EPIDEMIOLOGY, GENETIC DIVERSITY AND DIAGNOSTIC PROCEDURES OF *CRYPTOSPORIDIUM* SPECIES IN BUNGOMA COUNTY, KENYA

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Epidemiology, Genetic Diversity and Diagnostic Procedures of *Cryptosporidium* Species in Bungoma County, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This thesis is dedicated to my parents William Wasike and Ephamia Nanjala who have always encouraged me to achieve the ultimate goals in my live and to my wife and children who have brought a lot of joy in my life.

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Lastly I wish to thank everybody who contributed in one way or another towards the successful completion of this work. I may not have mentioned them by name, but that does not make them any less important. Thank you so much and may God bless you all.

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ABBREVIATION AND ACRONYMS

AIDS   Acquired Immune Deficiency syndrome

Crypto *Cryptosporidium*

DFA   Direct Fluorescent Antibody Assay

DNA   Deoxyribonucleic Acid

EIA   Enzyme Immuno Assay.

HIV   Human Immunodeficiency Virus

ICZN   International Code of Zoological Nomenclature

JKUAT   Jomo Kenyatta University of Agriculture and Technology.

PCR   Polymerase Chain Reaction

USA   United States of America

ZN   Ziehl Neelsen Staining Procedure.

ABSTRACT

Across-sectional study on prevalence of cryptosporidiosis was carried out in Bungoma County, Western region of Kenya. The main objective of the study was to determine the epidemiology, genetic diversity and diagnostic procedures of *Cryptosporidium* species in Bungoma County, Kenya. A total of 712 fecal samples from children up to five years of age were collected, from four Hospitals in Bungoma County, Western Kenya over a period of 30 months covering January 2011 to June 2013. Overall prevalence of cryptosporidiosis in children was 5.1 %. Peak prevalence was observed between June and July, with a smaller peak between October and November in both 2011 and 2012. Genotype analysis based on Polymerase Chain Reaction- restriction fragment length polymorphism (PCR-RFLP) of the 18S rRNA gene fragment revealed that among the *Cryptosporidium* positive patients of study group 38.89% (N=36) of the isolates were C. *hominis* 36.11% were *C. parvum*, while the prevalence of C. *meleagridis* and *C. canis* was 16.67% and 5.56% respectively. Cryptosporidiosis was significantly associated with diarrhea (OR=1.7793, CI=0.3670-1.6650, *P*=0.03164) and Abdominal swelling and pain, (OR=1.4711, CI=0.5680- 1.6202, *P*=0.04926). However, other symptoms such as headache, cough, fever and vomiting were not significantly associated with cryptosporidiosis, in the study groups. There were no significant sex related differences observed in the cryptosporidiosis prevalence in the children (*P*=0.119). However environmental factors such as water sources and treatment, or presence of livestock in homesteads were significantly associated with cryptosporidiosis. The results suggest that cryptosporidiosis prevalence is comparable to other regions of the world with C. *hominis* being the most common species circulating in the study area followed by *C. parvum* and C. *meleagridis* in that order. These results also suggest that both animals to human and human-to-human transmission are the main modes of spread of cryptosporidiosis in the region. The presence of animal species of *Cryptosporidium* such as *C. canis* in the study patients also suggested that animal reservoirs may still be an important link in the transmission dynamics of *Cryptosporidium spp.* It is therefore recommended that

diagnosis of *Cryptosporidium spp* be included as a mandatory test during routine examination of stool specimens for ova and parasites, and that mitigation effort against the disease should focus on preventive measures, research for an effective drug against the parasite, and that mitigation against the disease should focus on preventive measures that include research on effective drugs against *Cryptosporidium* and other intestinal parasites.

CHAPTER ONE

INTRODUCTION

This chapter deals with the background information about *Cryptosporidium* species including its biology, transmission, symptoms and management, statement of the problem, justification of the study, objectives and the scope of the study.

1.1. Background information

Cryptosporidium is a protozoan pathogen of the phylum Protozoa, sub-phylum Sporozoa and class Apicomplexa that causes a diarrheal illness called cryptosporidiosis. It was first discovered by Ernest Edward Tyzzer in 1907 (Marshall, Naumoritz, Ortega, & Sterling, 1997; Xiao, Bern, Sulaiman & Lal, 2004). *Cryptosporidium* was so named because of the absence of sporocysts within oocysts, which is a common characteristic of other Coccidia. Other Apicomplexan pathogens include *Plasmodium* and *Toxoplasma*. Unlike *Plasmodium* which is transmitted via a mosquito vector, *Cryptosporidium* does not utilize an insect vector and is capable of completing its lifecycle within a single host, resulting in microbial cyst stages that are excreted in feces (Carpenter, Ayer, Trout & Beach, 1999).

This protozoan parasite species is a leading cause of infectious diarrhea in humans and livestock (Wangeci *et al.,* 2006), with fecal-oral transmission by ingestion of oocysts. Infection is generally self-limiting, followed by variable protective immunity involving humoral and cell mediated responses, except in the immune suppressed, when infection may be prolonged and fatal (Marshall, Naumoritz, Ortega, & Sterling, 1997). *Cryptosporidium* oocysts remain viable in water and damp soils, for prolonged periods of up to two years and are resistant to disinfectants at concentrations usually used in

water treatment (Korich, Mead, Madore & Sinclair, 1990; Reinhardt, Casemore, & Miller, 2001).

Cryptosporidiosis accounts for 10.5% of nearly 8 million yearly deaths of children under five years (World Health Organization [WHO], 1997). It is the second biggest cause of diarrhea. For three out of the four leading causes there are drugs and vaccines but for *Cryptosporidium* there is no fully effective drug or vaccine for its treatment or immunization (Molbak *et al*., 1993;Tripoli & Ward, 2002; Xiao, Bern, Sulaiman & Lal, 2004; Abubakar, Aliyu, Arumugam, Hunter & Usman, 2007).Outbreak investigations have shown diverse modes of transmission, including contact with livestock (Casemore, Armstrong & Sands, 1985; Le chevalier, Norton, & Lee, 1991; Sayers, Dillon, &Connolly, 1996) person to person transmission in households and care settings (Cordel & 1996), consumption of contaminated foods and drinks, including milk (Gelleti, Stuart, Soltano, Armstrong & Nicols, 1997; Fayer, Morgan, & Upton, 2001).

Cryptosporidiosis has a worldwide distribution and in most surveys is among the four major pathogens causing diarrheal diseases in children. Notable outbreaks of cryptosporidiosis have been recorded in various parts of the world. In 1993, a water borne cryptosporidiosis outbreak occurred in south eastern Wisconsin, USA, during which an estimated four hundred and three thousand people became ill. Four thousand four hundred people were hospitalized and more than one hundred people died during the outbreak (Corso *et al.,* 2003).

In the summer of 1996, *Cryptosporidium* affected about 200 people in Cranbrook, Canada while in April 2001, an outbreak occurred in the city of North Battleford in Saskatchewan, Canada where between five thousand eight hundred and seven thousand one hundred people suffered from diarrheal illness (Public Health Agency of Canada, 2001).

In March 2007, a suspected cryptosporidiosis outbreak occurred in Galway Ireland, after a water source for much of the County was suspected to be contaminated with the disease. A number of studies from India have reported *Cryptosporidium* spp in diarrheal stool samples from children, with positivity rates of up to nearly 20% (Kaur *et al*., 2002) and asymptomatic infection rates of up to 10% (Mathan, Venkatesan, George, Mathew & Mathan,2005), using stool microscopy for detection.

A recent study on cryptosporidiosis conducted in Egypt by Abdel-Messih *et al.,* (2005), examined 1275 children, attending two hospitals and found prevalence of 17%. The study also found that children less than 12 months of age were most likely to get cryptosporidiosis and infection was significantly associated with diarrhea, vomiting and a need for hospitalization.

Another survey by Tumwine *et al.,* (2005) on the prevalence of cryptosporidiosis among Human Immune Deficiency Virus (HIV) infected and uninfected children with persistent diarrhea at Mulago hospital in Uganda showed 73.6% (67 of 91) of HIV infected children and 5.9% (9 of 152) of HIV negative children were infected.

Another recent study on cryptosporidiosis conducted in Kenya examined 4899 samples and showed an overall prevalence of 4%. The prevalence was highest in children 13-24 months of age (5.2%) and Lowest among those 48-60 months of age (Wangeci *et al.,* 2006).The main symptoms associated with the disease were abdominal pain, vomiting and abdominal swelling (Wangeci *et al.,* 2006).

In a cross- sectional study among 6- months to 15 year olds presenting to two Western Kenya Hospitals with acute diarrhea between 2011 and 2013, *Cryptosporidium* species had a prevalence of 3.7% (Pavlinac *et al.,* 2014). In a cross sectional study to investigating the epidemiological and clinical features of cryptosporidiosis, molecular

characteristics of the infecting species and serum antibody responses to *Cryptosporidium* specific antigen in HIV/AIDS patients at Kenyatta National hospital in Nairobi, Kenya, Wanyiri *et al* (2014) found a 34% prevalence, 36% of which had diarrhea while 33% were without.

In a study on burden of *Cryptosporidium* diarrheal disease among children less than 24 months old, that utilized data from Global Enteric Multicenter Study, Sow *et al.*, (2016), showed that *C. hominis* had a prevalence of 77.8% and *C. parvum* at 9.9%. 92% of *C. parvum* tested were anthropogenic genotypes. Annual *Cryptosporidium* Moderate to severe diarrhea (MSD) incidence was 4.48%. *Cryptosporidium* attributable deaths were about 59000 in excess of the expected if cases had been *Cryptosporidium* negative.

In another recent study on diversity of *Cryptosporidium* in children in urban informal settlements, Mbae *et al* (2015) found that *Cryptosporidium hominis* was the most common species of *Cryptosporidium* at 82.8% (125/151) followed by *C. parvum,* at 11.9%.

1.1.1. Biology and Life Cycle of *Cryptosporidium*

The life cycle of most *Cryptosporidium* species is completed within the gastrointestinal tract (primarily the small intestine) of the host, with developmental stages being associated with the luminal surface of the mucosal epithelial cells (Figure 1.1). Infection results from the ingestion of the resistant and infective stage of *Cryptosporidium*, the oocyst which releases four sporozoites that invade the epithelial cells.

In this process, the sporozoites induce the evagination of the host cell plasma membrane that surrounds the sporozoite and forms a parasitophorous vacuole. As a result, the parasite occupies an intracellular but extra-cytoplasmic localization, which is unique to

the genus *Cryptosporidium*. A feeder organelle allows the selective metabolic exchanges between the parasite and the cytoplasm of the host cell (CDC, 2012).

The sporozoites then differentiate into trophozoites that undergo asexual replication to form Type 1 meronts. The Type 1 meronts rupture releasing eight Type 1 merozoites that invade new cells, resulting in a second cycle of asexual replication that culminates into the production of Type 2 meronts, which contains four Type 2 merozoites. These can again invade host cells and form the sexual stages, the male microgamont or the female macrogamont (White, 2015).

Fertilization precedes the development of sporulated oocysts that are either thick or thin walled. Thick-walled oocysts are excreted by the host into the environment, whereas thin-walled oocysts can excyst endogenously, resulting in autoinfection, which helps to explain the mechanism of persistent infections (Gregory *et al.,* 2001; White, 2015).
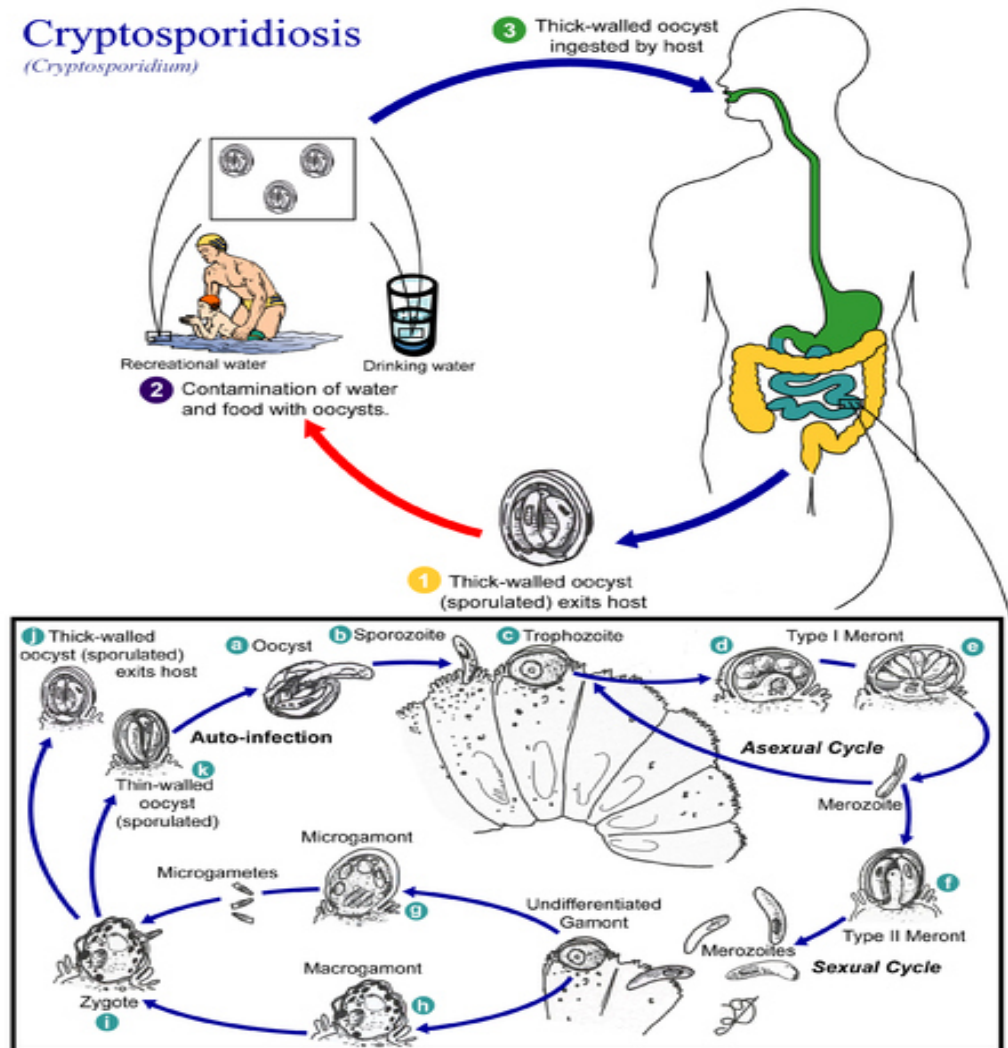
Figure 1.1:The life cycle of *Cryptosporidium*. Sporulated oocysts, containing 4 sporozoites, are excreted by the infected host through feces (1). Transmission occurs through exposure to infected animals or exposure to water contaminated by feces of infected animals (2). After ingestion by a suitable host (3), excystation occurs. (a) The sporozoites are released and parasitize epithelial cells (b, c) of the gastrointestinal tract, where the parasites undergo schizogony(d,e,f) and gametogony producing microgamonts (male) (g) and macrogamonts (female) (h). Upon fertilization of the macrogamonts by the microgametes (i), oocysts (j, k) develop that sporulate in the infected host. (CDC, 2012)

1.1.2. Transmission of Cryptosporidiosis

The protozoan parasite *Cryptosporidium* species is transmitted through the fecal-oral route by ingestion of oocysts (Goh *et al*., 2004). A major route of infection with *Cryptosporidium* is person-to-person transmission, as illustrated by outbreaks in day-care centres (Casemore 1990; Cordel & Adiss, 1996; Fayer *et al.,* 2001) and the spread of infection within the households of children attending these centres. Sexual practices involving oral-anal contact (rimming) also involves a high risk of exposure to the organism. Mammals, especially newborn animals such as calves and lambs may also be a source of infection, although they don't appear to be an important source of infection (Casemore 1990; Glaser, Saffrin, Reingold, & Newman, 1998).

Water borne outbreaks of Cryptosporidiosis have been attributed to contaminated drinking water from both surface water and ground water sources (Crawn, 1998; Mackenzie, Kazmiercza, & Davis, 1995), and to recreational water including swimming pools (Joce, 2002). Outbreaks caused by drinking water have been attributed to contamination of the water source by heavy rainfall or snow melt (Richardson, 1991) to sewage contamination of wells (D'Antonio *et al*., 1985; Kramer *et al*., 1996), to inadequate water treatment (Richardson, 1991; Badenoch, 1992).

The parasite lives in the intestine of infected humans or animals. Millions of *Cryptosporidium* parasites can be released in one bowel movement from an infected human or animal. Consequently the parasite may be found in soil, food, water or surfaces that have been contaminated with infected human or animal feces (Tarkhashvili, 2006). If a person swallows the parasite, they become infected.

The parasite may be spread by accidentally putting something in the mouth or swallowing something that has come into contact with feces of a person or animal infected with *Cryptosporidium*, or swallowing recreational water contaminated with

Cryptosporidium. Recreational water includes water in swimming pools, hot tubs, fountains, lakes, rivers, springs, ponds or streams that can be contaminated with sewage or feces from humans or animals. It should be noted that *Cryptosporidium* species can survive in swimming pools with adequate levels of chlorine (Clark & Diamond, 1991; Report, 2006), eating uncooked food contaminated with *Cryptosporidium* (Goh *et al.,* 2004; Tarkhashvili, 2006), accidentally swallowing *Cryptosporidium* parasites picked up on surfaces (bathroom fixtures, changing tables, diapers or toys) contaminated with feces from an infected person (Goh *et al*., 2004; Tarkhashvili, 2006).

1.1.3. Symptoms of Cryptosporidiosis

Majority of cryptosporidiosis infections are asymptomatic. In the symptomatic form, the most common symptom is watery diarrhea or loose stools. Other symptoms include dehydration, weight loss, stomach cramps or pain, fever, nausea and vomiting (Tarkhashvili, 2006; Wangeci *et al*., 2006). Symptoms of cryptosporidiosis generally begin 2-10 days (average 7 days), after becoming infected with the parasite. In persons with healthy immune systems, symptoms usually last about1-2 weeks. The symptoms may go in cycles in which one may seem to get better for a few days then feel worse again before the illness ends (Report, 2006).

People with severely weakened immune systems often cannot clear the parasite. They can suffer more severe diarrhea that can last long enough to be life threatening. People with HIV infection, cancer and transplant patients taking certain immune-suppressing drugs and persons with inherited diseases that affect the immune system have a higher risk of prolonged cryptosporidiosis (Tumwine *et al.*, 2005).

1.1.4. Diagnosis of *Cryptosporidium* Species

Various methods have been applied to detect oocysts in feces but difficulties of discriminating between non-Cryptosporidial bodies, acid-fast bodies like Cryptosporidia and *Cryptosporidium* remain. Screening by use of wet preparations has been found to be insensitive and not particularly helpful, although it may be useful in detecting cysts or ova (Casemore, Armstrong, & Sands, 1985; Omoruyi, Uchechukwu, Chukwuneke, & Okonkwo, 2014).

A modified Ziehl Neelsen staining method has been widely recommended and used. Its main limitation is that it has many stages involving concentration and staining, therefore unsuitable for handling large batches of specimens in routine laboratory examination. Overall, microscopic identification requires trained microscopists and involves time and labor for preparing, staining and examining (Mank, Zaat, Deelder, van Eijk, & Polderman, 1997; Marshall, Naumoritz, Ortega & Sterling, 1997).

As a result, immunoassays for detection of *Cryptosporidium* stool antigen have replaced microscopy as the routine diagnostic procedure of choice in hospitals and public health laboratories that routinely carry out this test (Fotedar *et al.,* 2007). Polymerase chain reaction (PCR) has been used to identify the various species and genotypes/strains of *Cryptosporidium*. This is a method used to analyze a short sequence of DNA or RNA even in samples containing only minute quantities of DNA or RNA by amplifying selected sections of the DNA or RNA.

