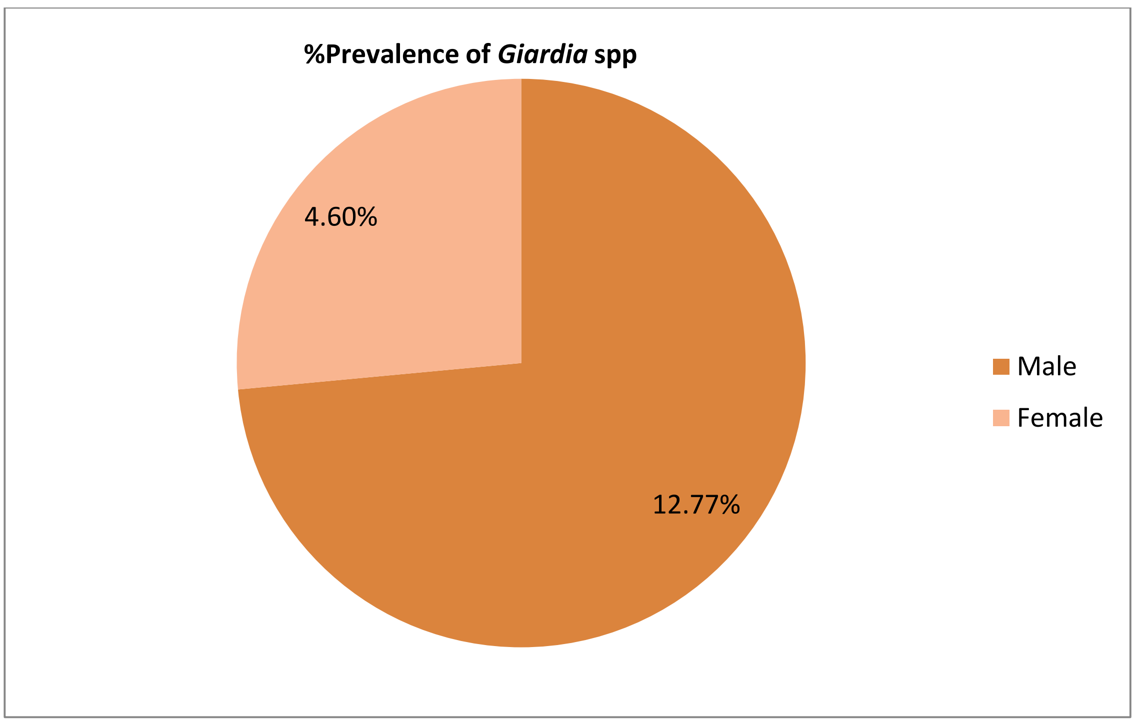


Fig 4: Sex wise prevalence of *Cryptosporidium* and *Giardia* spp. in cattle calves of Jammu region

4.1.5 Prevalence among cattle calves having mucus and blood in diarrhoeic faeces

The prevalence of *Cryptosporidium* and *Giardia* spp. infection in diarrhoeic cattle calves having mucus and blood in the faeces were examined (Table 12 and Fig 5). It was observed that the animals with *Cryptosporidium* infection having mucus in the faeces showed significantly (p<0.05) higher prevalence (37.33%) than those having no mucus and blood (17.79%) whereas 11.76% prevalence was recorded in faeces having blood.

The animals with *Giardia* infection having no mucus and blood showed non-significantly (p<0.05) higher prevalence(7.62%)than those having mucus in faeces (7.55%) and lowest prevalence in faeces stained with blood (5.22%) (Table 13).

4.1.6 Seasonal prevalence

According to Indian meteorological department, pune, the study period is divided into four seasons, viz. summer (March to June), Monsoon (July to September), post-monsoon (October to November) and winter (December to February). The seasonal prevalence of bovine cryptosporidiosis and giardiosis is shown in the Table 14 and Fig.6. Seasonal analysis of data suggested highest prevalence of *Cryptosporidium* infection in winter (34.65%) followed by post monsoon (26.57%), monsoon (22.06%) and summer (8.55%) whereas in case of *Giardia* infection, highest prevalence was found to be in monsoon (10.34%) followed by summer (6.57%), post monsoon (6.29%) and least in winter (5.11%). Further data revealed that in all the seasons, diarrhoeic animals showed higher prevalence than non diarrhoeic animals.

Statistical analysis suggests that prevalence of *Cryptosporidium* infection in winter season varied significantly (p<0.05) than other three seasons (Table 15) whereas prevalence of *Giardia* infection in all four seasons varied non significantly (p<0.05) (Table 16). The risk factors of *Cryptosporidium* and *Giardia* spp. infection in relation to various seasons were assessed (Table 17 and 18). The chances of occurrence of *Cryptosporidium* spp. in cattle calves in winter is 5.67 times higher than summer, whereas in *Giardia* spp. it was highest in monsoon (1.63 times) than in winter.

Table 12: Prevalence of *Cryptosporidium* spp. and *Giardia* spp. infection in cattle calves having mucus and blood in diarrhoeic faeces in Jammu region

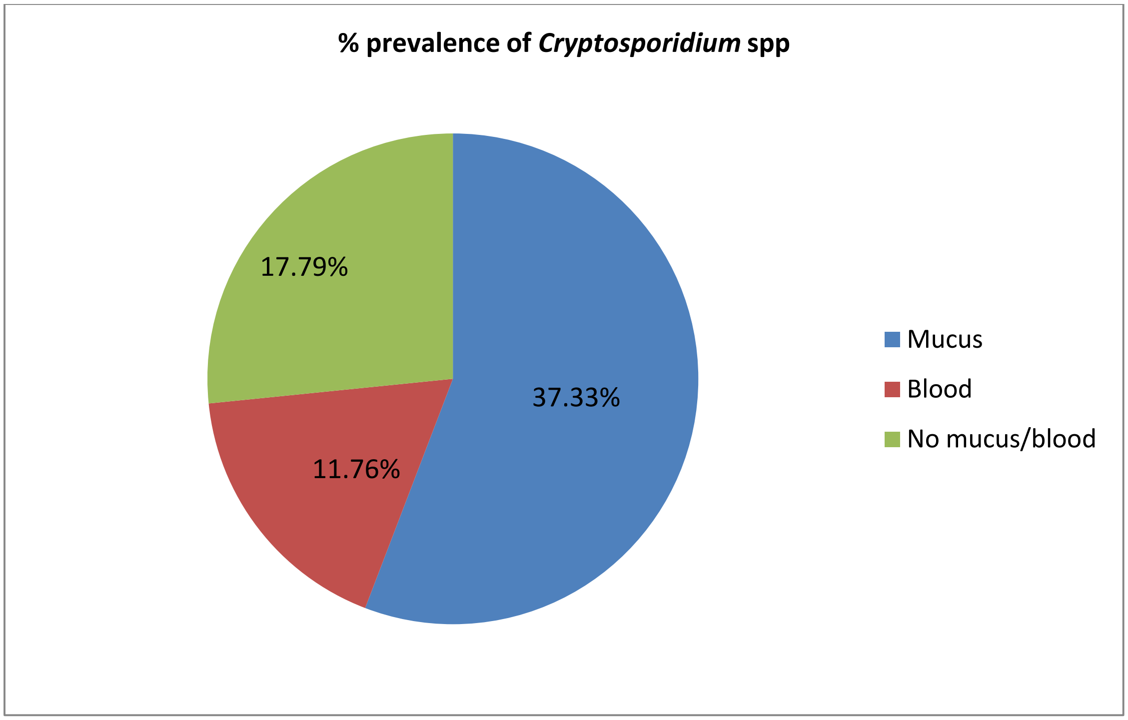
Particulars	*Cryptosporidium* spp		*Giardia* spp	
	Examined	Positive (%)	Examined	Positive (%)
Mucus	225	84 (37.33%)	225	17 (7.55%)
Blood	153	18 (11.76%)	153	8 (5.22%)
No blood /mucus	236	42 (17.79%)	236	18 (7.62%)
Total	614	144 (23.45%)	614	43 (7.00%)

Where E= Examined P= Positive P (%) = Percent prevalence

Table 13: Risk of *Cryptosporidium* spp and *Giardia* spp infection in cattle calves having mucus and blood in diarrhoeic faeces in Jammu region

Species	Risk Factor	Odd ratio	95% Confidence interval	χ^2- value
Cryptosporidium	Mucus	4.468	2.471-8.158	28.935**
	Blood			
Giardia	Mucus	1.481	0.584-3.856	0.466[ND]
	Blood			

**Significant at P<0.05; NS=Non-significant at P <0.05



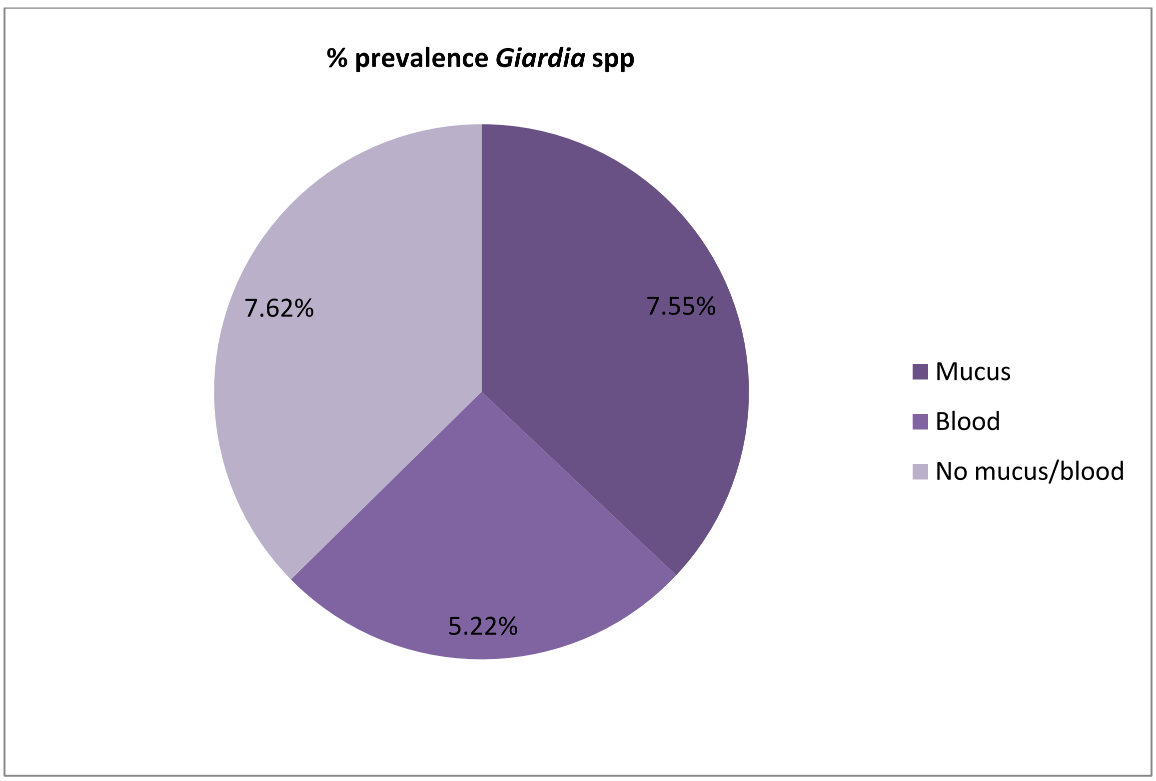


Fig 5: Prevalence of *Cryptosporidium* spp and *Giardia* spp. infections in cattle calves having mucus and blood in diarrhoeic faeces of Jammu region

Table 14: Season wise prevalence of *Cryptosporidium* spp. and *Giardia* spp. in cattle calves of Jammu region

Seasons	Number of diarrhoeic faecal samples						Number of non-diarrhoeic faecal samples						Number of total faecal samples					
	Cryptosporidium spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp			*Cryptosporidium* spp			*Giardia* spp		
	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)	E	P	P (%)
Summer	70	10	14.28%	70	6	8.57%	82	3	3.65%	82	4	4.87%	152	13	8.55%	152	10	6.57%
Monsoon	68	19	27.94%	68	10	14.70%	75	13	17.33%	75	5	6.66%	145	32	22.06%	145	15	10.34%
Post-monsoon	65	21	32.30%	65	7	10.76%	78	17	21.79%	78	2	2.56%	143	38	26.57%	143	9	6.29%
Winter	85	35	41.17%	85	8	9.41%	91	26	28.57%	91	1	1.09%	176	61	34.65%	176	9	5.11%
Total	288	85	29.51%	288	31	10.76%	326	59	18.09%	326	12	3.68%	614	144	23.45%	614	43	7.00%

Where E= Examined P= Positive P (%) = Percent prevalence

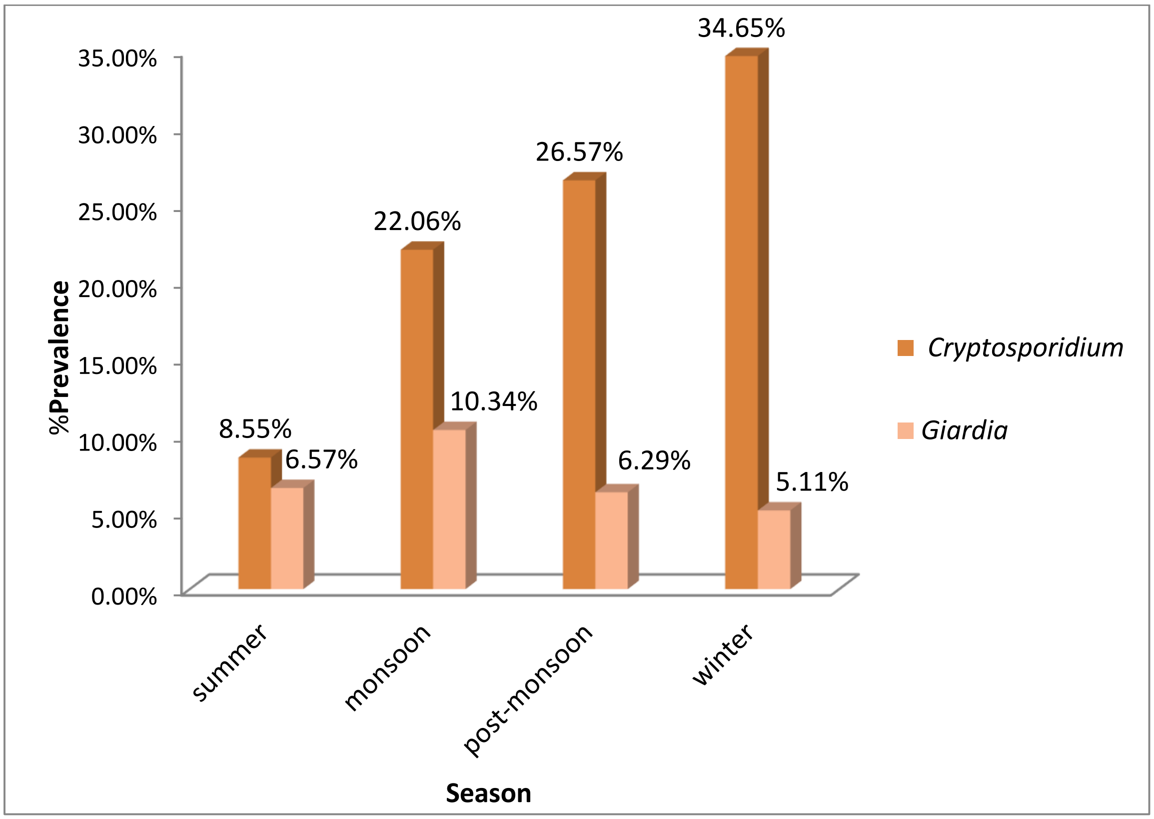


Fig 6: Season wise prevalence of *Cryptosporidium* spp. and *Giardia* spp. in cattle calves of Jammu region

Table 15: Statistical analyses (χ^2 - value) of *Cryptosporidium* spp. infection between different seasons in cattle calves of Jammu region

Season	Summer	Monsoon	Post-monsoon
Monsoon	9.520 **	-	0.568 NS
Post-monsoon	15.497 **	0.568NS	-
Winter	30.340 **	5.527 **	2.047 NS

**Significant at P<0.05; NS=Non-significant at P <0.05

Table 16: Statistical analyses (χ^2 - value) of *Giardia* infection between different seasons in cattle calves of Jammu region

Season	Summer	Monsoon	Post-monsoon
Monsoon	0.920 NS	-	1.062 NS
Post-monsoon	0.000 NS	1.062 NS	-
Winter	0.109 NS	2.434 NS	0.044 NS

NS=Non-significant at P <0.05

Table 17: Risk of *Cryptosporidium* spp. infection between different seasons in cattle calves of Jammu region

Risk Factor ↓	Summer	Monsoon	Post-monsoon	Winter
Summer	-	0.330 (0.156-0.690)	0.258 (0.124-0.533)	0.176 (0.087-0.350)
Monsoon	3.028 (1.449-6.412)	-	0.782 (0.440-1.390)	0.534 (0.314-0.907)
Post-monsoon	3.870 (1.877-8.088)	1.278 (0.719-2.273)	-	0.682(0.408-1.138)
Winter	5.672 (2.855-11.450)	1.873 (1.103-3.189)	1.466 (0.879-2.449)	-

95% Confidence Interval

Table 18: Risk of *Giardia* infection between seasons of in diarrhoeic, non-diarrhoeic samples in cattle calves of Jammu region

Risk Factor ↓	Summer	Monsoon	Post-monsoon	Winter
Summer	-	0.610 (0.245-1.504)	1.049 (0.379-2.911)	1.307 (0.475-3.614)
Monsoon	1.638 (0.665-4.087)	-	1.718 (0.678-4.426)	2.141 (0.849-5.494)
Post-monsoon	0.954 (0.344-2.636)	0.582 (0.226-1.475)	-	1.246 (0.440-3.534)
Winter	0.765 (0.277-2.106)	0.467 (0.182-1.178)	0.802 (0.283-2.275)	-

95% Confidence Interval

4.2 Intensity of *Cryptosporidium* spp. infection

The intensity of *Cryptosporidium* spp. oocysts present in the faecal smear (stained by mZN technique) of cattle calves was assessed in Jammu region. Among all the districts, maximum number of animals showed 1+ oocyst intensity, Whereas 2+ and 3+ intensity was observed in less animals. The same results has been shown in Fig 7. It was observed that 57.81% animals of <1month of age were having highest (3+) oocyst intensity, whereas 47.82% and 29.41% animals of 1-6 month age and >6 month age animals showed maximum intensity, respectively (Fig 8).The number of oocysts varied in diarrhoeic and non diarrhoeic faecal samples of cattle calves. It was observed that diarrhoeic animals had high oocyts intensity (3+) than non diarrhoeic animals. A total of 50.58% of diarrhoeic and 18.64% of non diarrhoeic cattle calves were positive for 3+ oocyst intensity. The number of oocysts present in non diarrhoeic animals was very less as 1+ grading was awarded to 21.17% and 50.84% in cattle calves (Fig 9).Higher number of animals (Male; 48.52%, Female; 46.05%) showed 2+ oocyst intensity whereas 27.94% of male and 25.0% of female showed highest oocyts intensity (3+). The same has been shown in Fig 10.On seasonal analysis, it was found that the maximum oocyst intensity (3+) of cattle cryptosporidiosis was highest in winter season (52.45%) followed by monsoon (39.06%), post monsoon (31.57%), while it was lowest in summer season (15.38%) (Fig 11).Higher number of animals (57.14%) having mucus in the faeces showed highest oocysts intensity (3+), whereas 22.22% animals having blood in the faeces showed the same oocyst intensity and the same has been shown in Fig 12.

4.3 Intensity of *Giardia* spp. infection

Mean *Giardia* cysts counts per gram faeces (by modified Mc Master Chamber technique) present in the faecal smear of cattle calves was assessed in Jammu region (Table 19). District wise intensity revealed that, there was almost similar mean cysts intensity in all the four districts of Jammu region (Rajouri, Samba, Kathua and Udhampur). The mean *Giardia* cysts intensity in Kathua (1323 ± 161.56), Udhampur (1235.7 ± 241.67), Samba (1417.8 ± 288.84) and in Rajouri (1058.9 ± 171.01) was recorded. The intensity of *Giardia* cysts among different districts varied non-significantly (Fig.13).Significantly ($p<0.05$) higher mean average number of cysts (1799.4± 189.42) was observed in 1-6 month of age group than >6 month age group (1024.15± 59.32).

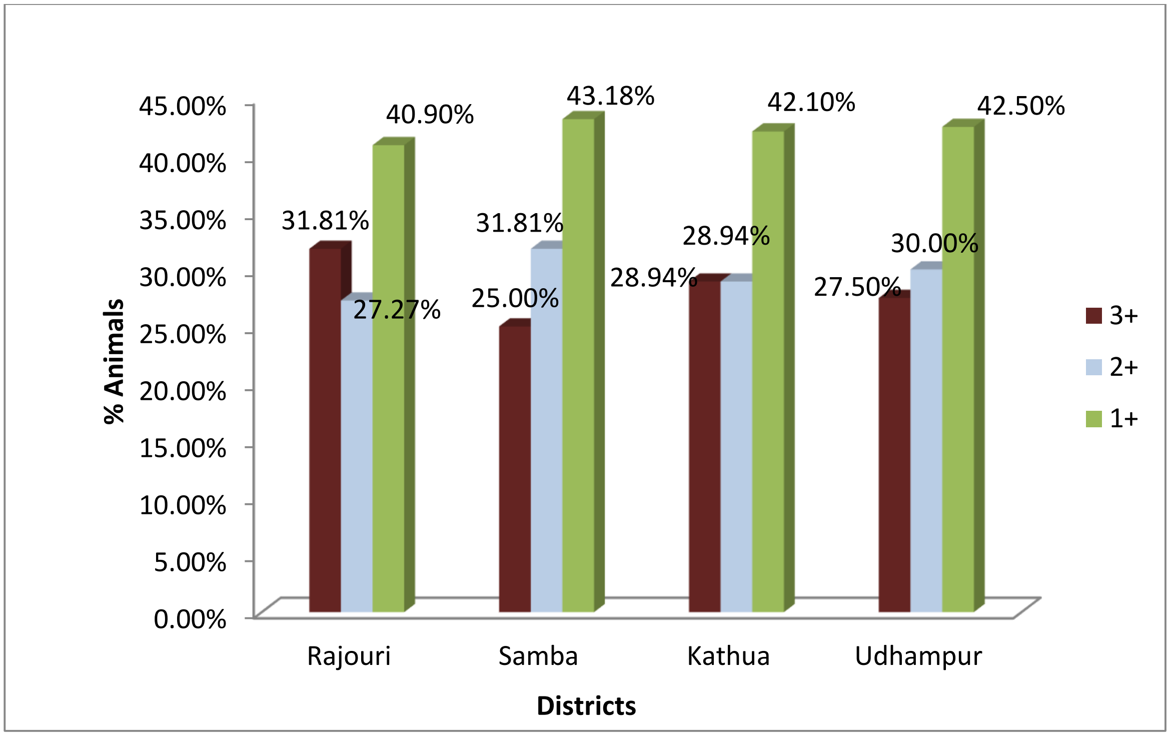


Fig 7: Intensity of *Cryptosporidium* oocysts in cattle calves among different districts of Jammu region

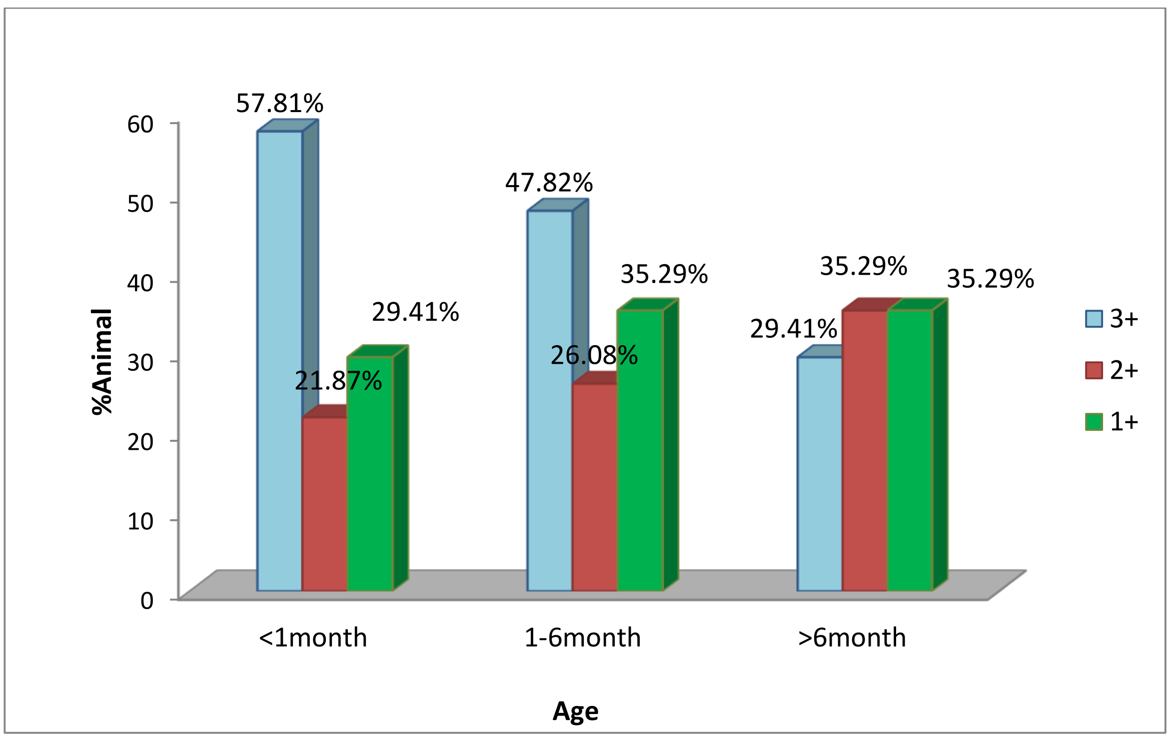


Fig 8: Intensity of *Cryptosporidium* oocysts in different age groups of cattle calves of Jammu region

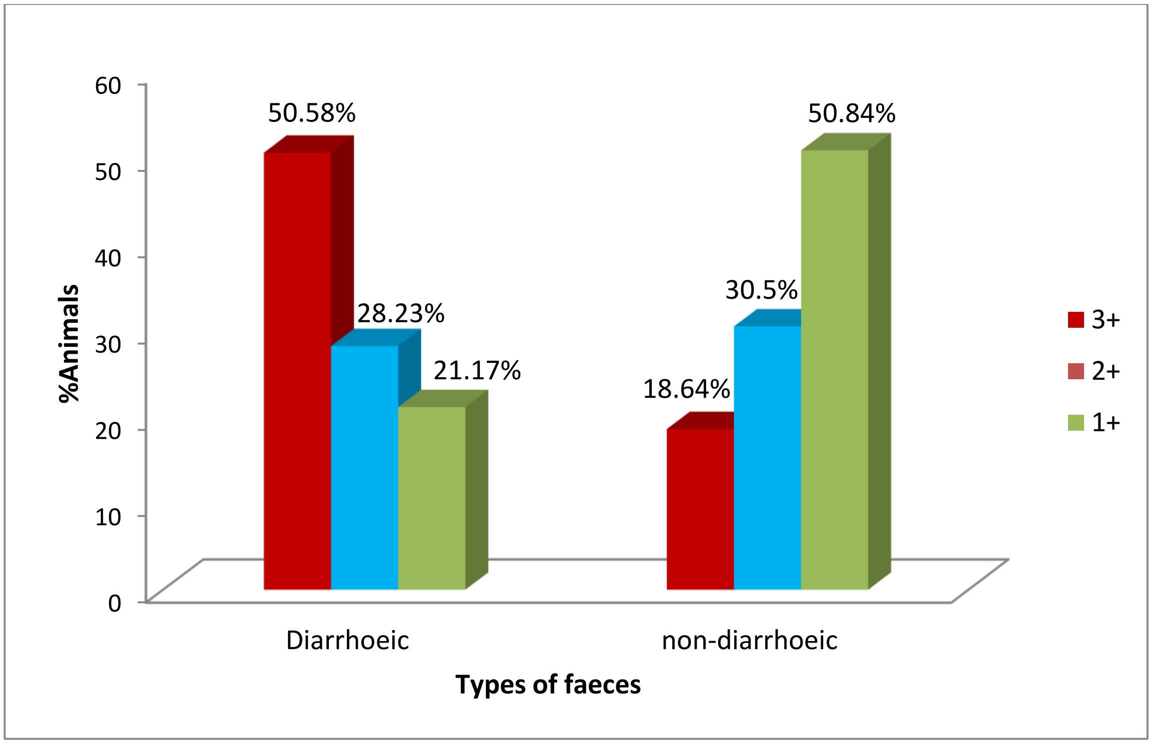


Fig 9: Intensity of *Cryptosporidium* oocysts in cattle calves among diarrhoeic and non-diarrhoeic samples of Jammu region

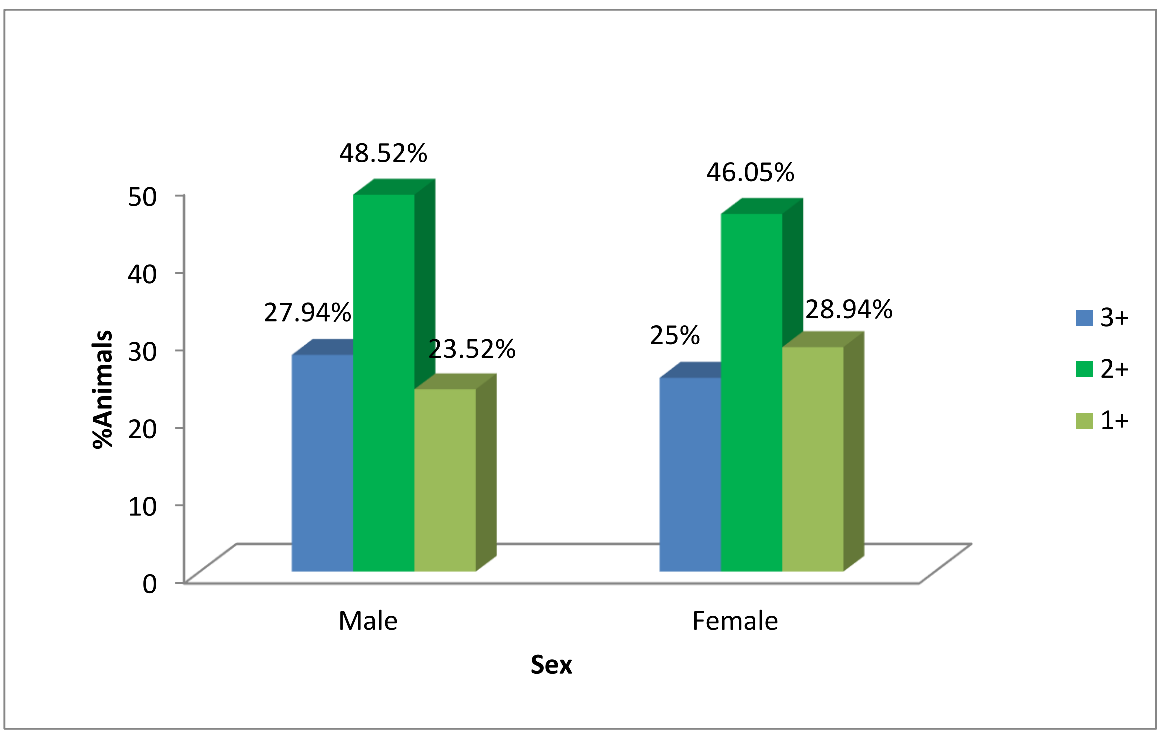


Fig 10: Sex wise intensity of *Cryptosporidium* oocysts in cattle calves of Jammu region

