University of Ghana http://ugspace.ug.edu.gh

# AETIOLOGY, MALNUTRITION AND FAECAL LACTOFERRIN LEVELS IN PAEDIATRIC DIARRHOEA

By

**JAPHETH AWULETEY OPINTAN**

Department of Microbiology

University of Ghana Medical School, Korle-Bu, Accra.

University of Ghana http://ugspace.ug.edu.gh

# AETIOLOGY, MALNUTRITION AND FAECAL LACTOFERRIN LEVELS IN PAEDIATRIC DIARRHOEA

**Japheth Awuletey Opintan, MPhil, BSc (Hons), Dip Stat (Ghana)**
**(ID # 10125294)**

This thesis is submitted to the University of Ghana, Legon in partial fulfillment of the requirement for the award of
**Doctor of Philosophy (PhD) Microbiology degree**

**Department of Microbiology**
University of Ghana Medical School, College of Health Sciences

**AUGUST, 2011**

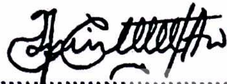
University of Ghana http://ugspace.ug.edu.gh

G 325308
bltc

University of Ghana http://ugspace.ug.edu.gh

# DECLARATION

This thesis has not been presented to any local or international university for the award of any degree. Data was collected from the Princess Marie Louise Children's Hospital in Accra, Ghana. Initial specimen processing and further molecular analyses were done at the Department of Microbiology, University of Ghana Medical School (UGMS), and the Centre for Global Health, Division of Infectious Diseases and International Health. University of Virginia, USA. The work was jointly supervised by the under-listed Professors.



14th Feb 2012

JAPHETH A. OPINTAN (Student) — Date

14th Feb 2012

PROFESSOR MERCY J. NEWMAN (Supervisor) — Date

14/02/2012

PROFESSOR MICHAEL D. WILSON (Supervisor) — Date

14/Feb/2012

PROFESSOR RICHARD L. GUERRANT (Supervisor) — Date

University of Ghana http://ugspace.ug.edu.gh

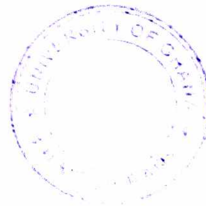
# DEDICATION

This PhD thesis is dedicated to;

all the children whose specimens and anthropometric data were used in the study,

my wife Gladys and daughter Marybeth, and to all the Opintan family.

To God is all the glory! Great thing He has done.



University of Ghana http://ugspace.ug.edu.gh

# ACKNOWLEDGEMENTS

This PhD research work was jointly sponsored by the University of Ghana Medical School (UGMS), Ghana, and the Center for Global Health/Pfizer Initiative in International Health (CGH/PIIH), University of Virginia, VA, USA.

I was blessed to have received support and encouragement from many special people, from the conception of the research to the time of putting the thesis together. I wish therefore to acknowledge the contributions of these important personalities.

I am most grateful to all faculty members. The warm reception and guidance from the Head of the Microbiology Department (UGMS), Dr. Patrick F. Ayeh-Kumi, is very much appreciated. I am very much indebted to Professor Mercy J. Newman, who I affectionately call 'Mummy'. Professor Newman has interest in hard work, and she did not relent to give me all the needed support. I am grateful to Professor Michael D. Wilson, from the Noguchi Memorial Institute for Medical Research (NMIMR), who guided me through various stages of my career.

I wish to thank Dr Rosina Gepi-Attee, Medical Director and Mr. Raymond Affrim, Head of the Laboratory Unit, of the Princess Marie Louise Children's Hospital, (PML) Accra, Ghana, for their contributions towards the design of the questionnaire and supervising specimen collection. I am also grateful to Mr. David Quarteng and the nurses at PML for administering questionnaires and collating anthropometric data. Mr. Samuel Obodai from the Geography

University of Ghana http://ugspace.ug.edu.gh

Department, University of Ghana helped with the plotting of the locations of the study subjects.

I am indebted to Professor Iruka N. Okeke from Haverford College, PA, USA, who mentored me in several ways. I also received consumables from grants Professor Okeke had from the Branco Weiss Fellowship, Society in Science ETHZ, Zurich, Germany.

I wish also to thank Professors Richard L. Guerrant and Michael Scheld, Director of the Center for Global Health (CGH) and Director of the Pfizer Initiative, respectively, for their immense support and supervision of my thesis. I am also indebted to the following special people at CGH, who mentored me in several ways; Professors Cirle Warren and Jim Roche, Doctors Jesus E.A.D Sevilleja and Glynis Kollings. I am also grateful to Ms Leah Barret, April Ballard and Charlotte Martins all of CGH, for making my stay and research in the US a memorable one.

I also wish to express my appreciation to Dr Patience Mensah, of the WHO Regional Office for Africa, for the encouragement and constant reminders to prioritize my activities.

Last and not the least, I wish to acknowledge the cooperation I enjoyed from my wife, Gladys and daughter, Marybeth. I had to leave them alone during difficult moments, spending long hours in the laboratory and on my work.

University of Ghana http://ugspace.ug.edu.gh

# TABLE OF CONTENTS

TITLE PAGE ........................................ i

DECLARATION ........................................ ii

DEDICATION ........................................ iii

ACKNOWLEDGEMENTS ........................................ iv

TABLE OF CONTENTS ........................................ vi

LIST OF FIGURES ........................................ x.

