"Evaluation of Lateral Flow Test for Detection of *Cryptosporidium* species in human fecal specimens"

A Dissertation submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Master of Pharmacy.

Submitted By

Rajashree Chowdhury

ID: 2014-3-79-021

Fall 2015

Department of Pharmacy

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Supervised by

Dr. Sufia Islam

Associate Professor

Department of Pharmacy

East West University

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This thesis paper

Is dedicated

To my beloved Parents…

DECLARATION BY THE CANDIDATE

I, Rajashree Chowdhury, hereby declare that this dissertation, entitled **"Evaluation of Lateral Flow Test for Detection of *Cryptosporidium* species in Human Fecal specimens"** submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Master of Pharmacy, is a genuine & authentic research work carried out by me under the guidance of Dr. Sufia Islam, Associate Professor, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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CERTIFICATION BY THE SUPERVISOR

This is to certify that the desertion, entitled **"Evaluation of Lateral Flow Test for Detection of *Cryptosporidium* species in Human Fecal specimens"** is a benefice research work done, under our guidance and supervision by Rajashree Chowdhury(ID: 2014-3-79-021), in partial fulfillment of the requirement for the degree of Master of Pharmacy.

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ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation, entitled **"Evaluation of Lateral Flow Test for Detection of Cryptosporidium species in human Fecal specimens"** is a bonafide research work done by Rajashree Chowdhury (ID: 2014-3-79-021), in partial fulfillment of the requirements for the degree of Master of Pharmacy.

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Approval Certificate

This is to certify that the dissertation entitled **"Evaluation of Lateral Flow Test for Detection of *Cryptosporidium* species in Human Fecal specimens"** was carried out by Rajashree Chowdhury, ID: 2014-3-79-021 for the fulfillment of the requirement for the degree, Master of Pharmacy for the session Summer-2015 of East West University, Dhaka, Bangladesh.

This work was carried out under our supervision and the style and content of the dissertation have been approved and recommended for the award of Master degree.

Dr. Rashidul Haque
Senior Scientist & Head,
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Abstract

Cryptosporidium is common cause of waterborne diarrheal disease worldwide, both in developing and developed countries as well as urban and rural areas; due to poor sanitation and urban crowding, *Cryptosporidium* infections are more prevalent in underdeveloped areas. There is need for a practical point-of-care diagnostic test that is rapid, reliable and feasible for use in the field. Since *Cryptosporidium* species causing invasive intestinal disease and infection, it is important to detect *Cryptosporidium* in diarrheal fecal efficiently and instantly. In clinical sites it is necessary to detect *Cryptosporidium* pathogen using a reliable rapid point of care test over other conventional diagnostic tools like microscopy. A new rapid lateral flow fecal antigen detection test for *Cryptosporidium* was evaluated using diarrheal stool samples from a cohort of children in Bangladesh. In this study, The *Cryptosporidium EZ VUE* test (developed and manufactured by TechLab®, Blacksburg, VA) was analyzed to establish it as an alternative method to detect *Cryptosporidium*-specific oocyst antigens in fecal specimens in clinical areas within 10 minutes and with less skilled personnel. In this study, fecal samples were collected from Mirpur slum area of Dhaka city. A total of 100 diarrheal samples were collected from100 children whose age ranges from 0-24 months. The diagnostic performance of recently released rapid test was compared with *Cryptosporidium* II Enzyme Linked Immuno-sorbent Assay (ELISA) from TechLab® *Cryptosporidium QUIK CHEK*™ , real time PCR, and *Cryptosporidium QUIK CHEK*™. The *Cryptosporidium EZ VUE* test was exhibited 100% sensitivity and 93.94% specificity and the 89.47% positive predictive value and 100% negative predictive value with compared to the *Cryptosporidium* II ELISA; were found to be 70% sensitivity and 94% specificity, and 92.1% positive predictive value and 75.81% negative predictive value as compared with RT-PCR; and were found to be 100% sensitivity and 98.41% specificity, accordingly, 97.37% positive predictive value and 100% negative predictive value as compared with *Cryptosporidium QUIK CHEK*™. Furthermore, *Cryptosporidium EZ VUE* test was evaluated with *E. histolytica* positive samples and *Giardia* positive samples by cross check reactions. All positive samples were exposed as negative by *Cryptosporidium EZ VUE* test. That means Lateral Flow method had no cross reaction with both *E. histolytica* and *Giardia* positive samples. These results indicate that *EZ VUE* assay can be successfully used for specific and rapid detection of *Cryptosporidium* oocysts in clinical fecal samples and can also be implemented as an alternative method of conventional microscopy in both research center and in clinical sites.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
APCs	Antigen presenting cells
C.	*Cryptosporidium*
CD4	(Cluster of differentiation 4) is a glycoprotein expressed on the surface of T helper cells.
CD8	(Cluster of differentiation 8) is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor.
cDNA	Complementary DNA
COWP	*Cryptosporidium* oocyst wall protein
CSL	Circumsporozoite-like glycoprotein
Ct	Threshold Cycle
dH2O	Di-Ionized Water
DNA	Di-Ribinucleic Acid
dsDNA	Double Stranded DNA
ELISA	Enzyme Linked Immuno-sorbent Assay
Gp60	Glycoprotein 60
NPV	Negative Predictive Value
NTZ	Nitazoxanide
OD	Optical Density
PCR	Polymerase Chain Reaction

PPV	Positive Predictive Value
RDT	Rapid Diagnostic Test
RFU	Relative Fluorescence Unit
RNA	Ribonucleic Acid
RT-PCR	Real-Time PCR
TNA	Total Nucleic Acid
TRAP-C1	Thrombospondin-related adhesive protein *Cryptosporidium* 1

Chapter One

Introduction

1.1 Background

Diarrheal diseases are extremely common in the developed and developing worlds and are major causes of morbidity and mortality, affecting millions of individuals each year (Liu *et al*., 2012). In Bangladesh, 1 in 30 children dies of diarrhea or dysentery by his or her fifth birthday (Mondal *et al*., 2009).The etiological agents of diarrhea include viruses, bacteria, and parasites. Infections with intestinal parasites are an important cause of diarrheal illness, with at least one-third of the world's population is believed to be infected with intestinal parasites (Huh *et al*., 2009).

The protozoan *Cryptosporidium* is a worldwide cause of enteric infections in both humans and animals, and, due to the zoonotic character of some of its species, is among the most relevant parasitic enteric agents in human. Human infections caused by *Cryptosporidium* spp. are termed cryptosporidiosis. It has major public health implications because infections can result from exposure to low doses of *Cryptosporidium* oocysts. Humans can acquire *Cryptosporidium* infections through several transmission routes, such as direct contact with infected persons or animals, and ingestion of contaminated food (food borne transmission) and water (waterborne transmission) (Xiao, 2010).

The greatest burden of this disease occurs in developing countries, *Cryptosporidium* has been recognized as a major cause of many waterborne and food-borne outbreaks of gastroenteritis in developed countries (Snelling *et al*., 2007).

The intestinal malabsorption caused by cryptosporidiosis in developing countries further affects particularly malnourished children (Desai *et al*., 2012). It was estimated that 1 to 10% of the populations in developing countries were infected with *Cryptosporidium*, wherein 1 to 9 year-old children and toddlers were the most affected groups (Lozano *et al*., 2012).

Protozoan parasites of the genus *Cryptosporidium*, primarily *Cryptosporidium parvum* and *Cryptosporidium hominis*, are increasingly recognized as common causes of persistent and chronic diarrhea in children, as well as immune-deficient adults worldwide contributing to the high diarrhea-related morbidity and mortality in these populations (White, 2010). *Cryptosporidium* spp. is a leading cause of diarrhea, particularly persisitant diarrhea in children in developing countries .In these areas of the world, *Cryptosporidium* spp. infection has been reported to be more common in malnourished children than in well-nourished children and the

consequences of the disease are more severe in the former than the latter. While infections do occur in immunocompetent hosts, immunocompromised hosts and children tend to have a more severe and prolonged disease course (Fayer, 2010) .There is need for a practical point-of-care diagnostic test that is rapid, reliable and feasible for use in the field.

Cryptosporidiosis has been reported from over 40 countries, in both immunocompetent and immunocompromised patients. The most severe clinical symptoms of cryptosporidiosis are observed in patients with acquired immune deficiency syndrome (AIDS) (Kurniawan *et al*., 2009).Thus, the ability of clinical laboratories to quickly and accurately diagnose infections with these parasites is crucial for the treatment of patients and to the prevention of further spread of disease.

Current diagnostic methods include microscopic examination of stool for Cryptosporidium species oocysts with acid-fast stains (modified Ziehl-Neelsen, Auramine-O, or Kinyoun's), Quik Chek Tests, antigen-based enzyme immunoassays (EIAs), PCR, and lateral-flow immunochromatographic ''strip'' tests (White, 2010). The sensitivity of these tests range from 10^3 to 10^5 oocysts/g of stool, with PCR being the most sensitive and acid-fast microscopy the least, often requiring examination of multiple stool samples in order to report a negative test result (White, 2010; Calderaro *et al*., 2011).

Real-time PCR is the most sensitive method, but it is expensive and requires skilled personnel, which limits its use. Fecal antigen detection is becoming more widely used, but requires technical expertise (Haque *et al*, 2007). Development of a rapid fecal antigen detection test would provide a simple method for diagnosis that may be easily applied in resource-limited settings.

With the exception of lateral-flow tests, which provide the advantage of a simple qualitative result (Garcia *et al*., 2009), existing diagnostic techniques remain confined to clinical laboratories because of the high instrumentation and reagent costs, and the need for a highly skilled technician or pathologist.

1.2 Classification and Nomenclature of *Cryptosporidium*

According to Integrated Taxonomic Information System, 2013,below is the taxonomic classification of the genus *Cryptosporidium:*

Kingdom – Protozoa

Phylum - Apicomplexa

Class – Conoidasida

Order - Eucoccidiorida

Suborder - Eimeriorina

Family - Cryptosporidiidae

Genus - *Cryptosporidium*

(Tyzzer, 1907)

Tyzzer discover *Cryptosporidium*, the host specificity, location in the host, and morphology characteristic have been the basis for taxonomy classification for species of the phylum Aplicomplexa (Fayer, 2010). From the decade of the 70s until the 90s, it was believed that only one species (*Cryptosporidium muris*) parasitized the gastric mucosa of mammals, while *Cryptosporidium parvum* parasitized the intestine of mammals (Fayer, 2010). The naming of a new species occurs now if the biological and genetic information is sufficient to identify an isolate as unique (Fayer, 2010).

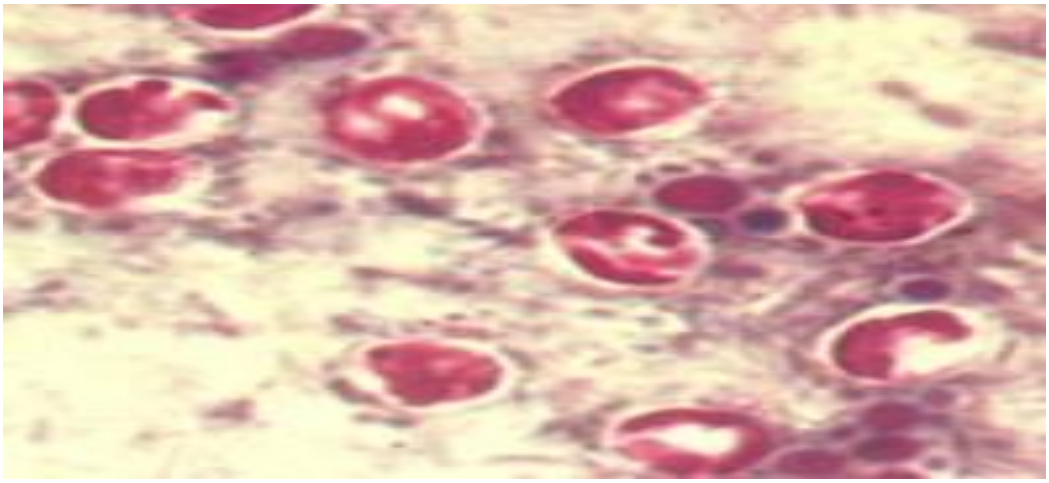


Figure1.1: *Cryptosporidium oocysts, under microscopy*

Traditionally, *Cryptosporidia* as protozoan parasites due to great similarities are classified in the Coccidia class of the phylum Apicomplex, although *Cryptosporidia* show features which differ them from all other Coccidia (Clopton,2009): among them are the intracellular, but extracytoplasmic localization, the forming of a "feeder" organ, the forming of two both morphological (thin- or thick- walled) and functional (auto versus new-infection) types of oocysts, the small size of oocysts, missing morphological characteristics like e.g. sporocysts or micropyles and the insensitivity of *Cryptosporidia* to all so far tested anti-coccidial compounds (Xiao & Cama, 2006).

Phylogenetic analyses using small-subunit (SSU) rRNA sequences have indicated that the genus *Cryptosporidium* does not form a monophyletic clade with either intestinal (*Eimeria, Isospora*) or cyst-forming (*Sarcocystis,Toxoplasma*) eucoccidia and that the genus is instead a sister-group of the Coccidia- Haematozoa (Fayer, 2010). In fact, the class Coccidia was shown to be monophyletic only if the genus *Cryptosporidium* was excluded from the analysis (Fayer, 2010).These studies suggest that *Cryptosporidium* may be an early emerging lineage among the apicomplexans (Lebbad *et al.*, 2013).

1.3 Species and host range

Currently, 26 morphologically, biologically and molecular-biologically confirmed different *Cryptosporidium* species are listed (Adamu *et al*., 2014), having mammals (primates, bovidae, equidae, carnivora, hares, rabbits, tapiridae and rhinocerotidae), amphibians, reptiles and birds as hosts.

Primarily, *C. parvum* is considered the zoonotic species of human cryptosporidiosis; infection in most cases is due to water contaminated from animal faeces and/or due to contact with animals. Further human cases are caused by *C. hominis* via the anthroponotic pathway (Lendner *et al.*, 2011). A true host specificity of the species in most cases, however, does not exist and after *C. baileyi, C. canis, C. felis, C. meleagridis, C. bovis, C.suis, C. andersoni* and *C. muris* also were detected in cases of human cryptosporidiosis,these species, further to *C. parvum,* also have to be considered potentially zoonotic (Helmy *et al.,* 2013). The zoonotic potential of these species has to be judged as lower, although not in immunosuppressed persons (Lendner *et al*., 2011).

Differences among *Cryptosporidium* species: their major hosts, oocyst sizes and locations in the gut include in table (1.1).

Table 1.1: Differences among *Cryptosporidium* species: their major hosts, oocyst sizes and locations in the gut(Caccio and Widmer, 2014)

***Cryptosporidium* spp.**	**Hosts**	**Oocyst size (µm)**	**Location**
C. parvum	Ruminants, humans, deer	4.5 x 5.5	Small intestine
C. andersoni	Ruminants, camel	5.5 x 7.4	Abomasum
C. hominis	Humans	4.5 x 5.5	Small intestine
C. meleagridis	Birds, humans	4.5-5.0 x 4.6-5.2	Intestine
C. suis	Pigs, humans	5.1 x 4.4	Small intestine
C. muris	Rodents, humans	5.6 x 7.4	Stomach
C. canis	Canids, humans	5.0 x 4.7	Small intestine
C. felis	Felids, humans	4.5 x 5.0	Small intestine

1.4 Life cycleof *Cryptosporidium*

Like related coccidian parasites, *Cryptosporidium* spp. feature asexual and sexual components in their life cycles and produce thick-walled, environmentally hardy oocysts. Unique features include the absence of a sporulation period outside the host (oocysts are infectious as they are shed in the stool), the lack of sporocysts within the oocyst (i.e., sporozoites are "naked" within the oocyst), and autoinfection (the life cycle recurs within the same host, a consequence of thin-walled oocyst production) (Hijjawi *et al*., 2009; Valenzuela *et al*., 2014).

The life cycle of *Cryptosporidium* is monoxenous (Requiring only one host to complete the life cycle), completed within gastrointestinal tract of a host (Putignani *et al*., 2009) or respiratory tract of such host (Hijjawi *et al*., 2009). Infection (and hence presumably excystation) has also been reported in other sites (often contiguous with the intestinal tract), such as the biliary tract, pancreatic tract, which are also lined with epithelial cells (Putignani*et al*., 2009).