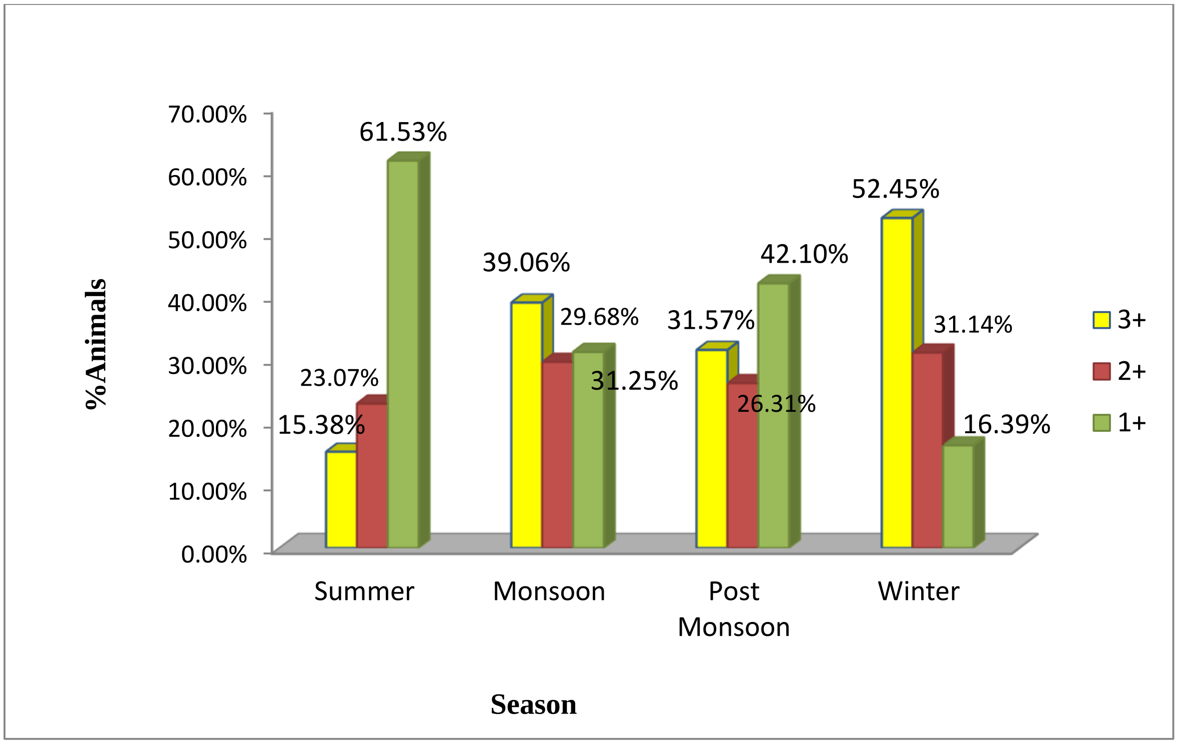


Fig 11: Seasonal intensity of *Cryptosporidium* oocysts in cattle calves of Jammu region

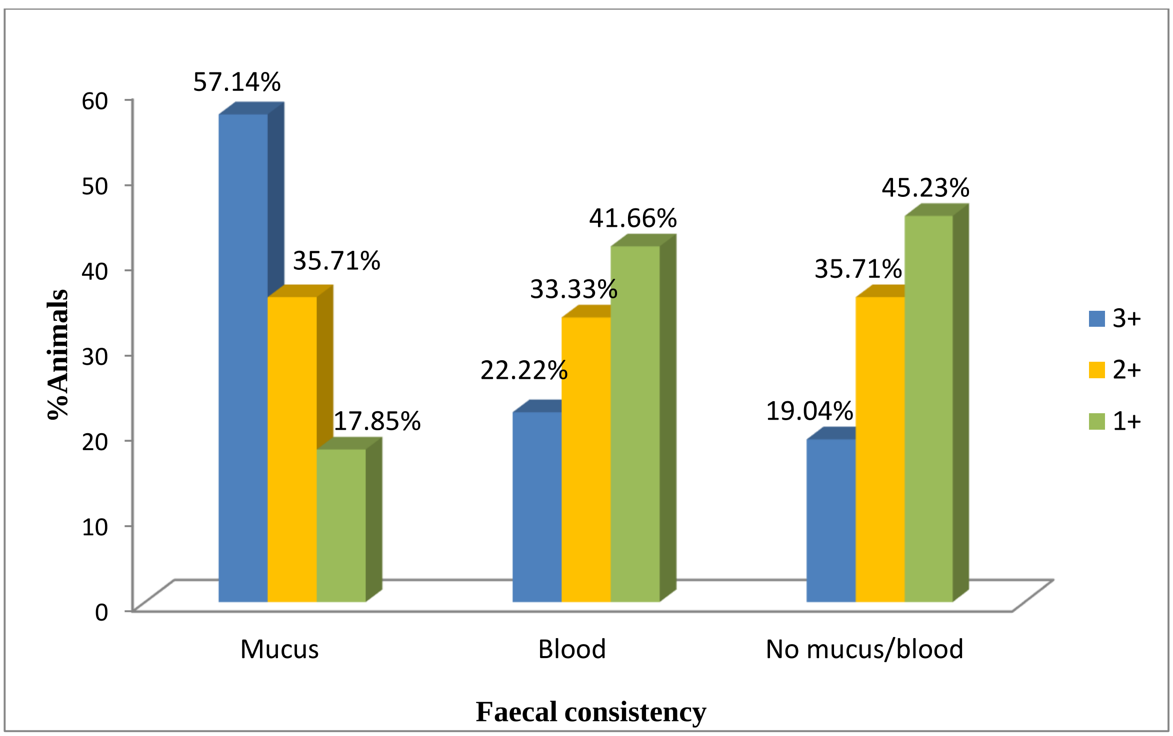


Fig 12: Intensity of *Cryptosporidium* oocysts in cattle calves of Jammu region with mucus and blood in faeces

Table 19: Statistical analysis of intensity of *Giardia* spp infection in cattle calves of Jammu region

Age wise	<1 month	1-6month	>6 month	
	834.12 ±5199.91[a]	1799.4±189.42[b]	1024.2±59.32[a]	
Faecal consistency	Mucus	Blood	No mucus/blood	
	1227.5±223.53[a,b]	932.88±77.52[a]	1675.3±114.26[b]	
District wise	Rajouri	Samba	Kathua	Udhampur
	1058.9±171.01[a]	1417.8±288.84[a]	1323±161.56[a]	1235.7±241.67[a]
Season wise	Summer	Monsoon	Post-monsoon	Winter
	1212.5±106.41[b,c]	1558.6±194.35[c]	939.67±132.34[a,b]	693.56±74.13[a]
Type of faeces	Diarrhoeic		Non-diarrhoeic	
	1290.2±224.91		918.41±135.83	
Sex wise	Male		Female	
	1222.1±72.70		1003.1±118.33	

Values with different superscripts (a,b) differs significantly ($p<0.05$) in the same row

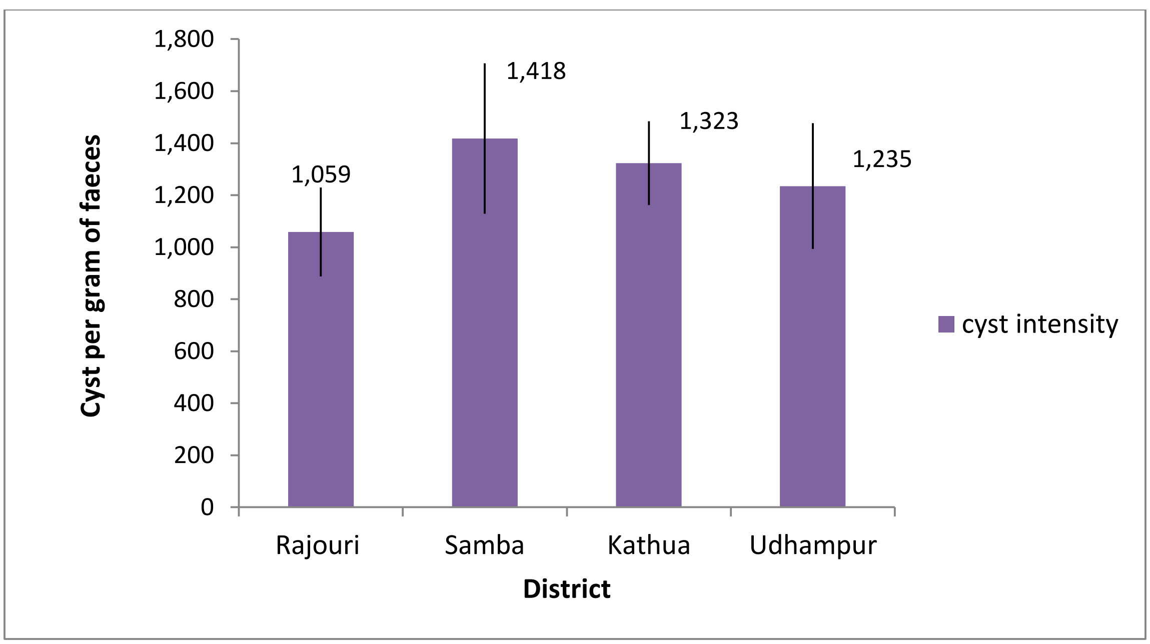


Fig 13: Intensity of *Giardia* cysts in cattle calves among different districts of Jammu region

Young animals of <1 month of age group were excreting less number of oocysts (834.13 ± 5199.91) (Fig.14). The mean number of cysts varied in diarrhoeic and non diarrhoeic faecal samples of cattle calves. It was observed that diarrhoeic (1290 .2 ± 224.91) animals had significantly ($p<0.05$) higher cyts intensity than non diarrhoeic (918.41 ± 135.83) animals (Fig 15). Higher number of cysts intensity was seen in male calves (1222.1 ± 72.70) than female calves which was non-significant ($p<0.05$) (1003.1 ± 118.33) (Fig 16). On seasonal analysis, it was found that the cyst intensity of cattle giardiosis was significantly ($p<0.05$) highest in monsoon season (1558.6 ± 194.35) followed by summer (1212.5 ± 106.41), post monsoon (939.67 ± 132.34), while it was lowest in winter season (693.56 ± 74.13) and same has been shown in Fig 17. The cattle calves having no mucus or blood in the faeces were having significantly ($p<0.05$) higher mean cysts intensity (1675.3 ± 114.26) than faeces with mucus (1227.5 ± 223.53) and with blood (932.87 ± 77.52) (Fig 18).

4.4 Sensitivity and specificity of techniques used for diagnosis

Examination of 75 faecal samples of cattle by various diagnostic assays for presence of *Cryptosporidium* and *Giardia* spp. infection revealed varying positivity. Examination of faecal samples by polymerase chain reaction test revealed highest infectivity of *Cryptosporidium* (46.66%) and *Giardia* (18.66%) infection. The least positivity was recorded by direct faecal smear staining (36%) and direct wet mount (5.33%) techniques for detection of *Crytosporidium* and *Giardia* spp. respectively in cattle faeces. The same has been recorded in Table 20 and 21.

4.4.1 *Cryptosporidium*

Overall 75 faecal samples were processed by different techniques viz. DFSS; direct faecal smear staining, DESS; diethyl ether sedimentation staining, SFSS; Sheather's floatation sedimentation staining, DFAT; direct fluorescent antibody test and PCR; polymerase chain reaction for detection of *Cryptosporidium* spp. oocysts in faeces. The sensitivity and specificity of all these five test procedures were calculated as described under materials and methods assuming the fact that PCR can detect as low as 1 oocyst in the faeces and is having 100 per cent sensitivity and specificity for the diagnosis of cattle cryptosporidiosis. The sensitivity and specificity of DFAT technique was 91.4% and 97.5%, respectively for the diagnosis of cryptosporidiosis (Plate 5 and 6).

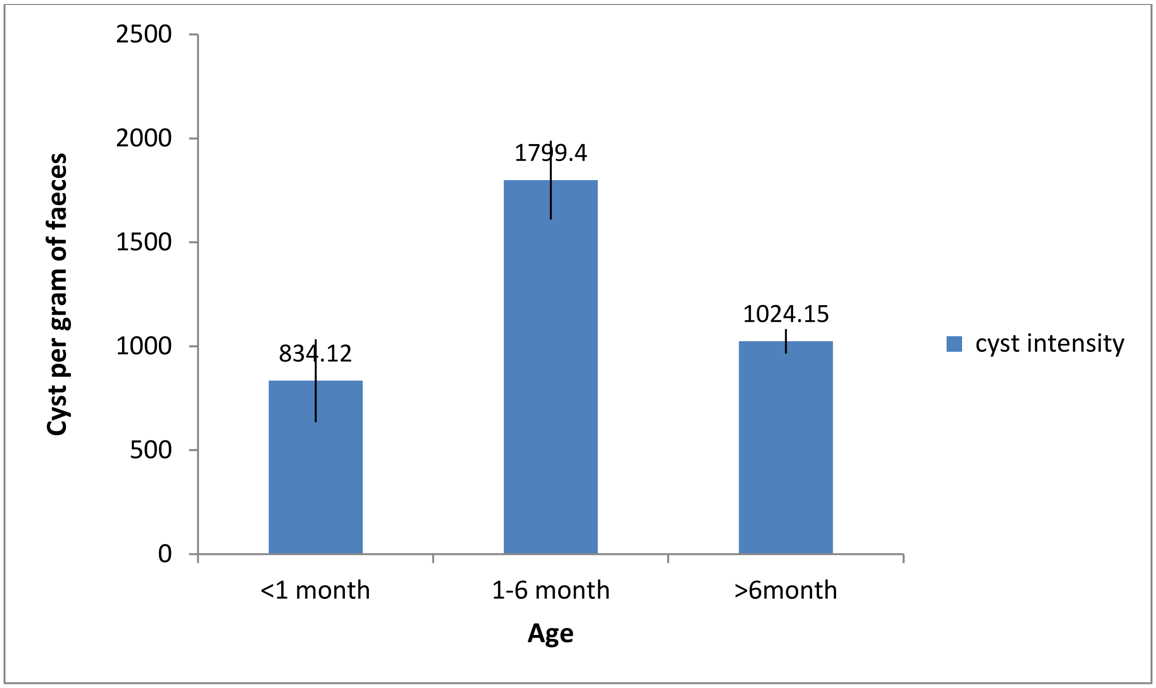


Fig 14: Intensity of *Giardia* cysts in different age groups of cattle calves in Jammu region

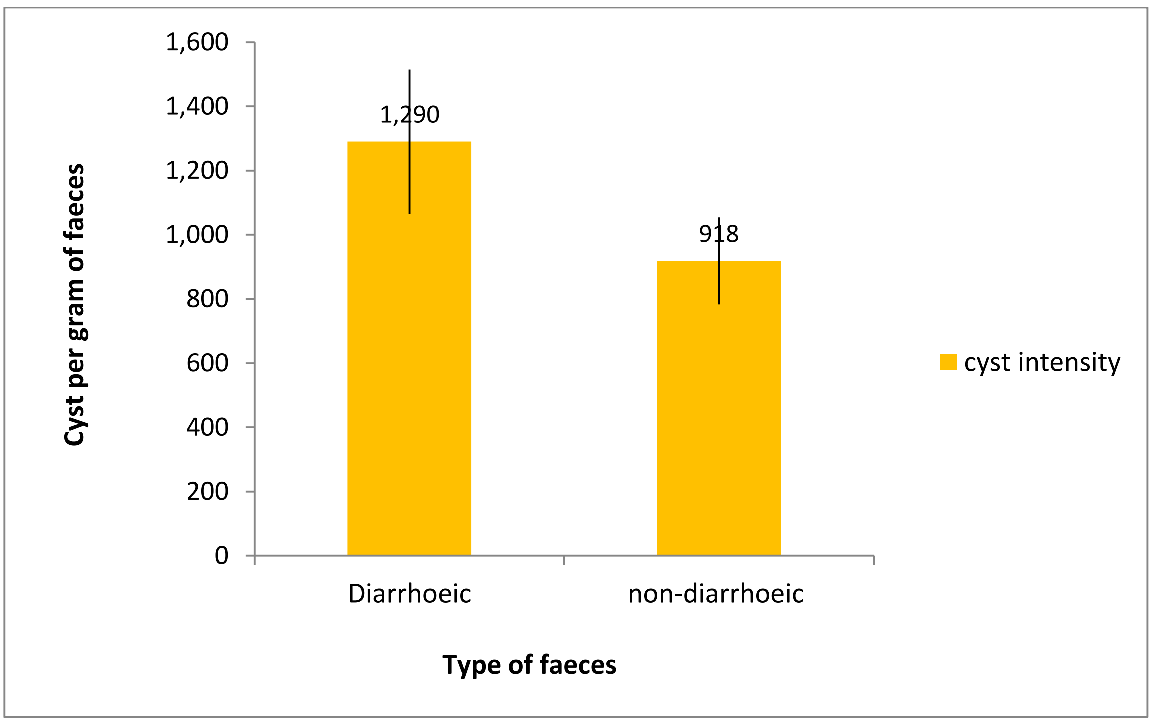


Fig 15: Intensity of *Giardia* cysts in cattle calves among diarrhoeic and non-diarrhoeic samples of Jammu region

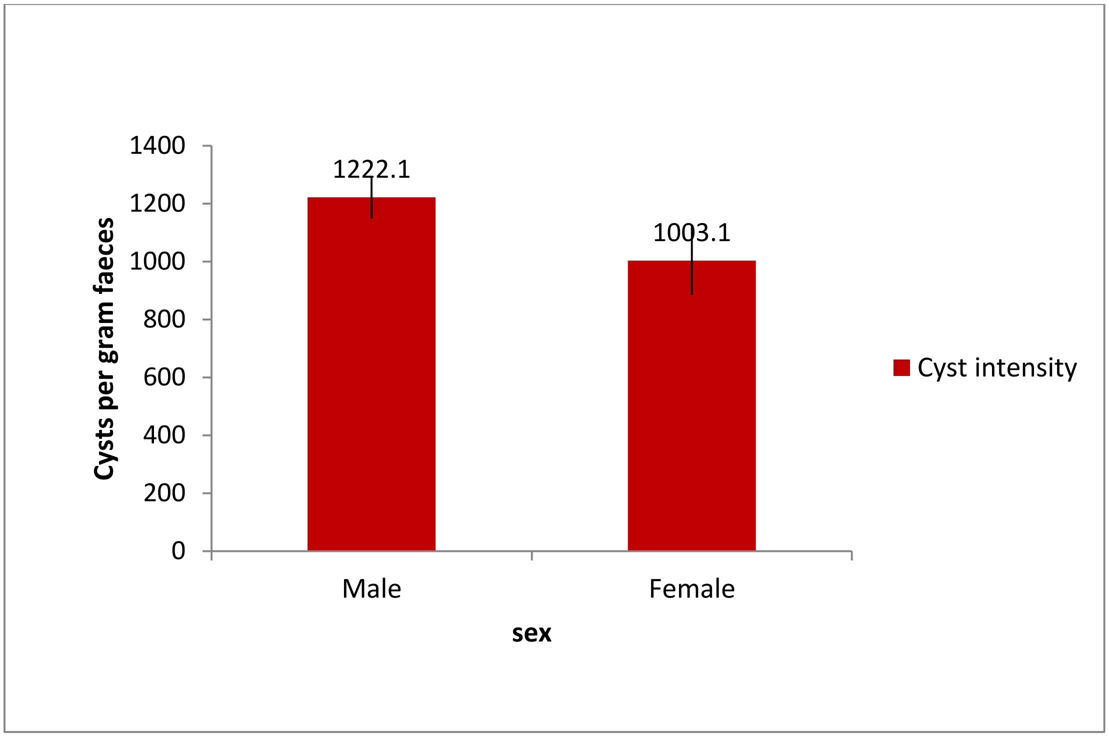


Fig 16: Sex wise intensity of *Giardia* cysts in cattle calves of Jammu region

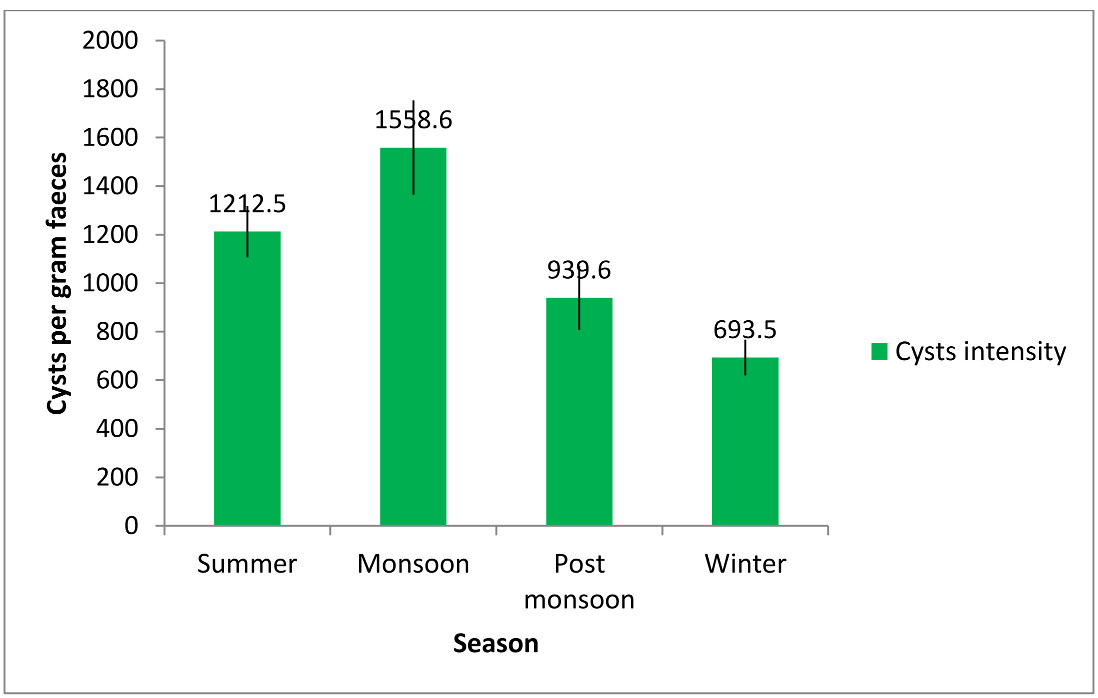


Fig 17: Seasonal intensity of *Giardia* cysts in cattle calves of Jammu region

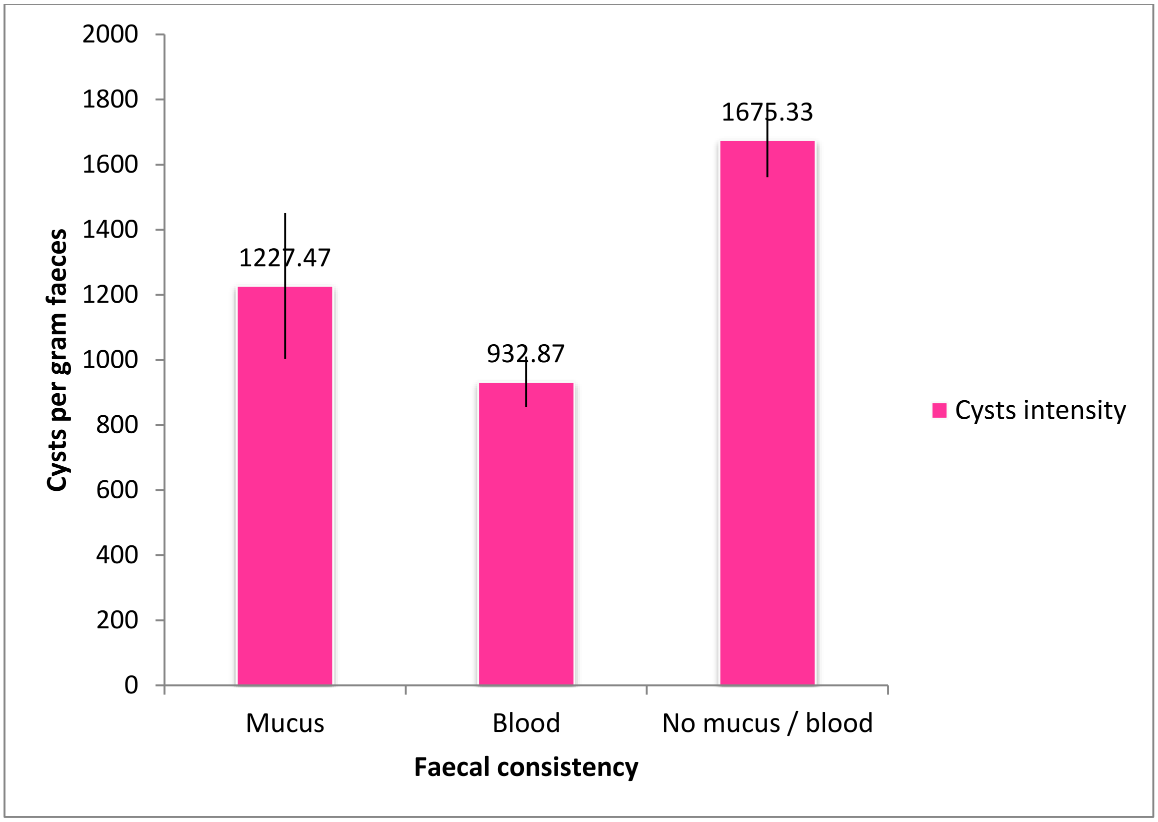


Fig 18: Intensity of *Giardia* cysts of cattle calves in Jammu region with mucus and blood in faeces

Table 20: Prevalence of *Cryptosporidium* spp in cattle calves of Jammu region as per diagnostic method

Diagnostic methods	No. of faecal samples		
	E	P	P (%)
DFSS	75	21	36.00%
DESS	75	26	34.66%
SFSS	75	30	40.00%
DFAT	75	32	42.66%
PCR	75	35	46.66%

Where E= Examined P= Positive P (%) = Percent prevalence

DFSS= Direct faecal smear staining; DESS=Diethyl ether sedimentation staining; SFSS=Sheather's floatation sedimentation staining, DFAT= Direct fluorescent antibody test; PCR= Polymerase chain reaction

Table 21: Prevalence of *Giardia* spp in cattle calves of Jammu region as per diagnostic method

Diagnostic methods	*Giardia*		
	E	P	P (%)
DWT	75	4	5.33%
FEST	75	6	8.00%
ZNSFST	75	8	10.66%
DFAT	75	11	14.66%
PCR	75	14	18.66%

Where E= Examined P= Positive P (%) = Percent prevalence
DWT=Direct wet mount; FEST= Formal ether sedimentation technique; ZNSFST=Zinc sulphate floatation Sedimentation technique; DFA=, Direct fluorescent antibody test.

The sensitivity and specificity of SFSS technique was 85.7% and 95.0%, respectively. The sensitivity of DESS technique was 74.3% and specificity was 92.5%. DFSS was least sensitive and specific method with 60% sensitivity and 90.0% specificity. The sensitivity and specificity of all the tests as compared with PCR is summarized in Table 22.

Table 22: Sensitivity and specificity of different diagnostic methods for detection of *Cryptosporidium* spp. w.r.t PCR

Diagnostic methods	**Sensitivity (%)**	**Specificity (%)**
DFSS	60%	90.0%
DESS	74.3%	92.5%
SFSS	85.7%	95.0%
DFAT	91.4%	97.5%

Where, DFSS=Direct faecal smear staining; DESS= Diethyl ether sedimentation staining;
SFSS= Sheather’s floatation sedimentation staining, DFAT=Direct fluorescent antibody test

The results of statistical analysis between different diagnostic tests conducted revealed that there is almost significant ($p<0.5$) relationship between the tests when PCR was kept as standard technique and non-significant ($p>0.05$) when DFSS compared with DESS, SFSS and SFSS. There was perfect agreement between PCR vs SFSS, DFAT with kappa values 0.81 and 0.89 as compared to PCR vs DESS with kappa value 0.67 as substantial agreement and PCR vs DFS with kappa value 0.50 as moderate agreement. The overall agreement between PCR vs DESS, SFSS and DFAT with Po value 0.84, 0.90, 0.94 as compared to agreement between PCR vs DFS with Po value 0.76. Therefore, the proportion of overall agreement indicates that there is significant substantial agreement between the other diagnostic techniques which is summarized in Table 23.

4.4.2 *Giardia* spp

Seventy five faecal samples were randomly selected and examined by five different techniques viz. DWT; direct wet mount, FEST; formal ether sedimentation technique, ZNSFST; zinc sulphate floatation Sedimentation technique, DFAT; direct fluorescent antibody test for detection of *Giardia* spp. oocysts in faeces. The sensitivity and specificity of all these five test procedures were calculated as described under materials and methods assuming PCR as 100 per cent sensitive and specific for the diagnosis of cattle giardiosis. The sensitivity and specificity of DFAT technique was 90.9% and 95.3%, respectively for the diagnosis of giardiosis (Plate 7 and 8).The sensitivity and specificity of ZNCT technique was 87.5% and 92.5%. The sensitivity of FEST technique was 66.7% and specificity was 91.3%. DWT was least sensitive and specific method with 50.0% sensitivity and 88.7% specificity. The sensitivity and specificity of all the tests as compared with PCR is summarized in Table 24.