LIST OF TABLES ........................................ xii

ABBREVIATIONS ........................................ xiii

ABSTRACT. ........................................ xvi

**CHAPTER ONE ........................................ 1**

**INTRODUCTION ........................................ 1**

1.1 General Introduction ........................................ 1

1.1.1 Rationale of study ........................................ 8

1.1.2 Working hypotheses ........................................ 12

1.1.3 Main objective ........................................ 13

1.1.3.1 *Specific objectives* ........................................ 13

**CHAPTER TWO ........................................ 14**

**LITERATURE REVIEW ........................................ 14**

2.1 The Global Burden of Childhood Diarrhoea ........................................ 14

2.1.1 Definition and forms of acute childhood diarrhoea ........................................ 18

2.2 Effect of pathogen-specific diarrhoeas on nutritional status ........................................ 20

2.3 Protein-Energy Malnutrition (PEM) ........................................ 24

University of Ghana http://ugspace.ug.edu.gh

2.3.1 Mild and moderate PEM ........................................................ 26

2.3.2 Epidemiology and causes of PEM ........................................................ 28

2.4 Aetiological Agents of Infectious Diarrhoea ........................................................ 30

2.5 Diarrhoeagenic *Escherichia coli* (DEC) ........................................................ 31

2.6 Enteroaggregative *E. coli* (EAEC) ........................................................ 33

2.6.1 Virulence profile of EAEC ........................................................ 34

2.7 Diagnostic Challenges in the Identification of EAEC ........................................................ 37

2.8 Pathogenesis of EAEC infections ........................................................ 42

2.9 Inflammatory Diarrhoea and its Assessment ........................................................ 47

2.9.1 Pathophysiology of Lactoferrin ........................................................ 49

2.9.2 Lactoferrin (LF) assays for enteric pathogens ........................................................ 50

2.10 Cryptosporidiosis: Background and Epidemiology ........................................................ 52

2.10.1 Diagnosis and genotyping of *Cryptosporidium* spp ........................................................ 57

2.11 Dysentery: Background and Epidemiology ........................................................ 59

2.11.1 Diagnosis and genotyping of dysentery ........................................................ 60

2.12 Giardiasis: Background and Epidemiology ........................................................ 61

2.12.1 Diagnosis and genotyping of giardiasis ........................................................ 62

**CHAPTER THREE ........................................................ 63**

**MATERIALS AND METHODS ........................................................ 63**

3.1 Ethical consideration, Study design, Population and settings ........................................................ 63

3.1.1 Interviews and diarrhoea definition ........................................................ 64

3.1.2 Anthropometric data and nutritional status assessment ........................................................ 65

3.2 Specimen Processing and Microbiological Analysis ........................................................ 66

University of Ghana http://ugspace.ug.edu.gh

3.2.1 Routine stool culture....66

3.2.2 Biochemical and serological identification of isolates....68

3.2.3 Archiving of bacterial strains....70

3.3 Bacterial DNA Extraction (*E. coli* Isolates)....71

3.4 Stool DNA Extraction....73

3.5 Optimization, Sensitivity and Specificity of Real-Time PCR (qPCR)....75

3.6 Detection of Enteric Pathogens from Stool DNA....83

3.7 Intestinal Inflammation Assessment....86

3.8 Real-Time PCR Detection of EAEC from Bacterial DNA....89

3.9 Genotyping of Amplified *Cryptosporidium*-positive stools....90

3.10 Data Storage and Analysis....92

**CHAPTER FOUR....93**

**RESULTS....93**

4.1 Study Population and Base-line Characteristics....93

4.2 Nutritional Status and Growth Faltering....96

4.3 Real-Time PCR Detection of Pathogens from Stool DNA....101

4.4 EAEC Virulence Genes Distribution (Stool DNA)....103

4.5 Multiple Infection (Stool DNA)....106

4.6 Conventional Stool Culture Results....108

4.7 Faecal Lactoferrin Levels....109

4.8 EAEC's Dispersin Gene Associates Better with Diarrhoea ($p<0.05$)....113

4.9 *Cryptosporidium* Genotypes: Preliminary results....118

4.10 Summaries of Additional Data from Questionnaires....119

University of Ghana http://ugspace.ug.edu.gh

**CHAPTER FIVE** .......... **121**

**DISCUSSION** .......... **121**

5.1 Nutritional Shortfalls among the Study Population .......... 121

5.2 PCR for Detection of Pathogens .......... 125

5.3 Co-infection .......... 127

5.4 Prevalence of EAEC Virulence-associated Genes in Stool DNA .......... 129

5.5 Lactoferrin levels .......... 132

5.6 Molecular Diagnosis of EAEC: Stool DNA versus Bacterial DNA .......... 134

5.7 Study Limitations .......... 137

5.8 Conclusions, Recommendations and Future Research Direction .......... 138

**REFERENCES** .......... **140**

**APPENDICES** .......... **184**

APPENDIX I: Questionnaires administered in this study .......... 184

APPENDIX II: Media and equipment used .......... 189

APPENDIX III: Cycling conditions of target genes screened .......... 195

APPENDIX IV: Sequenced data of *Cryptosporidium* positive stool DNA .......... 196

APPENDIX V: Abstracts and publication emanating from thesis .......... 207

University of Ghana http://ugspace.ug.edu.gh

# LIST OF FIGURES


| | | |
|---|---|---|
| Figure 1 | The cycle of malnutrition and enteric disease | 3 |
| Figure 2 | Worldwide distribution of deaths caused by diarrhoea in children < 5 years of age | 15 |
| Figure 3 | Skewed distribution of the global burden of diarrhoea towards Africa and Asia | 17 |
| Figure 4 | Diarrhoea linearly ablates catch-up growth | 21 |
| Figure 5 | Effect of undernutrition on EAEC-challenged weaned C57BL/6 mice | 23 |
| Figure 6 | An iceberg of Protein Energy Malnutrition (PEM) | 27 |
| Figure 7 | Adherence pattern of diarrhoeagenic *Escherichia coli* (DEC) to cultured epithelial cell | 38 |
| Figure 8 | A flowchart on processing of fresh stool specimen | 67 |
| Figure 9 | A flowchart on culture of stool specimens for bacteria | 69 |
| Figure 10 | A flowchart showing how bacterial DNA was extracted | 72 |
| Figure 11 | A flowchart showing how stool DNA was extracted | 74 |
| Figure 12 (*i-vi*) | Optimization curves obtained for real-time PCR for some of the genes screened | 77 |

