MOLECULAR DETECTION AND EPIDEMIOLOGY OF *SHIGELLA* SPP. AND ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC) INFECTIONS AMONG CHILDREN WITH ACUTE GASTROENTERITIS IN ACCRA, GHANA

BY

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JULY, 2013
DEDICATION

This research work is foremost dedicated to the Almighty God for His grace that has been sufficient for me throughout my academic life. This thesis is also dedicated to my resourceful supervisors for their enormous contribution and mentoring in this research work. Finally I dedicate this work to my dear mother, father and siblings for their love, care, support and understanding over the years.
DECLARATION

I, Albert Amenyedor of the Department of Medical Biochemistry of the University of Ghana Medical School, do hereby declare that, with the exception of quoted articles and references, this research work herein described was duly carried out by me and the results obtained are the true reflection of the work undertaken under the supervision of Dr. Bartholomew Dzudzor of the Department of Medical Biochemistry of the University of Ghana and Prof. George Armah of the Department of Electron Microscopy/Histopathology of Noguchi Memorial Institute for Medical Research (NMIMR) also of the University of Ghana.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>%</td>
<td>Percentage</td>
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<tr>
<td>±</td>
<td>Plus or minus</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>µ</td>
<td>Microliter</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CFA</td>
<td>Colonization factor antigens</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanidine monophosphate</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>DAEC</td>
<td>Diffusely adherent <em>Escherichia coli</em></td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate</td>
</tr>
<tr>
<td>DEC</td>
<td>Diarrhoeagenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>Escherichia coli</em></td>
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<td>EHEC</td>
<td>Enterohemorrhagic <em>Escherichia coli</em></td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
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<tr>
<td>HUS</td>
<td>Hemorrhagic uremic syndrome</td>
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<tr>
<td>IpaB</td>
<td>Invasion plasmid antigen B</td>
</tr>
<tr>
<td>KBTH</td>
<td>Korle-Bu Teaching Hospital</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile toxin</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>NMEC</td>
<td>Neonatal meningitis/sepsis associated <em>Escherichia coli</em></td>
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<td>PAGE</td>
<td>Polyacrylamide Gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PML</td>
<td>Princess Marie Louis Hospital</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>ST</td>
<td>Heat-stable toxin</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>UGMS</td>
<td>University of Ghana Medical School</td>
</tr>
<tr>
<td>VTEC</td>
<td>Verocytotoxin producing <em>Escherichia coli</em></td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Background: Diarrhoea remains an important public health problem and is the second leading cause of mortality and morbidity in children throughout the world, especially in developing countries. In Ghana, diarrhoea is the third common cause of hospital attendance of young children. It is responsible for 13.1% of hospitalizations among these children and an annual average of 5,193 deaths in children under 5 years of age. Bacterial enteropathogenic agents of diarrhoea and mechanisms responsible for disease pathogenesis are generally known. However, knowledge on the true prevalence and contribution of these bacteria to the disease burden is very limited. This study strives to optimize available PCR methods to facilitate accurate diagnosis of Enterotoxigenic E. coli and Shigella spp. infections and contribute baseline information on diarrhoeal bacterial enteropathogens.

Main Objective: To provide a new method for the routine screening and detection of Enterotoxigenic E. coli and Shigella spp. in diarrhoeal stool samples via PCR.

Design: Two hundred archived stool samples from previous diarrhoeal surveillance study were retrieved from the Department of Electron Microscopy/Histopathology of Noguchi Memorial Institute for Medical Research for analysis. Total DNA was extracted and conventional PCR used to identify Enterotoxigenic E. coli (ETEC) and Shigella spp. The incidence and prevalence was then computed.

Results: 4 (2%) samples screened were positive for heat-labile toxin producing ETEC with all detections occurring in the 0-12 month year group. Heat-stable toxin producing ETEC and Shigella were not detected.
Conclusion: The overall prevalence of ETEC-LT in this study was 2%. Conventional PCR may be used for the routine screening of diarrhoeal stool samples for ETEC-LT, ETEC-ST, and Shigella. This method of screening diarrhoeal stool samples is fast and specific. Taking into account the promptness with which results are made available for health care delivery and management, this method can be said to be relatively cheaper in comparison to the gold standard, culturing.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

In recent years, in spite of positive advances in economic growth, health care delivery and implementation of health service programmes (such as immunization and 6 months of compulsory exclusive breastfeeding) in Ghana, infant and under five mortality rates have still remained high. Most of these childhood deaths are caused by preventable or treatable health conditions such as malaria (26%), pneumonia (19%) and diarrhoea (18%) (GHS, 2012b).

Diarrhoea remains an important public health problem and is the second leading cause of mortality and morbidity in children worldwide (Bryce et al., 2005). Available figures peg the global average mortality from diarrhoea at 760 000 deaths annually (WHO, 2013). Regrettably, diarrhoea is more prevalent in the developing world (Bryce et al., 2005). Kosek and associates reported that 2 million children under 5 years of age die annually as result of diarrhoea in developing countries (Kosek et al., 2003). This high prevalence in the developing world may be due to the lack of safe drinking water, inadequate sanitation, poor hygiene and malnutrition, which particularly increases the risk of contracting diarrhoea (Bonkoungou et al., 2013). Improvements in sanitation, nutrition, education and early access to oral rehydrating salts (ORS) are believed to have contributed to the
reduction in mortality numbers from an estimated 1.5-2.5 million deaths in 2010 to the present estimated 760 000 child deaths (Black et al., 2010; Durley et al., 2004).

Diarrhoea is a common symptom of gastrointestinal infections caused by a wide range of pathogens. The enteropathogenic agents of diarrhoea are mainly microorganisms such as bacteria, viruses and protozoans as well as some macroorganisms such as helminthes (O’Ryan et al., 2010). Some specific examples of the microorganisms which are commonly isolated from diarrhoea stool samples include diarrhoeagenic Escherichia coli, Rotavirus, Shigella spp., Giardia lamblia and Entamoeba histolytica and Cryptosporidium. Among all these organisms only a handful are responsible for most acute cases of childhood diarrhoea (WHO, 1999). Additionally, most of the diarrhoea-causing pathogens share a similar mode of infection i.e. the faecal-oral transmission. There are however differences in the bacterial load needed to cause illness and the mode of transmission (Guerrant et al., 2008).

In Ghana, diarrhoea is the third common cause of young children visiting health centers (GHS, 2012a), nonetheless, knowledge of the etiological agents, prevalence and clinical significance of the agents are limited. A study published by Binka et al., 2003 showed that in parts of Northern Ghana, rotavirus was the main cause of childhood diarrhoea. Then again, numerous studies have reported diarrhoeagenic E. coli pathotypes and Shigella spp. as the most common bacterial pathotypes associated with childhood diarrhoea across the developing world (Nguyen et al., 2005). The incidence and prevalence of these pathogens however vary across geographic regions and is heavily dependent on the prevailing
socioeconomic status and sanitary conditions of the population. For instance, diarrhoeagenic \textit{E. coli} and \textit{Shigella} represented 24\% and 6\% respectively of enteric pathogens associated with childhood diarrhoea reported at Centre Medical avec Antenne Chirugicale (CMA) du Secteur 30 in Ouagadougou, Burkina Faso between January 2009 and January 2010 (Bonkoungou \textit{et al.}, 2013). Unfortunately, data on the contribution of these two pathogens to acute childhood diarrhoea in Ghana is currently unavailable.

\textit{Escherichia coli (E. coli)} are a group of typically non-pathogenic gram-negative bacteria that naturally occur in the lower intestine of warm blooded organisms including man (CDC, 2011). Most \textit{E. coli} strains are harmless but certain strains have been recognized as human pathogens since the 1940s when Bray (1945) hypothesized that \textit{E. coli} subtypes might account for common infantile diarrhoea of unknown etiology. Mathusa \textit{et al.}, (2010) also suggest a strong association between several food borne illness and certain \textit{E. coli} strains. Diarrhoeagenic intestinal \textit{E. coli} are classified on the basis of their epidemiological, clinical and pathogenic characteristics and specific virulence determinants into the following pathotypes: enteropathogenic \textit{E. coli} (EPEC), shiga-toxin producing \textit{E. coli} (STEC), enterotoxigenic \textit{E. coli} (ETEC), enteroinvasive \textit{E. coli} (EIEC), enteroaggregative \textit{E. coli} (EAEC) and diffuse adherent \textit{E. coli} (DAEC) (Nataro and Karper, 1998). Each serotype expresses a unique set of virulence and colonization factors encoded in the chromosome or in episomal structures, epidemiology, clinical manifestations and treatment (Huang \textit{et al.}, 2006). ETEC is the leading cause of diarrhoea in children 5years and below and traveler’s diarrhoea in developing and developed countries (Trabulsi \textit{et al.}, 2002). \textit{Shigella} is a genus of gram-negative, non-spore forming, non-motile, rod shaped bacteria closely related to \textit{E.}}
coli and Salmonella and responsible for human shigellosis. At infection, shigellosis presents as dysentery and affects only humans and apes and no other animals (Ryan and George, 2004). Post invasion, Shigella multiplies intracellular and spreads to neighbouring epithelial cells resulting in tissue destruction and characteristic pathology of shigellosis. Shigella species are classified under four main serogroups (A, B, C and D). Serogroup A known as S. dysenteriae has 12 serotypes. Serogroup B, S. flexneri has 6 serotypes. Serogroup C, S. boydii has 18 serotypes and serogroups D, S. sonnei has 1 serotype. With the exception of serogroup D which can be differentiated on the basis of biochemical metabolism assays, serogroups A, B and C are physiologically similar.

