KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI, GHANA

Cryptosporidium spp Contamination and Risk Associated with the Irrigation of Lettuce with Contaminated Water in the Kumasi Metropolis of Ghana

By

Angelina Ewusiwaa Sampson (BSc. Biochemistry)

A Thesis submitted to the Department of Biochemistry and Biotechnology,

College of Science

in partial fulfilment of the requirements for Master of Philosophy degree in Biochemistry

JUNE, 2015

DECLARATION

Except for references which I have duly acknowledged, I hereby declare that this thesis is entirely a record of my own original research carried out at the Department of Biochemistry (KNUST) and NMIMR, under the rincipal supervision of Dr. Felix Charles Mills-Robertson and Prof. Robert Clement Abaidoo of the Kwame Nkrumah University of Science and Technology (KNUST) Kumasi. I do further declare that this thesis has never been submitted in part or whole for any purpose elsewhere.

Angelina E. Sampson (PG9994613)	Date
Dr. Felix Charles Mills-Robertson (Supervisor)	Date
Prof. Robert Clement Abaidoo (Supervisor)	Date

Certified by:

Dr. Antonia Tetteh (Head of Department)	Date

ABSTRACT

Cryptosporidium is a protozoan parasite which can be transmitted via food and water. Some studies have shown irrigation water to be routes of transmission for *Cryptosporidium* into the food chain, however, little information is known about *Cryptosporidium* levels in water used for irrigation in the Kumasi Metropolis. The purpose of this study was to evaluate *Cryptosporidium* contamination and risk associated with the irrigation of lettuce with contaminated water in the Kumasi Metropolis of Ghana. The study considered *Cryptosporidium* detection and enumeration in water and on lettuce samples using light microscopy, fluorescent microscopy and molecular identification using nested polymerase chain reaction (PCR). A total of 54.10% of all water samples tested positive by using microscopy or nested PCR (HSP70) approach. A comparative assessment for microscopy and nested PCR (HSP70) showed that 37.5% of all water samples were positive for *Cryptosporidium* using microscopy whilst 41.67% showed positive presence using nested PCR approach. The study revealed that water samples from irrigation water contaminated with hospital waste upstream had the highest number of oocyst with a total of 263.15±0.92 oocysts /10 L on all rounds of sampling. Water contaminated with effluent from a waste stabilization pond had no detectable number of oocysts on all rounds of sampling. All irrigation water with the exception of water contaminated with effluent from a waste stabilization pond exceeded the recommendation of WHO/FAO for protozoan parasites (1 egg/L); however, there was no statistical difference between oocysts counts from the various water samples from the farms. Temperature had an inverse relationship with oocyst concentration from the farms whilst pH and turbidity had no significant relationship with oocyst concentration. Molecular detection of *Cryptosporidium* using nested PCR (HSP70) showed that 50.00% of all lettuce

samples analysed were positive for *Cryptosporidium.* Risk analysis indicated that all water sources for irrigation posed risk as possible sources of human infection through direct contact with these water sources. The risk was higher than the WHO accepted threshold of $10^{-5}\, or\, 10^{-4}$ threshold. However, risk analysed using the *E. coli* conversion for *Cryptosporidium* distribution resulted in an underestimation of risk. Based on the risk assessment, measures should be put in place to reduce pathogen concentration in water sources in order to reduce risk posed to farmers.

DEDICATION

This work is dedicated to my parents, Mr and Mrs Sampson.

ACKNOWLEDGEMENTS

I would never be able to finish this project without the Grace and Provision of God. Through it all HE has seen me through.

I would like to express my sincere gratitude to the following people for their involvement and support during my time as a student: A big thank you to my supervisors; Prof. Robert Clement Abaidoo, Prof. Anders Dalsgaard and Dr. F. C. Mills-Robertson for their useful comments, suggestions and guidance during the experimental procedure and thesis write-up.

A special thanks to Dr. Irene Ayi, (Head of Parasitology Department, NMIMR) Dr. Mitsuko Suzuki, Mr. Kofi D. Kwofie, Mr. Michael Amoa-Bosompem and Mr. Edward Dumashie (all of Parasitology Department, NMIMR) for their guidance and help through the laboratory work. My profound gratitude goes to Miss. Georgina Isabella Djameh, Mr. Faustus Azerigyik and Mr. Senyo Botchie for accommodating my mistakes and training me in the molecular aspects of my project. Special appreciation also goes to all fellows and members of Parasitology Department NMIMR for their keen interest and support in diverse ways.

I am also grateful to Mr. Kwadwo Sarfo Marfo for field assistance and to Mr. Emmanuel DeGraft Johnson for always keeping me on my toes. A special thanks to Mr. Tobias Boel-Peterson and Dr. Heidi Huus-Peterson for tolerating my questions and providing me with certain essential information when I needed it the most. To Mr. Emmanuel Awusah Blay, I say thank you for the initial training at NMIMR, your enthusiasm about my project, constructive scientific criticisms, keeping me on the right scientific track and believing in my competence. I am also grateful to my family for their prayers, words of encouragement and unflinching support.

Special gratitude goes to DANIDA for funding personal expenses involved in the project work. Monetary support for this project was provided by DANIDA funds from the Safe Water for Food project (SAWAFO).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AE	Elution buffer
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
AW1/AW2	Wash Buffer 1/2
CDC	Centres for Disease Control
DNA	Dioxyribonucleic acid
EIA	Enzyme Immuno Assay
FAO	Food and Agriculture Organisation
FISH	Fluorescent *in situ* Hybridization
FITS	Fluorescein Isothiocyanate
FTU	Formazin Turbidity Unit
HIV	Human Immunodeficiency virus
HSP 70	Heat Shock Proteins 70
IF	Immuno Fluorescence
IFA	Immuno Fluorescent Assay
IMS	Immuno-magnetic separation
IWMI	International Water Management Institute
KATH	Komfo Anokye Teaching Hospital
KMA	Kumasi Metropolitan Assembly
KNUST	Kwame Nkrumah University of Science and Technology
L	Litres
MMWR	Morbidity and Mortality Weekly Report
NaOH	Sodium Hydroxide
NMIMR	Noguchi Memorial Institute for Medical Research
PCR	Polymerase Chain Reaction
QMRA	Quantitative Microbial Risk Assessment
rDNA	Recombinant DNA
RNA	Ribonucleic acid
spp.	Multiple species
SPSS	Statistical Package for Social Sciences
TAE	Tris-Acetate-EDTA
UA	Urban agriculture
UK	United Kingdom
US	United States of America
WHO	World Health Organisation

CHAPTER ONE

1.0. INTRODUCTION

The United Nations estimate that 54% of the world's population lives in the urban areas and this is expected to increase to about 66% by the year 2050 (United Nations, 2014). The reports indicate that increasing population increases the competition for already scarce resources such as food and water. While agriculture was previously known as a rural phenomenon, it is becoming increasingly popular in urban areas and has improved access to fresh vegetables, fruits and animal products (Amoah *et al.*, 2007). Urban agriculture (UA) is defined as the production of crop and livestock goods within cities and towns (Zezza and Tasciotti, 2010). This has become sufficiently important in developing countries where there are heavy losses in transit of food products which increases scarcity and cost of agricultural products (FAO, 2010). Urban agriculture has become an important means of attaining a balanced local food supply and food security creating employment and income (Mensah *et al.*, 2002).

In Kumasi, farming systems are usually situated in lowlands where the land is not suitable for immediate construction purposes and are usually in close proximity to water bodies. Most farms in Kumasi cultivate food items with low shelf lives mainly vegetables. About 59 hectares of urban lands in Kumasi are invested in vegetable farming in the dry season and 48 hectares in the rainy season with lettuce being the commonest vegetable (Cornish and Lawrence, 2001; Keraita *et al.*, 2014).

About 85% of wastewater generated from urban centres ends up in the environment untreated with most grey water ending up in streams, rivers and ocean (Obuobie *et al*., 2006). Due to limited water resources, most farmers rely on this untreated surface water for irrigation. A study in Kumasi, it was identified that about 70% of farmers used polluted rivers and streams as the

main sources of water for irrigation but none of the farmers interviewed in that study used (raw) effluent directly from the source or a sewage treatment plant (Obuobie *et al*., 2006). The authors further revealed that there was also an extensive use of shallow dug wells on valley bottoms in the urban areas, which are generally more accessible, reliable and inexpensive (Obuobie *et al.*, 2006). Polluted irrigation water can serve as a source of pathogen entry into the food chain with these pathogenic organisms contaminating the food chain through their deposit on the surface of crops or vegetables during irrigation (Robertson and Gjerde, 2001). Consumption of contaminated vegetables could cause gastro enteric diseases commonly diarrhoea. Diarrhoeal causing micro-organisms could be of viral, bacterial, protozoan and parasitic origins such as *Norovirus*, *Rotavirus*, *E. coli* and *Giardia*. Studies in Ghana on wastewater and other contaminated water sources have been limited to faecal coliforms and helminths (Obuobie *et al.*, 2006). Investigations of vegetable consumption related to diarrhoeal prevalence have identified a range of organisms which include *Rotavirus* (Reither *et al*., 2007) and some *Salmonella* spp, *E. coli* and *Cryptosporidium* (Adjei *et al.*, 2004). Research conducted in certain parts of the world have also implicated wastewater irrigated plants as a potential source of transmission of protozoan diseases particularly cryptosporidiosis (Ajonina *et al.*, 2012; Koompapong and Sukthana, 2012). *Cryptosporidium* is widely associated with diarrhoeal diseases and like other protozoans such as *Giardia* and *Entamoeba*, has thus been used in quantitative microbial risk assessment (Mara *et al.*, 2007; WHO, 2009).

Food products, especially leafy vegetables, are becoming sources of pathogens such as *Cryptosporidium* (Millar *et al.*, 2002) with *C. parvum* being responsible for the majority of gastrointestinal parasitic infections. *Cryptosporidium* has been recognized as a human pathogen since 1976 and was initially associated with individuals with compromised immune systems but

has now been recognized to be an important source of diarrhoea in both immune compromised and immune competent individuals (Duffy and Moriarty, 2003).

Improved, efficient and highly sensitive diagnostic tools however are able to detect *Crytosporidium* infection in relatively immune competent individuals. Cryptosporidiosis in immune competent individuals is an acute, self-limiting diarrhoeal illness often characterized by nausea, abdominal cramps and low-grade fever, however, the disease is more severe and usually chronic in immune compromised individuals (Chalmers and Katzer, 2013).

Over the last two decades, increasing numbers of cryptosporidiosis (in particular water related) outbreaks have been recorded in developed countries like Denmark and the United States of America (Craun *et al.,* 2007). In 1993, the largest recorded *Cryptosporidium* outbreak in the history of United States of America was registered in Milwaukee, Wisconsin, where 403 000 people were infected through contaminated drinking water (MacKenzie *et al.* 1994). This outbreak was caused by *C. hominis* (Peng *et al.,* 1997; Xiao *et al*., 2006) and the total cost of outbreak-associated illness was estimated at a total of $96·2 million; $31·7 million in medical costs and $64·6 million in productivity losses (Corso *et al.* 2003).

Although outbreaks of food related cryptosporidiosis are less recorded as compared to waterborne outbreaks, *Cryptosporidium* which does not multiply in foods has the ability to retain their viability and thus infectivity under favourable moist and cool conditions for several months. Beyond a temperature of 65^{o}C, *Cryptosporidium* oocysts lose their infectivity (Dawson *et al.* 2004) and thus pasteurized foods do not pose risk. Studies on infectivity and dose have suggested that a single oocyst is capable of causing an infection (Rose *et al.,* 2002), however; the infective dose is species dependent.

Evidence that food products aid the mechanical transmission of *Cryptosporidium* to humans has been hindered by the lack of enrichment culture methods for determining small numbers of oocysts. Detection and prevalence of *Cryptosporidium* oocysts on vegetables like lettuce is dependent on the laboratory technique used to isolate the pathogen. Various methods have been developed and described for the detection of *C. parvum* in foods (Chalmers and Katzer, 2013). These methods have been categorized into two, namely; separation techniques and diagnostic techniques. Separation techniques include density centrifugation, immunomagnetic separation (IMS) and flow cytometry. Diagnostic techniques include immunofluorescent microscopy (IFA), fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR) (Chalmers and Katzer, 2013). Identification of *Cryptosporidium* may make use of agitation in detergents or mobilization of oocysts from solid matrices using immunomagnetic separation (IMS) for concentration of oocysts coupled with detection by immuno-fluorescent microscopy or polymerase chain reaction (Jiang *et al.,* 2005). Limitations in the use of these methods for isolation and detection of *Cryptosporidium* spp from food products include variations in recovery levels and difficulties resulting from the breakdown of the food product during detachment of the oocysts from the food matrix. This causes the aggregation of food debris which can interfere with IMS recovery processes, leading to an overall reduction in the final purified oocyst recovery rate (Robertson and Gjerde, 2001).

1.1 PROBLEM STATEMENT

The rise in non-communicable diseases such as obesity has a general increase in consumption of fresh and less processed fruits and vegetables (Zink, 2009). Food borne infection outbreaks associated with the consumption of raw vegetables have however been increasing worldwide

(Lynch *et al.*, 2009). For instance, about 2.2 million people get sick annually from eating contaminated leafy vegetables and this represents about 23% of the 9.6 million cases of food-borne illness each year in United States of America (Painter *et al.*, 2013). This has consequently resulted in an increase in the awareness and realization that fresh fruit or vegetables can be mechanical reservoirs of disease causing bacteria, viruses, protozoa and helminths. Irrigation with poor quality water is one way that vegetables can become contaminated with food borne pathogens. *Cryptosporidium* is one of the most chlorine resistant waterborne parasites that infect humans and can be transmitted via contaminated food and water. They cause morbidity in healthy individuals and more severe consequences among immune compromised individuals. Since *Cryptosporidium* oocysts cannot grow or multiply on the surface of vegetables, any oocyst present at the time of consumption is likely to have been transmitted through irrigation, fertilisation, contaminated soil on the farm or contamination during processing and handling (Millar *et al.*, 2002). A recent study showed the prevalence of cryptosporidiosis to be 8.20% in patients visiting the Komfo Anokye Teaching hospital in Kumasi, Ghana (Acquah *et al.*, 2012). Possible routes of these infections would possibly include contaminated water and food. With regards to food, contaminated vegetables are a notable cause for concern as they are most likely to be eaten raw. Oboubie *et al.*, (2006) indicated that most farmers utilise untreated surface waters for irrigation, nonetheless, little information is available on the levels of *Cryptosporidium* in surface water and wastewater used for irrigation in Kumasi which could serve as sources of vegetable contamination. This study was therefore designed to assess the possible contamination of lettuce through irrigation.

1.2 JUSTIFICATION

Studies on surface waters used for irrigation in the United States of America, Mexico and South Africa showed that 28.00%, 48.00% and 43.00%, respectively of the samples analysed were positive for *Cryptosporidium* (Thurston-Enriques *et al.*, 2002; Chaidez *et al.*, 2006; Duhain, 2011). A study done on some rivers and irrigation waters in Kumasi showed that 75% of water samples were positive for *Cryptosporidium* (Boel-Peterson, 2013). The study by Boel-Peterson (2013), focused on only farms whose source of irrigation water were streams and all study sites had similar potential sources of contamination and recommended that other sources of irrigation water should be investigated for the presence of *Cryptosporidium* spp. In line with the recommendations that have been outlined in past studies, this study was designed to estimate the oocyst levels in multiple irrigation water sources and also the lettuce cultivated on the farms that use these water sources for irrigation purposes. Public health studies generally have focussed on sources of drinking water in estimating quantitative microbial risk assessment because of the notion that drinking water provides the most feasible route for human infection. Results obtained from this study therefore will present evidence of possible risk of irrigation water in to *Cryptosporidium* infection routes in medical and veterinary contexts and application of Quantitative Microbial Risk Assessment (QMRA) model in this study will provide useful information the level of risk of infection associated with such farming, which is essential for policy formulation. Accurate identification of risks levels thus provides the foundation for the implementation of effective measures and control programmes that could potentially lower the risk and rate of infection and reduce the disease burden and invariably promote good health status of the populace.

1.3 HYPOTHESES

1. Sources of irrigation water can be potential route for *Cryptosporidium* transmission to lettuce

2. Water contaminated with hospital waste has higher concentration of *Cryptosporidium* than other sources of irrigation water.

3. Vegetables irrigated with water contaminated with hospital wastewater pose a higher risk to consumers as compared to other sources of irrigation water.

1.4 STUDY OBJECTIVES

1.4.1 Main objective

The main objective of this study was to establish *Cryptosporidium* spp. contamination in low quality water used for irrigation of lettuce and the health risks associated with such farming system.

1.4.2 Specific objectives

The specific objectives of this study were to

1. Establish the presence of *Cryptosporidium* spp. in low quality water for lettuce farming and the influence of selected water physical parameters on the levels of contamination
2. Determine the potential health risks associated with farmers' exposure to *Cryptosporidium* spp. contaminated irrigation water using the quantitative microbial risk assessment

CHAPTER TWO

2.0 LITERATURE REVIEW

This chapter deals with related research works on the study matter and identifies certain knowledge gaps in works done on *Cryptosporidium* spp. in relation to irrigation water and vegetable contamination. This study will seek to address some of these gaps thus creating new knowledge. The chapter covers urban agriculture, irrigation water and the protozoan, *Cryptosporidium.*

2.1 URBAN AGRICULTURE IN KUMASI

Urban agriculture is the production, processing and distribution of food items ranging from animals to crops in and around a town or city (Obuobie *et al*., 2006). Urban agriculture is in two main forms namely; open space production for urban market and backyard gardens (Obuobie *et al*., 2006). In Kumasi, such farming systems are usually located in lowlands where the land is unsuitable for construction purposes. Approximately 41 hectares of land in Kumasi are used for vegetable farming (Cornish and Lawrence, 2001), and these lands favour urban farming due to the availability of groundwater and their proximity to surface water bodies (Obuobie *et al*., 2006). Majority of these farmers are involved in the production of food items that have low shelf life periods with the commonest leafy vegetable being lettuce (Keraita *et al.*, 2014). Lettuce as well as other vegetables' consumed as salads is becoming a popular trend in urban areas and some studies have shown these salads to be of poor microbiological quality (Fung *et al.*, 2011)

Fung *et al*. (2011) showed a high contamination of salads with thermotolerant coliforms, *Salmonella* and helminth eggs.

2.2 WATER RESOURCES AND AGRICULTURE

Irrigation is an important factor in food security. About 17.00% of total arable land worldwide is irrigated and this contributes to 34.00% of crops that are produced (Jimenez, 2006). Approximately 192 million hectares of irrigated land is found in developing countries and as such there is high dependence on water for food production (United Nations, 2003). The demand on water resources in Ghana is increasing due to urban and agricultural development (Keraita *et al.*, 2014) and thus scarcity is imminent.

Due to the scarcity of fresh water and accessibility constraints, the use of low quality water in irrigation has become increasingly popular. According to Jimenez (2006), about 12,000 hectares of land in Ghana are irrigated with low quality water. In urban areas, surface water used for irrigation are rarely unpolluted as most waste generated in these areas ends up polluting rivers and streams (Obuobie *et al*., 2006). This raises the concern of the use of low quality water and food safety. Low quality water as defined by FAO (1992) is water that possesses certain characteristics which has a potential to cause problems when used for its intended purposes. This includes wastewater, polluted surface water and storm water that are used for irrigation

2.2.1 Quality of irrigation water

Wastewater and faecal sludge produced in Ghana are rarely treated before their release into the environment. International Water Management Institute (IWMI) recorded a total of 63

wastewater treatment plants and eight faecal sludge treatment plants across Ghana but this is awfully inadequate taking into consideration the per capita waste production according to Murray and Drechsel (2011). This implies that most of the waste generated ends up in the environment untreated and the situation will be worse in urban areas where population is dense and continues to increase. Keraita *et al.* (2014) have proposed a strong link between the lack of wastewater treatment and the use of polluted water for irrigation in urban agriculture. Wastewater-polluted streams and dug outs are the most common sources of irrigation in urban farming in Ghana (Sengupta *et al.*, 2012). In a study conducted in Kumasi, most farmers did not use waste stabilization pond effluent directly; however, in situations where there were no alternative sources of water, farmers used effluent from the waste stabilization pond and wastewater from other drains (Obuobie *et al*., 2006). Additionally, some farmers use shallow hand dug wells in urban irrigation.