University of Ghana http://ugspace.ug.edu.gh


| | | |
|---|---|---|
| Figure 13a | Photographs showing some reactions of the lactoferrin assays | 87 |
| Figure 13b | Standard curve fit of lactoferrin LF) using purified human controls | 88 |
| Figure 14 | An insert map of Ghana showing settlements of the enrolled children in the study | 94 |
| Figure 15 | Weight-for-age plot of (a) male and (b) female, infants and young children (<2 years) in the diarrhoea sub-population | 97 |
| Figure 16 | Weight-for-age plot of (a) male and (b) female, infants and young children (<2 years) in the non-diarrhoea sub-population | 99 |
| Figure 17 | Enteric pathogens detected and faecal lactoferrin levels | 110 |
| Figure 18 (i) | Stool DNA vs bacterial DNA in the molecular diagnosis of EAEC (all stool and bacterial DNAs) | 115 |
| Figure 18 (ii) | Stool DNA vs bacterial DNA in the molecular diagnosis of EAEC (bacterial DNA and their matching stool DNAs ) | 116 |
| Figure 19 | EAEC is better diagnosed in stool DNA as opposed to bacterial DNA | 117 |
| Figure 20 | HRM data analysis of *Cryptosporidium*-positive DNAs | 119 |

University of Ghana http://ugspace.ug.edu.gh

# LIST OF TABLES


| | | |
|---|---|---|
| Table 1 | Selected EAEC virulence factors and their description | 35 |
| Table 2 | Summaries of LF assays in relation to pathogen-specific diarrhoeas | 52 |
| Table 3 | Target genes screened from stool DNA | 84 |
| Table 4 | Baseline characteristics of study population | 95 |
| Table 5 | Organisms detected by real-time PCR from stool DNA | 102 |
| Table 6 | EAEC virulence factor positive in stool DNA | 104 |
| Table 7 | EAEC's genes in stool in association with and without malnutrition | 105 |
| Table 8 | Distribution of pathogens by age from stool DNA | 107 |
| Table 9 | EAEC genes detected an faecal lactoferrin levels | 111 |
| Table 10 | EAEC virulence factor-positive in bacterial DNA (*E. coli*) | 114 |

University of Ghana http://ugspace.ug.edu.gh

# ABBREVIATIONS

| | |
|---|---|
| µl | microlitre |
| µM | micro molar |
| AA | aggregative adherence |
| AAF | aggregative adherence fimbria |
| *aap* | gene encoding dispersin |
| *aatA* | gene encoding dispersin transporter protein |
| *aggR* | gene encoding master regulator |
| AIDS | Acquired Immune Deficiency Syndrome |
| bp | base pairs |
| CDEC | cell detaching *E. coli* |
| CGH | Centre for Global Health |
| cm | centimeter |
| Ct | melt temperature |
| CVD | center for vaccine development |
| DAEC | diffusely adherent *E. coli* |
| DEC | diarrhoegenic *E. coli* |
| EAEC | enteroaggregative *E. coli* |
| EHEC | enterohaemorrhagic *E. coli* |
| EIEC | enteroinvasive *E. coli* |
| ELISA | enzyme-linked immunosorbent assay |
| EPEC | enteropathogenic *E. coli* |

University of Ghana http://ugspace.ug.edu.gh

| | |
|---|---|
| ETEC | enterotoxigenic *E. coli* |
| FAO | Food and Agriculture Organization |
| h | hour |
| HAZ | height for age z-score |
| HEp-2 | human epithelia cell |
| HRM | high resolution melting |
| IBD | Inflammatory bowel disease |
| IBS | irritable bowel syndrome |
| IgM | Immunoglobulin M |
| *ipaH* | invasive plasmid antigen H |
| kb | kilo base |
| kg | kilogram |
| LEE | loci of enterocyte effacement |
| LF | lactoferrin |
| MAP | membrane-associated protein |
| MDG | Millennium Development Goals |
| min | minutes |
| ml | milliliter |
| $OD_{600}$ | Optical density at 600 nm wavelength |
| OMP | outer-membrane protein |
| ORT | Oral rehydration therapy |
| PEM | protein energy malnutrition |
| Pet | plasmid encoded toxin |

University of Ghana http://ugspace.ug.edu.gh

| | |
|---|---|
| PML | Princess Marie Louise Children Hospital |
| RFLP | restriction fragment length polymorphism |
| RFU | relative fluorescence unit |
| s | seconds |
| spp | species |
| UGMS | University of Ghana Medical School |
| UNICEF | United Nations Children's Fund (formerly United Nations International Children's Emergency Fund) |
| UVa | University of Virginia |
| WAZ | weight for age z-score |
| WHO | World Health Organization |
| WHZ | weight for height z-score |