After ingestion of the infective oocyst, excystation of the four sporozoites is triggered mainly by the change in temperature and pH. The sporozoites migrate along the surface of the epithelium until they find a place to attach. This process is driven by a complex biochemical mechanisms that include interaction of *Cryptosporidium* sporozoites with the host cell's cytoskeleton. This process has been called gliding motility (O'Hara & Chen, 2011). The formation of the parasitophorus vacuole occurs after being encapsulated by a parasite modified host membrane. This process is known as internalization (O'Hara & Chen, 2011). During internalization, the feeder organelle(located at the base of the parasitophorous vacuole) is formed between the parasite and host cytoplasm. This organelle confers selective transport properties between host and parasite for nutrients uptake (O'Hara & Chen, 2011).

Type I, followed by TypeII meronts develop next. These are derived from the asexual reproduction of the trophozoite in the process known as endopolygeny. The formation of the daughter cells occurs while still in the mother cell (O'Hara & Chen, 2011). The type I meront produce merozoites that are morphologically and biologically similar to the sporozoites. These merozoites invade the surrounding enterocytes and can produce meronts type I and II (O'Hara & Chen, 2011; Scorza & Lappin, 2012).

Merozoites, derived from Type II meronts, differentiate into gametocytes to complete the sexual stage of development. These gametocytes can be either male or female reproductive stages, known as microgametocyte and macrogametocyte respectively (O'Hara & Chen, 2011). The fertilizationof the macrogametocyte by the microgametocyte results in the only diploid stage of development (the zygote), which undergoes sporogony process (meiosis-like process) resulting in the production of a sporulated oocyst containing four sporozoites. This oocyst can be thin or thick-walled, the thick-walled oocysts are shed in the feces, and the thin-walled oocyst excysts within the intestinal lumen starting a process of autoinfection and escalating the infection level (O'Hara & Chen, 2011).

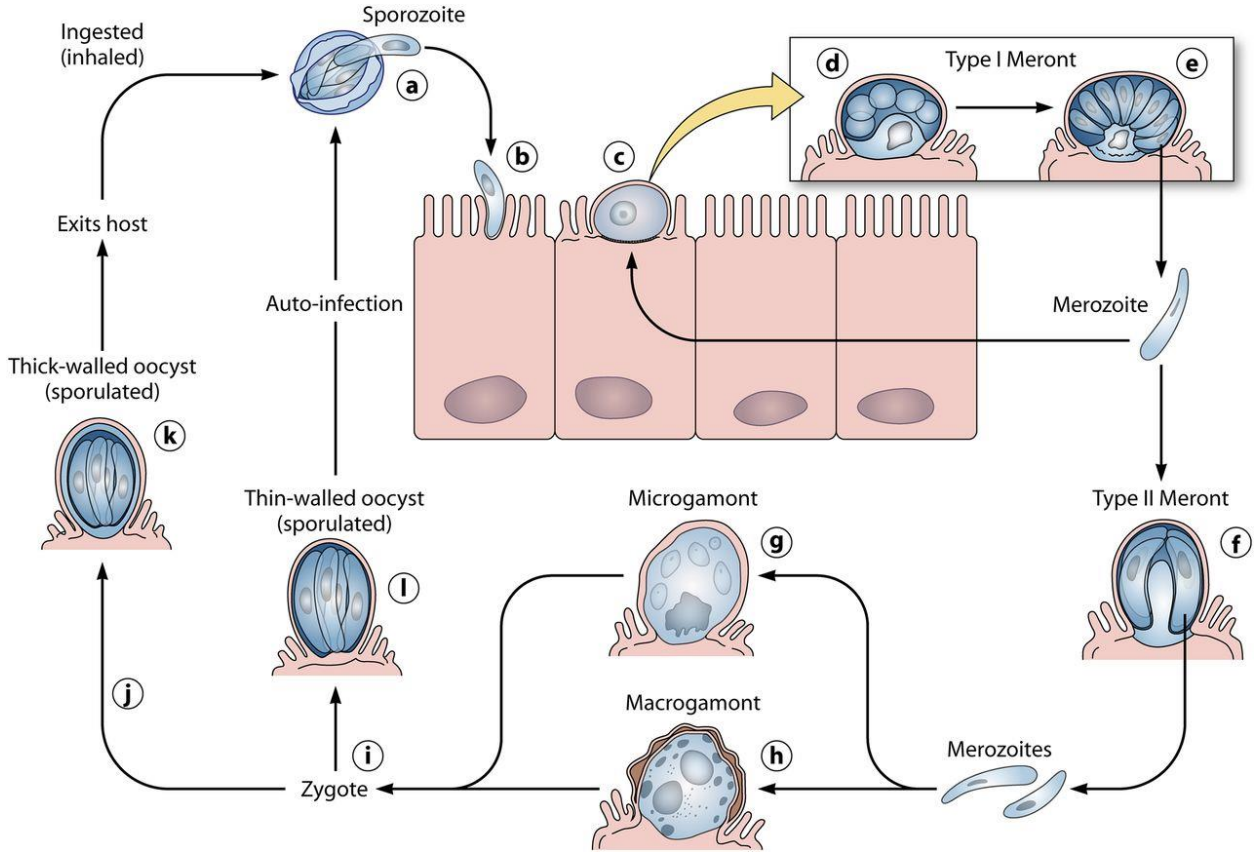


Figure 1.2: Life Cycle of *Cryptosporidium* spp.(Borowski *et al*., 2008).

1.4.1 Morphology of *Cryptosporidium* zoite

The typical zoites (merozoites or sporozoites) of *Cryptosporidium* are similar to other apicomplexans; they present crescent shaped cell body, apical rhoptry and micromeres, and dense granules distributed throughout the cytoplasm (O'Hara & Chen, 2011). The parasite surface (pellicle) is a multilayer membrane; the outer and inner membranes are each composed from two membranes and subpellicular microtubules (O'Hara & Chen, 2011).

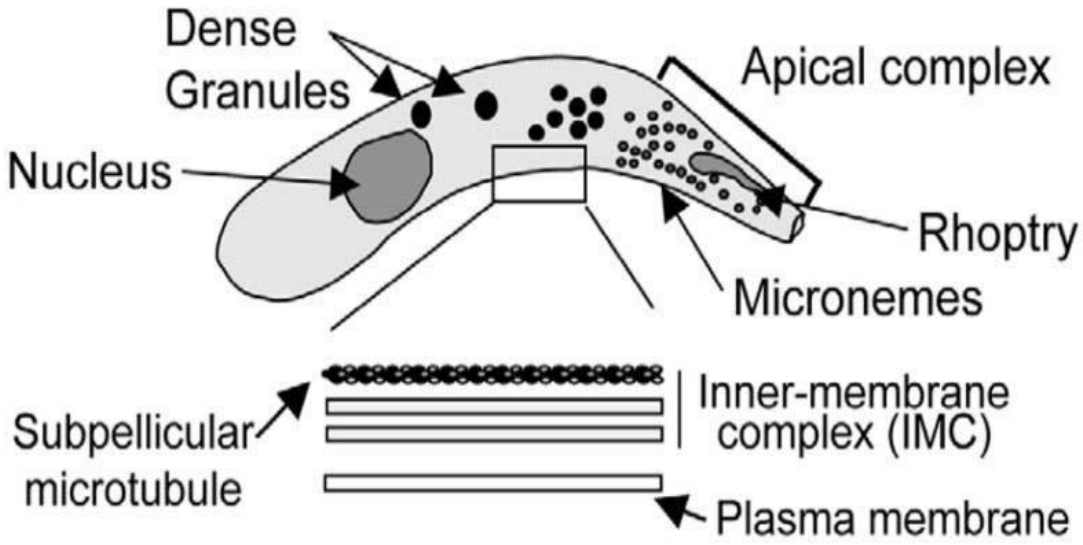


Figure 1.3: Scheme of the morphologic characteristics of a *Cryptosporidium* zoite.

The endogenous stages of the parasites are closely associated with the luminal surface of the epithelial cells; they protrude from the cell surface. These bodies have spherical or elliptical shapes with sizes ranging from 2 to 6µm. Their location has been determined to be intracellular but extracytoplasmic within the parasitophorus vacuoles membranes (O'Hara & Chen, 2011). The pellicle folds repeatedly forming a structure that adheres to the microvilli (O'Hara & Chen, 2011).

The oocyst is the exogenous, infective, and environmental-resistant form of the parasite. Mature oocysts contain 4 sporozoites enclosed within a oocyst. This configuration provides some of the characteristics for its visual classification. The oocysts vary in size and shape depending on the species, ranging from 4.5 to 8 µm in length by 4 to 6.5 in width (Yarlett *et al.*, 2007).

1.5 Route of Transmission of*Cryptosporidium*

The main way of transmission is the fecal-oral route; by ingestion of the infective oocysts contaminating water or food sources, grooming, or the ingestion of infected preys (Scorza & Lappin, 2012). The oocysts are resistant to several environmental conditions, as well as common disinfectants (Scorza & Lappin, 2012). In human populations, the contamination of public water supplies can lead to large outbreaks of cryptosporidiosis (Elwin *et al*., 2012).

Direct or indirect person-to-person transmission of infection is also a well recognised source of infection. Finally, zoonotic transmission has been widely recognised as an important route for *Cryptosporidium* infection particularly with *C. parvum* species (Elwin *et al*., 2007).

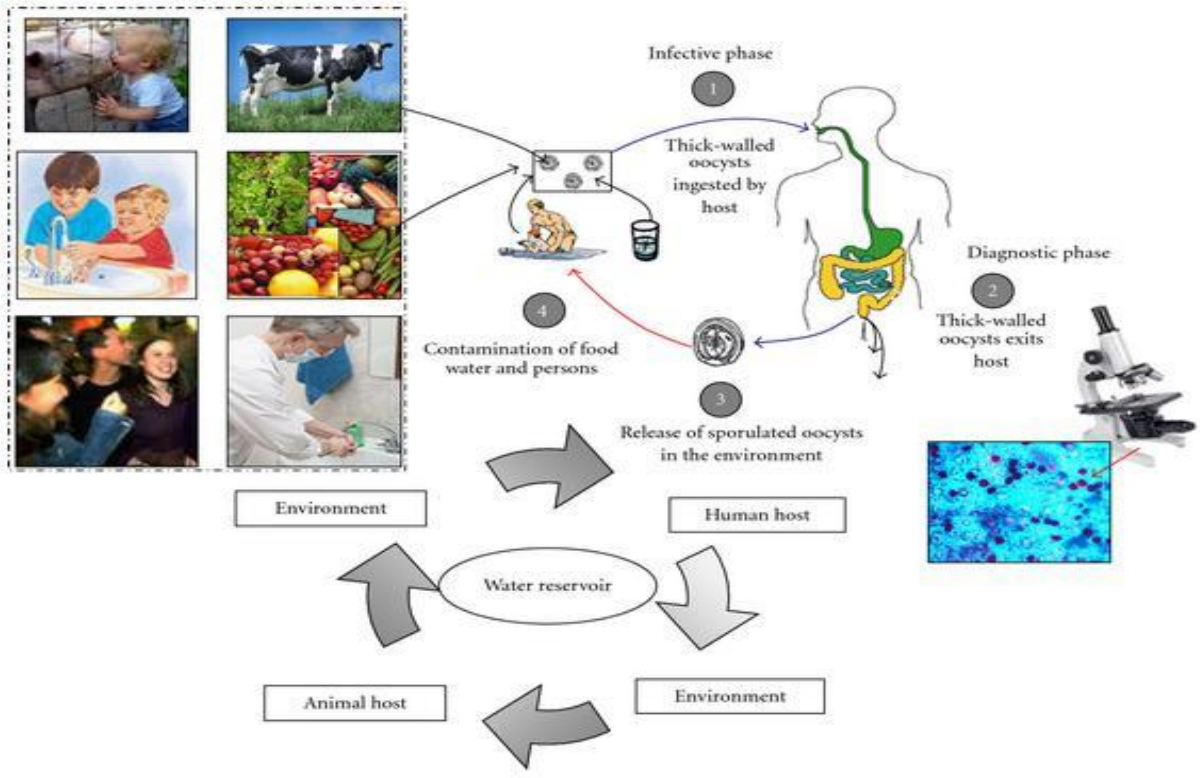


Figure 1.4: Description of transmission modes of *Cryptosporidium* (Putignani and Menchella, 2010)**.**

1.6 Infectivity

In addition to the many routes of transmission, there are five major characteristics that make these protozoa highly infectious pathogens. First, both oocysts/cysts are shed into the environment in relatively high numbers and in fully sporulated forms. The number of oocysts shed by a *Cryptosporidium* infected person is ~ 3.3×10^6 oocysts per ml of stool during symptomatic infection and ~ 3×10^5 oocysts per gram of stool for asymptomatic cases (Bushen *et al.*, 2007).

The second feature is that oocysts/cysts are highly resistant to the commonly used disinfectants. Unlike most of other pathogenic agents like bacteria and viruses, protozoan cysts/oocysts are highly protected from most of the antimicrobial agents used for water treatment. Moreover, the small sizes of these stages (4 – 15 µm in diameter) hinder their removal by the traditional filtration methods (Etzold *et al*., 2014). High concentrations of free chlorine, ozone or prolonged UV light exposure are required for decontamination to be effective (Li *et al.*, 2009). Another factor to be considered is that *Cryptosporidium* oocysts, can retain their infectivity for six months after being shed into the environment. Furthermore, oocysts/cysts continue to be shed by infected persons for a long period (O'Hara & Chen, 2011). Finally, ingestion of small number of oocysts/cysts can produce infection.

1.7 Pathogenesis

After excystation process, the free sporozoites adhere to the mucous membrane of the small intestine by a carbohydrate-lectin mediated mechanism (O'Hara & Chen, 2011). Multiple proteins, localized in the apical surface of the zoite, have been identified to be importantly involved in the attachment process; gp40, gp15, gp900, and Circumsporozite-like glycoprotein (CSL) are some (O'Hara & Chen, 2011). Furthermore, a Gal/GalNAc-specific lectin (p30) was identified having lectin activity. Another sporozoite protein (cp47) localized in the apical region of the parasite, was found to be highly correlated with the efficiency of in vitro infectivity. It has been demonstrated that this protein interacts with a 57kDa (p57) protein of the host cell which is abundant in the ileum. This explains, in part, its affinity for this tissue (O'Hara & Chen, 2011).

The motility possess of aplicomplexans undergoes a unique method that is defined by the absence of any obvious modification of the shape of the moving cell (O'Hara & Chen, 2011). The structural stability and polarity is maintained by the microtubules, while the locomotion and invasion mechanism is provided by the actomyosin system. The process of gliding motility, then, comprises three main steps: i) the secretion of adhesive molecules from the apical pole of the parasites that adhere to the host cell receptors; ii) the posterior translocation of the adhesive molecules; and iii) the proteolytic cleavage and release of the parasite molecules in motility trails (O'Hara & Chen, 2011).

After the zoite has found its niche in the luminal surface of the host, the process of invasion is initiated by the fusion of both parasite and host membranes. The rhoptry is in close relation with the site of attachment and other organelles associated with the process (micronemes and dense granules) migrate to the parasite-host interface. The cytoplasm of the zoite vacuolize and a tunnel-like structure is formed in this location (O'Hara & Chen, 2011). The process of internalization-invasion starts with the clustering of vacuoles that ultimately encloses the parasite. A unique condition is derived from this process; the zoites remains extracytoplasmic yet intramembranous (intracellular) (O'Hara & Chen, 2011). In addition, a structural support is formed at the base of the parasite-host interface by a network of recruited host actin (O'Hara & Chen, 2011). After internalization, the parasite also recruits the host cell channels and transporters to the parasite-host interface, which further serve to nourish and support the sporozoites (Scorza & Lappin, 2012).