1.1.5. Pathogenicity of *Cryptosporidium* Species

Much of the understanding of the patho-physiology of cryptosporidiosis has been the result of studies using calf, neonatal pig and primate. While cryptosporidiosis is most commonly a disease of the small intestine in immuno-competent individuals, extra-

intestinal gastric, hepatobilliary, pancreatic and pulmonary infections can cause signs, symptoms and even death in immuno-deficient or immunosuppressed individuals (Rivasi, Rossi, Righi & Pozio, 1999). Three major mechanisms have been proposed for the diarrhea seen with cryptosporidiosis: 1) mal-absorption resulting in an osmotic diarrhea; 2) parasite induced generation of inflammatory products and host neurohumoral secretogogues; 3) secretory diarrhea resulting from a parasite enterotoxin.

Infection studies in healthy human volunteers have demonstrated a clear relationship between probability of infection and the ingested oocysts dose of *Cryptosporidium* (Dupont, 1995). At the lowest dose of 30 oocysts, the probability of infection was 20%. However at a dose of 1000 oocysts, probability increased to 100% (Teunis & Havelar, 1999).The average incubation period varies widely but is usually about 7 days (Dupont, 1999). Watery diarrhea is the most prominent symptom of intestinal *Cryptosporidium* infections (Fayer, Speer & Dubey, 1997), and the frequent and copious bowel movements can cause dehydration and weight loss (Arrowood, 1997).

In immuno-competent individuals, the infection is limited by the immune response that clears the parasite. However infections in patients with defective cellular immune response (congenital or due to AIDS or chemotherapy) or humoral immune response (in congenital hypogammaglobulinaemia) are persistent and heavy, suggesting that both types of immune response are needed to limit and clear the infection. Several animal studies suggest that the immune response protects against re-infection (Gatei *et al.*, 2003), and protective immunity in humans is indicated by the large numbers of asymptomatic carriers in countries with a high prevalence of cryptosporidiosis.

The duration of the infection is generally 7–14 days in immunocompetent individuals, but a median duration of 23–32 days has also been reported (Van Asperen *et al.*, 1996). The peak intensity of oocyst shedding coincides with the peak intensity of clinical

symptoms. Relapses of diarrhea are common with up to five additional episodes in 40-70% of patients in population reporting relapses.

1.1.6. Treatment and Prevention of Cryptosporidiosis

There is no reliable treatment for *Cryptosporidium* enteritis. Certain agents such as Paromomycin, Atovaquine, and azithromycin are sometimes used but they usually have only temporary effects (Smith & Corcoran, 2004). Treatment is primarily supportive. Fluids need to be replaced orally especially where it results in diarrhea. A lactose-free diet should be taken as tolerated by the patients. In rare situations, intravenous fluids may be required.

People who are immunocompromised are at a higher risk for more severe and more prolonged illness. Cryptosporidiosis resolves slowly or not at all and frequently causes a particularly severe and permanent form of watery diarrhea coupled with a greatly decreased ability to absorb key nutrients through the intestinal tract. The result is progressively severe dehydration, electrolyte imbalances, malnutrition, wasting and eventual death. Spiramycin has been used and can help treat diarrhea in patients who are in the early stages of AIDS (Saez-Llorens, Odio, Umana & Morales, 1987).

Currently the best approach is supportive therapy to improve the immune status in immuno-deficient individuals. If one has diarrhea, then he/she should drink a lot of fluids to prevent dehydration. Rapid loss of fluids because of diarrhea can be life threatening in babies. Anti-diarrheal medicine may help slow down the diarrhea but a health care provider should be consulted first (Tarkhashvili, 2006).

A new drug, Nitazoxanide was approved in the USA for treatment of diarrhea caused by cryptosporidiosis in people with healthy immune system. However, the effectiveness of Nitazoxanide in immuno-compromised individuals is unclear (Tarkhashvili, 2006;

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Abubakar, Aliyu, Arumugam, Hunter, & Usman, 2007). Some drugs such as Paromomycin may reduce the symptoms of *Cryptosporidium* and new drugs are being tested (Saniel, Moriles & Monzon, 1995). However *Cryptosporidium* is usually not cured and may recur if the immune status worsens. For persons with AIDS, antiretroviral drugs (ARVS) therapy that improves immune status will also decrease or eliminate symptoms of *Cryptosporidium* infection (Tarkhashvili, 2006).

Prevention is primarily through maintaining good hygiene and sanitary conditions. This includes but is not limited to measures such as washing hands with soap and water which is the single most important step that can be taken to prevent Cryptosporidiosis and other illnesses, practice safe sex because infected people may have *Cryptosporidium* on their skin in the anal and genital areas including the thighs and buttocks. Rimming (kissing or licking the anus) is also likely to spread infection, hence should be avoided.

One should avoid touching farm animals particularly calves, lambs and other young animals. Equally the stool of any animals including that of pets should be avoided. Avoid swallowing water when swimming in oceans, lakes, rivers or pools and when using hot tubs. Wash and/or cook food: Fresh vegetables and fruits can be contaminated with *Cryptosporidium*. Raw fruits should be peeled after washing.

People should not drink un-pasteurized milk or dairy products unless they are well cooked. Water should be boiled at a rolling boil for one minute which kills the protozoa. Filter drinking water with filters with the label: Reverse osmosis (Tarkhashvili, 2006).

1.2. Statement of the Problem

Cryptosporidium has been shown to be a major cause of diarrhea and other enteric symptoms in children and in immuno-compromised individuals in various studies. Initially thought of basically as an animal parasite, there has been an increase in cases of human *Cryptosporidium* infection outbreaks since the discovery of the first human infection in 1976.

Although most of the human infections have been attributed to 'human genotypes 'such as *C. parvum* and *C. hominis,* there has been a rise in the number of infections with non-human genotypes such as *C. canis, C. meleagridis* and *C. felis*, which underlines the importance of animal reservoirs in the transmission dynamics of the parasite. Epidemiological studies done in other parts of the world have shown this hitherto little known protozoa is a more significant cause of morbidity.

However, despite this fact being known, not much has been documented or done about the genotypes and epidemiology of this parasite in some parts of Kenya, including the study area. There is also a dearth of information regarding the perceptions and knowledge of the general population regarding *Cryptosporidium* as a cause of diarrheal disease. At the same time, *Cryptosporidium* is not routinely diagnosed in most laboratories in Kenya during ova/parasite examinations. Where it is examined, the test is tedious, time consuming and requires experienced microscopists or it may be expensive, especially where PCR procedure is used. In Kenya PCR is not used for routine diagnosis of *Cryptosporidium* and it is mainly used for species identification in Research facilities. Knowing the epidemiology of cryptosporidiosis in various parts of the country, the genetic diversity and the validation of a cheaper and quicker method of diagnosis is important in controlling this disease should it become necessary.

1.3. Justification of the Study

This study was carried out to determine the epidemiology of *Cryptosporidium* in Bungoma County. The county was chosen for the study because it was one of the regions in Kenya where epidemiological studies of *Cryptosporidium* had not been done before.

Most communities in Bungoma County do not have piped water and rely mainly on borehole and spring water and water from rivers for domestic use. Majority are livestock farmers and due to shrinking sizes of land owned by individual households, coupled with economic hardships and occasional cattle rustling many families are keeping livestock in small plots adjacent to their residential houses. At the same time, many families in the rural settings live in mud walled houses with un-cemented floors. They therefore routinely use cow dung to pave their floors. In all these cases there is increased risk of people coming into contact with *Cryptosporidium* oocysts.

Because of all these factors it was necessary to carry out this study to determine the prevalence of *Cryptosporidium* in the County, the risk factors associated with it and the perceptions and level of Knowledge about the parasite in the study population.

1.4. Significance of the study

This study would provide information about the extent of *Cryptosporidium* problem in the study area, the possible sources of infection and the risk factors involved. This would help in drawing attention to the problem and in designing possible mitigation measures against the parasite.

Genotyping of *Cryptosporidium* species would help to identify the parasite species prevalent in the study area and therefore the disease burden attributable to each *Cryptosporidium* species. Again this would help in identifying the possible sources of

infection as some are predominantly animal species but can and do infect human beings while others are mainly anthroponotic.

Validation of the ImmunoCard STAT! Rapid assay would make diagnosis of *Cryptosporidium parvum* faster and reliable which is important for management of *Cryptosporidium* infections. The information may also assist public health laboratories and other related bodies in deciding whether to adopt the diagnostic procedure or not.

Lastly, the study would provide data on *Cryptosporidium* co-infections with other enteric parasites in the study group. This is important in establishing whether the presence of Cryptosporidium is influenced by the presence of other enteric parasites or vice versa. It may also assist in evaluating and re-designing programs geared towards eradicate enteric parasites in the County.

1.5. General Objective

To determine the epidemiology, genetic diversity and diagnostic procedures for detecting *Cryptosporidium* species in Bungoma County, Kenya.

1.5.1. Specific Objectives

The specific objectives for this study were:

1. To determine the epidemiology of *Cryptosporidium species* infections and disease pattern in children up to five years of age in Bungoma County,
2. To determine the genetic diversity of *Cryptosporidium species* prevalent in the study area and the risk factors associated with it.
3. To validate the ImmunoCard STAT! Rapid assay as an alternative and faster method of diagnosing *Cryptosporidium parvum* and *Giardia lamblia* in patients.

4. To determine the extent of co-infection of *Cryptosporidium species* with other intestinal parasites in the study population.

1.5.2. Scope of the Study

The study was carried out in selected hospitals in Bungoma County. It targeted children up to five years of age who visited Bungoma, Webuye, Kimilili and Chwele District Hospitals, who were referred to the laboratories specifically for ova and parasite examinations. Patients recruited into the study had to have been residents in the study area for the previous six months prior to the hospital visit. This study was confined to Bungoma County of Western Kenya which has its own unique social-cultural and economic characteristics

Although there are other methods for detecting *Cryptosporidium species*, this study focused on Ethyl Acetate Formalin Concentration method, Modified Ziehl Neelsen staining Method, ImmunoCard Stat! Assay and Nested Polymerase Chain Reaction targeting 18S ribosomal DNA fragment for detection and determination of molecular diversity of *Cryptosporidium* species.

1.5.3. Chapter Summary

This chapter has reviewed background information in terms of biology of *Cryptosporidium*, including its lifecycle, transmission, symptoms, diagnosis, Pathogenicity, and treatment. It has also explained the statement of the problem, significance of the study, objectives and scope of the study. Chapter two will review the literature available in relation to its epidemiology, genetic diversity, diagnosis and co-infections with other parasites and the knowledge gaps noted in the available literature.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

This chapter reviews the relevant literature available on prevalence of cryptosporidiosis genetic diversity, diagnosis and co-infections with other enteric parasites with reference to each objective.

2.2. The Epidemiology of *Cryptosporidium* Species Infections and Disease Pattern

2.2.1. Aetiology of Diarrhea in Children

Diarrheal diseases are some of the leading causes of childhood morbidity and mortality in developing countries. An estimated one billion episodes occur each year in children of up to five years of age. Diarrhea causes an estimated 5 million deaths in children up to five years of age per year (Snyder & Merson, 1982; Colford, Tager, & Hirozawa*.,* 1996; Report, 2006). About 80% of these deaths occur in children in the first two years of life. Most diarrheal illnesses are acute, usually lasting no more than 3-5 days and are secondary to other infections (bacterial, viral and parasitic). Infectious agents that cause diarrheal disease are usually spread by the fecal-oral route specifically by; a) Ingestion of contaminated food or water and, b) Contact with contaminated hands (Saniel Moriles, & Monzon, 1995).

The most common organisms responsible for diarrhea obtained from pooled data worldwide include rotavirus, enterotoxinogenic *Escherichia coli* (ETEC) *Shigella*, *Campylobacter* and *Vibrio cholerae*. Parasitic infections that cause diarrhea include *Giardia lamblia, Cyclospora cayetanensis*, *Isospora belli*, *Entamoeba histolytica, Strongyloides spp, Dientamoeba fragilis* and *Cryptosporidium species*. Minor or non-medical parasites (probable commensals) that may cause minor symptoms that do not

require specific therapy include *Endolimax nana, Entamoeba coli, Entamoeba dispar, Entamoeba hartimani, Entamoeba polecki, Iodamoeba buetschlii* and *Chilomastix mesnili* (Saniel, Moriles & Monzon, 1995; Gascon *et al*., 2000; Report, 2006).

2.2.2. **Epidemiology of** *Cryptosporidium*

Cryptosporidiosis is a type of gastroenteritis caused by the parasite *Cryptosporidium species*. *Cryptosporidium*, an intracellular (extra-cytoplasmic) protozoan once thought to be rare and host specific, is ubiquitous and has multiple hosts such as calves, lambs, foals, piglets, humans, and other vertebrates including fish, birds and reptiles (Fayer, *et al.,* 2001; Fotedar *et al*., 2007). It is responsible for acute self-limiting diarrhea in immuno-competent persons and life threatening diarrhea in immuno-compromised hosts particularly in persons receiving immuno-suppressive drugs and AIDS patients (Colford *et al.,* 1996).

Among the animal hosts, calves are the most important from a public health point of view as they may serve as a source of human infections (Fayer *et al.,* 2001). Because of the durability of its oocyst, *Cryptosporidium* is a significant water and food borne pathogen of humans as well as animals. There are three features of *Cryptosporidium* species that ensure a high level of environmental contamination and increase the likelihood of waterborne transmission. Firstly, they are responsible for disease in a broad range of hosts including man, have a low-infectious dose (10–30 oocysts) enhancing the possibility of infection. Healthy immune-competent people may be infected but remain asymptomatic carriers hence serving as a source of infection to susceptible hosts. Again infected diarrheic individuals may shed 10^8-10^9 oocysts in a single bowel movement and excrete oocysts for up to 50 days after cessation of diarrhea. Secondly, the (*Cryptosporidium*) infective stages, oocysts are small in size and environmentally robust and thirdly, they are insensitive to the normal disinfectants

commonly used in the water industry (Pradeep *et al.,* 2006; Reinoso, Becares, & Smith, 2008).

The vast majority of human cases of cryptosporidiosis worldwide are mainly caused by two species, *C. parvum* which infects both cattle and humans in a zoonotic cycle and *C. hominis* which infects humans mainly in an anthroponotic cycle (Morgan *et al.,* 1999).However other species, including *C. felis*, *C. meleagridis, C.canis* (which are mainly associated with diarrhea), *C. suis, C. muris* and more rarely *C. baileyi* can infect humans too, especially children under the age of 5 years and immuno-compromised individuals (Meinhardt, Casemore & Miller, 2001).

All *Cryptosporidium* species are transmitted in the various hosts by ingestion and inhalation of oocysts, irrespective of the species types. However, the clinical and epidemiological significance of various *Cryptosporidium* species and subtypes in humans is not yet clear. Results of recent genotyping studies nevertheless support the theory that *C. hominis* and *C. parvum* behave differently in humans especially with reference to the specificity of the clinical presentation (Morgan *et al.,* 1996).

According to Borad and Ward (2010), in *C. hominis* cases, non-gastrointestinal symptoms (such as joint pain, eye pain, headache, dizziness and fatigue) are seen more often than in cases of *C. parvum*. Furthermore in young children, infections with *C. hominis* and, if symptomatic, *C. parvum*, are often heavily associated with fecal lactoferrin and growth shortfalls. *C. hominis* appears to stimulate inflammation irrespective of age; this raises important questions regarding how it may specifically induce greater pro-inflammatory response.

During the last 30 years, the concept of cryptosporidiosis has changed from that of a rare, largely asymptomatic disease, to an important cause of diarrhea in animals and

humans worldwide. Significant disease first appeared in cattle. Subsequently, the zoonotic danger of the organism was recognized in HIV-infected persons and young children. *Cryptosporidium* species are now ubiquitous and the disease has been described in over 79 host species.

Cryptosporidiosis has become a major cause of childhood diarrhea worldwide. In humans it accounts for up to 20% of all cases of childhood diarrhea in developing countries and is a potentially fatal complication of AIDS (Xiao *et al.,* 1999). Waterborne contamination is a growing concern as a source of widespread outbreaks of the disease. Factors that have contributed to the emergence of cryptosporidiosis include biological features of the organism, the lack of an effective treatment or preventative methods, increased environmental contamination, and trends in livestock production. In humans, the zoonotic nature of *Cryptosporidium* infection and an increased population at-risk have contributed to increase in incidence of the disease.

2.2.3.   Risk factors associated with cryptosporidiosis

2.2.3.1.Waterborne Cryptosporidiosis

Out of the 71 *Cryptosporidium*-linked outbreaks described in the last decade, 40 (56.3%) appear to be correlated to waterborne diseases, with a distribution almost constant throughout the years, marked by peaks in 2000, 2001, 2002, and 2007. Worldwide environmental and veterinary surveillance data reveal the presence of *Cryptosporidium spp*. in entire water-treatment systems, which represents an unacceptable health risk, particularly in sensitive (pregnant women, children) and immuno-compromised populations (HIV-positive and transplanted patients) (Smith, Brown, Coulson, Morris, & Girdwood, 1993; Hague, Huston, Hughes, Houpt, & Petri, 2003). Such evidence suggests that focus ought to be placed on prevention of human and animal waste contamination especially in authorized recreational waters, and in a

few cases also in well-maintained community swimming pools treated by supplemental disinfection treatment.

2.2.3.2.Food-Related Cryptosporidiosis

In the analysis of *Cryptosporidium*-linked outbreaks, 15 out of 71 (21.1%) appear to be correlated to food borne transmission, with a higher number of outbreak episodes in 2006 and 2008. Geographically, the outbreaks seem to be concentrated in the USA, Canada, and Australia and in North Europe, especially Finland and Sweden (Corso *et al.,* 1993).

Many infection routes have been identified, such as consuming salad vegetables washed by contaminated water, eating raw meals, using contaminated water for making ice and frozen/chilled foods, or making products which receive minimum heat or preservative treatment (public Health Agency Canada, 2001). However, contact with contaminated feces transmitted by coprophagous transport hosts (for example, birds and insects), worker aerosols (from sneezes), and exposed hand lesions have also been associated with outbreaks (Todd, Greig, Bartleson & Michaels,2009) Transfer of pathogens has been documented through contaminated fabrics and carpets, rings, currency, skin surfaces, dust, and aerosols and though person-to-person transmission.

2.2.3.3.Immunity status and cryptosporidiosis

The prevalence of cryptosporidiosis in HIV-infected patients with diarrhea has been reported to range from 3 to 16% in developed countries, depending on the population studied, degree of immuno-suppression, and use of antiretroviral therapy although it is most frequent in men affected by gay-bowel syndrome. *C. parvum* is primarily responsible for watery diarrhoea, but it may also trigger biliary disease, hepatitis,

pancreatitis, arthritis, and possibly respiratory tract infections (Todd, Greig, Bartleson, & Michaels, 2009).

Diarrhoea is self-limited in immunocompetent individuals or in those whose CD_4 cell counts are >200/mm^3, but may be severe, and unremitting or relapsing in severely immuno-deficient patients (CD_4 cell counts <100/mm^3). In these cases chronic infection can lead to dehydration, malnutrition, mal-absorption, wasting and, frequently, death.

Biliary cryptosporidiosis is more frequent in patients with CD_4 cell counts of<50/mm3 and commonly presents with right upper quadrant pain, nausea, fever, vomiting and often with absence of diarrhea. Co-infection with cytomegalovirus or *Microsporidia* has been frequently found in biliary cryptosporidiosis (Montero, Sinnott, Holt, & Lloyd, 2001) All segments of the gastrointestinal tract may be involved, but the small bowel is the main target organ followed by the colon (Scaglia *et al.,* 1994).