University of Ghana http://ugspace.ug.edu.gh

# ABSTRACT

Diarrhoea is a major public health problem that affects the physical and cognitive development of young children. Anthropometric data was collected from 274 children, 170 with diarrhoea and 104 without diarrhoea. Stool specimens were analyzed by conventional culture, PCR for EAEC, *Shigella*, *Cryptosporidium*, *Entamoeba*, and *Giardia* species, as well as by ELISA for faecal lactoferrin levels. Additionally, all *E. coli* recovered from culture were PCR screened for EAEC, and compared with those obtained from the stool DNA. Multiple gene loci (*aaiC*, *aap*, *attA* and *aggR*) were sought for EAEC. About 50% of the study population was mildly to severely malnourished. Mild to severe malnutrition (WAZ <-1), moderate to severe stunting (HAZ < -2) and moderate to severe wasting (WHZ < -2) were associated with diarrhoea ($p$ = 0.023, 0.026 and 0.062, respectively). In only 1 of 170 diarrhoea stool specimen was *Shigella flexneri* recovered by culture. EAEC and *Cryptosporidium* were associated with diarrhoea ($p$ = 0.048 and 0.011, respectively), and malnourished children who had diarrhoea were often co-infected with both *Cryptosporidium* and EAEC. About 27 % (4/15) C. *parvum* genotypes were identified by HRM analysis. Faecal lactoferrin levels were higher in children with diarrhoea ($p$ = 0.019). Children who had EAEC infection, with or without diarrhoea had high mean lactoferrin levels irrespective of nutritional status. In conclusion, the current study identified high levels of growth deficits among the children with/without diarrhoea. The use of DNA-biomarkers revealed that EAEC and *Cryptosporidium* were common intestinal pathogens in Accra, and that elevated faecal lactoferrin was associated with

University of Ghana http://ugspace.ug.edu.gh

diarrhoea in this group of children. EAEC's dispersin gene (*aap*) was significantly associated with diarrhoea in both the faecal and bacterial DNAs, in the children studied ($p < 0.05$).

***Publication:*** *Part of the data presented in this thesis is published as follows:*

Opintan JA *et al*. (2010). Pediatric Diarrhea in Southern Ghana: Etiology and Association with Intestinal Inflammation and Malnutrition. *Am J. Trop Med Hyg.* 83; 936 – 943.

University of Ghana http://ugspace.ug.edu.gh

# CHAPTER ONE

# INTRODUCTION

## 1.1 General Introduction

Malnutrition in infants and young children has devastating effects on their performance, health and survival (Chang *et al.*, 2002; Guerrant *et al.*, 2008; Pelletier and Frongillo, 2003). It is estimated that about 53% of all deaths in young children are attributable to underweight, varying from 45% for deaths due to measles to 61% for deaths due to diarrhoea (Caulfield *et al.*, 2004). Thus, a specific target goal of the Millennium Development Goals (MDG) is to reduce by 50%, the prevalence of being underweight among children younger than 5 years between 1990 and 2015 (UN, 2002). Three commonly used indicators of under nutrition by the World Health Organization (WHO) are stunting (low height for age), thinness (low body mass index for age) and underweight (low weight for age). Stunting is an indicator of chronic under nutrition, the result of prolonged food deprivation and/or disease or illness. Thinness is an indicator of acute under nutrition, the result of more recent food deprivation or illness. Underweight, which is used as a composite indicator to reflect both acute and chronic under nutrition cannot distinguish between the two indexes (WHO, 1995a). Unfortunately, anthropometric measurements are not routinely performed to identify malnourished children in most clinics and hospitals in Ghana, thereby missing the opportunity for diagnosis and appropriate management (Antwi, 2008).

University of Ghana http://ugspace.ug.edu.gh

WHO estimated that 1.3 million deaths could be prevented in 42 countries with high rates of mortality, by increased levels of breast feeding among infants (Jones *et al.*, 2003; Lauer *et al.*, 2006). For this reason, exclusive breast feeding up to six months of age is promoted and practiced in many countries including Ghana. However, the appropriate timing of complementary feeding for breastfed infants is often controversial and some studies have shown that growth of such infants appears to falter as early as 2-3 months (Dewey *et al.*, 1992). Sub-optimal breast feeding and malnutrition in younger children has been identified as the most powerful shared risk factor of child death and poses a danger to children's development. Undernourished children have lowered resistance to infection and are more likely to die from common childhood ailments like diarrhoeal diseases and respiratory infections (Grotto *et al.*, 2003; Osumanu, 2008; Schmidt *et al.*, 2009). The risk of death is often directly correlated with the degree of malnutrition (Chen *et al.*, 1980; Van Den Broeck *et al.*, 1993).

A vicious cycle ensues between diarrhoea and malnutrition (Figure 1), and studies have shown that malnutrition with frequent diarrhoeal episodes slow cognitive and physical development of children (Guerrant *et al.*, 2008; Lima *et al.*, 2000). One mechanism for this is that diarrhoeagenic pathogens damage the intestinal epithelium and reduce its absorptive function, leading to nutrient depletion and malnutrition (Guerrant *et al.*, 2008). Malnourished individuals, especially young children living in developing countries, may be unable to repair mucosal damage,