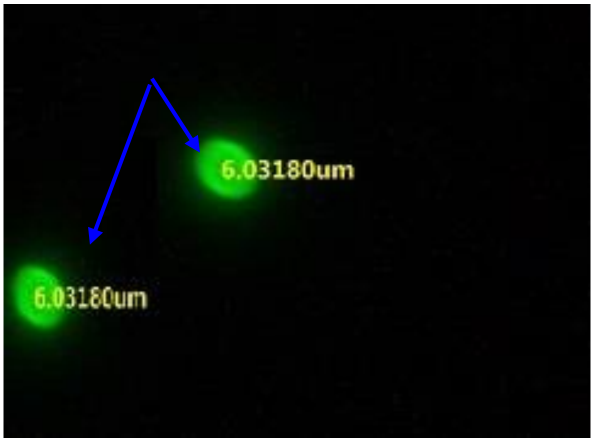


Plate 5: ***Cryptosporidium*** **oocyst (arrow marked) in faeces using direct immuno-fluorescent antibody tehnique x 1000**

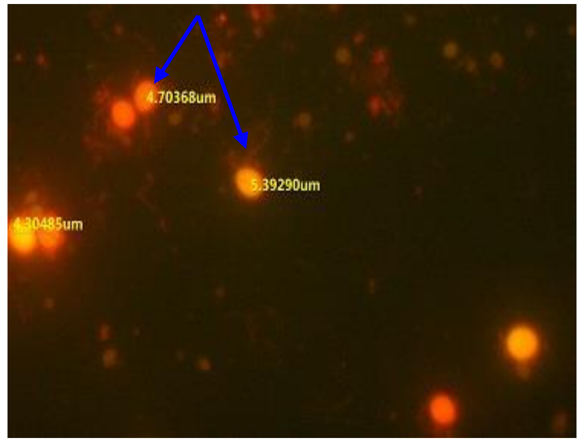


Plate 6: ***Cryptosporidium*** **oocyst (arrow marked) in faeces using direct immuno-fluorescent antibody tehnique x 400**

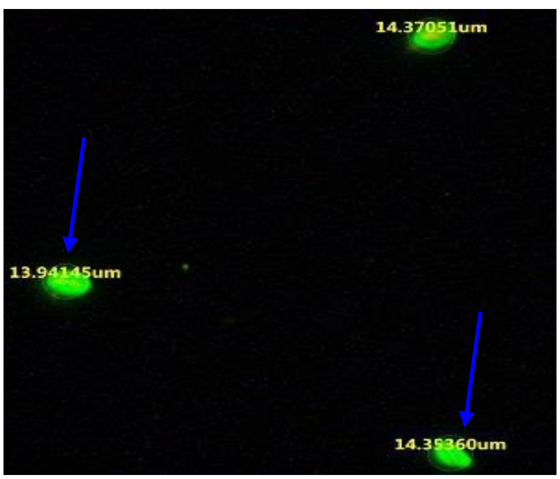


Plate 7: ***Giardia*** **cyst (arrow marked) in faeces using direct immuno-fluorescent antibody tehnique x 400**

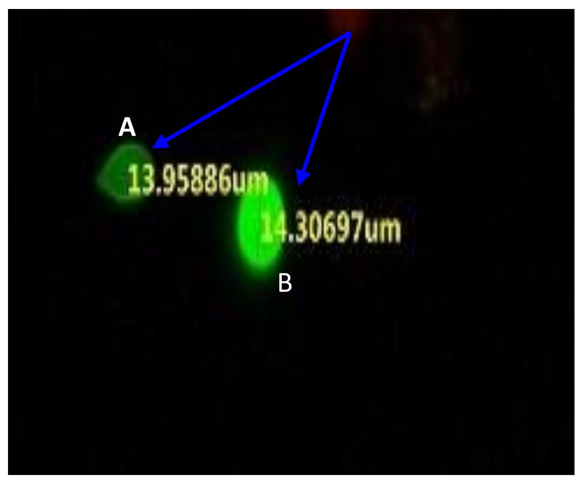


Plate 8: Trophozoite cyst (A) and ***Giardia*** **cyst (B) (arrow marked) in faeces using direct immunofluorescent antibody tehnique x 1000**

Table 23: Raw measures of agreement between each two tests to detect *Cryptosporidium* in cattle calve samples

Chi-square test (p-value)	DFS	DESS	SFSS	DFAT	PCR
PCR	18.81 ** (0.000)	32.34 ** (0.000)	46.46 ** (0.000)	56.35 ** (0.000)	---
DFAT	11.52 ** (0.001)	15.06** (0.000)	26.00 ** (0.000)	---	42.72 ** (0.000)
SFSS	6.38 ** (0.012)	9.12 ** (0.003)	---	16.09 ** (0.000)	27.83** (0.000)
DESS	3.16 NS (0.075)	---	6.75 ** (0.009)	10.93 ** (0.001)	21.34 ** (0.000)
DFS	---	1.17NS (0.279)	3.16NS (0.075)	6.18 NS (0.013)	9.01** (0.003)
Kappa test (k) value					
PCR	0.50	0.67	0.81	0.89	---
DFAT	0.41	0.47	0.61	---	0.78
SFSS	0.31	0.37	---	0.48	0.63
DESS	0.23	---	0.32	0.41	0.56
DFS	---	0.15	0.23	0.31	0.37
Po					
PCR	0.76	0.84	0.90	0.94	---
DFAT	0.72	0.74	0.81	---	0.89
SFSS	0.68	0.70	---	0.74	0.82
DESS	0.65	---	0.69	0.73	0.8
DFS	---	0.61	0.65	0.69	0.72
P_A					
PCR	0.84	0.89	0.93	0.96	---
DFAT	0.70	0.74	0.8	---	0.87
SFSS	0.61	0.65	----	0.69	0.79
DESS	0.5	----	0.55	0.61	0.70
DFS	---	0.44	0.5	0.56	0.6
P_N					
PCR	0.72	0.80	0.88	0.69	---
DFAT	0.73	0.75	0.82	---	0.90
SFSS	0.71	0.73	----	0.75	0.84
DESS	0.73	----	0.77	0.79	0.85
DFS	----	0.70	0.73	0.76	0.78

**Significant at P<0.05; NS=Non-significant at P <0.05, P_A= Positive agreement; P_N= Negative agreement; P_O= Overall agreement

Kappa value > 0.81 Almost perfect agreement, 0.61 - 0.80 Substantial agreement, 0.41 - 0.60 Moderate agreement, 0.21 - 0.40 Fair agreement, 0.01 - 0.20 Slight agreement, 0.00 Poor agreement

Table 24: Statistical analysis of different diagnostic tests used for diagnosis of *Giardia* spp. infection in cattle calves of Jammu region

Parameters	DWT vs PCR	FEST vs PCR	ZNSFST vs PCR	DFAT vs PCR
Positive predictive value	0.200	0.400	0.583	0.769
Negative predictive value	0.969	0.969	0.984	0.984
Sensitivity	50.0%	66.7%	87.5%	90.9%
Specificity	88.7%	91.3%	92.5%	95.3%
Overall agreement	86.66%	89.33%	92.00%	94.66%
Kappa value	0.22	0.44	0.65	0.80

**Significant at P<0.05; NS=Non-significant at P <0.05

Kappa value > 0.81 Almost perfect agreement, 0.61 - 0.80 Substantial agreement, 0.41 - 0.60 Moderate agreement, 0.21 - 0.40 Fair agreement, 0.01 - 0.20 Slight agreement, 0.00 Poor agreement

The results of statistical analysis between different diagnostic tests conducted revealed that there is almost significant (p<0.5) relationship between FEST, ZNSFST and DFAT when PCR was kept as standard technique and non-significant relationship between PCR vs DWT. Almost perfect agreement was observed between PCR vs DFAT with kappa values 0.80, substantial agreement between PCR vs ZNSFST with kappa value 0.65, moderate agreement between PCR vs FEST with kappa value 0.44 and fair agreement between PCR vs DWTVS with kappa value 0.22. Therefore, the proportion of overall agreement between different diagnostic tests conducted to study the prevalence of giardiasis revealed 94.66% of overall concordance of PCR vs DFAT, 92% of concordance of PCR vs ZNCTVS, 89.33% of concordance of PCR vs FEST and 86.66% of concordance in between PCR vs DWT and is summarized in Table 25.

4.5 Molecular characterization of *Cryptosporidium* spp. by 18S SSUrRNA

The prevalence of molecular characterization of *Cryptosporidium* spp. was carried on 120 samples (40 samples per age group) randomly selected from 144 samples found positive for *Cryptosporidium* oocysts by modified Ziehl-Neelsen staining. Confirmation of positivity was ascertained by nested PCR where an amplification of 1325 bp and 830 bp was obtained by primary and secondary PCR of 18S small subunit (SSU) rRNA (Plate 9 and 10).

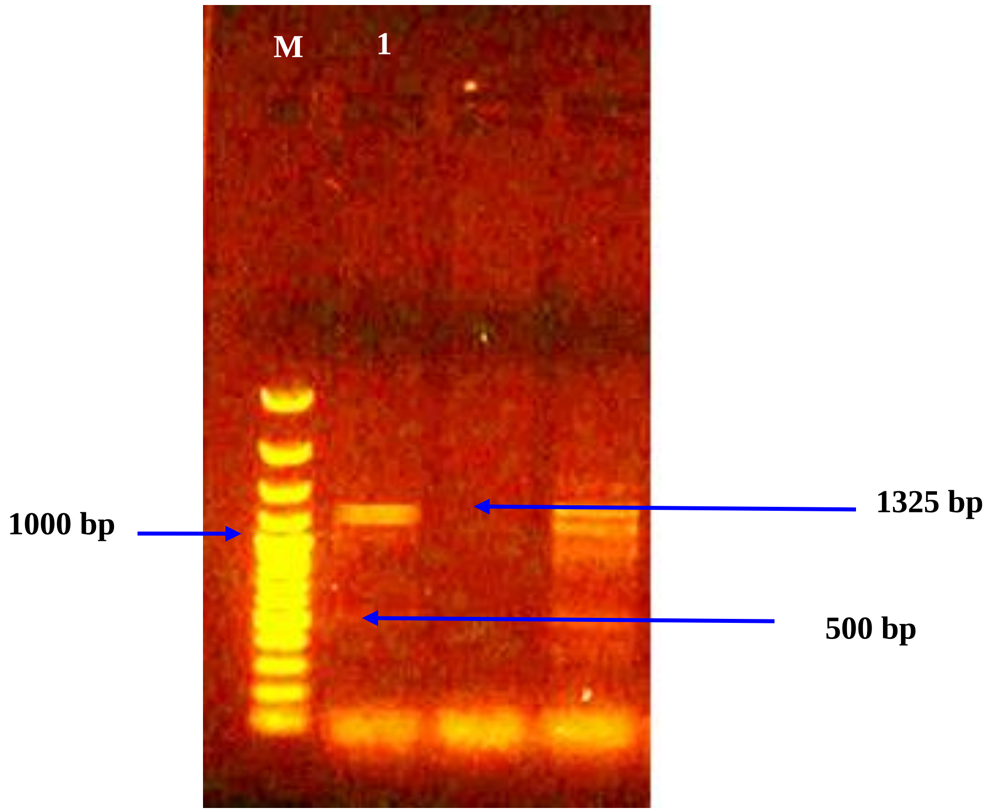


Plate 9: Amplification of *Cryptosporidium* spp. by 1st Set of Primer M-100 bp DNA ladder

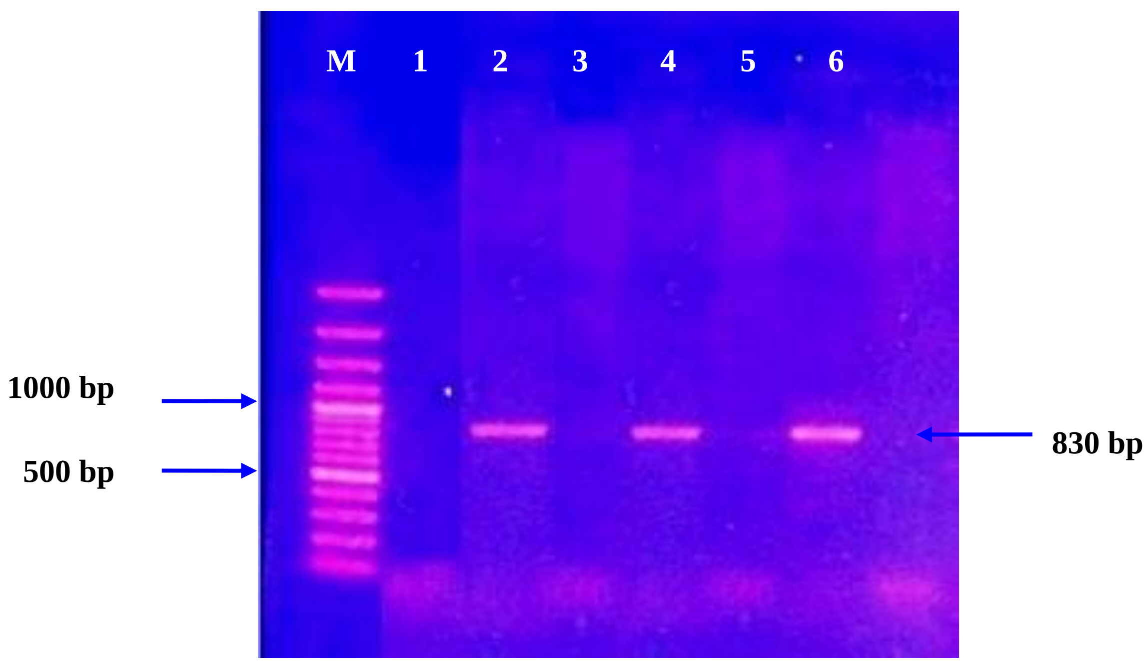


Plate 10: Amplification of *Cryptosporidium* spp. by 2nd set of primer M-100 bp DNA ladder, Lane 1, 3, 5-Negative sample, Lane 2, 4 -Positive test sample, Lane 6-Control positive

4.5.1 PCR-RFLP

As per available literature, the 830 bp nested product when digested by three restriction enzymes namely *Ssp*I, *Vsp*I and *Mbo*II, provides specific diagnosis of the species of *Cryptosporidium* involved. *C. parvum* yields three visible bands at 449 bp, 267 bp and 108 bp, *C. andersoni* yields 2 visible bands at 448 bp and 397 bp and *C. bovis* yields three visible bands at 432 bp, 267 bp and 103 bp with *Ssp*I digestion. Whereas, digestion with *Vsp*I enzymes, *C. parvum* yields 2 visible bands at 628 bp and 105 bp, *C. andersoni* show 2 visible bands at 730 bp and 115 bp and *C. bovis* show 2 visible bands at 616 bp and 115 bp. The three species when digested by the *Mbo*II two visible bands at 771 bp and 76 bp have to be observed for *C. parvum*. *C. andersoni* generates two visible bands at 769 bp and 76 bp and *C. bovis* show three visible bands at 485 bp, 185 bp, 162 bp. In our study, *Ssp*I restriction enzymes yielded two visible bands (449bp and267bp) for *C. parvum*, one band (448bp) for *C. andersoni* and two bands (432bp and 267bp) for *C. bovis*. RFLP with *Vsp*I enzyme yielded one visible band each for *C. parvum* (628bp), *C. andersoni* (730bp) and *C.*

Table 25: Diagnostic test wise risk of *Giardia* spp infection in cattle calves of Jammu region

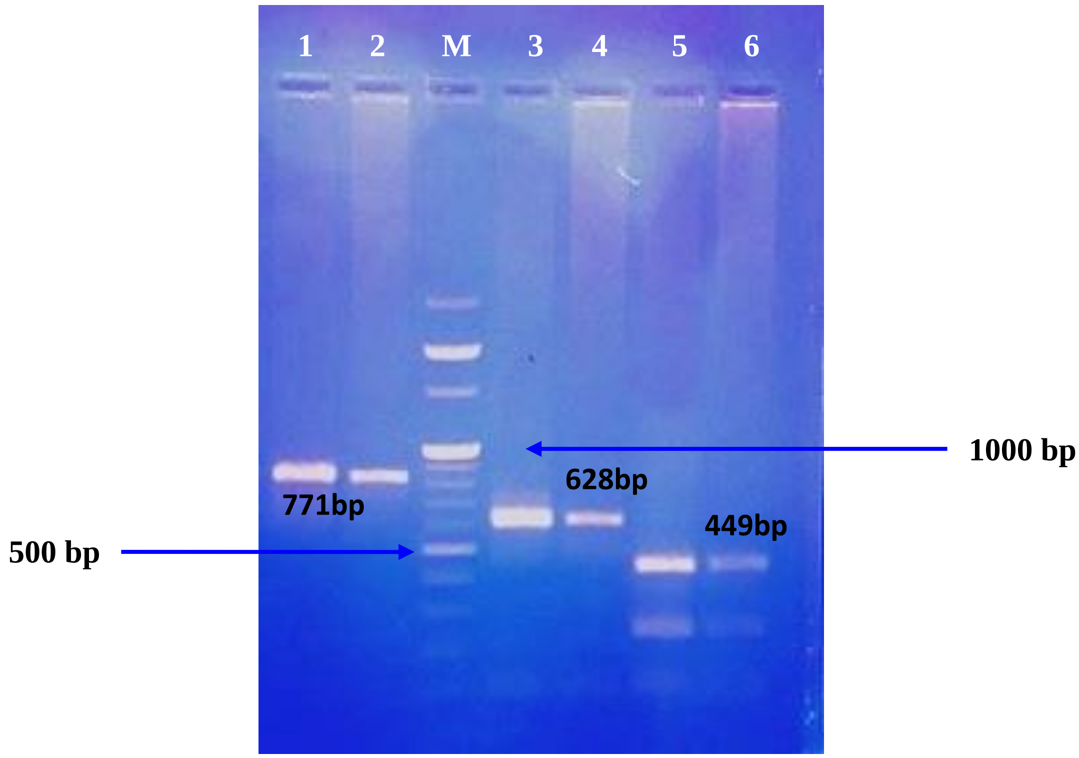
Diagnostic test	Odd ratio	95% Confidence Interval	Chi-square	(p-value)
DWT vs PCR	7.87	0.664-96.063	2.136 ^{NS}	0.14
FEST vs PCR	21.00	2.486-218.684	11.429**	0.00
ZNSFST vs PCR	86.80	7.643-2325.670	28.369**	0.00
DFAT vs PCR	203.33	16.017-6167.46	42.869**	0.00

95% Confidence Interval, **Significant at P<0.05; NS=Non-significant at P <0.05
Where, DWT= Direct wet mount; FEST= Formal ether sedimentation technique; ZNSFST= Zinc sulphate floatation Sedimentation technique; DFAT= Direct fluorescent antibody test.

bovis (616bp). Digestion of PCR product with *Mbo*II also yielded one band each for *C. parvum* (771bp), *C. andersoni* (769bp) and *C. bovis* (485bp). Other bands which have been cited in the literature but could not be observed in the present study might be because they have been washed during gel electrophoresis. The same has been shown in Plate 11, 12 and 13.

RFLP analysis of nested PCR product showed that a total of 74.07% samples examined were having *C. parvum* infection whereas, 16.66% were positive for *C. andersoni* and 9.25% were found to be positive for *C. bovis*. Cattle calves of very young age (<1 month) showed 100%positivity for *C. parvum* whereas, *C. andersoni* and *C. bovis* was not observed in any of the animal. In contrast, in older animals higher infection (55.55%) was observed by *C. andersoni* species as compared to other species. The same has been shown in Table 26and Fig. 19.

Gene sequence of identified *Cryptosporidium* species: The Jammu and Kathua isolates of *Cryptosporidium* spp. 18S small subunit (SSU) rRNA gene were custom DNA sequenced and the sequences were submitted to Genbank (Accession number MH183020.1 and MK241967.1 respectively). Sequence similarity searches in BLAST programme (blastn) of the National Center for Biotechnology Information (NCBI: http://blas.ncbi.nlm.nih.gov) revealed that the newly generated sequences of Jammu and Kathua isolates of genus *Cryptosporodium* were *C. parvum* 18S small subunit (SSU) rRNAgene. The sequences generated in our study (MH183020.1 and MK241967.1) showed 99.64% similarity among themselves. The sequences were aligned in Megalign of DNASTAR (Laser gene Suite 6.0) software for multiple sequence alignment with other isolates found in the database (KT151548.1 from Iran, MF671870 from China, and KU679364 from Czech Republic). In Jammu isolate there was a nucleotide deletion at position 34 while in others sequences at the same place nucleotide A was present. Also there was a change in position 35 from A to T in Jammu isolate compared to other isolates in multiple sequence alignment. On the contrary there was addition of nucleotide C at nucleotide position 822 in Jammu isolate. The multiple sequence alignment report has been attached as annexure I.



Pate 11: **RFLP Pattern of *C. parvum* M- 100Bp Ladder, Lane 1, 2-*Mbo*II, Lane 3, 4-*Vsp*1, Lane 5, 6-*Ssp*1**

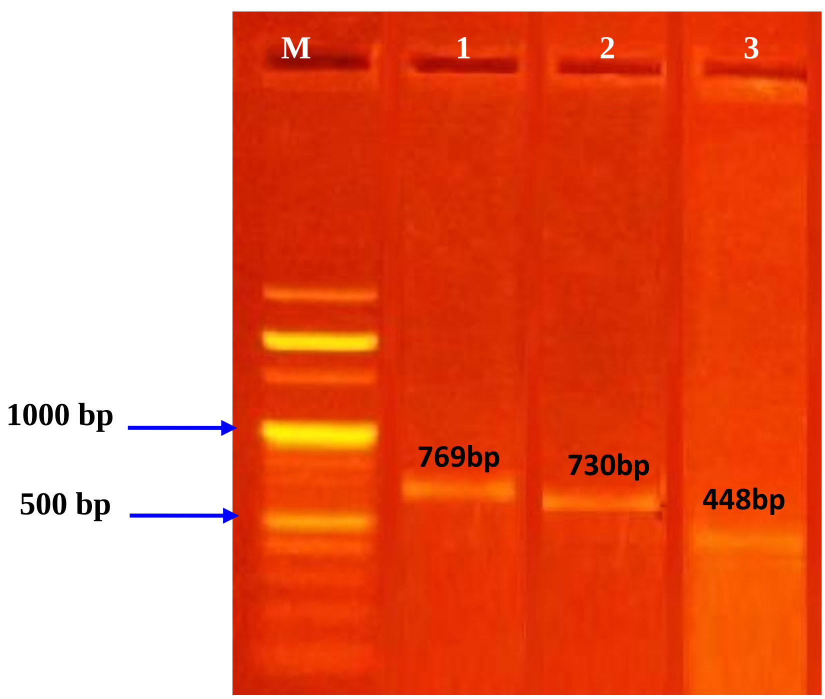


Plate 12: **RFLP Pattern of *C.andersoni* M-100 Bp Ladder, Lane 1-*Mbo*II Lane 2 -*Vsp*1, Lane 3-*Ssp*1**

Table 26: Genetic characterization of *Cryptosporidium* spp. in cattle calves of Jammu region

Age group	No. of cattles positive for *Cryptosporidium* spp.							
	Examined	Total Positive	*C. parvum*		*C. andersoni*		*C. bovis*	
			n	P (%)	n	P (%)	n	P (%)
<1 month	40	28	28	100%	0	0%	0	0%
1-6 month	40	17	10	58.82%	4	23.52%	3	17.64%
>6 month	40	9	2	22.22%	5	55.55%	2	22.22%
Total	120	54	40	74.07%	9	16.66%	5	9.25%

Where, n = No. of positive samples   P (%) = Percent prevalence

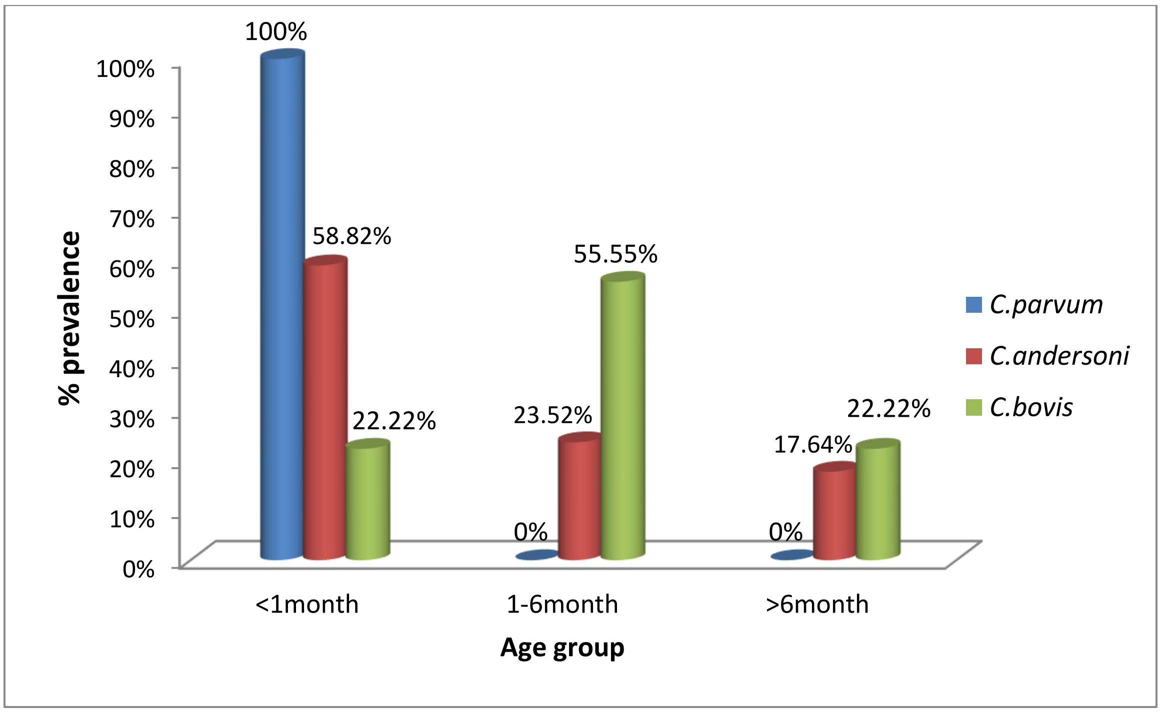


Fig 19: Percent prevalence of *C. parvum*, *C. andersoni* and *C. bovis* in different age groups of cattle calves

4.5.2 Sub-typing of *Cryptosporidium parvum*

DNA of 17 specimens was amplified targeting gp60 gene. The nested PCR produced product of 850 bp (Plate 14). The products obtained were cloned and sequenced. The sequences obtained were aligned with the sequences identified in previous studies. Phylogenetic analysis of the sequences showed that these *Cryptosporidium parvum* isolates belonged to one subtype family, i.e., IIa. Fifteen of the *C. parvum* specimens belonged to the subtype IIaA15G2R1, whereas two specimens belonged to subtype IIaA14G2R1.

Gene sequences of identified subtypes: Nucleotide sequence of identified subtypes in the present study have been mentioned in annexure II. Serine-coding trinucleotide repeats (TCA, TCG) and additional nucleotide repeat, represented as"R" (present after trinucleotide repeats) are highlighted for clarity. Subtype IIaA15G2R1 has 15 repeat regions of TCA (highlighted in red), 2 repeat regions of TCG (highlighted in green) and one repeat region of additional nucleotide "acatca" (highlighted in blue). Same is the case with subtype IIaA14G2R1 except it has 14 repeat regions of TCA.

4.6 Molecular characterization of *Giardia intestinalis* by β-giardin gene

The prevalence of Molecular characterization of *Giardia intestinalis* was carried on 120 samples (40 samples per age group) randomly selected from 43 samples found positive for *Giardia* cyst by iodine wet mount staining. Confirmation of positivity was ascertained by nested PCR where a band size of 511 bp was obtained by amplification of β-giardin gene (Plate 15). All samples found positive by iodine wet mount staining were also reported to be positive for *Giardia* spp. by nested PCR.