Esophageal cryptosporidiosis, with parasites attached to the squamous mucosa and the luminal borders of sub-mucosal glands and ducts, has been described both in adults and in children with AIDS. Recent evidence suggests that epithelial apoptosis mediated by cytotoxic host T cells might play a role in the development of colonic lesions in AIDS-related cryptosporidiosis (Montero *et al.,* 2001), suggesting a modified pathogenesis in HIV-positive patients. With the introduction of highly active antiretroviral therapy (HAART), the incidence of cryptosporidiosis has declined and chronic diarrhea and cryptosporidial infection often resolve with increases in CD_4 lymphocyte count.

2.2.3.4.Cryptosporidiosis in Children

In the early 1980s, diarrheal disorders were the biggest child killers, responsible for an estimated 4.6 million deaths worldwide every year. Despite widespread use of oral

rehydration therapies and an increased understanding of the pathogenesis of diarrhoea, 2.5 million children still die from these illnesses every year, almost all of them in developing countries (Kosek, Ben & Guerrant, 2003).

Parasites such as *Cryptosporidium* and *Giardia* are leading agents of chronic or persistent diarrhoea worsened by specific risk factors such as malnutrition or immune deficiency (Thapar & Sanderson, 2004). *C. parvum* is a leading pathogen in children in African developing countries. Here, as in other low-income areas, with no or limited access to highly active antiretroviral therapy (HAART), AIDS is rapidly expanding in infants. The fatality rate increased due to opportunistic infections, with *C. parvum* being one of the leading agents of severe diarrhea in infants affected by HIV/AIDS (Tumwine *et al.,* 2006).

2.3. Molecular diversity of *Cryptosporidium species* and associated risk factors
2.3.1. Diversity of *Cryptosporidium*

The genus *Cryptosporidium* is a member of the sub- phylum Apicomplexa, along with the related genera *Toxoplasma, Eimeria* and *Plasmodium*. Because of lack of clear diagnostic features that allow the differentiation of *Cryptosporidium species* there isn't a full understanding of infection sources in humans, the burden of disease attributable to different species and strains difference in virulence or transmission in humans (Griffiths, 1998).

Ernest Edward Tyzzer was the first individual to establish *Cryptosporidium* and recognize its multi-species nature (Xiao, Fayer, Ryan & Upton, 2004). Following the initial discovery there has been confusion with other Apicomplexan genera especially members of the coccidian genus *Sarcocystis*. There has also been an explosion of descriptions of new species of *Cryptosporidium* during the last three decades. This has been accompanied by confusion regarding the criteria for species designation largely because of lack of distinct morphologic differences and strict host specificity among

Cryptosporidium species. With the establishment of a framework for naming *Cryptosporidium* species and the availability of new taxonomic tools, there should be less confusion associated with the taxonomy of the genus *Cryptosporidium*.

Cryptosporidium parvum is usually considered the agent of human cryptosporidiosis. However only in the last few years, molecular biology based methods have allowed the identification of *Cryptosporidium* species and genotypes. Molecular characterization using Isozyme profiles, random amplified polymorphic DNA analysis, nucleotide sequence characterizations and PCR-restriction fragment length polymorphism analysis of several genes indicated that this genus is complex and contains at least 16 different species (Carraway, Tzipori and Widmer, 1996; Vasquez *et al.,* 1996).

Cryptosporidium hominis has been implicated as the major cause of cryptosporidiosis in humans in most areas (Morgan *et al.,* 2002). Other species that have been found in humans include *C. parvum*, C. *meleagridis*, *C. canis*, *C. muris* and *C. suis* (Tzipori & Ward, 2002). Others include *C. baileyi* from birds, *C. serpentis* from reptiles and *C. nasorum* from fish (Table2.1).

Table 2.1. Current recognized species of *Cryptosporidium*

Species	Major hosts	Minor hosts	Site of infection
C. hominis	Humans	Dugongs, sheep	Small intestine
C. parvum	Cattle, livestock, humans	Deer, mice, pigs	Small intestine
C. muris	Rodents	Humans, rock hyrax, mountain goat, Human	Stomach
C. suis	Pigs	s	Small and large intestine
C. felis	Cats	Humans, cattle	Small intestine
C. canis	Dogs	Humans	Small intestine
C. meleagridis	Turkey, humans	Parrots	Small intestine
C. wrairi	Guinea pigs		Small intestine
C. bovis	Cattle		Small intestine
C. andersoni	Cattle, Bactrian camel	Sheep	Abomasum
C. baileyi	Poultry	Quails, ostriches, ducks	Bursa
C. galli	Finches, chicken		Proventriculus
C. serpentis	Lizards, snakes		Stomach
C. saurophilum	Lizards	Snakes	Stomach and small intestine
C. scophthalmi	Fish		Intestine/ stomach

Source: CDC, 2012

Recently it has become increasingly clear that *C. parvum* is not a single species but is composed of a number of genotypes: a "human" genotype which has so far been found only in humans; a "cattle" genotype which is found in domestic livestock such as cattle, sheep, and goats and also infects humans; a "mouse" genotype which has been found in mice from around the world and "dog and ferret" genotype which have been found only in the respective hosts (Morgan, constantine, Forbes & Thompson, 1997). Only the human and cattle genotypes have so far been identified in immuno-compromised humans. The public health significance of the other remaining species is unknown.

2.3.2. Species concept in *Cryptosporidium*

One major reason for the long disputes in *Cryptosporidium* taxonomy is the difficulty in fulfilling the definition of a biological species. The classical definition of species as groups of interbreeding natural populations reproductively isolated from other groups is difficult to apply to many organisms such as *Cryptosporidium* because it is difficult to conduct genetic crossing studies with many *Cryptosporidium* species (Kariem, Robinson, Dyson & Evans, 1995; Award El Kariem, 1999).

Currently morphology, especially oocyst measurements represent the cornerstone of Apicomplexan taxonomy. Therefore oocyst structure is usually one of the requirements for establishing a new species. However for *Cryptosporidium*, morphology is not adequate by itself because oocysts of many species are virtually identical in size, which has even caused confusion about the historical validity of several *Cryptosporidium* species. As a result other characteristics have been included in the taxonomic description. In many cases experimental transmission followed by light microscopy and sometimes electron microscopy of endogenous species has proven useful. For example Current and Reese (1986) provided an excellent account of the life cycle of *C. parvum* in experimentally infected mice by using a combination of light and electron microscopy.

However, a strict requirement for life cycle studies in all taxonomic works seems impractical for two reasons. First distinct species may have similar endogenous development. Second and equally important, many host species fail to lend themselves easily to animal experimentation. This is especially true of oocysts derived from rare, exotic, venomous, excessively expensive or very large hosts; making animal studies prohibitive (Xiao, Bern, Sulaiman, & Lal, 2004).

Infectivity has sometimes been used to characterize and compare various *Cryptosporidium* isolates (Okhuysen, Chappell, Crabb, Sterling, & Dupont, 1999). Even though infectivity can be used as a good general indicator of host susceptibility and oocyst viability, real quantitative data are limited. Numerous variables affect parasite development including dosage, oocyst age, oocyst storage conditions, isolates employed, and chemical pre-treatments of the oocysts, the age, size and previous exposure history of the host (Upton & Gillock, 1996). Host specificity (the broad range of different hosts that can be infected by any one isolate) can prove highly useful when dealing with isolates derived from commonly encountered hosts (Lindsay *et al.,* 2000).

Biochemical differences can potentially be used as one criterion in defining *Cryptosporidium* species such as Restriction Fragment Length Polymorphism (RFLP), analysis of genomic DNA, isozyme analysis, two dimensional gel electrophoresis and protein/carbohydrate surface labeling of oocysts sporozoites or homogenates (Llovo, Lopez, Fabregas, & Munoz, 1993). Recent molecular studies have uncovered an overwhelming amount of genetic diversity within the genus *Cryptosporidium* (Carreno *et al.,* 2001).

In recent years, genetic differences have been a key essential element in defining several *Cryptosporidium* species such *as C. andersoni, C. canis, C. hominis*, and *C. galli*. Thus far genetic differences identified in the species or genotype level have correlated well with other biological characteristics such as the spectrum of natural hosts and infectivity in transmission studies. Because of the uncertainties associated with the extent of intra-specific allelic variation in *Cryptosporidium* taxonomy, numerous genotypes have been described without a designation of species being given or with them all being lumped into *C. parvum* (Lilia, Charles, Ynes, Robert & Lihua, 2014).

These entire genotypes designation scheme generally reflects significant genetic differences among *Cryptosporidium* isolates and tends to correlate well with biological differences wherever data is available. Not all genotypes differ from each other to the same extent. Thus some genotypes exhibit extensive nucleotide differences from co-generics whereas others are very similar (Xiao, Fayer, Ryan & Upton, 2004**).**

2.3.3. *Cryptosporidium* species of Mammals

Mammals represent the largest group of animals known to be infected with *Cryptosporidium*, probably due to the greater number of studies as a result of the perceived importance of these animals (Tilley, Upton & Freed, 1991). There is an enormous diversity in mammalian *Cryptosporidium* species and because of a plethora of molecular studies, multiple new species have been discovered and described.

(i) *Cryptosporidium muris* was described in 1907 by Edward Tyzzer in the gastric glands of laboratory mice.

(ii) *Cryptosporidium andersoni* was described by Lindsay *et al* in 2000 (Lindsay *et al.,* 2000). It infects the abomasum of cattle and produces oocysts that are morphologically similar to but slightly smaller to those of *C.*

muris. It was named after Bruce Anderson of the University of Idaho, the original finder of the parasite.

(iii) ***Cryptosporidium parvum,*** the most frequently reported species in mammals was first described by Edward Tyzzer in 1912. It was first found in mice and was differentiated from *C. muris* based on its smaller oocyst size and its location only in the villi of the small intestine. Over 150 species of mammals have been identified as hosts of *C. parvum* or *C. parvum* like parasites. *C. parvum* is known to infect mainly ruminants (cattle, sheep, goats and deer) and humans.

(iv) ***Cryptosporidium canis*** was first described by Fayer *et al* in 2001(Fayer *et al.,* 2001). Its oocysts have been observed in the feces of dogs worldwide. Based on its ability to infect humans and bovines but its inability to infect mice, as well as significant genetic differences from other *Cryptosporidium* species, the parasite was named *C. canis* (Fayer, Morgan, & Upton, 2001).

(v) ***Cryptosporidium hominis*** was first described by Morgan-Ryan *et al* in 2002. Previously designated *C. parvum* human genotype, genotype 1 or genotype H, it was delineated as a separate species based on molecular and biological differences (Morgan *et al.,* 2002). Genetic characterization of *C. hominis* and *C. parvum* has consistently demonstrated distinct differences between the two species at a wide range of loci (Alves, Matos, & Antunes, 2001). There are also fundamental differences in ribosomal gene expression between the two, since *C. parvum* constitutively expresses two types of rRNA genes whereas more than two transcripts have been detected in *C. hominis* (Le Blancq, Khramotsov, Zamani, Upton, & Wu, 1997; Xiao *et al.,* 1999).

2.4. Diagnosis of cryptosporidiosis

Various methods have been used to diagnose cryptosporidiosis infections all with different reliability outcomes. Some of the diagnostic procedures that have been used are discussed below.

2.4.1. Fecal smear: Ziel Neelsen Staining Method

Examination of fecal smears with acid-fast stains such as modified Ziehl Neelsen staining (after fecal concentration) is commonly used in diagnostic facilities. Recognition of oocyst morphological features via microscopy, after ZN staining, is the convention in the diagnosis of cryptosporidiosis. Khurana, Sharma, Sharma and Malla (2012) evaluated ZN staining method for detection of *Cryptosporidium* and found it to have sensitivity and specificity of 79.06% and 100% respectively. Tahvildar and Salehi (2014) evaluated the method against PCR and found it to have sensitivity of 94% and specificity of 100%. However, this technique is laborious and less sensitive and thus prone to error (Connelly, Nugen, Borejsza-Wysocki, Durst, & Montagna, 2008; Omoruyi Uchechukwu, Chikwuneke, & Okonkwo, 2014). *Cryptosporidium* oocysts are quite tiny, and consequently can easily be mistaken in stool debris as an artifact.

They may also be easily confused with other oocysts, such as those of *Cyclospora* species and yeast cells. Conversely, this method cannot differentiate between *Cryptosporidium* species oocysts because they similarly take up a red to pink colour as do other faecal components, which is the shortfall of this technique compared to others. However, it is cheap and affordable, hence resource-poor countries still rely on the technique as has been variously reported. Connelly, Nugen, Borejsza-Wysocki, Durst, & Montagna, 2008). From Peru, Bialek *et al* (2002) and Vitaliano *et al* (2008) reported 29.6% incidence of *Cryptosporidium*, while a 3.4% incidence was similarly reported in India. Other accounts include 2.7% from Tunisia (Bialek *et al.,* 2002), 12% from

Chennai (Lillia, Charles, Ynes, Robert, & Lihua, 2008)**,** 2.2% from Malaysia, 50% from Zambia, 2.2% and 37.3% from South Italy and France, respectively.

Resource-rich economies are not left out as this technique was used to report incidence in HIV-positive diarrhoeagenic individuals. Additionally, the ZN technique showed a 27.2% incidence in the study population which cannot be said to corroborate other studies as a prevailing factor in a population affects incidence at that particular point in time. (Garcia & Shimizu, 1997).

Most of the studies on various aspects *Cryptosporidium* in Kenya, such as those on prevalence, risk factors and genotypes, have often utilized ZN staining technique in the initial screening of stool specimen. Wangeci *et al.,* (2006), screened fecal samples using the procedure. Nyamwange, Mkoji and Mpoke (2012) and Mbae *et al.,* (2015) utilized ZN in the screening stool specimens during their research.

It should be noted that differential staining using modified Ziehl Neelsen staining technique and wet mount preparations have limited value for detection of *Cryptosporidium* in fecal samples where oocysts can easily be confused with other materials (Omuroyi, Uchechukwu, Chikwuneke, & Okonkwo, 2014).

2.4.2. Immunological Methods

Methods based on microscopic examination are being replaced with techniques which rely on molecular recognition of specific antigens for a target pathogen. Immunological based methods have been developed for use in both clinical and environmental monitoring. As a result, immunoassays have replaced microscopy as the routine diagnostic procedure of choice. These immunoassays are reported to be as sensitive and specific as traditional microscopy methods and increase laboratory efficiency by reducing labor, time and costs (Garcia, Brewer, & Brukner, 1987; Garcia & Shimizu,

1997). The most widely used antigen detection immunoassays for *Cryptosporidium* are the Direct Fluorescent Antibody (DFA) Assay which detects intact organisms, and Enzyme Immunoassays (EIA) which detect antibodies or antigens in fluid sample.

The sensitivity and specificity of most commonly used commercial DFA tests- the MERIFLOUR DFA Test has been reported to be 96 to 100% and 99.8 to100% sensitive and specific respectively (Ungar, Soave, Fayer & Nash, 1994; Kehl, Cicirello, & Havens, 1995). Commercially available EIAS use antibodies for qualitative detection of *Cryptosporidium* specific antigens in preserved stool specimens. The reported sensitivities of most EIAs range from 94-97% and specificities range from 99-100% (Kehl *et al*., 1995; Garcia & Shimizu, 1997). The advantages of EIAS are that numerous samples can be screened at one time and tests can be read objectively on a spectrophotometer instead of subjectively on a fluorescent microscope. However, problems with false positive and false negative results have been reported (Doing *et al*., 1999; Hanson & Cartwright, 2001).

Enzyme-linked immunosorbent assays (ELISAs) have been reported to be up to 10 times more sensitive than acid-fast staining (Katanik, Schneider, Rosenblatt, Hall, & Procop, 2001) making the ELISA method currently the "gold standard" for antigen detection in infected stool samples. Immunochromatographic lateral flow immunoassay (rapid assays) have become popular diagnostic tools because they eliminate the need for trained microscopists and costly equipment and can be completed in 10 minutes rather than the 1-2 hours required to perform DFA tests or EIAS (Garcia & Shimizu, 2000). These are simple 10 minute card Assays that have a reported sensitivity of greater than 97% and a specificity of 100% (Chan *et al*., 2000; Garcia & Shimizu, 2000).

2.4.3. The ImmunoCard STAT Assay

The Immuno-Card STAT! Test is a qualitative Immunochromatographic assay that diagnoses *Cryptosporidium parvum* and *Giardia lamblia*. The assay simultaneously detects and distinguishes between *Cryptosporidium parvum* and *Giardia lamblia* antigens in aqueous extracts of patient stool specimens. Like other immuno-assays, it detects similar antigens using a non enzymatic rapid immunoassay format.

The specimen, collected in sample transport or preservative medium is added to a tube containing treatment buffer. A biotinylated anti-*Giardia* capture antibody reagent is then added, followed by a pooled suspension of colloidal dye labeled monoclonal antibodies to *Giardia* and *Cryptosporidium*. The sample is then mixed and poured into the test device that contains a capture reagent (an avidin derivative) for *Giardia*, a capture antibody for *Cryptosporidium* and a control antibody that binds to excess colloidal dye conjugate. If a *Giardia* antigen is present in the sample, a black band appears at the GIAR position in the device window. If *Cryptosporidium* antigen is present, a black band will appear at the CRYPTO position of the device. The appearance of a black band at the CONT position is required for the test to be valid as it indicates that the colloidal dye conjugate is intact and that the proper capillary flow has occurred (Plate 3.1).

In one previous study, to compare the validity (a measure of actual positives which are identified as such, and actual negatives which are identified as such), of various immuno assays, the ImmunoCard assay was found to have a sensitivity and specificity of 96% and 97.5% respectively (Johnson, Ballard, Beach, Causer, & Wilkins, 2003).

2.4.4. Molecular Methods

Molecular characterization using Isozyme profiles, random amplified polymorphic DNA analysis, nucleotide sequence characterizations and PCR-restriction fragment length polymorphism analysis of several genes indicated that this genus is complex and contains at least 16 different species (Carraway Tzipori & Widmer, 1996; Vasquez*et al.,* 1996). *Cryptosporidium hominis* has been implicated as the major cause of cryptosporidiosis in humans in most areas (Morgan *et al.,* 2002). Other species that have been found in humans include *C. parvum*, C. *meleagridis*, *C. canis*, *C. muris* and *C. suis* (Alves *et al.,* 2001). Others include *C. baileyi* from birds, *C. serpentis* from reptiles and *C. nasorum* from fish.

Techniques based on nucleic acid amplification have proven to be essential for the detection and epidemiological tracking of members of the genus *Cryptosporidium.* PCR technology offers alternatives to conventional diagnosis of *Cryptosporidium* for both clinical and environmental samples. With development of sensitive primers for diagnosis of *Cryptosporidium*, it is now possible to directly differentiate between human and bovine genotypes of *C. parvum* on the basis of the size of the PCR product (Morgan *et al.*, 1997). Sensitivity of 97%-100% and specificity of 100% have been reported for diagnosis of *Cryptosporidium* by PCR (Morgan *et al.,* 1998; Omuroyi, Uchechukwu, Chikwuneke, & Okonkwo, 2014).

PCR techniques have the advantage of improved sensitivity and specificity. However current problems in molecular detection of *Cryptosporidium* oocysts include: the availability of only a limited number of tools for species differentiation, most of which are based on the small subunit rRNA gene; the non-specificity of some species differentiation tools such as primers that fail to bind to amplify targeted gene sections; the misinterpretation of data because of lack of information on recent findings; and, the existence of erroneous data in the databases and publications. They also have limited

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applicability at point of care or low resource settings due to their costs, infrastructure and high technical expertise involved.

2.5. Co-Infection of *Cryptosporidium* Species with other Intestinal Parasites

Humans are susceptible to infection with numerous protozoa that colonize the intestinal, some of which may occur in the same individual simultaneously. *Cryptosporidium* is known to coexist with other intestinal parasites. *Cryptosporidium* co-infections with other intestinal protozoa have been reported in various studies. In one study that involved screening patients for *Giardia* and *Cryptosporidium* in Oregon, USA, a 0.35% co-infection was reported. A case report in India also showed co-infection of the Cryptosporidium, *Isospora* and *Giardia* in a patient, (Chandora *et al.,* 2015). In Kenya, a study by Wangeci et al (2006) found a 2%,5% and 6% *Cryptosporidium* co-infection with *Giardia, Entamoeba coli* and *E. histolytica/dispar* respectively.