The gold standard for identification of diarrhoeagenic microorganisms in stool samples, with ETEC and Shigella not being an exception, has been the culture method (Nataro and Karper, 1998; Georges et al., 1983). Though effective, there are a few challenges such as easy sample contamination which may lead to falsification of results and inability to distinguish between specific serotypes of organisms present in the stool samples just to mention a few. These challenges may be overcome by exploiting the unique set of virulence genes of diarrhoeagenic E. coli and Shigella species using polymerase chain reaction assays. The objective of this study is thus to optimize the available PCR methods in order to establish a routine method of diagnosis for enterotoxigenic E. coli and Shigella associated with childhood diarrhoea in our various laboratories and hospitals across the country and determine the epidemiology of these two bacterial enteropathogens.
1.2 Problem statement

Childhood diarrhoea continues to be a principal cause of childhood morbidity and mortality in developing countries propagated by a self-perpetuating vicious cycle in which diarrhoea and malnutrition are synergistic (Opintan et al., 2010; Okeke, 2009). Frequent episodes of diarrhoea results in damaging of intestinal endothelium by actions of the pathogens of diarrhoea. A resulting decrease in absorptive function of the intestinal endothelium leads to nutrient depletion and malnutrition (Guerrant et al., 2008) which contributes to growth and cognitive impairment that may impact of school attendance and performance and development (Petri et al., 2008). The contribution of diarrhoea to malnutrition and growth impairment by diarrhoea is greater than other common infections and diarrhoeagenic E. coli induced diarrhoea may even be more detrimental than rotavirus in this regard (Mondal et al., 2009; Okeke, 2009). The disease also results in negative economic effects due to medical cost, loss of working hours by parents culminating in reduced income for the already overburdened parents in the developing world (Black et al., 2010). Cumulatively, this results in a lower quality of life for all and sundry. The longstanding gold standard for detection and screening for Shigella and ETEC, the culture method, is becoming less and less sensitive. This may be a consequence of the indiscriminate use of antibiotics by the populace resulting in antibiotic resistance and false negatives (Cars et al., 2001; Aminov and Mackie, 2007). Additionally for E. coli, identification of diarrhoeagenic E. coli by culture and biochemical tests is inefficient as they are indistinguishable from the non-pathogenic E. coli commonly found in human faeces. Also, specific serotyping is not always correlated with pathogenicity (Rappelli et al., 2005). Discrimination of pathogenic and non-pathogenic strains of E. coli requires DNA screening using molecular techniques.
Furthermore, epidemiological research is biased towards agents that are most easily detected such as Rotavirus, Salmonella spp. and Cryptosporidium spp. just to mention a few. Few studies look for supposedly minor pathogens such as diarrhoeagenic E. coli, which are difficult to differentiate from commensals (Brooks et al., 2006). There is thus deficient data on the contribution of Shigella spp. and ETEC to the diarrhoea burden in Ghana for any substantive policies to be formulated to remedy this dire situation. Without an alternative diagnostic tool for detection of ETEC and Shigella spp. infections, patients will be misdiagnosed and appropriate medications not administered.

1.3 Justification

This proposed conventional PCR method of diagnosis will provide a more specific, accurate and faster diagnostic tool for the detection of ETEC and Shigella from diarrhoeal stool samples. It is hoped that through this molecular method of diagnosis, false negatives will hopefully be reduced to the barest minimum. Moreover, patients and doctors alike would not have to wait for days on end before results are made available for treatment to be administered as is with the culture method. This will help save many of the over two million children lost to childhood diarrhoea in the developing world annually which would otherwise be lost in the turn-around time for the laboratory results or false reportage. Availability of adequate data on the role of rotavirus has led to the introduction in April 2012 of a rotavirus vaccination programme by Global Alliance for Vaccination Immunization (GAVI) in collaboration with the Ghana Health Service (GHS) (Armah et al., 2001). In light of this, it is hoped that data realized from this study will form baseline information for other studies and policy formulation with respect to these two very
important but neglected bacterial causes of diarrhoea. Recently, various PCR assays have been developed for the detection of diarrhoeagenic \textit{E. coli} and \textit{Shigella}. This present study is thus trying to optimize these PCR method for the detection of ETEC and \textit{Shigella} in the laboratories and hospitals across Ghana to surmount the problem of false negatives and enable efficient and early detection of these pathogens for appropriate medication to be administered.

1.4 Overall Objective

The main objective of the study was to optimize available polymerase chain reaction methods in order to establish a routine method for the detection of enterotoxigenic \textit{E. coli} and \textit{Shigella spp.} from diarrhoeal stool samples. Additionally, to determine the epidemiology of enterotoxigenic \textit{E. coli} and \textit{Shigella spp.} in the study subjects.

1.5 Specific Objectives

The specific objectives are to:

- detect the presence of \textit{Shigella} and ETEC in study samples.
- determine the prevalence of \textit{Shigella} and ETEC in the study subjects.
- determine the presence of mixed infections.
- determine the association of ETEC infection and the sex and ages of subjects
- determine the association of \textit{Shigella} infections and the sex and ages of subjects
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diarrhoea

Gastroenteritis is a medical condition characterized by diarrhoea with vomiting due to infection of the small and large bowel. This is a consequence of inflammation of the gastrointestinal tract involving both the stomach and small intestine (Mandell et al., 2010). The Ghana Ministry of Health’s Treatment Guidelines (2004) defines diarrhoea as the passing of frequent, loose, watery stools three or more times in a day which is usually accompanied by vomiting with the highest incidence amongst children. WHO (2005) defines diarrhoea as the passage of three or more unusually loose stools or liquid stools per day, or more frequent than normal for an individual. However, it is the consistency of the stools rather than the number that is most important. Frequent passing of formed stools is not diarrhoea as babies fed on only breastmilk often pass loose, pasty stools. Bowel frequency of a healthy individual ranges from three bowel movements per day to one bowel action every third day with a normal stool constituency range from porridge-like to hard and pellet stool (Haslett et al., 1999).

Diarrhoeal diseases are a paramount cause of childhood illness and death in developing countries and a major cause of malnutrition and malabsorption in children in these countries (Joosten and Hulst, 2008). It is the second leading cause of mortality of children under five years and is responsible for an estimated 760 000 deaths annually with about 80% of these deaths occurring in the first two years of life (WHO, 2013). In addition,
diarrhoea impedes weight gain in children, has adverse effects on their memory and their analytical skills and reduces their school attendances thereby crippling their future (UNDP, 2006). Most diarrhoeal deaths are caused by loss of fluids through watery stools, profuse sweating, vomiting, and excess urination culminating in dehydration and electrolyte disturbances and or other salt imbalances. Pivotal to the fight against diarrhoea has been the discovery that acute diarrhoea of any etiology, and at any age except when it is severe, can be efficiently managed by the simple method of oral rehydration in a single dose (Kane et al., 2003). In the past 25 years, over 50 million lives have been saved worldwide by treatment with Oral Rehydration Salts (ORS) with traces of salts and zinc tablets. In the absence of ORS, a solution of clean water, sugar and salt and zinc tablets has sufficed (WHO, 2005).

Advances in science over the past three decades has led to the discovery and isolation of many microbial causes of diarrhoea. Pathogens isolated cut across viruses, bacteria and protozoa with rotavirus and diarrhoeagenic Escherichia coli (DEC) being the most commonly isolated pathogens from stool samples in developing countries (Bonkoungou et al., 2013). Underlying conditions such as malnutrition, lack of potable water, inadequate sanitation and poor hygiene increases the risk of contracting diarrhoea in developing countries. Childhood malnutrition has been determined to be the dominant underlying cause of an estimated 35% of all deaths of children five years and below (WHO, 2012). Improved access to potable water and adequate sanitation, along with the promotion of good nutrition and good hygiene practices such as simply washing of hands with soap and water have contributed significantly to the reduction of childhood diarrhoea (Tagoe, 1995).
A study published by Tagoe (1995) in Ghana, draws a more than certain correlation between the risk of having diarrhoea and proper toilet facilities. In that study, it was determined that children living in houses with adequate toilet facilities were about 50% less likely to contract diarrhoea as against children who didn’t have adequate toilet facilities. A correlation he attributed to the faecal-oral transmission. Furthermore, the prevalence of diarrhoea was significantly lower among children of educated mothers than that of uneducated mothers. This supports the preposition that education of mothers is essential in the prevention of childhood diarrhoea (Tagoe, 2005). A comparative study of urban areas of Ghana, Egypt, Brazil and Thailand by Timaeus and Lush (1995) clearly indicates that a child’s health is affected by the socio-economic status of the household. According to that study, children from affluent households have lower diarrhoeal morbidity and mortality in these countries. Such differentials in diarrhoea by households due to economic status probably is due to differences in child care practices such as food preparation, weaning of children and proper sanitation and hygiene coupled with safe drinking water.

2.2 Clinical types of diarrhoea

Diarrhoea is classified under three main types with each depicting the underlying pathology and or altered physiology. These classifications require no laboratory diagnosis and form the basis of treatment of diarrhoea in children as prescribed by WHO (Hogue, 2000). Acute watery diarrhoea is the most common type of diarrhoea seen in hospital and health care facilities. It is characterized by significant fluid loss and rapid dehydration in an infected
individual and usually lasts for several hours or days. Pathogens responsible for this type of diarrhoea include *V. cholerae*, *E. coli* and Rotavirus (Armah *et al*., 2001). Acute watery diarrhoea includes travelers’ diarrhoea. These are usually an abrupt attack with abdominal cramps, anorexia and vomiting lasting for 3-5 days. Most affected demography are travelers, especially visitors to developing countries and endemic areas (Haslett *et al*., 1999).

Acute bloody diarrhoea, also referred to as dysentery, is marked by visible blood in the stools. This is usually associated with intestinal damage and nutrient loss in infected individuals. The pathogen responsible for bloody diarrhoea is the bacterial agent *Shigella* (Mock *et al*., 1995).

Finally, there is chronic or relapsing diarrhoea. This is an episode of diarrhoea, with or without blood, lasting for at least 14 days. Persons normally infected are undernourished children and patients with AIDS. Persistent diarrhoea rarely occurs in babies who are exclusively breastfed (Hogue, 2000). This chronic or relapsing diarrhoea, as it is commonly referred to as, is commonly caused by irritable bowel syndrome which can present with increased frequency of defecation and loose, watery or pellet stool. This type of diarrhoea rarely occurs at night and is most severe before and after breakfast. Stools often contain mucus but never blood with a 24 hour stool volume not exceeding 200g. Chronic diarrhoea is often categorized as disease of the colon or small bowel, or malabsorption (Haslett *et al*., 1999).
2.3 Disease burden of diarrhoea