4.6.1 PCR-RFLP

The 511 bp nested product when digested by *Hae* III restriction enzymes provided specific diagnosis of the species of *Giardia* involved. Assemblage specific patterns were obtained with two different expected fragments. Assemblage B with two visible bands at 150bp and 117bp were recorded. As per literature available, four other fragments of 110bp, 84bp, 26bp and 24bp are observed when PCR-RFLP product of β-giardin gene of

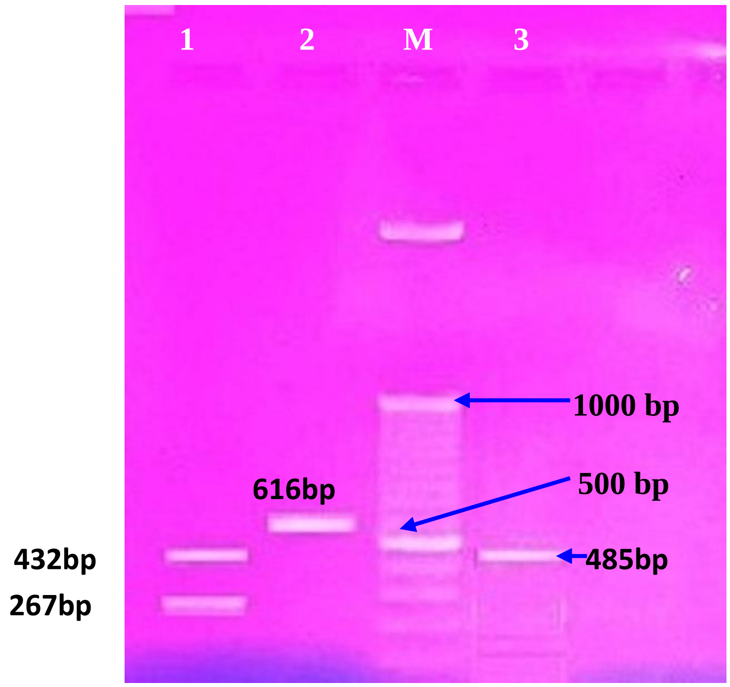


Plate 13: RFLP Pattern of *C. bovis* M-100 Bp Ladder, Lane 1- *Ssp*1, Lane 2-*Vsp*1 Lane 3- *Mbo*II

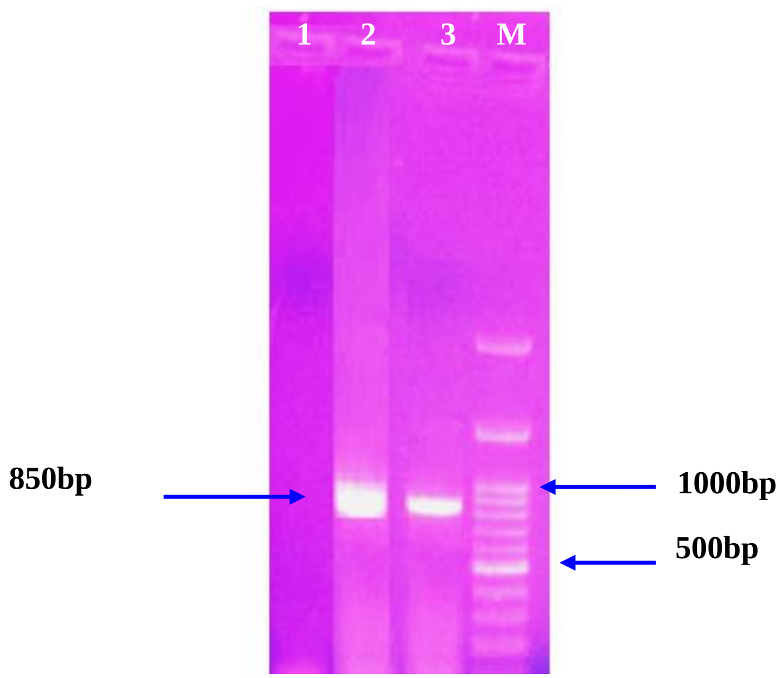


Plate 14: Amplification of *Cryptosporidium parvum* by Nested PCR of gp60 PCR, M-100 bp DNA ladder Lane 1- Negative sample, Lane 2 – Positive control, Lane 3-Test sample

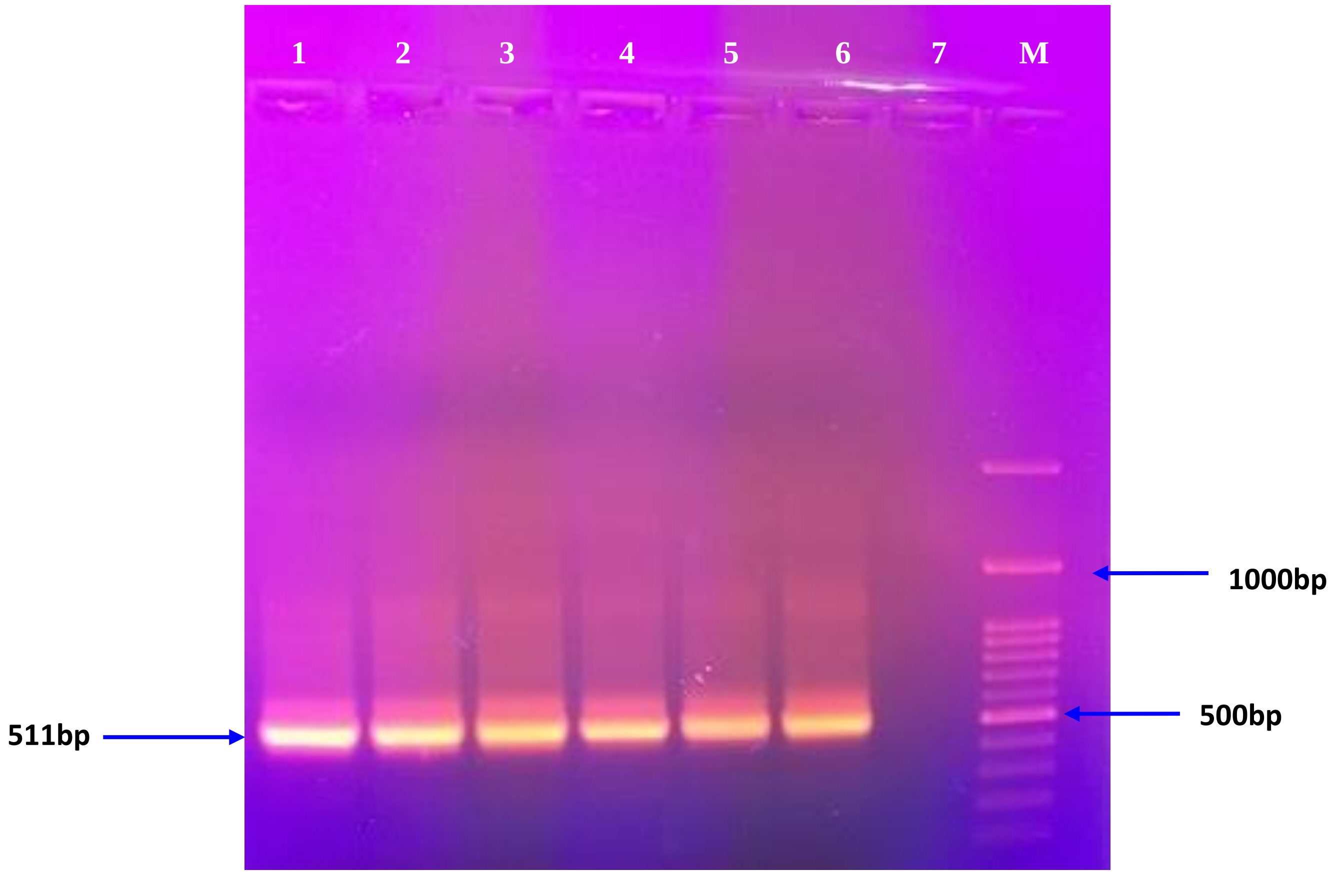


Plate 15: Nested PCR Amplification of *Giardia intestinalis by* β-giardin gene M-100 bp DNA ladder, Lane 1, 2, 3, 4, 5- Test samples Lane 6—Positive control, Lane 7—Negative control

Giardia spp. are digested with *Hae* III restriction enzyme. In our results, 110bp band probably coincided with 117bp band. So, could not be visualized and three other bands i.e. 84bp, 26bp and 24bp bands were not observed in the gel which could have been possibly washed off during gel electrophoresis.

Assemblage E was also detected where three bands of 186bp, 150bp and 110bp were observed in the, gel whereas other three bands (26bp, 24bp and 15bp) which have been cited in the literature could not be observed as they might have been washed during gel electrophoresis. The same has been shown in Plate 16 and 17.

RFLP analysis of nested PCR products revealed that a total of 1.6% samples examined were having Assemblage B infection whereas, 14.16% were positive for Assemblage E. The same has been shown in Table 27 and Fig. 20.

Gene sequence of identified *Giardia* species: The 511bp partial gene sequence of beta giardin gene of *Giardia* spp isolated from Cattle feces was custom sequenced. The gene sequence was analyzed for similarities in BLAST programme (blastn) of the National Center for Biotechnology Information (NCBI: http://blas.ncbi.nlm.nih.gov). The BLAST search revealed that the 511bp gene sequence belonged to *Giardia intestinalis* assemblage B. The gene sequence generated in our study had 97.68% similarity with *Giardia intestinalis* isolate 8 from Iran while it had 97.49% similarity with gene sequence from Egypt (MG736239.1) and 97.66% similarity with a Kenyan isolate (LC436576.1) available in the database. Further, the gene sequences were aligned in Megalign of DNASTAR (Laser gene Suite 6.0) software for multiple sequence alignment. Some major nucleotide substitutions were present in Jammu isolate from nucleotide position 474 to 475. Further there was a nucleotide substitution at position 505 from A to C and 511 from T to G. The multiple sequence alignment report has been attached as annexure III.

Table 27: Distribution of Assemblages in animal isolates according to β-giradin nested PCR assay

Age group	No. of cattle positive for *Giardia* spp.					
	Examined	Total Positive	Assemblage B		Assemblage E	
			n	P (%)	N	P (%)
<1 month	40	1	0	0%	1	2.5%
1-6 month	40	12	1	2.5%	11	27.5%
>6 month	40	06	1	2.5%	05	12.5%
Total	120	19	2	1.6%	17	14.16%

Where, n = No. of positive samples, P (%) = Percent prevalence

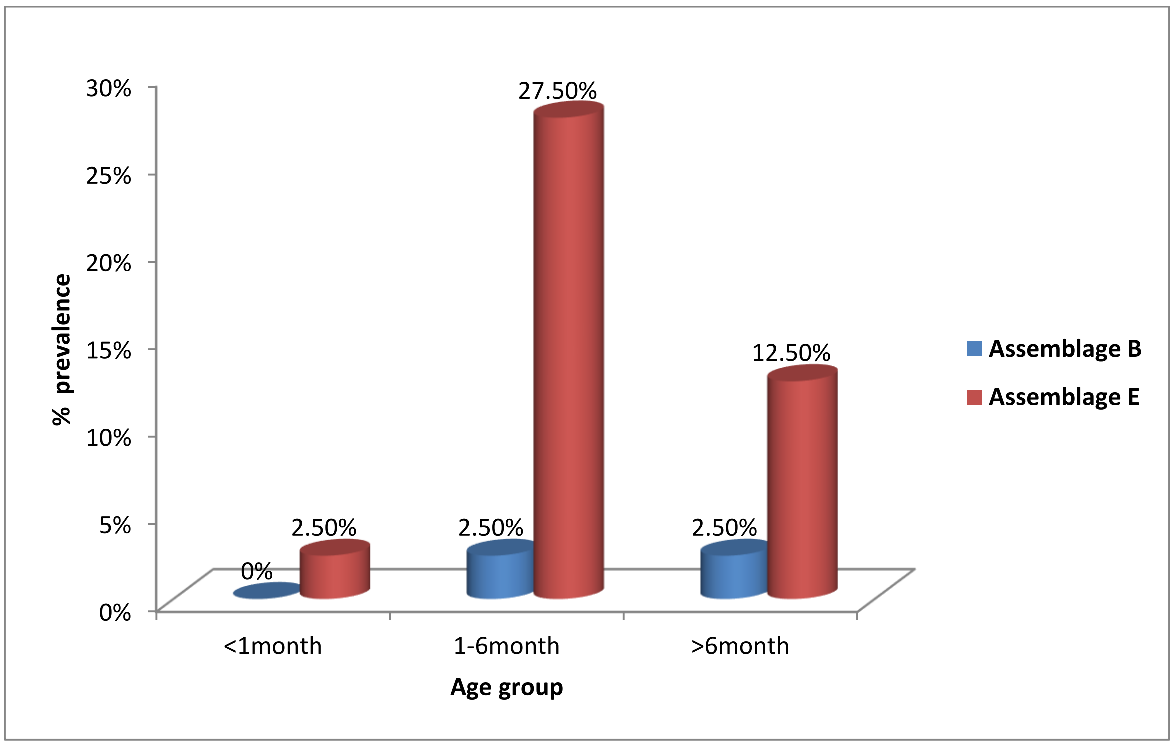


Fig 20: Percent prevalence of Assemblage B and Assemblage E in different age groups of cattle calves

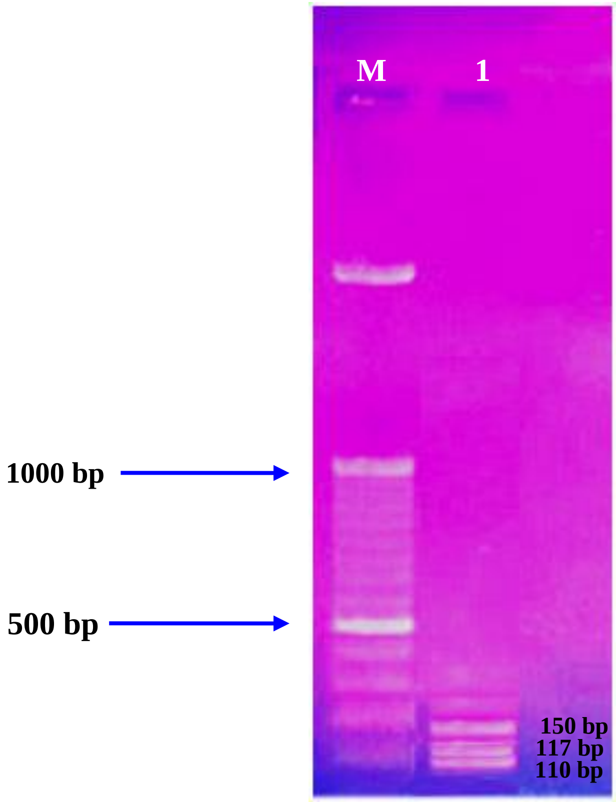


Plate 16: RFLP pattern of Assemblage B of *Giardia intestinalis* M-100 Bp Ladder, Lane 1 – *Hae*III

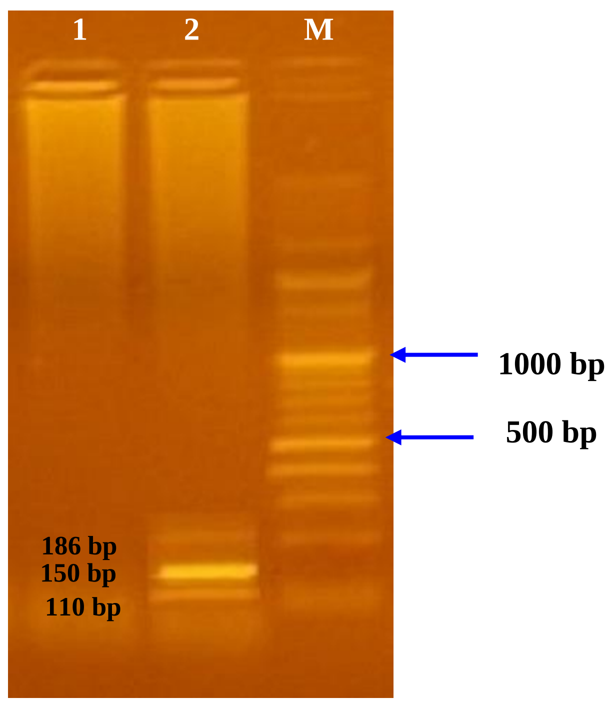


Plate 17: RFLP Pattern of Assemblage E of *Giardia intestinalis* M-100 Bp Ladder, Lane 1- Negative control, Lane 2- *Hae*III

4.7 Therapeutic efficacy of *Curcuma longa* against experimental cryptosporidiosis in mice

4.7.1 Percent extractability of *Curcuma longa* extract

Extractability percent of ethanolic extract of *Curcuma longa* (sigma-Aldrich, USA) was 6.4% as mentioned in material and method.

4.7.2 Analytic HPLC

The HPLC-PDA chromatogram at 423 nm showed peak of curcumin in a standard marker preparation and in the *Curcuma longa* extract sample (Table 28 and Fig. 21 and 22).Resolution of curcumin was clear and made their quantification easier.

Table 28: Concentration of curcumin per injection vs their respective mean area of peak

S. No.	Conc. (µg/mL)	Mean Area
1	0.1	469619
2	0.2	991854
3	0.5	2687820
4	1.0	5066547
5	2.0	9326270

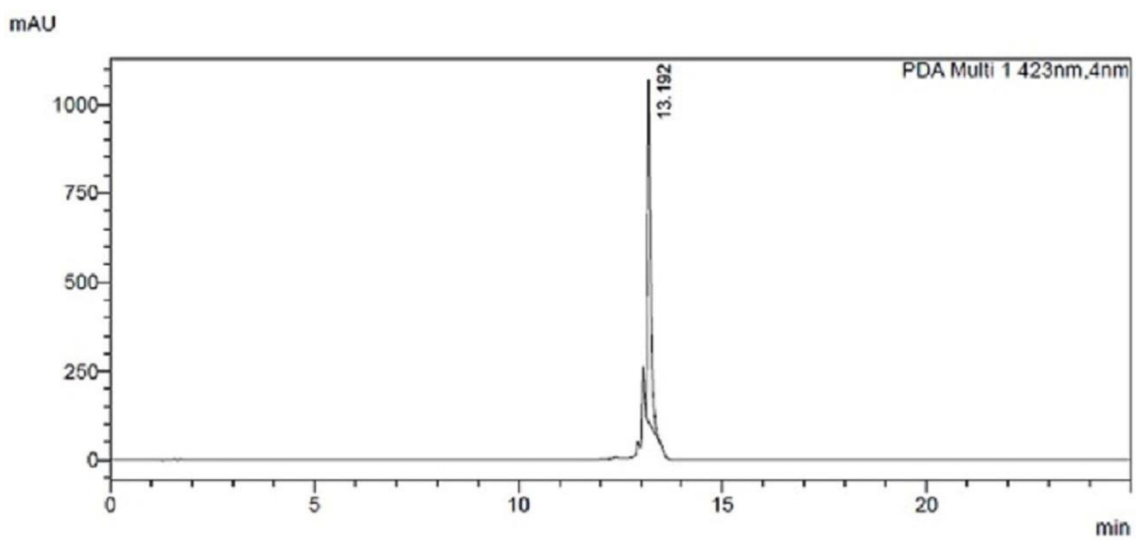


Fig 21: Chromatogram showing the peak of curcumin (from *Curcuma longa)* as a standard drug

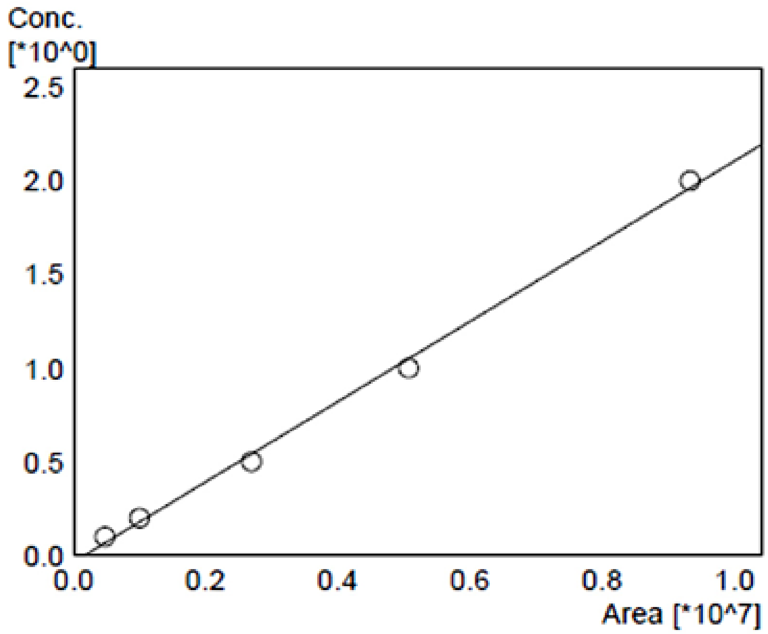


Fig 22: Standard curve of curcumin (from *Curcuma longa)* as a standard drug

Analysis revealed that the concentration of curcumin in extract was 1.6µg/µL and showed characteristic retention time of curcumin (13.192 min) as shown in Table 29 and Fig. 23. Curcumin was the major chemical constituent and this suggested twenty five percent (25%) of curcumin was present in the extract.

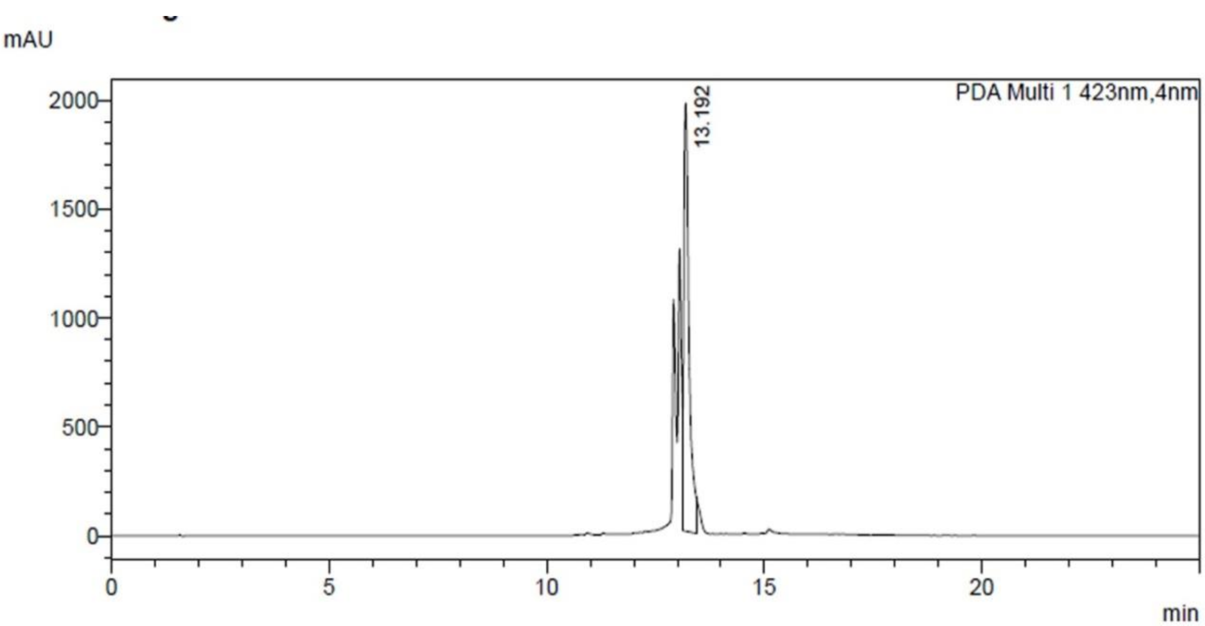


Fig 23: Chromatogram showing the peak of curcumin present in *Curcuma longa* extract

Table 29: Concentration of curcumin in extract per injection

S. No.	Ret. Time	Area	Height	Conc.	Inj. vol.
1	13.200	14019745	1827878	2.971	2
2	13.192	16265309	1964323	3.452	2
Average		15142527	1896100	3.211	
Std. dev.				0.340	
%RSD				10.601	

4.7.3 Pilot study

The results of pilot trial revealed that *Curcuma longa* extract @ 8 mg/kg b.wt resulted in maximum reduction in oocyt output in faeces of experimentally infected mice as compared to other two dose studied i.e. 2 and 4 mg/kg b.wt. So, *Curcuma longa* extract at dosage of 8 mg/kg b.wt was kept as a basic value for further evaluation of the drug in the final trial against cryptosporidiosis in mice.

4.7.4 Final study

In Final trial total 200 animals were divided into ten groups, i.e- Group I as a healthy control, Group II- uninfected and immunosupressed with dexamethasone @ 30µg/ml, Group III - infected control, Group IV- nitazoxanide as a standard drug, Group V to Group X with three variable doses of *Curcuma longa* and curcumin @ 4, 6 and 8 mg/kg body weight. Faeces from all the groups were checked daily by mZN staining from the time of arrival of animals till the end of experiment.

Two parameters viz. oocyst per gram faeces and average body weight were used for evaluating the effect of anticryptosporidial agents.

4.7.4.1 Oocyst per gram faeces (OPG)

The study revealed that no oocyst was observed in the faecal samples on day 0 and 3 post infection in experimental animals. The oocyst commenced in the faeces on 5th

day post infection, where mean OPG ranged from 6666.67±1370.320 to 6416.67±723.610 in animals of group III to X. The mean OPG count at day 7 post treatment being significantly (p<0.05) lowest in group IV animals (750±111.803) followed by group X (1666±307.318), IX (2000±223.607), VII (2416±436.208), VI (2533.34±338.296), VIII (2916.67±554.026) and V (3250±727.438). Among treatment group, maximum percent mean oocyst reduction (88.88%) was observed in group IV animals and least mean oocyst reduction was observed in group V animals (51.85%) on 7 day post treatment. The result of the present study revealed that the treatment with synthetic curcumin (group VIII, IX and X) resulted in significantly (p<0.05) higher reduction of mean oocyst count in mice than those which were administered with same dose of *Curcuma longa* (group V, VI and VII). The same has been shown in Table 30 and Fig 24.

Table 30: *Cryptosporidium* spp. oocyst count in mice of control and different treatment groups at different intervals

	Period			
Groups	5^{th} DPI (0 DPT)	7^{th} DPI (2 DPT)	9^{th} DPI (4 DPT)	11^{th} DPI (6 DPT)
Group I	0.000 ± 0.000^{A}	0.000 ± 0.000^{A}	0.000 ± 0.000^{A}	0.000 ± 0.000^{A}
Group II	0.000 ± 0.000^{A}	0.000 ± 0.000^{A}	0.000 ± 0.000^{A}	0.000 ± 0.000^{A}
Group III	$6666.67 \pm 1370.320^{B,a,b}$	$8916.67 \pm 1398.908^{C,b}$ (33.75%)	$6333.34 \pm 1229.273^{C,a,b}$ (4.99%)	$4216.7 \pm 981.63^{D,a}$ (36.75%)
Group IV	$6750 \pm 1022.660^{B,C}$	$4083.34 \pm 888.976^{B,b}$ (39.51%)	$2166.67 \pm 380.058^{B,a,b}$ (67.91%)	$750 \pm 111.803^{B,a}$ (88.88%)
Group V	6750 ± 1487.447^{B}	6000 ± 1310.216^{B} (11.11%)	5016.67 ± 1331.770^{C} (25.68%)	3250 ± 727.438^{D} (51.85%)
Group VI	6333.34 ± 1842.402^{B}	5166.67 ± 1301.708^{B} (18.42%)	$3866.67 \pm 1106.546^{B,C}$ (38.95%)	$2533.34 \pm 338.296^{C,D}$ (60%)
Group VII	$6500 \pm 1543.805^{B,\mathbf{b}}$	$4666.67 \pm 401.386^{B,a,b}$ (28.21%)	$3750 \pm 381.881^{B,a}$ (42.30%)	$2416.67 \pm 436.208^{C,D,a}$ (62.83%)
Group VIII	$6916.67 \pm 1135.904^{B,b}$	$5166.67 \pm 691.215^{B,a,b}$ (25.30%)	$4666.67 \pm 833.333^{B,C,a,b}$ (32.53%)	$2916.67 \pm 554.026^{C,D,a}$ (57.83%)
Group IX	$6500 \pm 1147.461^{B,c}$	$4666.67 \pm 586.894^{B,b,c}$ (28.21%)	$3500 \pm 258.199^{B,a,b}$ (46.15%)	$2000 \pm 223.607^{B,C,D,a}$ (69.23%)
Group X	$6416.67 \pm 723.610^{B,c}$	$4166 \pm 781.736^{B,b}$ (35.06%)	$3083 \pm 597.448^{B,a,b}$ (51.94%)	$1666 \pm 307.318^{B,C,a}$ (74.03%)

^{abc}Means bearing different superscripts within a row differ significantly and ^{A,B,C} Means bearing different superscripts within the column

Where, DPI- Days post infection and DPT- Days post treatment

Values in parenthesis indicate % reduction in mean oocyst count in comparison to day 0 of the group

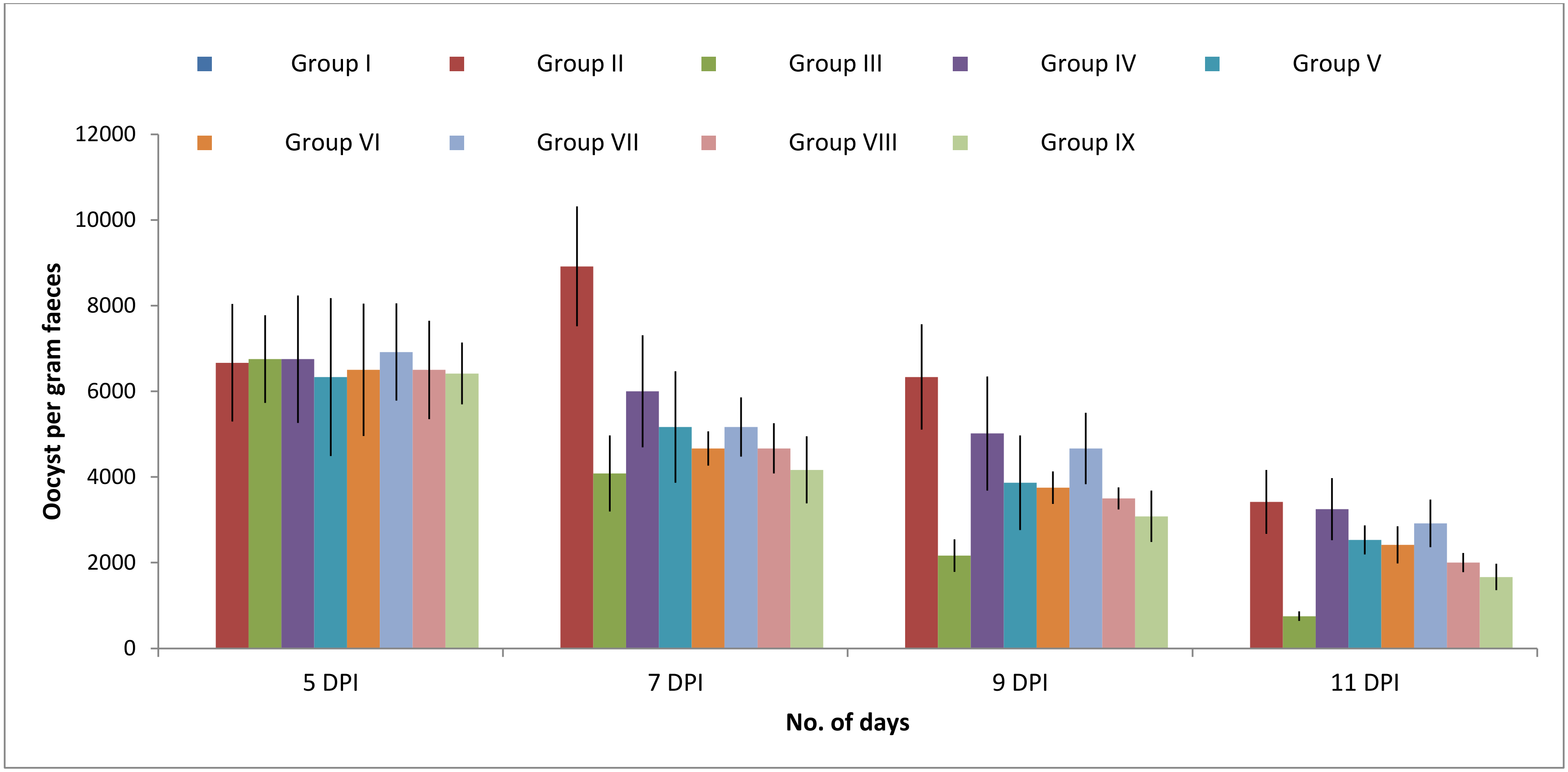


Fig 24: *Cryptosporidium* spp. oocyst count in mice of control and different treatment groups at 5th, 7th, 9th and 11thday PI

4.7.4.2 Average Body weight

The body weight of the animals was significantly (p<0.05) affected in animals of group III to X on 5thday PI with significantly higher average body weight in all treated groups which ranged from19.32 ±1.687 to 22.355 ±0.672. The average body weight at day 7 post treatment being significantly (p<0.05) highest in group IV animals (24.370 ± 2.420) followed by group X (23.915 ±2.224), IX (23.57 ± 2.185), VII (23.01 ± 2.057), VI (22.82 ±1.823), VIII (22.330±3.078) and V (21.97 ± 1.727). The body weight also showed significant (p<0.05) periodic increase in group I and II irrespective of treatment. The result of the present study revealed that the treatment with synthetic curcumin (group VIII, IX and X) resulted in slightly higher body weight in mice than those which were administered with same dose of *Curcuma longa* (group V, VI and VII). The rate of average body weight increase was however not significantly (p<0.05) affected with the treatment. The same has been shown in Table 31 and Fig 25.