2.5.1. *Giardia intestinalis/Giardia lamblia/ Giardia duodenalis*

This is a flagellated protozoan that has been found in more than 40 animal species. Five species of *Giardia* have been established in scientific literature- *G. muris* in rodents, birds and reptiles, G. *intestinalis* in mammals (including man), rodents and reptiles, *G. agilis* in amphibians, *G. ardue* in the great blue heron and *G. psittaci* in Budgerigar (Meyer, 1994).

Giardia has a simple typical fecal-oral life cycle. As with *Cryptosporidium*, the parasite is shed with faeces as an environmentally robust cyst which can then be transmitted to a new host. In the host's duodenum, the trophozoite emerges from the cyst and undergoes a mitotic division. Each of the two trophozoites produced attaches to the epithelial cells by means of an adhesive disc, feeding on epithelial cells. Some of the trophozoites

encyst during passage through the intestine and leave the host with faeces as cysts. In formed stools cysts are encountered more than trophozoites.

Transmission of *Giardia* involves fecal-oral transfer of cysts as indicated by the high prevalence in developing countries with poor sanitation and hygiene standards, in day care centres and nurseries (Black, Finch, Taghi-Kilani & Belosevic,1996). Food borne outbreaks are a result of contamination of food by infected workers or household members (Islam, 1990).

The role of animals in the transmission of human Giardiasis is still a matter of speculation. Although *Giardia* occurs in Domestic pets, farm animals and wild mammals, there is no unequivocal evidence that they cause infections in humans Waterborne outbreaks have been reported for some years (Erlandsen, 1994;. Crawn, 1998).

There is a relationship between dose of cysts and infection. The infection to illness ratio varies with isolates. Other factors such as age, nutritional status, predisposing illness and previous exposure determine the outcome of the infection (Flanagan, 1992).The mechanism by which *Giardia* causes diarrhea and mal-absorption is still unclear. There is no evidence for production of toxins. Its infections appear to affect the activity of gut enzymes, damage to the mucosal surface and leads to overgrowth of bacteria and yeasts in the small intestine (Hanson & Cartwright, 2001).

The time between infection and appearance of *Giardia* cysts in the stool is 12-19 days. Symptoms appear between 1-29 days after infection but generally at 6-15 days, coinciding with appearance of *Giardia* in stool (Farthing, 1993). Symptoms include diarrhea, weakness, weight loss, abdominal pain and to a lesser extent nausea, vomiting, flatulence and fever. In most cases the infection is acute and self-limiting with a

duration of 2-4 weeks. However about 30-50% of infections will then develop into chronic infection with intermittent diarrhea and weight loss (Farthing, 1994).

Both humoral and cellular immune responses are elicited by infection with *Giardia*. Secretory IgA and IgM appear to play a role in the clearance of intestinal infection by reducing the mobility of trophozoites and preventing their adhesion to the mucosa (Farthing, 1994). This provides more protection against re-infection as indicated by lower attack rates in chronically exposed populations (Rabbani & Islam, 1994).

Chemotherapeutic drugs for *Giardia* include Nitroimidazoles such as metronidazole (Flagyl), tinidazole, ornidazole and secnidazole. Metronidazole has been largely utilized because it is quickly and completely absorbed after oral administration and penetrates the body tissues and secretions such as saliva, breast milk, semen and vaginal secretions. The drug is metabolized in the liver and excreted in urine. Quinacrine and bacitracin zinc have also been used to successfully treat giardiasis in humans.

2.5.2. *Entamoeba* species

The genus *Entamoeba* contains many species, six of which (*Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli, and Entamoeba hartmanni*) reside in the human intestinal lumen. *Entamoeba histolytica* is the causative agent of amebiasis (amoebic dysentery) and is considered a leading parasitic cause of death worldwide in humans (Freedman, Maddison, & Elsdon-Dew, 1988). Although recent studies highlight the recovery of *E. dispar* and *E. moshkovskii* from patients with gastrointestinal symptoms, there is still no convincing evidence of a causal link between the presence of these two species and the symptoms of the host. (Clark & Diamond, 1991).

Entamoeba histolytica is the causative agent of amebiasis and is globally considered a leading parasitic cause of human mortality (Hague, Huston, Hughes, Houpt & Petri, 2003). Clinical features of amebiasis due to *E. histolytica* range from asymptomatic colonization to amoebic dysentery and invasive extra-intestinal amebiasis, which is manifested most commonly in the form of liver abscesses. Approximately 50 million people have invasive disease, resulting in 100,000 deaths per year (WHO, 1997; Hague *et al.,* 2003).

Although the parasite has a worldwide distribution, high prevalence rates of more than 10% of the population have been reported from various developing countries (Stanley Jr, 2003). *Entamoeba histolytica*-related diarrheal illnesses have recently been reported to have a negative impact on the growth of children (Mondal, Petri, Sack, Kirkpatrick & Hague, 2006). Despite the availability of effective therapy, morbidity and mortality associated with amebic infection have persisted, suggesting that interventions designed to limit or to eliminate disease are ineffective. As humans appear to be the only host, an appropriate control program could potentially eradicate amebiasis.

Although E. *dispar* was previously considered to be nonpathogenic and was regarded as a commensal species, intestinal symptoms in patients infected with this species have been reported (Jetter *et al*., 1997). In a recent study from India (Parija & Khairnar, 2005), 68 fecal specimens in which *Entamoeba species* were demonstrated on microscopy were tested using PCR. Eleven patients positive for *E. dispar* and *E. moshkovskii* (in association) had mild gastrointestinal discomfort, however, the study failed to clarify whether other parasites or bacterial or viral pathogens were detected in these 11 samples.

Entamoeba dispar can produce variable focal intestinal lesions in animals (Chadee, Smith, & Meerovitch*,* 1985) and can destroy epithelial cell monolayers in vitro. There is

also some evidence that following infection with E. *dispar*, pathological changes may occur in some humans (McMillan, Gilmour, McNeillage, & Scott, 1984). However Koch's postulates have not been fulfilled and no large case-controlled studies have been undertaken to assess the true pathogenic potential of this organism.

Entamoeba moshkovskii is another species of *Entamoeba* and is morphologically indistinguishable from *E. histolytica* and *E. dispar*. This species was first described from Moscow sewage by Tshalaia (1941) and was thereafter reported to occur in many different countries (Clark & Diamond, 1991). *Entamoeba moshkovskii* was initially thought to be a free-living environmental strain. However in 1961 an *E. histolytica*-like strain was isolated from a resident of Laredo, TX, who presented with diarrhea, weight loss, and epigastric pain. This strain was named the *E. histolytica* Laredo strain and shared many biological features with *E. moshkovskii*.

Both the Laredo strain and *E. moshkovskii* grow at room temperature, are osmo-tolerant, and are resistant to emetine. These characteristics distinguished them from *E. histolytica* and E. *dispar* (Clark & Diamond, 1991). Subsequent molecular studies have confirmed that the *E. histolytica* Laredo strain is a strain of *E. moshkovskii* (Clark & Diamond, 1991).The exact taxonomic classification of the species has yet to emerge, as *E. moshkovskii* seems to be a complex of at least two species

2.5.3. *Cyclospora cayatenensis*

First isolated in 1870 from intestines of moles, it is related taxonomically to other protozoan parasites *Cryptosporidium* and *Toxoplasma* who are members of the sub-phylum Apicomplexa. *Cyclospora* species infect enterocytes of the small intestines and can produce disease. *Cyclospora species* is transmitted through contaminated water and food. (Hoge *et al*., 1995). Both symptomatic and asymptomatic states have been

described. Symptoms of infection include watery diarrhea, fatigue, abdominal cramps, anorexia, weight loss, vomiting and nausea.

Incubation period is between 2-11 days (Hoge *et al*., 1995) with moderate numbers of un-sporulated oocysts being excreted for up to 60 days. Illness may last for weeks and episodes of watery diarrhea may alternate with constipation. In immunocompetent individuals, the symptoms are self-limiting and oocysts excretion is associated with clinical illness (Shlim *et al*., 1991). In immuno-compromised patients, diarrhea may be prolonged.

2.6. Research gaps
2.6.1. *Cryptosporidium* species

There has been an explosion of descriptions of new species of *Cryptosporidium* during the last two decades. This has been accompanied by confusion regarding the criteria for species designation, largely because of lack of distinct morphological differences and strict host specificity among *Cryptosporidium* species (Xiao *et al.*, 2002) A review of the biologic species concept, the International Code of Zoological Nomenclature (ICZN), and current practices for *Cryptosporidium* species designation calls for the establishment of guidelines for naming *Cryptosporidium* species.

All reports of new *Cryptosporidium* species should include at least four basic components: oocyst morphology, natural host specificity, genetic characterizations, and compliance with the ICZN rules. With the establishment of a framework for naming *Cryptosporidium* species and the availability of new taxonomic tools, there should be less confusion associated with the taxonomy of the genus *Cryptosporidium*. The clarification of *Cryptosporidium* taxonomy is also useful for understanding the biology of *Cryptosporidium* species, assessing the public health significance of

Cryptosporidium species in animals and the environment, characterizing transmission dynamics, and tracking infection and contamination sources.

2.6.2. Transmission of *Cryptosporidium* species

One major problem in understanding the transmission of *Cryptosporidium* infection is the lack of morphologic features that clearly differentiate one *Cryptosporidium* species from many others (Fall, Thompson, Hobbs, & Ryan-Morgan, 2003). Hence, one cannot be sure which *Cryptosporidium* species is involved when one examines oocysts in clinical specimens under a microscope.

Associated with the problems in taxonomy and nomenclature is the public health importance of various *Cryptosporidium* species. Without clear diagnostic features that allow the differentiation of *Cryptosporidium* species, we do not know the precise number of species infecting humans, the burden of disease (sporadic and outbreak related) attributable to different species or strains/genotypes, and the role of species and strains/genotypes in virulence or transmission in humans. These questions present challenges to our understanding of the epidemiology of cryptosporidiosis. Revision of *Cryptosporidium* taxonomy, therefore, is useful to our understanding of the biology, epidemiology and public health importance of various *Cryptosporidium* species. (Xiao, Bern, Sulaiman, & Lal, 2004).

2.6.3. Prevalence of *Cryptosporidium*

It is not known exactly how many cases of *Cryptosporidium* infections actually occur annually in many regions. Many people do not seek medical attention or are not tested for this parasite and so *Cryptosporidium* often goes undetected as the cause of intestinal illness. Health professionals in some countries are on the lookout for cases of

Cryptosporidium through surveillance programs at hospitals clinics and laboratories (Lindsay *et al.,* 2000).

Although oocysts are present in most surface waters (lakes and rivers) many of which supply public drinking water, only few laboratories have specialized capabilities to detect the presence of *Cryptosporidium*. Again current sampling methods are unreliable as it is difficult to recover oocysts trapped on the material used to filter water samples.

2.7. Chapter summary

This chapter has reviewed the available literature in relation to the objectives of this study such as prevalence and diversity, risk factors, diagnosis and co-infections of *Cryptosporidium* species. It has also outlined briefly the gray areas where we have knowledge gaps. Chapter three will describe the methods materials and procedures that were used to carry out the study. It will describe the research design, study site, population and sampling design, data collection methods, data analysis methods.

CHAPTER THREE

MATERIALS AND METHODS

3.0. Introduction

This chapter describes the methods and procedures that were used to carry out the study. It describes the research design, study site, population and sampling design, data collection methods and data analysis methods.

3.1. Study Site

The study was conducted in four Hospitals in Bungoma County- Bungoma District Hospital, Webuye District Hospital, Kimilili District Hospital and Chwele District Hospital (Figure 3.1). Bungoma County is found in Western Kenya. It is bordered on the South and East by Kakamega County, in the North by Trans-Nzoia County and Busia County and Uganda in the West. Bungoma district hospital was used as the main centre for collecting and storing processed stool specimens from other participating hospitals.

The four hospitals were chosen for the study for three main reasons. One, they are located in different sub-counties within the County and therefore could be used to collect samples from most areas of the County. Two, the lifestyle of people in the surrounding areas served by these hospitals often exposes them to potential sources of *Cryptosporidium* infections such as keeping livestock in their residential plots, keeping calves, lambs, and goats inside residences during nights and frequently using cow dung to pave floors of their houses, all of which are risk factors for *Cryptosporidium* infections. The hospitals were also picked because they were well equipped for collection and handling of stool specimens. Bungoma County was chosen for the study

because it was one of the regions in Kenya where epidemiological studies of *Cryptosporidium* have not been done before.
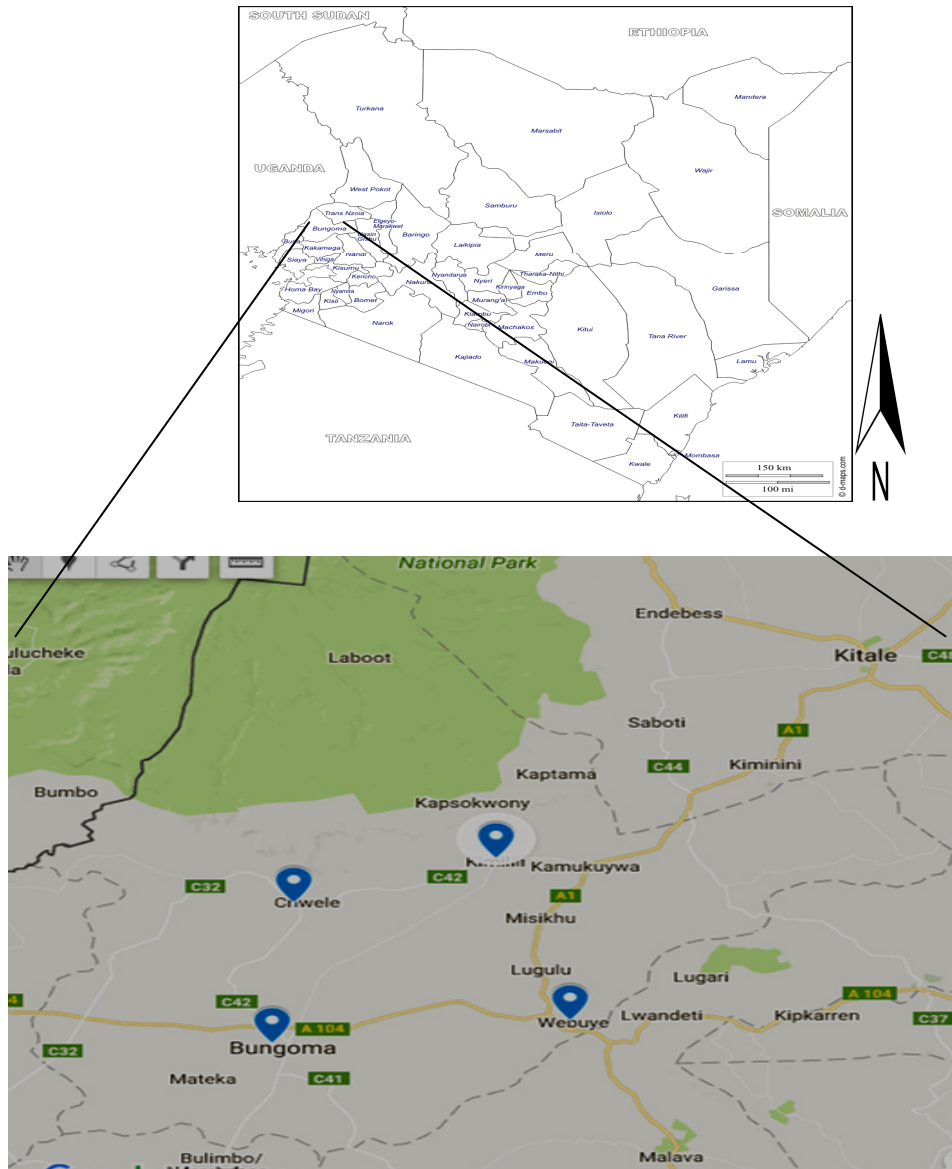
Figure 3.1: Map of Kenya Bungoma County and neighboring areas

The blue pins indicate the locations of the four participating hospitals (www.googlemaps/Kenya//western Kenya 2016)

3.2. Research Design

A prospective cross-sectional study design was employed in this study during which consecutive patients of up to five years of age presenting gastro-enteritis, and referred to the laboratory in the four participating hospitals for ova/cyst detection were enrolled into the study. Two stool specimen samples were taken from each patient on one single occasion, with no follow up samples.

The design was used to show prevalence and risk factors of *Cryptosporidium* and other enteric parasites. The design provided a 'snapshot' of prevalence of *Cryptosporidium* and the characteristics associated with it at specific points in time. This research design has been previously employed elsewhere in prevalence studies involving *Cryptosporidium* by Kaur, Rawat, Kakkar, Uppal & Sharma (2010), and Mathan, Venkantesan, George, Mathew and Mathan (2005) in India and by Wangeci *et a.,* (2006) and Nyamwange, Mkoji, Mpoke and Nyandieka (2012) in Kenya.

3.4. Study Population

The study population included children up to five years of age presenting gastroenteritis with or without diarrhea, abdominal pain abdominal swelling, vomiting and dehydration in the respective hospitals. The communities surrounding Kimilili and Chwele District Hospitals do not have piped water, are mainly farmers of livestock, and largely use pit latrines. Those around Webuye and Bungoma district hospitals have piped water which is treated and are mainly business people and salaried workers.

Majority in the rural settings have houses with mud floors. They therefore routinely use cow dung to pave the house floors. At the same time, due to shrinking sizes of land owned by individual households, coupled with economic hardships, occasional cattle rustling, more and more families are keeping livestock in ever shrinking small plots adjacent to the residential houses. In both cases the risk of coming in contact with *Cryptosporidium* oocysts is increased.

3.5. Inclusion criteria

a) All those children up to five years of age presenting gastroenteritis with (out) diarrhea in the respective hospitals during the study period were recruited into the study

b) Parents and guardians of the patients had to sign a written and informed consent for the patient to be recruited into the study.

Exclusion Criteria

a) Children above five years and other older patients who presented with gastroenteritis in the participating hospitals were not eligible for recruitment into the study

b) Children who met the other criteria but had been resident outside Bungoma County six months prior to the date of visiting the hospitals were also excluded from the study.

c) Children who were HIV positive (as voluntarily declared by parent/guardian were not recruited).

3.6. Sampling and Sample Size

A purposive sampling design was used in recruiting patients into the study. In this particular research, the characteristics of interest were children up to five years of age who presented gastro-enteritis defined by diarrhea, abdominal pain and/or swelling vomiting and dehydration in the four hospitals and had been referred to the laboratories for ova/ parasite examination. Gastroenteritis was identified by the consulting physicians who then referred the patients to the laboratories. The patients were then recruited into the study, subject to satisfaction of the inclusion criteria.

The minimum sample size for each hospital was 138, based on an assumed average prevalence of 10 %.(Wangeci *et al.,* 2006). This minimum sample size was arrived at using the formula below as described by Charan and Biswas (2013)

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$$N \geq \frac{z^2_{(1-\acute{a}/2)} P (1-P)}{d^2}$$

Where:

N =sample size

Z= standard normal deviate

P= assumed average prevalence of *Cryptosporidium* (expected proportion of the population showing the character of interest)

d= degree of precision or accuracy (error margin) =5%

$\acute{a}$= significance level

Confidence level= 95%.

Therefore n = $\frac{1.962 \times 0.1 (1-0.1).}{0.05^2}$ = 138.

The minimum total sample size for the four hospitals was 138 X 4 = 552. (138 patients for each of the four hospitals).However during the study a total of 712 patients were recruited into the study.