Major health risks are associated with the use of wastewater or polluted irrigation water and have been traced to viral, bacterial and parasitic origins. Farm-based methods such as the use of sedimentation ponds, sand filters and enhanced pathogen die-off on crops through cessation of irrigation before harvest have been found to be effective interventions for reducing risk associated with the use of contaminated water for irrigation. Careful collection of irrigation water without disturbing the sediment at collection point in hand dug or stream have also been found to reduce concentrations of pathogens such as helminths and *Cryptosporidium* (Searcy *et al.*, 2006; Keraita *et al*., 2008). Some risk assessments done in urban agriculture in Ghana have reported faecal and helminth contamination levels in irrigation water (Amoah *et al*., 2011), however, most studies done in Kumasi have been focused on faecal coliforms and helminths, with few studies on parasitic protozoa such as *Cryptosporidium* (Boel-Peterson, 2013) .

Putignani and Menichella (2010) stated that the most important enteric diseases in humans are caused by enterotoxigenic and enteropathogenic *E-coli*, *Rotavirus*, *Giardia lamblia* and *Cryptosporidium* and that approximately more than 58 million diarrhoea cases detected per year in children worldwide were associated with intestinal protozoa. Cryptosporidiosis is less recorded particularly in children thus the global burden of the disease is underestimated. This influenced the addition of *Cryptosporidium* to the WHO Neglected Diseases Initiative in 2004 (Savioli *et al.*, 2006).

2.3 *CRYPTOSPORIDIUM*

Cryptosporidium originally described by Tyzzer (1912) is an enteric pathogen which is considered a major agent of diarrhoeal diseases in man, farm animals and wild animals and has gained importance over the last twenty five years (Smith and Nichols, 2009; Ryan, 2010). They contaminate different sources of water such as drinking water, raw water, surface water, storm water, groundwater, wastewater, sewage and irrigation water (Steele and Odumeru, 2004). *Cryptosporidium* is responsible for many diarrhoeal outbreaks in the United States, the United Kingdom and other countries (Wohlsen *et al.*, 2004). Although person-to-person contact and domestic animals are some infection transmission routes, the environmental route of transmission especially through water, soil and food is becoming increasingly popular and gaining research attention. Research conducted in various geographical regions have implicated wastewater or contaminated surface water used for irrigating plants as a major potential source of transmission of protozoan diseases particularly cryptosporidiosis (Koompapong and Sukthana, 2012).

Cryptosporidium infects the epithelial cells in the digestive tract of humans, other mammals, birds and fish. The parasite forms thin-walled oocysts that are relatively small (4-6 µm) and almost spherical. Thin walled oocysts may encyst within the host resulting in severe diarrhoea whereas thick walled oocysts are easily shed by infected host into the environment (Fayer *et al.*, 2000). Faecal matters that contain oocyst end up in the environment and can contaminate foods by direct contact or contaminated surface water used for irrigation. Dose studies and models have suggested that as low as a single oocyst or cyst is capable of causing an infection (Rose *et al.,* 2002), however, the infective dose is species dependent. The oocyst stage is a critical stage for the dispersal, survival and infectivity of the parasite. Therefore the detection of the oocyst is particularly important.

2.3.1 Life cycle of *Cryptosporidium*

The life cycle of *Cryptosporidium* comprises of asexual, sexual and transmissive stages which are completed within one host. It takes 12 to 14 hours for the completion of the maturation per generation. *Cryptosporidium* is endogenous and occurs within the gastrointestinal tract of the host. It develops into a thin walled oocyst which is released and retained in the gut, developing and releasing sporozoites to auto infect the host (Caccio, 2003). This notwithstanding, about 20.00% of *Cryptosporidium* oocysts develop into an encysted stage, which has a thick two-layered resistant oocyst wall and escapes from the host gut within faecal matter to contaminate other sources and to infect a new host. The oocyst wall consists of an inner and an outer layer with a distinctive suture at the end of its structure. Due to their small size, difficulty in differentiating internal structures and similar morphological features, it is more difficult to differentiate between different species of *Cryptosporidium* as opposed to bacteria (Fayer *et al*.,

2000). The oocyst formation helps the organism to persist in the environment and later infect a new susceptible host. Relatively little information is known about the chemical composition of oocyst and how this affects their survival (Moore *et al.*, 2007).

Each oocyst has four infective units called sporozoites which have the ability of exiting through the suture and restart the process of infection once it is taken up by the host (Fayer, 1997; Caccio, 2003). The carbohydrate amylopectin has been shown to be responsible for production of energy used for excystation and penetration of host cells (Harris and Petry, 2003). Therefore *Cryptosporidium* is unable to infect the gastrointestinal cell wall once amylopectin is depleted (Vetterling and Doran, 1969; Harris and Petry, 2003; Duhain, 2011). The incubation period for *Cryptosporidium* is between 3 and 7 days (Tzipori and Ward, 2002).

2.3.2 Classification, taxonomy and host range

The genus *Cryptosporidium* is classified in the Phylum Apicomplexa just like *Plasmodium*, *Toxoplasma* and *Eimeria. Cryptosporidium* species are all intracellular parasites of vertebrates. There are two main sub-groups in the genus; the gastric and intestinal group. The gastric group consists of *C. muris*, *C. serpentis* and *C. andersoni* whereas the intestinal group consists of all *C. parvum* genotypes, *C. hominis, C. canis, C. felis, C. wrairi and C. meleagridis* (Fayer *et al.*, 2000).

Cryptosporidium parvum (*C. parvum)* infects a number of mammalian hosts whereas *C. hominis* infects only humans (Leav *et al.,* 2003). Despite their identical morphology, the two can be distinguished by molecular methods. Some studies have also shown a lot of heterogeneity among the genotype 1 (*C. hominis*) isolates. The heterogeneity is seen at the *Cpgp40/15* (also known as

gp60/45/15) locus which encodes for surface glycoproteins present during the invasive stages of the parasite (Strong *et al*., 2000).

Species that are zoonotic are also of major health importance. *Cryptosporidium meleagridis* is cross infectious between birds and a wide range of mammals. Naturally, *C. meleagridis* infects birds but cross-infection transmission routes occur when bird droppings are used as fertilizer in vegetable farming (Akiyoshi *et al.*, 2003). *Cryptosporidium parvum* is capable of infecting 152 mammalian species including cattle, dogs, goats (Quilez *et al.*, 2008) but *C. hominis* and *C. parvum* primarily infect humans (Insulander *et al.*, 2013). A study done in the United Kingdom showed that 96.00% of infection that were reported was caused by *C. hominis* (51.40%), *C. parvum* (44.00%) and *C. meleagridis* (0.60%) (Elwin *et al*., 2012). Table 1 shows various species of *Cryptosporidium* and their host. *Cryptosporidium parvum* has also been known to be the most dominant species in contaminated surface waters where potential sources of contamination included human and cattle (Xiao *et al*., 2001). The multi-host specificity of *Cryptosporidium* spp. increases their chances of transmittance in the environment thus aiding their potential widespread of infection to humans.

Table 1: List of *Cryptosporidium* species and their natural hosts

Cryptosporidium species **(genotype)**	**Major host**
C. andersoni	Cattle
C. baileyi	Poultry
C. bovis	Cattle
C. canis	Dogs
C. fayeri	Red kangaroos
C. felis	Cats
C. frageli	Toads
C. galli	Finches, chickens
C. hominis (genotype H, I or 1)	Humans, monkeys
C. macropodum	Eastern grey kangaroos
C. meleagridis	Turkeys, humans
C. molnari	Fish
C. muris	Rodents
C. parvum (genotype C, II or 2)	Cattle, other ruminants, humans
C. ryanae	Cattle
C. scophithalmi	Fish
C. serpentis	Reptiles
C. suis	Pigs
C. varanii	Lizards
C. wrairi	Guinea-pigs

(Courtesy: Health Canada, 2012)

2.4 *CRYPTOSPORIDIUM* OCCURRENCE IN WATER

Cryptosporidium has been associated with waterborne outbreaks and have been found in surface waters, ground water, irrigation water, treated drinking water, recreational water and bottled water worldwide (Franco and Neto, 2002; Steele and Odumeru, 2004; WHO, 2008). It is an important enteric protozoan which is considered a major causal agent of diarrhoeal diseases in humans and farm animals (Xiao and Fayer, 2008). According to Chaidez *et al*. (2006) irrigation water can be a potential transmission route for *Cryptosporidium* entry into the food chain. Studies done by Gracenea *et al.* (2011) supports this claim as 18.40% of major irrigation channels in Spain were found to be contaminated with *Cryptosporidium* spp. with concentration

ranging from 3 – 82 oocysts per Litre. Thurston-Enriquez *et al.* (2002) showed that 36% of irrigation water in Central America tested positive for *Cryptosporidium* spp. with a mean count of 227 oocysts per 100 L. Studies by Chaidez *et al.* (2006) in Mexico however, showed a higher prevalence in surface water (48%) with a lower concentrations ranging from 17 – 200 per 100 L. Other studies done in South Africa, showed a *Cryptosporidium* spp. prevalence of 43.00% in surface waters which were used for irrigation purposes (Duhain, 2011).

Transmission through the environment is facilitated by various factors. In natural waters, oocyst may occur in a freely suspended form, however due to large particle concentrations in wastewater and surface water, high proportion of oocyst in surface water could be attached to particles like clay, sand, algae and bioflocs (Medema *et al.*, 1997) and this could reduce the recovery rate when immuno magnetic separation is used to concentrate oocysts from water samples.

The extent of survival is also dependent on the characteristics of the water. Transport of infectious oocyst from source of water contamination to areas of exposure of oocyst to possible host is dependent on some hydro dynamical, chemical and biological factors. These factors include water flow, attachment of suspended oocyst to particles, sedimentation, re-suspension of free and attached oocyst and survival of the oocysts (Medema *et al.*, 1997). Very high turbid waters are known to have oocyst present and as such some studies have correlated turbidity to concentration of oocyst (House, 2011; Swaffer *et al.*, 2014). Studies by Lee *et al.* (2010) however, showed no correlation between turbidity and oocyst concentration. Other studies have revealed minor viability loss and inactivation of oocyst at pH below 4 and greater than 11. It has also been shown that ammonia can enhance inactivation at low and high pH and thus mostly employed in wastewater treatment (Kniel *et al.*, 2003).

The oocyst stage is stable and can survive in the environment from weeks to months. Oocysts have been shown to withstand a variety of environmental stress including freezing (Duhain, 2011). *Cryptosporidium* is resistant to disinfection by chlorine and can only be removed from a contaminated water source by filtration, inactivation by ozone, ultraviolet radiation and chlorine dioxide (Chen *et al.*, 2002). The structure of the oocyst wall which is made up of a double layer of protein-lipid-carbohydrate matrix makes the oocysts resistant to chlorine (Templeton *et al.*, 2004).

2.5 OCCURRENCE OF *CRYPTOSPORIDIUM* IN FOOD AND FOOD BORNE TRANSMISSION

Despite the fact that water is a well-recognized vector of *Cryptosporidium*, it has recently emerged that food may also play a more significant role in the transmission of *Cryptosporidium* to humans (Caccio, 2003). Studies on the occurrence of *Cryptosporidium* oocysts on food items have been performed in developing countries and most raw vegetables were found to be contaminated (Eraky *et al.*, 2014). Similar surveys conducted in Norway and Israel also showed contamination of fruits and vegetables. This goes to show that the contamination is not restricted to developing countries only (Robertson and Gjerde, 2001; Armon *et al*., 2002). Table 2 shows some of the studies that have been done on the presence of *Cryptosporidium* on foods.

Table 2: Incidence of *Cryptosporidium* in vegetables from different countries

Food type	Country	Detection method	Identified pathogen(s)	Mean concentrations	Reference
Basil, Cabbage, Celery, Cilantro, Green onions, Leeks, Lettuce, Parsley, Yerba Buena	Lima, Peru	Acid fast staining /IFA	*Cryptosporidium*	Not done	Ortega *et al*., (1997)
Dill, Lettuce, Mug bean sprouts, Radish sprouts	Norway (including imported food)	IMS/IFA	*Cryptosporidium* , *Giardia*	3 oocysts per 100 g produce	Robertson and Gjerde (2001)
Carrot, Cilantro, Cucumber, Lettuce, radish, Tomato	Costa Rica		*Cryptosporidium*	Not done	Monge and Arias (1996)
Zucchini	Israel	IFA	*Cryptosporidium* , Giardia	80 - 10,000 oocysts/0.5 kg	Armon *et al*., (2002)

(Courtesy: Caccio, 2003)

Regardless of some of these findings, reports on food borne cryptosporidiosis are scarce mostly due to difficulty in detection and documentation. Limited numbers of oocyst found in suspected samples and also the lack of sensitive methods of detection in food contribute to under-reporting of the disease are the predominant constraints.

Recent studies by Macarisin *et al.* (2010) proved the adherence and internalization of *Cryptosporidium* oocyst in spinach after contact with contaminated water. This study was the first to show attachment of oocyst on leafy vegetables thus pre-suggesting that oocyst could attach to other leafy vegetables such as lettuce. Factors such as presence of hairy structures are

thought to increase the amount of oocyst retained on vegetables (Armon *et al.,* 2002). Other than irrigation water, animal manure, presence of cattle near farms, unhygienic practices by farmers are all potential source of contamination of vegetables with *Cryptosporidium*. The moist and cool surfaces of vegetables such as lettuce could also enhance the survival and persistence of oocyst on their surfaces.

2.5.1 Foodborne outbreaks due to cryptosporidiosis

Outbreaks of cryptosporidiosis have been documented around the world with the greatest incident in the US and UK (Slifko *et al.,* 2002). Most of these outbreaks have implicated contaminated surface water and a significant amount with groundwater. In the United States, more than 10,000 people had contracted cryptosporidiosis before the year 2000 (Slifko *et al.*, 2002). The first outbreak of the disease occurred in 1984 in Texas and the source was suspected to be from an unfiltered ground water supply (D'Antonio *et al.*, 1985).

Although outbreaks of waterborne cryptosporidiosis are more common, some food related outbreaks have been recorded. *Cryptosporidium parvum* was identified as the causative organism for an outbreak in England and Wales between 1996 and 2000 (Adak *et al.,* 2005). *Cryptosporidium* was the fourth most common causative agent of food related infections in 2004 in the United States of America (CDC, 2006). In 2005, an outbreak was recorded in Denmark with 99 infected cases. Source of outbreak was attributed to salad bar (whole carrot, grated carrot and red pepper) (Insulander *et al.,* 2008). In 2008, similar findings were recorded in Finland and Sweden with sources being salad and parsley respectively (Ponka *et al.,* 2009; Ethelberg *et al.,* 2009)

2.5.2 Food and Vegetable Quality

The range of microorganisms associated with outbreaks linked to fresh produce encompasses bacteria, viruses and parasites (CDC, 2006). Most of the reported outbreaks have been associated with bacterial contamination, particularly members of the *Enterobacteriaceae*. Of these, *Salmonella* and *Escherichia coli* O157 in sprouted seeds and fruit juices are of particular concern. The viruses involved in outbreaks have a human reservoir (e.g. Norwalk-like and Hepatitis A) and can be associated with intact products grown in contact with the soil and/or water. Outbreaks linked to protozoa (e.g. *Cryptosporidium, Cyclospora, Giardia*) have been associated more with fruits than with vegetables (Adak *et al.,* 2005). Protozoa and viruses are most often associated with contaminated water or food handlers (Ponka *et al.,* 2009).

Fruits and vegetables normally carry a non-pathogenic epiphytic microflora. However, there are certain factors, which contribute to the microbiological contamination of these products with pathogens. Contamination can arise as a consequence of treating soil with organic fertilisers such as manure and sewage sludge and from irrigation water. Another aspect contributing to the microbial risk for the consumer is the increasing consumption of new products (e.g. sprouted seeds) or fruits and vegetables imported fruits and vegetables. Additionally, the application of technologies such as cutting, slicing, skinning and shredding will remove the natural protective barriers of the intact plant and open the possibility for providing a suitable medium for the growth of contaminating microorganisms (Garg et al., 1990). Beuchat (1995) referred to outbreaks of *Shigella flexneri* and hepatitis A which could be traced back to infected people working on the fields or in the packaging facility.

Some pre-harvest measures will reduce the microbial contamination of fruits and vegetables. However, the relative contribution of these measures is not always equal in terms of efficacy or

the level of safety achieved. Manure, bio-solids and irrigation water should be of a quality that does not introduce pathogens to the treated commodity. Special concern has to be taken to the risk with droppings from wild and domestic animals entering the production area and from fresh manure used as fertiliser. The potential of organic farming to contaminate fruits and vegetables with pathogens has to be investigated.

Harvesting at the appropriate time and storing the harvested products under controlled conditions will help retard growth of post-harvest spoilage and pathogenic microorganisms. Humid and warm storage conditions encourage the growth of microbial contaminants. The use of additional post-harvest procedures could reduce the contamination level of fruits and vegetables. Washing with water of potable quality can reduce the microbial load. Although a wide range of different agents is available for disinfecting/sanitizing fresh produce their efficacy is variable and none are able to ensure elimination of pathogens.

2.6 PATHOGENESIS OF CRYPTOSPORIDIOSIS

Cryptosporidiosis is produced by ingestion of food and water contaminated with oocysts. The disease is self-limiting in healthy individuals but chronic and potentially fatal in immune compromised individuals (Duffy and Moriarty, 2003; Chalmers and Katzer, 2013). The clinical manifestation varies from person to person but it is often characterised by watery diarrhoea. The diarrhoea is normally presented with abdominal pains and sometimes fever, malaise, nausea, vomiting and loss of appetite. Symptoms may take 5 -14 days after ingestion of oocysts and is dose-dependent. The period of illness is also variable and can last days to 5 weeks. The gastrointestinal epithelium generally recovers after symptoms cease but there are some indications showing that other diseases may arise. For instance *C. hominis* was associated with joint and eye pains, headaches and fatigue for about 2 months following infection (Chalmers and

Katzer, 2013). Many healthy individuals are asymptomatic (DuPont *et al.*, 1995; Chalmers and Katzer, 2013). Population with increased risk of infection includes children, immune compromised individuals and malnourished individuals (Fayer *et al.*, 2000). Other sources of immunodeficiency such as organ transplantation, x-linked hyper-IgM syndrome, severe combined immunodeficiency, selective IgA deficiency and IFN-γ deficiency are also believed to increase risk of infection with *Cryptosporidium* species (Leav *et al.,* 2003). The peak of the incidence of cryptosporidiosis occurs in children under the age of two particularly malnourished children (Ayuo, 2009). In Brazil, cryptosporidiosis and persistent diarrhoea was correlated with subsequent impairment physically and reduced cognitive function (Leav *et al*., 2003). People with serological evidence of previous infection are more resistant to the disease (Leav *et al*., 2003).

2.6.1 Epidemiological indicators of cryptosporidiosis

The disease in developing countries is endemic and one of the most common causes of diarrhoea in children (Leav *et al.*, 2003). The number of reported cases increased between - 2006 and 2008 with majority being reported in summer than winter (MMWR, 2010). A study in South Africa estimated a *Cryptosporidium* incidence of 18.00% in school going children and hospital patients (Samie *et al*., 2006). According to statistics, 32.00% of all AIDS patient develop cryptosporidiosis at one stage or another in their lifetime (Hunter and Nichols, 2002). In developing countries, diarrhoea accounts for 30.00 - 50.00% of all death cases for children less than five years and studies have shown that *C. canis*, *C. felis* and *C. meleagridis* have higher prevalence (Mak, 2004). In a study conducted at the Komfo Anokye Teaching Hospital, *Cryptosporidium* accounted for 19.00% (95/500) among HIV and AIDs patients in 2008

(Boaitey *et al*., 2012). A similar study done in primary school children in Kumasi showed a prevalence of 8.50% (Walana *et al*., 2014).