Notwithstanding large reductions in child mortality between 2000 and 2010, diarrhoea and pneumonia remains the foremost cause of avoidable deaths, accounting for about 30% of all child deaths globally (WHO, 2012). This toll is greater than deaths from malaria, AIDS and measles combined in the same period. Conservative estimates propose that in 2010, there were 1.731 billion episodes of diarrhoea of which 36 million of these progressed to severe episodes in children younger than 5 years of age. Estimates in 2011 recorded about 700,000 episodes of diarrhoea with a high proportion occurring in developing countries (WHO, 2012). Globally there is an estimated 1.7 billion cases of diarrhoeal disease annually with more than half of these cases occurring in Africa and South Asia (WHO, 2009; WHO, 2012). A high proportion of these deaths, 72%, occurs in the first 2 years of life. The World Health Organization’s health statistics profile for 2013 on Ghana reports that diarrhoeal diseases were responsible for 7% of all under five year child deaths. A similar report on Ghana reported under five mortalities due to diarrhoea at 9% in 2008. Data available from the Ghana Health Service 2011 Annual Report states that in the year under review, the total number of diarrhoea episodes reported in under five year olds was 111,786 of which 2,318 were with severe dehydration and culminated in 354 deaths (CFR=0.31%). The highest incident rate in Ghana was recorded at 3,611.2/100,000 population in the Upper East Region as against a national average of 2,217.6/100,000 population (GHS, 2012). The entire data for the cases per 100,000 population is in Figure 2-1.
Figure 2-1: Regional variation in diarrhoea cases amongst under 5 year olds per 100,000 cases in 2011 in Ghana (Source: Ghana Health Service 2011 Annual report) The highest number of cases per 100,000 population in 2011 was recorded in the Upper East region with the least occurring in Greater Accra region. The difference between the two extremes is higher than the national average of 2,217.6 cases/100,000. Generally, there was a higher recorded number of cases in the northern sector of Ghana relative to the south. This trend suggests the correlation between infrastructural development and level of sanitation and potable water and the prospect to develop diarrhoea.
Figure 2-2: Proportional distribution of cause of disease among children below 5 years of age in Ghana as at 2004. (Source: Based on WHO, Global Burden of Diseases estimate to the most recent estimates for the total number of under-five deaths (2007)).
Figure 2-3: Proportions of most common cause of death among children under five years of age globally in 2004. (Source: WHO, Global Burden of Disease estimates, 2004 update.)
2.4 Etiology of diarrhoea

2.4.1 Infectious causes

The gastrointestinal infections which characterize diarrhoea is caused by a wide range of pathogens ranging from viruses through bacteria and protozoa. Notable viruses include Rotavirus, Norovirus, Adenovirus and Astrovirus with rotavirus being implicated in about 70% of all diarrhoea in children in both the developing and developed world (Webb and Starr, 2005). Acquired immunity in adults makes them less susceptible to rotavirus infections (Eckardt and Baumgart, 2011). Studies by Patel et al., (2009) show that norovirus is the underlying cause of most adult viral diarrhoeal diseases.

Accounting for 15% of diarrhoeal diseases, bacteria including diarrhoeagenic E. coli, Shigella, Salmonella and Campylobacter spp. are commonly isolated from diarrhoeal stools from children. Additionally, cholera is a common cause of diarrhoea in sub-Saharan Africa and Asia (Charles & Ryan, 2011). Interestingly, Clostridium difficile causes diarrhoea in adults only, with infected children being asymptomatic. This bacteria is often associated with hospitalized individuals and individuals treated with antibiotics for unrelated conditions (Rupnik et al., 2009; Moudgal & Sobel, 2012). There is an apparent increase of exposure to Salmonella, Campylobacter and Clostridium difficile infection after usage of acid-suppressing medications such as Rabeprazole with a much greater risk in individuals taking proton-pump inhibitors as opposed to H2 antagonists (Leonard et al., 2007). Together, rotavirus and diarrhoeagenic E. coli are the most common pathogens isolated from diarrhoeal stool samples in developing countries (WHO, 2013). This is in accordance with studies recently conducted by Bonkoungou et al., (2010) in Burkina Faso,
West Africa which concludes that Rotavirus and diarrhoeagenic *E. coli* are the most common causes of childhood diarrhoea.

Protozoans are concomitant with about 10% of childhood diarrhoea with *Giardia lamblia* being the most common protozoan. Other commonly isolated protozoans are *Cryptosporidium* spp. and *Entamoeba histolytica* (Elliott, 2007). *Giardia lamblia* in particular is more prevalent in developing countries and travelers to high incident areas and also amongst day care attending children and homosexuals (Escobedo *et al.*, 2010). It is important to note that the load of pathogens required to cause infection and become symptomatic varies between pathogens from as many as $10^8$ for *Vibrio cholera* to as few as $10^0$ for *Cryptosporidium* spp. (Mandell, 2010). This notwithstanding, the mode of transmission is almost always fecal-oral transmission.

### 2.4.2 Non-infectious causes

Occasionally, diarrhoea results from non-infectious sources. Some medications such as NSAID and foods with lactose consumed by lactose intolerant individuals do elicit diarrhoeal symptoms. Diseases such as Inflammatory Bowel Disease and Irritable Bowel Syndrome all cause diarrhoea (O’Ryan *et al.*, 2010). Other causes of non-infectious diarrhoea are chronic ethanol ingestion, ischemic bowel disease, bile salt malabsorption and microscopic colitis. Certain hormones such as serotonin, when excreted in excess by hormone-secretion tumors also cause diarrhoea. Interestingly too, chronic mild diarrhoea
in infants and toddlers may arise without any apparent infection. This is known as Toddler’s diarrhoea. (Wedlake et al., 2009; Kasper et al., 2005).

2.5 Treatment of diarrhoea

Treatment of diarrhoea is usually supportive. Oral rehydration therapy has been the cornerstone of diarrhoea treatment programmes to prevent the life-threatening dehydration. Fluid replacements should begin at home and administered on the onset of a diarrhoeal episode (WHO, 2009). A new formula has been developed including a zinc regiment that improves the overall outcomes when compared to the original version. Addition of zinc reduces both the duration and severity of diarrhoea episodes as well as reduce stool volume and the need for advanced medical care. Additionally, children on this new ORS often tend to have greater appetites and are more active during the diarrhoeal episodes (Lazzerini & Ronfani, 2008). In the event that ORS is not available, other fluids prepared at home using readily available low-cost ingredients such as cereal-based drinks made from a thin gruel of rice, maize, potato or other readily available low-cost grain or root crop available at home. An excellent drink for fluid replacement is breastmilk and should continue to be given to infants with diarrhoea simultaneously with other oral rehydration solutions (WHO, 2013). Children who have inflammatory or bloody diarrhoea should not be given antimotility agents. Patients who have no laboratory evidence of hemolysis, thrombocytopenia or nephropathy 3 days after diarrhoea resolves, have a low risk of Hemolytic Uremic Syndrome (HUS). However, individuals with hemorrhagic colitis should have a careful follow-up consisting of complete blood cell count with smear, blood
urea nitrogen concentration and creatinine levels to detect changes that suggest HUS (CDC, 2004).

People with travelers’ diarrhoea with three or more loose stools in an 8-hour period with nausea, vomiting, abdominal cramps, fever or blood in stools may benefit from antimicrobial therapy. Antibiotics regimens are usually between 3-5 days. Commonly prescribed antibiotics include ciprofloxacin and norfloxacin. Patients suspected of having systemic infection should be given parenteral antimicrobial therapy (CDC, 2005).

2.6 *Escherichia coli*

*Escherichia coli* (*E. coli*) is a Gram-negative, rod shaped bacteria commonly associated with the lower intestine of warm animals as part of the normal flora of the gut where it produces Vitamin K₂ to the benefit of the host and also prevents the establishment of pathogenic bacteria within the intestine (Reid *et al.*, 2001). Most *E. coli* strains are harmless but some serotypes do cause serious food poisoning illness in humans and have been implicated in food poisoning and product recalls due to food contamination (Vogt & Dippold, 2005). Together with related bacteria, *E. coli* constitutes about 1% of gut flora with fecal-oral transmission being the foremost route of infection by pathogenic strains culminating in disease. The ability of cells to persist outside the body for a limited time facilitates the use of *E. coli* cells as an ideal indicator organism to test environmental samples for fecal contamination (Eckburg *et al.*, 2005; Feng *et al.*, 2002).
*E. coli* is facultative anaerobic and non-sporulating motile bacterium with its typically rod-shaped cells about 2.0µm long and 0.5µm in diameter with a cell volume of 0.6-0.7 (µm)³. *E. coli* is oxidative negative. It ferments glucose, sucrose and lactose with an optimum pH of 6.0-7.0 and temperature of 37°C. Some laboratories strains have however been known to reproduce at temperatures as high as 49°C (Fotadar *et al.*, 2005). Since many pathways in mixed-acid fermentation produce hydrogen gas, these require the levels of hydrogen to be low, as is the case where *E. coli* cohabitates with hydrogen-consuming organisms such as methanogens or sulphate-reducing bacteria (Madigan and Martinko, 2006). Growth in *E. coli* is known to be driven by either anaerobic or aerobic respiration via an array of redox pairs including the oxidation of pyruvic acid, formic acid, hydrogen and amino acid coupled with the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide and trimethylamine N-oxide (Ingledew and Poole, 1984). It has a protective outer membrane and an inner plasma membrane enclosing the cytoplasm and nucleoid. Between the inner and outer membranes is a thin but strong layer of peptidoglycans which gives the cells its shape and rigidity. The plasma membrane and the layers outside constitute the cell envelope. Differences in the cell envelope account for the variations in affinity for the Gentian Violet dye forming the basis for the Gram’s stain; Gram-positive bacteria retain dye while Gram-negative bacteria do not (Chakraborty, 2003). From the outer membrane of *E. coli* and some other eubacteria protrude short hair-like structures called pili by which the cells adhere to the surface of other cells. Some strains of *E. coli* in addition possess long peritrichous flagella that help the bacteria move through its aqueous surroundings (Chakraborty, 2003).
*Escherichia coli* exhibit the quality to transfer DNA through bacterial conjugation, transduction or transformation thus allowing genetic material to spread horizontally through an existing population. This largely account for the spread of genes encoding shiga-toxin from *Shigella* to *E. coli* O157:H7 carried by a bacteriophage (Brussow *et al.*, 2004).

*E. coli* possess a special subdivision system that is based on O (somatic), H (flagella) and K (capsular) surface antigen profiles. Though more than 175 O antigens and 53 H antigens are currently recognized, the number of serotype combinations responsible for the diarrhoeal disease is quiet small (Nataro & Karper, 1998).