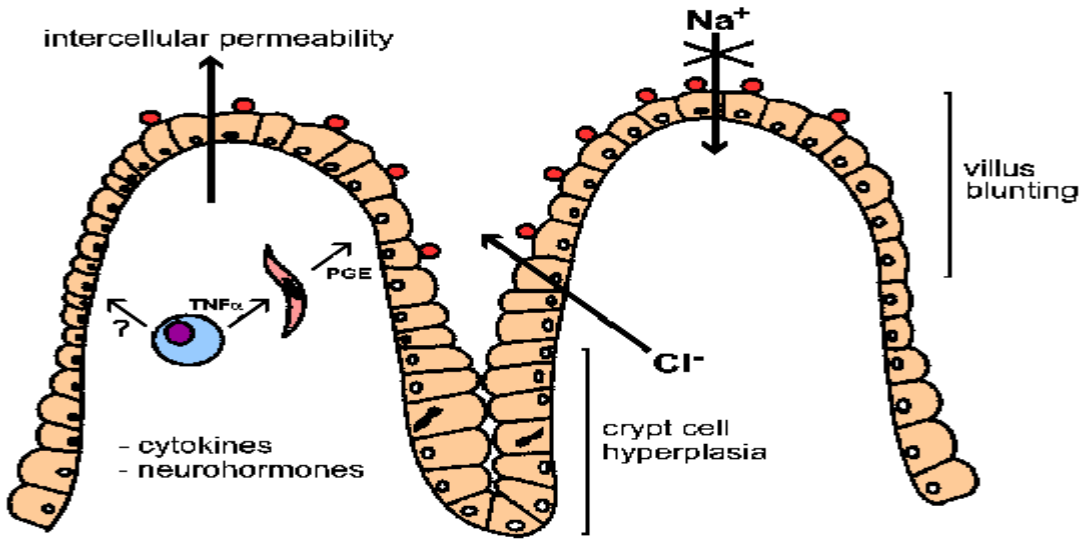


Figure1.5: *Cryptosporidium* involved in pathogenic infections (Fayer *et al*., 2009).

After sporozoite attachment, it has been hypothesized that the epithelial mucosa cells release cytokines that activate resident phagocytes (Tessema *et al*., 2009). These activated cells release soluble factors that increase intestinal secretion of water and chloride and also inhibit absorption. These soluble factors include histamine, serotonin, adenosine, prostaglandins, leukotrienes, and platelet-activating factor, and they act on various substrates, including enteric nerves and on the epithelial cells themselves (Xiao, 2010). Consequently, epithelial cells are damaged by one of two models:

1. Cell death is a direct result of parasite invasion, multiplication, and extrusion or
2. Cell damage could occur through T cell-mediated inflammation, producing villus atrophy and crypt hyperplasia

Either model produces distortion of villus architecture and is accompanied by nutrient malabsorption and diarrhea (Xiao, 2010). Experimental evidence supporting this pathogenic hypothesis exists in a pig model system, where decreased intestinal sodium absorption has been correlated with "both decreased villus surface area and inhibition by prostaglandin E2 produced by inflammatory cells" (Xiao, 2010).

1.8 Virulence factors of *Cryptosporidium*

Virulence is commonly defined simply as the ability of a microorganism to cause disease (Xiao & Fayer, 2008). Virulence and pathogenicity are often used interchangeably, but virulence may also be used to indicate the degree of pathogenicity, where pathogenicity is used solely to describe the ability of a pathogen to inflict damage to the host. Virulence is commonly used to describe the likelihood of an infected person becoming ill as well as the severity of symptoms.

Several studies have tried to determine the factors responsible for the initiation, establishment, and perpetuation of *Cryptosporidium* infection. *Cryptosporidium* does not normally cause a systemic infection or penetrate deep tissue; rather, the parasite establishes itself in a membrane-bound compartment on the apical surface of the intestinal epithelium (O'Brien *et al*., 2008). Nevertheless, it causes significant abnormalities in the absorptive and secretory functions of the gut. This damage could be the result of direct injury to the host epithelial cells or could be indirect through the effect of inflammatory cells and cytokines recruited to the site of infection (Tessema *et al*., 2009).

Virulence factors are considered to be the processes and substances by which the parasite initiates and maintains disease in the host; these factors can affect the host at any time during the life cycle from the time when the parasite enters the body until it is killed or completes the cycle and exits the host (Fayer *et al*., 2009). To date, *Cryptosporidium*-specific virulence factors have not been characterized to the point of unequivocally establishing their roles in causing damage to the host or proving that their deletion or inactivation results in a decrease of disease severity (O'Brien *et al*., 2008). This is mainly because, unlike other apicomplexan parasites such as *Toxoplasma* and *Plasmodium*, it remains difficult to employ *in vitro* cultivation and reverse genetics techniques with this parasite, meaning that genes cannot be readily knocked out or knocked down in experiments designed to examine virulence by a straightforward loss or gain of pathogenicity (Brady *et al*., 2010).

Putative virulence factors for *Cryptosporidium* have been identified as genes involved in the initial interaction processes of *Cryptosporidium* oocysts and sporozoites with host epithelial cells, including excystation, gliding motility, attachment, invasion, parasitophorous vacuole formation, intracellular maintenance, and host cell damage . There are currently over 25 putative

virulence factors, identified mainly by using immunological and molecular techniques. (Fayer *et al*., 2009; Nichols *et al*., 2010).

Table1.2: Putative *Cryptosporidium* virulence factors identified by immunological and molecular methods (Nichols *et al*., 2010**).**

Virulence factor	Putative function(s)
Serine protease	Excystation
Aminopeptidase	Excystation
CSL	Adhesion
gp900	Adhesion
P23	Adhesion, locomotion
TRAP-C1	Adhesion, locomotion
Cp2	Invasion, membrane integrity
Secretory phospholipase	Invasion, intracellular establishment
Hemolysin H4	Membrane lysis
CpABC	Transport, nutrient transport
CpATPase2	Biomembrane heavy metal transporter
CpATPase3	Biomembrane ion or phospholipid transporter
HSP70	Stress protection
HSP90	Stress protection
Cysteine protease	Immune/cytokine modulation
Acetyl cosynthetase	Fatty acid metabolism
CpSUB	Invasion

1.9 Epidemiology

Cryptosporidium is associated with diarrheal disease in all regions of the world, but is most common in less developed countries (Caccio and Widmer, 2014). The distribution of *C. hominis* and *C. parvum* in humans varies by geographic region. *C. hominis* tends to predominate in most parts of the world, especially in developing countries, while *C. parvum* is more frequent in the Middle East and both species are common in the Europe (Xiao, 2010).

Cryptosporidiosis is the clinical disease, usually presenting as a gastroenteritis-like syndrome, caused by infection with protozoan parasites of the Apicomplexan genus *Cryptosporidium*. Disease ranges in seriousness from mild to severe, signs and symptoms depend on the site of infection and nutritional and immune status of the host: evidence is also emerging that the clinical picture may vary with infecting species (Chalmers & Davies, 2010).

In the United States, cryptosporidiosis is widespread geographically, occurs more commonly during the warm, rainy months, and has a bimodal age distribution, with the greatest number of reported cases occurring among children 1–9 year and among adults aged 25–39 years. Risk factors associated with sporadic infection include contact with ill persons and cattle, travel abroad), and anal intercourse among homosexuals ((Yoder *et al*., 2010). Outbreaks in child care centers are also common, and can result in spread to the community (Xiao &Cama, 2006).

In developing countries, incidence of disease peaks in young children, who are often infected by the age of two years (Chalmers & Davies, 2010). Exclusive breastfeeding during the first 3 months of life, and partial breast feeding (compared to no breastfeeding) thereafter, appears to afford some protection (Bilenko *et al.,* 2008). Peaks usually occur during warm rainy months (Siwila *et al.,* 2010).

Persons most likely to be infected by *Cryptosporidium* are:

- infants and younger children in day-care centers
- those whose drinking water is unfiltered and untreated
- involved in farming practices such as lambing, calving, and muck-spreading
- engaging in sexual practices that brings a person into oral contact with feces of an infected individual

- patients in a nosocomial setting with other infected patients or health-care employees
- veterinarians who come in contact with farm animals
- travelers to areas with untreated water
- living in densely populated urban areas
- owners of infected household pets (rare) (Yoder *et al.*, 2010).

Within a population of immunocompromised individuals, severe and persistent disease has been associated with persons with CD4 counts of <180 cell/cubic mm (Siwila *et al.,* 2010).

1.10 Clinical Manifestations

A wide spectrum of disease severity is seen, influenced by the age, nutritional, and immune status of the host and possibly by the infecting species and subtype (Cama*et al.,* 2008). Many infections are asymptomatic or mild and self-limited and often go unrecognized. The cardinal symptom is diarrhea, which is typically watery, and accompanied by abdominal cramps, fatigue, nausea, and anorexia. Fever and vomiting may occur. Diarrhea tends to persist longer (median of 5 to 10 days) than that seen with other etiologies and may relapse. In industrialized countries, most cases are immunocompetent adults who experience a self-limited illness. Among children in developing countries, the diarrhea often lasts for 14 days or longer (Stockdale *et al*., 2008), making*Cryptosporidium* one of the most important causes of persistent diarrhea in this population. Several prospective studies have examined the complex bidirectional relationship between malnutrition and both symptomatic and asymptomatic *Cryptosporidium* infection in infants and children (Chalmers *et al*., 2009). Malnutrition is a risk factor for both diarrhea and prolonged diarrhea caused by *Cryptosporidium*, with significantly higher rates of infection in malnourished children controlling for HIV status (Moore*et al.,* 2010). Moreover, cohort studies have demonstrated that a single episode during infancy, even if asymptomatic, can lead to growth faltering that persists for months (Haque*et al*., 2009). Long term follow-up suggests an association with poor physical fitness, as children who had cryptosporidiosis during the first 2 years of life had Harvard Step Test fitness scores that were 10% lower than children who did not, when measured 4–7 years later (Saksirisampant *et al*., 2009).Cryptosporidiosis is also an independent risk factor of childhood mortality (Warren & Guerrant , 2008).

In persons with HIV/AIDS, it is not until the CD4 count falls below ~100 cells/ mm3 that the risk increases for severe, unrelenting disease accompanied by malabsorption, weight loss, and high case fatality , although asymptomatic or mild infection can occur even in this group. (Cabada*et al.*, 2010). Symptoms can be ameliorated and mortality rates diminished with immune reconstitution following antiretroviral therapy (Brady *et al.*, 2010). In developing countries where most HIV-infected people lack access to antiretroviral therapy, the burden of severe cryptosporididosis remains high (Yoder *et al*.,2010; Cabada*et al.*,2010).

Extra-intestinal manifestations of *Cryptosporidium* infection are seen. Biliary tract disease, including acalculous cholecystitis, pancreatitis, cholangitis, and stricture formation, is a well-documented complication in severely immunocompromised patients and carries a poor prognosis (Viriyavejakul *et al*., 2009)Respiratory cryptosporidiosis has been described, most often in children (Mor *et al*.,2010). Infection is often asymptomatic, but may manifest as pulmonary infiltrates and respiratory distress.

Several studies suggest that *C. hominis* produces more severe disease than *C. parvum* (Bushen *et al*., 2007; Mor *et al.*,2010). Evidence for a possible correlation between subtype and clinical manifestations is accumulating. In a birth cohort of children from Lima, Peru *C. hominis* subtype family Ib was associated with nausea, vomiting and malaise, whereas *C. hominis* subtype family Ia, Id and Ie and the other species were not (Cama*et al.*, 2008). Risk factors such as hygiene practices, presence of animals and economic variables were not associated with specific genotypes and subtypes. In a case series of nine HIV-infected patients from Italy, the four patients with the most severe disease, all who had a CD4+ T lymphocyte count <50 cells/mm^3, harbored *C. parvum* subtypes within the family IIc (Del Chierico *et al.*,2011).

1.11 Laboratory Diagnosis of *Cryptosporidium*

Among patients with cryptosporidiosis, the majority of immunocompetent patients have initially been symptomatic, with large numbers of oocysts present in their stools. In this situation, a number of diagnostic procedures would be acceptable (Dillingham *et al.*, 2009). However, as the acute infection resolves and the patient becomes asymptomatic, the number of oocysts dramatically decreases. Also, the number of oocysts passed by patients, including those with

AIDS, varies from day to day and week to week. It has also been established that the infective dose of *Cryptosporidium* oocysts in humans can be relatively low (Fayer, 2010).

There are considerable variations among clinical laboratories regarding the methods used for detection of intestinal protozoa. The choice of diagnostic method used depends mainly on the availability of the required resources and the suitably trained personnel, and the extent of infection in the population. Clinical laboratories with limited resources usually rely solely on microscopic examination of stool samples for ova/parasites or sometimes on serological assays Clinical laboratories with greater resources depend mainly on commercially available kits for protozoal antigen detection in stool samples (Antigen-Antibody detection kits) (Fayer and Santin, 2009).

1.11.1 Microscopic diagnosis

Several methods exist to detect *Cryptosporidium* in fecal samples. The most common method is microscopy for the detection of oocysts. This is because it is simple and cost-effective method in most cases. However, the sensitivity of the microscope varies from 10 – 60%. This low sensitivity is due to a number of reasons. Firstly, oocysts/cysts are shed intermittently in stool and therefore oocysts/cysts numbers vary greatly between samples. Finally, microscopic diagnosis is subjective and relies mainly on the skills of the person carrying it out (Libman *et al.*, 2008).

Fecal samples can be examined directly on slides or after concentration either by flotation or sedimentation to remove fecal debris or to concentrate the number of oocysts; the detection of oocysts in animals with low numbers of oocysts is facilitated (Fayer and Santin, 2009).

Visualization of *Cryptosporidium* oocysts by microscopy most commonly is done by direct smear and without any staining and by the modified Ziehl-Neelsen stain under light microscopy, whereby the oocysts stain purple with blue background. Most parasitological detection methods for *Cryptosporidium* do not distinguish between viable and nonviable oocysts. (Caccio and Widmer, 2014).

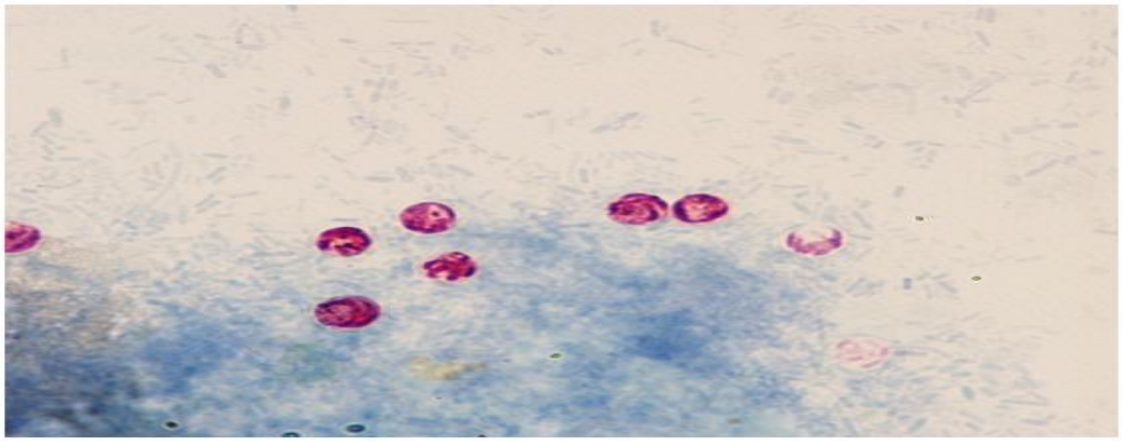


Figure 1.6: Representative bright field microscopic pictures for the *Cryptosporidium* oocysts stained with Modified Ziehl-Neelsen dye with x200 magnification.

1.11.2 Antigen-antibody detection assays

In Antigen-antibody detection assays, protozoal surface antigens present in fecal samples are used as targets for detection. Compared to microscopy, multiple stool specimens can be screened using these assays with higher sensitivities, less technical skill and faster turn-around times (O'Brien *et al*., 2008). However, when compared to PCR based detection methods, the sensitivity of several assays were found to be suboptimal (Power *et al*., 2009).

Several protozoal antigens with variable degrees of antigenicity have been used to develop a range of commercially available antigen-antibody detection kits. Two main types of copro-antigens assays are commercially available. The first type is an enzyme linked immunosorbent assays (ELISA) or enzyme-linked immunoelectrotransfer blots (EITB; Western blot) employing various aqueous extracts of *C. parvum* oocysts. Enzyme immunoassay (EIA) methods such as *Cryptosporidium* II test (TechLab, Blacksburg, VA) are fast, inexpensive, easy to be performed, and show sensitivity comparable to that of the immunofluorescence methods (Fayer and Santin, 2009). Rapid immunochromatographic (strip) tests can be also used. The other type is a lateral flow immunochromatographic assay such as *Cryptosporidium EZ VUE*™,RIDA® QUICK *Cryptosporidium*. These tests rely on the detection of cell wall proteins of the oocysts using monoclonal antibodies (Hawash, 2014).