2.7 CRYPTOSPORIDIUM DETECTION METHODS

2.7.1 Clinical Diagnosis of cryptosporidiosis

Diagnosis of Cryptosporidium infections in diarrhoeal patients is not a routine laboratory test in both developed and developing countries because rehydration and electrolyte balance is more crucial (Farthing et al., 2008). Before the 1980s, an expensive, invasive and time consuming histological diagnosis was carried out to find small spherical life cycle stages of *Cryptosporidium* in the microvillous region of the intestinal mucosa or in tissue obtained by necropsy. A variety of diagnostic methods have subsequently evolved to identify *Crytosporidium* oocysts mainly by examination of faecal specimens. Sputum and bile specimen have also been shown to alternative samples in the identification of oocysts (Current & Garcia, 1991).

Acute cryptosporidiosis is self-limiting and identification would be necessary in the event of an outbreak or if diarrhoea persist or becomes chronic. Reliance on the quality of stool samples, experience and skills of the microscopists and the resources available for stool evaluation is crucial in effective diagnosis of diarrhoea (O'Ryan et al., 2005). In developed countries where accurate and various diagnostic tests are available, underdiagnosis occur mostly because most laboratories do not examine faecal specimen for *Cryptosporidium* oocysts infections (Kosek et al., 2001). Alternative technologies for diagnosing cryptosporidiosis include ELISA, flow cytometry, UV-visible spectroscopy and PCR. Identification of various species and genotypes have been addressed by PCR base RFLP and DNA sequencing

2.7.1.1 Microscopy

Microscopic detection of *Cryptosporidium* in faecal specimens is the gold standard and widely used method in laboratory diagnosis. It is used to confirm suspected illnesses based on signs and symptom or clinical history of a patient. Techniques in microscopy include direct faecal smear, concentration techniques by flotation and sedimentation, conventional staining including faecal examination of smears by acid-fast stains such as modified Ziehl-Neelsen or auramine-rhodamine stains (Garcia et al., 1983; Scott, 1988; Current & Garcia, 1991). However, because microscopy is labour intensive, time consuming and requires expertise, misdiagnosis can occur especially in resource limited regions like Africa where there is the lack of adequate human resource and laboratory equipment (Ndao, 2009). This notwithstanding, microscopy is less expensive in comparison to other diagnostic techniques and very specific in identifying oocysts in faecal specimens but its sensitivity depends on infection intensity and specimen freshness (Pawlawski et al., 2009). Acid-fast staining method in particular is common in developing countries because it is relatively inexpensive and easy to use. Modified acid-fast staining is frequently used in developing countries because of its low cost, ease of use, and availability and ability to use a standard microscope for detection. The use of other methods together with microscopy to confirm diagnosis is significant for disease identification, treatment and disease surveillance.

2.7.1.2 Immunological Techniques

Immunological techniques used in clinical diagnosis of *Cryptosporidium* include the Enzyme Linked Immunosorbent Assays (ELISA), the enzyme immuno-assays (EIA) and immuno-fluorescent assays (IFA). Antigen capture ELISA for detecting *Cryptosporidium* antigen in faecal specimens offers a diagnostic substitute to direct microscopy. It saves time as many

samples can be tested at a time. Ungar (1990), achieved a specificity of 96.7% and sensitivity of 82.3% for the detection of *Cryptosporidium* antigen in faecal specimens in comparison with microscopy. ELISA may fail to detect infections in specimens fewer than five oocyst per 0.01 ml of concentrated faecal specimen because the antigen in the specimen was inaccessible to or not recognized by the polyclonal antibodies meaning the antigen level was lower than the detection limits of the assay (Ungar, 1990). On the other hand, ELISA is very sensitive and might detect *Cryptosporidium* antigens in microscopically negative samples which might suggest a real infection, or detection of disintegrating organism or their products which means individuals might have been previously exposed to infections. ELISA becomes an important technique in disease transmission because it is able to identify individuals who are not actively shedding oocysts during specimen collection. Isolates of *Cryptosporidium* may differ geographically; therefore the ELISA antisera prepared in one location and used in another may reduce sensitivity of detection. Immuno-flourescent assays and EIA kits have become commercially available for the detection of *Cryptosporidium* spp. These methods detect oocyst surface antigens but cannot differentiate between oocyst of different species.

2.7.1.3 Molecular Techniques

The major molecular technique that has proved effective in the identification and characterization of several *Cryptosporidium* genotypes is the Polymerase Chain Reaction (PCR) method and its variations. Methods such as PCR-restriction fragment polymorphism (PCR-RFLP), PCR-randomly amplified polymorphic DNA and PCR DNA sequencing have been used in identifying both human and animal transmissions of cryptosporidiosis.

2.7.1.3.1 Polymerase Chain Reaction (PCR) for the Detection of *Cryptosporidium* Oocysts

PCR, invented by Kary Mullis and colleagues in the 1980s, uses the enzyme DNA polymerase, primers and a DNA template to make copies of a defined DNA region (Mullis et al., 1986). Furthermore, heat stable polymerases such as Taq polymerase from the *Thermus aquaticus* bacterium are now being used for the separation of DNA strands. Although PCR is a more sensitive technique for detecting oocysts in faecal specimen than conventional microscopic and immunological assays, they are mostly restricted to research and specialized laboratories in developing countries.

Faecal specimen may contain PCR inhibitors that hamper the success of this technique. Bile salts, haemoglobin degradation products, polyphenolic compounds, heavy metals and complex polysaccharides are common PCR inhibitors in faecal specimen which exert their effects by either preventing amplification or block enzyme activity (Lantz et al., 1997; Stauffer et al., 2008). Purification of DNA is the best method for removing PCR inhibitors from faecal specimens and several commercial kits are available for the extraction and purification DNA which have proved to eliminate these inhibitors. The choice of DNA polymerase is also important in overcoming the effects of inhibitors that were not eliminated during extraction (Wiedbrauk et al., 1995; Stauffer et al., 2008).

Modifications have evolved over the years from the basic PCR technique give rise to methods that improve performance and specificity. Among these is the nested PCR which uses 2 sets of primers for a primary and secondary PCR and increases the sensitivity (detects small amounts of the target DNA) and specificity (DNA amplification that hybridize within the amplified fragment in the first PCR) (Johnson et al., 1995). Genus specific and species specific primers have been

developed to target cryptosporidium presence in faecal specimen and specific species respectively.

2.7.1.3.2 Nested-PCR based Restriction Fragment Length Polymorphism (RFLP) and DNA Sequencing for the Identification of Cryptosporidium Genotype

This is one of the genotyping techniques used to identify *Cryptosporidium* species in faecal specimen. Species identification and genotyping is important to identify sources of infection. Different genetic loci are targeted for genotyping techniques. Some of the gene loci targeted for amplification and detection include the Small Subunit ribosomal RNA (SSU rRNA; GenBank accession no. L16996), 70k-Da Heat Shock Protein (HSP) 70; GenBank accession no. AF221528), and 60k-Da glycoprotein (GP60; GenBank accession no. AF164489) (Ferguson et al., 2006) The SSU rRNA gene is a hyper-variable gene which improves sensitivity and is a preferred locus for genotyping because it has the largest historical database and has been targeted for most phylogenic analyses. For all species and over 30 genotypes described, there are genus specific primers that amplify the SSU rRNA gene,

2.7.2 Detection of *Cryptosporidium* species in environmental samples

Detection of *Cryptosporidium* oocysts in environmental samples such as water and soil mainly relies on methods that are used in clinical diagnosis such as microscopy, immunological techniques and molecular identification. However, the major difference is the sample processing method. Mostly in environmental samples, it is important to apply methods that enable the concentration or isolation of oocysts before detection. The most widely used method for detection is the detection of oocysts in environmental samples using the Modified Ziehl Neelson method (acid fast stain), Auramine O staining or Kinyoun stain (Rossle and Latif, 2013) and this requires experience in the identification of *Cryptosporidium* oocyst. Other detection tools such as

the enzyme immuno-assays (EIA) and immuno-fluorescent assays (IFA) have also been employed in the detection of *Cryptosporidium* oocysts. The PCR based technique have been used to accurately detect oocyst in environmental samples, however, like their use in clinical diagnosis, they are yet to be standardised and used for routine clinical use and as such are used generally for research purposes (Leav *et al.*, 2003). Molecular detection of *Cryptosporidium* has not been adopted for regulatory purposes because only oocyst containing sporozoite DNA is detected. Small oocyst numbers in environmental samples presents a challenge in quantification of oocysts. Standard methods of identification such as microscopy and PCR do not give reliable information about virulence of oocyst, as such in water monitoring; all oocysts detected are assumed to present a public health risk (Chalmers and Katzer, 2013). Again, the approach of sequencing DNA from nested PCR products of the SSU rRNA gene, or using limiting PCR primers according to Chalmers and Katzer (2013) tends to improve differentiation of multiple species or genotypes in a single sample. This approach is however technically demanding.

2.8 PREVENTION AND TREATMENT

In the absence of effective specific therapy against infection, preventive measures are essential especially among children and immune-compromised individuals. Preventive measures that need to be practiced to prevent cryptosporidiosis infection are generally related to keeping good personal hygiene and thus bad hygienic practice during production, transport preparation and handling of vegetables like lettuce spread cryptosporidiosis (El Said Said, 2012; Walana *et al.*, 2014). With infection route of *Cryptosporidium* being predominantly faeco-oral, it is important to ensure that ingested substances are safe and not contaminated.

No drug therapy had been useful in the treatment of cryptosporidiosis and thus treatments were merely aimed at symptomatic manifestations (Fayer *et al.,* 2000). However, an anti-parasitic drug with broad spectrum known as Nitazoxanide was introduced in 2002 in the United States as a treatment for cryptosporidiosis (MMWR, 2010). This drug is effective in immune competent individuals, however, its efficiency in immune compromised individuals is not clear (CDC, 2006). Symptomatic therapy includes fluid replacement, proper nutrition and treatment of diarrhoea. Anti-diarrhoeal drugs like loperamide, diphenoxylateatropine, somatostatin analogues or opiate are often used in combination with anti-parasitic drugs (Ayuo, 2009). Due to the absence of chemotherapeutic agents that are directed at asymptomatic infections, it is important that preventive measures outline above are given much more importance since they are relatively cheaper and inexpensive and poses less risk in comparison with treatment for symptomatic infections.

Cryptosporidium studies in irrigation water which can serve as a route for transmission into the food chain is limited. Methods used in this study will provide additional baseline information of *Cryptosporidium* contamination in surface water and their produce.

2.9 WATER QUALITY

Natural ground water sources (such as rivers, lakes, springs, waterfalls etc.) in its natural state are generally considered to be of good quality because rocks and their derivatives such as soils act as bio-filters and reduce the microbial load quality. However, not all soils are equally effective in this respect and therefore pathogens contained in human excreta such as parasites, bacteria and viruses are likely to be transmitted through the soil and aquifer matrix to water bodies (Lewis *et al.,* 1982). In Ghana, the major source of water to majority of the urban centres

is by the purification of naturally occurring water and transported as tap water (Mintah, 2011) Natural water sources can be contaminated and outbreaks of disease such as Cryptosporidiosis and Giardiasis have reported from countries at all levels of economic development (Lewis *et al.*, 1982). Some water sources naturally contain constituents of health concern such as arsenic and fluoride. However, understanding the impact of natural occurring ground water on public health is often difficult and the interpretation of health data is complex due to various factors. These include limited surveillance mechanisms and difficulty in identifying the primary cause of the outbreak. Throughout the world, there is evidence of contaminated groundwater leading to outbreaks of disease and contributing to background endemic disease in situations where groundwater sources used for drinking have become contaminated. However, diarrhoeal disease transmission is also commonly due to poor excreta disposal practices and the improvement of sanitation is a key intervention to reduce disease transmission (Curtis *et al*., 2000). Furthermore, water may be re-contaminated during withdrawal, transport and household storage. This may then require subsequent treatment and safe storage of water in the home (Sobsey, 2002).

Several chemical contaminants which are potentially toxic to human and other animals have been identified in natural water sources. Water quality suffers significantly due to several anthropogenic fluxes such as discharge of industrial wastes, excessive use of fertilizers, pesticides, spillage of oil and gases, mine wastes, and landfills (Howard *et al.,* 2006). On the other hand, natural geochemical reactions in the aquifers may release metals and other toxic chemicals in groundwater, and result in health risk when present at high levels. There are a number of naturally occurring contaminants in the ecosystem such as arsenic, lead, zinc, copper, cadmium, radium, radon, uranium, selenium, barium, thallium, iron, manganese, fluoride,

sulphate, chloride, boron, microbial contaminants, and many others which are potentially toxic for humans (Jacks and Bhattacharya, 2009)

2.9.1. pH of water

The pH is a measure of the activity of the hydrogen ion [H+]; also, it is the reciprocal of the logarithm of the hydrogen ion concentration (Silberberg, 2000). The pH scale ranges from 0 to 14. In general, water with a pH less than 7 is considered acidic, soft and corrosive. pH more than 7 is considered basic (Mintah, 2011).

The pH of pure water is 7 at 25 ^{o}C (Silberberg, 2000), but when exposed to the carbon dioxide in the atmosphere, equilibrium results in the pH of approximately 5.2. The WHO optimum limit of pH values is between 6.5 and 8.5. Because of the association of pH with atmospheric gases and temperature, it is strongly recommended that the water be tested as soon as possible.

Water with a low pH could contain elevated levels of toxic metals, cause premature damage to metal piping, and have associated aesthetic problems such as a metallic or sour taste, staining of laundry and the characteristic "blue-green" staining of sinks and drains. Water with a pH more than 8.5 could indicate that the water is hard. Hard water does not pose a health risk, but can cause aesthetic problems. These problems include formation of a "scale" or precipitate on piping and fixtures causing water pressures and interior diameter of piping to decrease, causes an alkali taste to the water and can make coffee taste bitter, formation of a scale or deposit on dishes, utensils, and laundry basins, difficulty in getting soaps and detergents to foam and formation of insoluble precipitates on clothing (Mintah, 2011).

The primary method to treat the problem of low pH water is with the use of a neutralizer. The neutralizer feeds a solution into the water to prevent the water from reacting with the house

plumbing or contributing to electrolytic corrosion; a typical neutralizing chemical is soda ash. Neutralizing with soda ash increases the sodium content of the water.

2.9.2 Turbidity

Turbidity is an optical property where suspended and dissolved materials such as silt, clay, finely divided organic and inorganic cause light to be scattered rather than penetrate in a straight lines. Turbidity is a measure the amount of light scattered by suspended particles and can be considered as the "cloudiness" of a water sample (Stanistski et al., 2000). Turbidity is contributed mainly by suspended sediment and /or plankton, which are solid particles of inorganic or biological origin. Human activities, including logging, grazing, agriculture, mining, road building, urbanization and commercial construction contribute to periodic pulse or chronic levels of suspended sediment in streams and other water bodies (Zoeteman, 1980). In drinking water the higher the turbidity level, the higher the risk that people may develop gastrointestinal diseases. This is especially problematic for immuno-compromised people, because contaminant like viruses or bacteria can become attached to the suspended solids (Stanistski et al., 2000).

2.9.3 Temperature

Temperature is the measure of the warmth and coldness of water with a thermometer in degree Celsius or Fahrenheit. The temperature of water to a large extent determines the extent of microbial activity. Optimal temperature levels for various organisms may promote the survivability of the organisms or render them unviable. For *Cryptosporidium* oocysts temperatures exceeding 65 °C generally inactivates the viability of the organism (Stanistski et al., 2000)

2.9.4 Total Dissolved Solids

Dissolved solids refer to any minerals, salts, metals, cations or anions dissolved in water. Total Dissolved Solids (TDS) comprises of inorganic salts (principally, calcium, Magnesium, Potassium, Sodium, Bicarbonate, Chlorides and Sulphates) and some small amount of organic matter that are dissolved in water (EPA, 2006) TDS originate from natural sources such as, sewages, urban run-offs industrial waste water and chemicals used in the water treatment process. Total Dissolved Solids concentration is the sum of the cations and the anions in the water. An elevated TDS concentration is not a health hazard. The TDS concentration is a secondary drinking standard and therefore is regulated because it is more of an aesthetic rather than health hazard (Nkansah et al., 2010). An elevated TDS also indicate the concentration of the dissolved ions may cause the water to be corrosive, salty or brackish taste, result in scale formation and interfere and decrease efficiency of hot water heaters. It also shows that it contain elevated levels of ions that are above the primary or secondary drinking water standard such as an elevated level of nitrate, arsenic, aluminium copper and lead

2.9 QUANTITATIVE MICROBIAL RISK ASSESSMENT

The use of contaminated water for irrigation raises concerns about health risks associated; however, these concerns cannot be justified through traditional hypothesis testing in science: The infection rates through irrigation water are so low that the sample sizes needed for adequate statistical power render such studies impracticable (Hamilton *et al*., 2006). Quantitative microbial risk assessment (QMRA) is an impact assessment that draws on the concept of chemical risk assessment to estimate the consequences from a planned or actual exposure to infectious microorganisms (Haas *et al.* 1999). It is a powerful tool for estimating order-of-magnitude risks associated with specific scenarios. QMRA has been extensively applied for developing and establishing standards, guidelines and other recommendations regarding

wastewater and excreta reuse in agriculture (WHO, 2006). QMRA has been applied to assess the occupational health risk associated with wastewater irrigation (Mara *et al*. 2007). The approach involves, hazard identification, exposure assessment, dose-response relationship and risk characterization.

Hazard identification involves the selection of microbial pathogens for which there is adequate information on their epidemiology and spectrum of diseases in the local context In the exposure assessment, the size and nature of the population exposed as well as the rout (single or multiple), frequency, duration and magnitude of pathogens associated with the routes are assessed. The dose-response assessment estimates the probability of infection following exposure to a dose of organisms. Infection has been defined as a situation in which the pathogen, after ingestion and surviving all host barriers, actively grows at its target site (Haas *et al.* 1999). The likelihood of infection is dependent on two main factors: the probability that the pathogen is ingested and the probability that the ingested organism will survive in the host to initiate an infection.

Risk characterization integrates all these information to obtain a level of risk for different exposure scenarios. Here the infection risk can be expressed as daily or annual depending on the exposure scenario being modeled (Haas *et al.* 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

This chapter deals with all materials and methods used for the study. This includes the study design, sampling methods, laboratory techniques used for various determinations and statistical tools used for analysis of the results.

3.1 STUDY DESIGN

The study was a cross sectional study on irrigational water sources of vegetable farms located in Kumasi. The farms were selected based on the common sources of irrigation water used in Kumasi. Four rounds of sampling were done for both irrigation water and lettuce with the measurement of temperature and pH done each time for water samples. All sampling was done over a period of two months in the dry season.

3.2 STUDY AREA

The study area is the Kumasi Metropolis of the Ashanti region. The Metropolitan area is located on latitude (6^o 35'-6^o40N) 1'30W and on longitude (1^o30'-1^o35') 6W, 40N, with an approximate area of 254 km^2. The area shares boundaries with Kwabre and Sekyere Districts on the North, Atwima Nwabiagya district to the west, Ejisu-Juaben district to the east and Atwima Kwanwoma Bosomtwi district to the south (KMA, 2014).