4.7.5 Clinical signs

Observable clinical signs were seen in mice of groups III, V, VI, VII, VIII and IX respectively. No clinical signs could be seen in mice of group I, II, IV and X.

Diarrhoea was first seen in group III mice on 5th DPI which became profuse on day 7th and animals were having loose faeces even on 11th DPI. Similar, trend and severity of diarrhoea was also seen in mice of group V, VI, VII, VIII and IX.

4.7.6 Patho-morphological observations

4.7.6.1 Gross lesions

Histopathological observations was done on 12 mice which were euthanized humanely by ether on day 11 of post infection and remaining 8 mice were sacrificed on 20th day post infection. Upon necropsy, in group III animals showed severe congestion of serosa and mucosa along with catarrhal exudate in the lumen of intestinal tract. In mice of Group V and VIII, similar pattern of clinical disease was seen. Also, intestinal tract didn’t show any gross abnormalities except for mild congestion in some animals of group VI,

Table 31: Average body weight (gms) of mice in control and different treatment groups at different intervals

	Period				
Groups	**0 DPI**	**5th DPI (0 DPT)**	**7th DPI (2 DPT)**	**9th DPI (4 DPT)**	**11th DPI (6 DPT)**
Group I	20.43 ± 0.583[a]	21.69±0.666[a,b]	22.51 ± 0.688[b,c]	23.22 ± 0.471[b,c]	24.78 ± 0.710[c]
Group II	19.92 ± 0.785 [a]	21.17 ±0.982[a,b]	22.07 ± 1.027[a,b]	22.80 ± 1.113[a,b]	24.07 ± 0.847[b]
Group III	19.95 ± 0.533	19.32 ±1.687	18.81 ± 0.959	18.25 ± 1.745	18.31 ± 1.96
Group IV	22.010 ± 1.050	22.835 ± 0.879	23.280 ± 2.106	23.900 ±2.549	24.370 ± 2.420
Group V	19.75 ± 0.603	20.380 ± 0.731	20.93 ± 1.034	21.32 ± 1.759	21.97 ± 1.727
Group VI	20.65 ± 0.658	21.04 ± 0.874	21.570 ± 1.372	21.91 ± 1.568	22.82 ±1.823
Group VII	20.92 ± 0.554	21.720± 0.695	22.47 ± 1.115	22.92 ± 1.904	23.01 ± 2.057
Group VIII	20.79 ± 0.519	21.030 ± 0.975	21.470 ±1.420	21.900± 2.554	22.330±3.078
Group IX	21.13 ±0.736	21.91 ± 0.993	22.55 ± 1.840	22.94 ±1.938	23.57 ± 2.185
Group X	21.850 ±0.678	22.355 ±0.672	22.970 ±1.881	23.360 ±2.166	23.915 ±2.224

[abc]Means bearing different superscripts within a row differ significant

Where, DPI- Days post infection and DPT- Days post treatment

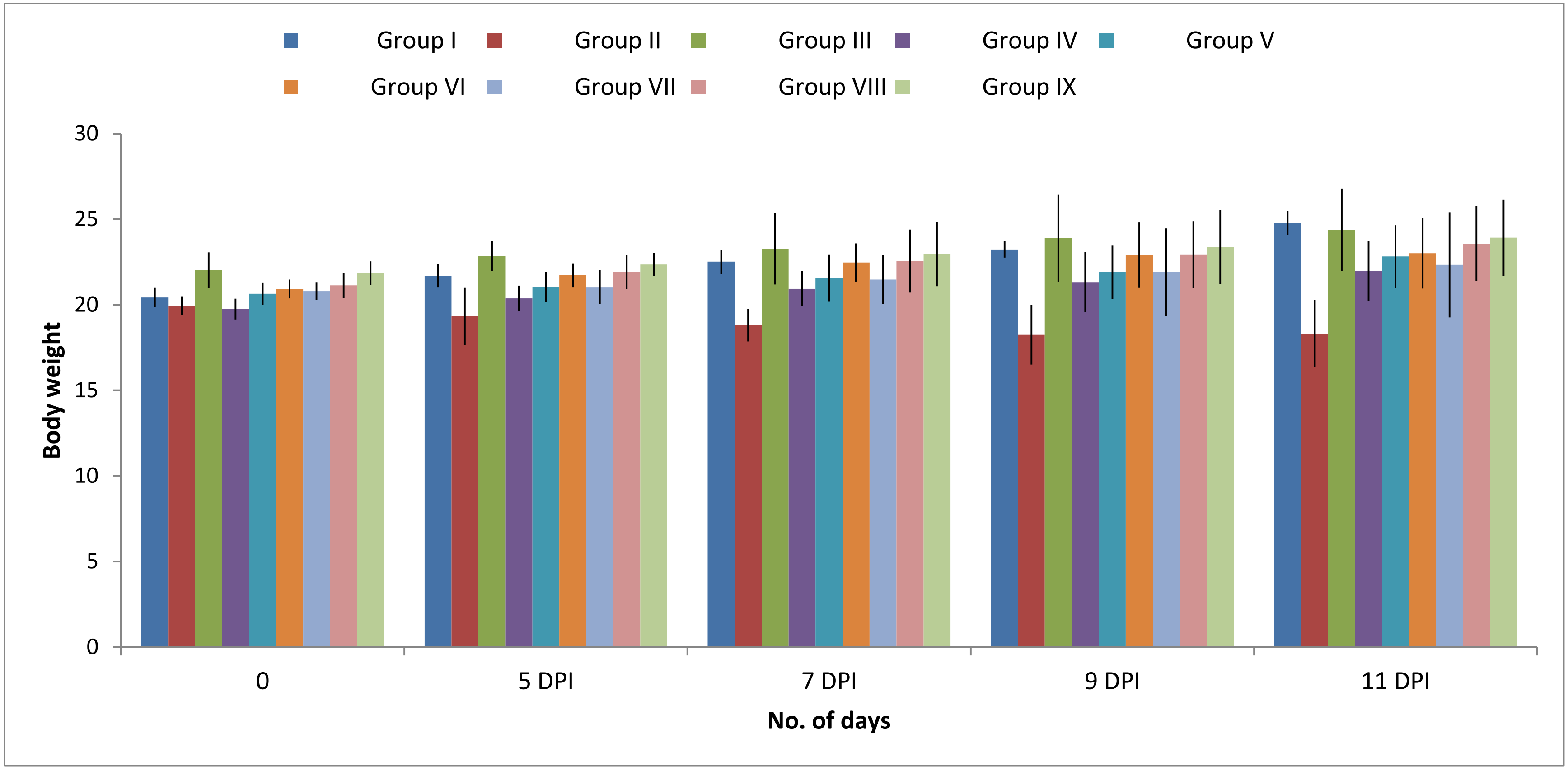


Fig 25: Average body weight (gms) of mice in control and different treatment groups at 0, 5th, 7th, 9th and 11thday PI

VII and IX. On the contrary, intestinal tract of group IV and X animals didn't reveal any pathological changes upon gross examination.

4.7.6.2 Histopathological changes

On day 11 PI, in group I and II animals, the intestinal sections didn't reveal any microscopic abnormalities and intestinal villi were lined by intact columnar epithelial cells (Plate 18 and 19).

In contrast, in group III, severe disruption of intestinal histology was seen. The villi were severely stunted and fused. There was necrosis and desquamation of villi into the intestinal lumen. Lamina propria and submucosa were severely infiltrated by inflammatory cells. Also, oocysts were seen adhering to the enterocytes of the villi (Plate 20 and 21). Severe thickening of wall of intestine was seen wherein the serosa was markedly congested and infiltrated by inflammatory cells (Plate 22).

Mice in group IV showed mild inflammation of lamina propria but no oocysts were seen attached to the villi. Also, intestines appeared normal as compared to the intestinal sections of animals in group V, VI, VII, VIII, IX and X (Plate 23).

On the other hand, in group V severe enteritis was seen with fragmentation and necrosis of intestinal villi and infiltration of inflammatory cells in lamina propria (Plate 24). Also, oocysts adhering to villi could be seen (Plate 25).

In group VI and VII mice, enteritis was seen which was less severe as compared to the lesions seen in group V and group III (Plate 26).

Occasionally oocysts were seen attached to the enterocytes in mice of group VIII (Plate 27). Lesions of comparable severity with mild enteritis with that of group IX was seen (Plate 28). This mild enteritis was characterized by slight desquamation of intestinal epithelial and infiltration of few inflammatory cells in lamina propria seen in group VIII and IX.

In group X, animals had minimal intestinal lesions upon microscopic examination which were nearly comparable to the animals in group IV (Plate 29).

On day 20 PI, group I and II did not show any microscopic lesion of any pathological significance in intestinal sections. In group III, mild enteritis with desquamation, sloughing of intestinal villi was seen (plate 30). Whereas animals in all treated groups (IV,V,VI,VII,VIII,IX and X) showed no appreciable gross lesion but intestinal sections revealed mild enteritis characterised by sloughing of intestinal villi and no oocyst attached to the villi (Plate 31).

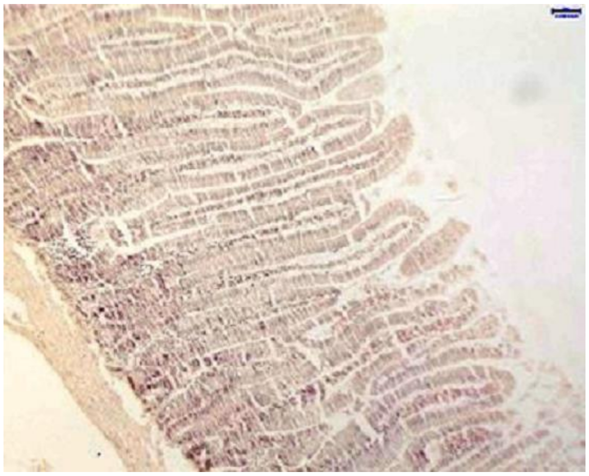


Plate 18: Group I (control): Normal intestinal epithelium. H&E×100

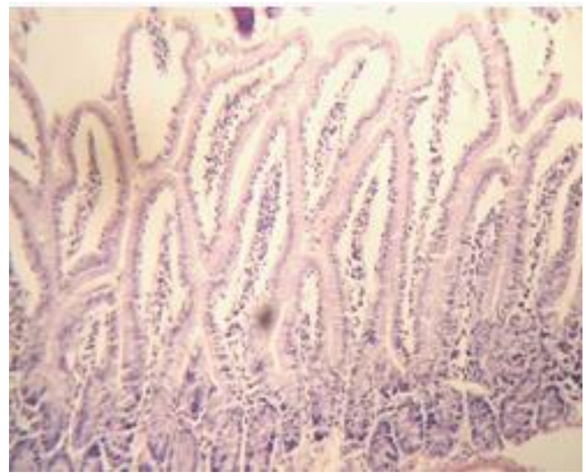


Plate 19: Group II (immunosupressed): Normal intestinal villi lined by intact columnar epithelial cells. H&E×100

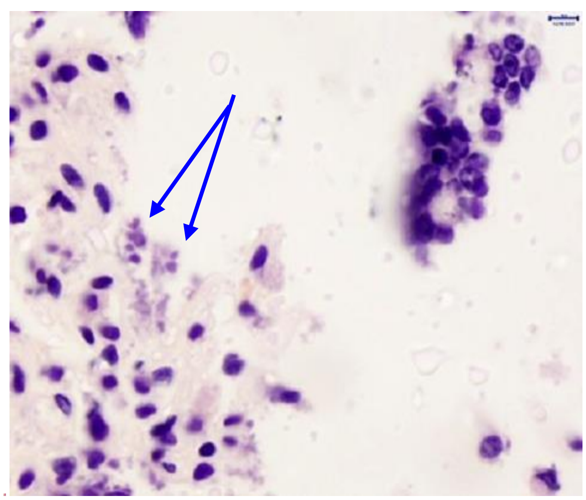


Plate 20: Group III at 7 DPI (infected): Presence of few parasitic stages (arrow) of *C. parvum*. H&E×1000

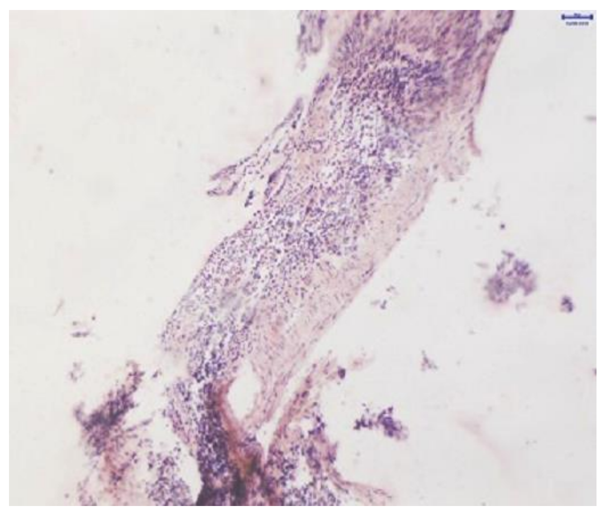


Plate 21: Group III at 7 DPI (infected): Necrosis and desquamation of villi into the intestinal lumen. H&E×100

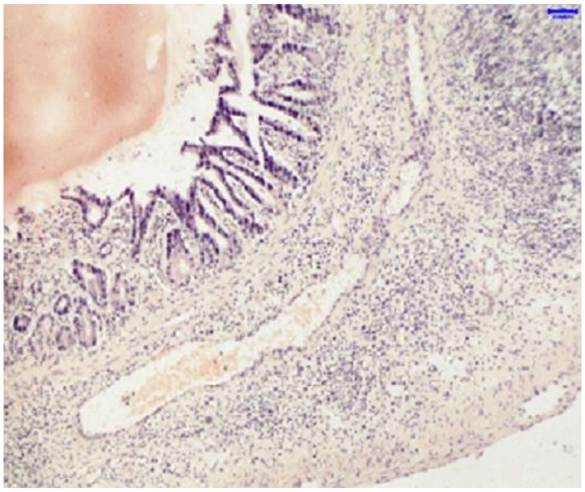


Plate 22: Group III at 7 DPI (infected): Severe thickening of wall of intestine and infiltrated by inflammatory cells. H&E×100

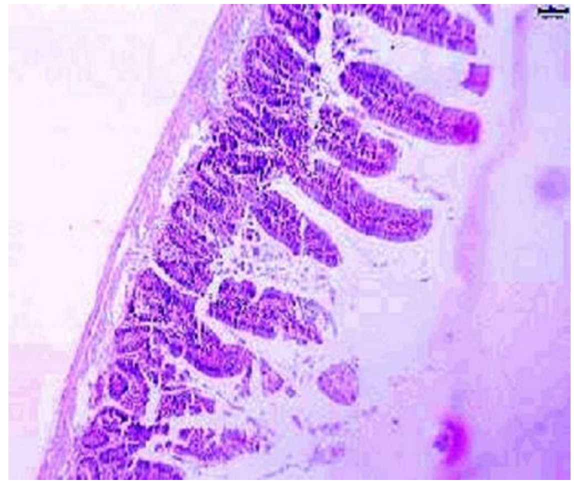


Plate 23: Group IV at 11 DPI (nitazoxanide): Mild inflammation of lamina propria. H&E×100

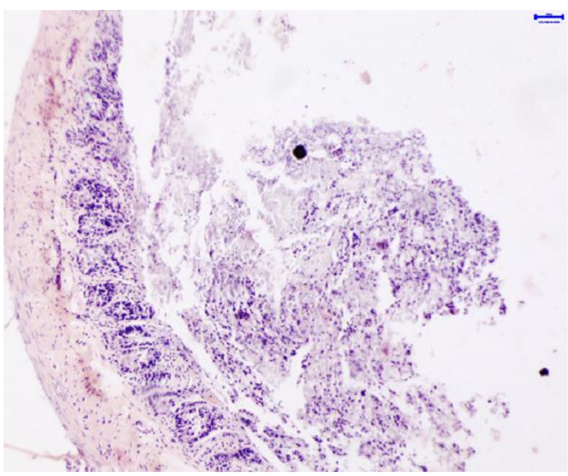


Plate 24: Group V at 11 DPI (*C. longa* @ 4mg/kg b.wt): Severe enteritis with infiltration of inflammatory cells. H&E×100

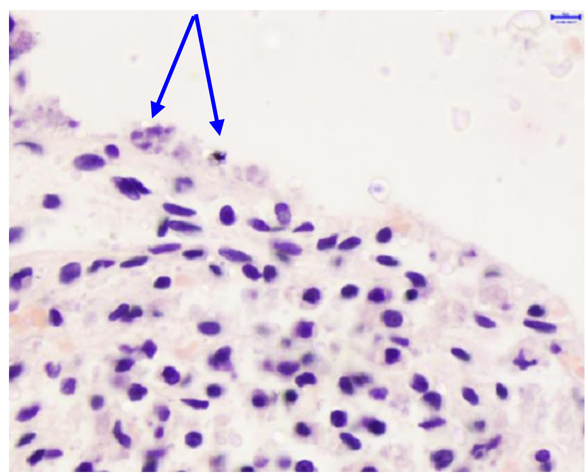


Plate 25: Group V at 11 DPI (*C. longa* @ 4mg/kg b.wt): Oocysts of *C. parvum* adhering superficially to the intestinal villi. H&E×1000

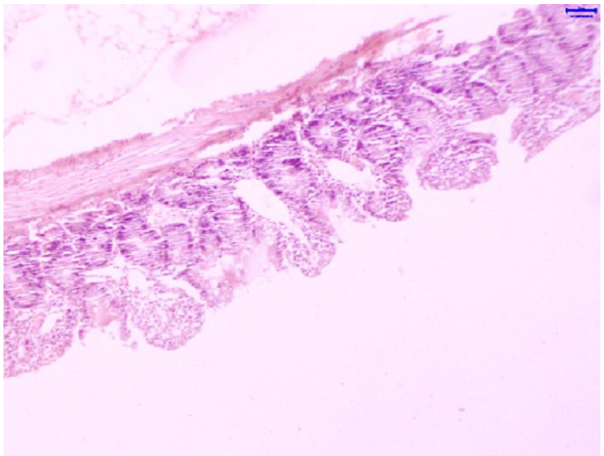


Plate 26: Group VI and VII at 11 DPI (*C. longa* @ 6 and 8 mg/kg b.wt): Less severe enteritis. H&E×100

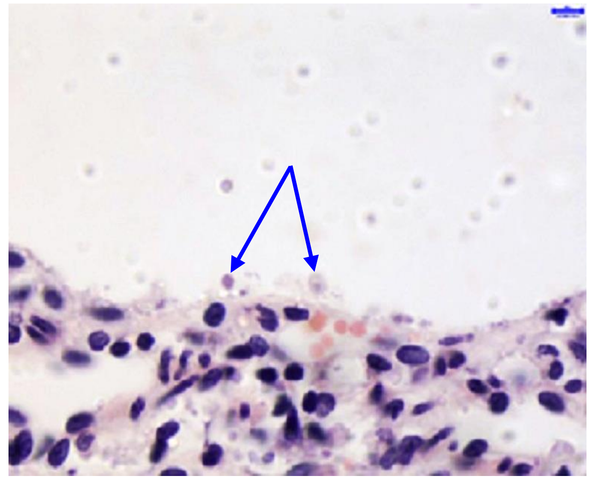


Plate 27: Group VIII at 11 DPI (Curcumin @ 4mg/kg b.wt): Oocysts of *C. parvum* adhere to enterocytes. H&E×1000

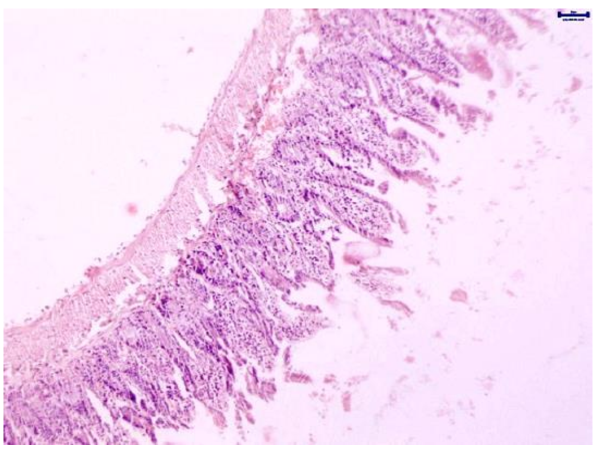


Plate 28: Group IX at 11 DPI (Curcumin @ 6mg/kg b.wt): Less severe lesions with mild enteritis. H&E×100

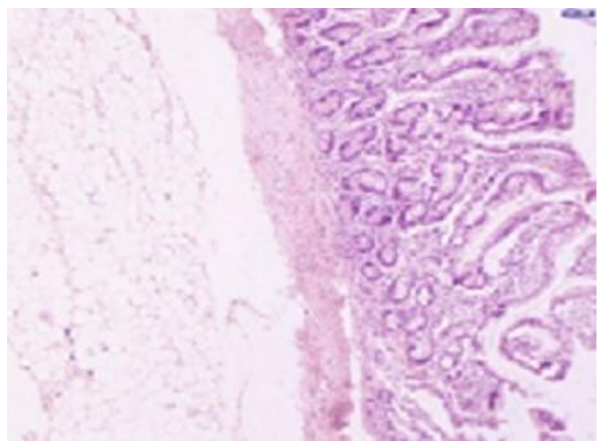


Plate 29: Group X at 11 DPI (Curcumin @ 8mg/kg b.wt): Minimal intestinal lesions. H&E×100

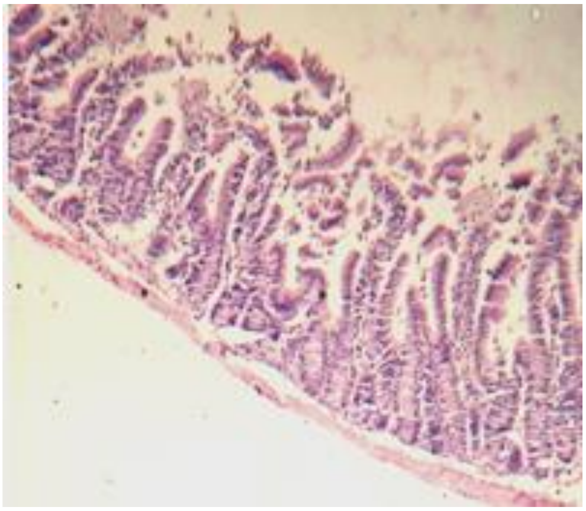


Plate 30: Group III at 20 DPI: Mild enteritis, desquamation, sloughing of intestinal villi. H&E×100

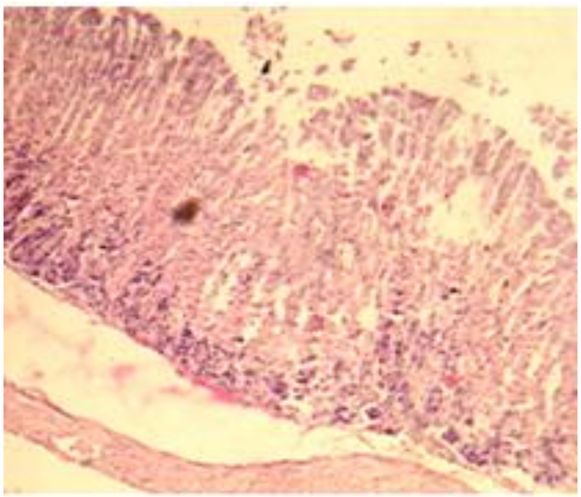


Plate 31: Group IV to X at 20 DPI: Mild enteritis and no oocysts adhere to the intestinal villi. H&E×100

Chapter-5

Discussion

CHAPTER–V

DISCUSSION

Parasitic zoonotic diseases are prevalent in India, including the northeastern states. Proper epidemological data are lacking from our country on zoonotic parasitic diseases, and newer diseases are emerging in the current scenario (Das *et al*., 2014). During the last 20 years, 70% of the emerging diseases have been found to be zoonotic in nature and about 300 diseases are common to man and animals (Thapliyal, 1999).

Cryptosporidium spp. and *Giardia intestinalis* are protozoan parasites which infect a wide host range, including various livestock and humans (Feng and Xiao, 2011) and is one of the most common intestinal parasites in livestock. Because of the impact on the socio economic development, especially in developing countries, *Giardia* spp. was included in the "Neglected Disease Initiative" of the World Health Organization (Savioli *et al*., 2006). Being a zoonotic disease, in Asia, Africa and Latin America, about 200 million people have symptomatic giardiosis with some 500,000 new cases reported each year.

The genus *Cryptosporidium*, which constantly causes outbreaks in humans and also affects a wide range of mammalian animals, is one of the major zoonotic problem in healthy individuals, domesticated animals and also immunosuppressed patients (Checkley *et al*., 2015; Shahiduzzaman and Daugschies, 2012). The recognition of *Cryptosporidium* spp. as a public health problem came in 1993 with the world's largest recorded outbreak of water-borne disease in Milawaukee, Winconsin, USA (Mckenzie *et al*., 1994). *Cryptosporidium* was discovered 100 years back but till date no effective curative drug or vaccine against either human or animal cryptosporidiosis has been developed and in spite of the prominent developments in different aspects of the parasite, its proper treatment still exists as an unresolved issue (Ryan *et al*., 2016 and Ward, 2017).

Molecular diagnostic tests to detect parasites have been developed in last decade. Their specificity and sensitivity have gradually increased to detect the parasites that were previously difficult to diagnose using conventional techniques (Tavares *et al*., 2011).

Cryptosporidium is notoriously diverged from other apicomplexan parasites by some peculiarities in biology, distinct structure, and biochemical composition that could be related to natural drug resistance. Over 200 chemotherapeutic agents have been evaluated against *Cryptosporidium* and some of them are active against other apicomplexa, but none of them is able to clear the parasite from the host (Shahiduzzaman *et al*., 2009).

The study included the research work done to establish the prevalence of *Cryptosporidium* and *Giardia* spp. infection in cattle calves of Jammu region. The prevalence studies were based on identification of *Cryptosporidium* oocysts and *Giardia* cysts in faecal samples by diethyl ether sedimentation technique followed by conventional techniques (modified Ziehl-Neelsen staining technique and iodine wet mount technique). Further, to establish sensitivity and specificity of various diagnostic techniques, 75 faecal samples (25 samples from each age group viz. <1 month, 1-6 months and > 6 months) were randomly selected from total 614 faecal samples collected from Jammu region. All the 75 samples were subjected to five techniques. Along direct smear techniques, SFSS (Sheather's floatation sedimentation staining), ZNSFST (zinc sulphate floatation Sedimentation technique), DFAT (direct fluorescent antibody test), nucleic acid based (PCR) technique was also carried out. Sensitivity and specificity of various techniques of faecal samples were also made considering 100 per cent sensitivity and specificity of PCR. Statistically various risk factors of the infection were also analysed. During the study, a total of 614 faecal samples were collected from cattle calves of Jammu region out of which 23.45% were found positive for *Cryptosporidium* spp. infection and 7.00% were found to be positive for *Giardia* infection. The rate of prevalence of infection in cattle as recorded by other workers in India agreed to the present finding. However, difference in rate of infection was also recorded which could be due to application of more sensitive and specific PCR technique. In the survey of infection adopting modified acid fast staining of faecal smears, Dubey *et al.* (1992) recorded 17.7 per cent infection whereas, Jeyabal and Ray (2005) recorded 35.5 per cent infection in bovine calves from Izatnagar, UP. In the same manner a prevalence rate of 11-23 per cent was recorded in the Eastern part of India by Chattopadhyay *et al.* (2000) and Das *et al.* (2004b). Similarly Kumar *et al.* (2004) reported 25% infection from south

India. PCR based diagnosis has reported higher prevalence of *Cryptosporidium* spp. Paul *et al.* (2008) reported 30.20 % infection from neonatal bovine calves across the different regions of India. Singla *et al.* (2013) from five organized dairy farms of Ludhiana (Punjab) reported *Cryptosporidium* positivity in 23.52% and 12.50% respectively in buffalo and cattle calves by lateral flow immunoassay, whereas by modified Ziehl-Neelsen stain, it was 17.65% and 6.25% respectively. On the other hand Bhat *et al.*, (2014) from Ludhiana district (Punjab) reported 25.0% and 47.0% positivity for the oocyst of *Cryptosporidium* spp. by direct faecal smear technique and nested PCR, respectively. In contrary, Paul *et al.* (2008) detected only 12.5 per cent infection in 6-24 month old bovines in the state of Andhra Pradesh by PCR assay, which was due to availability of less number of samples for examination. Further Khan *et al.* (2010) reported lower prevalence of *Cryptosporidium* in cattle calves of Kolkata as 11.7% through nested PCR, whereas higher prevalence (39.65%) of *Cryptosporidium* spp. was reported using nested PCR of 18S SSU rRNA from South India by Venu *et al.* (2012). The study revealed that the infection was highest in Puducherry (86.67%) and lowest in Kerala (17.65%). The prevalence of 48.5% *Cryptosporidium* infection in cattle calves examined from dairy farms of Jammu region in the present study varies from earlier study carried by Yadav (2010) from Jammu district only. In the previous study, although an overall prevalence was 26.52%, but it was 48.39% in organized farms as compared to 21.31% in unorganized farms or individual animal units. It has also been observed that overall prevalence of cryptosporidiosis can vary remarkably depending on the age of calves (Santin *et al.*, 2004, 2008) and the detection method used because molecular methods are more sensitive and accurate than microscopic methods.