1.11.3 Molecular Biology-Based Diagnostic Tests and PCR

To circumvent the problems of microscopic or culture-based diagnosis and to increase the sensitivity, specificity, and simplicity over other two techniques, molecular biology-based technology has become commonly used (Parija*et al*., 2014). It potentially becomes the "gold standard" by which other diagnostic techniques (microscopy, antibody detection, etc.) are measured.

Nucleic acid amplification assays particularly polymerase chain reaction (PCR)-based assays have initiated a revolution in detection and characterization of many pathogens including enteric protozoa. Due to the incredible sensitivity, specificity, and speed of amplification, PCR has been widely used for detection of *Cryptosporidium, G. lamblia* and *E. histolytica* DNA in stool specimens. However, the majority of these PCR-based assays were originally developed as research tools only.

Protozoal DNA extraction directly from stool is a challenging procedure due to a number of factors. One factor to be considered is the complexity and non-uniformity of stool specimens **(**Smith *et al*., 2009). Furthermore, in contrast to many other enteric pathogens, the genetic material of protozoa, to be isolated, is enclosed mainly in oocysts/cysts which posses very robust cell memberanes. Lastly, some constituents of stool such as heme, bilirubins, bile salts, and complex carbohydrates interfere with DNA amplifications. These substances impair cell lysis, degrade the nucleic acid and/or inhibit polymerase activity if co-extracted with the target pathogen DNA (Goncalves *et al*., 2008).

Detection of cryptosporidiosis using PCR-based methods is more sensitive than by conventional microscopical and serological methods for detecting oocysts in feces. Molecular methods can also identify the species/genotypes and subtypes of *Cryptosporidium*, important for determining the epidemiology of *Cryptosporidium* and predicting transmission routes (Parija*et al*., 2014). Since the description of the first PCR-based tool for the differentiation of *C. parvum* and *C. hominis* (Goncalves *et al*., 2008), molecular techniques in the diagnosis of cryptosporidiosis became popular, especially due to their genotyping capabilities. The target genes of *Cryptosporidium* that can be detected using molecular diagnosis include, but are not limited to, 18S rRNA, KDa, HSP70, COWP and the actin gene. Specific PCR assays targeting the 18S rRNA gene are very useful for the detection (targeting a conserved region in the gene) or

differentiation between *Cryptosporidium* spp. (targeting variable nucleotide stretches) (Fayer and Santin, 2009).

Another method to determine different species is by direct DNA sequencing using DNA, purified and amplified by internal primers and labeled with colored nucleotide bases which omit light at different wavelengths; this property is used to analyze the gene sequence (Morrison *et al*., 2008).

1.12 Prevention and control

No effective vaccine is available to prevent cryptosporidiosis. Vaccination might be an effective preventive strategy comes from observations of age-related declines in infection among children from developing countries (which presumably reflects acquisition of immunity), and human challenge studies showing protection associated with previous exposure (Xiao, 2010). Efforts to develop vaccine are limited by insufficient understanding of the immune responses mediating protection. The surface-associated immunodominant antigens (gp15, gp40, p27) present on the invasive stage of the organism is one target of interest (McDonald, 2011; Ebrahimzadeh *et al*., 2009). Antibody to the p23 antigen is observed in children with cryptosporidiosis compared to those with non-cryptosporidial diarrhea and the *p23* sequence appeared to be relatively conserved among infection species and subtype families, making it another promising vaccine antigen (Borad *et al*., 2012). Antigens associated with the intracellular and sexual stages are also of interest and incorporation of multiple antigens into a vaccine may eventually be required (Mead,2010). A recently developed reproducible model of cryptosporidiosis in weaned mice may provide a useful tool for vaccine development (Costa *et al*., 2012). Passive and other novel immunotherapies are also being explored (McDonald, 2011; Ebrahimzadeh *et al*., 2009).

1.13 **Treatment**

No safe and effective therapy for cryptosporidial enteritis has been successfully developed. Since cryptosporidiosis is a self-limiting illness in immunocompetent individuals, general, supportive care is the only treatment for the illness. Oral or intravenous rehydration and replacement of electrolytes may be necessary for particularly voluminous, watery diarrhea. Oral rehydration treatment can include Gatorade, bouillon, or oral rehydration solution, containing glucose, sodium bicarbonate, and potassium (Stockdale *et al*., 2008).

Over 100 compounds have been evaluated for the treatment of cryptosporidiosis. However, none of them has shown clear remission of signs or elimination of infection (Scorza & Lappin, 2012). Thus, the primary goal of the treatment should be to stop diarrhea. Palliative support should be given, according to practitioner discretion. The use of high digestible diet, hydration solutions, mucosal protectors, and antibiotic for secondary bacterial infection may be necessary as part of the treatment of cryptosporidiosis (Scorza & Lappin, 2012).

Immunocompetent individuals typically recover without intervention (Chalmers & Davies, 2010). Treatment of cryptosporidiosis may involve oral or intravenous liquid to replace water and electrolytes if patient are suffering from dehydration (Rossignol,2010). Re-establishing a healthy immune system is the most effective method for combating chronic cryptosporidiosis in immunocompromised paitents (Chalmers & Davies, 2010). However , this may be difficult or impossible for severly immunocompromised individuals. Antiretrovial therapies for AIDS patients, if successful in restoring the immune system (increase CD4 counts) , can led to recovery from cryptosporidiosois (Rider Jr. & Zhu, 2008) .

For immunocompromised patients with cryptosporidiosis, several antimicrobial agents have been tested as possible treatments for the illness. Until recently, treatment of cryptosporidiosis using antimicrobials such as spiramycin, paromomycin and azithromycin was only marginally effective (Stockdale *et al*., 2008).

Antibiotics such as spiramycin and dicalzuril sodium have produced partial responses in patients (a partial decrease in diarrhea or partial decrease in stool oocyst number), but have not yielded reliable, reproducible results (Stockdale *et al*., 2008). However, one particular antimicrobial agent, paromomycin, has been shown to decrease the intensity of infection and improve intestinal function and morphology (Scorza & Lappin, 2012). Paromomycin is a poorly absorbed, broad spectrum antibiotic similar to neomycin, and in a review of 12 patients with 23 episodes of gastrointestinal cryptosporidiosis, 16 of the 23 epidoses had a complete response to therapy (symptom improvement, diarrhea eradication, and weight gain) and the other seven episodes had a partial response (Savioli,Smith,andThompson,2006). However, relapse after treatment is common and fairly expensive maintenance therapy is often necessary.

The thiazole compound, nitazoxanide is the only drug FDA approved for the treatment of cryptosporidiosis. Nitazoxanide has been shown to improve both clinical and microbiological

cure rates and decrease the duration and severity of symptoms in immunocompetent patients. Nitazoxanide inhibits the growth of sporozoites through the reduction of metabolic enzymes and has proven effective in in the treatment of Cryptosporidosis in immunocompetent children and adults (Cabada and White, 2010).Diarrhea was resolved in 80% of adults and children within 7 days of being randomized to receive a 3 day course of nitazoxanide compared to only 41% of those randomized to placebo. Elimination of oocysts shedding occurred in 75% of nitazoxanide recipients compared to 20% of those receiving placebo ((Rossignol, 2010). Several other thiazoles have been shown to have*in vitro* activity against *C. parvum* and may serve as candidates for future drug development (Gargala *et al*., 2010). Conversely, nitazoxanide is ineffective in HIV-infected patients (Cabada and White, 2010), even when high doses and prolonged treatment are used (Rider Jr. & Zhu, 2008). Paromomycin, a nonabsorbable aminoglycoside with some activity against *Cryptosporidium* in immunocompetent people, is also not curative in HIV/ AIDS patients (Cabada and White, 2010). Resolution of symptoms relies on restoration of immune status using combination antiretroviral therapy. Combination anti-parasitic and antiretroviral therapy, especially with a protease inhibitor based therapy which seems to have some additional anti-parasitic activity, seems to benefit patients with cryptosporidiosis (Cabada and White, 2010).

Nitazoxanide is used uncommonly in developing countries. In Peru, children receiving empiric nitazoxanide had a shorter duration of diarrhea associated with multiple etiologies compared with those receiving placebo (Rossignol, 2010). This benefit was also seen in the large group of patients with no identified enteropathogen. No cases of cryptosporidiosis were detected, but diagnosis relied upon stool microscopy, which may have lacked sufficient sensitivity to detect the organism.

Azithromycin in combination with Paromomycin has been used experimentally to treat cryptosporidiosis in AIDS patients with several trials given different results. Other drugs , including some antiretroviral agents , have been tested against cryptosporidiosis with some promising results (Rider Jr. & Zhu, 2008).

However, NTZ and paromomycin remains as the two most well-recognized treatment option. Additionally, anticoccidia drugs have been tested or used in animals, but their utility remains unclear (Rider Jr. & Zhu, 2008).

1.13.1Water treatment

About 56% of the 71 *Cryptosporidium*-linked outbreaks in the last decade appear to be correlated to waterborne diseases and worldwide environmental and veterinary surveillance data revealed the presence of *Cryptosporidium* spp. in entire wastewater, surface water and water-treatment systems (Putignani and Menichella, 2010). Therefore, control of the parasite is a major challenge to water treatment professionals. *Cryptosporidium* testing has a role, but the prevention of cryptosporidiosis as waterborne disease is the result of proper water treatment, not pathogen testing. Infectious oocysts pass through different filtration processes and are unaffected by chlorine and chlorine-based disinfectants; outbreaks occur even in water from plants meeting all water quality and operational standards. In developing countries, different filtration methods (conventional, direct, slow-sand, diatomaceous earth, bag or cartridge, membrane) will have to be introduced or made effective in nonfunctional water purification stations and ineffective water treatment systems (Muraleedharan, 2009).

1.14 Objective of the Study

Cryptosporidium spp. is considered responsible for the majority of human infections, called Cryptosporidiosis, which is an acute self-limiting gastroenteritis in immune-competent humans. It occurs worldwide, and in all age groups, although children especially those under 2 years old are most frequently and severely affected, especially in children in developing countries, like Bangladesh. In developing countries where there is low hygiene level, poor sanitation, no good water management, and frequent contact with animals, the burden of cryptosporidiosis remains to be a major health problem. Infection with other *Cryptosporidium* species complicates its diagnosis. Therefore, it is important to detect *Cryptosporidium* infection in diarrheal fecal samples efficiently and instantly in clinical sites and in remote areas. Timely and accurate diagnosis of *Cryptosporidium* is important to properly manage infected individuals and understand its epidemiology for effective prevention.

General Aim

General objective of this study was to evaluate and establish a rapid immunochromatographic assay (*Cryptosporidium EZ VUE™*) for the detection of *Cryptosporidium* infection in fecal samples *in children*.

Specific Aim

- *To find the Cryptosporidium infection in different age groups in both male and female patients.*
- *To find specificity and sensitivity in detection of Cryptosporidium infection by using RT-PCR, ELISA, QUIK CHEK and Cryptosporidium EZ VUE and to compare them.*
- *To find the positive predictive value and negative predictive value using RT-PCR, ELISA, QUIK CHEK and Cryptosporidium EZ VUE and to compare them.*
- *To determine the efficacy of Cryptosporidium EZ VUE by cross checking it with E. histolytica positive fecal samples.*
- *To determine the efficacy of Cryptosporidium EZ VUE by cross checking it with Giardia lamblia positive fecal samples.*

Chapter Two

Rationale of the study

Cryptosporidiosis is a frequent cause of diarrheal disease in humans caused by the protozoan parasite *Cryptosporidium*. The often nonspecific clinical presentation of the infections makes it difficult to choose more specific and sensitive methods for the detection of these pathogens. In developing countries, infection with *Cryptosporidium* can cause watery diarrhea, abdominal pain, low-grade fever, nausea and/or vomiting in humans **(Haque *et al.,* 2007).***Cryptosporidium* spp. infections occur mostly in children younger than 5 years of age. Furthermore, the morbidity and mortality rate in children due to diarrheal diseases is also very high in developing countries (Mondal*et al.,* 2009). Therefore, early detection of *Cryptosporidium* infection and differentiate from other parasites both in adult and children in either rural or urban area is very important.

There is a greater demand for efficient diagnostic methods for *Cryptosporidium* in developing nations where the infection is prevalent and most consequential. In an attempt to find alternative testing methods to traditional microscopy, ELISA, and PCR, which has limited diagnostic suitability in resource-limited countries. Among other methods such as ELISA, Quik Chek and PCR, the Lateral Flow Test can rapidly detect Cryptosporidium infections. We can use Lateral Flow Test as Rapid Diagnostic Kit for detecting *Cryptosporidium* infection.

2.1 Lateral Flow assay (*Cryptosporidium EZ VUE*)

Lateral Flow methods utilize a solid-phase qualitative immunochromatographic assay to binds antigen specific for each parasite using an antibody-conjugate complex. With the addition of substrate, colored bars indicate a positive result when antigen is present. Testing cartridges are single use and come with internal controls (Agnamey *et al.,* 2011).

Cryptosporidium lateral flow is an immunochromatographic assay that qualitatively detects *Cryptosporidium* antigen in fecal specimens. It is a dipstick that uses a monoclonal antibody sandwich design to detect *Cryptosporidium* oocyst antigen. This data represents the first field test of the *Cryptosporidium* lateral flow as we evaluated the sensitivity and specificity of this rapid dipstick test (Chalmers *et al.,* 2011).

Rapid chromatographic methods used to detect the presence of antigen are commercially available from a variety of manufacturers.Available alternative rapid antigen detection dipstick tests include the Crypto Uni-Strip (CorisBioConcept) (Goni*et al.,* 2012), RIDA QUICK Cryptosporidium (R-Biopharm), and Crypto + Giardia dipstick (CLONIT) (Hawash, 2014). All of these tests have comparable time to results and easy visual result interpretation. However, the

other available rapid antigen detection tests above involve at least one additional step in comparison to the *Cryptosporidium* lateral flow test. Reported sensitivities for rapid lateral flow immunoassay methods range from 56-100% for *Giardia* and from 58-98.8% for *Cryptosporidium*. Specificities for *Giardia* range from 44% to 100% with *Cryptosporidium* specificities ranging from 50% to 100% (Garcia *et al*., 2009).

2.1.1 Rapid Lateral Flow Assay Advantages

As their name suggests, rapid methods can be performed quickly. One advantage in rapid testing is decreased testing time compared with current methods. Some assays require as little as ten minutes of hands-on time per specimen. As with traditional ELISA, rapid methods do not rely on parasite visualization for diagnosis, and thus these methods do not require expertise in parasitology. A spectrophotometer is not required for these rapid immunoassays because unlike standard ELISA, visually reading of results is sufficient.

2.1.2 Rapid Lateral Flow Assay Disadvantages

A significant disadvantage of the rapid detection methods compared with traditional EIA's is cost: Test kits come with individually wrapped test cartridges rather than the 96-well plates seen with traditional ELISA. Although these cartridges are single use only and are often double the expense of an ELISA, the testing costs are comparable to microscopy because of decreased testing time per specimen.

In comparison with Microscopy, there is some concern about the limit of detection, which could be significant in asymptomatic populations (Garcia *et al*., 2009). When low parasite numbers are present, antigen levels fall below the detection limit of these methods, which causes the reporting of false negative results (Garcia *et al*., 2009).

Furthermore, rapid methods are specifically designed to detect the two most common species of *Cryptosporidium* reported in humans, *C. parvum* and *C. hominis*. When immunoassay methods have been tested against species other than *C. parvum* and *C. hominis* a decrease in the sensitivity of the assays has been observed (Chalmers *et al*., 2011).

2.2 Parameters used for the evaluation of Lateral Flow assay (*Cryptosporidium EZ VUE*)

2.2.1 Specificity and Sensitivity

For any test, there is a tradeoff between sensitivity and specificity of the test. The point on continuum between normal and abnormal which is known as the location of cutoff is a random one. Thus, for any particular test one characteristic (sensitivity) can be increased at the expense of another (specificity) (Fletcher and Fletcher, 2005).