University of Ghana http://ugspace.ug.edu.gh

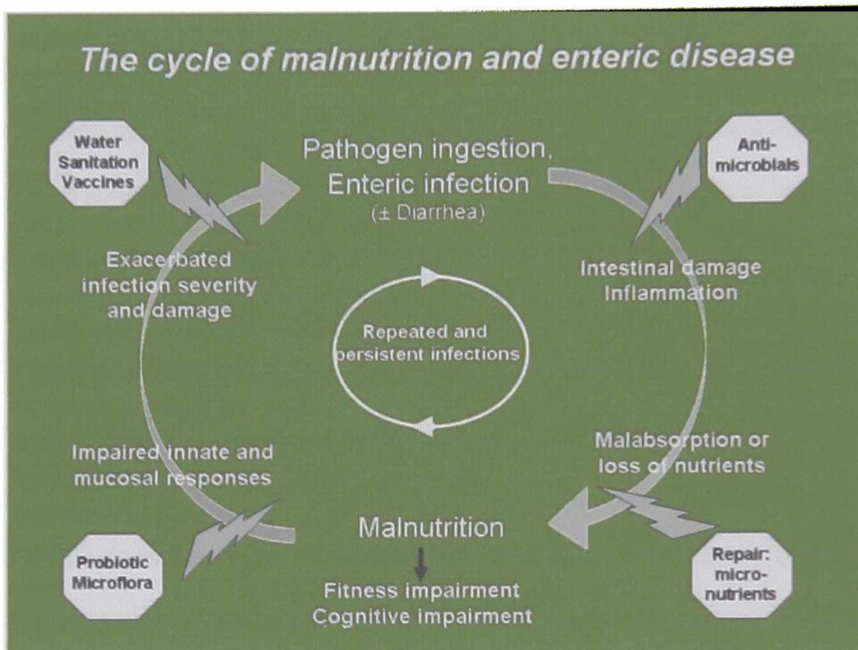



**Figure 1: The cycle of malnutrition and enteric disease.** Enteric infection disrupts the gut's absorptive function leading to malnutrition, and malnutrition in turn leads to more infection. Source: (mal-ed, 2010) http://mal-ed.fnih.org/: The interactions of Malnutrition and Enteric Infections: Consequences for child health and development.

University of Ghana http://ugspace.ug.edu.gh

and thus may become prone to persistent or chronic diarrhoea (Adachi *et al.*, 2002; Guerrant *et al.*, 2008; Huppertz *et al.*, 1997; Nataro and Kaper, 1998).

The agents capable of causing infectious diarrhoea and the mechanisms responsible for disease pathogenesis are generally known, but the true prevalence of these agents in developing countries is poorly understood (Gomez-Duarte *et al.*, 2009). The present study is particularly interested in the relative importance of EAEC, (an emerging pathogen), as compared to other enteropathogens. In most sub-Saharan African countries including Ghana, microbiological methods for clinical investigation of diarrhoeal diseases are usually restricted to identifying conventional enteric bacteria such as *Salmonella* and *Shigella*. *Escherichia coli* (*E. coli*) isolates are often not fully characterized because of the lack of resources. EAEC, a category of the diarrhoegenic *E. coli* (DEC), is however associated with diarrhoea in several contexts: traveler's diarrhoea (Adachi *et al.*, 2001; Cabada and White, 2008), paediatric diarrhoea (Cennimo *et al.*, 2009; Paul *et al.*, 1994), food borne out-breaks (Kaur *et al.*, 2010; Scavia *et al.*, 2008), human immunodeficiency virus infection (Samie *et al.*, 2007b), symptomatic and asymptomatic cases (Nataro *et al.*, 1987; Olesen *et al.*, 2005), acute and persistent diarrhoea (Lima *et al.*, 2000; Okeke *et al.*, 2003), among others. Additional diarrhoeal pathogens of potential importance include *Cryptosporidium* spp, *Entamoeba histolytica* and *Giardia lamblia*, which are parasitic causes of diarrhoea (Ajjampur *et al.*, 2008; Gutierrez-Cisneros *et al.*, 2010; Haque *et al.*, 2003a; Kosek *et al.*, 2001; Ortega and Adam, 1997). Unfortunately, investigation

University of Ghana http://ugspace.ug.edu.gh

of diarrhoea caused by these parasites in most developing countries largely depends on expert microscopy, where technical competence is necessary (Yakoob *et al.*, 2010).

Diarrhoeagenic *E. coli* (DEC), particularly EAEC, which is not often sought for in developing countries, may be an important unrecognized bacteriological agent associated with acute and persistent diarrhoea in children less than 5 years in the sub-region (Bangar and Mamatha, 2008). A recent study in Accra suggests that asymptomatic carriage of EAEC by adults may place younger children at risk of diarrhoea (Opintan *et al.*, 2010) . The 'gold standard' for diagnosis of EAEC is the human epithelial cell (HEp-2) adherence assay (Nataro *et al.*, 1987). In this assay, EAEC strains form a characteristic 'stacked brick' pattern. Unfortunately, the lack of specialized facilities and skills required for this type of assay, limits many laboratories in Africa to use the adherence assay for identifying EAEC. This obviously is causing a disproportionate investigation and diagnostic insufficiency, particularly in resource-poor countries where diarrhoea is endemic and resources need to be prioritized (Okeke, 2006).

A molecular probe, CVD432, was developed as a diagnostic tool and is highly specific for EAEC strains carrying the somewhat variable aggregative adherence (pAA) virulence plasmid (Baudry *et al.*, 1990). While the CVD432 probe made it possible for more investigators to seek EAEC, it varies in sensitivity. As reviewed by Okeke 2009, the sensitivity variation was between 20% to 89%, when

University of Ghana http://ugspace.ug.edu.gh

comparing to the HEp-2 adherence assay (Okeke, 2009). Far fewer *E. coli* strains were available from Africa in the earlier tests using the CVD432 probe (Baudry *et al.*, 1990). It was initially hoped that dispersin, an anti-aggregative protein (aap) secreted by many EAEC strains, could serve as a possible target for diagnosis (Sheikh *et al.*, 2002; Velarde *et al.*, 2007). A recent report however demonstrates that *aap* is produced by some non-EAEC strains (Monteiro *et al.*, 2009). To increase the chances of recovering EAEC using molecular probes, some authors recommended seeking for multiple gene loci (Monteiro *et al.*, 2009). To date, several dozens of gene probes have been designed and used for screening EAEC, but increasingly, the molecular definition of this pathogen is perplexing. As yet, there is no all-encompassing genetic marker for EAEC, as the organism is often identified in individuals with and without diarrhoea (Okeke, 2009; Opintan *et al.*, 2010; Regua-Mangia *et al.*, 2009).