3.2.1 Study sites

Four study sites with various sources of irrigation water were selected. The sample sites were chosen based on their geographical location and type of vegetable produced. The routines from seeding to harvest were similar for all farms selected that are the lettuce seeds were sowed on one bed and later transferred to harvest beds, chicken manure was applied as fertilizer once per crop life cycle just after the seedlings were transferred to harvest beds. Irrigation was generally done in the morning and the evening, given that no rainfall occurred the previous night. In case of rainfall, the farmers found it unnecessary to irrigate the following day. Insecticides were sprayed on the lettuce once a week, regardless of weather conditions.

Study site one (1) [6 40 625N 1 36 9W] is a farm (F-1) situated at Ahodwo near the Georgia hotel. The farm produces lettuce only. Irrigation water was from a stream where wastewater from the Komfo Anokye Teaching Hospital (KATH) joins upstream without any formal treatment. Irrigation was performed using a pump as water source is a little far from beds. Water from the stream was also stored in a barrel which was sometimes used for irrigation. Site two (2) [6 40 12N, 1 34 3W] is a farm situated at Chirapatre Estate. Irrigation water was mostly from a stream close to the farm where effluent from a waste treatment plant joins upstream hand dug wells were also used. Samples were taken from both the hand dug well (F-2A) and stream joined upstream by waste treatment plant effluent (F2-B). The areas supplying the dugout with water were mainly groundwater, run off from private housing and from nearby green areas. Study site three (3) [6 40 24N, 1 32 1W] is situated at Twumduase behind a school facility. Source of irrigation water was mainly hand dug wells. A stream was also used but only for beds near it as the hand dug wells provided enough irrigation for most of the beds. Two main hand dug wells were used and both were sampled from (F-3A and F-3B). The last site, [640 12N 1 32 45W] is

located at Boadi. Irrigation water source was a stream (F4) which was joined by various streams from surrounding communities such as Oduom and Anwomasi. No hand dug wells were used.

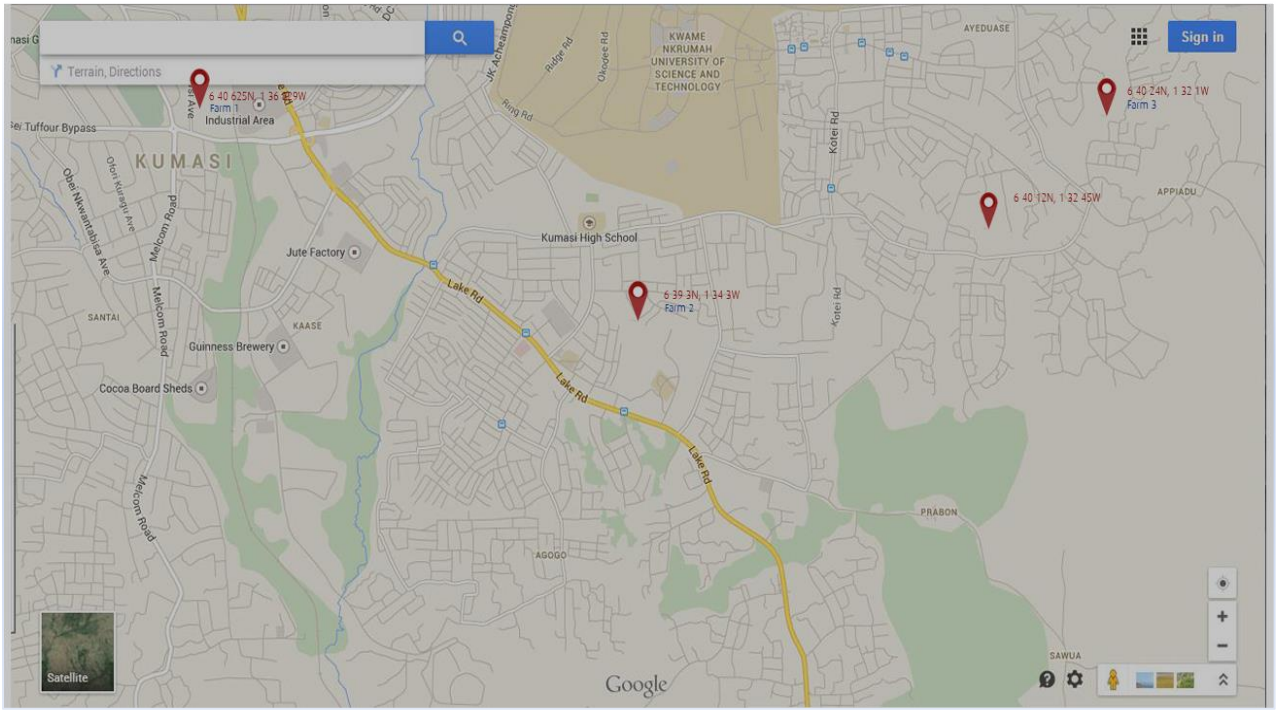

Figure 1: The geographical locations of the sample sites
(Courtesy: Google maps)

3.3 IRRIGATED WATER SAMPLING

Water samples were taken every week from the four sampling sites over a period of four weeks. Twenty (20) litre water samples was collected using two 10 L clean transparent graduated plastic containers for each sample site for *Cryptosporidium* analysis as recommended by Duhain (2011). Samples were taken from the water source 20 to 30 cm beneath the water surface and transported to the Biochemistry Department of Kwame Nkrumah University of Science and Technology (KNUST) under optimal conditions to perform immuno-magnetic separation (IMS) in order to

obtain purified oocysts. Purified oocysts were transported to the Parasitology Department of the Noguchi Memorial Institute for Medical Research (NMIMR) in thermos flask containing ice packs for further analysis.

3.3.1 Determination of physical parameters of water

The temperature of the water samples were measured on site during sampling using a field thermometer. The pH and turbidity of each of the water samples were taken in the laboratory using the pH meter (Mettler Toledo) and turbidimeter (Hanna instrument LP 2000), respectively.

3.4 LETTUCE SAMPLING

Four randomly chosen lettuce plants were collected from each farm per sampling for four weeks. Lettuce closest to harvest (3-4 weeks old) were taken. Outer leaves were discarded to reduce other contamination by soil. Sterile containers were used for collection and taken to the laboratory for analysis and storage. Thirty (30) grams of lettuce from each farm was pulsified in 200 ml of 0.01% Tween-20 solution. Filtration and IMS were carried out and the purified oocysts were then sent to the Parasitology Department of the Noguchi Memorial Institute for Medical Research for further analysis.

3.5 CONCENTRATION OF *CRYPTOSPORIDIUM* FROM SAMPLES

3.5.1 Concentration of *Cryptosporidium* spp. from water samples

Cryptosporidium was isolated from water samples using sedimentation, immuno-magnetic separation and immunofluorescence assay. The 10L containers were left on the table for 48 hours at room temperature, promoting sedimentation as *Cryptosporidium* spp. oocysts settle (Medema *et al*., 1997). The supernatants were then removed by pump-suction system leaving approximately 0.75 L water in the containers. The remaining solutions were transferred to 3 L plastic containers, followed by a 3 x 150 ml distilled water cleaning cycle (manual vortexing) of the 10 L containers (Boel-Peterson, 2013). The 3 L containers were placed at a 30° angle and left on the table to sediment for another 48 hours. Consequently, the supernatants were removed, leaving approximately 90 ml in the containers. The remaining solutions were transferred to 50 ml tubes, followed by a 3 x 20 ml tap water clean-up cycle of the 3 L containers. The 50 ml tubes were centrifuged at 1583 g for 10 min and the supernatant removed leaving approximately 5 ml. Pellets were collected in one of the 50 ml tubes followed by a 3 x 5 ml clean-up cycle with 0.01% Tween-20 in distilled water. The 50 ml tube containing the pellets and clean-up solution were centrifuged at 1585 g for 10 min and the supernatant removed leaving approximately 5-10 ml of filtrate.

3.5.2 Concentration of *Cryptosporidium* spp. oocysts on lettuce

Thirty (30) gram portion of inner lettuce leaves were pooled from samples per site. The pooled 30 g of lettuce was placed in a stomacher bag, covered by 200 ml of 0.01% Tween-20 water and pulsified for 1 min (Microgen bioproducts PUL 100 E). This was done in duplicates. The extract

was then divided among 15 ml tubes and centrifuged at 1600 g for 10 min. The supernatant was then removed to approximately 2 ml and all pellets were collected in four 15 ml tubes, filtering through a single layer of gauze to hold back lettuce pieces. The remaining 15 ml tubes were subjected to a 3 x 2 ml clean up cycle with 0.01% Tween-20 water and transferred to the 15 ml tubes containing the pellets, while rinsing the gauze. The 15 ml tubes containing pellet and clean-up solution (water used for cleaning 15 ml tubes), were centrifuged at 1600 g for 10 min and the supernatant was removed to approximately 1 ml. The pellets were transferred to a 15ml tube and the remaining 15 ml tubes were cleaned with 0.5 ml tap water and transferred to the tube containing the pellets according to Robertson and Gjerde (2000) with slight modifications.

3.5.3 Immuno-magnetic separation (IMS) protocol

Pellets from the water and lettuce samples were transferred to Dynal L10 tubes and 1 ml of 10× SL buffer A, 1 ml of 10× SL buffer B and 100 µl of anti-*Cryptosporidium* bead conjugates (Life Technologies) were added. Samples were then rotated for 1 hour at room temperature. The magnetic beads (with attached *Cryptosporidium* spp. oocysts) were recovered from the Dynal L10 tube using a magnetic particle concentrator. The recovered bead-bound parasites were then re-suspended in 1 ml of 1× SL buffer A and transferred into a 1.5 ml eppendorf tube with further separation using a magnetic particle concentrator. The oocyst complexes were dissociated from beads using 50 µl of 0.1 N HCl and the magnetic particle concentrator (Dynal beads). Dissociated oocysts were transferred to a new 1.5 ml eppendorf tube and the pH neutralized with 5 µl of 1 N NaOH. The magnetic beads were subsequently washed with 45µl 0.1% Tween-20 (Merc milipore®) according to the manufacturers' protocol.

3.6 CLASSICAL IDENTIFICATION OF *CRYPTOSPORIDIUM* SPP FROM SAMPLES USING MICROSCOPY

3.6.1 Modified Ziehl Nelson

After IMS, samples (100 µl) were smeared on glass slides and allowed to air dry followed by fixation in methanol for 3 min. The slides were then stained in carbol fuchsion for 15-20 minutes and later rinsed with tap water. The slides were decolourised in acid alcohol (1% HCl in methanol) for about 15-20 seconds followed by thorough rinsing with tap water. Counterstaining was done with malachite green for 30 seconds followed by rinsing and air drying. Slides were examined under X40 magnification using light microscope (Nikon Eclipse 80i). Each sample was done in triplicates.

3.6.2 Immuno-fluorescent assay

After immuno-magnetic separation (IMS), samples (100 µl) were transferred to 3-well slides (3 samples per slide) and left to dry, followed by fixation with acetone (20 µl). Anti-*Cryptosporidium* spp. fluorescein isothiocyanate (FITS)-labelled antibody mix (Crypto-Cell IF test, CellLabs, Australia) was added to each well (25 µl), the slide was placed in a self-constructed humidity chamber and incubated for 45 min. at 37 °C. FITS was removed from slides and wells by washing twice with a 100 µl of MiliQ water per wash. A 12 µl of mounting fluid (Crypto-Cell IF test, CellLabs, Australia) plus a cover was added to the slide and the oocyst counted by epifluorescence (Olympus BX 53) at ×200 magnification microscopy (495 nm excitation and 519 nm emission wavelength).

3.7 MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *CRYPTOSPORIDIUM*

3.7.1 Genomic DNA extraction

Genomic DNA was extracted from processed water and lettuce samples using the Qiagen kit. Briefly, 200 µl of Buffer AL (lysis buffer) was added to 100 µl of sample and 20 µl of proteinase K and vortexed briefly. The resulting solution was incubated at 70 °C for 30 min in a water bath. Absolute alcohol (250 µl) was added and vortexed after the incubation period and the resultant mixture was transferred to DNeasy spin column and spun at 8000 g for 1 min. The eluted flow-through was discarded. The spin column was then washed with 500 µl of AW1 (wash buffer 1) and spun at 8000 g for a minute and flow-through discarded followed by washing with 500 µl of AW2 (wash buffer 2) and spun at 8000 g for a minute and flow-through discarded. After washing, the spin column was made dry by spinning at maximum speed (14000 g) for 5 min to ensure all droplets of wash buffer were discarded completely. The spin column was placed in a labelled sterile 1.5 ml eppendorf tube and 150 µl of AE (elution buffer) added. This was incubated at room temperature for 2 min and spun at 8000 g for 1 min to elute DNA. The eluted genomic DNA was stored at -40 °C until use.

3.7.2 Polymerase Chain Reaction (PCR)

For molecular detection of *Cryptosporidium* (species/genotypes), polymerase chain reaction (PCR) amplification of the HSP70 gene (325 bp) was done according to Langkjar *et al.* (2007) with slight modifications. The primer sequence for nest two of the PCR was nested HSP3m instead of HSP3. The PCR mix contained 12.5µl of Gotaq and 12.5 pmol of each primer (primer

sequences are shown in Table 3) in a total reaction volume of 25 µl. The PCR mix included 5 ml of purified DNA as template for primary steps and 1 µl of primary PCR product for secondary steps.

The PCR was carried out in a Takara thermocycler with an initial hot start (95 °C for 3 min) and a final extension (72 °C for 10 min). For the nested PCR of the HSP70 gene, 30 cycles (94 °C for 30 sec, 58 °C for 20 sec, 72 °C for 30 sec) cycling conditions were used using the HSP4 primers for the primary step and 45 cycles (94 °C for 25 sec, 58 °C for 18 sec, 72 °C for 25 sec) using HSP3m primers in the secondary step. Positive and negative controls were included in every reaction. Both control mix contained Gotaq (PROMEGA) and primer sequences but the positive control (oocysts purified from the feaces of calves from a previous study) contained positive *Cryptosporidium* DNA whereas the negative contained distilled water.

Table 3: Primer sets used and the gene sequence

Primer	Sequence (5'- 3')
HSPF4	GGT GGT GGT ACT TTT GAT GTA TC
HSPR4	GCC TGA ACC TTT GGA ATA CG
HSPF3m	GCT GGT GAY ACT CAC TTG GGW GG
HSPR3m	CTC TTR TCC ATA CCA GCA TCC

3.7.3 Gel electrophoresis

A 2% (w/v) agarose gel was used for electrophoresis. Each gel was cast by dissolving 2 g of agarose in 100 ml of tris-acetate-EDTA (TAE). The resulting solution was microwaved for 2 minutes to ensure efficient dissolution. The agarose was allowed to cool down and 1µl of ethidium bromide added. The gel was then poured into casting tray and allowed to solidify. The

gel was transferred into the electrophoresis chamber containing 1× TAE buffer (enough to cover gel). The first well of each gel was filled with 3 µl of 100bp hyper-ladder (Bioline) and the remaining wells filled with 7 µl of PCR product. A positive and negative control was included in each run. The gel was run at constant 100 V for approximately 40 minutes.

3.8 QUANTITATIVE MICROBIAL RISK ASSESSMENT

3.8.1 Exposure assessment using *E. coli* conversion

Exposure assessment was built with the use of stochastic (random) technique for the input parameters shown in **Table 4**. The pathogen dose (number/ml) ingested at each exposure for a farmer accidentally ingesting irrigation water assumed to be contaminated was modified from earlier work by Mara *et al.* (2007) represented by

$$dose = \frac{10^M}{100\ X\ 10^5} V \quad \text{----------(1)}$$

$$i.e\ dose = 10^{M-7} V$$

Where 10^M is the number of *E. coli* per 100 ml of irrigation water, ($\frac{10^M}{100}$) is the number per ml and $\frac{10^M}{100\ X\ 10^5}$ is the number per units of 10^5 *E. coli* per ml), $\boldsymbol{M}$ is the number of oocyst per 10^5 *E. coli*. The range of parameter values for conversion of oocyst was 0.01-0.1 *Cryptosporidium* oocyst per 10^5 *E. coli* as used in a study by Mara *et al.* (2007). This approach was chosen in line with the policy guidelines of the use of wastewater (WHO, 2006).$\boldsymbol{V}$ is the volume of irrigation water accidentally ingested by farmers during irrigation. The accidental ingestion of water and aerosol by farmers during irrigation was assumed to be 1-5 ml to account for the use of improvised equipment for irrigation practices, whiles the total exposure of farmers were also

estimated to be a little over 2 months for planting to harvesting of vegetable produces, thus 60-70 days (Seidu *et al*., 2008). All input parameters are indicated in Table 4.

3.8.2 Exposure assessment using *Cryptosporidium*

Exposure assessment was built with the use of stochastic (random) technique for the input parameters shown on **Table 4**. The pathogen dose (number/ml) ingested at each exposure for a farmer accidentally ingesting irrigation water assumed to be contaminated was modified from earlier work by Mara *et al.* (2007) and represented by the formular

$$dose = C\ X\ V\ X\ I \quad \text{----------(2)}$$

Where, $C = C_{raw} + [C_{raw}$ X R] and C_{raw} is the (oocyst) raw detectable in irrigation water, recovery efficiency R of the detection method was determined based on Petterson *et al.* (2007).

C is the concentration of ((oo)cyst/ml).

I is the percentage of infectious (oo)cyst.

V is the volume of irrigation water accidentally ingested by farmers during irrigation. This was assumed to be 1-5 ml to account for the use of improvised equipment for irrigation practices, whiles the total exposure of farmers were also estimated to be a little over 2 months for planting to harvesting of vegetable produces, thus 60-70 days (Seidu *et al.*, 2008). Moreover, as done in previous studies (Hamilton *et al.*, 2006; Ryu and Abbaszadegan, 2008; Mota *et al*., 2009), all oocyst detected were assumed to be equally transferrable during accidental ingestion of water. Two scenarios were assumed for the infectivity of the detectable oocyst. Firstly, where all oocyst were assumed to be infectious (worst scenario) and secondly, where infectivity I was taken to be 0.41 (Ryu and Abbaszadegan, 2008). All input parameters are indicated in **Table 4**.

Table 4: Input parameters for dose response model and risk characterisation

Parameter	Distribution type	Notation	Units	Reference
Volume of irrigation water accidentally ingested/aerosol inhalation	Uniforml(1,5)	V	ml	(Seidu *et al.,* 2008) (WHO 2006)
Total Exposure	60-70	j	$d(year)^{-1}$	(Seidu *et al.,* 2008)
Cryptosporidium constant (r)	0.00419	r		(Haas, *et* al., 2014; Ryu and Abbaszadegan, 2008)
*Recovery	Calculated	R	-	(Petterson *et al.*, 2013)
Infectivity	[1, 0.41]	I		(Ryu and Abbaszadegan, 2008; Mota *et al.*, 2009)
*No of *Cryptosporidum* oocyst/10L of irrigation water	Range < 52 -105			

*Recovery Infectivity and number of *Cryptosporidium* oocyst /10L of irrigation water were not input into model for risk using *E. coli* conversion

3.8.3 Dose response assessment

The dose response model used for *Cryptosporidium* infection was the exponential model given by

$$P_{inf} = 1 - \exp(-r \times dose) \quad \text{----------(3)}$$

Where r is the dose parameter for *Cryptosporidium* (Haas, *et al*., 2014) (WHO 2006).

3.8.4 Risk characterization

The annual probability of infection was estimated as

$$P = 1 - \prod_{j=1}^{d} \left(1 - P_{\text{inf},j}\right) \quad \text{----------(4)}$$

Where P_j is the jth probability of infection caused by *Cryptosporidium* and *d* is the number of events exposure per year (Karavarsamis and Hamilton, 2010) and *P*, the annual risk of infection. All the models were constructed in Microsoft Excel and calculated with Monte Carlo simulation at 10,000 iterations using the @ Risk 6.3 (Palisade Corporation) software add-on to Excel.