Specificity is defined as the fraction of people without the disease who have a negative test result. It limits the chances of incorrectly classifying a person as having disease when he/she is not infected. It is particularly useful in confirming diagnosis suggested by other data. It rules out the chances of classifying a highly specific test as positive in absence of disease, it reduces the chances of false positive results (Rajul *et al.,* 2008).

$$\text{specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}}$$

Sensitivity is defined as the fraction of people having the disease who have been tested positive for the disease. It reduces the chances of missing out people with disease. A sensitive test is usually chosen when accurate determination of disease is required (Rajul *et al.,* 2008).

$$\text{sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}}$$

2.2.2 Predictive value

Predictive value is the probability of a disease when the results of a test are given. Positive predictive value indicates the probability of disease in patient with positive test result whereas negative predictive value indicates the probability of not having disease when the result is normal; negative. Predictive value shows the chances of a patient with positive/negative result does/doesn't have disease. It is also known as posterior or posttest probability. That means the probability of disease once the result is known (Fletcher and Fletcher, 2005).

Determinants used for calculation of Predictive value

Sensitivity and specificity of a test and the prevalence of the disease within a population that is being tested are used for calculating predictive value. Prevalence is also known as prior or pretest probability.

Bayes's theorem of conditional probabilities helped in deriving mathematical formula which includes specificity, sensitivity and prevalence to predictive value.

Positive predictive value:

$$PPV = \frac{\text{Number of True Positive}}{\text{Number of True Positive + Number of False Positive}}$$

Negative predictive value:

$$NPV = \frac{\text{Number of True Negative}}{\text{Number of True Negative + Number of False Negative}}$$

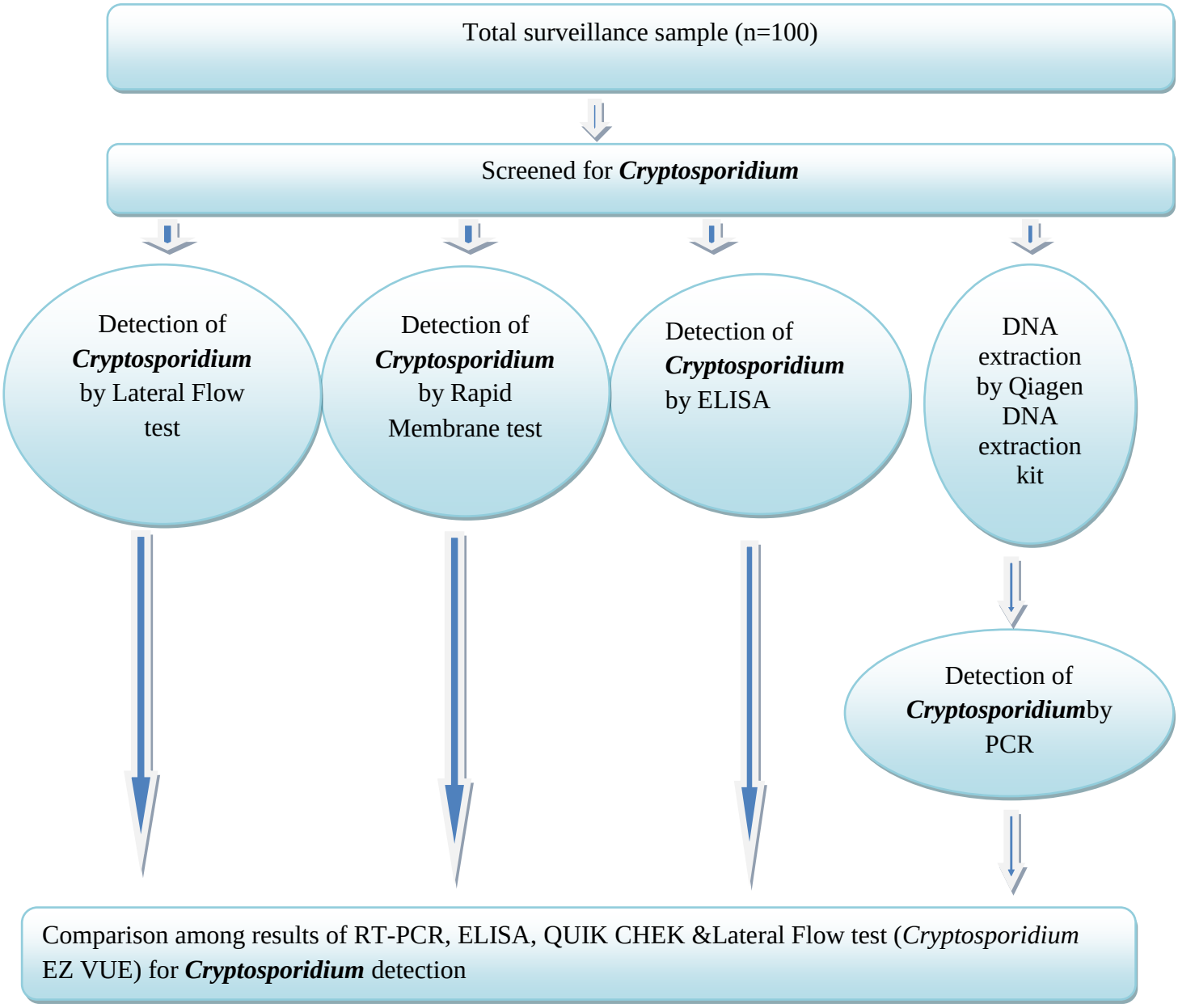
More specific test gives a better positive predictive value and a more sensitive test gives a better negative predictive value. Prevalence also plays a huge role in their determination because even if a test is very specific then positive results for patients having less chances of having the disease will be false positives. Negative results for highly sensitive tests of patients having high chances of having the disease will most probably be false negatives. When prevalence is relatively high within a population tested, the performance of the test turns out well but at lower prevalence the positive predictive value decreases to zero and therefore the test becomes inaccurate. Prevalence value has more effect on predictive value when the sensitivity and specificity of a test decreases(Fletcher and Fletcher, 2005).

Chapter Three

Materials & Methods

3.1 Experimental Design

The experimental design is represented by the following follow chart



3.2 Brief Overview of Samples

This study was conducted in the Parasitology laboratory of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B). The samples were collected from the children in Provide study living in an urban slum of Mirpur, Bangladesh in 2012-2013. From 18 January,2012 to 9 November,2013 total 2681 Fecal specimens were collected under Provide study, among those 100 fecal specimens were used during the study period. The study period extends from September'2015 to March'2016. The specimen that is collected from children is called Child diarrheal Stool (CDS).

3.3 Sample collection

Fecal sample was collected in fecal collection pot and from this fecal microscopic examination, ELISA, DNA extraction and Polymerase Chain Reaction (PCR) were done.

The fecal samples were collected in a clean, leak proof, transparent container. No antiseptic was used. Fecal samples were not contaminated with urine. No de-worming medication was given before taking these samples. Identification number labels were put on the container (patient's number, laboratory number and date of collection). The fecal specimens were collected by the technicians from Mirpur slum area and then they had sent the specimens to the Parasitology Laboratory. From 18 January,2012 to 9 November,2013 total 2681 Fecal specimens were collected under Provide study, among those 100 fecal specimens were used during the study period. In the Laboratory, about 100 mg of frozen fecal specimens (thawed at room temperature) was transferred to a 1.5 ml microcentrifuge tubes (two copies- one for DNA extraction and another for ELISA).The population of the study was infants aged between 0-24 months enlisted in Provide study during the study period.

Multiple fecal samples from 100 children were tested for the study, here 60specimens were collected from female child and 40 specimens were collected from male child.

3.4 Sample Criteria

Here, all frozen *Cryptosporidium* positive and negative samples were collected in 2012-2013 and stored at -20 °c. In this study 50 positive samples and 50 negative samples were taken

which were previously detected by *Cryptosporidium*specific RT-PCR. Here,50 *Cryptosporidium*positive and 50 *Cryptosporidium* negative frozen samples were taken to test *Cryptosporidium* EZ VUE. These samples were stored at -20°c.

3.5 Study Location

The study area was Mirpur (Sector-11, avenue-5), an urban slum of Dhaka city. The majority of the inhabitants are of Bihare ethnic origin. Mirpur area is densely populated with more than one lakh of people. This area was selected as the unhygienic living condition where the impoverished children are mostly affected by diarrheal disease than the others. The Parasitology Laboratory of icddr,b: International Centre of Diarrheal Disease Research' Bangladesh, runs a diarrhea surveillance system here. This surveillance system collects information on clinical and demographic features at the same time. All the laboratory works were done in the Parasitology Laboratory of icddr,b Dhaka, Bangladesh.

3.6 Detection of *Cryptosporidium* in Human Fecal Sample by a Lateral Flow Test (*Cryptosporidium EZ VUE*) ™

The *Cryptosporidium EZ VUE* (developed and manufactured by TechLab®) is a rapid immunochromatographic assay for the qualitative detection of *Cryptosporidium* antigen in fecal specimens. It is a dipstick that uses a monoclonal antibody sandwich design to detect *Cryptosporidium* oocyst antigen. The membrane is pre-coated with antibodies against *Cryptosporidium* antigens on the test line region. During testing, the sample reacts with the particle coated with anti-*Cryptosporidium* antibodies which were pre-dried on the test strip. The mixture moves upward on the membrane by capillary action. In the case of any positive result the specific antibodies present on the membrane will react with the mixture conjugate and generate one coloured lines. A green coloured band always appears in the control line and serves as verification that sufficient volume was added, that proper flow was obtained and as an internal control for the reagents(Goni*et al.,* 2012).

3.6.1 Sample preparation

The *Cryptosporidium EZ VUE* test was supplied by TechLab, Inc. (Blacksburg, VA), and was specifically designed to identify *Cryptosporidium*-specific oocyst antigen in fecal samples. For this study, Test Strips, Specimen Dilution Tubes containing 600 µl Diluent, Wooden Applicator Sticks, Positive Control was used for per specimen. The foil bags containing these Kit contents were brought to room temperature before opening. These were used immediately after opening.

Fresh/ Frozen Fecal: Frozen fecal specimens were thawed. All reagents as well as the required number of devices should be brought to room temperature before use. The fecal sample was mixed thoroughly by stirring or vortexing the sample. All specimens should be mixed thoroughly before transferring.

The cap from the Specimen Dilution Tube containing 600 µl Diluent was removed. 50 µl of feces was transferred with the wooden applicator stick or pipette to the tube and mixed well. With preserved sample 200 µl of specimen was transferred to the specimen dilution tube.

A test strip was removed from the canister. Minimize the amount of time the canister is open and make sure that the canister is fully closed after removing the test strip. Drop the sample end of the test strip into the tube so that the arrows on the test strip are pointing down.

Set a timer for ten (10) minutes. At ten (10) minutes, read the results. Do not read after more than ten (10) minutes have passed. Strong positive results may be visible before ten (10) minutes.

3.6.2 Interpretation of *Cryptosporidium EZ VUE* Results

The results must be interpreted in the following way:

POSITIVE

If both test line and control line are present, the result is positive. The color of the lines will range from a dark red to a light pink Blue.

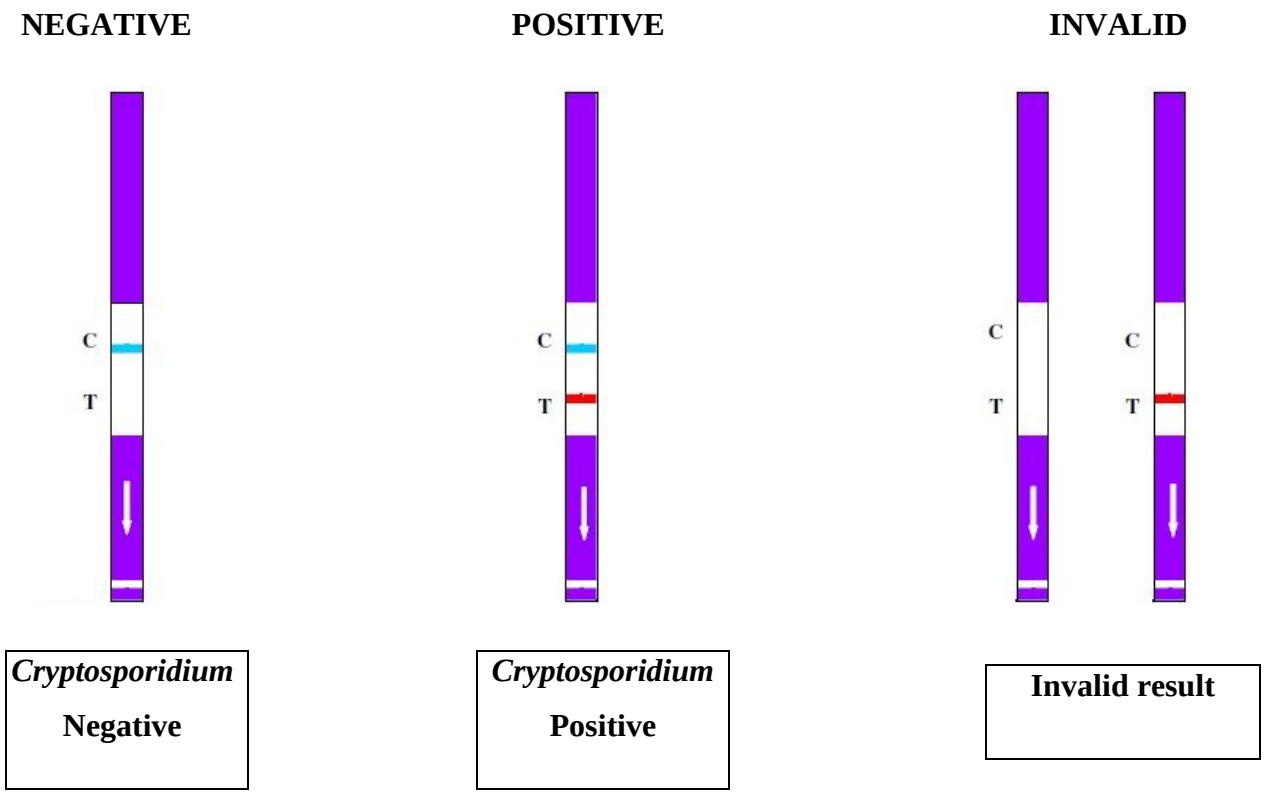


Figure 2.1: Results of *Cryptosporidium EZ VUE*

NEGATIVE

If only the control line is visible, the result is negative.

INVALID

If the control line does not appear, the test is invalid or the test procedure was not followed properly. Verify the test procedure and repeat the test with a new test strip.

3.7 Detection of *Cryptosporidium* in Human Fecal Sample by a Rapid Membrane Enzyme Immunoassay (QUIK CHEK) ™

The *Cryptosporidium QUIK CHEK*™ test uses monoclonal and polyclonal antibodies to cell-surface antigens of the organisms. The device contains a Reaction Window with two vertical lines of immobilized antibodies.

The *Cryptosporidium QUIK CHEK*™ test (developed and manufactured by TechLab®) is a rapid assay for the qualitative detection of *Cryptosporidium* in fecal samples and targets an oocyst unique to *Cryptosporidium*. The test can be used for quick and reliable diagnosis of Cryptosporidiosis (Garcia, 2009).

3.7.1 Sample preparation and procedure

Fresh/ Frozen Fecal: Frozen fecal specimens were thawed. All reagents as well as the required number of devices should be brought to room temperature before use. 25 µl of solid specimen was added to the eppendorf tube and 500 µl diluent was added to each tube. Then 1 drop of conjugate was added to each tube. All specimens should be mixed thoroughly before transferring.

500µl of the diluted sample-conjugate mixture was transferred from each tube into the Sample Well of a membrane device using a new transfer pipette. Liquid sample was made certain to expel on to the wicking pad inside of the Membrane Device. The Tip of the transfer pipette was angled towards the Reaction Window during loading the sample into the sample well. The device is incubated for 15 minutes at room temperature. After the incubation 300µl wash buffer was added to the reaction window. Graduated white dropper was used for adding wash buffer. Wash buffer was allowed to flow through the reaction window membrane.2 drops of substrate were added to the reaction window. Results was read and recorded visually after 15 minutes.

3.7.2 Interpretation of *Cryptosporidium QUIK CHEK*™ Result

Interpretation of result is most reliable when the device is read immediately at the end of the reaction.