Diarrhoea is both a cause and an effect of malnutrition (Guerrant *et al.*, 1992b), and this hypothesis has at least been proven in murine models (Coutinho *et al.*, 2008; Roche *et al.*, 2010). These murine models mirror what happens in real human infections, under the condition of malnutrion (Petri *et al.*, 2008). While giardiasis has been consistently associated with nutrient malabsorption and stunting in children, the effects of several bacteria and other protozoan on nutritional status and gastrointestinal morbidity are less clear (Boeke *et al.*, 2010). Data is required, especially from sub-Saharan Africa, to interrupt at all possible points, the bi-directional nature of diarrhoea and malnutrition (Checkley *et al.*,

University of Ghana http://ugspace.ug.edu.gh

2003; Checkley *et al.*, 2002; Guerrant *et al.*, 1992b; Mata, 1992). Some authors alude that infection with specific enteric pathogens such as EAEC and *Cryptosporidium* spp. can affect growth even in the absence of overt diarrhoea (Guerrant *et al.*, 1999a; Huang and Dupont, 2004).

Faecal lactoferrin is readily available in the stool, and has the potential of being used routinely in the assessment and management of diarrhoea. Especially in cases of persistent diarrhoea, the lining of the intestional mucosa may be compromised, leading to malnutrition (Guerrant *et al.*, 2008; Guerrant *et al.*, 1992b). In patients with inflammatory bowel disease (IBD), faecal lactoferrin has been shown to be a sensitive and specific marker of intestinal inflammation (Kane *et al.*, 2003; Sugi *et al.*, 1996; Uchida *et al.*, 1994; Vaishnavi *et al.*, 2000). Data on the relationship between pathogen-specific diarrhoea and IBD is scanty, especially in developing countries (Greenberg *et al.*, 2002; Kane *et al.*, 2003; Samie *et al.*, 2007b). In a large cohort of children with/without diarrhoea, the current study also sought to investigate any relationship between specific enteropathogen(s) and intestinal inflammation.

University of Ghana http://ugspace.ug.edu.gh

### 1.1.1 Rationale of study

The prevalence of acute malnutrition among children under five years is a sensitive and objective crisis indicator, and reflects the wider situation of at-risk populations (Young and Jaspars, 2006). Additionally, the plight of sub-optimally breast fed and malnourished children is often largely invisible because they are only mildly or moderately undernourished (UNICEF, 2009).

Malnutrition and diarrhoeal diseases are linked in a complex, vicious cycle. Under-nutrition contributes to the severity of disease caused by intestinal infections, and infection affects the gut's capacity to absorb nutrients, thus contributing to further malnutrition [Figure 1]. Important strategies which could be used to break this vicious cycle include the identification of specific enteropathogens capable of inducing intestinal inflammation and malnutrition in young children. This will help prioritize scarce resources in endemic populations.

EAEC, *Cryptosporidium* spp, *Entamoeba histolytica* and *Giardia* spp are important enteric pathogens, able to induce repeated and/or prolonged diarrhoea in young children, with potential consequences of growth stunting and reduction of intellectual development (Guerrant *et al.*, 2008; Lima *et al.*, 2000; Lorntz *et al.*, 2006). For many of these pathogens, optimal diagnostic testing is unavailable, impractical, or prohibitively expensive for developing countries like Ghana. In most sub-Saharan Africa countries including Ghana, microbiological methods for clinical investigation of diarrhoeal diseases are usually restricted to identifying

University of Ghana http://ugspace.ug.edu.gh

conventional enteric bacteria such as *Salmonella* and *Shigella*. DEC are often not fully characterized because of the lack of resources. Few studies in Ghana which investigated DEC used serological methods (Addy *et al.*, 2004; Agbodaze *et al.*, 1988; Mensah *et al.*, 2002). However, majority of diarrhoea causing strains of *E. coli* do not fall into standard or even predominant serotypes (Campos *et al.*, 2004).

There are geographic variations in the epidemiology of the different DEC pathotypes and their sub-types, and surveillance for DEC remains weak. Although some pathotypes show promise for vaccine development, not enough is known about predominant subtypes to ensure that vaccines will be effective in the places where they are most needed.

EAEC is one of the most recently identified DEC and is associated with diarrhoeal disease in different contexts, including paediatric and traveler's diarrhoea (Huang *et al.*, 2007; Scavia *et al.*, 2008). The major problem today is not the identification of EAEC as a pathotype, but the identification of the true virulent clones especially the ones that are involved in persistent illness. Unfortunatly, the molecular definition of EAEC is confusing, and almost all studies recover EAEC from patients as well as controls without diarrhoea (Ochoa *et al.*, 2009; Okeke *et al.*, 2003; Opintan *et al.*, 2010; Samie *et al.*, 2007b). Very little is known about EAEC's epidemiology in West Africa and there is scanty

University of Ghana http://ugspace.ug.edu.gh

information from Ghana (Opintan *et al.*, 2010). In particular, the relative importance of EAEC versus other pathogens has not been assessed. Such knowledge would be helpful for prioritizing targeted interventions. As EAEC are often recovered from healthy individuals (without diarrhoea) as well as patients with diarrhoea, it is important to determine whether specific virulence factors are associated with intestinal inflammation, as well as whether any risk factors predispose children to EAEC infection.

Asymptomatic carriage of EAEC can result in evidence of low-level enteritis (Steiner *et al.*, 1998). Several studies have suggested that patients infected with EAEC manifest intestinal inflammation, in which the presence of faecal lactoferrin and proinflammatory cytokines, notably interleukin (IL)-8, is observed (Greenberg *et al.*, 2002; Jiang *et al.*, 2003). Over the last 20 years, adhesions, enterotoxins and cytotoxins encoded on plasmid and chromosome of EAEC, with well-documented deleterious effects on cells *in vivo* have been identified. In these studies, a variety of mechanisms of action of these factors at the cellular level have been elucidated (Boisen *et al.*, 2009; Harrington *et al.*, 2006; Harrington *et al.*, 2005). Still unclear and quite challenging to elucidate, however, is which of these EAEC-generated factors drives the most clinically relevant and consequential manifestation in children over time leading to prolonged and recurrent diarrhoea. Human volunteer studies relying on pathogen challenge to define this are ethically difficult and potentially harmful. An alternative approach, less direct but likely to arrive at the same conclusion, is to investigate stool

University of Ghana http://ugspace.ug.edu.gh

specimens from large cohorts of young children presenting for diarrhoea, with and without growth shortfalls. Such an approach requires strong demographic and clinical data (type of diarrhoea, its duration, height/weight for age, etc.) together with means for quantification of the biomarkers in the stool.