### 2.6.1 Pathogenic *E. coli*

Some strains of *E. coli* exhibit pathogenicity via possession of virulence factors thus allowing them to cause disease. These virulence factors include toxins (heat labile and heat stable), adhesion or colonization factors, Type 2 secretion systems (T2SSs), Type 3 secretion systems (T3SSs) and plasmids. These virulence determinants facilitate their role as causative organisms in both humans and animals (Kaper *et al.*, 2004). These pathogenic *E. coli* are responsible for the three main classes of clinical infection namely; (i) enteric or diarrhoeal disease (ii) urinary tract infections and (iii) meningitis/septicemia. Alternatively, based on their peculiar virulence and clinical presentations of the host, pathogenic *E. coli* may be typed as Diarrhoeagenic *E. coli* (DEC), Uropathogenic *E. coli* (UPEC), Neonatal meningitis/sepsis associated *E. coli* (NMEC) (Dawson *et al.*, 1999).
Diarrhoeagenic *E. coli* (DEC), are amongst the most frequently isolated and versatile pathogenic group amongst bacteria that cause diseases in man (Campos *et al.*, 2004). Transmission of most DEC occurs from the consumption of food and water contaminated with human or animal faeces. Thus person-to-person transmission from an infected symptomatic individual or asymptomatic carrier can thus be an important mechanism for secondary spread (Russo, 2006). DEC is divided into six well-characterized classes on the basis of clinical manifestation, phenotypic traits, specific virulence properties and pathogenesis. These classes are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), verocytotoxin producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC) (Nataro & Karper, 1998). Associated clinical manifestations comprise bloody diarrhoea and hemolytic uremic syndrome (EHEC), bacillary dysentery-like diarrhoea (EIEC), childhood and traveler’s diarrhoea (ETEC), infantile diarrhoea (EPEC) and acute and persistent diarrhoea in children and adults (EAEC) (Gascon *et al.*, 1998). Some DEC require fewer than 1000 colony forming units to facilitate pathogenesis while others require a great deal more. Laboratory diagnosis of DEC is generally by serotyping from stool samples, enzyme immunoassay (e.g. Immunoblotting), culturing or by other molecular methods such as PCR and colony blots (Lopez-Saucedo, 2003). Identification of pathotypes is via detection of their peculiar respective virulence-associated factors. Regrettably, diagnosis of DEC is challenging for most clinical laboratories due to the difficulty in differentiation of pathogenic strains of *E. coli* from non-pathogenic *E. coli* present in stool. Such diagnosis techniques with differential prowess is the preserve of reference laboratories and research settings (Voetsch, 2004). Differentiation of DEC pathotypes from commensal *E. coli* isolated from stool samples is done by screening for
peculiar genes which include: *eaeA* gene which mediates intimate attachment to epithelial cells and the *bfpA* which expresses bundle forming pili for EPEC; pCVD432 encoding for EAEC; *stx1* and *stx2* for EHEC; *IPA* of EIEC; and *ST* and *LT* of ETEC (Schmidt *et al.*, 1995; Nada *et al.*, 2010). EAEC pathogenesis and epidemiology is less clear but are associated with the presence of a large 60-kD plasmid encoding different virulence factors and toxins (Presterl *et al.*, 2003). These include: *aggR*, a transcriptional activator; *aggA*, fimbriae AAF I; *aafA*, fimbriae AAF II; *agg3A*, fimbriae AAF III; *astA*, aggregative stable toxin 1 (EAST 1); *pet*, plasmid-encoded heat-labile toxin; *aap*, anti-aggregation protein; and *pic*, a protein involved in colonization (Yamamto and Nakazawa, 1997; Kahali *et al.*, 2004).

2.6.2 Enterotoxigenic *Escherichia coli* (ETEC)

This is one of the most studied pathotypes of DEC with an established association with infantile diarrhoea especially after weaning. Epidemiological studies implicate contaminated food and water as the most common route of infection. A high infectious dose, ranging from 106 to 1010 CFU, of ETEC has been demonstrated in humans with a large number of serogroups of ETEC being associated with diarrhoea (Oberhelman *et al.*, 1998).

ETEC strains elicit cholera-like watery diarrhoea via the amplification and action of LT (heat-labile) and/or ST (heat-stable) enterotoxins. The ETEC strain is defined by the ability to produce LT and/or ST enterotoxins leading to diarrhoeal illness. To perpetuate diarrhoea
by LT and/or ST, ETEC must first adhere and colonize the intestinal mucosa by attaching with one or several colonization factor antigens (CFAs). These are antigenically diverse and usually encoded by plasmids (Vila et al., 1998). The heat-labile toxins of ETEC are oligomeric toxins that are functionally and structurally similar to the cholera toxin (CT) expressed by *Vibrio cholerae*, serogroups O1 and O139 (Sixma et al., 1993). Two variants of LT occur in humans and animal isolates; LT-I and LT-II. However, the term LT primarily refers to LT-I which is associated with disease in both humans and animals alike whereas LT-II is expressed only in animals and rarely associated to disease (Nataro & Karper, 1998). Constitutionally, LT-I is approximately 80% amino acid identical with CT containing a single A subunit and five identical B subunits. The enzymatic activity is coded by the A subunit while the B subunits are responsible for the toxin binding to the cell surface ganglioside. After endocytosis, the A subunit stimulates a series of intracellular processes leading to an increased level of cyclic adenosine monophosphate (cAMP) culminating in an increased phosphorylation of chloride channels. Hence a reduced absorption sodium chloride. This increased extracellular ions content results in osmotic diarrhoea (Oberhelman et al., 1998).

*E. coli* heat-stable toxins are small peptides of two unrelated classes; STa and STb. These differ in structure and mechanism of action. Genes for both classes, *estA* and *estB*, have been found either on plasmids or on transposons. Similarly, only toxins of STa class has been associated with disease in humans and animals. Studies show that the STa receptor is located on the apical surface of enterocytes and binding to the receptors leads to an increased intracellular cyclic guanidine monophosphate (cGMP) level. This affects the
electrolytic balance similar to the action of LT (Kaper et al., 2004; Nataro & Karper, 1998). STb in contrast is associated with diarrhoea in piglets. Some human ETEC isolates expressing STb have however been reported (Schulz et al., 1990). STb can elevate cytosolic Ca\(^{2+}\) concentrations, stimulating both the release of prostaglandin E2 and serotonin, which lead to increase ion secretion (Kaper et al., 2004).

The clinical features of diarrhoea from ETEC infection are consistent with the pathogenic mechanisms of its enterotoxins, and characterized by watery diarrhoea without blood, mucus or pus, fever and vomiting. The onset of the diarrhoea is characteristically abrupt but may vary from mild, brief and self-limiting to a severe disease similar to in *Vibrio cholerae* infection (DuPont, 2009; Levine et al., 1977). Available data suggests a prevalence of between 10 to 30% with clinical presentation and prevalence, as is observed amongst other DECs, varies across different geographical areas (Mangia et al., 1993). The mode of transmission of ETEC is through the consumption of faecally contaminated food or water (Quadri, 2005).

### 2.6.3 Detection of ETEC

Detection of ETEC is primarily by detection of LT and/or ST enterotoxins. ST was originally detected in a rabbit ligated ileal loop assay (Evans et al., 1973). However, the cost involved and the lack of standardization caused the test to be replaced with the suckling-mouse assay which became the gold standard for the detection of STa for many years. This assay entails the measurement of intestinal fluid in infant mice after
percutaneous injection of culture supernatants (Gianella, 1976). In recent past, immunoassays including radioimmunoassay and ELISAs have been developed for the detection of ST. Both these tests tally well with results of the suckling-mouse assay and require significantly less expertise (Cryan, 1990). Amongst the pathogenic organisms for which molecular diagnostic techniques were developed were the ETEC strains. As early as 1982, DNA probes were found to be useful in the detection of LT and ST encoding genes in stool and environmental samples. Years down the line, several advances in ETEC detection has occurred but genetic techniques continue to attract the most attention and use. Though there is no perfect test for the detection of ETEC, the detection of LT and/or ST defines an ETEC isolate although many such isolates will express colonization factors specific for animals and thus lack human pathogenicity (Vila et al., 1998). Several PCR assays for ETEC detection are very sensitive and specific when used on clinical samples or isolated bacterial colonies. A useful adaptation of PCR is multiplexing. However, most amplicons are usually differentiated easily by product size, a second detection step is generally required to identify the respective PCR products definitely (Okeke et al., 2000).

### 2.7 Shigella species

*Shigella* is a genus of gram-negative, non-spore forming, non-motile, rod shaped bacteria closely related to *E. coli* and *Salmonella* and responsible for human shigellosis. At infection shigellosis presents as dysentery and affects only humans and apes and no other animals (Ryan & George, 2004). Individuals with shigellosis develop diarrhoea, fever and stomach cramps within a day or two after infection. The diarrhoea is characteristically bloody, fever, nausea, vomiting, stomach cramps and flatulence and resolves between 5 to 7 days post
infection. However, in immunocompromised individuals such as children and the elderly, the diarrhoea may aggravate and patients might require hospitalization. Severe shigellosis compounded with high fever often results in seizures in children under 2 years of age. Some infected individuals are however asymptomatic but may act as carriers and infect others. Traditionally, shigellosis is diagnosed by the laboratory tests that may identify *Shigella* in stools samples of infected individuals (Clark, 2012).

Transmission of *Shigella* is primarily via the faecal-oral route by the passing of soiled fingers to the mouth of another person. A consequence of inadequacies in basic hygiene and sanitation especially in the developing world. Infection usually occurs in toddlers who are generally not toilet trained and more rampant at day care centers where children from divergent backgrounds come together. Transmission is also foodborne and waterborne. The efficiency of the transmission largely has to do with the little amount of organisms (≤100 bacterial cells) needed to cause illness. Food handlers are a major source of contamination as well as vegetables grown on fields with contaminated sewage. It is also possible for the transmission of *Shigella* by flies. Swimming or drinking contaminated water is a sure way to get infected with this enteropathogen (Hogue, 2000).
2.7.1 Biology and biochemistry of *Shigella spp.*

Upon ingestion, the bacteria survive the gastric environment of the stomach and makes its way to the large intestine. Where it attaches and penetrates the epithelial cells of the intestinal mucosa. Post invasion, *Shigella* multiplies intracellularly and spreads to neighbouring epithelial cells resulting in tissue destruction and representative pathology of shigellosis. Generally, *Shigella* adheres to the membrane of the cell and is internalized by an endosome which it subsequently lyses to gain access to the cytoplasm where multiplication occurs (Presterl *et al.*, 2003). *Shigella* invades the host through the M-cells in the gut epithelia of the small intestine, as they cannot enter directly through the epithelial cells. Using a Type III secretion system acting as a biological syringe, the bacterium injects Invasion plasmid antigen D (IpaD) proteins into cells, triggering bacterial invasion and the subsequent lyses of vacuolar membranes using IpaB and IpaC proteins (Kotloff *et al.*, 1999). Extracellular *Shigella* is not motile but intracellularly it is able to move occupying the entire cytoplasm of the infected cell and between cells. It uses a mechanism for its motility by which its *IcsA* and *IcsB* proteins trigger actin polymerization in the host cell (via N-WASP recruitment of Arp2/3 complexes) in a rocket propulsion fashion for cell-to-cell spread (Levinson, 2006). Specifically, movement between adjacent cells is facilitated by the *IcsA* protein.