3.9 STATISTICAL ANALYSIS

Data obtained was collated and entered into Microsoft Excel and imported into Statistical Package for Social Sciences (SPSS) software version 20 for statistical analysis. Descriptive and inferential statistics such as percentage calculation of prevalence, correlation of oocyst concentrations with physical parameters and Analysis of variance (ANOVA) to determine significance difference among the concentration of oocyst in irrigation water used on various farms were employed. A recovery rate of 1.9% (Boel-Peterson, 2013) from a previously conducted study using a negative binomial distribution model to estimate the number of oocyst in each sample of water collected from each farm.

The negative binomial model which is a derivative of a Poisson distribution model and is given as $Xc = 1 - \frac{1}{(1+\propto CWe^{n})}$ (Crainiceanu *et al.*, 2003).

Where C= then defined as the probability of detecting one or more oocysts per assay given that oocysts were present. This is equivalent to saying that sensitivity is equal to 1 minus the probability that no oocysts were detected

W = Mass or volume of sample examined

α = is an ancillary parameter for modeling dispersion.

e^{n} = the percent recovery of the diagnostic assay as a function of the covariates,

With values obtained from the previously conducted study, the recovery rate percentage was then calculated as $r = {}^{k}/_{X}$

Where *x* = the observed number of oocysts counted for each sample and

k = the total number of oocysts added to the sample.

CHAPTER FOUR

4.0 RESULTS

This chapter presents the outcome of executing the methodology previously outlined (Chapter 3) in order to aid the achievement of the aim and objectives of the study. Four samples were taken from the four farms with two of the selected farms having two points of sampling each. Four pooled samples of lettuce were taken from all the sampling points. All samples were analysed for *Cryptosporidium* oocyst concentration and identification of the various types/subtypes of the *Cryptosporidium* spp. present.

4.1 PHYSICAL PARAMETERS OF THE IRRIGATION WATER

Physical parameters such as temperature, turbidity and pH were assessed on all irrigation water used on the Farms and the data have been presented in **Table 4**.

Temperature of irrigation water measured for all the Farms ranged from 21^{o}C - 28^{o}C. The highest temperature was recorded for Farm-2 on all occasions with a mean temperature of 26.7^{o}C ± 0.96. There was a significant difference (p value ≤ 0.001) between temperatures of the irrigation water from the various farms. For multiple comparisons, temperatures of irrigation water from Farm-1 was significantly different [p values ≤0.05 (CI=95%)] from Farms 2 and 3.

Turbidity measured in the units of FTU (Formazin Turbidity Unit) of all irrigation water ranged from 7.1 FTU to 230 FTU. Irrigation water from Farm-4 generally had a higher turbidity on all occasions compared to the other Farms. Turbidity of water from the various Farms were also significantly different with p value of ≤ 0.05 (CI=95%). There was a significant difference between the turbidity of irrigation water from Farm-4 compared with the irrigation water from

other Farms with p values of =0.01 (F-1), 0.031 (F-2), 0.002 (F-3). The pH of irrigation water from the Farms ranged between 5.65 and 6.96 on the pH scale indicating mild acidity (Table 5).

Table 4: Measured physical parameters of irrigation water

Farm	Temperature (^{o}C)		Turbidity (FTU)		pH	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
F1	21	22	36.2	46.1	6.28	6.46
F2-A	26	28	53.1	63.7	5.59	5.94
F2-B	25	27	52.8	57.0	6.03	6.96
F3-A	23	24	7.1	13.3	6.30	6.47
F3-B	24	24	12.3	14.1	6.30	6.59
F4	22	23	75.8	230	6.28	6.58

Results obtained by analysis of variance between the pH and turbidity measured for the water samples on the various farms and the oocysts concentrations showed no significant associations **(Table 6)**. However, there was an inverse relationship between temperature and oocysts concentrations within and between the different farms.

Table 5 : Relationship between physical parameters of water and the oocyst concentrations

Variable	Estimated oocyst concentrations	Sum of Squares	F Value	P value	Pearson's R value	P value
Temperature	Between Farms 1-5	10.330	1.148	0.551	-0.949	0.02
	Within Different Sampling days on each farm	4.500				
pH	Between Farms 1-5	0.028	0.688	0.649	0.5	0.26
	Within Different Sampling days on each farm	0.020				
Turbidity	Between Farms 1-5	8156.648	4.028	0.332	0.21	0.35
	Within Different Sampling days on each farm	1012.500				

Note: Sampling point F-2B where no oocyst was detected for all sampling rounds was excluded*

4.2 *CRYPTOSPORIDIUM* OOCYST CONCENTRATION IN WATER

A total of 24 water samples were collected from irrigation water from the study farms and examined for the presence of *Cryptosporidium* spp. oocysts. Based on microscopic examination, nine (37.5%) water samples had detectable numbers of oocysts; with all the farms having detectable number of oocyst at one point in the sampling times with the exception of irrigation water from the stream on Farm-2 (F-2B). Farm-1 had detectable oocyst count in all rounds of sampling. The oocyst recovered ranged from an average of 52 - 105 oocysts per 10L. Recovery efficiency and precision of the method used to purify oocysts from water samples used was 1.9%

(Boel-Peterson, 2013). Table 6 shows a multiple comparison showed that Farm-1 was significantly different from Farm-4 (p=0.36)

Table 6: Estimated oocyst concentration in irrigation water on the different farms

Irrigation source	Week 1 Oocyst/10L	Week 2 Oocyst/10L	Week 3 Oocyst/10L	Week 4 Oocyst/10L
F1	52	52	52	105
F2-A	< 52	< 52	< 52	52
F2-B	< 52	< 52	< 52	< 52
F3-A	< 52	< 52	52	< 52
F3-B	105	< 52	105	< 52
F4	< 52	52	< 52	< 52

Recovery efficiency = 1.9%

4.3 POLYMERASE CHAIN REACTION (PCR) FOR THE IDENTIFICATION OF *CRYPTOSPORIDIUM* IN IRRIGATION WATER

A nested PCR was performed to amplify the HSP70 gene band on the *Cyptosporidium* spp. gene sequence from genomic DNA extracted from the water samples. Ten (10) out of the 24 representing 41.67% were positive with a band size of approximately 325bp corresponding to the expected band size (Figure 2).

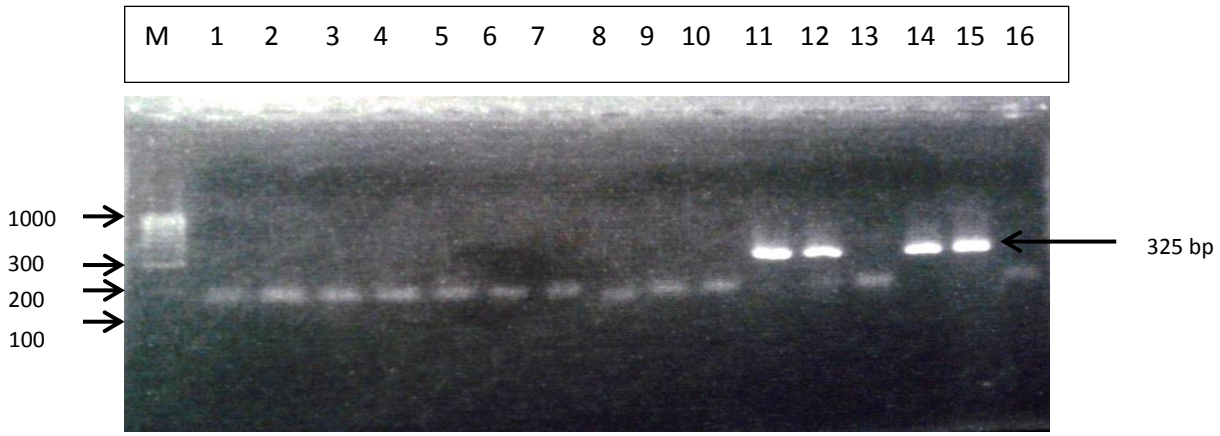

Figure 2: An agarose gel representative of *Cryptosporidium* based on the HSP70 gene (HSP70- 325bp) for some processed water samples

Lane M= 100 base pair ladder (Bioline) Lane 11, 12 and 14 represent samples that showed positive for *Cryptosporidium* ; F3B (week 3), F3B (week 4), F4 (week 1) and F4 (week 2) respectively
Lane 15 and 16 represents a positive and negative control respectively

4.3.1 Comparison of PCR results and microscopy

A comparative assessment was done on the two diagnostic methods used for the identification of *Cryptosporidium* spp. Results obtained indicate that, microscopy identified 37.50% of samples as positive compared to the 41.67% by PCR. A total of 13 samples tested positive to at least one of the diagnostic methods used representing a total prevalence of 54.2 percent.

Table 7: Comparison of PCR and microscopy detection of *Cryptosporidium*

Farm	Water Source	Week	Microscopy	PCR
F1	Stream joined upstream by waste form a teaching hospital	1	+	+
		2	+	-
		3	+	-
		4	+	+
F2A	Hand dug well	1	-	-
		2	-	+
		3	-	-
		4	+	+
F2B	Stream joined upstream by effluent of waste stabilization pond	1	-	-
		2	-	+
		3	-	-
		4	+	-
F3A	Hand dug well	1	-	-
		2	-	-
		3	-	-
		4	+	-
F3B	Hand dug well	1	+	+
		2	-	+
		3	+	+
		4	-	+
F4	Stream	1	-	-
		2	+	+
		3	-	-
		4	-	-

Table 8: Comparison of sensitivities of Microscopy and PCR

Method	Number of samples examined	Number of positives detected	Sensitivity (%)[a]	Specificity (%)[b]
PCR	24	10	76.92	73.33
Microscopy	24	9	69.23	78.57

[a] Calculated as follows:

[number of true positives/(number of true positives + number of false negatives)] × 100

[b] Calculated as follows:

[number of true negatives/(number of true negatives + number of false positives)] × 100

4.4 POLYMERASE CHAIN REACTION (PCR) FOR DETECTION OF *CRYPTOSPORIDIUM* IN LETTUCE SAMPLES

A nested PCR was performed to amplify the HSP70 gene band on the *Cyptosporidium* spp gene sequence from genomic DNA extracted from the lettuce samples. Twelve (12) out of the 24 representing 50.00% showed positive for the presence of *Cryptosporidium* as indicated in **Table 9** and the presence of *Cryptosporidium* spp. in an irrigation water source did not always correspond with a detection of *Cryptosporidium* spp. on lettuce irrigated with the water. The **Figure 3** also shows a representative of some samples after PCR amplication with a band size of approximately 325bp corresponding to the expected band size.

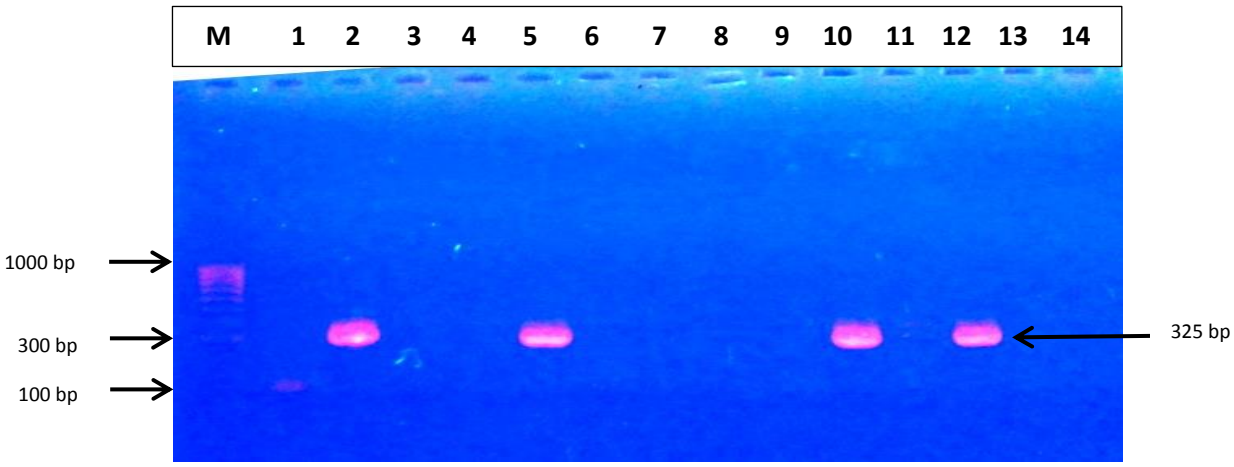

Figure 3: An agarose gel representative of *Cryptosporidium* based on the HSP70 gene for processed lettuce samples

Lane M= 100 base pair ladder (Bioline)
Lane 2, 5, and 10 represent some samples that were positive for *Cryptosporidium;* F2B (Week 4), F3A (Week 3) F4 (Week 2) respectively
Lane 12 and 13 represent positive and negative control respectively

Table 9: Comparison of PCR detection of *Cryptosporidium* spp. in irrigation water and lettuce being irrigated

Farm	Water Source	Week	PCR	
			Water	Lettuce
F1	Stream joined upstream by waste form a teaching hospital	1	+	+
		2	-	-
		3	-	-
		4	+	-
F2A	Hand dug well	1	-	-
		2	+	-
		3	-	-
		4	+	+
F2B	Stream joined upstream by effluent of waste stabilization pond	1	-	+
		2	+	-
		3	-	-
		4	-	+
F3A	Hand dug well	1	-	-
		2	-	-
		3	-	+
		4	+	+
F3B	Hand dug well	1	+	+
		2	+	+
		3	+	+
		4	+	-
F4	Stream	1	-	-
		2	+	+
		3	-	+

		4	-	+

(+) is for positive PCR detection (-) is for no PCR detection

4.5 IMMUNO FLUORESCENT ASSAY OF WATER AND LETTUCE SAMPLES

Attempts of verifying the presence of *Cryptosporidium* spp in water and lettuce samples using immuno-fluorescent microscopy were unsuccessful. There was a lot of cross-reactivity and possible oocysts could not be easily differentiated from the background of the slide being read.

4.6 DOSE AND DAILY RISK OF INFECTION

The dose and probability of infection for the cryptosporidium infection for both with and without infectivity constant is as shown in **Table 10**. In all cases, the lower concentration mean (52 oocyst/10L) was lower than for the distribution fitted dose (crypto oocyst data distribution), whereas the upper mean concentration served the upper limit of the both dose ingestion and probability of infection (105 oocyst/10L). The dose ingestion in all scenarios falls within the limit of 1.50×10^{-2} to 3.29×10^{-2} for those without infectivity constant(worst case scenario), 6.15×10^{-3} to 1.35×10^{-2} for infectivity constant, whereas for the probability of infection also falls within 6.28 ×10-5 for worst case scenario and 2.58×10^{-5} to 5.67×10^{-5} for those with infectivity constant.

Table 10: The dose and daily risk of *Cryptosporidium* spp. infection of farmers associated with the accidental ingestion of contaminated irrigation water

	Scenario	Range		*Cryptosporidium* spp. Oocyst data Distribution
		52 oocyst/10L	105 oocyst/10L	
Dose	Without Infectivity Constant (Worst Case)	$1.50X10^{-2}$	$3.29X10^{-2}$	$2.29X10^{-2}$
	Infectivity Constant	$6.15X10^{-3}$	$1.35X10^{-2}$	$9.38X10^{-3}$
Daily risk of infection	Without Infectivity Constant (Worst Case)	$6.28X10^{-5}$	$1.38X10^{-4}$	$9.58X10^{-5}$
	Infectivity Constant	$2.58X10^{-5}$	$5.67X10^{-5}$	$3.93X10^{-5}$

4.6 ANNUAL RISK OF INFECTION `

The median risk of infection of worst case scenario (without inclusion of *Cryptosporidium* oocyst infectivity constant) were $4.08 \text{ X } 10^{-3}$, $8.95 \text{ X } 10^{-3}$ and $6.15 \text{ X } 10^{-3}$ for lower limit, upper limit and oocyst distribution data, respectively. Moreover, the annual probability of infection for farmers exposure model was found to have a medial daily infection of $1.67 \text{ X } 10^{-3}$, $3.68 \text{ X } 10^{-3}$ and $2.53 \text{ X } 10^{-3}$ for lower limit, upper limit mean concentrations and *Cryptosporidium* distribution, respectively as indicated in **Table 11**. The median estimate of annual probaility of infection for the exposures were in all cases found to be of ≤ 1 order of magnitude higher than the WHO acceptable threshold as indicated (Fig 4). All the estimated risk were within $1.0 \text{ X } 10^{-3}$ to $1.0 \text{ X } 10^{-2}$ and far above the threshold of $1.0 \text{ X } 10^{-4}$.

Table 11: Yearly risk of *Cryptosporidium* infection of farmers associated with accidental ingestion of contaminated irrigation water

Yearly risk of infection given under *Cryptosporidium* oocyst in irrigation water in Kumasi Ghana			
Scenario	Range		*Cryptosporidium.* oocyst data distribution
	52 oocyst/10L	105 oocyst/10L	
Without Infectivity Constant (Worst Case)	$4.08\ X\ 10^{-3}$	$8.95X\ 10^{-3}$	$6.15\ X\ 10^{-3}$
Infectivity Constant	$1.67\ X\ 10^{-3}$	$3.68\ X\ 10^{-3}$	$2.53\ X\ 10^{-3}$

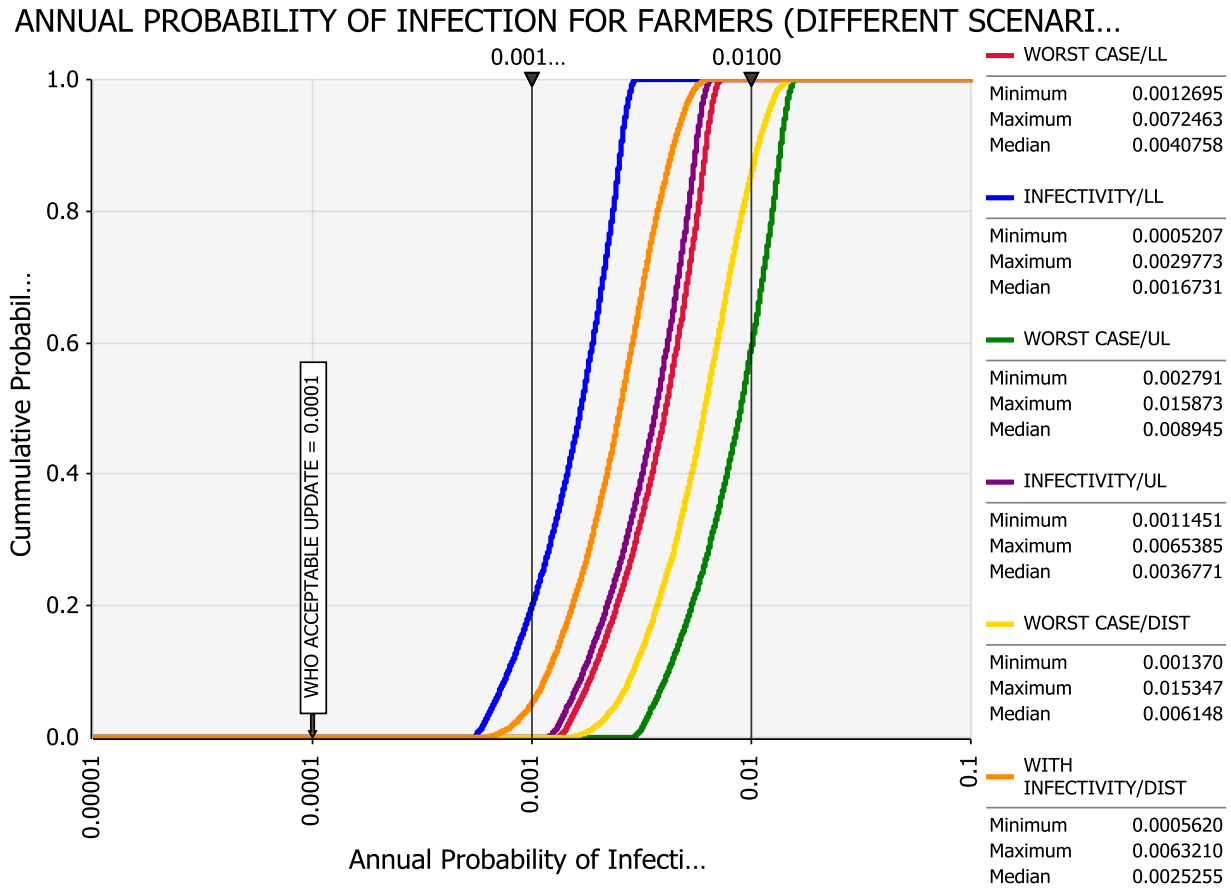

Figure 4: Annual Probability of Infection for farmers of *Cryptosporidium* spp

The annual probability of infection for farmers' exposure model using the *E. coli* conversion was found to have a medial daily infection of $4.32\ X\ 10^{-6}$ with the maximum and minimum estimated risk of infection as $4.32\ X\ 10^{-5}$ and $7.73\ X\ 10^{-10}$ respectively. The mean annual risk of infection for exposure was estimated as $6.65\ X\ 10^{-6}$ which was close to the median risk of infection (Figure 5).