The myriad variations of clinical symptoms of EAEC infection has been attributed to factors such as; 1) host genetic susceptibility, 2) host immune response, 3) heterogeneity of virulence among EAEC strains and 4) the amount of bacteria ingested by the infected host (Adachi *et al.*, 2002; Kaur *et al.*, 2010).

The current study seeks to identify biomarkers in the stool of children that will be indicative of enteric infection. Clinical applicability will be enhanced since biomarkers proposed for the study are present in stool, readily available in the field without the need for a blood draw or stool culture.

University of Ghana http://ugspace.ug.edu.gh

### 1.1.2 Working hypotheses

For this study it is hypothesized that:

1. Enteric infection with specific pathogens leads to malnutrition by causing intestinal inflammation.
2. Under the condition of malnutrition and enteric infection, a biomarker of intestinal inflammation is elevated.
3. The virulence profile of the dominant EAEC strain (amount of virulence factor-positive) in the stools of children less than 5 years initiates clinical disease and growth shortfall.

University of Ghana http://ugspace.ug.edu.gh

### 1.1.3 Main objective

The current study seeks to identify biomarkers in the stool of young children that will be clinically useful to determine enteric infection, inflammatory enteropathy, and growth faltering due to specific enteropathogens.

#### *1.1.3.1 Specific objectives*

1. To assess the nutritional status of children less than 5 years old with/without diarrhoea, attending a secondary hospital in Ghana.
2. To determine the prevalence of specific enteropathogens in the children, and to assess any association (s) with diarrhoea and nutritional status.
3. To characterize, by genotyping the most prevalent enteropathogen(s) in the children with diarrhoea.
4. To quantify faecal lactoferrin levels in the stools of the children and to determine its association with diarrhoea and nutritional status.
5. To assess the utility of stool DNA and bacterial DNA in the diagnosis of EAEC-associated diarrhoea.

University of Ghana http://ugspace.ug.edu.gh

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 The Global Burden of Childhood Diarrhoea

Between the 1950's and the mid 1980's, estimates of global mortality from diarrhoea declined from approximately 4.6 million annual deaths to 2.6 million (Bern *et al.*, 1992; Rohde and Northrup, 1976; Snyder and Merson, 1982). This decline in mortality is attributable to the promotion and use of oral rehydration therapy (ORT) for acute childhood diarrhoea (UNICEF, 2002). In the last two decades however, the overall annual incidence has remained relatively stable, between 1.6 – 2.1 million (Bern *et al.*, 1992; Kosek *et al.*, 2003). Despite the different methods and sources of information for these estimates, each successive review of the diarrhoea burden over the last two decades has demonstrated declining mortality but relatively stable morbidity (Bern *et al.*, 1992; Durley *et al.*, 2004; Kosek *et al.*, 2003). The incidence of diarrhoeal diseases varies greatly with seasons and the child's age. The youngest children are most vulnerable, and incidence is highest in the first two years of life and declines as the child grows older (Abba *et al.*, 2009; Agbodaze *et al.*, 1988; Albert *et al.*, 1999; Bryce *et al.*, 2005).

The global prevalence of diarrhoea is disproportionally skewed towards Africa and South Asia. Africa and South Asia are home to more than 80 per cent of child deaths due to diarrhoea [Figure 2] (Petri *et al.*, 2008; Wardlaw *et al.*, 2010).

University of Ghana http://ugspace.ug.edu.gh

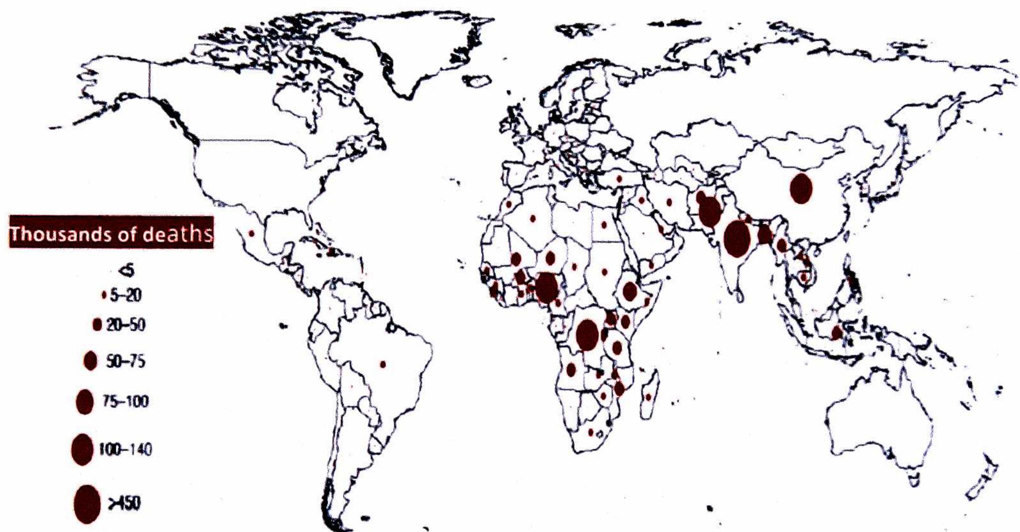



**Figure 2: Worldwide distribution of deaths caused by diarrhoea in children less than five years of age in 2000.** In the last 2 decades, global mortality from diarrhoea has remained relatively stable between 1.6-2.1 million per year. Adapted from (Petri *et al.*, 2008).