For the follow up questionnaire regarding perceptions and knowledge about *Cryptosporidium,* participants, were randomly sampled among the parents and guardians of the children who had been recruited into the study. For each hospital out of the 138 to be sampled 35 parents were randomly chosen using the online random integer generator (Random.org). They were different in terms of age socio-cultural, educational and economic backgrounds. A total of 138 adults were recruited but responses were received from 126 participants with 12 participants not responding. This is because some participants insisted on filling the questionnaires later on and returning them to the interviewer at another time, of which they failed to return.

3.7.Methods and Assays

3.7.1. Samples collection

Stool specimens were collected by qualified laboratory technicians in the respective hospitals from legible patients after obtaining consent from their parents/guardians. All the samples were obtained from children up to five years of age presenting gastroenteritis in the participating centers. The specimens were collected over the same period from the four participating hospitals and immediately preserved in 10% formalin before being processed.

The Immuno-chromatographic Rapid Assay was immediately performed on the un-concentrated fecal specimens. Parts of the fecal specimens were then aliquoted in specimen bottles and then transported to Bungoma District Hospital for processing where all the other procedures except PCR were performed. Only those found to be positive for *Cryptosporidium* were further processed and transported to KEMRI laboratory in Kisian, Kisumu (initially but later to JKUAT laboratory in Nairobi) for molecular identification of *Cryptosporidium* species involved.

3.7.2. Administration of Questionnaires

Before fecal specimen collection, parents/guardians were informed about the research and those who agreed to have their children participate gave a signed consent (Appendix i). They were then asked for their consent to fill a patient information form and questionnaires (Appendix ii and iii). The patient information form provided personal details of the patient such as name, sex, age, place of residence and so on. The first questionnaire on the other hand sought information relating to symptoms that prompted a hospital visit, risk factors such as presence of livestock and pets, nature of floors in their residential houses, and source and treatment of water for domestic use. A second questionnaire sought information on perceptions of parents/guardians on cause

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of diarrheal disease in children, prior knowledge of *Cryptosporidium* and the sources of the same information. Two questionnaires were used because each sought slightly different information from the respondents. In both cases, the questionnaires were orally administered by the interviewer. This was necessary because in some cases it involved translating the questions into Kiswahili or the local dialect as the case could be. However, there were some cases where the interviewees insisted on self administering the questionnaires. As a result, some interviewees failed to return the filled questionnaires.

3.7.3. Immunochromatographic Rapid assay.

The ImmunoCard STAT! Rapid assay was performed on un-concentrated formalin fixed stool specimens as specified by the manufacturer (Meridian Bioscience Inc., Appendix v) at the collection centers. In brief, two drops of sample treatment buffer were added to a tube containing approximately $60\mu l$ of stool specimen followed by conjugate A and B respectively. The contents were then poured into the sample part of the Immuno-Card device. Results were visualized after ten minutes.

A positive control line was visible on the device each time a test was completed successfully. A positive test was a grey black band visible in the test windows. No reaction in the test window and a positive control line was interpreted as negative (Plate 3.1). Any of the assays above that gave discrepant results were tested twice for accuracy and the original tests were confirmed in all incidences. The ZN concentrated wet smear was used as a gold standard during the validation of this immuno assay.

Plate 3.1: An unused ImmunoCard device

The prepared sample was poured in the sample part of the immuno card device. (Marked as SPL on the device). A positive control line would be visible every time a test is complete. Two grey lines at the GIAR and CONT would be a positive test for Giardia lamblia while two grey lines at the CRYP and CONT would be a positive test for *Cryptosporidium parvum* (plate 4.1). Three grey lines would be an indication that the sample was positive for both parasites

3.7.4. Fecal Concentration, Wet Preparations and Modified Ziehl Neelsen Staining

The fecal samples were concentrated in formalin ethyl acetate to obtain a concentrated pellet (appendix vi). A thick smear of the concentrated pellet was made on the glass slide and air dried. It was then fixed using methanol and stained with strong Carbol Fuchsine stain for 15 minutes. It was then rinsed in distilled water before being decolorized in 1% hydrochloric acid in alcohol (Acidified alcohol). After rinsing again in distilled water, the slides were counterstained with 0.4% Malachite green for 30 seconds. After being rinsed well in distilled water and air drying, they were observed under high power of X100 and confirmed under oil immersion of the microscope (Appendix vi). The *Cryptosporidium* oocysts appeared as round objects with a degree of red staining of the internal structure (Plate 2). Part of the concentrated pellet was also used to prepared concentrated wet smears for microscopic examination for presence of other enteric parasites (Appendix vii).

3.7.5. Polymerase Chain Reaction Amplification (PCR)

Those samples that were found to be positive for *Cryptosporidium* by microscopy were further processed for molecular genotyping (Appendix ix). This procedure was carried out at JKUAT laboratory in Nairobi (19 samples) and at KEMRI in Kisumu (17 samples). Those positive samples taken to JKUAT were then further analyzed at the University of Venda in South Africa. All the samples that were positive for *Cryptosporidium* by ZN staining were preserved in 75% ethanol. Approximately 200µLof fecal suspension was then washed three times in distilled water before DNA extraction. Genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, Appendix ix).

Supernatants containing DNA were stored at −20°C until use later. Nested PCR was used to amplify an approximately 300 base pair (bp) long fragment corresponding to the *Cryptosporidium* 18S rRNA gene that encompassed a hyper-variable region using two sets of oligonucleotide primers: 5'-CTCATAAGGTGCTGAAGGAGTA-3' and 5'CCCATTTCCTTCGAAACAGGA-3' for primary PCR and 5'-GGAAGGGTTGTAT TTATTAGATAAAG-3' and 5'-CTCATAAGGTGCTGAAGGAGTA-3' for secondary PCR (*Xiao et al.,* 2001). The PCR reactions were carried out in 25 µl volumes containing 12.5 µl DreamTaq Master Mix (Promega, Madison, WI), 0.5 µl of each primer (10 µM), 6.5 µl nuclease-free water (Promega) and 5 µl DNA template. For amplification, templates were subjected to a hot start at 95°C for 15 min followed by 35 cycles of 94°C for 45 s, 53°C for 75 s and 72°C for 1 min followed by a final extension at 72°C for 7 minutes. The PCR products were stored indefinitely at 4°C. Secondary PCR products were analyzed by 2% agarose gel electrophoresis and Ethidium bromide staining (Appendix x).

3.7.6. DNA Sequencing and Analysis

Positive secondary PCR products were subjected to two directional sequencing with secondary primers by the Shanghai Biotechnology Co. Ltd. (Shanghai, China).
Amplified sequences were blasted against sequences in the NCBI database and then deposited in GenBank. *Cryptosporidium* isolates identified were compared phylogenetically using the MEGA 4.1 software.

Polymerase Chain Reaction amplification products of respective *Cryptosporidium* isolates were subjected to 2% agarose gel electrophoresis. To differentiate *Cryptosporidium spp*. and *C. parvum* genotypes, 20 μl of each secondary PCR product was digested in a 50-μl (total volume) reaction mixture containing 20 U of SspI (New England BioLabs, Beverly, Mass.) 20 U of VspI (GIBCO BRL, Grand Island, N.Y.) and 5 μl of the appropriate restriction buffer at 37°C for 1 hour, as described previously (Xiao *et al*., 2004).Each participant's results for concentrated wet smears for both *Cryptosporidium* and other enteric parasites and DNA genotyping were all recorded in a test results form (Appendix xi).

3.8.Data Management and Analysis

The data was entered and analyzed using SPPS version 11.0 (Microsoft Inc. USA). Data was presented in form of tables, histograms and graphs as was appropriate using MS Excel. Sensitivity, specificity, positive and negative predictive values for the ImmunoCard STAT! Rapid assay were calculated against the Modified Ziehl Neelsen staining procedure as the Gold standard.

To determine associations between infection with cryptosporidiosis, diarrhea or other symptoms, a Pearson's Chi square test was used to compare proportions of independent samples after making contingency tables. Normality was assumed and a P value <0.05

was considered statistically significant. Prevalence by centers and by seasons was also calculated as well as overall prevalence of cryptosporidiosis for Bungoma County.

3.9. Ethical considerations

Permission

Permission to carry out the study was obtained from the National Council for Science Technology and Innovation NACOSTI (Research permit No CST/RRI/12/1/MED/223) and all participating Hospitals' Management Committees.

Consent

Patients were enrolled into the study only after receiving informed consent from their parents/guardians. (Appendix i).

Confidentiality

All information obtained about patients was handled with utmost confidentiality and used only for intended purposes.

Risks

There were minimal risks involved with collecting and handling of fecal specimens from patients both to the technicians and the patients.

Benefits

The patients' parents/guardians were given results for all the tests undertaken and appropriate information about management of cryptosporidiosis and other enteric parasites where the tests was positive was given to the care givers in the respective hospitals.

3.10. Chapter summary

Chapter three has described the methodology used to carry out the research. In the main it has discussed the research design, the study site and population and data collection procedures employed during this research. Chapter four will discuss the results.

CHAPTER FOUR

RESULTS

4.0.Introduction

This chapter presents the summarized results of the study. The results presented include the prevalence of cryptosporidiosis in the target population, the risk factors involved, genotypes, the validity of the ImmunoCard STAT! Assay and co-infections of cryptosporidium with other enteric parasites.

4.1.General information

This study was carried out in four hospitals in Bungoma County. It involved as its subject, children of up to five years who presented with gastroenteritis in the four hospitals and were referred to the laboratory for ova/cyst examination. Although the calculated minimum sample size was 552, a total of 712 stool specimen samples were ultimately collected from children up to five years of age during a 30 month period in four hospitals in Bungoma County. Slightly more female participants were recruited as compared to males at 52.1% and 48.9% respectively (Table 4.1).

Table 4.1: Patient distribution in participating hospitals

Hospital	Percentage of Males patients	Percentage of Female patients(%)	Percentages per hospital
Bungoma	12.6%	14.4%	27.0%
Chwele	12.1%	11.6%	23.7%
Webuye	10.4%	12.5%	22.9%
Kimilili	12.8%	13.5%	26.3%
Total	**47.9**	**52.1%**	**100% (N=712)**

The table shows the distribution of patients recruited in the in the four hospitals. Bungoma hospital had the highest number of recruited patients while Webuye hospital had the least. Slightly more female patients were recruited (52.1%) than males (47.1%)

4.2. Epidemiology of *Cryptosporidium* species infections in children
4.2.1. Prevalence of *Cryptosporidium* by hospitals

Out of the 712 samples collected, 36 (5.1%) were found to be positive for *Cryptosporidium* by microscopy after fecal concentration and ZN staining. Chwele Hospital had the highest prevalence of 6.51% (n=169) while Webuye District Hospital had the least prevalence of 3.74% (n=187) (Table 4.2 and plate 4.2). However there was no significant difference in *Cryptosporidium* prevalence in the four hospitals (Fischer's exact test=0.8756).

Table 4.2: Prevalence of *Cryptosporidium* by Centres

Hospital	Total samples	Cryptosporidium cases	% prevalence
Bungoma	193	08	4.15
Chwele	169	11	6.51
Kimilili	163	10	3.74
Webuye	187	07	5.05
Total	**712**	**36**	**5.06**

The table shows *Cryptosporidium* prevalence by centres. There were no significant differences in prevalence in the various hospitals (Fischer's exact test=0.8756).

4.2.2. Distribution of *Cryptosporidium* by age

There was a prevalence of 4.97% (n=181) in children aged 12 months and below, 7.18% in those 13-24 months and 6.06% in those 24-36 months. The least prevalence was seen in the 49-60 month age bracket (Table 4.3). When comparing the prevalences in between the age groups, using the prevalence in children of 1-12 months as a reference, there was no significant difference in infection between the reference group and children aged between 36-48 months and 49-60 months (OR=1.5466, CI=0.6519-3.2025, *P*=0.3512 and OR=1.9157, CI=0.6781-5.4121, *P*=0.2193 respectively). However the difference was statistically significant between the reference group and those aged between 12-24 months (OR=2.00 CI=0.8345-3.9560, *P*=0.02431).

Table 4.3 *Cryptosporidium spp* distribution by Age

Age group (Months)	Frequency	*Cryptosporidium* prevalence	OR	95% CI	*P* value
1-12	181	4.97% (9/181)	1.7205	0.9247-3.426	0.0656
13-24	185	7.18% (13/185)	2.0000	0.8345-3.9560	0.02431
25-36	132	6.06% (8/132)	3.2324	0.6619 – 3.2052	0.1302
37-48	113	3.54% (4/113)	1.5466	0.6519-3.2025	0.3512
49-60	101	1.98% (2/101)	1.9157	0.6781 -5.4121	0.2199
Total	**712**	**5.1% (36/712)**			

The table shows cryptosporidium prevalence by age categories. Using the 1-12 month age group as a reference group, *Cryptosporidium* was significantly associated with the 13-24 month age group.

4.2.3. Prevalence of *Cryptosporidium* by Seasons

On average, prevalence of *Cryptosporidium* was highest during the months of June-July and October –November, in years, 2011 and 2012, which coincided with the tail end of the long and short rains respectively (Figure 4.1). Univariate analysis showed

that cryptosporidiosis was seasonal with more infections likely to occur during the wet season (OR-1.73, CI=1.23-241, P<0.001).

Prevalence of *Cryptosporidium* in 2011
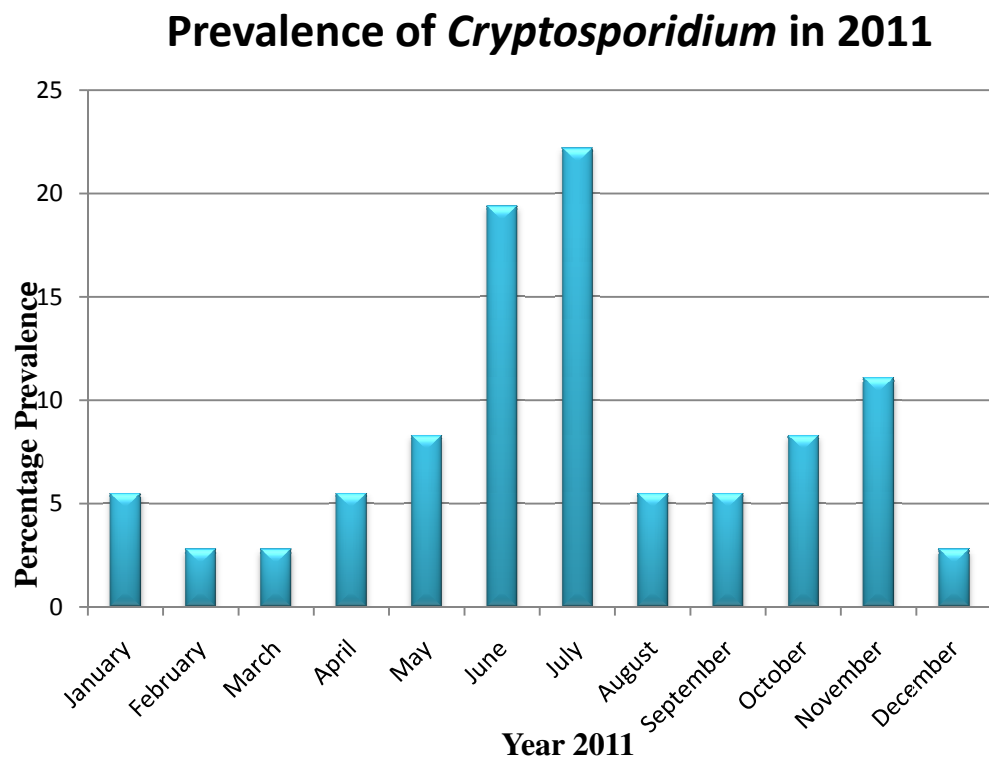
Figure 4.1a: Distribution of *Cryptosporidium spp* by months in 2011.

There was peak prevalence in June- July and November of that year. Compare the prevalence with that in 2012.

Prevalence of *Cryptosporidium* in 2012
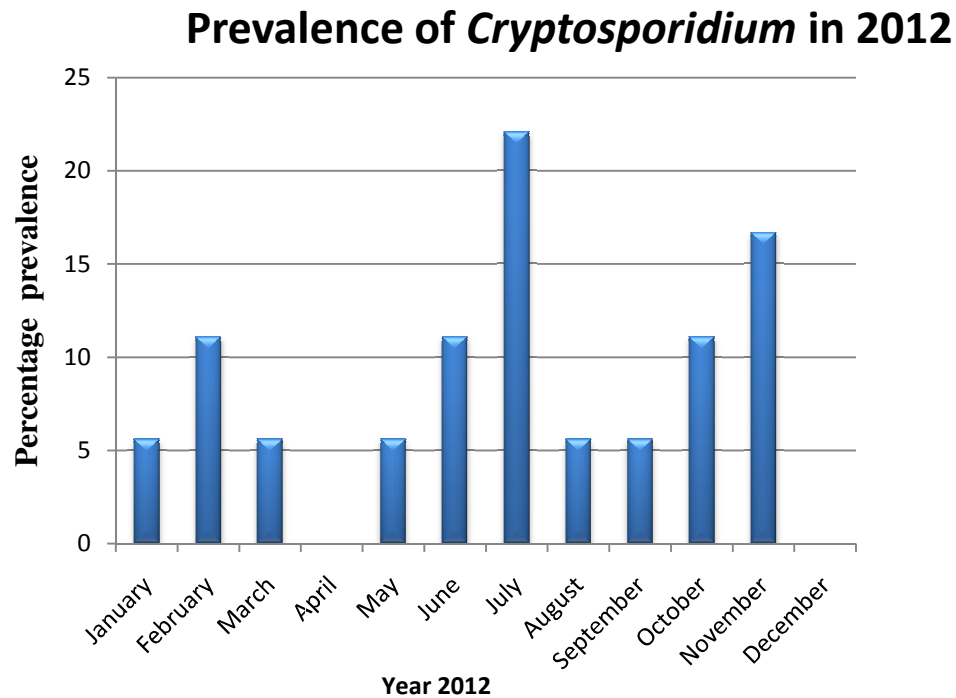
Figure 4.1b: Distribution of *Cryptosporidium spp* by months in 2012.

There were peak prevalences in July and November in both years of the study that coincided with the end of the long and short rains respectively in the study area.

4.2.4. Symptoms associated with *Cryptosporidium* infections

Diarrhea was the most common symptom in *Cryptosporidium* infected patients at 22.26% followed by abdominal pain/swelling/stomach muscle cramps at 16.9%. The least symptom shown by patients was vomiting at 3.6%. Cryptosporidiosis was significantly associated with diarrhea (OR=1.7793, CI=0.3670-1.6650, *P*=0.03164) and abdominal pains and swelling (OR=1.4711, CI=0.5680- 1.6202, *P*=0.04926) (Table 4.4).

Table 4.4: Symptoms associated with *Cryptosporidium* infections

Symptom	Status	Prevalence		Odds ratio	95%CI	P-value
		Symptom	***Cryptosporidium***			
Diarrhea	Yes	67.3%	22.3%	1.7793	0.3670-	0.03164
	No	33.7%	4.2%		1.6650	
Abdominal pains/swelling	Yes	58.4%	16.9%	1..4711	0.5680-	0.04926
	No	42.6%	11.2%		1.6202	
Dehydration	Yes	61.2%	13.6%	0.5607	0.2647-	0.1309
	No	39.1%	15.6%		1.1877	
Vomiting	Yes	47.5%	2.2%	0.7319	0.2553 –	0.7901
	No	52.5%	3.6%		3.5790	

The table shows the symptoms associated with *Cryptosporidium* infections. *Cryptosporidium* was significantly associated with diarrhea and abdominal pains

4.2.5. Perception about the cause of children's gastroenteritis

When parents/guardians were asked about the possible causes of their children's gastroenteritis, the main categories (and respective subcategories) of causes identified were: water (drinking water, swimming, and other for example, "dirty puddle"), food (raw vegetables, cooked meat, unspecified, for example, fruits), contagion (from other , from domestic animals, and from other or multiple sources for example, insects and rats), lack of hygiene (usually "not washing the hands"), and uncommon causes (that were mentioned by two or fewer respondents), for example, "low immune system", "gardening"). The place where the food was eaten (at home, outside for example, restaurant, or unspecified) was also considered. (Table 4.5). The most frequently mentioned causes were eating of contaminated food at73% (n=126) especially when

eaten out of home (16%), water (58.7%), lack of hygiene (34%) and contagion (29%). About half (52%) of the respondents mentioned at least one of these causes. Regarding joint beliefs, the most frequent combination of causes were "water and food" (6.6%, n=30,), "water and contagion" at 4.1% (n=17,), and "food and hygiene" at 3.6% (n=16).