Figure A **Figure B**

Figure 2.2: Results of *Cryptosporidium* QUIK CHEK™ device

In Figure A, *Cryptosporidium* Quik Chek assay shows positive result. Here, two vertical blue lines were visible, the control line on the 'c' side of the Reaction Window and the test line on the "T" side of the Reaction Window. A positive result indicated the presence of *Cryptosporidium* antigen, and there was a properly reactive positive control line.

In Figure B, *Cryptosporidium* Quik Chek assay shows negative result. Here, a single vertical blue line was visible on the "C" side of the reaction window and no test line was visible on the "T" side of the reaction window. A negative result indicated that *Cryptosporidium* antigen was either absent or below the detection limit of the test, and that there was a properly reactive positive control line.

The test is invalid if a control line is not present at the completion of the test reaction. A single line on the "T" side of the reaction window or no lines are visible in the reaction window also indicates invalid result.

3.8 Detection of *Cryptosporidium* by using ELISA

The ELISA which was used for the detection of *Cryptosporidium* called *Cryptosporidium* II, a 2nd generation Monoclonal ELISA for detecting *Cryptosporidium* oocyst antigen in fecal specimens. The *Cryptosporidium II* (TechLab®, Blacksburg, VA) is a monoclonal antibody-based ELISA for the rapid detection of the organism in feces as a diagnostic aid. It is the only test on the market that specifically detects *Cryptosporidium.* The TechLab® (Blacksburg, Virginia) *Cryptosporidium* II kit is the only Food and Drug Administration-approved test that is specific and sensitive for the detection of *Cryptosporidium* in feces (Haque *et al*., 2007).

At first1.5 ml eppendorf tube were taken and labeled according to the ID. 100mg fecal samples were taken into each eppendorf tube. For formed fecal about 400µl*Cryptosporidium diluent* was added to the eppendorf tubes and for liquid fecal, about 200µl*Cryptosporidium diluent* was added to the eppendorf tubes. The fecal specimens were thoroughly mixed through vortex.

Figure 2.3: Kits for *Cryptosporidium* ELISA

1 drop of conjugate (red cap bottle in figure 2.3) was added to all the wells of polystyrene assay well strips. 1 drop of *Cryptosporidium* positive control reagent was added to a well as a positive control and 100 µl of diluent was added to another well as negative control. 100 µl of diluent specimen was added to the test well. The wells were covered with adhesive plastic sheet and incubated for one hour at room temperature. After incubation the contents of the

well strips were shaken and emptied out and washed 5 times in the wash solution. After washing, the residual liquid was then removed by striking the plate onto a dry paper towel until no liquid came out. Then two drops of substrate were added to each well and incubated for 10 minutes at room temperature. After incubation, add one drop of stop solution to each well. A yellow color (in figure 2.4) was shown to positive control and also positive sample. After 2 minutes, the optical density (O.D.) of the colored solution was read at 450 nm on a microplate ELISA reader (Elx, Germany). A positive result had an OD of 0.150 or greater and a sample is considered negative if the reading is <0.150.

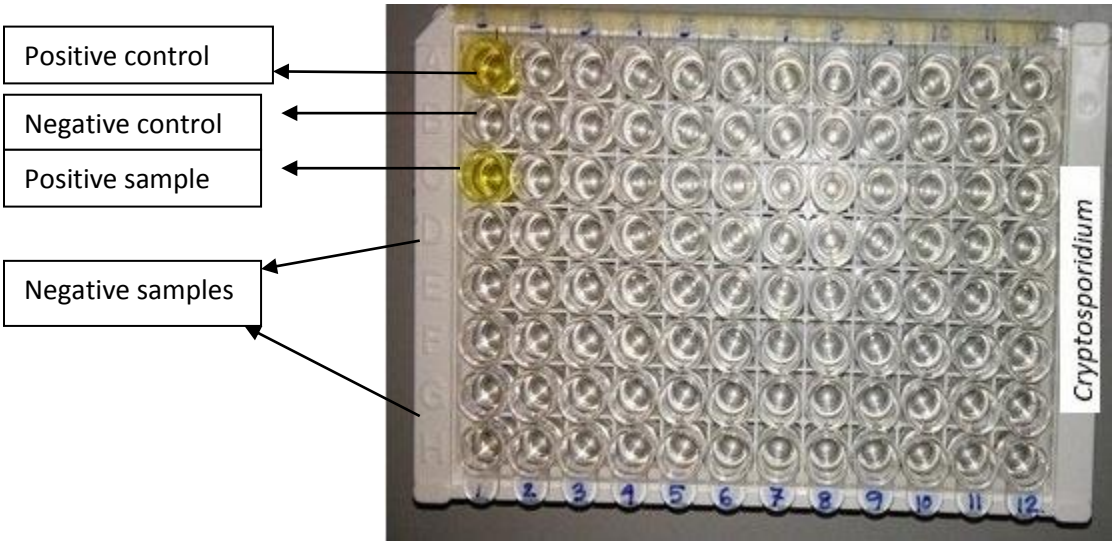


Figure 2.4: Microassay Plate showing positive control at 1st column 1st row, negative control at 1st column 2nd row. Yellow color at 1st column 3rd row shows positive results and colorless wells mean negative results.

Cut-off is the point at which one considers the test to change from negative to positive. The cut-off value of these ELISA was <0.150 at 450 nm wavelengths (Garcia, 2009).

3.9 Detection of *Cryptosporidium* by Molecular Based Technique RT-PCR

3.9.1 Extraction of DNA by QIAGEN method

The DNA was extracted by using QIAamp® Fast DNA Stool Mini Kit (QIAGEN). Purification requires no phenol-chloroform extraction or alcohol precipitation. DNA was eluted in low-salt buffer and was free of protein, nuclease, and other impurities or inhibitors. The purified DNA was ready for use in PCR and other enzymatic reaction, or can be stored at -20°C for later use.

Fecal samples typically contain many compounds that can degrade DNA and inhibit downstream enzymatic reactions. InhibitEX Bufferefficiently adsorbs these substances early in the purification process so that they can easily be removed by a quick centrifugation step. Another Buffer is used (buffer AL, 33ml), this was a lysis buffer, contained guanidine hydrochloride. Buffer AW1 (Concentrated), 19 ml was a wash buffer and contained guanidine hydrochloride. Before using for the first time, 25 ml ethanol (96-100%) was added to obtain 44 ml AW1. Buffer AW2 (Concentrated), 13 ml was also a wash buffer. Before using for the first time, 30 ml, ethanol (96-100%) was added to obtain 43 ml AW2. Elution buffer (Buffer AE, 12 ml) was used. Proteinase K, QIAamp spins columns, collection tubes (2 ml), all were provided by QIAamp® Fast DNA Stool Mini Kit. All the reagents were stored at room temperature between 15-25˚C.

Other required materials which were used but not provided with QIAamp® Fast DNA Stool Mini Kit -

1. Ethanol (96-100%)
2. 1.5 ml and 2 mlmicro centrifuge tubes
3. Micropipettes
4. Filter tips for micropipette
5. Microcentrifuge
6. Vortexes
7. Water bath
8. Deionized distilled water

The DNA extraction methods and materials were provided by QIAamp® Fast DNA Stool Mini Kit (QIAGEN). For this study, about 180-220 mg of frozen fecal samples (thawed at room temperature) or 200 µl of liquid fecal samples was transferred to a 2 ml microcentrifuge tube and

was placed on ice.1 ml InhibitEX Buffer was added to each stool sample and vortex continuously for 1 min or until the stool sample is thoroughly homogenized. It is important to vortex the samples thoroughly. This helps ensure maximum DNA concentration in the final eluate. The suspension was bead beated at maximum speed for 2-3 minutes.. The suspension was incubated for 5 minutes at 95°C. This heating step helps to lyse bacteria and other parasites. The lysis temperature can be increased for cells that are difficult to lyse (such as Gram-positive bacteria)and then vortexed for 15s. Samples were centrifuged at full speed(14000 rpm) for 1 minutes to pellet the stool particles.25 µl of proteinase K was pipette into a new 2 ml microcentrifuge tube. Proteinase K helps to digest the unnecessary proteins.

600 µl supernatant was pipette into the 2 ml microcentrifuge tube containing 25 µl proteinase K and then 600 µl of Buffer AL was added and vortexed for 15 seconds. The sample and Buffer AL were thoroughly mixed to form a homogeneous solution.

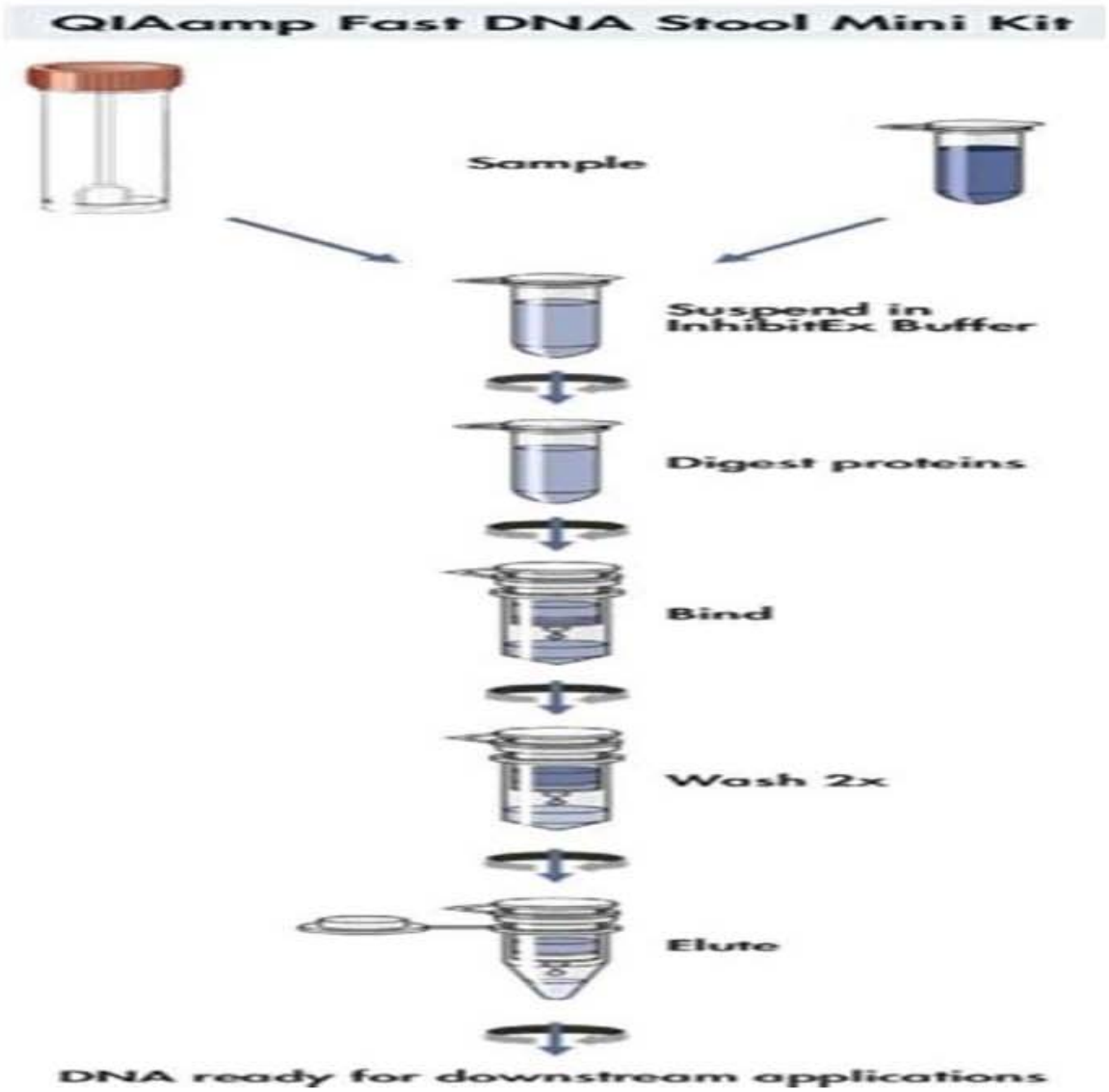
This solution was incubated at 70˚C for 10 minutes. A brief centrifugation was done to remove drops from the inside of the tube lid. 600 µl of ethanol (96-100%) was added to the lysate, and mixed by vortexing. A brief centrifugation was used to remove drops from the inside of the tube lid. The lid of new QIAamp spin column placed in 2 ml collection tube that was labeled. The complete lysate from previous step was carefully applied to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 minute. The QIAamp spin column was placed in new 2 ml collection tube and the collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500 µl of buffer AW1 was added, centrifuged at 14000 rpm for 1 minute.

The QIAamp spin column was placed in a new 2 ml collection tube, and the collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500 µl of Buffer AW2 was added. Centrifuged at full speed for 3 minutes, the collection tube containing the filtrate was discarded.

The residual buffer AW2 in elute might cause problems in downstream applications. To overcome this, the QIAamp spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 3 min. The collection tube containing the filtrate was discarded. The QIAamp spin column was transferred into a new, labeled 1.5 ml micro-centrifuge tube and 2 µl of Buffer AE (supplied with QIAGEN kit) was pipette directly on to the QIAamp

spin column membrane, incubated for 1-3minutes at room temperature, then centrifuge at full speed for 1 min to elute DNA.

The DNA extraction method of QIAamp Fast DNA Stool Mini is shown in the following flowchart-



3.9.2 Real time-PCR (RT-PCR)

Real time-PCR (RT-PCR), a technique that follows the general basics of conventional PCR however it allows the measurement of PCR products in real time as the reaction progresses. Here, RT-PCR was considered to be the gold standard.

Principles of RT-PCR

As with conventional PCR, in RT-PCR, double strand DNA (ds-DNA) is denatured at approximately 95°C, this high temperature breaks the hydrogen bonds that bind one side of the helix to the other allowing the two strands separate. The sample is cooled to between 50ºC to 60°C to allow annealing of primers that are complementary to a specific site on each strand. The temperature is raised to 72ºC and the addition of the heat-stable Taq polymerase extends the DNA from the primers generating four cDNA strands.

A major improvement in this system is the addition of a dual labeled probe, which contains a flurophor on one end and a quencher molecule on the other. The shape of the un-bound probe allows the flurophor and quencher molecules to sit in close proximity to each other preventing the premature release of fluorescence. After each cycle, the level of fluorescence released is measured when bound to the ds-DNA i.e., the PCR product.

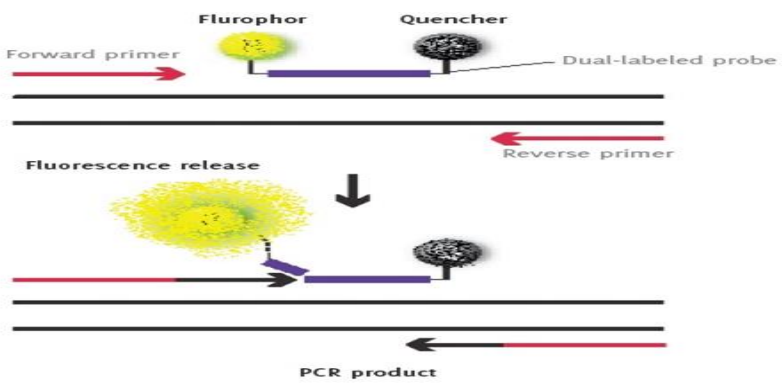


Figure 2.5: Plot of RT-PCR with dual labeled probe

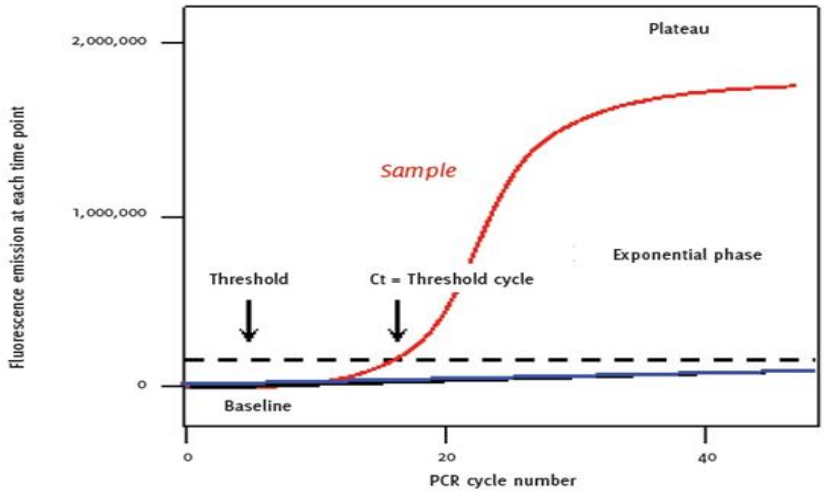


Figure 2.6: Quantitative plot of RT-PCR by the intensity of the fluorescence signal versus cycle number.