After successful epithelial cell invasion and penetration of the colonic mucosa by the bacteria, there is degeneration of the epithelium and inflammation of the lamina propria culminating in desquamation and ulceration of the mucosa and subsequent leakage of blood, inflammatory elements and mucus into the intestinal lumen (Kotloff *et al.*, 1999).
Thus the characteristic passage of frequent, and scanty dysenteric stool mixed with blood and mucus. Absorption of water by the colon is inhibited under these conditions (Yang, 2005).

Some strains of *Shigella* produce enterotoxins and shiga-toxins similar to the verotoxin of *E. coli* O157:H7. The toxin has a molecular weight of 68kDa and is a multi-subunit protein consisting of one molecule of an A subunit (32,000 MW) and five molecules of the B subunit (7,700 MW). Both shiga-toxins and verotoxins are associated with hemolytic uremic syndrome (HUS), Hemolytic colitis and dysentery (Levinson, 2006). The names of these conditions are dependent on the causative organism and symptoms range from severe diarrhoea, abdominal pain, vomiting and bloody urine. Each of the *Shigella* genomes includes a virulence plasmid that encodes conserved primary virulence determinants. The *Shigella* chromosomes share most of their genes with those of *E. coli* K12 strain MG1655 (Yang, 2005). No antidote exists for these toxins. Thus supportive care requires maintenance of fluid and electrolyte levels and monitoring and support for kidney function. Inactivation of the toxin is achieved by steam treatment, oxidizing agents such as bleach and chemical sterilizing agents such as glutaraldehyde. The toxin acts on the lining of the blood vessels, the vascular endothelium. The B subunits of the toxin bind to a cell membrane component, Gb3, and the complex enters the cell. Once inside, the A subunit interacts with the ribosomes to inactivate them. The A subunit of the shiga-toxin is an N-glycosidase that modifies the RNA component of the ribosome to inactivate it and thereby bring a halt to protein synthesis of the cell leading to cell death (Ito *et al.*, 1991). The vascular endothelium has to continually renew itself. Hence, cell death leads to breakdown
of the lining leading to hemorrhage. The primary response is characteristically bloody diarrhoea. For unexplained reasons, the toxin is seemingly effective against small blood vessels such as found in the digestive tract, kidneys and lungs but not against vessels such as the arteries or major veins. A specific target for the toxin appears to be the vascular endothelium of the glomerulus destroying the structures and concluding in kidney failure and the development of the often deadly and frequent debilitating hemolytic uremic syndrome. Food poisoning with shiga-toxin often has an effect on the lungs and the nervous system. (Ito et al., 1991).

2.7.2 Types of *Shigella* spp.

*Shigella* species are classified under four main serogroups (A, B, C and D). Serogroup A known as *S. dysenteriae* has 12 serotypes. Serogroup B, *S. flexneri* has 6 serotypes. Serogroup C, *S. boydii* has 18 serotypes and serogroups D, *S. sonnei*, has 1 serotype. With the exception of serogroups D which can be differentiated on the basis of biochemical metabolism assays, serogroups A to C are physiologically similar. *S. flexneri* is the most frequently isolated species worldwide accounting for 60% of cases in the developing world with *S. sonnei* accounting for 77% of cases in the developed world. *S. sonnei* is also responsible for 15% of cases in the developing world. *S. dysenteriae* is believed to be the cause of epidemics of dysentery, especially in confined populations such as refugee camps and slums (Hale & Keusch, 1996; WHO, 2012).
About 3% of infected individuals with *Shigella flexneri* may go on to develop eye irritations, joint pains, and painful urination which may last anywhere from a month to many years. This condition is known as Reiter’s syndrome. This can further develop into chronic arthritis (Hill & Lillicrap, 2003). Persons with the Human Leukocyte Antigen B27 (HLA-B27) have a genetic predisposition of developing this syndrome as a late complication of *S. flexneri*. HLA-B27 has been strongly associated with a set of autoimmune diseases referred to as seronegative spondyloarthropathies. A complication of *S. dysenteriae* is the hemolytic uremic syndrome (HUS). This presents as convulsions in children resulting from a rapid increase of temperature coupled with metabolic alterations. It is also associated with production of the Shiga toxin. (Ram et al., 2008).

### 2.7.3 Treatment of Shigella

Treatment of Shigella is by antibiotics. Commonly prescribed antibiotics include ampicillin, nalidixic acid and the fluoroquinolone, ciprofloxacin. These work by killing the bacteria in the gastrointestinal tract and shorten and resolving the illness. Regrettably, *Shigella* has developed resistance to many of these antibiotics the direct consequence of inappropriate use of antibiotics to treat shigellosis and other bacterial infections. Individuals with mild infections do not require antibiotics as the diarrhoea resolves within a day or two (Clark, 2012). Antidiarrhoeal agents such as Imodium or diphenoxylate with atropine (Lomotil) should not be administered during infections as they tend to worsen the illness. Antibiotics should only be administered in severe cases. Though complete recovery after treatment occurs, it may take several months before normal bowel habits are restored. Also, an amount of immunity is conferred on an individual from a specific type of *Shigella*
for several years after treatment of shigellosis. This immunity is presumably due to IgA. Currently no licensed vaccine is available on the market although there are several candidates at various trial stages (Christopher et al., 2010).
CHAPTER THREE

3.0 METHODOLOGY

3.1 Research design, population and setting

The study was cross-sectional with laboratory molecular analysis of stool samples collected between January 2008 and December 2009, of children younger than 5 years of age hospitalized for longer than 24 hours with acute gastroenteritis at the Child Health Department of Korle Bu Teaching Hospital (KBTH) and Princess Marie Louise Children’s Hospital (PML), Accra, Ghana. Samples had been collected and used by Enweronu-Laryea et al., (2012) for a survey on the prevalence of severe acute rotavirus gastroenteritis and intussusceptions in Ghanaian children under 5 years of age. Gastroenteritis was defined as \( \geq 3 \) watery stools voided within 24 hours for \( \leq 7 \) days.

3.2 Sample size

A total of 200 archived stool samples were used. These were randomly sampled from the total samples collected for the survey by Enweronu-Laryea et al., (2012).

The sample size was determined using the equation below;
\[ n = Z^2 \frac{(P)(1 - P)}{\text{Error}^2} \]

\[ Z = 1.96; \ P = 0.04 \text{ (Bonkoungou et al., 2013); Error} = 0.05 \]

\[ n = \frac{1.96^2(0.04)(1 - 0.04)}{0.05^2} \]

\[ n = 59.007 \]

The minimum sample size was approximately 59 patients. However, a total number of 200 samples were used for the study. This was to ensure statistically significant results and also rule out chance variations.

### 3.3 Data on study samples

Clinical information on the gender and age of randomly chosen subjects were recorded. Additional information on dates stool samples were collected and residence/location of subjects were also obtained from file and recorded. Finally, published results of the rotavirus survey by Enweronu-Laryea et al., (2013) on the samples were also sought, obtained and recorded. All recorded data were entered into a pre-designed data sheet for subsequent analysis by SPSS version 20 with a confidence interval of 95% and \( p < 0.05 \).
3.4 Sample collection and preparation

Stool samples from participating children had been collected by trained healthcare personnel using sterile wide-mouthed containers and transferred to the Virology Laboratory of the Korle-bu Teaching Hospital where after screening by ELISA for Rotavirus were sent to the Department of Electron Microscopy/Histopathology for storage at -20°C. Stool samples were obtained from -20°C storage freezers and allowed to thaw on the bench prior to DNA extraction. A 10-fold dilution in sterile distilled water was made for processing with FastDNA SPIN kit (MP Biomedicals, Santa Ana, CA) as described by Layton et al., (2006).

3.5 Fecal DNA Extraction

The FastDNA SPIN kit (MP Biomedicals, Santa Ana, CA) was used to extract genomic DNA from the stool samples. Nine hundred and eighty micro litres (980 µl) of Sodium Phosphate Buffer was added to 300 µl of fecal slurry and mixed well. The suspension was then added to a Lysing Matrix E tube and 122 µl of MT Buffer added. These were then shaken to mix properly. It was ensured that the volume of sample and Lysing Matrix does not exceed more than 7/8 of the tube thus improving chances for better homogenization. Each tube was subsequently vortexed at maximum agitation for 60 s ensuring that the beads in the tube are thoroughly agitated throughout the entire tube. The Lysing Matrix E tube was then centrifuged at 14,000 g for 30 s and supernatant transferred into a clean tube. Two hundred and fifty microliters of PPS reagent was now added to the tube and mixed by shaking the tube by hand 10 times after which it was centrifuged at 14,000 g for 5 min to
pellet any precipitates. The supernatant was decanted into a 15 ml tube and 1 ml of resuspended Binding Matrix suspension added. The tubes were then placed on a rotator for 2 min to enable binding of DNA after which the tube were placed in a rack on the bench for 3 min to allow settling of silica matrix. Seven hundred and fifty microlitres (750 µl) of the supernatant was then removed and discarded without disturbing the settled Binding Matrix. The Binding Matrix was resuspended in the remaining amount of supernatant and 750 µl of the mixture transferred into a SPINTM filter and centrifuged at 14,000 g for 1 min. The catch tube was emptied, the supernatant and suspension mixed again, added to the SPINTM Filter and spun again. This was repeated until all of the suspension had been loaded onto the filter. Five hundred microliters (500 µl) of SEWS-M was then added to the SPINTM filter and centrifuged at 14,000 g for 1 min after which the flow-through was decanted and the SPINTM filter in the catch tube replaced and centrifuged a second time at 14,000 g for 2 min to “dry” the matrix of the residual solution. The catch tube was then discarded and replaced with a new, clean catch tube. The SPINTM Filter was subsequently air dried for 5 min at room temperature on the bench. One hundred microliters (100 µl) of DES (DNase/Pyrogen Free Water) was then added and the matrix on the filter paper gently mixed by finger flips to resuspend the silica for efficient elution of the DNA. Finally, the samples were centrifuged at 14,000 g for 1 min to transfer eluted DNA to the catch tube and the SPIN filter discarded. Extracted DNA was subsequently stored in a -20°C storage freezer until needed.
3.6 Singleplex Polymerase Chain Reaction

The extracted DNA was amplified by conventional PCR using primers specific to the genes of interest for the identification of ETEC and *Shigella*. Three separate PCRs reactions were employed for the amplification of the DNA fragments.