Table 4.5: Perceptions about causes of diarrheal illness in children

Cause (category)	(subcategory)	Frequency (n=126)	Percentage
Water	Drinking	51	40.5
	swimming	0	0.0
	Other	23	18.3
	Total	**74**	**58.7**
Food	Raw vegetables (Total)	6	4.7
	Cooked meat	33	26.2
	Unspecified	53	33.1
	Total	**92**	**73.0**
Place eaten	Out	21	16.6
	Home	12	9.5
	Total	**33**	**26.2**
Contagion	From other people	15	11.9
	From cattle/pets	13	10.3
	Unspecified	9	5.4
	Total	**37**	**29.3**
Lack of hygiene		44	34.9

The most frequently mentioned causes were eating of contaminated food (73% n=126) especially when eaten out of home (16%), water (58.7%), lack of hygiene (34%) and contagion (29%).

4.2.6. Prior knowledge about *Cryptosporidium Ascaris* and Hookworm

Out of the 126 participants who responded to the second questionnaire regarding some intestinal parasites, 13.49%, 51.56% and 83.33% had heard of *Cryptosporidium,* Hookworm and *Ascaris lumbricoides* respectively. *Cryptosporidium* was the least

known parasite as 69.05% and 17.46% of the respondents asserted that they had no prior knowledge about it or were not sure respectively (Table 4.6).

Of those who had heard about *Cryptosporidium*, majority (70.6%, N=17) asserted that they had heard about it from doctors and nurses (Table 4.7). Of particular note was the fact that none had received information about the parasite from media or from laboratory test results.

Table 4.6: Prior information of *Cryptosporidium,* Hookworm *and Ascaris*

Prior knowledge	*Cryptosporidium*	*A .lumbricoides*	**Hookworm**
Yes	13.5% (17/126)	83.3% (105/126)	51.6% (65/126)
No	69.1% (87/126)	11.1%914/126)	38.9 (49/126)
Don't know	17.5% (22/126)	5.6% (7/126)	9.5% (12/126)

The table shows responses on prior knowledge on Cryptosporidium hookworm and *Ascaris*. Of the 126 participants who responded to the second questionnaire, 13.49%, 51.56% and 83.33% had heard of *Cryptosporidium,* hookworm and *Ascaris lumbricoides* respectively

Table 4.7: Sources of prior information about *Cryptosporidium*

Source of information	Number (n)	Percentage
Test results	0	0.0%
Environmental health officers	3	17.7%
Doctors/nurses	12	70.6%
Media	0	0.0%
Professional knowledge	2	11.8%

The table shows sources of information about *Cryptosporidium*. Of those who had heard about *Cryptosporidium*, majority (70.6%, N=17) asserted that they had heard about it from doctors and nurses (Table 4.7). Of particular note was the fact that none had received information about the parasite from media or from laboratory test results.

4.3. Genetic Diversity of *Cryptosporidium species* and associated risk factors

4.3.1. Occurrence and Genetic Diversity of *Cryptosporidium* species

Thirty six (36) *Cryptosporidium spp* positive samples were identified by microscopy. These positive samples were then subjected to molecular analysis by PCR to identify the *Cryptosporidium* genotypes/species. The PCR reaction targeted the conservative 18S Ribosomal RNA for amplification. *Cryptosporidium hominis* had the highest prevalence of 38.9% (14/36), followed by *C. parvum* (both cattle and human genotypes) at 36.1% (9/36), C. *meleagridis* 16.7% (6/36) and *C. canis* at 5.6 % (2/36). One sample was not amplified and therefore could not be identified. (Table 4.8, Figure 4.2 & Appendix xii).

Table 4.8: *Cryptosporidium* genotypes

Genotype	Total	Prevalence %
Cryptosporidium hominis	14	38.9
Cryptosporidium parvum	13	36.1
Cryptosporidium meleagridis	6	16.7
Cryptosporidium canis	2	5.6
Unknown genotype	1	2.8

C. hominis had the highest prevalence while *C. canis* had the least prevalence after sequencing and analysis of the 18S ribosomal DNA. One sample failed to amplify.
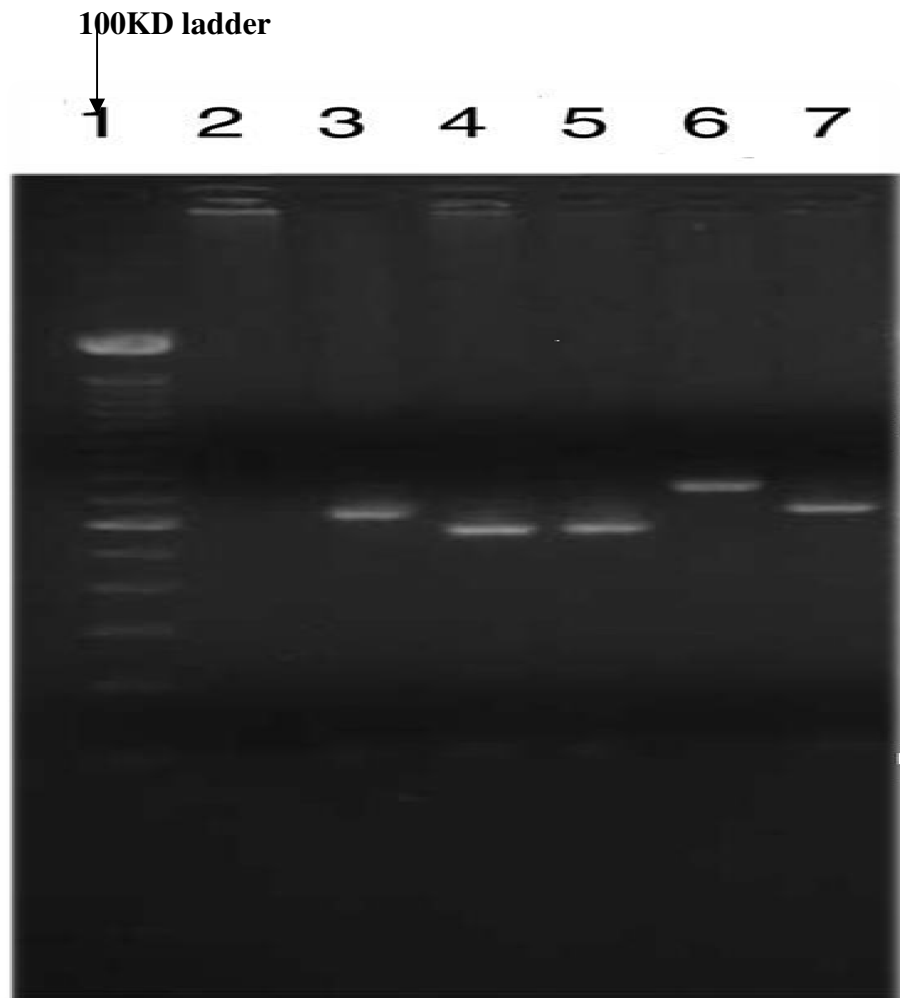
100KD ladder

Figure 4.2: Gel electrophosis results for PCR amplification and restriction enzyme digestion of a segment within the 18S rRNA of *Cryptosporidium* species.

Lane 1 is 1000KD ladder. Lane 2 was a negative control. Lanes 3, 4, 5 6 and 7 were identified as *C. hominis* (290bp), *C. canis* [4, 5, (279bp)], *C. parvum* (293bp) and *C. meleagridis* (283bp).

4.3.2. Water Source and Positivity of Cryptosporidiosis

The main sources of water for drinking and domestic use were 12.40% (N=712) surface or river water, 70.73% (N=712) spring or borehole water and 16.71% (N=712) tap water. The prevalence of Cryptosporidiosis stratified in relation to water sources was 3.96% (N=505), 6.72% (N=119), 9.09% (N=88) in patients who reported using spring/ borehole water, tap water and Surface and river water for drinking and other domestic use respectively. The results showed that tap water may also be contaminated with *Cryptosporidium* as those who reported using tap water had the second highest *Cryptosporidium* infection (Table 4.9). Use of surface/river water and tap water were significantly associated with and *Cryptosporidium* infection (Mann Whitney test P=0.0432, 0.0390 respectively).

Table 4.9: Water Sources and *Cryptosporidium* occurrence

Water source	Crypto present	Crypto absent	Total	X^2	*P* value
Surface/river water	9.09% (8/88)	90.0% (80/88)	88	0.2196	0.045
Spring/borehole water	3.96%(20/505)	96.0% (485/505)	505	23.3291	0.519
Tap water	6.72% (8/119)	93.3% (111/119)	119	0.3027	0.03902
Total	**36 (5.1%)**	**676**	**712**		

The table shows relation between type of water source and *Cryptosporidium* positivity. The main sources of water for drinking and domestic use were 12.40% surface or river water, 70.73% spring or borehole water and 16.71% (N=712) tap water. Use of surface/river water and tap water were significantly associated with *Cryptosporidium* infection**.**

The major methods of water treatment were chemical treatment at 3.5%, boiling at 34.4% and no treatment, at 62.1%. The prevalence of cryptosporidiosis in relation to

water treatment was 0.4% (1/245), 6.6% (29/442) and 24.0% among those who boiled drinking water, who did not treat water at all and those who used chemicals respectively (Table 4.10). There was significant difference between those who boiled water or those who chemically treated water and those who did not treat water at all when the group boiling water was used as a reference group (OR=0.9559, CI- 1.2553-3.5791, P=0.0436).

Table 4.10: Water treatment and *Cryptosporidium* occurrence

Water treatment	Crypto present	Percent(N=712)	OR	CI	*P*-value
No treatment	Yes 6.7%	62.1%	0.9558	1.2553-	0.0436
	No 92.2%			3.5791	
Boiling/filtration	Yes 0.4%	34.4%	1.4566	0.6619–	0.0235
	No 99.6%			3.2052	
Chemical treatment	Yes 24.0%	3.5%	1.9157	0.6781-	0.01251
	No 76.0%			5.4121	
Total	**5.05% (N=36)**	**100%**			

The table shows association between *Cryptosporidium* positivity and water treatment. There was significant difference between those who boiled water or those who chemically treated water and those who did not treat water at all when the group boiling water was used as a reference group (OR=0.9559, CI- 1.2553-3.5791, P=0.0436).

4.3.3. Association between Livestock and *Cryptosporidium* infections

Cryptosporidium spp especially *Cryptosporidium parvum* was initially considered as an animal parasite. Therefore contact with livestock is considered a major risk factor for contraction of cryptosporidiosis. This study found a significant difference between *Cryptosporidium* infections and livestock keeping (*P*=0.0340; Tables 4.11 &4.13).

However, there was no significant difference in relation to *Cryptosporidium* infections and type of livestock or pets kept (*P*=0.0534, Table 4.12&13).

Table 4.11: Livestock keeping and *Cryptosporidium* infections

Livestock	Crypto present	Crypto absent	Total	Overall Prevalence (%)N=712
Livestock present & kept in residence	23	425	448	5.1%
No livestock kept	13	251	264	4.9%
Total	**36**	**676**	**712**	**5.1%**

The table shows livestock keeping and *Cryptosporidium* positivity. There was a significant difference in infection between patients who came from livestock keeping homesteads and those who didn't have livestock.

Table 4.12: Type of livestock and *Cryptosporidium* infections

Type of livestock	Crypto present	Crypto absent	Total	% prevalence
Pets(dogs, rabbits/cats)	7	157	164	4.3%
Livestock(cattle/sheep/goats)	16	268	284	5.6%
Total	23	425	448	5.1%

The table shows Cryptosporidium infections and type of livestock. There was no significant difference in relation to *Cryptosporidium* infections and type of livestock or pets kept (P=0.0534).

Table 4.13: Two tailed T test statistic for differences in *Cryptosporidium* prevalence among livestock keepers and those without and the type of livestock

Presence/ absence of livestock	p_1	p_2	n_1	n_2	(P)*	SE	Z-score	Sig. level	P-value
	0.0513	0.0492	448	264	0.0502	0.0167	0.1257	0.05	0.0341
Type of livestock	0.0427	0.0563	164	284	0.0513	0.0980	0.1388	0.05	0.0532

*Pooled sample Proportion

The table shows Z-score test showing differences in proportions in *Cryptosporidium* infections in relation to livestock presence and type of livestock. There was a significant difference in infections between the livestock keepers and non keepers. However there was no difference in infection in relation to type of livestock.

4.4. Validity of the *Giardia /Cryptosporidium* ImmunoCard STAT! Rapid Assay
4.4.1. Validity of the *Giardia/* Crypto ImmunoCard Stat! Assay

The detection of *Cryptosporidium* by PCR was also compared with detection using the Immuno card Assay (Table 4.14, plate 4.2). The assay only detected *C. parvum* and not the other genotypes. The results showed the Assay had sensitivity and specificity of 92.8% (13/14) and 90.5% (19/21) respectively. It had a positive predictive value of 87% and a negative predictive value of 95%.

Table 4.14: Detection of *Cryptosporidium parvum* by ImmunoCard Stat! Assay

	IMMUNOCARD STAT! ASSAY	
	POSITIVE	NEGATIVE
POSITIVE	13	2
NEGATIVE	1	19
	14	21

(PCR DETECTION)

The detection of *Giardia lamblia* by microscopy (concentrated wet smear) was compared with detection by the ImmunoCard Assay. (Table 4.15, plate 4.2). The former was used as the Gold standard. The ImmunoCard STAT! Assay was found to have Sensitivity and specificity of 95% (n=39) and 90% (n=21) respectively. It had a positive predictive value of 95% and negative predictive value of 90%.

Table 4.15: Detection of *Giardia intestinalis* by the ImmunoCard Assay

		IMMUNOCARD STAT! ASSAY	
		POSITIVE	NEGATIVE
CONCENTRATED WET SMEAR	POSITIVE	37	2
	NEGATIVE	2	19
		39	21

The table shows validity of ImmunoCard assay in detection of *C. parvum*. The Assay was found to have Sensitivity and specificity of 95% (n=39) and 90% (n=21) respectively.

Plate. 4.1: The Immuno card Assay

4.1a: A used immuno card assay showing a positive test for *Giardia lamblia*, as indicated by two grey lines in the GIAR test window and a positive CONT line.

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4.1b: A used immuno card showing a positive test for *Cryptosporidium parvum*. Note the grey lines at the CRYP window and the CONT line.

4.5. Co-infection of *Cryptosporidium* species with other intestinal parasites

4.5.1.   *Cryptosporidium* co-infections with other enteric parasites

Cryptosporidium species, were found to coexist with *Giardia intestinalis* (*Giardia lamblia),* at 30.5%. Other parasites were *Entamoeba coli/dispar* at 27.78%`, and *Entamoeba histolytica*, at 16.6%. Of the other enteric parasites *Ascaris Lumbricoides* had the highest prevalence of 9.41%, while *Entamoeba coli/dispar* had the lowest prevalence of 2.63% (N=712) (Table 4.16, plates 4.2- 4.5).

Table 4.16: *Cryptosporidium* co-infections with other enteric parasites

Parasite	Prevalence	Percents of co-infections with *Cryptosporidium*
Ascaris Lumbricoides	9.4% (67/712)	0.00%
Entamoeba histolytica	8.9% (63/712)	16.6% (6/36
Giardia lamblia	5.5% (39/712)	30.5% (11/36)
Hookworm	4.6% (33/712)	0.00%
E. coli/ E. dispar	3.8% (27/712	27.8% (10/36)

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The table shows *Cryptosporidium* species co-infection with other intestinal parasites. *Giardia lamblia* had the highest co-infection with *Cryptosporidium*, while *A. lumbricoides* was the most prevalent parasite in the study group.
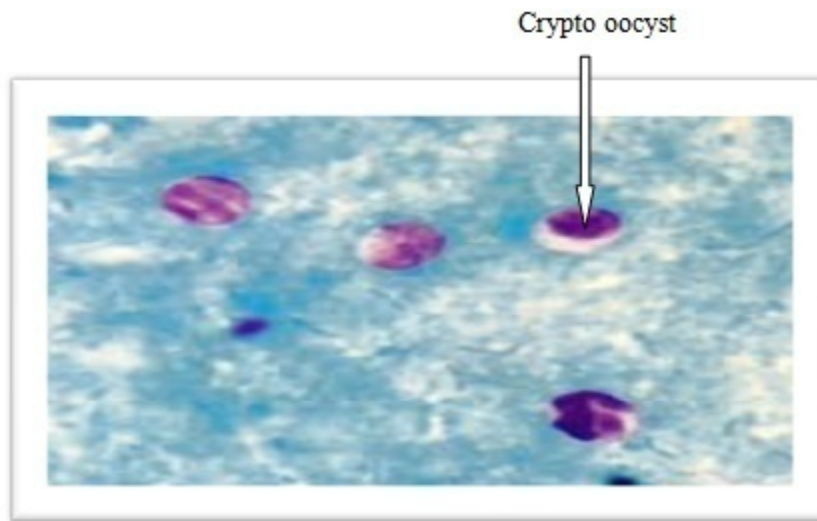
Plate 4.2: Appearance of *Cryptosporidium spp* Oocysts following Modified Ziehl Neelsen staining

The oocysts presented as round objects with some degree of red/purple staining of the internal structure varying from an amorphous mass filling the oocyst, to obvious multiple crescentic sporulated forms.