The results of the present study revealed almost similar prevalence of *Cryptosporidium* spp. infection was found in four districts (Samba, Udhampur, Kathua and Rajouri) of Jammu region. The difference can be attributed to demographic location of the districts. But among animals of all four districts, slightly higher prevalence was observed in Samba district which is located on plain irrigated belt with less area for open grazing for animals. Therefore, animals are mostly kept in sheds which results in more close contact with more chances of spread of infection. Kathua district shares the border with Samba and almost similar prevalence was found in this area. On contrary, Udhampur and Rajouri districts blocks are located mainly on non-irrigated foot hills i.e. kandi area where farming is rain dependent and animals are kept on open grazing which reduce chances of direct contact between animals. It has been already established that calves kept on pasture throughout the year had lower probability of *Cryptosporidium* infection than kept in cow shed (Kvac *et al.,* 2006). Also, similar block wise prevalence of *Cryptoporidium* spp. infection was recorded by Yadav (2010) with difference was estimated on the basis of demographic locations of Jammu region.

The higher prevalence of *Cryptosporidium* spp. in cattle calves has already been reported from India (Paul *et al.,* 2008) and other countries (Becher *et al.,* 2004; Santin *et al.,* 2004; Geurden *et al.,* 2007). Moreover, in the earlier study, animals upto 6 months of age were screened using three diagnostic technique viz. mZN staining, ELISA and PCR, whereas in the present study, cattle calves of three age groups i.e <1month, 1-6 month and > 6 month were screened based on mZN stain for prevalence study. It has also been observed that overall prevalence of cryptosporidiosis can vary remarkably depending on the age of calves (Santin *et al.,* 2004, 2008) and the detection method used because molecular methods are more sensitive and accurate than microscopic methods. Although cryptosporidiosis in cattle has been reported throughout the globe, but there are very few reports regarding the prevalence and risk factors of *Cryptosporidium* spp. in cattle calves.

The result of age wise distribution in the current study demonstrated that the majority of *Cryptosporidium* spp. infection was highest in cattle calves upto one month of age group. The diarrhoeic animals of this age showed very high prevalence of *Cryptosporidium* infection as compared to non diarrhoeic animals. The constant association of diarrhoea and presence of oocyst of *Cryptosporidium* in the faeces has been recorded by other workers (Lise *et al.,* 2005; Roy *et al.,* 2006; Singh *et al.,* 2006; Kaur and Kaur, 2008; Venu *et al.,* 2013 and Bhat *et al.,* 2014). Occurrence of *Cryptosporidium* in clinically asymptomatic animals indicated that the particular age group of animals might be reservoir for the parasites. This suggests that very young calves are more susceptible and vulnerable for cryptosporidiosis and managemental practices at the farms are needed to minimize this infection. Similarly, Quilez *et al*. (1996), Bendali *et al*. (1999), Del cocco *et al.* (2008), Helmy *et al.* (2013), Rieux *et al.*

(2013), Mirzai *et al*. (2014) and Adamu *et al*. (2015) reported that calves under 1 month of age are frequently infected with *Cryptosporidium* spp. which results in economic losses. The present observation also supports the earlier finding of Kumar *et al.* (2004), Shobhamani and Singari (2006), Roy *et al.* (2010), Khan *et al*. (2010), Venu *et al.* (2012) and Bhat *et al*. (2014), Sivajothi *et al*. (2014), Joute *et al*. (2016) and Hingole *et al*. (2017) from India. The rate of prevalence decreased proportionally with the increase in age of the target animals and lowest prevalence was recorded in adult animals of above 2 years of age. Contrary to the present study Maddox-Hyttel *et al*. (2006) recorded higher prevalence in older calves. Fayer *et al.* (1998) and Castro-Hermida *et al.* (2002) stated that prevalence of cryptosporidiosis is underestimated because of the low number of samples taken during the preweaning period. Similarly, Villacorta *et al.* (1991) recorded prevalence of 93.0% in 3-6 days old calves selected randomly and examined twice weekly for one month. In contrast, when only one or two faecal samples per calf were examined during the preweaning period, less than 30.0% of the calves were found to have oocysts in their faeces (Maldonado-Camargo *et al.*, 1998). Also, as per age, the infection rate were 12.5% in pre-weaned calves, 10.4% in post-weaned calves, 22.1% in heifers and 10.7% in adults.

In the present study, the prevalence of *Cryptosporidium* with the sex of the host showed higher infection in male calves as (46.34%; diarrhoeic, 30.61%; non-diarrhoeic) as compared to females (22.81%; diarrhoeic, 12.71%; non-diarrhoeic). The present results are in accordance with the findings of Paul *et al.* (2008) but are in contrary to Shobhamani *et al.* (2006), Prakash *et al.* (2009) and Singh *et al*. (2018) who observed higher prevalence in female calves. In India, the male calves are generally neglected as present day agriculture has become more mechanized, so males are not used for draught purpose. Further, due to religious sentiments their slaughter is prohibited in many states. All these reasons predispose male bovine calves to poor feeding and management, which in turn lowers the immunity and predispose them to various pathogens of biological origin.

Association of mucus, blood and normal faeces of *Cryptosporidium* spp. infection has been reported in the present study, where prevalence of *Cryptosporidium* infection in the faeces with mucus was higher (37.33%) than those having no mucus and blood (17.79%) and lowest prevalence in faeces with blood (11.76%). Similarly Del Coco *et al.* (2008) reported that 37.5% of diarrhoeic faeces without blood were positive, showing presence of mucus of 83.3% and 66.7% of samples with mucus showed an average of 10 oocyst/field. The co-existence of *C. parvum*, rotavirus, coronavirus and *Salmonella* in calves is already on records (de la Fuente, 1999) and these factors may also be responsible for association of blood and *Cryptosporidium* infection as observed in few calves in present study.

The season wise prevalence of *Cryptosporidium* spp. in cattle calves during the study had significant effect. The overall prevalence of infection was highest in winter (34.65%) followed by post monsoon (26.57%), monsoon (22.06%) and summer (8.55%). This data suggests that the prevalence of infection of *Cryptosporidium* is not only related to the presence of animals at risk but is also related to the presence of suitable climatic condition for viability and spread of the parasite. In winter, the temperature in study area is suitable for viability and survival of *Cryptosporidium* oocysts. Similar findings were recorded by many workers (Tzipori *et al.*, 1983; Lefay *et al.*, 2000; El-Khodrey and Osman, 2008, Hingole *et al*., 2017). The present findings are also supported by observation of Fayer *et al.* (1998) and Jenkins *et al.* (2003) who observed that *Cryptosporidium* oocysts can remain viable and infective for 4-5 months at 5 to 20^0c. Garber *et al.* (1994) attributed the high prevalence of cryptosporidiosis in winter (December to February) to presence of large number of calves at risk as a result of concentration of calving in winter months. Further, it was observed that animals in organized farms were overcrowded in winter months, resulting in easy availability of infective *Cryptosporidium* oocysts to susceptible calves. The overcrowding has also been suggested for increased prevalence of *Cryptosporidium* by many workers (Garber *et al.*, 1994; Quigley *et al.*, 1994 and Mohammed *et al.*, 1999). They reported that higher the density of animals, greater the chances of infection and consequently the higher prevalence. On contrary, in unorganized farms the higher infection was recorded in monsoon months (July to September) and this finding is in agreement with other reports from India (Roy *et al.*, 2006, Paul *et al.*, 2008 and Bhat *et al*., 2012) and abroad (Chai *et al.*, 2001). The calves were at high risk of contracting infection by *Cryptosporidium* spp.

during monsoon months because of greater contact with source of infection. As these animals drink water from canals and ponds and during rains faecal material run off to these water bodies and increase the contact between infective oocysts and host. The earlier study of Jammu district by Yadav (2010) reported that in organized farms like present study highest prevalence was in winter season whereas in unorganized farms it was in monsoon season. Wade *et al.* (2000) contrary to the present report observed that seasons do not affect the prevalence of cryptosporidiosis in bovine calves. On the other hand, Castro-Hermida *et al*. (2002) suggested that the seasonal effects can only be evaluated correctly if studies are made over several consecutive years. Similarly, Brankston *et al*. (2018) reported that the peak of cryptosporidiosis in cattle were observed in late winter to early spring.

The sensitivity and specificity of the various diagnostic techniques (DFAT, SFSS, DESS, DFSS and PCR) were carried out in the present study assuming PCR as 100 percent sensitive and specific for diagnosis of cattle cryptosporidiosis. The highest prevalence as per diagnostic method by (PCR) polymerase chain reaction (46.66%) followed by (DFAT) Direct fluorescent antibody test (42.66%), (SFSS) Sheather's floatation sedimentation staining (40.00%), (DESS), Diethyl ether sedimentation staining (34.66%) and least positivity was recorded by (DFSS) direct faecal smear staining (36%) technique. The probable reason for different prevalence rate by diagnostic methods may be because of variable detection limit of oocysts present in the faecal samples. Anusz *et al.* (1990) reported a detection limit of 10^6 oocysts per ml of faeces using the Kinyoun modification of mZN on unconcentrated faecal smears. Variations in faecal consistency influence the ease of detection, with oocysts being more easily detected in watery, diarrheal specimens than from formed stool specimens (Weber *et al.*, 1991). It has been found that PCR can detect as low as 1 oocyst per gm of faecal sample (Xiao *et al.*, 1999) and that probably explains detection of high number of samples positive for *Cryptosporidium* spp. oocysts by PCR. On contrary, in a study by Leetz *et al*. (2007) concluded that PCR detection protocols for *Cryptosporidium* are not capable of detecting all isolates particularly in samples with low number of oocysts. Also, the present concluded the study on sensitivity and specificity of DFAT technique in relation to PCR was 91.4% and 97.5% respectively. On contrary Mirhashemi *et al*. (2015) from Ireland combined the PCR and DFAT techniques to screen livestock to maximize the chance of *Cryptosporidium* detection in asymptomatic cases. Also DFAT was found to be more specific test while PCR is most sensitive test. In addition, the sensitivity and specificity of SFSS was 85.7% and 95.0% and that of DESS in relation to PCR technique was 74.3% and 92.5%. On contrary, Paul *et al*. (2009) studied by considering the PCR assay 100 % sensitive and specific but SFSS exhibited highest sensitivity (82.6%) and specificity (93.76%) among the conventional techniques, SFSS and DESS were far less time and money consuming and the most economical (15 cents per sample) followed by NSSS (19.6 cents per sample), SF (23.6 cents per sample) and SFSS (33.9 cents per sample) than PCR technique. On the other hand modified Ziehl-Neelsen staining technique (Henricksen and Pohlenz, 1981) was found to be very useful method for microscopical detection of the oocysts in diarrhoeic animals. The oocysts appeared as pink spherical bodies against a green background of malachite green/methylene blue. The wall of *Cryptosporidium* spp. oocyst was acid fast i.e. it retained the colour of carbol fuchsin even after decolouration by 10 per cent sulphuric acid. The faecal debris, bacteria and yeast cells could not retain the colour of carbol fuchsin and took up the counter stain green/blue. In accordance to the previous studies, present study revealed 60 % sensitivity and 90.00 % specificity of DFSS technique (mZN) in relation to PCR. The present findings also supported the observations made by Fayer *et al.* (1997). A faint staining and distorted morphology of organisms has been suggested to be responsible of the lower sensitivity of the acid fast stains, especially in those samples with a low number of oocysts (Mtambo *et al.,* 1992). In contrast to other studies, it was also observed that prevalence results could have been higher if mZN technique would have been used after concentration of *Cryptosporidium* oocyst from faeces. Arrowood and Sterling (1989) have also reported the problems of false-positive results by using acid fast staining technique. Some fungal spores have been found to be acid- fast, but these can be distinguished by size alone (Casemore, 1991). On contrary, no false-positive results were recorded by using the mZN staining technique, in accordance with previous surveys concluding that the acid fast stains are 100% specific (Garcia *et al.,* 1992 and Quilez *et al.,* 1996). Thus, mZN staining must be included in routine diagnostic procedures for diagnosis of *Cryptosporidium* oocysts as a causative agent of diarrhoea in clinically symptomatic animals as being practiced in many other countries (Webster *et al.,* 1996).

The Kappa statistics is a measure of agreement of diagnostic tests with the agreement owing to chance alone removed. In the present study, there was perfect agreement between PCR vs SFSS, DFAT with kappa values 0.81 and 0.89 as compared to PCR vs DESS with kappa value 0.67 as substantial agreement and PCR vs DFS with kappa value 0.50 as moderate agreement. The current study also revealed that overall agreement between PCR vs DESS, SFSS and DFAT with Po value was 0.84, 0.90, 0.94 as compared to agreement between PCR vs DFS with Po value 0.76. Reasons for variation between diagnostic tests are often attributed to the measurement of different antigens or forms of pathogens, or to the measurement of antigenically or morphologically similar organisms (Mc Cluskey *et al.,* 1995). As per Mirhashemia *et al*. (2015) there is an association between the results obtained from both DFAT and PCR in cattle and sheep at 0.05 level of significance and kinyoun's and DFAT also showed to be associated with each other.

The number of *Cryptosporidium* spp. oocysts present in the faecal smear (stained by mZN technique) of cattle calves was assessed in Jammu region. In the present study, it was observed that more number of male cattle calves with mucus in there faeces especially of <1month of age were having highest (3+) oocyst intensity in acid fast smear particularly in winter season demonstrates that these animals are important from an epidemiological point of view and play an important source of contamination to water and soil of the area, which has resulted in several water borne outbreaks of cryptosporidiosis in man and animals (Fayer *et al.,* 2000). The results are also in accordance with observation of Singh *et al.* (2006) and Del Coco *et al.* (2008).

The present study determines the various genotypes of *Cryptosporidium* which were differentiated by PCR-RFLP technique targeting the amplified 830bp fragments of SSU rRNA gene in last decade. Screening of the literature published throughout the globe in last decade revealed that there is widespread use of the SSU rRNA gene in *Cryptosporidium* genotyping (Khan *et al.,* 2010; Imre *et al.,* 2011; Helmy *et al.,* 2013; Murakoshi *et al.,* 2013; Rieux *et al.,* 2013 Bhat *et al.,* 2014 and Mirzai *et al.,* 2014). It 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. Earlier, genotyping tool based on the oocyst wall protein (COWP) gene have been used but it only amplify DNA of *C. parvum*, *C. hominis*, *C. meleagridis* and species/genotype closely related to *C. parvum* and have been used in limited reports. Various studies that have used this tool have usually showed fewer *Cryptosporidium* species and genotype than expected (Meamar *et al.*, 2007; Wolska-Kusiners *et al.*, 2007; Duranti *et al.*, 2008 and Giangaspero *et al.*, 2009).

For genetic characterization the amplified 830 bp nested PCR product from all the 120 samples (40 samples per age group) randomly selected from 144 samples of the present study were subjected to digestion with the three restriction enzymes *Ssp*I, *Vsp*I and *Mbo*II. Similarly, Xiao (2010), Venu *et al.* (2012) and Rieux *et al.* (2013) observed that nested PCR protocol was used to amplify 830 bp fragments of SSUrRNA gene and the different species of *Cryptosporidium* can be distinguished by three restriction enzymes viz. *Ssp*I, *Vsp*I and *Mbo*II. In the present study three species of *Cryptosporidium* i.e. *C. parvum* and *C. andersoni* and *C. bovis* were recorded in different age groups of animals.

In the present study, PCR-RFLP with *Ssp*I restriction enzymes yielded two visible bands (449 bp and 267 bp) for *C. parvum*, one band (448bp) for *C. andersoni* and two bands (432 bp and 267 bp) for *C. bovis*. The similar findings have been reported in previous studies by Spano *et al.* (1997), Sulaiman *et al.* (1998), Xiao *et al*. (1999 and 2001) and Paul *et al.* (2009). In the present study, RFLP with *Vsp*I restriction enzyme yielded one visible band each for *C. parvum* (628 bp), *C. andersoni* (730 bp) and *C. bovis* (616 bp). Similarly, Das *et al*. (2011) identified two visible bands at 628 bp and 104 bp when nested PCR products of bovine *Cryptosporidium* strain was digested with *Vsp*I restricted enzyme. This result has similarity with the study of Xiao *et al.* (1999). On the other hand, Paul *et al*. (2009) showed two visible bands at 730 bp and 155 bp with *Vsp*I enzyme and isolated *C. andersoni*.

Till 2007, only two enzymes *Ssp*I and *Vsp*I were used for species differentiation of *Cryptosporidium*. Feng *et al*. (2007) for the first time used new restricted enzyme, *Mbo*II for the easy differentiation of the species of *Cryptosporidium*. The similar findings have been reported by Khan *et al*. (2010), Imre *et al.* (2011), Venu *et al*. (2012) and Helmy *et al*. (2013). The present study concluded that digestion of PCR product with *Mbo*II also yielded one band each for *C. parvum* (771 bp), *C. andersoni* (769 bp) and *C. bovis* (485 bp). Therefore, other bands which could not be observed in identification of *C. parvum*, *C. andersoni* and *C. bovis* but have been described in the literature by many researchers might have been washed during gel electrophoresis or the DNA ladder used during electrophoresis was of 100 bp size, so the unidentified bands which were below 100 bp could not be visualized.

PCR-RFLP analysis for *Cryptosporidium* species by nested PCR in the present study reported a total of 74.07% of *C. parvum* infection whereas, 16.66% were positive for *C. andersoni* and 9.25% were found to be positive for *C. bovis*, respectively. Cattle calves of very young age (<1 month) showed 100% positivity for *C. parvum* whereas, *C. andersoni* and *C. bovis* was not observed in any of the animal of this age. In contrast, in older animals higher infection (55.55%) was observed by *C. andersoni* species as compared to other species.

Four species of *Cryptosporidium* viz. *C. parvum*, *C. bovis*, *C. rynae* and *C. andersoni* usually infect cattle. But, in the present study only *C. parvum*, *C. andersoni* and *C. bovis* were reported to infect cattle calves of Jammu region. The present findings are in accordance with the observation of Venu *et al.* (2012) from South India where examination of cattle calves upto 6 months of age reported that *C. andersoni* was chiefly observed in Tamil Nadu, Karnataka and Puducherry. However, *C. parvum* and *C. bovis* were noted at Gannavaram of Andhra Pradesh and Tuticorin of Tamil Nadu, respectively. The study highlighted that the prevalence of a *Cryptosporidium* genotype varies in a definite geographical region. On contrast many studies throughout the world has recorded observation of all the three species of *Cryptosporidium* in the animals of a defined geographical area (Silverlas *et al.*, 2010, Follet *et al.*, 2011, Imre *et al.*, 2011, Helmy *et al.*, 2013, Rieux *et al.*, 2013, Amer *et al.*, 2013 and Mirzai *et al.*, 2014)

The findings of the present study revealed that *C. parvum* was found to be the major species prevalent in cattle calves of Jammu region. Similarly other studies (Das *et* *al.*, 2004b, Roy *et al*., 2006, Paul *et al.*, 2008, Khan *et al.*, 2010 and Roy *et al.*, 2010) in India has reported the same but Venu *et al*. (2012) reported sporadic infection of *C. parvum* from Andhra Pradesh state only. The present study revealed that as per age, *C. parvum* was found to be the only species affecting very young animals and predominant genotype less than one month of age. Similarly, earlier studies in India (Das *et al.*, 2004b, Roy *et al.*, 2006, Paul *et al.*, 2008 and Khan *et al.*, 2010) and world (Geurden *et al.*, 2006, Coklin *et al.*, 2007, Feng *et al.*, 2007, Imre *et al.*, 2011, Helmy *et al.*, 2013) observed existence of *C. parvum* in young animals. Similarly, throughout the globe *C. parvum* has been reported as a major species affecting cattle (Elwin *et al.*, 2001, Santin *et al.*, 2004, Feng *et al.*, 2007, Keshavarz *et al.*, 2009, Imre *et al.*, 2011, Helmy *et al.*, 2013, Rieux *et al.*, 2013 and Mirzai *et al.*, 2014). The high prevalence of *C. parvum* in cattle of Jammu region indicates its zoonotic significance. The previous study carried by Yadav, (2010) from Jammu district has reported 40.0% and 76.47% prevalence of *C. parvum* by PCR-RFLP technique in HIV patients and children, respectively.

Cryptosporidium andersoni (formerly known as *Cryptosporidium muris* which is an established species in rodents now), the abomasal species which infects cattle, has been associated with gastritis, reduced milk yield and poor weight gain in adult cattle (Anderson, 1998 and Lindsay *et al*., 2000). *C. andersoni* was reported as minor species affecting bovines which are in accordance with Santin *et al.* (2004), Feltus *et al*. (2008) and Imre *et al.* (2011). In contrast Paul *et al.* (2009_a) and Venu *et al.* (2012) from India and Ondrackova *et al*. (2009) from Czech Republic reported *C. andersoni* as the predominant species affecting cattle. *C. andersoni* was observed to be predominant species infecting post weaned and adult animals. In India similar finding has earlier been reported by Paul *et al.* (2009) where an overall prevalence of 12.85% (45/350) infection of *C. andersoni* was reported in cattle of over 6 months of age. In another study from South India, Venu *et al.* (2012) observed 28.13% prevalence of *C. andersoni.* The present study observed that the prevalence of the infection decreased with increase in age which is in accordance with Paul *et al.* (2009) and may be attributed to the fact that the immunological competence of the host strengthened with increase in age and thereby supporting the infection to a latent stage. Similar findings have been reported from the United States (Fayer *et al.*, 2006 and Santin *et al.*, 2004). On contrary, including larger dairy farms in Kafr El Sheikh province in the Nile delta of Egypt (Amer *et al.*, 2013) *C. andersoni* was identified from very young calves (<6 weeks of age).

C. bovis are not much associated to host age related susceptibility and have been reported in all age groups (Feng *et al.*, 2007). In India, Khan *et al*. (2010) reported 11.7% prevalence of *C. bovis* in young calves. In other studies, post –weaned calves were mostly infected with *C. bovis* (Fayer, 2010). On contrary, studies in China, India, and Georgia western North Dakota have reported the *C. bovis* are most commonly found in pre-weaned calves (Feng *et al*., 2007 and Feltus *et al*., 2008). The present study observed that the prevalence of infection increases with increase in age which is in accordance with Robertson *et al*. (2014) and concluded that *C. bovis* are found to be in older as well as young calves. On another study (Wegayehu *et al*., 2016) in China concluded that *C. bovis* was the dominant species (70.0%) in calves younger than 1 month of age.

The other species of *Cryptosporidium* like *C. ryanae* although not reported in the present study, but have been found to infect cattle in India (Khan *et al*., 2010 and Venu *et al*., 2012) and throughout the globe (Imre *et al.*, 2011; Rieux *et al.*, 2013 and Helmey *et al.*, 2013).

For further confirmation of the species of *cryptosporidium*, nested PCR products identified as *C. parvum* by PCR-RFLP were sequenced. Sequence analysis of nested PCR products confirmed that they belong to *C. parvum*. Isolates from cattle calves of Jammu and Kathua district in Jammu region of *Cryptosporidium* spp. by 18S small subunit (SSU) rRNA gene was custom sequenced. The sequences generated in our study showed 99.64% similarity among themselves. Phylogenetic analysis revealed that the 18S small subunit (SSU) rRNA sequences of *C. parvum* isolates, identified in the present study, clustered, together with already published sequences of *C. parvum* from different parts of the world, which further confirmed presence of *C. parvum* in the isolates identified in this study. Phylogenetic relationship of the sequences were studied with the reference published sequence information available in GeneBank, as a final step for the confirmation of the species or genotype identified by PCR-RFLP by Fayer and Xiao (2008), who utilized the molecular taxonomy studies to identify multiple species of *Cryptosporidium*. Karanis *et al.* (2010) used phylogenetic analysis of the PCR amplified sequences of 18S rRNA gene to confirm 33 isolates of *Cryptosporidium* as that of *C. parvum*. Paul *et al*. (2008) have also reported *C. parvum* as the only species of *Cryptosporidium* prevalent in cattle calves in three different geographical regions of India and Roy *et al*. (2006) confirmed that *C. parvum* is responsible for bovine cryptosporidiosis in India. Infected calves can act as a great source of zoonotic cryptosporidiosis. Similarly, Keshavarz *et al*. (2009) analysed 51 positive samples which were sequenced using 18S SSU rRNA gene and confirmed *C. parvum* among 98 post weaned calves. Rieux *et al*. (2013) confirmed 32 isolates by amplifying an 830 bp segments of the 18S SSU rRNA gene and isolated *C. parvum* from one day old calf. Thus, the purpose of sequencing of the PCR amplified fragments of *Cryptosporidium* isolates followed by their alignment with the reported reference sequences of various species of the same organism is a sort of proof reading to confirm the genotypic results obtained from PCR-RFLP

To characterize the transmission dynamics and zoonotic potential of *C. parvum* in farm animals, especially calves, most of the studies employed gp60 sequence analysis and were done in industrialized nations. In the present study, the PCR-RFLP analysed products were further subjected for genotype analysis. The RFLP products were characterized by cloning and DNA sequencing, wherein the glycoprotein 60 (gp60 locus) gene was amplified and sequenced. DNA sequence analysis of 60 kDa glycoprotein (gp60) gene is one of the most popular subtyping tools used extensively in studies of the transmission of *Cryptosporidium parvum* infection in humans and ruminants. Broglia *et al.* (2008) and Quilez *et al.* (2008b) have successfully subtyped *C. parvum* isolates from dairy calves by sequence analysis of gp60 gene. In the present study, *C. parvum* yielded clear band at 850 base pairs, which further confirmed the presence of *Cryptosporidium parvum*.

The gene sequences obtained in the present study were aligned corresponding to the sequences obtained in the previous studies and the molecular phylogenetic analysis of the sequences indicated that these *C. parvum* isolates belonged to one subtype family, IIa, with 15 out of 17 positive specimens having subtype IIaA15G2R1 and two positive specimens having subtype IIaA14G2R1. Both subtypes reported are zoonotic in nature.

These results have similarity with the findings of Feng *et al.* (2007) who reported prevalence of zoonotic subtypes IIaA15G2R1, IIaA13G2R2, IIaA14G2R1a and IIaA14G2R1b by sub-genotyping of *C. parvum* isolates of cattle calves from India. Rieux *et al.* (2013) also reported subtype IIaA15G2R1 of *C. parvum* as zoonotic subtype. Geurden *et al.* (2007) detected four subtypes of *C. parvum* isolates of cattle calves (by sequence analysis of gp60 gene), IIaA15G2R1, IIaA13G2R1, IIaA16G2R1 and IIaA14G2R1. Xiao *et al.* (2007), Brook *et al*. (2009), Diaz *et al.* (2010), Imre *et al.* (2010) and Rahmouni *et al.* (2014) also reported IIaA15G2R1 subtype as highly prevalent subtype of *C. parvum* isolates in cattle calves throughout the study area. Contrary to the present study, Amer *et al.* (2010) reported two subtypes of *C. parvum* isolates in cattle calves, among which subtype IIdA20G1 was highly prevalent subtype and subtype IIaA15G2R1 was less prevalent subtype. Similar findings were reported by Wang *et al.* (2011) who identified subtype IIdA19G1 as highly prevalent subtype of *C. parvum.* The findings are also supported by Cui *et al.* (2014) reported subtype IIdA15G1 as highly prevalent subtype of *C. parvum* isolates in cattle calves. On the other hand, Abeywardana *et al.* (2014) carried out a study in Sri Lanka, wherein no subtype of *C. parvum* with zoonotic potential was reported after performing DNA sequence analysis of gp60 gene. The present study indicated that subtype IIaA15G2R1 of *C. parvum* isolates in cattle calves is highly prevalent in Jammu and Kashmir region.

Examination of 614 faecal samples of cattle by diethyl ether sedimentation technique followed by iodine wet mount technique revealed an overall positivity 43 animals (7.00%) for *Giardia* spp. infection. In India, Deshpande and Shastri (1981) from Maharastra reported the first incidence of *Giardia* infection in calves. Thereafter, Singh *et al*. (2008), Khan *et al*. (2011) and Sabu *et al*. (2011) reported *G. intestinalis* infections in cattle from three states of India viz. Punjab, West Bengal and Kerala, respectively. The prevalence of *G. duodenalis* infection in cattle ranges from 3 to 64% world-wide (Geurden *et al*., 2008). In the present study, the prevalence of *G. intestinalis* infection in cattle was comparable to that of Wade *et al*. (2000) and Singh *et al*. (2008), who reported 21.68 and 34.78% infection in heifer and young calves, respectively. On the other hand Das *et al*. (2015) reported 17.94% prevalence of *Giardia intestinalis* infection in dairy cattle present in Assam by using Formol-Ether technique and zinc sulphate (33%) floatation method.

In the present study age related susceptibility of *Giardia* spp. infection in calves was found to be highest in 1-6 month age animals with 11.2% prevalence with non-significant higher prevalence (9.02%) in diarrhoeic animals. Similarly, Ahmed *et al*. (2015) in Iraq reported 85.71% infection in calves (< 1year) with a significant difference and in calves (< 6 months) and (6 months to 1 year) with non-significant prevalence. Higher prevalence in younger animals were also in accordance with the findings of Ehsan *et al.* (2015) and Wang *et al*. (2014) from Bangladesh and China, who reported 22% and 22.7% infections in calves, respectively. Some findings reported that due to decreased level of acquired immunity in younger animals they become more susceptible to infections (Reinemeyer, 1995). Ralston *et al*. (2003) observed that the infection rate (15%) and the number of *Giardia* cysts in the cows faeces (38.49 cysts/g) numerically increased at 1 week post calving compared to levels at calving and thus they act as a carrier of infections for calves. Higher infection among the diarrhoeic (83.41%) animals in the present findings was in accordance with the findings of Singh *et al.* (2008).

Findings of the present study revealed prevalence of *Giardia* infection varied non significantly among all the four districts of Jammu region with highest prevalence of *Giardia* spp. recorded in Kathua (7.78%) followed by Rajouri (7.43%), Samba (6.49%) and Udhampur (6.39%) districts. The study correlates with other studies showing variations in the prevalence due to geographical, topographical locations and climatic changes of different regions in a particular place. Variations have been reported from different geographical areas worlwide viz. 5.0% from Thailand (Inpankaew *et al*., 2015), 7.2% from Germany (Gillhuber *et al*., 2014), 14% from Canada (Budu-Amoako *et al*., 2012) and 34.5% from Argentina (Tiranti *et al*., 2011). The variation in different geographical localities may be due to differences in the levels of management practices employed at farm level, housing-related factors and nature of the study (Swai and Schoonman, 2010). As compared to other countries and other studies of India, a moderate percentage of animals (17.94%) were found to be infected with *G. duodenalis* in Assam (Das *et al*., 2015).