The primers *LT-1* and *LT-2* which amplify the 132 bp *elt* gene for ETEC-LT toxin and primers *ST-1* and *ST-2* which amplify the 190 bp *est* gene for ETEC-ST toxin were used for the detection of Enterotoxigenic *E. coli*. Amplification reactions for LT-PCR assays contained 5X Green GoTaq Reaction Buffer, 10 µM of each dNTP and 2.5 µM of each primer, 5U GoTaq DNA Polymerase (PROMEGA, Madison WI, USA) and 3 µl of template DNA in a final reaction volume of 25 µl. Samples were amplified in an Eppendorf Mastercycler Personal 5332 (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturing step at 95°C for 3 min, followed by 30 cycles beginning with a 10 sec denaturing for 95 °C, primer annealing at 60 °C for 20 sec, 72 °C elongation for 30 sec and a final extension at 72 °C for 7 min. The 25 µl ST-PCR assays contained 5X Green GoTaq Reaction Buffer, 10 µM of each dNTP and 2.5 µM of each primer, 5U GoTaq DNA Polymerase (PROMEGA, Madison WI, USA) and 3 µl of template DNA. Temperature conditions performed in the Eppendorf Mastercycler Personal 5332 (Eppendorf) were an initial denaturing step at 95 °C for 3 min, followed by 30 cycles beginning with a 10 sec denaturing for 95 °C, primer annealing at 55 °C for 20sec, 72 °C elongation for 30 sec and a final extension at 72 °C for 7 mins.
To detect the *Shigella spp.*, the *IpaB-F* and *IpaB-R* primer set, forward and reverse primers respectively, which amplify the 120 bp *ipaB* (invasion plasmid antigen B) gene of *Shigella spp.* was used. Each 25 µl reaction mix contained 5X Green GoTaq Reaction Buffer, Bovine Serum Albumin (BSA), 10 µM of each dNTP and 2.5 µM of each primer, 5U GoTaq DNA Polymerase (PROMEGA, Madison WI, USA) and 3 µl of template DNA. Thermocycling conditions were 95 °C for 3 min and 30 cycles of 95 °C for 10 sec, 60 °C for 20 sec and 72 °C for 30 sec and a final extension of 72 °C for 7 min. Table 3-1 shows the nucleotide sequence of the primer pairs used for the PCR and the expected amplicon sizes.

Hypure Molecular Biology Grade Water (Hyclone laboratories Inc. South Logan, Utah) was used to make up the volumes of all PCR assays to 25 µl. A master mix was made without the template DNA, vortexed to mix and pulse centrifuged before aliquoting out into the PCR tubes after which the DNA template was added. Both the negative control and positive control were prepared according to the same protocol.
Table 3-1: Primer pairs used in PCR indicating nucleotide sequence and expected amplicons sizes

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> LT</td>
<td>LT – 1</td>
<td>5’ AGCAGGTTTCCCCACCGGATCACCA 3’</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>LT – 2</td>
<td>5’ GTGCTCAGATTCTGGGTCTC 3’</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ST</td>
<td>ST – 1</td>
<td>5’ GCTAATGTTGGCAATTTTTATTTCTGTA 3’</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>ST – 2</td>
<td>5’AGGATTACAACAAAGTTTCACAGCAGTAA 3’</td>
<td></td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>Ipa – B F</td>
<td>5’ GACGCCCAAGCCTTGAGCA 3’</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Ipa – B R</td>
<td>5’ AGCAGCGACCAGGGAATTCCT 3’</td>
<td></td>
</tr>
</tbody>
</table>

3.7 Agarose gel electrophoresis

After amplification reactions, 10 µl of amplicons each were separated on an ethidium bromide stained 1.5 % agarose gel (AGTC Bioproducts Ltd, UK) in 1X TAE buffer through gel electrophoresis at 120 volts for 45 min. A 100 base pair nucleotide sequence molecular size marker (PROMEGA, Madison WI, USA) was run alongside the amplicons on the gel. The gel was photographed using the Benchtop 2UV Transilluminator (Upland, CA USA) and pictures analyzed.
3.8 Ethical considerations

The study was approved by the Ethical and Protocol Review Committee (EPRC) of the UGMS. Permission was also obtained from the Ethical and Review Committee (ERC) and the Institutional Review Board (IRB), both of NMIMR, for the use of the archival stool samples and bench space.

3.9 Statistical analysis

To avoid any experimental biases, stool samples were coded before testing and only decoded for purposes of analysis. The data obtained from the tests were analyzed using IBM Statistical Package for Social Sciences (SPSS) Statistics version 20. Qualitative variables were summarized by proportions and percentages while a test of association between ETEC and Rotavirus infections and the subjects’ demographic data were summarized by odds ratio (OR). Statistical significance was defined as $p < 0.05$. 

CHAPTER FOUR

4.0 RESULTS

4.1 General characteristics and demography of study population.

A total of 200 archived stool samples were retrieved from storage at the Department of Electron Microscopy/Histopathology of NMIMR and used for the study. All the stool samples were watery of varying consistency from mucoid to very watery. None of the stool samples were bloody. Clinical features of watery diarrhoea (87%) and vomiting (42%) were reported in the study population. No cases of bloody diarrhoea were reported. Fever was reported to have occurred in 47% of the study population. The distributions of sex and age are shown in Figure 4-1 and Table 4-1 respectively. A total of 124 (62%) were males and 76 (38%) were females. The children were aged between 0-42 months with 68.5% of the children falling in the 0-12 month category. The mean age (months) was 10.93 ± 7.27 with the modal age of 9 months. The youngest child was only 5 days old with the eldest aged 42 months. Figure 4-2 shows the distribution of the sexes of the patients with respect to the age categories.

Available data on the residence of the patients indicate that majority of the patients reside in and around the two health centers in places such as Korle Gonno, Kaneshie, Chorkor, Mamprobi, James Town, Laterbiokorshie, Dansoman, Bukom, Odorkor and Sukura just to mention a few. Few patients however, were recorded to have come from as far as Kasoa in the Central Region and Taifa, Dome, Tantra Hill and even Kutunse which are on the eastern limit of Greater Accra.
Figure 4-1: A bar graph of frequency of the sex of children admitted for diarrhoea at KBTH and PML between November 2011 and December 2012. The greater proportion of the children admitted for diarrhoea at the two health centers were males.
Table 4-1: Age distribution of subjects used in the study

<table>
<thead>
<tr>
<th>AGE CATEGORY (MONTHS)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 12</td>
<td>137(68.5)</td>
</tr>
<tr>
<td>13 – 24</td>
<td>53(26.5)</td>
</tr>
<tr>
<td>25 - 59</td>
<td>10(5)</td>
</tr>
<tr>
<td><strong>ALL</strong></td>
<td><strong>200(100)</strong></td>
</tr>
</tbody>
</table>

The majority of diarrhoea cases fell within the 0-12 month’s age group with a steady decline as the subjects approached their fifth birthday.
**Figure 4-2:** Distribution of the sex of subjects within the age categories
4.2 Electrogram of Polymerase Chain Reaction products

The amplified *elt*, *est* and *ipaB* genes were detected with the primer pairs described in Table 3.1. The nucleotide sequences, specific region of organisms amplified in the 25µl reaction mixtures in the 30 PCR cycles as well as the size of the amplicons are all available in the table below. The amplicons were neatly separated on the 1.5% agarose gels stained with ethidium bromide. The PCR assays could not be multiplexed because of the difference in annealing temperatures of the respective primers. Primers *LT-1* and *LT-2* anneal at 60°C with no bands forming at temperatures below this. *ST-1* and *ST-2* primers only annealed between 55-57°C with band streaks forming above this temperature range. *Ipa-B* primers specific to *Shigella* equally anneals at 60°C as *LT* primer. However, amplicons from the *Ipa-B* amplification reaction and *LT* amplifications are 120bp and 132bp respectively. A difference of only 12bp. These bands were indistinguishable when the ETEC-LT and *Ipa-B* assays were multiplexed and amplicons viewed on the agarose gel under UV light.
**Figure 4-3:** A sample gel electrogram of an ETEC- LT PCR amplicon.

Lane 1 contains the 100bp molecular weight marker. Lanes 2-7 contained amplicons of samples from PCR. Lane 4 shows a band of 132bp indicative of the correct amplicons size for the elt gene of E. coli- LT. Lane PC contained positive control (purified E. coli DNA). NC contained negative control (no DNA).

**Figure 4-4:** A sample gel electrogram of a *Shigella spp.* PCR amplicon.

Lane 1 contains the 100bp molecular weight marker. Lanes 2-7 contained amplicons of samples from PCR. Lane PC contained positive control (purified *Shigella* DNA). The 120bp band in lane PC is indicative of the correct amplicon size for the *IpaB* gene for *Shigella spp.* NC contained negative control (no DNA).
4.3 Occurrence of enteric pathogens

Of the 200 stool samples screened, multiple pathogens were detected in 4 (2%) of the stool samples and no pathogen was detected in 16 (8%) of the stool samples. Rotavirus had been detected in 182 of the stool samples from the gastroenteritis survey study as reported by Enweronu-Laryea et al., (2012). All ETEC samples detected via the PCR were of the heat labile strain (ETEC-LT). No ETEC pathogen of the heat-stable (ETEC-ST) strain was detected from the stool samples. The IpaB gene specific to Shigella spp. was not detected in any of the samples. The positive controls for the ETEC-ST and Shigella however showed clearly on the gel when viewed under UV light certifying the fidelity of their respective assays. The distribution of pathogens in the stool samples is shown in Table 4-2.

All four ETEC-LT pathogens occurred in the group aged 0-12 months. Rotavirus and E. coli LT were simultaneously detected in 4 samples only. Concurrent presence of pathogens in a sample was determined to be statistically insignificant (p>0.05). None of the samples contained genes for all the pathogens concurrently. Sixteen samples were negative for all three pathogens despite being clinically certified as diarrhoeal stools. The distribution of the five pathogens showed no association with sex (p>0.05).
Table 4-2: Frequency distribution of pathogens detected in stool samples

<table>
<thead>
<tr>
<th>ENTEROPATHOGEN</th>
<th>N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> ST</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em> LT</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Shigella</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rotavirus**</td>
<td>184</td>
<td>92</td>
</tr>
<tr>
<td>Mixed pathogens (ETEC LT and Rotavirus)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>No pathogens</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

** Results obtained from ELISA previously run on during previous diarrhoea surveillance by Enweronu-Laryea *et al.*, (2012).
Table 4-3: Age groups and enteropathogen characteristic of the 200 diarrhoea samples

<table>
<thead>
<tr>
<th>Age group(months)</th>
<th>Enteropathogens in stool samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>E. coli LT</strong></td>
</tr>
<tr>
<td>0 - 12</td>
<td>4</td>
</tr>
<tr>
<td>13 – 24</td>
<td>0</td>
</tr>
<tr>
<td>25 - 59</td>
<td>0</td>
</tr>
</tbody>
</table>

** Results obtained from ELISA previously run during the diarrhoea surveillance study by Enweronu-Laryea et al., (2012). All ETEC-LT infections occurred in the 0-12 month year group. Rotavirus infections were recorded in all three age categories with the highest frequency in the 0-12 month group. There was then a gradual decline in the number of subjects with rotavirus infection as the subjects grew older with the least number of infections in the 25-59 months’ group.