The progress of PCR amplification can be continuously monitored in real time by acquiring fluorescence signals in each amplification reaction cycle. Thus, after 30 to 40 cycles, quantitative information of the PCR process is obtained by plotting the intensity of the fluorescence signal versus cycle number. Automated assays are relatively easy to perform and they are not only valuable in reducing chances of contamination, but also enable reproducibility and rapid processing of multiple samples.

The oligonucleotide primers and Taqman probes for *Cryptosporidium spp*. were designed on *Cryptospridium*Oocyst Wall Protein (COWP; accession no. AF248743). The amplified target was 151-bp for *Cryptosporidium* spp.

The *Cryptosporidium spp* -specific Oligonucleotide primers was used for real-time PCR assay for the detection of *Cryptosporidium spp.*

Cp-583F	CAA ATT GAT ACC GTT TGT CCT TCT G
Cp-733R	GGC ATG TCG ATT CTA ATT CAG CT

Oligonucleotide probes for real-time PCR assay for the simultaneous detection of *Cryptosporidium* spp.

Oligonucleotide sequence (5′–3′)

Cp-TRT Texas Red-TGC CAT ACATTGTTGTCCTGACAA ATT GAA T-DDQ2

For *Cryptosporidium spp*., 0.8 µmol/L of each primer (Cp-583F and Cp-733R primers) and 0.4µmol/L Cp-TRT probe for Cryptosporidium spp. and 3 µL of the extracted DNA were used in each reaction.

Amplification reactions were performed in a volume of 25 µL with Qiagen master mix (containing 100 mmol/L KCL; 40mmol/L Tris-HCL, Ph 8.4; 1.6 mmol/L deoxynucleoside triphosphate; iTaq DNA polymerase, 2 mmol/L $MgCl_2$) with an additional 3 mmol/L $MgCl_2$ added; 0.4 µmol/L of each primers (Eh-f, Eh-r primers) and 0.08 µmol/L Eh-YYT probes for *E. histolytica.*

Amplification consisted of 3 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. Amplification, detection, and data analysis were performed with i-Cycler real-time detection system (BioRad). Fluorescence was measured during the annealing step of each cycle. The ramping of the machine was 3.3°C/s in every step. Fluorescence emitted at, 575and 530 nm was measured for*Cryptosporidium* and*E. histolytica* spp. respectively.

Before setting the experimental plate for Real-Time PCR, well factor was collected by setting external well factor plate for the subtraction of background RFU.

The 10 X External Well Factor Solution was diluted to make 1X solution with ddH2O. 25 µl of 1X solution was transferred to each well of external well factor plate. The number and position of the well of external well factor plate were same as the wells of experimental plate of Real-Time PCR.

The plate was sealed with optically clear sealing tape and then plated into i-Cycler. The filter pair for FAMfluorophore was selected and then ran with an automated 3 cycle protocol and the well factors were collected.

Setting of Experimental plate for Real-Time PCR

The Real-Time PCR was done in a total reaction volume of 25 µl. 22 µl of freshly prepared master mix was added to the well of the experimental plate. 3µl of sample DNA was added to the well containing water and mastermix. Composition of reaction mixture and mastermix for a single reaction was:

Reaction mixture	
Mastermix	19.25 µl
dH2O	2.75µl
Sample DNA	3.00 µl
Total volume	25.00 µl

After adding mastermix to the well, experimental plate was sealed with i-Cycler Optical Sealing Tape and the plate was briefly spun to bring all the reagents to the bottom of the well. The plate was placed into i-Cycler and for the activation of Taq polymerase and initial detanuration at 95°C for 3 minutes was done. Then a 55 cycles of PCR with denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 15 seconds was performed. Following amplification, the fluorescence reading was taken after each extension step.

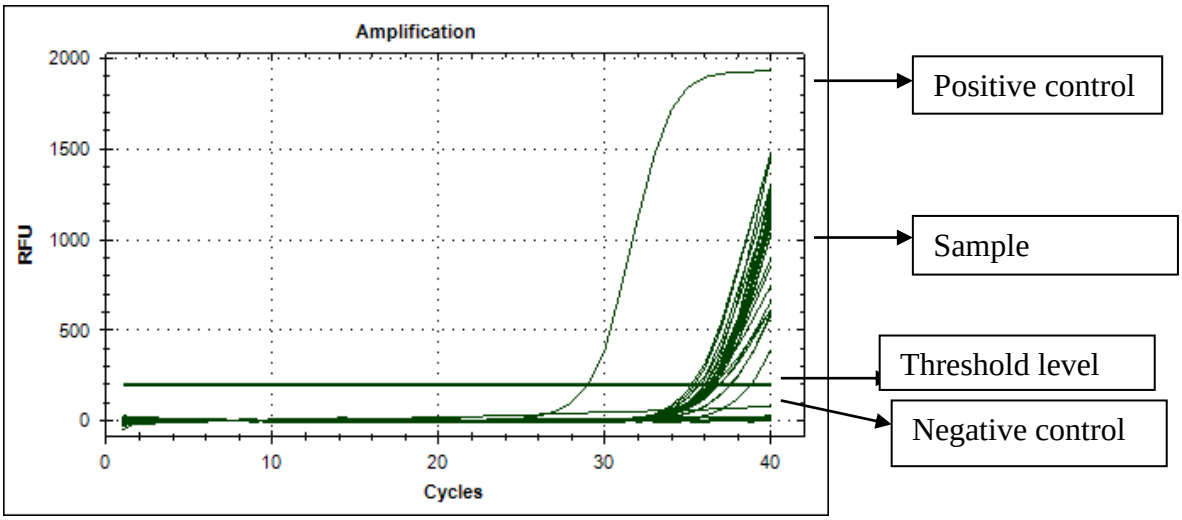


Figure 2.7: Graphic representation of RT-PCR quantitative plot in test is obtained by plotting the intensity of the fluorescence signal versus cycle number after 35 cycles**.** . Here, threshold level is present at 200RFU. Under the threshold value all samples are negative samples and upper the threshold level, all samples are positive. This graph also shows the positive control and the negative control.

Chapter Four

Results

4.1 General Features of the Study Population

This study was approved by the Ethical Review Committee at the International Centre for Diarrheal Disease Research, Bangladesh. Informed consent was obtained from parents or guardians of the children. Children ranged in age from one month to twenty four months.

Total 100 diarrheal fecal specimens from 100 subjects (children) were examined in this study, among them 60 (60%) were male and 40(40 %)were female child. Out of 100 specimens 60 (60%) specimens were collected from male child and 40(40 %)specimens were collected from female child (Table 3.1). Age ranges of these children were 0-24 months.

Table 3.1 The number of tested samples during the study period

Criteria	Number	Percentage
Total samples	100	100%
Collected from	100 diarrhea patients.	100%
Collected from Male Children	60	60%
Collected fromFemale Children	40	40%
Cryptosporidium positive	50	50%
Cryptosporidium negative	50	50%

100 samples were tested by Real-Time PCR (RT-PCR), ELISA, Quik Chek and *EZ VUE* to detect the *Cryptosporidium* infection in fecal samples and stored 50 *Cryptosporidium* spp. positive samples and 50 *Cryptosporidium* spp. negative samples were examined during the study period.

4.2 Age distribution of *Cryptosporidium* infection determined by RT-PCR

The age range of the infected population was 0-24months. This population was divided into 4 age groups (0-6; 7-12; 13-18; 19-24) in 6 months interval.

Table 3.2: Age variations in *Cryptosporidium* positive samples

Age group in months	**No. of *Cryptosporidium* Positive samples**
0-6	**28**
7-12	**13**
13-18	**06**
19-24	**03**
	Total=50

Among the 50 *Cryptosporidium* Positive samples 28 samples were in 0-6 age groups, 13 were in 7-12 age groups, 6 were in 13-18 age groups and 3 were in 19-24 age groups. Prevalence of *Cryptosporidium* infection was found to be highest in patients belonging to the age group of 0-6 months, whereas, minimum infected patients were held in 19-24months old. *Cryptosporidium* positive samples starts from 0 to 24months as shown in table 3.2, Thus it has been said that *Cryptosporidium* usually infects children younger than 5 years of age, with a peak in children younger than 2 years of age.This result is contrasted to the study of Haque *et al.*, 2007 where *Cryptosporidium* infection was present within 2-5 years old children from an urban slum of Dhaka, Bangladesh (Haque *et al*., 2007).

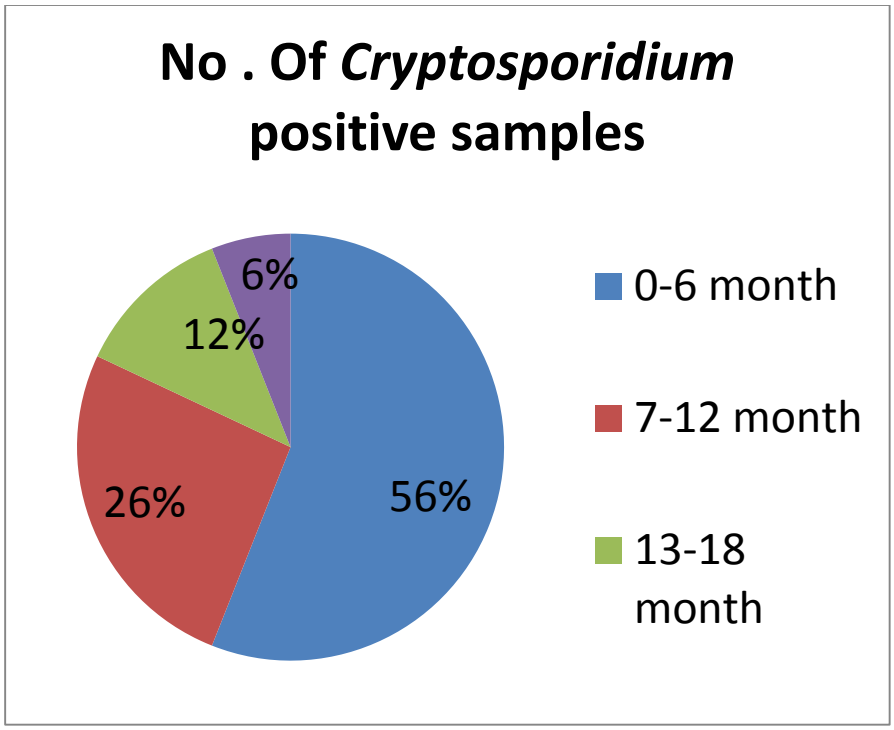


Figure 3.1:Age variations in *Cryptosporidium* positive samples

4.3 Results of *Cryptosporidium EZ VUE*, QUIK CHEK™, ELISA and Real Time PCR (RT-PCR)in fecal samples for detection of *Cryptosporidium* infection

As already mentioned, RT- PCR assay was considered to be the gold standard for this study as it has high sensitivity. RT-PCR assay employs fluorescent labels to enable continuous monitoring of PCR product formation throughout the reaction. The detection period of RT-PCR assay was 100oocysts of *Cryptosporidium* per milliliter, while the antigen detection test required 50 parasites per reaction. A third-generation fecal rapid antigen detection test (*Cryptosporidium EZVUE*; TechLab), was also done which is specific for *Cryptosporidium* oocyst antigen.

Table 3.3 Results of *Cryptosporidium* Real Time PCR (RT-PCR), ELISA ,QuikChek, and *EZ VUE* in fecal samples

	RT-PCR	ELISA	Quik Chek	*EZVUE*
Positive	50	34	37	38
Negative	50	66	63	62
Total	100	100	100	100

Result of *Cryptosporidium* RT-PCR, ELISA and Quik Chek in 100 fecal samples for detection of *Cryptosporidium* infectionis shown in table 3.3. Of these 100 fecal samples, 50 samples are positive and 50 are negative by RT-PCR assay. On the other hand, 34 samples are positive and 66 are negative by ELISA, 37 samples are positive and 63 are negative by Quik Chek and *EZ VUE* reveal positive results 35 out of 100 samples and negative results 65.

4.4 Comparison of *Cryptosporidium EZ VUE* with RT-PCR ,ELISA, and QUIK CHEK™ Assay of *Cryptosporidium infection*in Fecal Samples

4.4.1 Analysis of Sensitivity, Specificity and Positive predictive value (PPV), negative predictive value (NPV) between Real Time PCR, *Cryptosporidium* II ELISA , Quick chek and *EZ VUE*

Sensitivity and specificity are statistical measures of the performance. Sensitivity (also called the true positive rate) measures the proportion of real positives which are correctly identified as having the condition. Specificity (sometimes called the true negative rate) measures the proportion of negatives which are correctly identified as not having the condition.

Sensitivity and Specificity of *EZ VUE* of *Cryptosporidium* infection were calculated with 100 fecal samples using *Cryptosporidium*-specific RT-PCR assay as Gold Standard because of its superior performance.

Table 3.4 Sensitivity, Specificity, Positive Predictive Value (PPV) and, Negative Predictive Value (NPV) of *Cryptosporidium EZ VUE* when *Cryptosporidium*-specific RT-PCR assay considered as Gold Standard'

EZ VUE	***Real time PCR***		**Total**	**Sensitivity %**	**Specificity %**	**Predictive Value**	
	Positive	***Negative***				**Positive**	**Negative**
Positive	35	03	38	70.0		92.1%	
Negative	15	47	62		94.0		75.81%
Total	50	50	100				

Total 100 diarrheal samples were examined for the detection of *Cryptosporidium*, in which 38 were revealed positive by *EZ VUE* and 50 were found positive by specific Real Time RT-PCR. Among 38 positive samples determined by *EZ VUE*, 3 samples were exposed negative by RT-PCR. A true positive result was defined as a specimen identified as positive by use of one or more of the diagnostic techniques. Out of 100 samples examined in the study35 were revealed as true positive (TP) and 3 samples were false positive (because these were detected as negative during real time RT-PCR assay) by *Cryptosporidium EZ VUE*. In this result, the RT-PCR method was used as a "Gold Standard". On the other hand, 47 samples were true negative as these were detected negative by both RT-PCR assay and *Cryptosporidium EZ VUE* assay, 15 samples were positive by "Gold Standard" RT-PCR method, and thus known as false negative. The calculated sensitivity and specificity of *EZ VUE* was 70.0% and 94.0% respectively (Table 3.4).

The positive predictive value (PPV) and Negative Predictive value are the proportions of positive and negative results in statistics and diagnostic tests that are true positive and true negative results respectively. These values are used to describe the performance of a diagnostic test or other statistical measures.

Here, table (3.4) shows that the positive predictive value and negative predictive value between *Cryptosporidium EZ VUE* and RT-PCR were 92.1% and 75.81% respectively. As there were three false positive value, that increased the positive predictive value. As there

were fifteen false negative value; here, negative predictive value was lower than the positive predictive value.

Table 3.5 Sensitivity, Specificity and Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of *Cryptosporidium EZ VUE* when *Cryptosporidium* II ELISA assay as Gold Standard

EZ VUE	***Cryptosporidium II ELISA***		**Total**	**Sensitivity %**	**Specificity %**	**Predictive Value**	
	Positive	***Negative***				Positive	Negative
Positive	34	4	38	100		89.47%	
Negative	0	62	62		93.94		100%
Total	34	66	100				

Sensitivity and Specificity of *EZ VUE* of *Cryptosporidium* infection were tested with 100 fecal samples when specific *Cryptosporidium* II ELISA assay was considered as Gold Standard, because of its superior performance over other traditional detection tool.