\
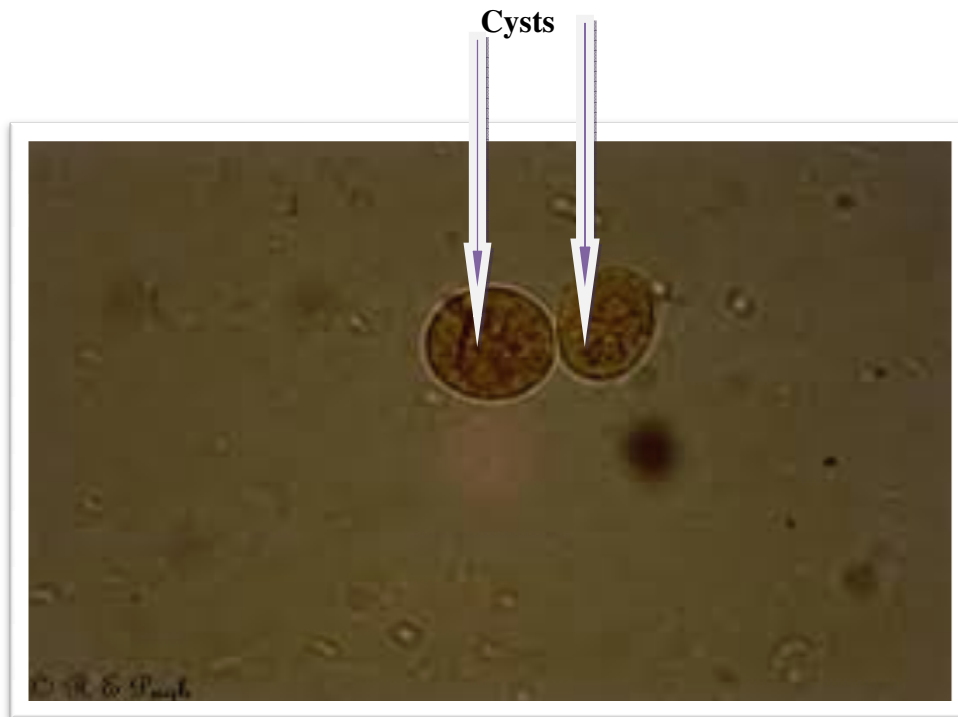
Cysts

Plate 4.3: Cysts of Entamoeba histolytica as seen under the light microscope following concentrated wet smear

Note the presence of prominent chromatin bars that distinguish the cyst from other species of *Entamoeba.*
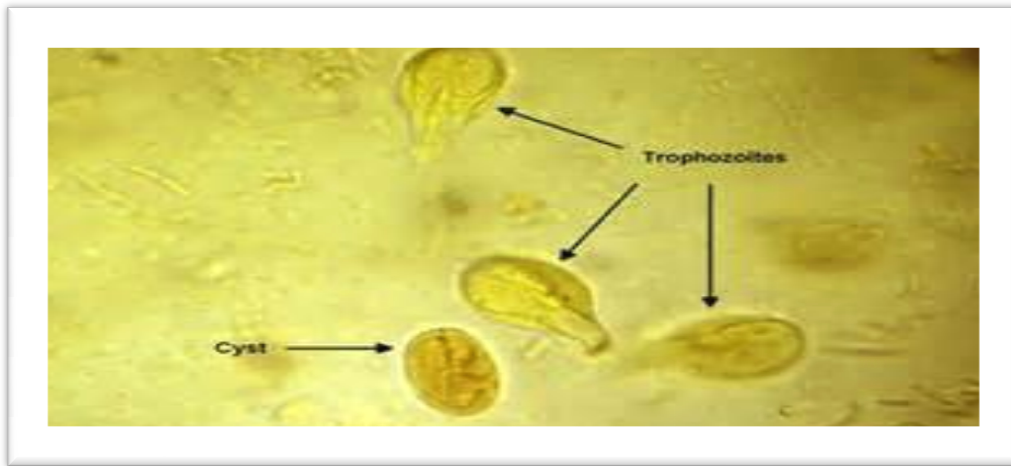
Plate 4.4: Concentrated fecal wet smear, showing *Giardia lamblia* cyst and trophozoites in fully formed stools.

Note the shape and prominent flagella in the trophozoite that distinguishes it from the cyst that is oval in shape.
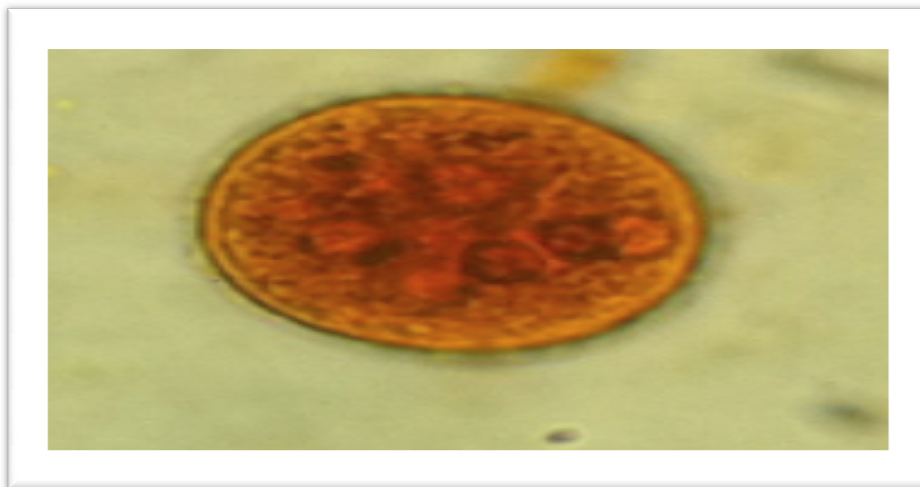
Plate 4.5: *Entamoeba coli* cyst in a concentrated wet smear examined under light microscope

The cyst was distinguishable from other *Entamoeba* cysts due to presence of more than four nuclei in the cytoplasm

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. Introduction

This study focused on the current state of *Cryptosporidium* infections in children up to five years of age, who presented gastroenteritis in the four participating hospitals and were referred to the laboratories for ova and cyst examination. Over a 30 month period, 712 samples were examined with patients' catchments that included all socio-economic settings in the region, ranging from urban, peri-urban and rural settings.

5.2. Discussion

5.2.1. Epidemiology of *Cryptosporidium* species infections

The results showed that *Cryptosporidium* is among the most common enteric parasites that cause diarrhea and abdominal pains. Tests for this parasite are however not routinely performed during ova and parasite diagnosis in many laboratories. Because it was a cross-sectional study, only one sample per patient was examined resulting in an overall *Cryptosporidium* prevalence of 5.1%.

This prevalence was slightly higher than results from previous studies in the country. For example, a 2006 study in Nairobi, Kenya focusing on the same age range showed an overall prevalence of 4% (Wangeci *et al.*, 2006). A 2014 cross sectional study on high risk enteric pathogens in Western Kenya found an overall prevalence of 3.7% which was again lower than the prevalence found in this study even though the cohort group in this study was different: 6months- 15 years (Pavlinac *et al.,* 2014). On the other hand, a similar prospective study by Nyamwange, Mkoji, Mpoke and Nyandieka (2012) found a higher overall prevalence of 9.8% among a similar age cohort seen at an outpatient clinic at Moi Teaching and Referral hospital in Eldoret Kenya.

A higher prevalence of more than 5.1% has also been previously reported in patients whose immunity was impaired, for instance in HIV infected children in Mulago Hospital, Uganda where the prevalence was 73.6% (Tumwine, *et al.*, 2005) and 17% in a study in Egypt (Abdel-Messih *et al.*, 2005). A prevalence of 34% in HIV/AIDS patients was reported at Kenyatta National hospital in Nairobi, Kenya (Wanyiri *et al.,* 2014). However the highest prevalence in a non-high risk group was recorded in a study in children of up to 12 years of age in Taiz District Yemen, where an overall prevalence of 43.7% was recorded (Al-Shamir, Al-Zubairy, & Al-Mamari, 2010).

This higher prevalence observed in this study may be an indication of the changing nature of *Cryptosporidium* parasite, implying that more and more immune-competent individuals are becoming susceptible to the parasite. This should be a cause for concern to public health authorities, bearing in mind that there is no known cure for the parasite. Treatment aimed directly at *Cryptosporidium* such as use of Paromomycin and Atovaquine are only partially effective and rarely eliminate the organism (Tarkhashvili, 2006; Abubakar, Aliyu, Arumugan, Hunter, & Usman, 2007).

Cryptosporidium infections were significantly found to be seasonal with peaks and lows. The highest prevalences as seen here were in the months of July and November, which coincided with the tail ends of the long and short rains respectively in the study area. This seasonality in infection was also found in similar studies (Pradeep *et al*., 2006; Kathure *et al.,* 2013). This peak prevalence during the end of the rain season coincided with results of two other studies carried out in Malawi and Kolkata, India, which also had peak prevalences during the rainy season between March and October (Peng, *et al*., 2003; Pradeep, *et al*., 2006; Wangeci *et al*., 2006). However the peak prevalence in this study differed from the peaks in other studies conducted in the country, which concurred with the dry season of November- February (Wangeci *et al*.,

2006). One possible hypothesis for this high prevalence during the rainy season could be the fact that surface run-off or percolation of surface water may have resulted in contamination of the water sources for domestic use (MacKenzie, Kazmiercza, & Davis, 1995).

Diverse causes of gastroenteritis were mentioned by the parents/guardians of the participants, ranging from food and water to contagions. Majority of the participants mentioned multiple causes, an indication that most lacked precise information about *Cryptosporidium* and other enteric parasites. This in turn would affect their ability to control the disease. This is because perceptions about the cause of disease are important as they are linked to protective behaviour and to the kind of treatment they may seek. This observation seemed to confirm the fact that tests for *Cryptosporidium* were rarely included in the routine ova/parasite examinations in laboratories in the study area. It also served to confirm that media may not be playing a major role in the dissemination of health information, especially on emerging diseases such as *Cryptosporidium.* The findings of this study corresponded to results of a similar study carried out by Doria, Abubakar, Syed, Hughes and Hunter (2006), who found that the most frequent perceived causes of cryptosporidiosis were water, contagions and contaminated food.

5.2.2. Molecular diversity of *Cryptosporidium species* and associated risk factors

Cryptosporidium infections were found to be significantly associated with diarrhea, and abdominal pains and swelling of abdomen but less so with other symptoms which included, dehydration and to a lesser extent vomiting. These results were consistent with results in a similar study by Sow *et al.,* (2016), who in their Global Enteric Multicenter study found a prevalence of 34.1% of *Cryptosporidium* attributable diarrhea in collated results for Sub-Saharan Africa and Asia. The results were also in agreement with studies carried out elsewhere, where persistent diarrhea and abdominal pains were

the commonest symptoms associated with cryptosporidiosis (Colford, Tager, & Horozawa, 1996; Wangeci *et al.,* 2006).

However, these results were not in agreement with studies carried out by Nyamwange, Mkoji, Mpoke and Nyandieka (2012) who found no significant association between Cryptosporidiosis and the symptoms presented by patients such as diarrhea, fever, vomiting and cough.

PCR analysis of genotypes showed that *Cryptosporidium hominis* and *C. parvum* (both human and cattle subtypes) had the highest and second highest prevalence respectively thus contributing to a heavy burden of cryptosporidiosis in humans. These results are consistent with results in other studies (Tumwine *et al.,* 2005; Wangeci *et al*., 2006; Sirisena, Iddawala, Noordeen & Wickramasinghe, 2013; Wanyiri *et al.,* 2014; Mbae *et al.,* 2015). *C. hominis* is predominantly a human genotype indicating a human to human transmission. However the presence of zoonotic species such as C. *meleagridis* and *C. canis* in the study population may be an indication that animal reservoirs are still playing an important part in the transmission pathways of Cryptosporidiosis. Since the immune status of the patients was not established during the study, it may not be very clear if the immune status may have predisposed some of the patients to the zoonotic species of *Cryptosporidium.*

Some of the samples that had been found to be positive by microscopy and ImmunoCard Stat! Assay failed to amplify during PCR analysis. It is because of this inconsistent diagnosis that data on distribution of the parasite is sometimes limited and inaccurate (WHO, 1997). Hawash, Ghonaim and Al-Hazmi (2015) noted that various constituents in clinical specimens particularly feces can inhibit the PCR assay and lead to false negative results. To ensure that negative results of a PCR assay are true, they have constructed and validated a cloning vector harboring a modified target DNA sequence ($\approx$ 375bp) that can be used as a competitive internal amplification control for a

conventional PCR that detects ≈550bp of *Cryptosporidium* oocyst wall protein (COWP).

Patients who reported using surface and/or river water for drinking had the highest *Cryptosporidium* infection, while those who reported using spring/borehole water had the least infection. There was a significant association between surface/river water source and *Cryptosporidium* infection. There was also a correlation between positivity of *Cryptosporidium* and type of drinking water. These results were consistent with results of other similar studies (Park *et al.,* 2006, Wanyiri *et al.,* 2014). Surface water becomes contaminated through the discharge of untreated and treated sewage and run-off of manure. Rivers receive both agricultural run-off and treated and untreated waste water. As a result *Cryptosporidium* oocysts of various genotypes are ubiquitous in surface and river waters (Park *et al.*, 2006; Muchiri *et al.,* 2009). Several characteristics of *Cryptosporidium* facilitate waterborne transmission. The high resistance of *Cryptosporidium* oocysts against chlorine disinfection renders this process ineffective for oocyst inactivation in drinking water. This explains why prevalence was second highest in patients who reported using piped water and those who treated water by chlorination. The oocysts are also persistent in the environment and can survive for months in surface water (Molbak *et al*., 1993).

Boiling appeared to be the most effective way of water treatment to eliminate *Cryptosporidium*, as only one case was reported out of the 245 cases who reported boiling drinking water. Similar results supporting boiling of water were obtained in Sri Lanka (Sirisena, Iddawala, Noordeen & Wickramasinghe, 2013). Chemical treatment by chlorinating did not seem effective on the *Cryptosporidium* oocysts as already mentioned, with those who reported chemically treating water having the highest prevalence. Although water is probably not the most important source of infection, (in this study infection seems both human to human- (anthroponotic) and zoonotic) many, waterborne outbreaks of cryptosporidiosis have been described (Crawn *et al*., 1995).

A higher number of those exposed to livestock had *Cryptosporidium* than the non-exposed. There was a statistically significant difference between *Cryptosporidium* infections among those who reported keeping livestock in the residence area and those who had no livestock. This may be attributed to the fact that transmission especially of the human genotypes was both anthropogenic and zoonotic. Among those positive cases who reported to keep livestock, the prevalence was highest in males than females. This may be due to the fact that male children are likely to play in gardens and farms' outdoor areas and with soil and animals all of which increase the risk of parasite transmission. These results are in agreement with results of another study where men were infected at a higher rate at 1.9% than women at 1.2% (Park *et al*., 2006). Contact with domestic animals may act as a risk factor in zoonotic infections. Zoonotic transmission has been well documented with various reports of outbreaks or cases of cryptosporidiosis in school children after exposure to calves or lambs (Casemore, Armstrong & Sands, 1985; Casemore, 1990).

The higher prevalence of *Cryptosporidium* in sheep and cattle and their higher numbers of oocysts shed by infected animals, especially newborns make cattle and sheep important sources of environmental contamination with *Cryptosporidium* oocysts that are capable of infecting humans. Pet animals which are also infected with *Cryptosporidium* oocysts may be an important source of human infection, as evidenced by presence of animal species such as *C. canis* in the study group. Other studies have also found 'animal species in humans especially *C. felis,* (Cats*) C. canis* (dogs) and *C. meleagridis* (bird species) that are occasionally found in immuno-compromised humans (Pedraza-Diaz, Amar, Nichols & McLauchlin,2001; Wangeci *et al.,* 2006). However one particular study noted that there was no statistically significant difference in the odds of *Cryptosporidium* infection between those with and those without animals within homesteads (Nyamwange, Mkoji, Mpoke & Nyandieka, 2012).

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Univariate analysis did not show any significant differences in infection between males and females. Results of a study by Nyamwange, Mkoji, Mpoke and Nyandieka (2012) did not also find a significant difference in infections in males and females. However these results are in contrast with results of another study where men were infected at a higher rate (1.9%) than women (1.2%), (Park *et al*., 2006). Another study in Brazil also found that males were 2.2 times more at risk of infection with *Cryptosporidium parvum* when compared with females (Pereira *et al*, 2002). Age on the hand was found to be significantly associated with *Cryptosporidium* infection, whereby the prevalence was highest in children 13-24 months. This was the age group that was more likely to acquire Cryptosporidiosis. These results are in agreement with results of other studies elsewhere (Pradeep *et al*., 2006; Wangeci *et al*., 2006).

5.2.3. Validity of the ImmunoCard STAT! Rapid assay for detection of *Cryptosporidium parvum* and *Giardia lamblia*

The ability to simultaneously detect and distinguish between *Giardia* and *Cryptosporidium* antigens in fixed and unfixed stool specimens using the ImmunoCard STAT! Assay is a novel idea. The detection of *Giardia lamblia* by microscopy and detection of *Cryptosporidium parvum* by PCR were compared with detection by ImmunoCard STAT! Assay. The assay was found to have sensitivity and specificity of 95% and 90% respectively for *Giardia*. For detection of *Cryptosporidium parvum*, the sensitivity and specificity were 92.8% and 90.5% respectively. A study by Schuurman, Lankamp, Van Belkum, Kooistra-Smid &Van Zeet (2007), found sensitivity and specificity of 98-100% and ≥ 97% respectively for detection of both *Cryptosporidium parvum* and *Giardia lamblia*. In another study to evaluate three commercial assays for detection of *Giardia* and *Cryptosporidium parvum*, the ImmunoCard Assay was found to have sensitivities of 81% and 78% respectively, and specificities of above 96% in both cases (Johnson *et al*., 2003). However the sensitivity and specificity of the method

as reported in this study are lower than other immunoassays such as the Direct Fluorescent Antibody (DFA) tests that detect intact organisms that had 100% and 98% sensitivity and specificity respectively, and Enzyme Immunoassay (EIA) tests which detect stool antigens with sensitivity and specificity of 95.5% and 98% respectively (Chan *et al.*, 2000; Johnson *et al.*, 2003). Although the test kit is relatively expensive (a unit consisting of 30 test kits costs kshs.52800 or kshs.1760 per test), the test procedure is relatively easy to perform, requires minimal training and multiple samples can be run as a batch at the same time.

5.2.4. Co-infection of *Cryptosporidium species* with other Intestinal Parasites

Cryptosporidium spp had the highest co-infection with *Giardia lamblia*, while *Entamoeba dispar*, a commensal protozoa had the least co-infection. However there was no statistical significance in terms of association between occurrence of *Giardia lamblia* and *Cryptosporidium spp*, to conclude that the presence of one *Giardia*/ *Cryptosporidium* indicated the presence or influenced infection by the other.

Of the other enteric parasites, *Ascaris lumbricoides* had the highest prevalence, while *Entamoeba coli/dispar* had the lowest prevalence. The prevalence of the other enteric parasites was much lower as compared to prevalence recorded in other similar studies. For example a study in Thika Sub-county, Kenya found an overall intestinal parasite prevalence of 43.7% (Ngonjo *et al.*, 2012). A similar study on primary school children in Ethiopia found an overall prevalence of 57.3% (Workneh, Esmael, & Ayichiluhm, 2014). The high prevalence of the other enteric parasites may be an indication that perhaps the primary school based de-worming campaign by the Ministry of Health (in collaboration with the Ministry of Education) may not be having the desired effect. There may therefore be the need to rethink the strategy since it may not be having the desired effect. It should be noted however that the different prevalence obtained in

various studies may have to a greater extent been influenced by the diagnostic methods used in detecting the parasites in the study population.

In summary, this study has shown that *Cryptosporidium spp* and other enteric parasites are common in Bungoma County, Kenya. There is therefore the need for public health education to raise the public awareness of the disease especially in relation to its seasonality risk factors and seemingly anthropogenic and zoonotic sources of infection. This will go a long way in mitigating the effects of the disease since there is currently no known drug for its treatment. The drug Nitazoxanide that was approved by the USA's Food and Drug Administration (FDA) is currently being used but its efficacy and safety in immuno-compromised people is still in doubt.

5.3. Conclusion

Based on the results discussed in the previous chapter the following conclusions are drawn

- **Epidemiology of *Cryptosporidium* species Infections in Children**

Cryptosporidium species is one of the parasites that cause child hood watery diarrhea and abdominal pain in the study area. The infections were marked with seasonality, with peaks that coincided with the end of the rain seasons possible due contamination of drinking water by flood waters carrying animal and human wastes. There were no significant differences in prevalence in patients recruited in each of the four participating hospitals. Parents and Guardians of children recruited into the study had varied perceptions of the causes of gastroenteritis in their children with majority never having had prior knowledge about *Cryptosporidium.*

- **Genetic Diversity of *Cryptosporidium species* and Associated Risk Factors**

Cryptosporidiosis was attributed to several species of the parasite, four of which were identified in the study area. Sources of infections appeared to be both anthroponotic and zoonotic as indicated by the presence of several animal species such as *C. canis* and *C meleagridis* in the study population.

- **Validity of the ImmunoCard STAT! Rapid assay for Detection of *Cryptosporidium parvum* and *Giardia lamblia***

The ImmunoCard Stat! Assay is a valid diagnostic kit for rapid detection of *Cryptosporidium parvum* and *Giardia lamblia..*

- **Co-infection of *Cryptosporidium* species with other intestinal parasites**

  Co-infections of *Cryptosporidium* and other enteric parasites were common in the study population especially with other protozoan parasites such as *Giardia lamblia Entamoeba coli* and *Entamoeba histolytica.*

5.4. RECOMMENDATIONS

Arising from the results and the conclusions made at in this study, the following recommendations are made.