Table 4-4: Sex characteristic of ETEC-LT and Rotavirus infections

<table>
<thead>
<tr>
<th>SEX</th>
<th>ETEC-LT</th>
<th>Rotavirus**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>3</td>
<td>113</td>
</tr>
<tr>
<td>Females</td>
<td>1</td>
<td>71</td>
</tr>
</tbody>
</table>

** Results obtained from ELISA previously run on during previous diarrhoea surveillance study by Enweronu-Laryea et al., (2012). More males were detected to have a rotavirus infection as well as ETEC-LT infection. However it was determined that there was no association between rotavirus infection and sex (p=0.91). Similarly, it was determined that there was no association between ETEC-LT infections and sex (p= 0.60).
Table 4-5: Odds of association of ETEC-LT infection with age category

<table>
<thead>
<tr>
<th>Age Category (months)</th>
<th>Odds Ratio</th>
<th>95% C.I</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>4.2809</td>
<td>0.2270 – 80.7369</td>
<td>0.3318</td>
</tr>
<tr>
<td>13-24</td>
<td>0.2980</td>
<td>0.0158 – 5.6296</td>
<td>0.4194</td>
</tr>
<tr>
<td>25-59</td>
<td>1.9735</td>
<td>0.00995 – 39.1355</td>
<td>0.6556</td>
</tr>
</tbody>
</table>

Data from this study suggests no association between ETEC-LT infection and any particular age category.
5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

This study in addition to highlighting the burden of gastroenteritis in children under the five years of age, especially those less than fifteen months old, also illustrates the potential use of PCR as an alternative diagnostic technique for the routine detection of *Enterotoxigenic E. coli* and *Shigella spp.* Diarrhoea is the third commonest cause of visit of children under five years to health centers in Ghana and responsible for 13.1% of hospitalizations among children under five years of age (GHS, 2012; Enweronu-Laryea *et al.*, 2012). Available statistics from the Ministry of Health indicate 84 000 annual deaths in Ghana are attributed to gastroenteritis with about 25 per cent being children under five years of age (Ghana News Agency, 2003). This incidence of 25% is comparable to inpatient data from other African countries but much high as compared to similar studies from developed countries (Payne *et al.*, 2008; Waggie *et al.*, 2008). Gastroenteritis thus continues to be an important public health concern in Ghana and among many African and developing nations despite greater understanding of the bacteriology, epidemiology and public health aspects associated with the disease for more than a century (Opare *et al.*, 2012). Mortality aside, the long term effects of diarrhoeal disease on child health are particularly serious with far reaching negative consequences on cognitive and growth development which impacts on school performance (Brown, 2003).

In this present study, although all patients were 5 years of age or below, a greater proportion belonged to the 0-24 month group (95%). This is similar to findings by Presteri and others (2003) in their work in Germany. Many studies have also reported that diarrhoea is more
prevalence in children in this age group in both the developed and developing countries (Antikainen et al., 2009). The higher prevalence of diarrhoea in children under 18 month may be attributed to breastfeeding practices, poor hygienic conditions and lack of potable water. The World Health Organization recommends exclusive breastfeeding for the first six months of a baby’s life before complementary foods are introduced. This is however not the case in many developing countries with Ghana being no exception. In Ghana for instance, most infants are exclusively breastfed for three to four months after which complementary foods are introduced (Plenge-Bonig et al., 2010). This practice is due to certain socio-cultural norms which have been passed down from generations resulting from the misconception that breastmilk alone isn’t sufficient for the proper development of the baby thus the need to complement the diet of the infant (Antwi, 2008). This practice unfortunately exposes Ghanaian children to gastroenteritis infections since their immune system are not matured to manage the enteropathogens from the contaminated water and complementary foods (Gyimah, 2003). Transmission of diarrhoea is primarily through the fecal-oral route via contaminated foods and drinks, contact with contaminated objects and utensils and flies landing directly on children (van Ginneken and Teunissen, 1992).

Gyimah (2003) and Boadi and Kuitunen (2005) among many others have amply demonstrated the relationship between access to quality drinking water and sanitation in relation to the incidence of childhood diarrhoea. Ghana is recently touted to have attained middle income status. Paradoxically, many parts of Ghana including the national capital, Accra, where the subjects in this current study reside lack quality potable water and sanitation facilities. These households thus depend on water vendors, boreholes, wells and rain water. Additionally, about 33%, 30% and 58% of households still use traditional pit
latrines in Accra, Kumasi and Northern Ghana respectively. A smaller percentage simply engage in open defecation (Songsore, 2003). Numerous studies have shown a high incidence of childhood diarrhoea with households who depend on alternate sources of water such as water vendors and boreholes as the likeliness of contamination is very high. Storage of water in pots and barrels have also been found to be associated with high diarrhoea prevalence. The Millennium Development Goals (MDG) aspires to reduce the population without access to safe water and sanitation by half by 2015 (WHO, 2003). While the MDGs consider piped water as an improved water source, the transmission of gastroenteritis enteropathogens may still occur if periodic maintenance is absent or few and far between (Opare et al., 2012). Some behavioural factors that potentially result in high diarrhoea prevalence include failure to wash of hands after defecation, failure to wash hands before cooking and the buying of foods from street vendors. These are all consistent with findings in different parts of Ghana (Benneh et al., 1993; Mensah et al., 2002) and other African countries (Roberts et al., 2001). Of the 200 subjects, 124 (62%) were males with the remaining 76 (36%) being females. The male to female ratio thus being 1.63:1. This is also similar to finding of Bonkoungou and associates (2013), where they found the male to female ratio to be 1.13:1. There is however no immediate explanation for this observation. Unpublished data by Tidsskrift and Osumanu (2007) however suggest some socio-cultural underpinnings for this observation.

In this study, data collected from the diarrhoea surveillance study by Enweronu-Laryea et al., 2012 was used with permission. The advantage of this approach was that a large data set was realized, collected under routine diagnostic conditions. Results can thus be
described as indicative of the true assay performance since they were not biased due to study protocols using selected research technicians (Dragsted et al., 2004). Enteropathogens were detected in 184 of the 200 stool samples. Coinfection were found in 2% of the samples. Sixteen samples were negative for both ETEC and *Shigella spp*. Interestingly, these samples had also been negative for rotavirus during the gastroenteritis survey although they were clinically certified diarrhoea samples. Such variations in the prevalence of enteropathogens is expected as the occurrence of diarrhoea is reliant on factors such as age, seasonality, epidemicity, methods of sample collection and tools used detection just to mention a few (Antikainen et al., 2009).

Enterotoxigenic *Escherichia coli* of the LT strain was the only bacterial enteropathogen detected in the study with a prevalence of 2% with all positive cases occurring in the 0-12 month group. Recent studies have documented ETEC to frequently cause diarrhoea in infants younger than two years of age (Qadri et al., 2005). An assertion which is in accordance with findings of this present study. The susceptibility of infants and young children may be attributed to failure of most mothers in African countries to adhere to the exclusive 6 months breastfeeding as advised by the WHO, unhygienic weaning foods and food preparation wares and methods and the general poor sanitary and hygienic conditions ubiquitous in many African and developing countries (Rao et al., 2003). The incidence of ETEC infections however decrease between the ages of 5 to 15 years and then surprisingly increases beyond 15years into adulthood. (Qadri et al., 2000). The decrease in infection of ETEC after infancy and increase at adulthood may be attributed to both immunological and environmental factors. Immunogenetics and diversity which predispose an individual to
ETEC infections coupled with an increase immune response due to repeated infections in early childhood which may decrease due to fewer infections during adolescence might suggest the pattern of ETEC incidence across the different ages (Ako-Nai et al., 1990). About 25% of ETEC illness are thus seen in adults (Clemens et al., 2004).

The 2% prevalence of ETEC in this present study is comparable to a prevalence of 4% obtained from a study of diarrhoeal enteropathogens in Ouagadougou, Burkina Faso, between January 2009 and January 2010 (Bonkoungou et al., 2013). Kabir (2011) however detected ETEC in 39% of samples in a similar work done in Bangladesh. The difference in prevalence rate amongst these developing countries may largely be due to the level of infrastructure especially clean drinking water and access to health care facilities. The level of education or literacy rates of the people also facilitates the spread and management of ETEC and diarrhoea as a whole. Last but not least, the socio-economic status may also be pivotal to the prevalence of ETEC in these developing countries (Addy, 2004). Thus with these parameters alone under consideration, it might just suffice that the lowest rate recorded in this present study conducted in Ghana over the prevalence rate of 4% in Ouagadougou and 39% in Bangladesh may simply hinge on the level of development of the people. No ETEC-ST was detected in the stool samples in this present study. LT is known to be expressed in about 67% of ETEC strains, either alone or in combination with ST (WHO, 2009). Thus it is significantly responsible for majority of the worldwide disease burden of ETEC (Nguyen et al., 2005). This corroborates findings of this study. Quadri et al., (2005) reports an association of 14% of ETEC with diarrhoea cases in Bangladesh. Antikainen et al., (2009) reports of a 11% prevalence of ETEC associated diarrhoea in
India. Pankaj and Ali, (2010) in a similar report from India also reported a 10% ETEC associated diarrhoea. This is much higher than the study findings in this present study. This might be due to geographical, study duration and distribution of the different strains of diarrhoeagenic E. coli that influence bacterial prevalence (Albert et al., 1995). Shigella spp. was not detected in any of the 200 samples. This is not surprising as watery diarrhoea and vomiting were the main complaints of patients. None of the samples screened were bloody stools.