This table shows that out of 100 diarrheal fecal samples 34 were positive by *Cryptosporidium* II ELISA assay for *Cryptosporidium* infection, which was defined as an optical density value > 0.150. 38 samples were positive by the *Cryptosporidium EZ VUE*, 4 samples were exposed negative by *Cryptosporidium* II ELISA. Out of 100 samples examined in the study 34 were revealed as true positive (TP) and 4 samples were false positive (because these were detected as negative during *Cryptosporidium* II ELISA assay) by *Cryptosporidium EZ VUE*. In this result, the *Cryptosporidium* II ELISA assay was used as a "Gold Standard". On the other hand, 62 samples were true negative as these were detected negative by both *Cryptosporidium* II ELISA assay and *Cryptosporidium EZ VUE* assay. Thus the *Cryptosporidium* EZ VUE had a sensitivity of 100% and a specificity of 93.94% when compared to ELISA. Therefore this rapid antigen detection test can be completed within 30minutes as early mentioned, appears to be just as sensitive and specific as the commercially available *Cryptosporidium* II ELISA.

Table 3.5 shows that the positive predictive value and negative predictive value between *Cryptosporidium EZ VUE* and ELISA were 89.47% and 100% respectively. The *Cryptosporidium EZ VUE* appears to be just as sensitive and specific. The positive predictive value and negative predictive value of *Cryptosporidium EZ VUE* was 100% while compared with the commercially available *Cryptosporidium* II ELISA.

As there were four false positive value, that increased the positive predictive value. As there was no false negative value; here, negative predictive value was higher than the positive predictive value.

Table 3.6 Sensitivity, Specificity and Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of *Cryptosporidium EZ VUE* infection when *Cryptosporidium* Quick chek as Gold Standard

EZ VUE	***Cryptosporidium Quick chek***		**Total**	**Sensitivity %**	**Specificity %**	**Predictive Value**	
	Positive	***Negative***				Positive	Negative
Positive	37	1	38	100		97.37%	
Negative	0	62	62		98.41		100%
Total	37	63	100				

Sensitivity and Specificity of *EZ VUE* of *Cryptosporidium* infection were calculated with 100 fecal samples using Crypto-Quick chek, because of its superior performance.

Total 100 diarrheal samples were examined for the detection of *Cryptosporidium*, in which 38 were revealed positive by *EZ VUE* and 37 were found positive by Crypto-Quick chek and 1 sample was exposed negative by Crypto-Quick chek. A true positive result was defined as a specimen identified as positive by use of one or more of the diagnostic techniques. Out of 100 samples examined in the study 37 were revealed as true positive (TP), 1 sample was positive by *EZ VUE* method, and thus known as false positive. On the other hand, 62 samples were true negative as these were detected negative by both Crypto-Quick chek and

Cryptosporidium EZ VUE assay. The calculated sensitivity and specificity of *EZ VUE* was 100% and 98.41% respectively (Table 3.6).

Here, table (3.6) shows that the positive predictive value and negative predictive value between *Cryptosporidium EZ VUE* and Crypto-Quick chek were 97.37% and 100% respectively. As there was no false positive value, that increased the positive predictive value. As there were no false negative value; here, negative predictive value was higher than the positive predictive value.

4.5 Analysis of Cross-reactivity of *Cryptosporidium EZ VUE* with *E. histolytica* and *Giardia lamblia* positive (+) samples

4.5.1 *E. histolytica* positive (+) samples

E. histolytica infections occur mostly in children with 2-5 years of age (Haque *et al*., 2009).Here**,** *E. histolytica*50 positive frozen samples were taken to test *Cryptosporidium EZ VUE*. These samples were stored at -20°c.

These fecal samples were detected by RT-PCR, *CRYPTOSPORIDIUM* II ELISA test and *CRYPTOSPORIDIUMEZ VUE* before storage. *Cryptosporidium* Oocyst Wall Protein (COWP) was amplified by singleplex RT-PCR using primer and Taqman probe. The amplified target was 151 bp. On the other hand, *CRYPTOSPORIDIUM* II ELISA and *CRYPTOSPORIDIUM* QUIK CHEK was used to detect *Cryptosporidium* Oocyst antigen in fecal samples, similar antigen was detected in the same fecal samples by using rapid membrane enzyme immune assay *CRYPTOSPORIDIUMEZ VUE*.

In this study, all positive *E.histolytica* were tested by using *CRYPTOSPORIDIUMEZ VUE* to evaluate this newer rapid Immunochromatographic assay method whether it cross reacts with *E. histolytica* or not.

Table 3.7 Cross-reaction test of *Cryptosporidium EZ VUE* with *E. histolytica* positive samples

No. of *E. histolytica* positive samples	No. of *E. histolytica* negative samples(with *Cryptosporidium EZ VUE*)
50	50

This result shows that 50 positive samples which were determined by RT-PCR, ELISA and CRYPTO-QUIK CHEK, were exposed as negative by *Cryptosporidium EZ VUE* (Table 3.7). That means *Cryptosporidium EZ VUE* had no cross reaction with *E. histolytica* positive samples.

4.5.2 Giardia positive (+) samples

A previous study has found *Giardia lamblia* in 68% of children aged between 2 to 8 months in Bangladesh (Haque *et al.* 2007) reported 11.08% *Giardia lamblia* infection in 2–5 years old group in Mirpur, an urban slum area in Dhaka. In Bangladesh several studies has been performed to determine the prevalence of *Giardia lamblia* (Haque *et al.*, 2007) by direct microscopic examination.

Here, *Giardia* 50 positive frozen samples were taken to test *Cryptosporidium EZ VUE*. These samples were stored at -20°c. RT-PCR, *Giardia* II ELISA test and *Giardia* QUIK CHEK were the detection tools used to detect *Giardia*. In this study, highest number of children infected with *Giardia* was in 13-18 age range (30 *Giardia* positive patients). Second highest number of *Giardia* infected children was in 7-12 age range (11 Giardia positive patients); next 07 infected children were in 19-24 age range.

Small subunit ribosomal (16S-like) RNA was amplified by single-plex RT-PCR using primer and Taqman probe. The amplified target was 62 bp. On the other hand, *GIARDIA* II ELISA was used to detect *Giardia* cyst antigen in fecal samples, similar antigen was detected in the

same fecal samples by using rapid membrane enzyme immune assay, *GIARDIA* QUIK CHEK.

In this study, all positive Giardia were tested by using *Cryptosporidium EZ VUE* to evaluate this newer rapid Immunochromatographic assay method whether it cross reacts with Giardia or not.

Table 3.8 Cross-reaction test of *Cryptosporidium EZ VUE* device with Giardia positive samples

	No. of Giardia positive samples	No. of Giardia negative samples(with *Cryptosporidium EZ VUE*)
	50	50

This result in table 3.8 shows that 50 positive samples which were determined by RT-PCR, GIARDIA II ELISA assay and Giardia-QUIK CHEK, were exposed as negative by *Cryptosporidium EZ VUE*. That means *Cryptosporidium EZ VUE* had no cross reaction with Giardia positive samples.

Chapter Five

Discussion

The major finding of our evaluation of the *Cryptosporidium EZ VUE* assay is that the rapid Lateral Flow detection method has comparable performance with *cryptosporidium* specific RT-PCR, *Cryptosporidium* II ELISA and *Cryptosporidium* QUIK CHEK.

In this study, fecal samples were observed by *Cryptosporidium* specific RT–PCR, *Cryptosporidium* II ELISA, *Cryptosporidium* QUIK CHEK as well as *Cryptosporidium EZ VUE* to detect *Cryptosporidium* infection in fecal samples. This is the first report on evaluation of *Cryptosporidium EZ VUE* assay with *Cryptosporidium* specific Real Time-PCR, *Cryptosporidium* II ELISA antigen detection tests and *Cryptosporidium* QUIK CHEK assay.

In the present study, a total of 100 fecal samples were examined where 100 were children. The age range of children was 0 month to 24months. Most of the *Cryptosporidium* positive samples were 0-12months (41 positive samples out of 50 positive samples, 82% were in this age group). Even though the samples were taken from children 0 month to 24months age, most of the *Cryptosporidium* positive samples were from children who were younger than 5 years old. These findings are contrasted to those reported by Haque *et al*., 2007, where *Cryptosporidium* infected children were in 2-5 years old.

This research was conducted in order to find out the efficacy of the new *Cryptosporidium EZ VUE* assay. For the evaluation of this technique, parameters such as sensitivity, and specificity, positive predictive value, negative predictive value were used. Specificity and sensitivity are inter-related with positive predictive value and negative predictive value. More specific test gives a better positive predictive value and a more sensitive test gives a better negative predictive value (Fletcher and Fletcher, 2005).

While *Cryptosporidium EZ VUE* assay was compared with *Cryptosporidium* specific RT-PCR assay, 70.0% was sensitive and 94.0% was specific. Out of 100 samples examined in this study, 50 samples were revealed as true positive where 3 samples were false positives, and 47 samples were true negative while 15 samples were false negative by RT-PCR. A number of reasons can be accounted for 3 false positive results i.e., cross contamination; degraded or impure DNA might limit the efficiency of the RT reaction and reduce the amplicon (DNA product) yield. DNA should be prepared from fresh fecal samples, however, this study was done with samples

collected in 2012-2013 and stored in -20◦ c since then. Partially degraded DNA might not give an accurate representation of gene expression. A Reproducibility of RT-PCR result could also highly influenced by pipetting variation. Electronic pipetting is preferable to manual pipetting. Typical pipetting rules and precautions should be followed to control pipetting error. Once the solution is drawn, it is important to be sure that any excess volume on the outside of the tip is not carried over. Poor primer and probe design might be another cause of less DNA amount to identify infection. Thus use of primer design software is highly recommended for primer and probe design.

In *EZVUE* testing, *Cryptosporidium*-specific oocyst antigen of *Cryptosporidium* was detected; other antigens present in stool samples may be cross-contaminated and showed positive results in *EZ VUE* device, which was not detected by PCR due to absence of DNA of oocyst antigen. DNA is more degradable than stool, as DNA is used for RT-PCR and direct stool is used for *EZ VUE*. This may be another cause of less DNA product to detect infection.

On the other hand, DNA extraction is also an important part of RT-PCR. This process is much time consuming, it needs four and half hour by expertise to obtain Total Nucleic Acid (TNA) from stools. There were twenty four steps among these steps nine times centrifuge was needed. Two time incubations were also included. Thus cost effective and less time consuming *EZ VUE* process can be alternatively used as detection tool for indentifying *Cryptosporidium* infection.

During *Cryptosporidium* II ELISA was considered as Gold Standard, 34 were positive by both *Cryptosporidium EZ VUE* and *Cryptosporidium* II ELISA. And 62 samples were revealed as negative by both *Cryptosporidium EZ VUE* and *Cryptosporidium* II ELISA. Therefore, *Cryptosporidium EZ VUE* assay was 100% sensitive and 93.94% specific with compared to *Cryptosporidium* II ELISA. This result was similar to the previous study done by Agnamey *et al.*,2011, reported that *Cryptosporidium EZ VUE* assay was 100% sensitive and specific with *Cryptosporidium* II ELISA.

During *Cryptosporidium* QUIK CHEK, 37 were positive by both *Cryptosporidium* EZ VUE and *Cryptosporidium* QUIK CHEK. And 62 samples were revealed as negative by both *Cryptosporidium EZ VUE* and *Cryptosporidium* QUIK CHEK. Therefore, *Cryptosporidium EZ*

VUE assay was 100% sensitive and 98.41% specific with compared to *Cryptosporidium* QUIK CHEK.

Cryptosporidium II ELISA is a reliable and widely used detection method, however, it takes two and half hours for providing complete result and *Cryptosporidium* QUIK CHEK method takes 30 minutes for providing complete result . On the other hand *Cryptosporidium EZ VUE* assay takes only 10 minutes for giving as accurate result as ELISA and QUIK CHEK gives. Fieldworkers can easily apply this method in clinical site without being expert what is necessary during RT-PCR as well as during ELISA test.

Positive predictive value helped to determine the number of *Cryptosporidium* infected samples that were actually positive out of the samples found to be positive. It describes the percentage of patients with a positive test who actually have the disease, if the positive predictive value is higher (as close to 100 as possible) then it suggests that this new test is doing well as "gold standard". Negative predictive value helped to calculate the number *Cryptosporidium* infected samples that were truly negative out of the samples found to be negative(Fletcher and Fletcher, 2005). Positive predictive value of *Cryptosporidium EZ VUE* was found to be 92.1% and negative predictive value was less than positive predictive value, which was 75.81% when *Cryptosporidium* infection was searched by *Cryptosporidium* specific RT-PCR assay. And positive predictive value and negative predictive value of *Cryptosporidium EZ VUE* was 89.41% and 100% while compared with ELISA that means *EZ VUE* is as well as *Cryptosporidium* specific ELISA.

On the other hand, positive predictive value and negative predictive value of *Cryptosporidium EZ VUE* was 97.37% and 100% while compared with *Cryptosporidium* QUIK CHEK, which means *EZ VUE* is as well as *Cryptosporidium* QUIK CHEK.

Our study was extended with cross-reactive analysis of *Cryptosporidium EZ VUE* assay. Cross reaction has a negative impact on accurate diagnosis. Same antigenic components are exhibited in the cross reaction among parasites. As *Cryptosporidium EZ VUE* assay was identified antigen in *Cryptosporidium* and this specific antigen was absent in both *E. histolytica* and *Giardia lamblia* and therefore there was no cross-reaction with these two parasites. Total 50 *E. histolytica* positive samples and 50 *Giardia lamblia* positive samples were used for cross reaction and

among 100 samples were negative during *Cryptosporidium EZ VUE* assays. Thus, it can be said that it is 100% cross reaction free rapid point of care test. However, the use of this rapid point of care test is still unimplemented in most of the clinical sites in Bangladesh.

The *Cryptosporidium EZVUE* can be used for quick and reliable diagnosis of Cryptosporidiosis, and as an alternative to labor-intensive methods such as ELISA and PCR. The *Cryptosporidium EZ VUE* may be more practical for use in the developing world than the commercially available *Cryptosporidium* II ELISA because the later requires expensive equipment, skilled personnel, and takes more than two hours to perform. The *Cryptosporidium EZ VUE* can be performed and interpreted within 10 minutes, without any additional equipment, and with minimal technical training. Thus, the *Cryptosporidium EZ VUE* is an ideal point-of-care test that can be performed in resource-limited settings for rapid and accurate diagnosis of Cryptosporidiosis. In remote areas and field sites ,Fieldworkers can use this method to detect *Cryptosporidium* infection rapidly and timely and immediately take steps to cure this infection that increase morbidity and mortality in developing countries. This *Cryptosporidium EZ VUE* technique has already been used in different clinical sites correlated with International Center for Diarrheal Disease and Research (ICDDR'B) as an alternative of conventional microscopy.

In this study, *Cryptosporidium* specific RT-PCR had error during DNA extraction, old storage samples (from 2012-2013) were used for DNA extraction. This could be made less amount of PCR product and this shortage of amplicons (DNA product) could not reach the amplification level. Use of stored samples for real time PCR in this study may limit the true detection of *Cryptosporidium* infection. Cross reactivity test of *Cryptosporidium* EZ VUE was limited with *E. histolytica* and *Giardia* spp. While other parasites and viruses are also responsible for diarrheal diseases. It will be interesting in future studies to estimate the cross-reactivity of this assay with other microbes. The limitations for this study also include the tests employed for diagnosis of *Cryptosporidium* did not include more accurate methods such as PCR and Microscopy, against these comparisons of the RDT test is imperative. Moreover, failure to characterize the *Cryptosporidium* parasite using molecular techniques in those false negative samples might be a missed opportunity to better elucidate the findings in a way to offer inputs towards continuous improvement of RDT's performance.

Lateral flow Test improves the efficiency of laboratories by reducing labor, time and resources; thus, has great significance in screening large populations such as HIV infected people, children, and in outbreak scenarios. It should be noted that an RDT with such performance is not to replace microscopy, but rather to serve as a diagnostic option in situations where microscopy is not suitable.

In remote areas and field sites, Fieldworkers can use this method to detect *Cryptosporidium* infection rapidly and timely. Timely and accurate diagnosis of *Cryptosporidium* is important to properly manage infected individuals and understand its epidemiology for effective prevention.

We concluded that the *Cryptosporidium* lateral flow has a comparable sensitivity & specificity to the *Cryptosporidium* II ELISA, *cryptosporidium* specific RT-PCR, *Cryptosporidium* QUIK CHEK and is rapid, reliable, and easy to use in the field.

Chapter Six

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