The prevalence of *Giardia* spp. infection in the present study was recorded significantly higher in males (15.85%). The present study is in accordance with Suman *et al.*, 2011 which also stated that male calves (14.3%) have slightly higher prevalence of *Giardia* spp. than female calves (12.5%). Mendiratta and Kondaiah (2015) reported in IVRI Izatnagar, India that the growth in population of male calves as compared to female calves is almost half (53.6%) indicating the country level mortality in male buffalo calves about 50 percent additional and are kept with apathy and they are not fed properly and die before they reach 1 year of age. The male calves below 2 years of age comprise 67.5% of all males, which clearly indicates the usage of male calves is very limited and the farmers don't want to keep them for various reasons but are used for draught purposes. Therefore, because of the negligence of male calf regarding feeding, management and treatment, lead them with reduced immunity and make them susceptible to various parasitic diseases.

The prevalence of *Giardia* infection having no mucus and blood in faeces showed non- significantly higher prevalence (7.62%) in the present study which is in agreement with Colville and Berryhill (2007) revealed that usually no fever and no blood in the diarrhea and feces are usually formed, not watery, but are mixed with mucus.

The season wise prevalence of *Giardia* spp. in cattle calves during the study had non-significant results. The highest prevalence of *Giardia* infection was found to be in monsoon (10.34%) season which coincides with the findings of Das *et al*. (2015) with highest infection during monsoon (30.90%) season. On contrary, Ayaz *et al.* (2012) in Pakistan observed highest infection (36.66%) in autumn (August, September, October) followed by 34.58% infections during summer (May, June, July), spring (30.83%) and winter (25%). A lowest percentage of infection during winter might be due to less availability of water thereby less organisms in contaminated water. Similarly, Castro-Hermida *et al.* (2009) in Spain detected more *G. duodenalis* cysts in faecal samples of cows during spring (16.0%) and summer (6.9%). High temperature and humidity along with frequent rains in the monsoon season enabled the transmission of cysts faster by contaminating feed and water of the animals. Thus, the warm and humid monsoon season was observed to be the most amicable season for the propagation of the disease than other seasons.

Sensitivity and specificity of various diagnostic techniques (DWT, FEST, ZNSFST, DFAT and PCR) for detection of *Giardia* spp. cysts in faeces was carried out on randomly selected samples of Jammu region. The highest prevalence as per diagnostic method was recorded by (PCR) polymerase chain reaction (18.66%) followed by (DFAT) direct fluorescent antibody test (14.66%), (ZNSFST) zinc sulphate floatation Sedimentation technique (10.66%), (FEST) formal ether sedimentation technique (8.00%) and least in positivity was recorded by (DWT) direct wet mount staining (5.33%) technique. The study recorded the sensitivity and specificity of DFAT technique in relation to PCR was 90.9% and 95.3%, respectively where PCR is kept as a standard technique. Also, the sensitivity and specificity of ZNCT technique was 87.5% and 92.5% and that of FEST technique was 66.7% and 91.3% and least was recorded in DWT with 50.0% sensitivity and 88.7% specificity. Other studies concluded that PCR allows semiautomation of the diagnostic procedure, and PCR permits the detection of several different parasite species (Bruijnesteijn van Coppenraet and Wallinga, 2009; Mary *et al*., 2013; Stensvold and Nielsen, 2012) in one working process. Similarly, Rasmussen *et al*. (2016) had kept the IFA technique as a reference and sensitivity and specificity when compared with qPCR was 50% and 99.5% but when comparison of qPCR was done with IFA, the sensitivity and specificity recorded was 91% and 95.1%. Also, FEA (formol ethyl acetate) and SSF when compared with qPCR, sensitivity recorded was 31% and 67% with 100% sensitivity for both the tests. Other researchers have compared all the techniques used in the current study, microscopy after concentration, to fluorescence labeling of monoclonal antibodies and PCR, respectively (Nazeer *et al*., 2013; Winiecka-Krusnell and Linder, 1995). In agreement with our data, these studies obtained sensitivities of both IFA and qPCR which were higher compared to the concentration techniques, iodide staining, and microscopy. In contrast, Schuurman *et al*. (2007) found qPCR, rapid immunoassay, and microscopy to be equally sensitive in a study that compared samples initially detected positive for *G. duodenalis* by microscopy. Similarly, Ahmad *et al*. (2016) detected the *Giardia* spp. infection by microscopic examination with 29.03% prevalence. Others also studied conventional SFSS technique which has been used to concentrate and quantify *Giardia* spp. cysts in fecal samples from animals (Maddox-Hyttel *et al*., 2006). On contrary, Garcia *et al*., 1992 and Rose *et al*., 1989 reported that Immunofluorescence assays (IFAs) can be used to stain fecal smears without initial concentration which has an increased sensitivity compared to formol ethyl acetate (FEA) method.

The diagnostic techniques in the present study revealed non-significant relationship between PCR vs DWT and almost perfect agreement was observed between PCR vs DFAT, ZNSFST (substantial agreement), FEST (moderate agreement) and DWT(fair agreement) with kappa values 0.80, 0.65, 0.44 and 0.22. Therefore, the proportion of overall agreement in concordance of PCR vs DFAT to study the prevalence of giardiosis revealed 94.66% of PCR vs DFAT and that with ZNCTVS, FEST and DWT the prevalence was recorded as 92%, 89.33% and 86.66%. Lee *et al.* (2016) results from PCR and ELISA testing methods with an agreement of 84.9 % and κ value of 0.34, which suggests fair agreement.

The quantitative examination of *Giardia* cysts in the current study was done by modified Mc Master Chamber technique in the faecal smear of cattle calves in Jammu region. Mean *Giardia* cysts intensity were recorded among different districts of Jammu region viz; Rajouri (1058.9 ± 171.01) Samba (1417.8 ± 288.84), Kathua (1323 ± 161.56) and Udhampur (1235.7 ± 241.67) which varied non-significantly. In the survey of *Giardia* spp. infection, many researchers studied the variable range of shedding intensity of cysts in calves 4.1 x 10^3 and 3 x 10^5cysts/gram of faeces (Taminelli *et al*., 1989), 3.8 x 10^7cysts/gram of faeces (Nydam *et al*., 2001), 110 to 270 cysts/gram of faeces (Nizeyi *et al*., 2002) and 2230 cysts/gram of faeces at 5 week of age and then decreased to 2 cysts/gram of faces at 25-27 weeks of age (Ralston *et al*., 2003). This variation might be due to the different geographical conditions and parasitic burden in the infected animals. A higher infection rate and cysts shedding intensity of *Giardia* spp. could be attributed to the presence of other concomitant infection. Higher mean average number of cysts intensity (1799.4± 189.42) was observed in 1-6 month of age group and in male calves (1222.1 ± 72.70) during the study. On contrary, the cyst counts in faeces of buffaloes of ≤ 1 year was 4.5x10^3 followed by 2.8x10^3 (1-3 years) and 1.7x103 (≥ 3 years) cysts per gram of faeces (Goraya *et al*., 2004). Similarly, Hamnes *et al*., 2006 reported that intensity of *Giardia* spp. infection declines with age, in agreement with the current results where calves of >6 month of age had mean cyst intensity (1024.15± 59.32) less than in < 1month age of animal. On the other hand Fischer (1983) reported the highest infection rate and intensities of *Giardia* in calves of 5 to 6 weeks of age. In the present study, diarrhoeic (1290.2 ± 224.91) animals had significantly higher cyts intensity. Also, giardiosis was significantly highest in monsoon season (1558.6 ± 194.35) and in faeces with no mucus and blood (1675.3 ± 114.26). Similary, Goraya *et al*. (2004) reported mean shedding intensity of cysts per gram of normal faeces ($2.7x10^2$) which was less than intensity in abnormal faeces ($4.3x10^3$). Also, other studies reported that higher intensity of cyst per gram faeces were found in rainy season and in autumn in Bangladesh, India and Pakistan (Roy *et al*., 2006 and Maqbool *et al*., 2008). The warm and humid conditions are usually favorable for cyst to survive in heavy rainfall and flooding facilitates transmission of the infection.

The present study represents the molecular characterization of *Giardia intestinalis* by β-giardin gene carried on 120 faecal samples (40 samples per age group) randomly selected from 43 samples found positive for *Giardia* cyst by iodine wet mount staining. Amplification by nested PCR of the *Giardia* isolates yielded 511 bp fragment of β-giardin gene. Few reports are available which correlate this study with nested PCR of *Giardia* spp. by using β-giardin gene as it provides consistent and reproducible results (Lalle *et al*., 2005 and Sulaiman *et al*., 2003). Similarly, Khan *et al.* (2011) recorded overall prevalence of *G. duodenalis* in cattle calves with 12.2% dairy farms of West Bengal by PCR analysis of β-giardin gene of *Giardia* spp. However, certain reporters suggests that the molecular characterization of *Giardia* cyst and molecular data on nested PCR analysis represents an objective means for determining the source of infection in outbreaks and in single cases of cattle claves (Isaac-Renton *et al*., 1993; Hopkins *et al*., 1997 and McIntyre *et al*., 2000). Direct analysis of the cyst from faeces by using amplification technique with several PCR assay have been developed (Weiss *et al*., 1992; Caccio *et al*., 2002 and Sulaiman *et al*., 2003).

PCR-RFLP results in amplification of 511 bp nested product when digested with *Hae* III restriction enzymes and provides specific diagnosis of the species of *Giardia* involved. Assemblage B and E with specific band patterns were identified in the current study which relates to Hong *et al*. (2014) who successfully performed a molecular analysis of β –giardin gene and pattern analysis using PCR-RFLP assay with *Hae* III restriction enzymes on *Giardia* DNA from faecal analysis.

In the present study, Assemblage B was observed when PCR-RFLP product of β-giardin gene of *Giardia* spp. are digested with *Hae* III restriction enzyme. In our results, 150 and110bp band pattern was visualized and this 100 bp band probably coincided with 117bp band and could not be visualized whereas, three other bands i.e. 84bp, 26bp and 24bp bands were not observed. Assemblage E was also detected when digested with *Hae* III restriction enzyme in the present study where three bands of 186bp, 150bp and 110bp were observed whereas other three bands (26bp, 24bp and 15bp) were not seen. The unidentified bands which could not be seen and have been cited in other reports would have been washed during gel electrophoresis. Similarly, Khan *et al*. (2011) also reported Assemblage E which could identify only three visible bands at 186 bp, 150 bp and 110 bp upon restriction enzyme *Hae* III. This study on visibility of the three bands of Assemblage E correlates with Lalle *et al*. (2005). Also, survey conducted in Australia (Becher *et al*., 2004), Canada (Appelbee *et al*., 2003) and the United States (Trout *et al*., 2004) a higher prevalence of the non-zoonotic, host-specific Assemblage E was reported. The same pattern of Assembalge E have been found by other workers (Xiao *et al*., 1993; Wade *et al*., 2000a and Berrilli *et al*., 2004).

RFLP analysis of nested PCR product and sequencing of the amplification products revealed that a total of 1.6% samples examined were having Assemblage B infection whereas, 14.16% were positive with Assemblage E, respectively. Our results showed almost similar prevalence of Assemblage B with Lalle *et al*. (2005) who identified 13.5 % containing Assemblage B. Assemblage E is specifically found in cattle which have been found in north western china with overall prevalence of 92.8% of samples (Qi *et al*., 2016). High occurrence (60%) of *G. duodenalis* was found in dairy calves, the existence of high genetic heterogeneity of Assemblage E within a small geographical area which has reported from Shanghai, China (Wang *et al*., 2017). On contrary to our results, Coklin *et al*., 2007 have reported high overall prevalence of Assemblage B (24.5%) as compared to Assemblage E (17.5%) in eastern Ontario, Canada. A study done by PCR-RFLP demonstrated that all the faecal samples from cattle belong to *G. duodenalis* Assemblage A except for only one calf which was Assemblage B (Van Keulen *et al*., 2002).

The sequencing of amplicon from *Giardia* positive samples revealed that the 511bp gene sequence belonged to *Giardia intestinalis* assemblage B isolates from cattle calves of Jammu region. The gene sequence of beta giardin gene of *Giardia* spp. isolated from cattle faeces was custom sequenced. The gene sequence generated in our study had 97.68% similarity with *Giardia intestinalis* isolate 8 from Iran while it had 97.49% similarity with gene sequence from Egypt and 97.66% similarity with a Kenyan isolate available in the database. These results have similarity with the findings of Coklin *et al*. (2007) reported Assemblage B in 25 samples of cattle calves with 100% homology. Similarly, Lalle *et al*. (2005) revealed Assemblage B found in five isolates out of 24 isolates. A study on molecular characterization and sequencing of *Giardia* spp. in cattle in Portugal demonstrated Assemblage B (Mendonca *et al*., 2007). *Giardia intestinalis* Assemblage B were detected in dairy cattle calves in the current study echoing studies of dairy animals in New Zealand (Learmonth *et al*., 2003 and Winkworth *et al*., 2008). On the other hand several studies on gene sequence belonging to *Giardia intestinalis* Assemblage B was demonstrated by Hunt *et al*., 2000, Sprong *et al*., 2009a and Johnston *et al*., 2010. Thus, in the present study, sequencing of *Giardia* spp. could assign only Assemblage B and other Assemblage E was not sequenced due to either because fragments of only one locus was amplified or because repeated analysis using more Assemblage specific typing assays failed due to low sensitivity of DNA samples and this observation in the present study have been recorded by Helmy *et al*., 2014.

Many compounds have been tested for their potential anticryptosporidial activity, but there is no efficient therapy to prevent or treat cryptosporidiosis, particularly in the immunocompromised host (Mead, 2002 and Kayser, 2001). During the last 20 years, 70% of the emerging diseases have been found to be zoonotic in nature and about 300 diseases are common to man and animals (Thapliyal, 1999). *Cryptosporidium* is notoriously diverged from other apicomplexan parasites by some peculiarities in biology, distinct structure, and biochemical composition that could be related to natural drug resistance. The parasite's unique location in the host cell may affect the drug concentration, and the existence of transport proteins or efflux pumps that transport drugs out of the parasite or into the host cells provides the *Cryptosporidium* organisms with resistance to drug therapy. Despite encouraging anecdotal data, the drug does not appear to be uniformly effective in suppression of oocyst excretion or resolution of clinical signs (Giacometti *et al.,* 2000). In spite of the prominent developments in different aspects of the parasite, its treatment still exists as an unresolved issue (Ryan *et al*., 2016 and Ward, 2017). Since there is lack of sufficiently effective therapeutic drugs and oocyst have a high tenacity (including resistance to many chemicals) and tiny size (they easily pass standard physical filtration), it is quite difficult to securely prevent and control cryptosporidiosis and thus many therapies are not effective against the parasite.

The present study was planned to evaluate an invivo efficacy of different concentrations of turmeric (*Curcuma longa* extract) and curcumin (active compound) against cryptosporidiosis in experimentally infected swiss albino mice by assessing oocyst per gram of faeces, average body weight and histopathological changes in mice. Our results showed that oocyst shedding was started at day 5 PI and percent oocyst reduction of *Cryptosporidium* oocysts were recorded at $5^{th,}$ 7^{th}, 9^{th} and 11^{th} day PI. In this trial *Curcuma longa* and curcumin was given @ 4, 6 and 8 mg/kg body weight to the experimental animals which is in accordance with the findings of Asadpour *et al*. (2018) and Cervantes-Valencia *et al*. (2016). In the current study, at day 7 PI the mean OPG was at peak (8916±139) with % oocyst shedding of 33.74% and then decreases till the end of 11^{th} day (3416±746) with % oocyst shedding of 48.75% of post treatment. Our findings showed that treatment started from 5 day PI and oocyst shedding stopped without reoccurrence after 11 day PI.

In the current work, dexamethasone, a synthetic glucocorticoid, was used to induce chemical immunosuppression in all the groups of mice except healthy control group I. Animals which were uninfected but were administered with dexamethasone in drinking water only (group II) showed slight variation in the body weight of mice. Immunosuppressed mice serve as an excellent model to study experimental *C. parvum* infection (Certad *et al*., 2007 and Ndao *et al*., 2013). Mice administered dexamethasone in drinking water could be readily infected with *C. parvum* experimentally (Abdou *et al.*, 2013; Certad *et al*., 2012; Aguirre *et al.*, 1994; Costa *et al*., 2011; Rasmussen *et al.*, 1992; Theodos *et al.*, 1997). This relates to the study revealing that glucocorticoids are known to have an effect on the priming of the innate immune response and could suppress IFN-g-regulated expression (Franchimont, 2004). Our findings coincides to some extent with Chai *et al*., 1999 and Certad *et al*., 2012 who reported that the intensity of oocyst shedding was higher in immunosuppressed mice than in immunocompetent ones throughout the duration of the experiment.

Nitazoxanide and other nitrothiazole salicylamide compounds have broad antiparasitic activities (Adagu *et al*., 2002). In the present study analysis of mean OPG count at day 7 post treatment was significantly lower in group IV animals (750±111.803) treated with nitazoxanide drug with maximum percent of oocyst reduction (88.88%). The present study is in accordance with Blagburn *et al.* (1998) who orally administered the injectable formulation of nitazoxanide at a dose of 150 mg/kg and resulted in improved efficacy of oocyst shedding in faeces of mice (oocyst output, <5% of that in controls). These results agree with those of Bailey and Erramouspe *et al*. (2004) and Fox and Saravolatz (2005) who found that the responses to the nitazoxanide drug were lower in immunosuppressed animals. Whereas, Rossignol (2010) reported the variability of the effectiveness of NTZ, which depended on the degree of immunosuppression and CD4 counts. The effects of nitazoxanide on reduction of oocyst shedding in immunocompetent and immunosuppressed animals were to some extent in agreement with Ollivett *et al*., 2009 who revealed that 85% of the nitazoxanide treated calves stopped oocysts shedding by the end of the observation period. In immunosuppressed animals, similarly a significant reduction in oocysts excretion was obtained in immunosuppressed gerbil at dose of 200 mg/kg for 12 days (Baishanbo *et al*., 2005). On the contrary, Theodos *et al*., 1998 mentioned that no significance difference in oocyst shedding was observed between groups of mice treated with NTZ and placebo control group.

The present study revealed that the treatment with synthetic curcumin on day 7 post treatment in group X (1666±307.318) resulted in significantly higher reduction of mean oocyst count with percent oocyst reduction of 74.03% in mice than those which were administered with same dose of *Curcuma longa* extract in group VII (2416.67±436.208) with 62.83% mean oocyst reduction. The dose selected for synthetic curcumin and *Curcuma longa* extract was selected in agreement with the findings of Asadpour *et al*., 2018. In other studies, anti-coccidial efficacy of curcumin (Cui *et al*., 2007; Khalafalla *et al*., 2011; Cervantes-Valencia *et al*., 2015) and its in vitro anti-cryptosporidial potential have been reported (Shahiduzzaman *et al*., 2009). The present findings showed that oocyst shedding was started at 7 days PI and ileum was more parasitized and these results are in agreement with other studies (Al-Mathal and Alsalem *et al*., 2013; Perrucci *et al*., 2006 and Surl and Kim, 2006).

The present study revealed that among the treated groups, the average body weight of mice at day 7 post treatment was significantly highest in group IV animals (24.370 ± 2.420). The animals treated with synthetic curcumin in group X resulted in slighly higher body weight (23.915 ±2.224) in mice than those which were administered with same dose of *Curcuma longa* in group VII (23.01 ± 2.057). However, the average body weight increase was however not significantly affected with the treatment. Costa *et al.* (2011) found an additional 20% weight loss in malnourished mice after *C. parvum* challenge. Present observations are also in agreement with the findings of Castro *et al*. (2012); Tarazona *et al.* (1998); Mirza-Qavami and Sadraei (2011); Certad *et al*. (2012) who have also reported weight loss because of cryptosporidiosis in mice. However, there are not much reports on the effect of Turmeric (*Curcuma longa*) against cryptosporidiosis in mice and improvement in body weight of animal by this treatment but few reports on dietary supplementations with the *C. longa* against other protozoans revealed significant effect on body weight gain of *Eimeria maxima* and *Eimeria tenella* infected chickens between day 0 to 10 postinfection compared with infected birds given a nonsupplemented control diet. Similarly, Lee *et al*., 2010 reported that feeding broiler chickens with diet containing *C. longa* did not show any toxic effect on host but enhanced body weight gain and reduce oocyst shedding as compared to non-infected control groups.

The present study showed that the administration of curcumin could stop oocyst shedding without recurrence at 11th days after drug withdrawal. Histopathological findings confirmed that treatment with curcumin could improve the lesions and decreases the number of oocyst on the villi.

In the present study, histopathological changes were observed where in group I and II did not show any microscopic abnormalities but maximum intensity of infection in the intestinal sections was found on 11th day PI in group III animals. Gross lesions in intestines of group III mice on 11th day PI revealed severe congestion of serosa and mucosa along with catarrhal exudate in the lumen of intestinal tract. Other changes in the intestine included severe degeneration, catarrhal and haemorrhagic enteritis, necrosis of intestinal epithelium and crypts, goblet cell hyperplasia, severe stunting, blunting and fusion of the villi, Lamina propria and submucosa were severely infiltrated by inflammatory cells and oocysts adhering to the enterocytes were also seen. Group IV animals, intestinal sections showed mild inflammation of lamina propria but no oocysts were seen attached to the villi and appeared normal as compared to other treated groups. Animals in group VII showed enteritis which was less severe. Whereas in group X, animals had minimal intestinal lesions upon microscopic examination and nearly comparable to group IV animals. The intestinal enteritis was resolved by 20th day PI but only mild enteritis was seen in group III mice at this time. The present study revealed that maximum mean oocyst shedding was obsereved at day 11 PI which coincided with most severe gross and histopathological lesions and maximum intestinal colonization by various stages of *C. parvum* on same day. Similar reporters studied these observations which were in agreement with reports of Mirza-Qavami and Sadraei (2011) who have reported peak severity of intensity occurred on 9th day post infection and then infection gradually decreased on 12th day post infection and cleared by 16th day post infection, whereas, Verdon *et al*. (1998) have observed that oocysts intensity of faecal samples of infected mice was cleared between 14 and 21 days PI. Castro *et al.* (2012) reported peak infection on day 14th PI and afterwards infection subsides. Tarazona *et al.* (1998); Rasmussen *et al*. (1992) revealed that oocyst count was higher in immunosuppressed infected mice as compared to non-immunosuppressed mice. Whereas, Aguirre *et al.* (1994) reported *C. parvum* intensity was higher in adult mice of 5-6 weeks of age as compared to mice of 3-5 days of age. Similarly histopathological, reports were in agreement with Ndao *et al*. (2013) who have described severe enteritis, villous blunting, and abundant intracellular parasitic stages in mice infected with *C. parvum*. Castro *et al*. (2012) also observed ileal crypt hyperplasia, villus blunting, severe enteritis. Aguirre *et al*. (1994); Costa *et al*. (2011); Rasmussen *et al*. (1992); Theodos *et al.* (1997) reported histological presence of *C. parvum* within the villous and crypt mucosa of the intestines alongwith mononuclear cell infiltration in the lamina propria, blunting of the villi, crypt dilatation and necrosis. Evaluation of the oocyst shedding pattern and the number of oocysts adherent to the surface of the villi have been considered in many studies (Al-Mathal and Alsalem, 2012; Perrucci *et al*., 2006 and Viu *et al*., 2000).

Chapter-6

Summary and Conclusion

CHAPTER–VI

SUMMARY AND CONCLUSIONS

Cryptosporidium and *Giardia* are important gastrointestinal protists with a wide spectrum of hosts, including humans, livestock, companion animals and wildlife. Infection acquired via the faecal oral route following the ingestion of infective oocysts or cysts, by either direct contact or the ingestion of contaminated food or water. Diarrhea along with dehydration, fever, nausea and anorexia are the typical common symptoms in animals infected with cryptosporidiosis and giardiosis.

The present study carried out in Jammu districts used conventional, immunological and molecular methods for identification of *Cryptosporidium* oocysts and *Giardia* cysts in faecal samples of cattle calves. Characterization using molecular methods helped in identification of *Cryptosporidium* species, establishing zoonotic potential of *Cryptosporidium parvum* and its subtypes along with identification of *Giardia* Assemblages. Zoonotic potential of both protozoa has become clearer with the use of molecular techniques to genotypes isolates (*Cryptosporidium parvum*- IIa subtype and *Giardia intestinalis*-Assemblage B). Cattle are mammalian species commonly infected with *Cryptosporidium* and cattle calves are considered the most important reservoir for zoonotic infection. In terms of economic losses *Cryptosporidium parvum* is usually considered as the most important species in cattle calves. The prevalence of zoonotic species, *Giardia intestinalis* and genotypes Assemblage B is found in cattle calves and Assemblage E which have been detected in a wide range of animals mostly in cattle.

One of the most intriguing clinically frustrating features of cryptosporidiosis is its resistance to antimicrobial drugs. *Cryptosporidium* spp. establishes a parasitophorous vacuole within the host cell, which shelters the parasite from antimicrobial agents. The clinical course of cryptosporidiosis depends largely on the immune status of the host and thus treatment options vary accordingly. Therefore, cryptosporidial drug therapy includes various anticryptosporidial agents, immunization and supportive treatment. In the current study, mice model for chronic cryptosporidiosis was used to identify effective anticryptosporidial activity of *Curcuma longa* extract and curcumin to study the immunosuppressed animals that allow persistent infections to develop.

The prevalence studies were based on identification of *Cryptosporidium* oocysts and *Giardia* cysts in faecal samples by Diethyl ether sedimentation technique followed by conventional techniques (modified Ziehl-Neelsen staining technique and iodine wet mount technique).

The overall prevalence of *Cryptosporidium* spp. in cattle calves of Jammu district was observed to be 23.45 %. Among four districts of Jammu the highest prevalence of *Cryptosporidium* was recorded in Samba district (28.57%) and least prevalence in Rajouri district (18.18%). Statistically risk factors were analysed and it was observed that cattle calves were significantly ($p<0.05$) at higher risk (2.210 times) in Udhampur as compared to other districts. It was highest in < 1 month of age animals (43.24%) and lowest prevalence in >6 months of age animals (12.54%). *Cryptosporidium* spp. infection in young calves varied significantly ($p<0.05$) with other age groups.

The prevalence of infection was greater among diarrhoeic animals (29.51%) than non diarrhoeic animals (18.09%). Diarrhoeic animals revealed significantly higher prevalence in different districts, age groups, sex and season than non diarrhoeic calves. Male animals showed higher incidence of *Cryptosporidium* infection (46.34%; diarrhoeic, 30.61%; non-diarrhoeic) as compared to female (22.81%; diarrhoeic, 12.71%; non-diarrhoeic) animals. As per season the highest prevalence was recorded in winter (34.65%) season from December to February and lowest in summer season (8.55%). Moreover chances of occurrence of *Cryptosporidium* infection was assessed and revealed that in all the seasons, diarrhoeic cattle calves showed higher prevalence than non diarrhoeic calves. Statistical analysis suggests that prevalence of *Cryptosporidium* infection in winter season varied significantly ($p<0.05$) than other three seasons. Also, the chances of occurrence of *Cryptosporidium* spp. in cattle calves in winter were 5.672 times higher than summer months. Cattle calves having mucus in the faeces showed significantly ($p<0.05$) higher prevalence (37.33%) whereas least prevalence was recorded in faeces having blood (11.76%).

Highest intensity of 3+ was observed in Udhampur district of Jammu region as compared to other districts but maximum number of animals showed 1+ oocyst intensity among other districts. High numbers of calves (57.81 %) of < 1 month age group were having 3+ oocysts intensity, whereas the older animals (>6 months age) were excreting quite less number of oocysts. Diarrhoeic animals had 3+ oocyts intensity than non diarrhoeic animals. A total of 50.58% of diarrhoeic and 18.64% of non diarrhoeic cattle calves were positive with 3+ oocyst intensity. Higher oocyst intensity was found in male animals than in females. On seasonal basis, it was found that the oocyst intensity of cattle cryptosporidiosis was highest (3+) in winter season (52.45%) while it was lowest in summer season (15.38%). The animals having mucus in the faeces (57.14%) showed highest oocysts intensity (3+).

As per diagnostic methods, the overall prevalence recorded by DFSS, DESS, SFSS, DFAT and PCR was 36.00%, 34.66%, 40.00%, 42.66% and 46.66%. The sensitivity of DFAT, SFSS, DESS, DFSS technique was 91.4%, 85.7%, 74.3% and 60%, whereas 97.5%, 95.0%, 92.5% and 90.0% was recorded as specificity by various techniques, respectively for the diagnosis of cryptosporidiosis. There was perfect agreement between PCR vs SFSS and DFAT with kappa values 0.81 and 0.89 as compared to PCR vs DESS with kappa value 0.67 as substantial agreement and PCR vs DFS with kappa value 0.50 as moderate agreement. The overall agreement between PCR vs DESS, SFSS and DFAT with Po value 0.84, 0.90, 0.94 as compared to agreement between PCR vs DFS with Po value 0.76.

The zoonotic potential of cattle cryptosporidiosis was assessed by characterization of *Cryptosporidium* positive calve samples and subjected to digestion by *Ssp*I restriction enzymes which yielded two visible bands (449bp and267bp) for *C. parvum*, one band (448bp) for *C. andersoni* and two bands (432bp and 267bp) for *C. bovis*. RFLP with *Vsp*I enzyme yielded one visible band each for *C. parvum* (628bp), *C. andersoni* (730bp) and *C. bovis* (616bp). Digestion of PCR product with *Mbo*II also yielded one band each for *C. parvum* (771bp), *C. andersoni* (769bp) and *C. bovis* (485bp). Other bands which could not be observed in the present study might have been washed during gel electrophoresis. RFLP analysis of nested PCR product showed that a total of 74.07%

samples examined were having *C. parvum* infection whereas, 16.66% were positive for *C. andersoni* and 9.25% were found to be positive for *C. bovis*. Gene sequence of identified *Cryptosporidium* species by RFLP of 18S small subunit (SSU) rRNA gene revealed that the newly generated sequences of Jammu and Kathua district isolates of genus *Cryptosporidium* were *C. parvum*. The RFLP products were characterized by DNA sequencing, wherein the glycoprotein 60 (GP60 locus) gene was amplified and sequenced. Subtyping of *Cryptosporidium parvum* positive isolates by sequence analysis of 60kDa glycoprotein gene indicated the presence of 2 allele IIa subtypes, IIaA15G2R1 and IIaA14G2R1. Among these two identified subtypes, IIaA15G2R1 was found to be most prevalent in calves.