Mixed infection of ETEC and other enteropathogens is common thus making it difficult to determine whether the presenting symptoms of a case are primarily due to ETEC infection and thus better appreciate the pathogenesis of infection (Steinsland et al., 2003). Incidence of mixed infections have been noted to increase with age in studies in Bangladesh and fewer copathogens were seen in infants than in older children and adults with ETEC diarrhoea (Qadri et al., 2000). In cases of mixed infections in children, rotavirus is the most common followed by other bacterial enteropathogens such as V. cholerae, Campylobacter, Shigella, Salmonella and Cryptosporidium (Gaastra et al., 1996). The occurrence of ETEC infections in the cool dry seasons of Ghana is in accord with several studies that equally report ETEC diarrhoea in warm periods of the year (Abu-Elyazeed et al., 1999). Such distribution may be facilitated by climate and spread by environmental factors. The warm atmospheric temperature facilitates increase growth of bacteria and upon the advent of rains, there is enhanced contamination of surface water with faecal material (Rowland, 1986).
This present study shows the potential of using molecular methods to overcome the shortcomings of the gold standard including false negatives which has become rampant due to the abusive use of antibiotics in Ghana. This is because this PCR assay detects the presence of specific virulent genes of the organism irrespective of it being dead or alive. This is unlike the culture method which requires the enteropathogen to be alive to enable growth when plated and thus detection. Improper transportation or storage thus renders the sample useless for culture for the enteropathogen, notwithstanding the already daunting problem of antibiotic usage. Thus this PCR assay is a more suitable, fast and reliable method for the detection of ETEC and *Shigella spp.* by amplifying its virulence genes across the country where the facilities exist.

### 5.2 Conclusion

The use of specific DNA-biomarkers were able to effectively determine the presence of enterotoxigenic *Escherichia coli* (ETEC) in the diarrhoeal stool samples. Of the samples positive for ETEC, the PCR was able to adequately specify the infection as that of the heat-labile strain. ie ETEC-LT. The overall prevalence of ETEC-LT in this study was determined to be 2%. No samples were determined positive for *Shigella spp.* This was no surprise as no bloody stools were observed. ETEC lack the invasion plasmid antigen (Ipa) effectors that facilitate the invasion of eukaryotic cells and movement of the bacteria from cell to cell *in vivo* (Prunier *et al.*, 2007). However, the ability of the PCR assay to effectively detect the positive standards for the ETEC-ST and *Shigella spp.* goes to suggest
the efficacy of the PCR method as a favourable method for the routine detection of ETEC-ST, ETEC-LT and *Shigella* spp. There was coinfection between ETEC and rotavirus but this was however determined to be statistically insignificant. In all cases, the majority of enteropathogens were detected in males than females although no direct association between the infection and sex of the subjects was established. All ETEC-LT infections occurred in the 0-12 month age group. Sixteen diarrhoea samples were negative for all three enteropathogens screened suggesting the presence of other enteropathogens aside rotavirus, ETEC and *Shigella*. Thus this molecular method of screening diarrhoeal stool samples provides a rapid, simple and sensitive technique for the routine detection of ETEC and *Shigella* spp. from stool samples and would greatly expedite the laboratory detection of these enteropathogens (Holland *et al.*, 2000). Although in Sub-Saharan Africa the cost involved is high and thus cannot be borne by patients alone, the efficiency and promptness with which results are made available to medical personnel for prompt action to be taken, makes PCR and overall a cheap option as countless young and innocent lives will be saved.
RECOMMENDATIONS

This study has demonstrated the presence of ETEC as a contributing bacterial enteropathogen for gastroenteritis in Ghana. Information gathered from this study alone is not enough to make any definite statement. More work on a much larger scale has to be done in the field as the number of diarrhoea reported cases at our health centers is only a fraction of the true disease burden in the society. The greater proportion of diarrhoea cases go unreported and are managed at homes. Thus to get the true influence of ETEC, *Shigella* and other enteropathogens on diarrhoea in Ghana, its these unreported cases that needs to be analyzed. Also, since 16 of the diarrhoeal stools samples which tested negative for all the pathogens investigated needs further analyses for other enteropathogens. It is recommended that all samples be tested for all the other diarrhoeagenic *E. coli* to get a holistic view of the true contribution of pathogenic *E. coli* to diarrhoea in Ghana. The history of dehydration status of patients reporting to the various health centers should be adequately recorded and filed and general record keeping at our various health centers be improved. Lastly, this PCR method for detection of ETC and *Shigella* spp. should be rolled out across laboratories and health facilities to ensure a more efficient health care delivery system for the nation with a possible cost sharing system between patients and health care providers explored as every life is valuable.
REFERENCES


detected by 16-plex PCR in children with and without diarrhoea in Burkina Faso.

*Clinical Microbiology and Infection*, 18, 901-916.


diagnosed by SeHCAT scanning in patients with diarrhoea-predominant irritable bowel syndrome. *Alimentary pharmacology and therapeutics*, 30(7): 707-17


APPENDICES

APPENDIX I

DNA sequence detail of oligonucleotide primers used for ETEC PCR assay

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli LT</strong></td>
<td>LT – 1</td>
<td>5’ AGCAGGTTTCCCACCGGATCACCA 3’</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>LT – 2</td>
<td>5’ GTGCTCAGATTCTGGGTCTC 3’</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli ST</strong></td>
<td>ST – 1</td>
<td>5’ GCTAATGTTGGCAATTTTTATTTCTGTA 3’</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>ST – 2</td>
<td>5’AGGATTACAACAAAGTTTCACAGCAGTAA 3’</td>
<td></td>
</tr>
</tbody>
</table>

APPENDIX II

DNA sequence detail of oligonucleotide primers used for *Shigella spp.*

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shigella spp.</strong></td>
<td>IPA – B F</td>
<td>5’ GACGCCCACAAGCTCGAGCA 3’</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>IPA – B R</td>
<td>5’ AGCAGCGACCAGCAATTCCT 3’</td>
<td></td>
</tr>
</tbody>
</table>
### APPENDIX III

ETEC-ST PCR reaction mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>X 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X Buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>10µM dNTP Mix</td>
<td>0.4</td>
</tr>
<tr>
<td>10µM forward primer</td>
<td>0.5</td>
</tr>
<tr>
<td>10µM reverse primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>15.4</td>
</tr>
<tr>
<td>5U/µl Taq Polymerase</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA Template</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>25µl</strong></td>
</tr>
</tbody>
</table>
## APPENDIX IV

### ETEC-LT PCR reaction mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>X 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X Buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>10µM dNTP Mix</td>
<td>0.4</td>
</tr>
<tr>
<td>2.5µM forward primer</td>
<td>0.5</td>
</tr>
<tr>
<td>2.5µM reverse primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>15.4</td>
</tr>
<tr>
<td>5U/µl Taq Polymerase</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA Template</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>25µl</td>
</tr>
</tbody>
</table>

---

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# APPENDIX V

*Shigella* PCR reaction mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>X 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>2.0</td>
</tr>
<tr>
<td>5 X Buffer</td>
<td>10</td>
</tr>
<tr>
<td>10µM dNTP Mix</td>
<td>0.5</td>
</tr>
<tr>
<td>2.5µM forward primer</td>
<td>0.5</td>
</tr>
<tr>
<td>2.5µM reverse primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>8.0</td>
</tr>
<tr>
<td>5U/µl Taq Polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>DNA Template</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>25µl</strong></td>
</tr>
</tbody>
</table>
APPENDIX VI

Materials used

1. 10X PCR buffer (Promega, Madison WI, USA)
2. FastSoil DNA Extraction Kit
3. 1.7 Eppendorf tubes
4. 15ml Eppendorf tubes
5. 100μl pipette tips
6. 1000μl pipette tips
7. Taq Polymerase (Promega, Madison WI, USA)
8. dNTPs (Promega, Madison WI, USA)
9. Agarose (AGTC Bioproducts Ltd, UK)
10. 100bp DNA ladder (Promega, Madison WI, USA)
11. Electrophoresis set up
12. Ethidium bromide (Promega, Madison WI, USA)
13. Benchtop 2UV Transilluminator gel photography system (Upland, CA, USA)
14. Centrifuge (Eppendorf, Hamburg, Germany)
15. Eppendorf Mastercycler personal PCR Machine (Eppendorf, Hamburg, Germany)
16. Nuclease free water (HyClone Lab Inc., South Logan, Utah, USA)
APPENDIX VII

Reagent s preparation

1. Working concentration of dNTPs from stock concentration

Stock concentration of dNTPs (C1) = 100mM

Volume of stock concentration needed to prepare working concentration (V1) =?

Working stock concentration (C2) = 10mM

Volume of working concentration needed for 50 samples (V2) = 20µl

Since C1 xV1 = C2 x V2,

V1= (C2 x V2)/C1

V1 = (10mM x 20µl)/100mM

V1=2µl

Thus the dNTP stocks were obtained from the freezer, thawed and vortexed after which 2µl was pipetted into an Eppendorf tube and made up to 20µl with nuclease free water.

10µl dNTP mix was made by taking equal volumes of the individual dNTPs into a single tube and pulse vortexed to uniformly mix the cocktail.
2. **Working concentration of primers from stock concentration**

Stock concentration of primers (C1) = 100mM

Volume of stock concentration needed to prepare working concentration (V1) =?

Working stock concentration (C2) = 10mM

Volume of working concentration needed for 20 samples (V2) = 10µl

Since C1 x V1 = C2 x V2,

\[ V1 = \frac{(C2 \times V2)}{C1} \]

\[ V1 = \frac{(10\text{mM} \times 10\mu\text{l})}{100\text{mM}} \]

\[ V1 = 1\mu\text{l} \]

Thus from the thawed vortexed stock primer, pipette 1µl and make up to 10µl with nuclease free water.
3. 1X Tris acetate EDTA (TAE) buffer

Stock concentration of TAE buffer (C1) = 20X

Volume of stock concentration needed to prepare 1X (V1) = ?

Working concentration (C2) = 1X

Volume of working concentration needed (V2) = 1000ml

\[ V1 = \frac{(C2 \times V2)}{C1} \]

\[ V1 = \frac{(1X \times 1000ml)}{20X} \]

\[ V1 = 50ml \]

Thus 50ml of the stock TAE buffer was obtained and topped up to 1000ml in a 1L measuring cylinder using double distilled water.

4. Preparation of 1.5% agarose gel and casting

The edges of the gel plate was taped and checked for leakages. 1.5g of agarose gel was weighed into a conical flask containing 100ml of 1X TAE buffer. The solution was warmed in a microwave to facilitate the dissolving of the agarose in the TAE. After allowing to cool for a few seconds, 5µl of ethidium bromide was then pipetted into the solution and swirled to mix. The resulting solution was then cast into the plate and comb placed into it to make
wells. The agarose was subsequently left to set at room temperature. On completion of the PCR, 10µl of the amplicons was loaded into the wells on the agarose gel and electrophoresed at 120 volts. Five microliters of 100bp DNA molecular marker was loaded alongside the amplicons.