- It is recommended that diagnosis of *Cryptosporidium spp* be included as a mandatory test during routine examination of stool specimens for ova and parasites in public hospital laboratories.

- Mitigation efforts against the effects of the disease should focus on preventive measures. Campaigns for example by the Ministry of Health should focus on educating people on avoiding exposure risks by boiling drinking water, avoiding exposure to animals and their excreta and maintaining sanitary conditions especially for 1-2 year olds who are the most vulnerable

- Since the Immuno-card Assay can only detect *Cryptosporidium parvum,* it is recommended that more research be done to come up with an all-encompassing rapid Immuno-Assay that can detect all the *Cryptosporidium* species

- It is recommended that efforts be intensified towards mitigating the morbidity caused by the other intestinal parasites besides *Cryptosporidium species*. Perhaps, besides targeting school going children for de-worming programs, a campaign programme (modeled along similar lines as the polio vaccination program) targeting house to house de-worming could be implemented to capture the 1-2 year old who would not have started going to school.

- The prevalence of *Cryptosporidium* reported in this study was restricted to those children who made hospital visits. *Cryptosporidium* infections are known to be asymptomatic and may not necessitate hospital visits. Therefore further research on prevalence in the general population is recommended to give a more accurate picture of *Cryptosporidium* prevalence in the general population in Bungoma County.

- Currently there is no recommended drug for treatment of *Cryptosporidium,* neither are there disinfectants effective against the parasite. Research should therefore focus on developing effective drugs and disinfectants against the parasite
  Current diagnostic procedures for *Cryptosporidium* are either insensitive and time consuming or are expensive and may not be used in routine screening for *Cryptosporidium* in health centres' laboratories. The study recommends more research towards the development of less costly but valid diagnostic methods *Cryptosporidium.*

- More research needs to be done to come up with more sensitive primers that could amplify the wide array of *Cryptosporidium* species that have been described so far. There is also need to develop internal amplification controls to avoid false negative results due to internal inhibition during PCR assays.

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APPENDICES

Appendix I: Consent form.

As part of the fulfillment of the requirements for the award of a Doctor of Philosophy (PhD) degree in Zoology (Medical Parasitology) at Jomo Kenyatta University, I am carrying out a study to investigate the epidemiology of Cryptosporidiosis and other enteric parasites in children under Five years of age and evaluate a diagnostic method that may be quicker and cheaper in diagnosing cryptosporidiosis.

I will ask you to fill a patient information form/questionnaire to enable me obtain information about the age and sex of your child, your residence and the risk factors, and the symptom(s) that prompted you to seek medical attention for your child.

I shall also ask you to allow me to take a sample of your child's stool /fecal matter to enable me to determine the presence/absence of various enteric parasites. The procedure for taking a stool sample from your child will cause no harm at all to your child.

The results of this investigation including the child's cryptosporidiosis status, other enteric parasites, and how to manage the condition where it is positive will be made available to the health facility in the normal manner so that appropriate advice can be given on treatment.

If you have understood what I have explained to you, I ask you to confirm and sign up for your voluntary consent to your child's participation in the study.

Declaration by parent/guardian

I understand that the participation of my child in this study is purely voluntary on my part as his/her parent/guardian, and I hereby accept to fill the questionnaire and allow you to take the stool specimen for the purpose explained to me herein

Name_____

Signature_____Date_____

Interviewer_____Date_____

Appendix II:Patient information form and questionnaire.

Date…………………..

Hospital………………………………………………

Code of patient………………………………………… Age (in months)……………

Sex………….1) Male

2) Female

*a) what is the source of water for domestic use (drinking and cooking) for your family? (Tick whichever is applicable)

i) Surface/river water.

ii) Spring water.

iii) Tap water

b) Is the water treated in any way before using?

i) No

ii) By boiling

iii) Using chemicals/filtering /chlorination

*b) Do you keep any animals in your home/where you live with the patient? (Tick applicable answer)

i) Yes

ii) No

*If yes, what types of animals are kept where you live? (Tick whichever is applicable)

i) Pets (Dogs Cats Rabbits etc.).

ii) Livestock (Cows, Sheep, Goats etc.).

*c) What symptoms in the child/patient prompted you to seek medical care for him/her? (Tick whichever is applicable)

i) Acute diarrhea.   ii) Persistent diarrhea.

iii) Recurrent diarrhea   iv) abdominal pain

v) Abdominal swelling   vi) Vomiting

**Consistency and Gross appearance of stool specimen at the time of collection

Consistency	Gross appearance
i) Soft	i) Conspicuously fibrous
ii) Mushy	ii) Fibres scanty
iii) Loose	iii) Colloidal/homogenous
iv) Diarrhearic	iv) Scanty mucus
v) Watery	v) Much mucus
vi) Formed/semi formed	vi) Mucus with scanty blood

* To be answered/filled by patient's mother/guardian.

** To be filled by medical staff taking/receiving stool specimen from patient's mother/ guardian

Appendix III: Questionnaire about perceptions on *Cryptosporidium* and other enteric parasites

Consent form

As part of a wider study to profile the prevalence and epidemiology of Cryptosporidium and other enteric parasites, I am carrying out a study on the perceptions and knowledge of Cryptosporidium in rural settings with special reference to Bungoma County

Towards this end your village and you as a member of the village have been selected to voluntarily participate in this study by answering questions in the attached questionnaire. The questionnaire seeks information on your perception and knowledge on emerging Zoonotic diseases (diseases transmissible between animals and humans) the information you will give will be treated with confidentiality for the purpose explained above only.

Declaration by participant

I understand that my participation in this study is purely voluntary on my part as a consenting adult and hereby accept to fill/answer the questionnaire as requested

Signature of participant_____Date_____

Interviewer_____Date_____

<div align="center">

Questionnaire

</div>

Tick appropriate answer where applicable

1. Do you keep any livestock in your home?

   Yes   [ ]

    No   [ ]

2. Are the animals kept in the same compound as your residential house

   Yes   [ ]

   No   [ ]

3. Did you know that livestock can be a source of diseases/parasites that are transmissible to human beings?

    Yes   [ ]

    No   [ ]

4. Do you know of any parasites/parasitic diseases that are transmitted from livestock to humans?

    Yes   [ ]

    No   [ ]

5. If yes list

    them_____

    _____

    _____

    _____

6. Have you heard of the following animal/human parasites?

    Cryptosporidium.   Yes [ ]   No/Never[ ]. Don't know [ ]

    Ascaris lumbricoides Yes [ ]   No/ Never [ ].   Don't know [ ]

    Hookworm   Yes [ ]   No/ Never [ ].   Don't know [ ]

7. From what source did you about any/all the parasites mentioned above?

    From doctors/nurses   [ ]

    From lab test reports   [ ]

    Professional knowledge   [ ]

    From media/TV/Radio   [ ]

8. Have you or your family members ever suffered from Diarrhea?

    Yes   [ ]

    No   [ ]

9. If yes, which of the following could have been the cause of the diarrhea?

a) Water: Drinking water [ ]. During swimming [ ] Other [ ]

b) i) Food: Raw vegetables [ ], cooked meat[ ], unspecified [ ]

   ii) Place food was eaten: At home [ ]. Out   [ ]

c) Contagion: From other people [ ] From animals (cattle/pets) [ ]

   Unspecified [ ]

Appendix IV: Random Selection of Parents/ Guardians for Questionnaire II

Bungoma District Hospital				
9	49	83	118	29
10	138	112	103	58
21	95	129	109	12
26	87	78	16	67
114	76	40	61	97
127	106	24	55	33
99	117	93	23	60
Webuye District Hospital				
33	35	59	131	84
21	85	136	64	119
18	51	122	89	51
99	40	50	86	118
45	114	5	102	37
10	46	47	131	65
35	135	98	6	20
Chwele District Hospital				
130	82	31	88	93
74	13	92	135	101
12	83	118	7	31
90	107	126	79	94
52	26	38	39	91
33	92	29	116	133
114	22	87	57	
Kimilili District Hospital				
131	129	74	114	91
2	63	60	139	31
68	92	68	49	88
96	91	26	28	126
100	67	45	23	7
62	61	78	86	33
87	20	116	87	

Source: Random integer generator (True Random number service -www. random.org, 2011)

Appendix V: ImmunoCard STAT! Rapid Assay

1. The test can be performed on fresh, frozen or fixed stool specimens (10% formalin, SAF, Cary-Blair and Stuart's Media.

2. Add 2 drops of sample Treatment Buffer to tube with 60µl of specimen.

3. Add 2 drops of conjugate reagent A to the tube.

4. Add 2 drops of Conjugate Reagent B to the tube.

5. Pour the tube content into the sample port of the device

6. Read the results after 10 minutes.

Interpretation of results

Positive result: 1 band in the control zone + 1 band in the *Cryptosporidium* zone.

Negative Results: 1 band in the control zone only.

Storage

The kit consists of test cards, sample treatment buffer, conjugate A and B, plastic droppers and tubes for specimen dilution.

The kit must be stored at 2-8∘C

(Meridian Bioscience, USA)

Appendix VI: Formalin-Ethyl Acetate Concentration procedure.

1. Strain the stool specimen through a filter containing a double layer thickness of gauze into a disposable conical centrifuge tube, large enough to hold at least 12ml. of contents

2. Add normal saline to the 12ml.mark on the tube and mix well.

3. Centrifuge the tube twice for 10 minutes at 500x g to concentrate. Decant the supernatant. About 1to1.5ml of sediment should be left in the tube.

4. Add 9ml. of 10% formalin to the sediment and mix thoroughly.

5. Add 3ml.of ethyl acetate, stopper the tube, and shake the mixture vigorously in an inverted position for at least 30 seconds.

6. Centrifuge the tube at 200RPM for 1 minute. Four layers will form in the tube. From the top to the bottom, they are as follows: layer of ethyl acetate, plug of specimen debris, layer of formalin and sediment.

7. Remove the stopper and with an applicator stick, gently rim the plug of debris to loosen it from the sides of the tube. Carefully decant the top three layers.

8. Add a few drops of formalin to the sediment and mix well. This will preserve the specimen, which may be helpful if an additional sample will be needed at a later stage.

9. Make concentrated wet smears on glass slides for observation under the microscope or for Ziehl Neelsen staining procedure.

Appendix VII: Modified Ziehl Neelsen Staining Procedure.

1. Concentrate the fecal sample in formalin-ethyl acetate to obtain a concentrated pellet.
2. Make a thick smear of the concentrated pellet on a glass slide and air-dry it.
3. Fix the smear using Methanol for 3 minutes
4. Stain the smear using strong carbol fuchsine for 15 minutes.
5. Thoroughly rinse the slides using distilled water.
6. 7. Decolorize the stain in 1% hydrochloric acid in alcohol for 10-15 seconds.
7. Rinse again in distilled water.
8. Counter stain in 0.4% malachite green for 30 seconds.
9. Rinse well in distilled water and dry in air.
10. Observe under medium power and high power of X400 oil immersion of the microscope

Note: *Cryptosporidium* oocysts present as round objects with some degree of red staining of the internal structure varying from an amorphous mass filling the oocyst, to obvious multiple crescentic sporulated forms.

Appendix VIII: Concentrated wet preparation protocol

1. Place a small drop of 0.85% saline on a glass slide

2. Mix the saline with a very small portion of the concentrated stool specimen, using a wooden applicator stick

3. The resulting slide should be thin enough for newspaper print to be read through the smear.

4. Place a cover slip on the slide before microscopic examination under low and medium power.

Appendix IX: extraction of DNA from fecal samples using the QIAamp DNA Stool Mini Kit

1. Add 1.4ml of Buffer ASL to each of the sample tubes (Add the buffer in two 700ul increments because the upper limit of the P1000 pipette is 1.0ml

2. Change tips before each entry into Buffer ASL stock bottle.

3. Vortex at full speed for 1 min to homogenize the stool sample. (Secure the caps during vortexing so that caps do not pop open

4. Return tubes to floating rack and incubate at 95oC for 5min (Change temperature to 70oC after incubation)

5. Vortex the tubes for 15 seconds at full speed. Secure caps during vortexing.

6. Centrifuge tubes at 13400 rpm for 1min.

7. Prepare new 2ml tubes and add one InhitEx tablet. (Note: Push the tablet through the foil and guide it into the tube without touching it with your fingers.)

8. Remove the tubes from the centrifuge. Add all of supernatant to the inhibitEx tablet.

9. Vortex tablet and supernatant for 1 minute at full speed

10. Incubate at room temperature for I minute

11. Centrifuge at 13,400 rpm for 3 minutes

12. Pipet all of supernatant into a 1.5 mL microfuge tube.

13. Centrifuge at 13,400rpm for 3 minutes

14. During the centrifugations pipet 25µl of proteinase K to a 2.0mL tube for each sample

15. Add ALL of supernatant to the proteinase K tubes

16. Add 600µl of Buffer AL to each tube. Vortex for 15 seconds
    DO NOT add buffer AL directly to the proteinase K

17. Incubate at 70C water bath for 10 minutes

18. Remove tubes from water bath. Turn off water bath

19. Add 600µl of 100% absolute alcohol. Vortex briefly to mix

20. Centrifuge for 15 seconds at 13, 400 rpm
21. Add entire contents of tube to the spin column, being careful not to get any liquid on the rim of the tube.
22. Centrifuge spin column in collection tube for 1 minute at 13,400 rpm
23. Place spin into a new collection tube. Discard old collection tube and filtrate.
24. Add 500µl of wash buffer 1 to the centre of the spin column
    Note: Be careful not to get any liquid on the rim of the tube
25. Centrifuge spin column in collection tube for 1 minute at 13,400 rpm
26. Place spin column into a new collection tube. Discard the old collection tube and filtrate
27. Add 500µl of Wash Buffer 2 to the centre of the spin column
    Note: Be careful not to get any liquid on the rim of the tube
28. Centrifuge spin column in collection tube for 1 minute at 13,400 rpm
29. Pour out filtrate and place spin column in the same collection tube
30. Centrifuge spin column a second time in the collection tube for 1 minute at 13,400 rpm.
31. Place spin column into labelled1.5mL microfuge tube. Discard collections tube and filtrate
32. Add 200µl of Buffer AE to the centre of the spin column
33. Incubate at room temperature for 1 minute
34. Centrifuge at 13,400 rpm for 1 minute
    Note: microfuge cup will need to be open during this spin
35. Discard the spin column and KEEP the filtrate. The filtrate contains the DNA. Can be stored frozen at -20C

Appendix X: Gel Electrophoresis protocol.

Materials/Equipment Needed

Electrophoresis apparatuses, electrodes, and power supplies, Micropipette, Micropipette tips, Loading dye, 0.8% agarose gel, Molecular weight markers (1 tube per gel).Water bath at 55°C or hot plate, TAE buffer, Ethidium bromide paper (1 piece per gel), Staining tray, Gloves (for handling Ethidium bromide), UV light box, UV light Polaroid set up (including camera, film, camera connector, and light shield), Biohazard bag.

Procedure

Pouring an Agarose Gel

1. Get the electrophoresis apparatus and seal both ends of the gel tray with tape or stoppers.

2. Make sure one comb is in place at the negative electrode (black end of the gel).

3. Pour melted agarose into the gel space until the gel is about 5 mm deep.   Let the agarose harden, which should take 5-10 minutes.   Don't touch/move your gel until it's hard. In the meantime, prepare your PCR reactions (for electrophoresis.

Electrophoresis of PCR reactions

1. Using the micropipette with a clean tip, pipet 5 µl gel loading dye into the PCR reaction tube. Load both the PCR reactions and standard DNA markers sample into the gel.

2. Draw a picture of the gel and label in which wells you will load which samples (PCR reaction(s), DNA marker).

3. When the gel has hardened, remove the tape or stoppers.

4. Load your samples into the wells making sure you keep track of which samples are being loaded in which wells.

5. Pour TAE buffer carefully so it fills the electrophoresis apparatus and just covers the gel.

6. Run that gel! Plug the electrodes into your electrophoresis apparatus (red to red, black to black), being careful not to bump your gel too much.

7. Plug the power source into an outlet and set the voltage to about 100 V (max = 120 Volts).

8. Let the gel run until the dye migrates about 5-6 cm from the wells (about 20-25 minutes).

9. Turn off the power supply, disconnect the electrodes, and remove the top of the electrophoresis apparatus.

10. Carefully remove the gel. The gel can be wrapped in plastic wrap and stored in the refrigerator or placed it in the staining tray for DNA staining.

Staining gels to examine PCR reactions

1. Place gel in staining tray

2. Using gloves remove the plastic from the Ethidium bromide sheet and place the Ethidium bromide paper on the gel. Gently rub the paper with your fingers to make sure it is contacting the gel all over.

3. Stain for about 10 minutes.

4. Put the gel on the UV light box and, with the UV shield down, view your gel.

5. Take a Polaroid picture of your gel.

Appendix XI: Test Results Form

Name of patient……………….......................code………………………..

Hospital………………………………………………………………………..

Results of Concentrated wet smear.

Tick whichever is present in the smear.

Giardia lamblia, Isospora belli, Endameba histolytica, Dientamoeba fragilis Endolimax nana, Entamoeba coli, Entamoeba dispar, Entamoeba hartimani, Entamoeba polecki, Iodamoeba buetschlii and *Chilomastix mesnili*.

(Indicate below any other infective parasitic and/or helminthic agent found in the concentrated wet smear.

……………………………………………………………………………………

……………………………………………………………………………………

……………………………………………………………………………………

Results of Ziehl Neelsen staining procedure

Positive (Crypto present).

Negative (Crypto absent)

Tick whichever is applicable

Results of ImmunoCard STAT! Test.

1 a) Positive for *Cryptosporidium* parvum (crypto present).

b) Negative for *Cryptosporidium* parvum (crypto absent). Tick whichever is

      applicable.

2 a) positive for *Giardia lamblia*

b) Negative for *Giardia lamblia*. (Tick whichever is applicable).

PCR analysis results.

(To be performed on positive samples by ZN staining only)

Indicate the species of *Cryptosporidium* present_____

_____

Appendix XII: Sequence alignments of the *Cryptosporidium* 18S rRNA gene fragments for *C. parvum* (cattle genotype), *C. parvum* (human genotype), *C. hominis*, *C. meleagridis* and *C. canis* respectively.

```
0                                                           60
ACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGG
ACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGG
ACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGG
ACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGG
ACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGG
61                                                          120
AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTT
AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTT
AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTT
AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTT
121                                                         180
GTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTA - ATAATTTATATAAAATATTTTGA
GTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTA - ATAATTTATATAAAATATTTTGA
GTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTA - ATAATTTATATAAAATATTTTGA
GTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTA - ATAATTTATATAAAATATTTTGA
GTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTA - ATAATTTATATAAAATATTTTGA
181                                                         240
- - - - - - -ATATTTA - -TATATAACATTAACATAATTCATATT- - - - - - ACTATATTTTT- - - T
T - - - GAATATT - - TATATAATATTAACATAATTCATATT - - - - - - ACTATTTTTTT - - - T
T - - - GAATATT - - TATATAATATTAACATAATTCATATT - - - - - - ACTATATATTT - - - T
T - - - GAATATT - - TATATAATATTAACATAATTCATATT - - - - - - ACTAAATATAT- - - T
TTTTAAATATTAATATGTAAGATTAACATAATTCATATTTTTAAGACTGAATTTTTAGTT

241                 265
- - - AGTATATGAAATTTTACTTTGA
TTT AGTATATGAAATTTTACTTTGA
- - - AGTATATGAAATTTTACTTTGA
- - - AGTATATGAAATTTTACTTTGA
TTGATAATATGAAATTTTACTTTGA
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Dashes represent alignment gaps. The GenBank accession numbers for the above sequences are AF093494, AF228682,AF093491, AF112574 and AF15113 respectively