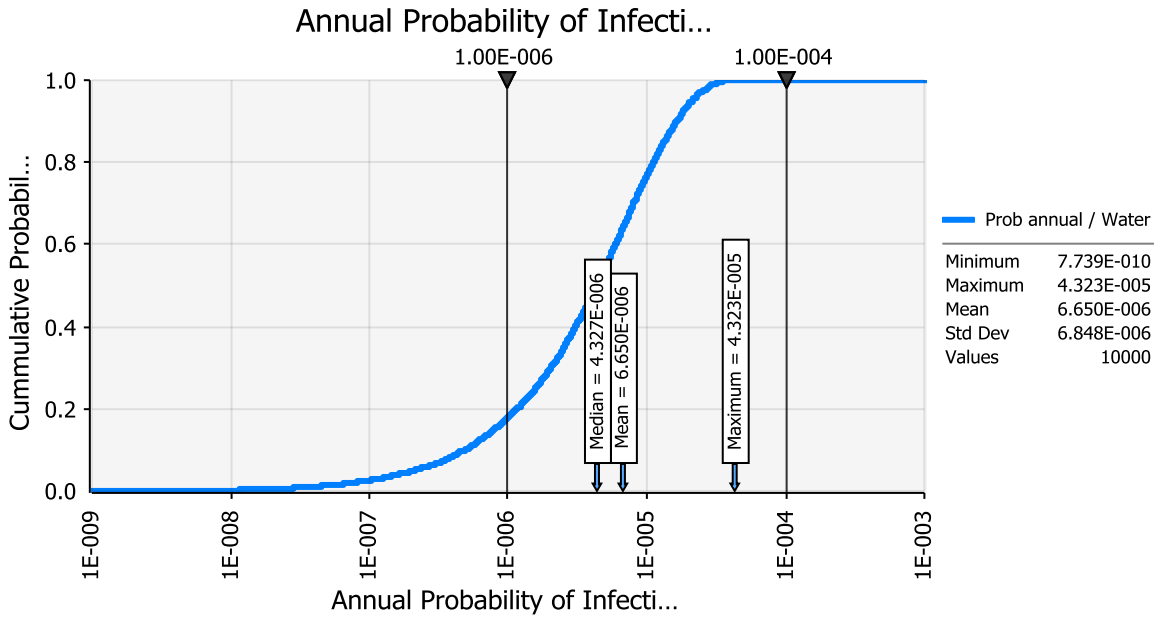

Figure 5: Cumulative probability distribution of estimated annual risk of infection for farmers' exposure to *Cryptosporidium* spp. in irrigation water using the *E. coli* conversion approach

4.6.1 Sensitivity analysis

The sensitivity analysis was used to identify the model parameters with significant impact on the risk output. It was observed that the annual probability of infection for both models was very sensitive to *Cryptosporidium* spp. (Crypto) concentration in irrigation water. The daily accidental ingestion of contaminated irrigation water and the total exposure to contaminated irrigation water for each irrigation period did not have significant impact on the risk output whereas the *E. coli*

conversion used for distribution of *Cryptosporidium* had a significant impact on risk analysis as indicated in **Figure 6**.

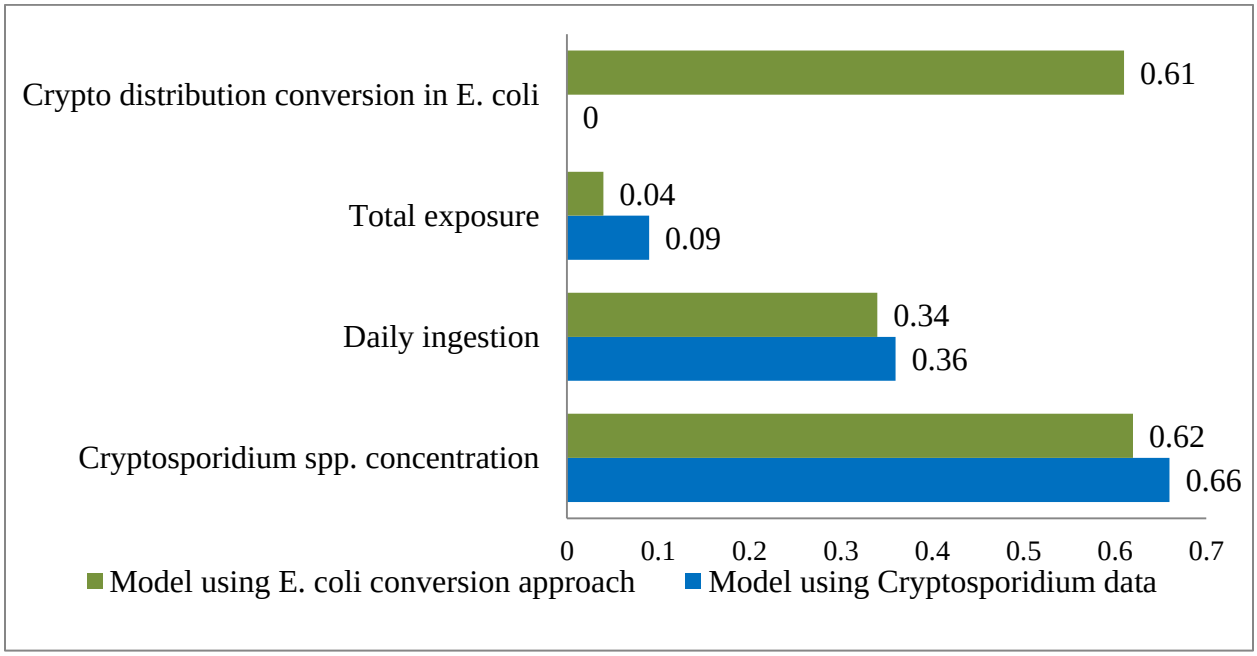

Figure 6: Spearman correlation coefficient for sensitivity analysis of models' input parameters

CHAPTER FIVE

5.0 DISCUSSION

Recent studies have reported Cryptosporidiosis as an ubiquitous health problem and identified the premium route for infection to be anthroponotic (Ranjbar-Bahadori *et al*., 2011). Anthroponotic diseases are infectious diseases in which the disease causing agent carried by humans is transferable to other animals. The prominent routes for transmission for the disease are waterborne and person to person. In recent advanced studies, the consumption of fruits and vegetables have also been cited as possible infection routes thus implicating water for irrigation water as a possible source of contamination to these fruits and vegetables (Koompapong and Sukthana, 2012).

This study was designed to research into whether or not sources of water for irrigation and lettuce cultivated on the selected farms were contaminated with *Cryptosporidium* spp. The physical parameters, turbidity and pH of water used on all farms showed no significant correlation with the oocysts concentration in the water. This confirms the outcomes of several studies where turbidity and pH did not have any correlation with oocysts concentration (Lee *et al.,* 2010). Nonetheless, few studies have stated that high turbidity waters contains high oocysts concentrations as oocysts bind to solids during rainfall and eventually end up in surface water resources (House, 2011). Swaffer *et al*. (2014) also showed that there is a positive correlation of statistical significance between turbidity levels and *Cryptosporidium* oocyst levels during rainy seasons. This shows that the relationship between turbidity and oocyst concentrations may vary with seasonal variation and geographical location. The agricultural activity level and how the agricultural waste in proximity to surface water is managed may also affect turbidity and oocyst concentration. It is therefore possible to postulate that there could be a possible relationship

between turbidity and oocyst concentration in rainy seasons where there is high flow of water, however, the actual mechanism underlying this association remains unclear.

The acceptable pH for irrigation water for most fruits and vegetables are within the range of 6-7 on the pH scale. All pH values recorded were within the acceptable range with the exception of Farm-2 A (hand dug) which was generally slightly lower. Temperature, on the other hand had an inverse correlation with oocysts concentration thus oocyst numbers decreased as temperature increased. Oocysts are generally inactivated at temperatures above 65 °C and the general temperature range of water samples ranged from 21 °C to 29 °C which are observably lower than 65 °C, thus may promote the persistence of the oocyst in all water samples.

Results obtained indicated an overall prevalence of 54.17% for detection of *Cryptosporidium* in irrigation water using the two detection methods. Microscopy (classical method) and PCR (HSP70), however, recorded individual prevalence of 37.50% and 41.67%, respectively. Further analysis revealed that, 12.50% of samples were exclusively positive for microscopy whiles 16.67% tested positive for PCR (HSP70) exclusively.

The overall prevalence of *Cryptosporidium* positive water samples in this study was quite higher than other studies, which recorded prevalence as low as 18.4% for irrigation water (Gracenea *et al.,* 2011). The disparity in this result could be due to multiple factors such as geographical location, seasonal variations, human activities and methods used in oocysts concentration which are all known to contribute to variability of the determination of *Cryptosporidium* prevalence in water samples analysis. The study design and sampling frame could also contribute to the variations in statistical analysis. In this study, the sampling frame was generally low and this might have affected the statistical power of the results obtained. Notwithstanding, the study design ensured minimal bias in sampling method and processing of the samples obtained to

ensure accurate results. In a study by Ranjbar-Bahadori *et al.*(2011), as low as 6.60% of all irrigation water samples were positive for *Cryptosporidium* oocyst and noticeably the water samples that were negative were mostly found in the industrial areas of Tehran. In a study on water samples from irrigation channels in Spain, positive samples were obtained from irrigation channels that were close to farmlands and open to contamination water from farm fields whilst no positive sample was obtained from reservoirs that had no farming activity in close proximity. As high as 50.00% of all samples from the reservoir (Pinyana) which was closest to the various farm sites were contaminated (Gracenea *et al.*, 2011). Thus geographical locations and differences in oocysts purification methods used for both water and lettuce samples could contribute to differences in prevalence. Studies by Robertson and Gjerde (2001) have shown different methods to have different recovery rates and this could affect prevalence variation in different studies. Liu *et al.* (2013), also argued that when discussing contamination of water and vegetables like lettuce, environmental conditions influenced by geographical area and climate should be considered as these factors can influence the genetic, phenotypic and physiological characteristics of pathogens that are responsible for contamination. Thus, in the interpretation of such results, there must be a contextual basis and careful analysis since the conditions existing in other geographical areas can not necessarily be extrapolated to other areas. It must also be noted that other multiple factors such as human and animal water contact rate and socio-economic activities near the water sources can contribute to the contamination routes (Wells *et al.*, 2015).

Methods of *Cryptosporidium* identification have generally relied on microscopy, the classical method of identification (Chalmers and Katzer, 2013) but though highly specific, the sensitivity is much lower than molecular identification protocols (Chalmer and Katzer, 2013). The sensitivity of PCR in this study was higher than microscopy. As expected; however, it (76.92%)

was generally lower than other studies where sensitivity as high as 96.00%-100% was reported (Xiao *et al.*, 2006). Most studies have also revealed immunologically based methods such as immunofluorescence to be as sensitive as PCR based methods. Nonetheless, there have been disparities between which of these methods is more sensitive. Reasons for these inconsistencies have been attributed to factors such as cross reactivity and antigen variability which affects sensitivity of immunofluorescence and PCR inhibitors that affect PCR.

A variety of PCR primers have been developed for the detection of *Cryptosporidium* spp in environmental samples. In this study, primers specific for HSP70 gene which is a conserved region specific for *Cryptosporidium* spp that infect humans were employed. The HSP70 protein functions as molecular chaperones which facilitate the folding of proteins during secretion and transport. The expression of the HSP protein is up regulated under environmental stress and is involved in the protection of the *Cryptosporidium* (Sulaiman *et al*., 2000). HSP70 is thus very effective in the detection of possible viable oocyst that infect humans (*Cryptosporidium parvum, Cryptosporidium hominis and Cryptosporidium meleagridis*) as opposed to microscopy that detects all *Cryptosporidium* spp. This could explain why some oocysts were not detected by PCR but detected by microscopy.

The PCR results were not always reproducible when repeated and some samples were positive in one PCR cycle but negative in another PCR cycle due to low oocysts concentration. This agrees with some studies where samples which were known positives did not produce positive results in all replicates of PCR (Xiao *et al*., 2006). Again, some samples which were positive for microscopy were negative for PCR. Although PCR is known to be able to detect a single oocyst, studies by Ochiai *et al.* (2004), shows that the PCR detection limit is dependent on recovery rate

and the detectable limit in nested PCR is about 50 oocysts per cycle. Thus below 50 oocysts, PCR may give a false negative.

Again, although immuno magnetic separation (IMS) method increases the chances of retaining oocysts number that could increase concentration of DNA extracted from IMS purified oocyst, other studies have also shown DNA extracted from IMS purified oocysts to contain residual PCR inhibitors (Jiang *et al*., 2005) which could contribute to false negatives. Methods like quantitative real time PCR and Loop mediated isothermal DNA amplification are much more sensitive due to increased sensitivity of detection and more amplified DNA respectively and thus can detect below the detection limit of conventional PCR. This decreases the number of false negatives that can occur (Bakheit *et al.*, 2008; Hadfield *et al*., 2010). These methods though efficient are relatively expensive to be used for routine analysis and epidemiological studies and thus could not be used for this study.

For microscopy staining to be effective, the morphological integrity of the organism should be intact; however, this is not a necessity for PCR identification since DNA extraction disrupts the cellular integrity of the microbe (Wells *et al.,* 2015). This reason can therefore be adduced as to why samples that tested positive for PCR could test negative for microscopy.

A total of 50% of all lettuce samples showed positive for PCR detection approach. This result thus is in conformity to similar studies in Norway and Spain where there was evidence to show prevalence of 26.00% and 63.00%, respectively of lettuce samples contaminated with *Cryptosporidium* (Robertson and Gjerde, 2001). A study done in Zaria Metropolis of Kaduna State in Nigeria (Maikai *et al*., 2013) on the contamination of raw vegetables indicated the highest contamination of *Cryptosporidium* on lettuce (48.00%) using sucrose flotation medium of 1.21 specific gravity and modified Ziehl Neelsen staining technique. A study by El Said Said

(2012) in Egypt on the contamination levels of vegetables with intestinal protozoan parasites ova and oocysts revealed that lettuce was second highest (46.70%) contaminated vegetable with *Cryptosporidium* being the most abundant parasite recording a prevalence of 29.30%. Centrifugation and modified Ziehl Neelson method of staining were employed in the concentration and detection of oocysts (El Said Said, 2012). This confirms that vegetables with particular mention of lettuce, could serve as potential routes for *Cryptosporidium* infection. Household consumption of raw salad is less common in the dietary contents of Ghanaian cuisines in comparison to its francophone neighbouring countries (Klutse, 2006), however, with urbanization and modernization, there is an overwhelming upsurge of fast-food consumption especially in urban centres. The so-called "exotic" vegetables, like lettuce, which hitherto were uncommon, are today a common feature of urban diets (Amoah *et al.,* 2007). Results obtained from this study therefore suggest that there is possible risk of lettuce being a source of cryptosporidiosis infection. Further studies on risks assessment on consumers' exposure to contaminated lettuce are, however, needed to be conducted to ascertain this hypothesis.

Approximately half of all lettuce samples showed positive for *Cryptosporidium* after PCR (HSP70). Attempts at using the immuno fluorescent assay for the detection of oocysts in water samples were, however, unsuccessful due to difficulty in differentiating possible oocysts counts from background of slides. Enumerations of oocyst therefore could not be done. This could be due to the fact that the integrity of the purified oocysts may have been altered due to freeze and thaw processes. There has been evidence to prove that processes that produce stress such as freeze and thawing may disrupt the morphology of the oocyst beyond the point of reliable identification (Kuczynska and Shelton, 1999). Also studies have shown different temperature storage conditions to have an effect on the morphology of *Cryptosporidium* oocysts and their

ability to withstand ultrasonics (Inoue *et al.*, 2005). Due to possible cross reactivity that occurred, there were difficulties in distinguishing possible oocysts from the background.

Farm-1 (irrigation water joined upstream by hospital waste) had oocyst count on all occasions of sampling; however, there was no significant statistical difference ($p \geq 0.05$) between Farm-1 and the other farms with different sources of irrigation water with respect to oocyst counts. This suggests that there is not much difference in *Cryptosporidium* contamination levels in the water sources used for irrigation and thus these irrigation sources may pose a fairly equal possible risk with respect to contamination of vegetables (lettuce) being irrigated For Farm 1, the relatively longer distance between the irrigation point and the point of contamination upstream could reduce *Cryptosporidium* spp concentration as pathogen concentration can decrease with distance along stream flow path.

All farms with the exception of Farm-2 B (water joined upstream by effluent from waste stabilization pond) had oocyst counts between 52.63 and 105.26 /10L which is above the WHO/FAO recommended threshold (1 egg/L) for protozoan parasites. Farm-2B had no oocyst count which could be due to the oocysts numbers that were too low and thus lost along the recovery process. *Cryptosporidium* oocysts due to their small size and low specific gravity are thought to have little interaction with sediment. Contrary to this idea, coupling of surface and pore water flow has shown a transfer of dissolved and suspended solids across the water-sediment interface (Elliot and Brooks, 1997; Searcy *et al.*, 2006). This coupling process known as hyporheic exchange can result in the high rates of deposition on sediment bed regardless of the size and specific gravity (Ren and Packman, 2002). The hyporheic exchange could impact on oocysts in surface water and cause accumulation of oocyst on the beds of streams and hand dug wells used for irrigation. These sediments thus become a source of pathogen during events that

disrupt the water-sediment interface to cause sediment re-suspension. This could be the reason for elevated concentration of oocyst after periods of heavy rainfall as reported in some studies (Schijven and de Roda husman, 2005). For most farmers from this study, with the exception of the few that used pumps, irrigation was done manually using watering cans. Farmers thus walked into the water sources frequently to fetch water to irrigate their large farms. This practice when done constantly can disturb the water-sediment surface thus sediment serving as a source of pathogen distribution into the food chain system by irrigation.

Farm-1 (stream contaminated upstream with hospital waste) which had the oocyst counts on all occasions for irrigation water samples had the least contaminated lettuce samples as compared to the rest of the farms. Due to the distance between the stream on Farm-1 and the beds, the water from the pump which is put in the stream is sometimes poured into barrels which are then fetched into water cans for watering on days that the pump is not used. This kind of system improves the sedimentation of parasitic oocyst thereby decreasing the contamination levels. Studies have proved that farm-based methods like the use of sedimentation ponds, sand filters, enhanced pathogen die-off on crops by cessation of irrigation before harvest are effective interventions for reducing risks associated with the use of contaminated water for irrigation (Keraita *et al.*, 2008). Farm-2B (stream contaminated upstream with effluent from WSP) had no detectable oocyst counts for irrigation water using microscopy but showed the presence of *Cryptosporidium* on the second round of sampling using PCR (HSP70). This could be attributed to the higher sensitivity of PCR when PCR inhibitors are low. Despite the low contamination of water from Farm-2B, there was parasite detection on two occasions from lettuce sampled from this point. This shows that there could be other sources of contamination such as manure, on

farm practices such as raising watering can during irrigation and general hygiene of farmers could lead to crop contamination.