The overall prevalence of *Giardia* spp. infection in cattle calves was 7.00%. Prevalence of *Giardia* spp. infection varied non-significantly ($p<0.05$) among all the four districts (Udhampur, Kathua, Rajouri and Kathua) of Jammu region. *Giardia* infection was found to be highest in 1-6 month age animals (11.2%) and lowest was recorded in > 6 month age group (4.79%). *Giardia* infection was non-significantly ($p<0.05$) higher in diarrhoeic animals (9.02%) than non diarrhoeic animals (5.21%). Higher incidence of *Giardia* infection (15.85%; diarrhoeic, 10.20%; non-diarrhoeic) was recorded in males as compared to females (6.31%; diarrhoeic, 3.07 %; non-diarrhoeic). Faecal samples having no mucus and blood showed non- significantly ($p<0.05$) higher prevalence (7.62%) than those with blood and mucus. On season wise analysis, highest prevalence was found to be in monsoon (10.34%) and least in winter (5.11%). However, the chances of occurrence of *Giardia* spp. was 1.63 times higher in monsoon than in winter season.

Mean *Giardia* cysts counts per gram faeces was identified by modified Mc Master Chamber technique and cysts intensity in all the four districts of Jammu region (Rajouri, Samba, Kathua and Udhampur) varied non-significantly. Significantly ($p<0.05$) higher mean average number of cysts (1799.4± 189.42) was observed in 1-6 month of age group and least seen in young animals of <1 month of age (834.13 ± 5199.91). Diarrhoeic (1290 .2 ± 224.91) animals had significantly ($p<0.05$) higher cyts intensity than non diarrhoeic (918.41 ± 135.83). Male calves showed higher cyst intensity (1222.1 ± 72.70) than female calves (1003.1 ± 118.33) which varied non-significantly ($p<0.05$). On season wise analysis, it was found that cyst intensity of cattle giardiosis was significantly ($p<0.05$) highest in monsoon season (1558.6 ± 194.35) and lowest in winter season (693.56 ± 74.13). The cattle calves having no mucus or blood in the faeces were having significantly ($p<0.05$) higher mean cysts intensity (1675.3 ± 114.26).

The overall prevalence recorded as per diagnostic techniques by DWT, FEST, ZNCT, DFAT and PCR was 5.33%, 8.00%, 10.66%, 14.66% and 18.66%. In the current study, DFAT technique was recorded with high sensitivity and specificity (90.9% and 95.3%) as compared to ZNCT (87.5% sensitivity and 92.5% specificity), FEST (66.7% sensitivity and specificity was 91.3%) and least was seen in DWT (50.0% sensitivity and 88.7% specificity), respectively for the diagnosis of giardiosis. Almost perfect agreement was observed between PCR vs DFAT (kappa values 0.80), substantial agreement between PCR vs ZNSFST (kappa value 0.65), moderate agreement between PCR vs FEST (kappa value 0.44) and fair agreement between PCR vs DWTVS (kappa value 0.22). Therefore, the proportion of overall agreement between different diagnostic tests conducted to study the prevalence of giardiosis revealed 94.66% of overall concordance of PCR vs DFAT, 92% of concordance of PCR vs ZNCTVS, 89.33% of concordance of PCR vs FEST and 86.66% of concordance in between PCR vs DWT.

Molecular characterization of *Giardia intestinalis* by β-giardin gene was ascertained by nested PCR amplification. Further digestion of PCR products by *Hae* III restriction enzymes provided specific diagnosis of the species of *Giardia* involved. Assemblage B with two visible bands at 150bp and 117bp were recorded and Assemblage E was also detected where three bands of 186bp, 150bp and 110bp observed in the gel whereas other bands which could not be observed have got coincided with each other or might have been washed during gel electrophoresis.

RFLP analysis of nested PCR products revealed that a total of 1.6% samples examined were having Assemblage B infection whereas, 14.16% were positive for Assemblage E. RFLP products were further characterized by DNA sequencing, wherein beta giardin gene was amplified and sequenced which analyzed the presence of *Giardia intestinalis* Assemblage B found to be prevalent in cattle calves of Jammu region.

The use of curcumin against induced cryptosporidiosis in experimental mice at the dose rate of 8 mg/kg body weight provided better reduction of oocysts excretion and helps in maintaining body weight of animal as compared to *Curcuma longa* extract. The treatment with synthetic curcumin at 7th day post treatment resulted in significantly higher reduction of mean oocyst count with percent mean oocyst reduction of 74.03% in mice than those which were administered with same dose of *Curcuma longa* with 62.83% mean oocyst reduction. But when compared to a standard drug nitazoxanide, maximum percent mean oocyst reduction (88.88%) was observed in comparison to treatment with curcumin and *Curcuma longa* drug. The rate of average body weight was not significantly affected with the treatment but was maintained as compared to healthy control animals. Histopathological changes showed that on day 11 PI, healthy control and immunosuppressed animals (group I and II) revealed no microscopic abnormalities. The intestinal sections of infected control (group III) animals had severe disruption of intestines with stunted and fused villi, severely infiltrated by inflammatory cells and oocysts were seen adhering to the enterocytes of the villi. Animals treated with nitazoxanide (group IV) showed mild inflammation of lamina propria and no oocysts were seen attached to the villi. Administration of oral curcumin (group X) in animals had minimal and improved intestinal lesions upon microscopic examination which were nearly comparable to the animals in group IV. Whereas, in group of animals treated with *Curcuma longa* extract (group VII) showed enteritis which was less severe as compared to infected control animals. Oocysts shedding stopped in all treated animals (group IV, V, VI, VII, VIII, IX and X) without reoccurrence after day 7 drug withdrawal (after 11th day PI). On day 20 PI no gross lesions were seen and intestinal sections revealed mild enteritis except the animals in infected control (group III) showed mild enteritis, desquamation, sloughing of intestinal villi.

The study concludes that

1. Cattle calves of Jammu region are infected with *Cryptosporidium* spp. and *Giardia* spp. infection.
2. Young diarrhoeic calves (<1 month) showed high prevalence of *Cryptosporidium* spp. infection and calves (1-6 month) showed prevalence of *Giardia* spp. infection.

3. Cattle calves of Jammu region showed high prevalence of *Cryptosporidium* spp. in winter season and that of *Giardia* spp. infection in monsoon season.
4. Mucus in faeces was associated with high prevalence of cryptosporidiosis, whereas faeces with no mucus and blood had prevalence of giardiosis.
5. Major zoonotic species affecting young calves was *Cryptosporidium parvum* and *Giardia intestinalis*.
6. Potentials and subgenotype IIaA15G2R1, observed in the study, is an important zoonotic subtype of *Cryptosporidium parvum* affecting cattle calves in Jammu region whereas *C. andersoni* was more prevalent in adult animals than other age groups and *C. bovis* was prevalent in young as well as in adult animals.
7. The zoonotic *G. intestinalis* Assemblage B in cattle calves greatly increases the potential reservoir of infection for animals which suggests the zooanthroponotic source of infection in cattle calves of Jammu region.
8. Curcumin at dosage of 8 mg/kg b. wt. with many valuable properties is an exceptional herbal drug and applicable agent which has promising anti cryptosporidial activity.

The study recommends that

1. For the diagnosis of causative agent of diarrhoea in animals, modified Ziehl-Neelsen and iodine wet mount stain should be used for routine examination in laboratory.
2. Prevalence of zoonotic species of *Cryptosporidium* and *Giardia* in Jammu region, so awareness in general public, professionals who are dealing with animal and human health regarding zoonotic potential of bovine cryptosporidiosis is necessary.
3. *Curcuma longa* has promising anti cryptosporidial activity against experimental cryptosporidiosis in mice.

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Appendix

APPENDIX

Ammonium acetate (10M)

Ammonium acetate	77.08 g
Distilled water	100 ml

Antibiotic solution

Crystalline Penicillin G	100,000 IU/ 100 ml
Streptomycin	0.5g/ 100 ml
Gentamycin	0.25g/ 100 ml

Carbol fuchsin

Carbolic acid	2.5 ml
Absolute alcohol	5 ml
Basic Fuchsin	0.5 g
Distilled water	upto 50 ml

Filterd and stored at room temperature

5% Malachite green (w/v)

Malachite green (crystals)	5 g
Distilled water	100 ml

10% Sulphuric acid

Sulphuric acid (conc.)	50 ml
Distilled water	upto 500 ml

2.5 % Potassium dichromate (w/v)

Potassium Dichromate	2.5 g
Distilled water	100 ml

Sheather's sugar solution (normal)

Sucrose	500 g
Distilled water	320 ml
Phenol	6.5 g

Sheather's sugar solution (for modified Sheather's floatation)

Sucrose	500 g
Distilled water	300 ml
Phenol	6.8 g

Sheather's sugar solution (for discontinuous sucrose floatation)

Sucrose	500 g
Distilled wate	300 ml
Phenol	9 ml

1% Tween 80 (v/v)

Tween 80	1 ml
Distilled water	upto 100 ml

0.2% Tween 20 (w/v)

Tween 20	200 mg
Distilled water	100 ml

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Phenol (Equilibrated with 0.1M Tris-HCl)	250 ml
Chloroform	240 ml
Isoamyl alcohol	10 ml

1M Tris Cl (Tris-hydroxymethyl aminomethane) pH-8

Tris Base	121 g
Distilled water	800 ml

Adjust the pH to 8 by conc. HCl and add water to make 1000 ml

Lysis Buffer (for disruption of oocyst wall)

NaCl	138.6 mg
EDTA (0.5M)	2 ml
Tris (1M)	250 ml
Sarcosyn (1%)	1 ml
Polyvinyl Pyrollidone (PVPP 1%)	1 ml

Gel Loading Dye (6X)

Tris-HCl (pH 7.6)	10 mM
Bromophenol blue	0.03%
Xylene cyanol FF	0.03%
Glycerol	60%
EDTA	60 mM

Ethidium Bromide (10mg/ml)

Ethidium bromide	10 mg
Distilled Water	1 ml

Phosphate Buffered Saline

Sodium Chloride	8 g
Potassium Chloride	0.2 g
Disodium Hydrogen Phosphate	1.44 g
Potassium Dihydrogen phosphate	0.24 g
Distilled water to make up to	1000ml

Adjust pH to 7.2 or 7.4

0.5M EDTA

EDTA.2H_2O	18.16 g
Distilled water	80 ml

Stirred vigourously on a magnetic stirrer. Adjust pH to 8 and make up the volume to 100 ml, autoclave and store at 4^oC.

5M NaCl

NaCl	29.22 g
Distilled water	100ml

Autoclave and store

5X TBE buffer

Tris base	54 g
Boric Acid	27.5 g
0.5M EDTA (pH 8.0)	20 ml

Distilled water to make up to 1000ml

Tris EDTA (TE) buffer 1M (pH 8.0)

Tris-Hcl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Vita

VITA

Name of the student	Alveena Ganai
Father's name	Mr. Faiz Ahmad Ganai
Mother's name	Mrs. Rashida Ganai
Nationality	Indian
Date of birth	02-11-1988
Permanent home Address	H.No. EP/BC, 336 wazarat road, Near D.C Office, Jammu
Telephone no.	9419781318

EDUCATIONAL QUALIFICATION

EXAMINATION	YEAR OF PASSING	UNIVERSITY/ BOARD	PERCENTAGE
BVSC	2012	SKUAST-J	66.7%
MVSC	2014	SKUAST-J	81.7%
Ph.D	2019	SKUAST-J	81.6%

Title of Master's Thesis: Molecular characterization and Chemotherapeutic management of bovine cryptosporidiosis

1. Qualified ICAR-NET (October 2014).Indian council of agricultural research.
2. University scholarship during M.V.Sc
3. Qualified MANF (Maulana Azad National Fellowship) scholarship for Doctoral Studies in 2016.

MEMBERSHIP OF PROFESSIONAL SOCIETIES, AWARDS AND HONOURS:

1. Member of Indian association for the advancement of veterinary parasitology.
2. Awarded merit scholarship during MVSC programme by SKUAST JAMMU
3. Awarded best poster presentation on "first report of xenopharynx spp. from gall bladder of rat snake(ptyas korros) in jammu" in 2nd national IAVNAV conference on nutrition health interaction for optimum livestock production and human welfare, September 2013.

4. Awarded best poster presentation on “Molecular characterization and chemotherapeutic management of bovine cryptosporidiosis in jammu region” in Society for bioinformatics and biological sciences,October,2017.

CONFERENCES AND WORKSHOPS ATTENDED (NATIONAL/INTERNATIONAL)

1. National symposia ISVPT-2013 (NOV 20-22) 7TH annual conference(four abstracts)
2. International symposium on 21st century road map for vety practice education and research in india(Indian society for vety medicine-14-16 feb,2014)-(four abstract)
3. 2nd national symposium on interdisciplinary sciences (27-28feb,2015)GGM science college(Two abstract)
4. 2nd national IAVNAW conference (2013) vety nutrition(six abstracts)
5. Management of Coccidiosis in sheep and goat, current concept in sheep and goat production management and heath care.(article in book)
6. One world one health on 27th march, 2015 at FVSC and AH, R.S Pura jammu-two chapters (parasitic zoonoses: An overview and sustainable control of helminth parasites in jammu region).
7. Recent updates in small ruminants health and production-one chapter (Diagnosis of helminthic infections of small ruminants).
8. Society for bioinformatics and biological sciences, oct 12-13,2017(four abstract).
9. Innovative technological interventions for doubling farmers income, feb 8-10, 2018 (two abstracts).
10. XXVII National congress of veterinary parasitology and national symposium, feb 12-14,2018(two abstracts).
11. zoonotic parasites of livestock , diagnosis and control -An overview of helminthes of zoonotic and public health importance-one chapter.

PUBLICATIONS

1. Efficacy of Azithromycin in treatment of Cryptosporidiosis in naturally infected cattle calves (accepted), journal of animal research (NAAS RATING:4.49)
2. Incidence of *Buxtonella sulcata* in bovines in R.S Pura, Jammu, Journal of parasitic disease(NAAS RATING:4.65)

Certificate-IV

Certified that all the necessary corrections as suggested by the external examiner/ evaluator and the advisory committee have been duly incorporated in the thesis entitled **"Molecular studies on *Cryptosporidium* and *Giardia* species in cattle calves and therapeutic evaluation of *Curcuma longa* against experimental cryptosporidiosis in mice"** submitted by **Alveena Ganai**, Regd. No. **J-14-D-74-V**.

Dr. Anish Yadav
Chairman Advisory Committee

No: AU/FVSJ/19-20/VPA/226
Dated: 15-10-19

Dr. Rajesh Katoch
Head of Division

Annexure-I

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    --------------------------------------------------  Majority
             10        20        30        40        50
1   AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCC  KT151548.1.seq
1   --------------------------------------------------  KU679364.1.seq
1   --------------------------------------------------  MF671870.1.seq
1   --------------------------------------------------  MH183020.1 Jammu.seq
1   --------------------------------------------------  MK241967.1 Kathua .seq

    --------------------------------------------------  Majority
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1   --------------------------------------------------  MF671870.1.seq
1   --------------------------------------------------  MH183020.1 Jammu.seq
1   --------------------------------------------------  MK241967.1 Kathua .seq

    --------------------------------------------------  Majority
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1   --------------------------------------------------  KU679364.1.seq
1   --------------------------------------------------  MF671870.1.seq
1   --------------------------------------------------  MH183020.1 Jammu.seq
1   --------------------------------------------------  MK241967.1 Kathua .seq

    ----------------------------------------ATGGAAGGGT  Majority
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1   -------------------------------CTCGACTTTATGGAAGGGT  MF671870.1.seq
1   ------------------------------------------GGAAGGGT  MH183020.1 Jammu.seq
1   ------------------------------------------GGAAGGGT  MK241967.1 Kathua .seq

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751  GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGT  KT151548.1.seq
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     AACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAA  Majority
              860       870       880       890       900
851  AACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAA  KT151548.1.seq
661  AACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAA  KU679364.1.seq
670  AACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAA  MF671870.1.seq
658  AACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAA  MH183020.1 Jammu.seq
659  AACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAA  MK241967.1 Kathua .seq

     AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC  Majority
              910       920       930       940       950
901  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC  KT151548.1.seq
711  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC  KU679364.1.seq
720  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC  MF671870.1.seq
708  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC  MH183020.1 Jammu.seq
709  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC  MK241967.1 Kathua .seq

     GAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAG  Majority
              960       970       980       990      1000
951  GAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAG  KT151548.1.seq
761  GAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAG  KU679364.1.seq
770  GAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAG  MF671870.1.seq
758  GAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAG  MH183020.1 Jammu.seq
759  GAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAG  MK241967.1 Kathua .seq

     AGATTGGAGGTTGT-TCCTTACTCCTTCAGCACCTTATGAGAXXXXXXXX  Majority
             1010      1020      1030      1040      1050
1001 AGATTGGAGGTTGT-TCCTTACTCCTTCAGCACCTTATGAGAAATCAAAG  KT151548.1.seq
811  AGATTGGAGGTTGT-TCCTTACTCCTTCAGCACCTTATGAGA          KU679364.1.seq
820  AGATTGGAGGTTGT-TCCTTACTCCTTCAGCACCTTATGAGAAATCAAAG  MF671870.1.seq
808  AGATTGGAGGTTGTCTCCTTACTCCTT                         MH183020.1 Jammu.seq
809  AGATTGGAGGTTGT-TCCTTACTCCTT                         MK241967.1 Kathua .seq
```

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       X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X   Majority
                           1060                1070                1080                1090                1100
1050   T C T T T G G G T T C T G G G G G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G G A A T   KT151548.1.seq
851                                                                                                         KU679364.1.seq
869    T                                                                                                    MF671870.1.seq
834                                                                                                         MH183020.1 Jammu.seq
834                                                                                                         MK241967.1 Kathua .seq

       X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X   Majority
                           1110                1120                1130                1140                1150
1100   T G A C G G A A G G G C A C C A C C A G G A G T G G A G C C T G C G G C T T A A T T T G A C T C A A   KT151548.1.seq
851                                                                                                         KU679364.1.seq
869                                                                                                         MF671870.1.seq
834                                                                                                         MH183020.1 Jammu.seq
834                                                                                                         MK241967.1 Kathua .seq

       X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X   Majority
                           1160                1170                1180                1190                1200
1150   C A C G G G A A A A C T C A C C A G G T C C A G A C A T A G G A A G G A T T G A C A G A T T G A T A   KT151548.1.seq
851                                                                                                         KU679364.1.seq
869                                                                                                         MF671870.1.seq
834                                                                                                         MH183020.1 Jammu.seq
834                                                                                                         MK241967.1 Kathua .seq

       X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X X   Majority
                           1210                1220                1230                1240                1250
1200   G C T C T T T C T T G A T T C T A T G G G T G G T G G T G C A T G G C C G T T C T T A G T T G G T G   KT151548.1.seq
851                                                                                                         KU679364.1.seq
869                                                                                                         MF671870.1.seq
834                                                                                                         MH183020.1 Jammu.seq
834                                                                                                         MK241967.1 Kathua .seq

       X X                                                                                                  Majority

1250   G A                                                                                                  KT151548.1.seq
851                                                                                                         KU679364.1.seq
869                                                                                                         MF671870.1.seq
834                                                                                                         MH183020.1 Jammu.seq
834                                                                                                         MK241967.1 Kathua .seq
```

Decoration 'Decoration #1': Shade (with solid dark green) residues that differ from the Consensus.

Sequence pair distances of Untitled ClustalW (Slow/Accurate, IUB)

Friday, March 29, 2019 4:57 PM

Percent Identity

Divergence

	1	2	3	4	5		
1		68.0	69.5	66.5	66.7	**1**	KT151548.1.seq
2	0.0		100.0	97.8	98.0	**2**	KU679364.1.seq
3	0.0	0.0		95.7	96.0	**3**	MF671870.1.seq
4	0.1	0.1	0.1		99.8	**4**	MH183020.1 Jammu.seq
5	0.0	0.0	0.0	0.1		**5**	MK241967.1 Kathua .seq
	1	**2**	**3**	**4**	**5**		

Annexure-II

Sequence one: Subtype IIaA15G2R1

```
  1 gatgttcctg ttgagggctc atcatcgtca tcgtcatcat catcatcatc atcatcatca
 61 tcatcatcaa catcaaccgt cgcaccagca aataaggcaa gaactggaga agacgcagaa
121 ggcagtcaag attctagtgg tactgaagct tctggtagcc agggttctga agaggaaggt
181 agtgaagacg atggccaaac tagtgctgct tcccaaccca ctactccagc tcaaagtgaa
241 ggcgcaacta ccgaaaccat agaagctact ccaaaagaag aatgcggcac ttcatttgta
301 atgtggttcg gagaaggtac cccagctgcg acattgaagt gtggtgccta cactatcgtc
361 tatgcaccta taaaagacca aacagatccc gcaccaagat atatctctgg tgaagttaca
421 tctgtaacct ttgaaaagag tgataataca gttaaaatca aggttaacgg tcaggatttc
481 agcactctct ctgctaattc aagtagtcca actgaaaatg gcggatctgc gggtcaggct
541 tcatcaagat caagaagatc actctcagag gaaaccagtg aagctgctgc aaccgtcgat
601 ttgtttgcct ttacccttga tggtggtaaa agaattgaag tggctgtacc aaacgtcgaa
661 gatgcatcta aaagagacaa gtacagtttg gttgcagacg ataaaccttt ctataccggc
721 gcaaacagcg gcactaccaa tggtgtctac aggttgaatg agaacggaga cttggttgat
781 aaggac
```

Sequence two: Subtype IIaA14G2R1

```
 1  gaactttaaa  ggatgttcct  gttgagggct  catcatcgtc  atcgtcatca  tcatcatcat
61 catcatcatc atcatcaaca tcaaccgtcg caccagcaaa taaggcaaga actggagaag 121
acgcagaagg  cagtcaagat  tctagtggta  ctgaagcttc  tggtagccag  ggttctgaag 181
aggaaggtag  tgaagacgat  ggccaaacta  gtgctgcttc  ccaacccact  actccagctc  241
aaagtgaagg  cgcaactacc  gaaaccatag  aagctactcc  aaaagaagaa  tgcggcactt 301
catttgtaat  gtggtccgga  gaaggtaccc  cagctgcgac  attgaagtgt  ggtgcctaca 361
ctatcgtcta  tgcacctata  aaagaccaaa  cagatcccgc  accaagatat  atctctggtg 421
aagttacatc  tgtaaccttt  gaaaagagtg  ataatacagt  taaaatcaag  gttaacggtc 481
aggatttcag  cactctctct  gctaattcaa  gtagtccaac  tgaaaatggc  ggatctgcgg 541
gtcaggcttc  atcaagatca  agaagatcac  tctcagagga  aaccagtgaa  gctgctgcaa 601
ccgtcgattt  gtttgccttt  acccttgatg  gtggtaaaag  aattgaagtg  gctgtaccaa 661
acgtcgaaga  tgcatctaaa  agagacaagt  acagtttggt  tgcagacgat  aaacctttct 721
ataccggcgc aaacagcggc actaccaatg gtgtctacag gttgaa
```

Annexure-III

GAACGAACGAGATCGAGGTCCGCCGCGTCGACGACGACACGCGCGTGAAG Majority

10 20 30 40 50

1 GAACGAACGAGATCGAGGTCCGCCGCGTCGACGACGACACGCGCGTGAAG MG736239.1.seq
1 GAACGAACGAGATCGAGGTCCGCCGCGTCGACGACGACACGCGCGTGAAG 472824.1.seq
1 GAACGAACGAGATCGAGGTCCGCCGCGTCGACGACGACACGCGCGTGAAG Jammu isolate.seq
1 GAACGAACGAGATCGAGGTCCGCCGCGTCGACGACGACACGCGCGTGAAG KY320576.1.seq

ATGATCAAGGACGCCATCGCACACCTCGACAGGCTCATCCAGACGGAGTC Majority

60 70 80 90 100

51 ATGATCAAGGACGCCATCGCACACCTCGACAGGCTCATCCAGACGGAGTC MG736239.1.seq
51 ATGATCAAGGACGCCATCGCACACCTCGACAGGCTCATCCAGACGGAGTC 472824.1.seq
51 ATGATCAAGGACGCCATCGCACACCTCGACAGGCTCATCCAGACGGAGTC Jammu isolate.seq
51 ATGATCAAGGACGCCATCGCACACCTCGACAGGCTCATCCAGACGGAGTC KY320576.1.seq

GAGGAAGCGCCAGGCCTCGTTCGAGGACATCCGCGAGGAGGTCAAGAAGT Majority

110 120 130 140 150

101 GAGGAAGCGCCAGGCCTCGTTCGAGGACATCCGCGAGGAGGTCAAGAAGT MG736239.1.seq
101 GAGGAAGCGCCAGGCCTCGTTCGAGGACATCCGCGAGGAGGTCAAGAAGT 472824.1.seq
101 GAGGAAGCGCCAGGCCTCGTTCGAGGACATCCGCGAGGAGGTCAAGAAGT Jammu isolate.seq
101 GAGGAAGCGCCAGGCCTCGTTCGAGGACATCCGCGAGGAGGTCAAGAAGT KY320576.1.seq

CCGCCGACAACATGTACCTAACGATCAAGGAGGAGATCGACACCATGGCT Majority

160 170 180 190 200

151 CCGCCGACAACATGTACCTAACGATCAAGGAGGAGATCGACACCATGGCT MG736239.1.seq
151 CCGCCGACAACATGTACCTAACGATCAAGGAGGAGATCGACACCATGGCT 472824.1.seq
151 CCGCCGACAACATGTACCTAACGATCAAGGAGGAGATCGACACCATGGCT Jammu isolate.seq
151 CCGCCGACAACATGTACCTAACGATCAAGGAGGAGATCGACACCATGGCT KY320576.1.seq

GCAAACTTCCGCAAGTCCCTTGCGGAGATGGGCGACACACTCAACAACGT Majority

210 220 230 240 250

201 GCAAACTTCCGCAAGTCCCTTGCGGAGATGGGCGACACACTCAACAACGT MG736239.1.seq
201 GCAAACTTCCGCAAGTCCCTTGCGGAGATGGGCGACACACTCAACAACGT 472824.1.seq
201 GCAAACTTCCGCAAGTCCCTTGCGGAGATGGGCGACACACTCAACAACGT Jammu isolate.seq
201 GCAAACTTCCGCAAGTCCCTTGCGGAGATGGGCGACACACTCAACAACGT KY320576.1.seq

TGAGACAAATCTCCAGAACCAGATCGCCATCCATAACGACGCCATCGCGG Majority

260 270 280 290 300

251 TGAGACAAATCTCCAGAACCAGATCGCCATCCATAACGACGCCATCGCGG MG736239.1.seq
251 TGAGACAAATCTCCAGAACCAGATCGCCATCCATAACGACGCCATCGCGG 472824.1.seq
251 TGAGACAAATCTCCAGAACCAGATCGCCATCCATAACGACGCCATCGCGG Jammu isolate.seq
251 TGAGACAAATCTCCAGAACCAGATCGCCATCCATAACGACGCCATCGCGG KY320576.1.seq

CTCTCAGGAAGGAGGCCCTCAAGAGCTTGAACGACCTCGAGACGGGCATT Majority

310 320 330 340 350

301 CTCTCAGGAAGGAGGCCCTCAAGAGCCTGAACGACCTCGAGACGGGCATT MG736239.1.seq
301 CTCTCAGGAAGGAGGCCCTCAAGAGCTTGAACGACCTCGAGACGGGCATT 472824.1.seq
301 CTCTCAGGAAGGAGGCCCTCAAGAGCTTGAACGACCTCGAGACGGGCATT Jammu isolate.seq
301 CTCTCAGGAAGGAGGCCCTCAAGAGCTTGAACGACCTCGAGACGGGCATT KY320576.1.seq

GCCACGGAGAACGCAGAAAGGAAGAAGATGTACGACCAGCTCAACGAGAA Majority

360 370 380 390 400

351 GCCACGGAGAACGCAGAAAGGAAGAAGATGTACGACCAGCTCAACGAGAA MG736239.1.seq
351 GCCACGGAGAACGCAGAAAGGAAGAAGATGTACGACCAGCTCAACGAGAA 472824.1.seq
351 GCCACGGAGAACGCAGAAAGGAAGAAGATGTACGACCAGCTCAACGAGAA Jammu isolate.seq
351 GCCACGGAGAACGCAGAAAGGAAGAAGATGTACGACCAGCTCAACGAGAA KY320576.1.seq

```
     GGTCGCAGAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGACGA  Majority
                         410       420       430       440       450
401  GGTCGCAGAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGACGA  MG736239.1.seq
401  GGTCGCAGAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGACGA  472824.1.seq
401  GGTCGCAGAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGACGA  Jammu isolate.seq
401  GGTCGCAGAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGACGA  KY320576.1.seq

     TCGCCCGCGAGAGGGCCGTTAGTGCTGCCACGACAGAAGCGCTCACAAAC  Majority
                         460       470       480       490       500
451  TCGCCCGCGAGAGGGCCGTTAGTGCTGCCACGACAGAAGCGCTCACAAAC  MG736239.1.seq
451  TCGCCCGCGAGAGGGCCGTTAGTGCTGCCACGACAGAAGCGCTCACAAAC  472824.1.seq
451  TCGCCCGCGAGAGGGCCGTTAGTCAAGACGTGCTGGACGCGCTCACAAAC  Jammu isolate.seq
451  TCGCCCGCGAGAGGGCCGTTAGTGCTGCCACGACAGAAGCGCTCACAAAC  KY320576.1.seq

     ACGAAGCTCGTCGAGX  Majority
                         510
501  ACGAAGCTCGTCGAG   MG736239.1.seq
501  ACGAAGCTCGTCGAGA  472824.1.seq
501  ACGACGCTCGGCGAG   Jammu isolate.seq
501  ACGAAGCTCGTCGAG   KY320576.1.seq
```

Decoration 'Decoration #1': Shade (with solid bright yellow) residues that differ from the Consensus.

Sequence pair distances of Untitled ClustalW (Slow/Accurate, IUB)
Friday, March 29, 2019 4:14 PM

Percent Identity

Divergence

	1	2	3	4		
1		99.8	97.5	99.8	**1**	MG736239.1.seq
2	0.2		97.5	99.8	**2**	472824.1.seq
3	2.6	2.4		97.7	**3**	Jammu isolate.seq
4	0.2	0.0	2.4		**4**	KY320576.1.seq
	1	**2**	**3**	**4**		