The use of contaminated irrigation water sources raise concerns about the farmers who are in direct contact with the water and consumers via contaminated vegetables. It has been impossible to justify such concerns via the traditional scientific hypothesis testing as infection rates are low and huge sample sizes are needed for such epidemiological studies. Quantitative Microbial Risk Assessment (QMRA), a probabilistic tool has thus become important in estimating the order of magnitude of risk to both farmers and consumers.

It is worthy to note that, the QMRA cannot determine whether an individual becomes infected more than once per year, through a translation of waning protective effect to become susceptible again within the year. Mara *et al*. (2007) argued that, taking all other points into account, there is an agreeable per estimates between QMRA procedure and that of epidemiological studies and hence the QMRA provides a confidence when the epidemiological data is not available. In the scenarios presented in this study (infectivity constant and worst case scenario), the annual probability of infection could not establish the safety of farmers' exposure for the lower limit (52 oocyst/10L), upper limit (105 oocyst/10L) and uniform distribution. All estimated risk was within $1.0 \text{ X } 10^{-3}$ to $1.0 \text{ X } 10^{-2}$ and far above the threshold of $1.0 \text{ X } 10^{-4}$. This implies that between 0.10-1.00 percent of farmers were likely to get infected per year. The WHO guideline states that, "if the overall burden of diseases from other exposures is very high, setting a less stringent level of acceptable risk of 10^{-5} or 10^{-4} threshold may be more realistic as was argued by Mara and Hamilton (2010). The updated threshold of $\leq 10^{-4}$ (0.01%) was still not met by any of the limits taking into consideration the infectivity constant where 41% of oocyst are infectious (Mota *et al.,* 2009) and the worst case scenario where all oocyst accidentally ingested by farmers

are infectious, thus all year round exposure cannot be guaranteed for an acceptable level. In contrast, the maximum risk of infection of farmers when the *E. coli* conversion was employed was 4.32 x 10^{-5} which fell within the acceptable WHO threshold ($\leq 10^{-4}$) for risk. A maximum risk level of 0.004% per year using the *E. coli* conversion is relatively far lower than 0.10% per year which is the lower limit for risk range using the actual parasites for distribution. The conversion to *E. coli* for the risk estimate significantly affected risk output as indicated by the sensitivity analysis. This finding suggest that, the call for the use of appropriate pathogen of interest without conversion in risk modelling is needed to minimise underestimation of risk as a result of the use of conversion ratio methodology (Silverman *et al.,* 2013).

Quantitative microbial risk assessment can help in identifying critical control points (Seidu *et al*., 2008). The sensitivity analysis for the model parameters also indicated another key parameter for the risk estimate which was the initial level of *Cryptosporidium* spp. contamination level in irrigation water. Accidental ingestion of contaminated irrigation water and total exposure had little or no significant impact on risk output. This implies that exposure of farmers to contaminated irrigation water did not increase their risk significantly as opposed to the concentration of *Cryptosporidium* spp present in the water. Thus, farmers who are in direct contact with *Cryptosporidium* contaminated irrigation water could be at risk irrespective of the exposure times. This risk could probably increase as the concentration of oocysts increase. Therefore measures to decrease risk would be more effective if they aim at decreasing pathogen concentration. For this study, though the presence of *Cryptosporidium* spp on lettuce on the farms were established, risk posed to consumers as a result of ingestion of these contaminated vegetables could not be estimated due to inability to enumerate oocyst numbers on the vegetables.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study has confirmed the occurrence of *Cryptosporidium* spp in water sources used in irrigation. There is a high probability that low quality water used in lettuce irrigation contains high levels of *Cryptosporidium* spp. which could be a source of contamination along the food chain Water physical parameters such as pH and turbidity are less likely to influence the concentrations of *Cryptosporidium spp.* in low quality water used in lettuce irrigation than temperature.

Cryptosporidium spp. oocysts found in irrigation water with direct contact to farmers are a potential risk for infection to both farmers and consumers. Although there was no direct data on consumption of raw vegetables in this study, it is assumed that the lettuce would be ingested raw because of its uses in Ghanaian culinary. Furthermore, there was no statistically significant difference between contamination levels of *Cryptosporidium* spp in the commonly used sources of water for irrigation. This implies that all the water sources used for irrigation purpose could pose equal risk with regards to *Cryptosporidium* contamination.

The estimate of annual probability of infection for all scenarios presented in this study was higher than the updated WHO standard of 1.0×10^{-4}. Thus low quality water should not be used for unrestricted irrigation without prior treatment in order to achieve the WHO acceptable pathogen level for unrestricted irrigation as enshrined in the WHO policy document (WHO, 2006). Since the *E. coli* conversion model underestimated the risk, it is thus essential to use the

appropriate pathogen of interest without conversion in risk modelling in order to minimise the uncertainty that could arise as a result of underestimation or overestimation of risk.

6.2 RECOMMENDATIONS

Further studies on viability and genotyping of oocysts found in both water and on lettuce samples would help improve the general knowledge on *Cryptosporidium* spp. in the area and improve the QMRA model. This study can also be extended to other vegetables, consumed in their raw state, to provide baseline information about the presence of *Cryptosporidium* spp. oocysts on commonly consumed vegetables in Kumasi.

Again, the development of standard methods for purification of *Cryptosporidium* spp. oocysts from environmental samples, based on cheap and available materials, would enhance the study with a larger sampling frame in other to garner more data that would give a more accurate presentation of the *Cryptosporidium* contamination along the food chain in the Kumasi Metropolis.

REFERENCES

Acquah, F., Tay, S.C.K., Frimpong, E.H. (2012). Prevalence of *Cryptosporidium* and *Isospora belli* in HIV/AIDS Patients at Komfo Anokye Teaching Hospital in Kumasi-Ghana. *International Journal of Pure and Applied. Science Technology*, 8(2), 26-33.

Adak, G.K., Meakins, S.M., Yip, H., Lopman, B.A., O'Brien, J.J. (2005). Disease risks from foods, England and Wales, 1996-2000. *Emerging Infectious Diseases*, 11(3), 365-372.

Adjei, A.A., Armah, H., Rogrigues, L., Renner, P., Borketey, P., Ayeh-Kumi, P., Adiku, T., Sifah, E., Lartey, M. (2004). *Cryptosporidium* spp: A frequent cause of diarrhea among children at the Korle-Bu Teaching Hospital, Accra, Ghana. *Japan.Journal.Infectious Disease*, 57, 216-219.

Ajonina, C., Buzie, C., Ajonina, I.U., Basner, A., Reinhardt, H., Gulyas, H., Liebau, E. Otterpohl, R. (2012). Occurrence of *Cryptosporidium* in a waste water treatment plant in north Germany. *Journal of Toxicology and Environmental Health*, Part A 75, 1351-1358.

Akiyoshi, D.E., Dilo, J., Pearson, C., Chapman, S., Tumwine, J., Tzipori, S. (2003). Characterization of *Cryptosporidium meleagridis* of human origin passaged through different host species. *Infection and Immunity*, 71 (4), 1828-1832.

Amoah, P., Drechsel, P., Henseler, M., Abaidoo, R.C. (2007). Irrigated urban vegetable production in Ghana: microbiological contamination in farms and markets and associated consumer risk groups. *Journal of Water and Health*, 5, 455–466.

Amoah, P., Keraita, B., Akple, M., Drechsel, P., Abaidoo, R.C., Konradsen, F. (2011). Low-cost options for reducing consumer health risks from farm to fork where crops are irrigated

with polluted water in West Africa. *International Water Management Institute* Research Report 141, p. 45.

Armon, R., Gold, D., Brodsky, M., Oron, G. (2002). Surface and subsurface irrigation with effluents of different qualities and presence of *Cryptosporidium* oocysts in soil and on crops. *Water Science and Technology*, 46, 112-122.

Ayuo, P.O. (2009). Human cryptosporidiosis: a review. *East Africa Medical Journal*, 86, 89-93.

Bakheit, M.A., Torra, D., Palomino, L.A., Thekisoe, O.M.M., Mbati, P.A., Ongerth, J., Karanis, P. (2008). Sensitive and specific detection of *Cryptosporidium* species in PCR negative samples by loop-mediated isothermal DNA amplification and confirmation of generated LAMP products by sequencing. *Veterinary Parasitology*, 158, 11-22.

Beuchat, C.R. 1995. Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection* 59(2): 204-216

Boaitey, Y.A., Nkrumah, B., Idriss, A., Tay, S.C.K. (2012). Gastrointestinal and urinary tract pathogenic infections among HIV seropositive patients at the Komfo Anokye Teaching Hospital. *BMC Research Notes,* 5, 454.

Boel- Peterson, T. (2013). Occurrence of *Cryptosporidium* spp. Oocysts in low quality water and on vegetables in Kumasi, Ghana, Masters' thesis, University of Copenhagen, Denmark.

Caccio, M.S. (2003). Molecular techniques to detect and identify protozoan parasites in the environment. *Acta Microbiologica Polonica*, 52, 23-34.

Carmena, D. (2010). Current research, technology and education topics in applied microbiology and microbial biotechnology (2010 edition). U.S.A: Formatex research center, p 3-11.

CDC. (2006). FoodNet surveillance report for 2004 (Final Report). Pp 32-33.

Chaidez, C., Soto, M., Gortares, P., Mena, K. (2006). Occurrence of *Cryptosporidium* and *Giardia* in irrigation water and its impact on the fresh produce industry. *International Journal of Environmental Health Research,* 15, 5.

Chalmers R., Katzer, F. (2013). Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. *Trends in parasitology*, 237, 51.

Chen, X.M., Keithly, J.S, Paya, C.V., LaRusso, N.F. (2002). Cryptosporidiosis. *New England Journal of Medicine,* 346 (22), 1723-31.

Cornish, G.A. and Lawrence, P. (2001).Informal irrigation in Per-urban areas: A summary of findings and recommendations, DFID's Water KAR Project R7132, report OD 144, HR Wallingford, Wallingford, UK, 54pp.

Corso, S.P., Kramer, M.H., Blair, K.A., Addiss, D.G., Davis, P.J., Haddis, A.C. (2003).Costs of Illness in the 1993 Waterborne *Cryptosporidium* Outbreak, Milwaukee, Wisconsin. *EID,* 9, 4.

Crainiceanu, C. M., Stedinger, J. R., Ruppert, D., Behr, C. T. (2003). Modeling the U.S. national distribution of waterborne pathogen concentrations with application to *Cryptosporidium parvum*. *Water Resource. Research,* 39(9), 1235- 1245.

Craun, G.F., Calderon, R.L., Craun, M.F. (2007). Outbreaks associated with recreational water in the United States. *International Journal of Environmental Health Research*, 15, 4 (243-262).

Current WL, Garcia LS (1991). Cryptosporidiosis. *Clin. Microbiol. Rev*. 4(3): 225-258.

Curtis V, Cairncross S and Yonli R, 2000, Domestic hygiene and diarrhoea - pinpointing the problem, *Tropical Medicine and International Health*, 5(1): 22-32

Dallingham RA, Lima AA, Guerrant RL (2002). Cryptosporidiosis: Epidemiology and Impact. *Microbes Infect. 4: 1059-1066.*

D'Antonio, R.G., Winn, R.E., Taylor, J.P., Gustafson, T.L., Current, W.L., Rhodes, M.M., Gary, G.W., Zajac, R.A. (1985). A waterborne outbreak of cryptosporidiosis in normal hosts. *Annals of Internal Medicine,* 103, 886-888.

Dawson, D.J., Samuel, C.M., Scrannage, V., Atherton, C.J. (2004). Survival of *Cryptosporidium* species in environments relevant to foods and beverages. *Journal of Applied Microbiology,* 96, 6 1222-1229.

Duffy, G., Moriarty, E.M. (2003). *Cryptosporidium* and its potential as a food-borne pathogen. *Animal Health Research Review,* 4(2), 95-107.

Duhain, G.L.M.C. (2011). Occurrence of *Cryptosporidium spp* in South African irrigation waters and survival of *Cryptosporidium parvum* during vegetable processing, Masters' thesis, University of Pretoria, South Africa.

DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B., Jak- ubowski, W. (1995). The infectivity of *Cryptosporidium* parvum in healthy volunteers. *New England Journal of Medicine,* 332, 855- 865.

El Said Said, D. (2012). Detection of parasites in commonly consumed raw vegetables. *Alexandria Journal of Medicine,* 48, 345-352.

Elliott, A. H., and N. Brooks. (1997). Transfer of nonsorbing solutes to a streambed with bed forms: laboratory experiments. *Water Resource. Research,* 33, 137-151.

Elwin, K., Hadfield, S.J., Robinson, G., Chalmers, R.M., (2012). The epidemiology of sporadic human infections with unusual *Cryptosporidium* detected during routine typing in England and Wales, 2000-2008. *Epidemiology and Infection*, 140 (4), 673-683.

Environmental Protection Agency (EPA) (2006). //http: www.epa.gov/ogwdw assessed on 27/07/2015.

Eraky, M.A., Rashed, M.S., Nasr, M.E., El-Hamshary, M.S., El-Ghannam, S.A. (2014). Parasitic contamination of commonly consumed fresh leafy vegetables in Benha, Egypt. *Journal of Parasitology Research,* 2014, 61390.

Ethelberg, S., Lisby, M., Vestergaard, L.S., Enemark, H.L., Olsen, K.E.P., Stensvold, C.R., Nielsen, H.V., Porsbo, L.J., Plesner, A.M., Molbak, K. (2009). A foodborne outbreak of *Cryptosporidium hominis* infection. *Epidemiology of Infection,* 1, 9.

Farthing M, Lindberg G, Dite P, Khalif I, Salazar-Lindo E, Ramakrishna BS, Goh K, Thomson A, Khan AG (2008). World Gastroenterology Organization Practice Guideline: Acute Diarrhoea.

Fayer, R. (1997). *Cryptosporidium* and Cryptosporidiosis. Boca Raton, CRC Press. pp: 1–41.

Fayer, R., Morgan, U., Upton, S.J. (2000).Epidemiology of *Cryptosporidium*: transmission, detection and identification. *International Journal of parasitology*, 30, 1305-1322.

Franco, R.M.B., Neto, R.C. (2002). Occurence of Cryptosporiodial oocysts and *Giardia* cysts in bottled mineral water commercialized in the city of Campinas, State of Sao Paulo, Brazil. *Memorias do InstitutoOswaldo, Cruz* 97, 205-207.

Food and Agriculture Organization. (1992). Wastewater treatment and re-use in agriculture. Food and Agriculture Organization. Irrigation and Drainage Paper 47, Rome Italy.

Food and Agriculture Organization. (2010). Policy briefs: Fighting poverty and hunger, the role of urban agriculture, http://www.fao.org/economic/es-policybriefs/briefs-detail/en/, (accessed 15th August 2014).

Fung, J., Keraita, B., Konradsen, F., Moe, C., Akple, M. (2011). Microbiological quality of urban-vended salad and its association with gastrointestinal diseases in Kumasi, Ghana', *Int. J. Food Safety, Nutrition and Public Health*, 4, (2/3/4), 152–166.

Di Giovanni, G.D., Hoffman, R.M., Sturbaum, G.D. (2010). *Cryptosporidium* genotyping method for regulatory microscope slides. Denver: Water Research Foundation. pp 9-22.

Garcia LS, Bruckner DA, Brewer TC, Shimizu RY (1983). Techniques for the Recovery and identification of *Cryptosporidium* oocysts from specimens. *Journal of Clinical Microbiology* 18:185-190.

Garg, N., Churey, J.J. and Splittstoesser, D.F. 1990. Effect of processing conditions on the microflora of fresh-cut vegetables. *Journal of Food Protection* 53: 701-703

Gracenea, M., Gomez, M.S., Ramirez, C.M. (2011). Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in water from irrigation channels in Catalonia (NE Spain). *Reviews Ibero-Latinoam. Parasitology.* 70(2), 172-177.

Haas, C.N., Rose, J.B., and Gerba, C.P. (1999). Quantitative Microbial Risk Assessment. New York, John Wiley and Sons Inc.

Haas, C.N., Rose, J.B., Gerba, C.P. (2014). Conducting the dose-response assessment. *Quantitative Microbial Risk Assessment*, 2, 267–321.

Hadfield, S.J., Robinson, G., Elwin, K., Chalmers, R.M. (2010). Detection and differentiation of *Cryptosporidium* spp in human clinical samples by use of real-time PCR. *Journal of Clinical Microbiology,* 49, 918-924.

Hamilton, A.J., Stagnitti, F., Premier, R., Boland, A., Hale, G. (2006). Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water. *Applied and Environmental Microbiology,* 72 (5), 3284 – 3290.

Harris, J.R., Petry, F. (2003). Amylopectin: a major component of the residual body in *Cryptosporidium parvum* oocysts. *Parasitology,* 128, 269-282.

Health Canada (2012). Guidelines for Canadian Drinking Water Quality: Guideline Technical Document -Enteric Protozoa: *Giardia* and *Cryptosporidium*. Water, Air and Climate Change Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario. (Catalogue No H129-23/2013E-PDF).

House, A. (2011). *Cryptosporidium* in water supplies. *Foundation for Water Research* 3, 1-17.

Howard G., Schmoll O., Chilton J., and Chorus I. 2006 World Health Organization. *Protecting Groundwater for Health: Managing the Quality of Drinking-water Sources*. ISBN: 1843390795. Published by IWA Publishing, London, UK.

Hunter, P.R., Nichols, G. (2002). Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. *Clin Microbioogy, Rev* 15(1), 145-54.

Inoue, M., Uga, S., Oda, T., Rai, S.K., Vesey, G., Hotta, H. (2005). Changes of physical and biochemical properties of *Cryptosporidium* oocysts with various storage conditions. *Water Research,* 40, 4 (881-886).

Insulander, M., de Jong, B., Svenungsson, B. (2008). A food-borne outbreak of cryptosporidiosis among guests and staff at a hotel restaurant in Stockholm country, Sweden, September 2008. *Eurosurveillance*, 13 (51), 19071.

Insulander, M., Silverlas, C., Lebbad, M., Karlsson, L., Mattsson, J.G., Svenungsson, B. (2013). Molecular epidemiology and clinical manifestations of human cryptosporidiosis In Sweden. *Epidemiology and Infection*, 141 (5), 1009-1020.

Jacks G. and Bhattacharya P. (2009) Drinking Water– Sources, Sanitation and Safeguarding. The Science of Total Environment.. doi: 10.1016/j.scitotenv.2014.05.064.

Jiang, J., Alderisio, K.A., Singh, A., Xiao, L. (2005). Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors. *Applied Environental Microbiolgy*. 71, 1135-1141.

Jimenez, B. (2006). Irrigation in developing countries using wastewater. *International Review for Environmental strategies*, 6, 2 (229-250).

Karavarsamis, N., Hamilton, J. (2010). "Estimators of annual probability of infection for Quantitative Microbial Risk Assessment." *Journal of Water and Health*, 8 (2), 365–73.

Keraita, B., Dreschel, P., Konradsen, F. (2008). Using on-farm sedimentation ponds to improve microbial quality of irrigation water in urban vegetable farming in Ghana. *Water Science & Technology*, 57, (4), 519–525.

Keraita, B., Silverman, A., Amoah, P., Asem-Hiablie, S. (2014). Quality of irrigation water used for urban vegetable production . In Drechsel, P., Keraita, B., *Irrigated urban vegetable production in Ghana; characteristics, benefits and risk mitigation*. Sri Lanka: IWMI. (pp. 62-74)

Keraita, B., Dreschel, P., Klutse, A., Cofie, O. (2014). On-farm treatment options for wastewater, greywater and fecal sludge with special reference to West Africa (Vol 1). IWMI.

Klutse, A. (2006) Circuit and practices in washing gardening products from production to consumption. Report submitted to the Challenge Program on Water and Food (CPWF) by CREPA (Centre Regional pour l'Eau Portable et l'Assainissement a` Faible Cout), Ouagadougou.

Kosek M, Alcantara C, Lima AAM, Guerrant RL (2001). Cryptosporidiosis: An Update. *The Lancet Infectious Diseases* 1:262-69.

Kniel, K.E., Sumner, S.S., Lindsay, D.A., Hackney, C.R., Pierson, M.D., Zajac, A.M., Golden, D.A., Fayer, R. (2003). Effect of organic acids and hydrogen peroxide on *Cryptosporidium parvum* viability in fruit juices. *Journal of Food Protection,* 66, 1650-1657.

Koompapong, K., Sukthana, Y. (2012) .Seasonal variation and potential sources of *Cryptosporidium* contamination in surface waters of Chao Phraya River and Bang Pu Nature Reserve pier, Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, 43, 832-840.

Kuczynska, E., Shelton, D.R. (1999). Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feaces, manures and soils. *Applied and Environmental Microbiolgy*, 65, 2820-2826.

Kumasi Metropolitan Assembly. (2014). About the Kumasi Metropolis, www.kma.ghanadistricts.gov.gh/?arrow=atd&_=6, (accessed on 5th June, 2014).

Langkjer, R.B., Virge, H., Enemark, H.L., Maddox-Hyttel, C. (2007). Molecular and phylogenetic characterization of *Crytoporidium* and *Giardia* from pigs and cattle in Denmark. *Parasitology,* 134, 339-350.

Lantz PG, Matsson M, Wadstrom T, Radstrom P (1997). Removal of PCR Inhibitors from Human Faecal Samples through the use of and Aqueous two-phase System for Sample Preparation Prior to PCR. *Journal of Microbiological Methods* 28(3): 159-167.

Leav, A.B., Mackay, M., Ward, D.H. (2003). *Cryptosporidim* species: New insights and old challenges. *Clinical Infectious Diseases,* 36, 903-908.

Lee, M.Y., Cho, E.J., Lee, J.H., Han, S.H, Park, Y.S. (2010). A survey of *Cryptosporidium* oocysts in water supplies during a 10-year period (2000-2009) in Seoul. *Korean Journal Parasitology,* 48(3), 219-224.

Lewis, W.J., Foster, S.S.D. and Drasar, B.S. (1982) The risk of groundwater pollution by on-site sanitation in developing countries: A literature review. *International Reference Centre for Wastes Disposal*, 01, 82.

Liu, C., Hofstra, N., Franz, E. (2013). Impacts of climate change on the microbiological safety of pre-harvest leafy green vegetables as indicated by *Escherichia coli* O157 and *Salmonella* spp. *Int Journal of Food Microbiology*, 163, 119-128.

Lynch, M.F., Tauxe, R.V., Hedberg, C.W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemeology and Infection,* 137, 3 (307-315).

Macarisin, D., Bauchan, G., Fayer, R., (2010). *Spinacia oleracea* L. leaf stomata habouring *Cryptosporidium parvum* oocysts: a potential threat to food safety. *Applied and Environmental Microbiology,* 76, 555-559.

MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R. and Rose, J.B. (1994). A massive outbreak in Milwaukee of *Cryptosporidium* infection through the public water supply. *The new England Journal of Medicine,* 331, 161-167.

Maikai, B.V., Baba-Onoja, E.B.T., Elisha, I.A. (2013). Contamination of raw vegetables with *Cryptosporidium* oocysts in markets within Zaria metropolis, Kaduna State, Nigeria. *Food Control,* 31, 45-48.

Mak, J.W. (2004). Important zoonotic intestinal protozoan parasites in Asia. *Tropical Biomedicine,* 21, 39-50.

Mara, D.D., Sleigh P.A., Blumenthal, U.J., Carr, R.M. (2007). Health risks in wastewater irrigation: Comparing estimates from quantitative microbial risk analyses and epidemiological studies. *Journal of Water and Health*, 5, 39-50.

Mara, D., Sleigh, A. (2009). "Understanding and Updating the 2006 WHO Guidelines for the Safe Use of Wastewater in Agriculture," no. June: 1–2.

Mara, D., Hamilton, A., Sleigh, A., Karavarsamis, N. (2010). Options for Updating the 2006 WHO Guidelines.

Medema, G.J., Bahar, M., Schets, F.M. (1997). Survival of *Cryptosporidium parvum, Escherichia coli,* faecal enterococci and *Clostridium perfringens* in river water: influence

of temperature and autochthonous microorganisms. *Water Sci. Technol*., 35(11–12), 249–252.

Mensah, P., Yeboah-Manu, D., Owusu-Darko, K., Ablordey, A. (2002). 'Street foods in Accra, Ghana: how safe are they?' *Bulletin of the World Health Organization*, 80 (7), 546–554.

Millar, B.C., Finn, M., Xiao, L., Lowery, C.J., Moore, J. E. (2002).*Cryptosporidium* in foodstuff-an emerging aetiological route of human food borne illness. *Trends in Food Science and Technology,* 6, 168-187.

Mintah P. V. (2011) The quality of water from hand dug wells in the Akim Oda Township Kwame Nkrumah University of Science and Technology, Department of Theoretical and Applied Biology.

MMWR (Mortality and Morbidity Weekly Reports). (2010). CDC Surveillance summaries June 11: 59 no SS-6.

Monge, R., Arias, M.L. (1996). Presence of various pathogenic microorganisms in fresh vegetable in Costa Rica. *Arch Latinoam Nutr,* 46, 192-294.

Moore, J., Cherie Millar, B., Kenny, F., Lowery, C.J., Xiao, L., Rao, J.R., Nicholson, V., Watabe, M., Heaney, N., Sunnotel, O., McCorry, K., Rooney, P.J., Snelling, W.J., Dooley, J.S.G. (2007). Detection of *Cryptosporidium parvum* in lettuce. *International Journal Food Science & Technology,* 42 (4), 385-393.

Morgan, U.M., Pallant, L., Dwyer, B.W., Forbes, D.A., Rich, G., Thompson, R.C.A. (1998). Comparison of PCR and microscopy of *Cryptosporidium parvum* in human feacal specimens: Clinical trial. *Journal of Clinical Microbiology,* 36 (4), 995-998.

Mota, A., Mena, K.D., Soto-Beltran, M., Tarwater, P.M., Cháidez, C. (2009). "Risk assessment of *Cryptosporidium* and *Giardia* in water irrigating fresh produce in Mexico." *Journal of Food Protection,* 72 (10), 2184–88.

Mullis K, Faloona F, Scharf S, Saiki RK, Horn GT, Erlich HA (1986). Specific Enzymatic Amplification of DNA in vitro: the Polymerase Chain Reaction. Cold Spring Harbor Symposium. *Quantitative Biology* 51: 263-273.

Murray, A. and Drechsel, P. (2011). Why do some wastewater treatment facilities work when the majority fail? Case study from the sanitation sector in Ghana. *Waterlines,* 30(2), 135–149.

Ndao M (2009). Diagnosis of Parasitic Diseases: Old and New Approaches Interdisciplinary Perspectives into Infectious Diseases. doi: 10.1155/2009/278246.

Nkansah M.A., Owusu Boadi N., and Badu M (2010). Assessment of the quality of water from hand-dug wells in Ghana, *Environmental Health Insight* Vol 4.pp 7-12

Obuobie, E., Keraita, B., Danso, G., Amoah, P., Cofie, O.O., Raschid-Sally, L., Drechsel.P. (2006). Irrigated urban vegetable production in Ghana: Characteristics, benefits and risks. IWMI-RUAF-CPWF, Accra, Ghana: IWMI, pp150.

Ochiai, Y., Takada, C., Hosaka, M. (2004). Detection and discrimination of *Cryptosporidium parvum* and *C. hominis* in water samples by immunomagnetic separation-PCR. *Applied and Environmental Microbiology,* 71, 2.

Ortega, Y.R., Roxas, C.R., Gilman, R.H., Miller, N.J., Cabrera, L., Taquiri, C., Sterling, C.R. (1997). Isolation of *Cryptosporidium parvum* and *Cyclospora cayetanensis* from

vegetables collected in markets of an endemic region in Peru. *The American Journal of Tropical Medicine and Hygiene,* 57, 683-686.

O'Ryan M, Prado V, Pickering LK (2005). A millennium update on pediatric diarrheal illness in the developing world. *Seminar on Pediatric Infectious Diseases* 16: 125-136.

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., Griffin, P.M. (2013). Attribution of foodborne illnesses, hospitalizations and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases,* 19, 3.

Pawlowski SW, Warren CA, Guerrant R (2009**).** Diagnosis and treatment of acute or persistent diarrhea. *Gastroenterology* 136(6): 1874-1886.

Peng, M.M., Xiao, L., Freeman, A.R. (1997). Genetic polymorphism among *Cryptosporidium parvum* distinct human transmission cycles. *Emerging Infectious Diseases,* 3, 567-573.

Petterson, S.R., Signor, R.S., Ashbolt, N.J. (2007). Incorporating method recovery uncertainties in stochastic estimates of raw water protozoan concentrations for QMRA." *Water and Health*, 5(1), 51–65.

Ponka, A., Kotilainnen, H., Rimhanen-Finne, R., Hokkanen, P., Hanninen, M.L., Kaarna, A., Mer, T., Kuusi, M. (2009). A foodborne outbreak due to *Cryptosporidium parvum* in Helsinki, November 2008. *Eurosurveillance,* 14, 1-3.

Putignani, L., Menichella, D. (2010). Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. *Interdisciplinary Perspectives Infectious Diseases*, 2010. Id 753512.

Quilez, J., Torres, E., Chalmers, R.M., Hadfield, S.J., del Cacho, E., Sanchez-Acedo, C. (2008). *Cryptosporidium* genotypes and subtypes in lambs and goat kids in Spain. *Applied Environmental Microbiology,* 74 (19), 6026-6031.

Ranjbar-Bahadori, S.H., Sangsefidi, H., Shemshadi, B., Kashefinejad, M. (2011). Cryptosporidiosis and its potential risk factors in children and calves in Babol, north of Iran. *Tropical Biomedicine,* 28(1), 125-31.

Reither, K., Ignatius, R., Weitzel, T., Seidu-Korkor, A., Anyidoho, L., Saad, E., Djie-Maletz, A., Ziniel, P., Amoo-Sakyi, F., Danikuu, F., Danour, S., Otchwemah, R.N., Schreier, E., Bienzle, U., Stark, K., Mockenhaupt, F.P. (2007). Acute childhood diarrhoea in northern Ghana: epidemiological, clinical and microbiological characteristics. *BMC Infectious Diseases,* 7, 104.

Ren, J., and A. I. Packman. (2002). Effects of background water composition on stream-subsurface exchange of sub-micron colloids. *Journal of. Environmental. Engineering*, 128, 624-634.

Robertson, L.J. and Gjerde, B. (2000). Isolation and enumeration of *Giardia* cysts *Cryptosporidium* oocysts and *Ascaris* eggs from fruits and vegetables. *Journal of Food Protection,* 63, 775-778.

Robertson, L.J., Gjerde, B. (2001). Occurrence of parasites on fruits and vegetables in Norway. *Journal of Food Protection,* 64, 1793-1798.

Rose, J.B., Huffman, D.E., Gennaccaro, .A. (2002). Risk and control of waterborne cryptosporidiosis. *FEMS Microbiological. Reviews,* 26, 113.123.

Rosssle, N.F., Latif, B. (2013). Cryptosporidiosis as threatening health problem: a review. *Asian Pacific Journal of Tropical Biomedicine,* 3 (11), 916-924.

Ryan, U. (2010). *Cryptosporidium* in birds, fish and amphibians. *Experimental Parasitology,* 124, 113-120.

Ryu, H., and Abbaszadegan, M. (2008). "Long-term study of *Cryptosporidium* and *Giardia* occurrence and Quantitative Microbial Risk Assessment in surface waters of Arizona in the USA." *Journal of Water and Health,* 6 (2), 263–73.

Samie, A., Bessong, P.O., Obi, C.L., Sevilleja, J.E.A.D., Strop, S., Houpt, E., Guerrant, R.L. (2006). *Cryptosporidium* species: preliminary descriptions of the prevalence and genotype distribution among school children and hospital patients in the Venda regions, Limpopo Province, South Africa. *Experimental Parasitology,* 114, 314-322.

Savioli, L., Smith, H., Thompson, A. (2006). *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. *Trends in Parasitology,* 22(5), 203-208.

Schijven, J. F., and de Roda Husman. A. M. (2005). Effect of climate changes on waterborne disease in The Netherlands. *Water Science. Technology,* 51, 79-87.

Scott CD (1988). Screening Faecal Smears for *Cryptosporidium* and *Isospora belli* using a Modified Ziel-Neelsen Stain. *Australian Journal of Medical Laboratory Sciences.* 9: 80-82.

Searcy, K. E., Packman, A. I., Atwill, E. R., and Harter, T. (2006). Deposition of *Cryptosporidium* oocysts in streambeds. *Applied. Environmental. Microbiology.* 72, 1810–1816.

Seidu, R., Heistad, A., Jenssen, P. D., Drechsel, P. and Stenström, T.A. (2008) 'Quantification of the health risks associated with wastewater reuse in Accra, Ghana: A contribution toward local guidelines'. *Journal of Water and Health,* 6 (4), 461–471.

Sengupta, M.E., Keraita, B., Olsen, A., Boateng, O.K., Thamsborg, S.T., Palsdottir, G.R., Dalsgaard, A. (2012). Use of *Moringa oleifera* seed extracts to reduce helminths egg numbers and turbidity in irrigation water. *Water Research,* 46, 11 (3646-3656).

Silberberg, MS (2000). Chemistry, the Molecular Nature of Matter and Change, 2nd Ed. McGraw- Hill Companies, USA.

Silverman, A. I., Akrong, M.O., Amoah, P., Drechsel, P., Nelson, K.L. (2013). Quantification of human *Norovirus GII*, human *Adenovirus*, and fecal indicator organisms in wastewater used for irrigation in Accra, Ghana. *Journal of Water and Health,* 11 (3), 473–88.

Slifko, T.R., Huffman, D.E., Dussert, B., Owens, J.H., Jakubowski, W., Haas, C.N., Rose, J.B. (2002). Comparison of tissue culture and animal models for assessment of *Cryptosporidium parvum* infection. *Experimental Parasitology,* 101(2–3), 97–106.

Smith, H.V., Nichols, R.A. (2009). *Cryptosporidium*: detection in water and food. *Experimental Parasitology,* 124, 61–79.

Sobsey M D, 2002, Managing water in the home: accelerated health gains from improved water supply. WHO, Geneva, Switzerland.

Steele, M., Odumeru, J. (2004). Irrigation water as a source of foodborne pathogens on fruits and vegetables.*Journal of Food Protection,* 67, 2839-2849.

Stanistski C L, Eubanks LP, Middlecamp CH, Stratton WJ (2000) Chemistry in context Third Ed. McGraw-Hill Com

Stauffer SH, Birkenheuer AJ, Levy MG, Marr H, Gookin J L (2008). Evaluation of Four DNA Extraction Methods for the Detection of *Tritrichomonas foetus* in Feline Stool Specimens by Polymerase Chain Reaction. *Journal of Veterinary Diagnostic Investigations* 20: 639-641.

Strong, W.B., Gut, J., Nelson, R.G. (2000). Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilo- dalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. *Infection and Immunity* 68: 4117–34.

Sulaiman, I.M., Morgan, U.M., Thompson, R.C.A., Lal, A.A., Xiao, L. (2000). Phylogenetic relationships of *Cryptosporidium* parasites based on the 70 kilodalton Heat Shock Protein (HSP70) gene. *Applied and Environmental Microbiology*, 66, 6 (2385-2391).

Swaffer, B.A., Vial, H.M., King, B.J, Daly, R., Frizenschaf, J., Monis, P.T. (2014). Investigating source water *Cryptosporidium* concentration, species and infectivity rates during rainfall-runoff in a multi-use catchment. *Water Research.* Dec 15 (67), 310-20.

Templeton, T.J., Lancto, C.A., Vidorovich, V., Liu, C., London, N.R., Hadsall, K.Z., Abrahamsen, M.S. (2004). The *Cryptosporidium* oocyst wall protein is a member of a multigene family and has a homolog in *Toxoplasma. Infection and Immunity,* 72, 980-987.

Tzipori, S., Ward, H. (2002).Cryptosporidiosis: biology, pathogenesis and disease. *Microbes and Infection*, 4, 1047-1058.

Thurston-Enriquez, J. A., Watt, P., Dowd, S.E., Enriquez, R., Pepper, I.L., Gerba, C.P. (2002). Detection of protozoan parasites and microsporidia in irrigation waters used for crop production. *Journal of Food Protection*, 2, 251-435.

Tyzzer, E.E. (1912). *Cryptosporidium parvum* (sp.nov.), a coccidium found in the small intestine of the common mouse. *Archives Für Protistenkunde*. 26, 394-412.

Ungar BL. (1990) Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. *Journal of Clinical Microbiology* ;28(11):2491-5.

United Nations. (2003). Water for people, water for life. The United Nations world water development report. Barcelona: UNESCO.

United Nations. (2014). Revision of the world urbanisation prospects, www.un.org/en/development/desa/publications/2014-revision-world-urbanization-prospects.html, (accessed 12th February, 2015).

Vetterling, J.M., Doran, D.J. (1969). Storage polysaccharide in coccidial sporozoites after excystation and penetration of cells. *Journal of Protozoology,* 16, 772-775.

Walana, W., Tay, S.C.K., Tetteh, P., Ziem, J.B. (2014). Prevalence of intestinal protozoan infestation among primary school children in urban and peri-urban communities in Kumasi, Ghana. *Science Journal of Public. Health*, 2, 52- 57.

Wells, B., Shaw, H., Hotchkiss, E., Gilray, J., Ayton, R., Green, J., Katzer, F., Wells, A., Innes, E. (2015). Prevalence, species identification and genotyping *Cryptosporidium* from livestock and deer in a catchment in the Cairngorms with a history of a contaminated public water supply. *Parasites & Vectors,* 8, 66.

Wiedbrauk DL, Werner JC, Drevon AM (1995). Inhibition of PCR by Aqueous and Vitreous Fluids. Journal of Clinical Microbiology 33(10): 2643-2646.

Wohlsen, T., Bates, J., Gray, B., Katouli, M. (2004). Evaluation of five membrane filtration methods for recovery of *Cryptosporidium* and *Giardia* isolates from water samples. *Applied and Environmental Microbiology*, 70 (4), 2318–2322.

World Health Organization. (2006). "WHO Guideline for the Safe Use of Wastewater, Excreta and Greywater." *Safe Use of Wastewater in Agriculture*. Vol. II. Geneva, Switzerland.

World Health Organization. (2008). Protozoan parasites (*Cryptosporidium, Giardia, Cyclospora)*, www.who.int/water_sanitation_health/dwq/admicrob5.pdf, (accessed 15th August 2014).

World Health Organization. (2009). Risk assessment of *Cryptosporidium* in drinking water. WHO/HSE/WSH/09.04.

Xiao, L., Alderisio, K., Singh, A. (2006). Development and standardization of a *Cryptosporidium* Genotyping tool for water samples. Denver, U.S.A: Awwa Research Foundation. pp 40-50.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H., Lal, A.A. (2001). Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. *Journal of Infectious Diseases,* 183, 492–7.

Xiao, L., Fayer, R. (2008). Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. *International Journal of Parasitology,* 38, 1239–1255.

Zezza, A., Tasciotti, L. (2010). Urban agriculture, poverty and food security: empirical evidence from a sample of developing countries. *Food Policy,* 35(4), 265-273.

Zink, D.L. (2009). The impact of consumer demands and trends on food processing, http: //www.cdc.gov/ncidod/EID/vol3no4/zink.htm, (accessed 6th January, 2014).

Zoeteman, B.C.J. (1980). Sensory Assessment of Water Quality. Pergamon Press, Oxford.