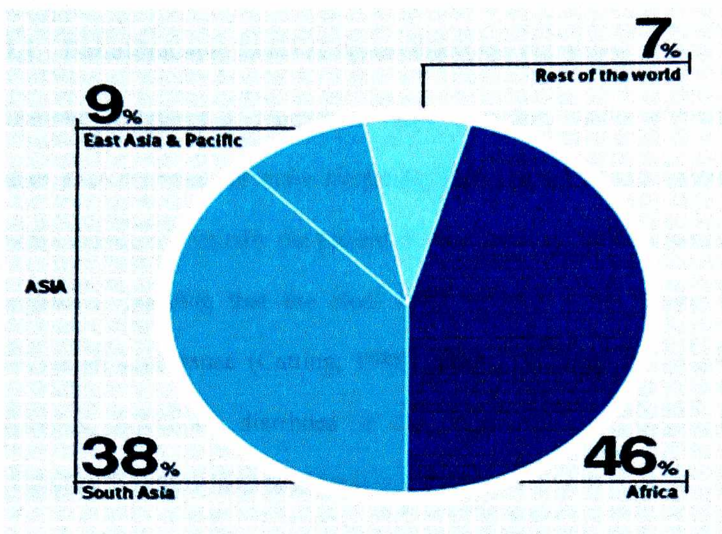
University of Ghana http://ugspace.ug.edu.gh

Just 15 countries account for almost three quarters of all deaths from diarrhoea among children under five years of age annually [Figure 3] (Wardlaw *et al.*, 2010).

Globally, diarrhoea closely follows pneumonia as the second leading cause of death among children under five years. Together, pneumonia and diarrhoea account for an estimated 40 per cent of all child deaths globally each year. Nearly one in five child deaths is due to diarrhoea, a loss of about 1.5 million lives each year. The toll is greater than that caused by AIDS, malaria and measles combined (Wardlaw *et al.*, 2010).

The causes of diarrhoea are several. Displacement of populations into temporary overcrowded shelters is often associated with contaminated water sources, inadequate sanitation, poor hygiene practices, contaminated food and malnutrition – all of which affect the spread and severity of diarrhoea. The lack of adequate health services and transport reduce the likelihood of prompt and appropriate treatment of diarrhoea cases. For example, in 1994, between 500,000 and 800,000 Rwandan refugees fled into areas around Goma in what is now the Democratic Republic of the Congo. An estimated 50,000 deaths occurred in the first month alone, with 85 per cent of them attributed to diarrhoea (GEP, 1995). The scarcity of water was cited as the main cause of the outbreak. Malnutrition is also common in emergencies and tends to be heightened when feeding practices are disrupted and sanitation deteriorates (Ezzati *et al.*, 2002; GEP, 1995).

University of Ghana http://ugspace.ug.edu.gh




| RANK | COUNTRY | TOTAL NUMBER OF ANNUAL CHILD DEATHS DUE TO DIARRHOEA |
|---|---|---|
| 1 | India | 386,600 |
| 2 | Nigeria | 151,700 |
| 3 | Democratic Republic of the Congo | 89,900 |
| 4 | Afghanistan | 82,100 |
| 5 | Ethiopia | 73,700 |
| 6 | Pakistan | 53,300 |
| 7 | Bangladesh | 50,800 |
| 8 | China | 40,000 |
| 9 | Uganda | 29,300 |
| 10 | Kenya | 27,400 |
| 11 | Niger | 26,400 |
| 12 | Burkina Faso | 24,300 |
| 13 | United Republic of Tanzania | 23,900 |
| 14 | Mali | 20,900 |
| 15 | Angola | 19,700 |

**Figure 3: Skewed distribution of the global diarrhoea burden towards Africa and Asia.** More than 80 percent of child deaths due to diarrhoea occur in Africa and South Asia, and nearly three quarters of the deaths occur in just 15 countries. Adapted from (Wardlaw *et al.*, 2010).

University of Ghana http://ugspace.ug.edu.gh

### 2.1.1 Definition and forms of acute childhood diarrhoea

Diarrhoea is defined as passage of loose or watery stools at least three times within a 24 h period, or more frequently than normal for an individual (WHO, 1995b). Often, it is usually the patient or the mother/guardian who first diagnoses diarrhoea – noticing that the stool have become more liquid, frequent and different in appearance (Cutting, 1988). Diarrhoea could be infectious or non-infectious. Infectious diarrhoea is the more common cause of diarrhoea worldwide (Casburn-Jones and Farthing, 2004).

There are four main types of diarrhoea namely osmotic (malabsorption), secretory, inflammatory and motility related diarrhoeas. The review here will only consider secretory and inflammatory diarrhoea. Most fatal consequences of diarrhoea are due to the loss of water and salts in the stool and therefore medical supervision is usually required to manage diarrhoea in infants and young children.

Though most episodes of childhood diarrhoea are mild, acute cases can lead to significant fluid loss and dehydration, which may result in death or other severe consequences if fluid is not replaced at the first sign of diarrhoea. There are three main forms of acute childhood diarrhoea, all of which are potentially life-threatening and require different types of treatment:

1) *Acute watery diarrhoea*: is associated with significant fluid loss and rapid dehydration in the infected individual. It usually lasts for several hours or

University of Ghana http://ugspace.ug.edu.gh

days. The pathogens that generally cause acute watery diarrhoea include *V. cholerae* or *E. coli*, as well as rotavirus.

2) *Bloody diarrhoea*: often referred to as dysentery, is marked by visible blood in the stools. It is associated with intestinal epithelial damage and nutrient losses in an infected individual. The most common cause of bloody diarrhoea is *Shigella dysenteriae* type 1a.

3) *Persistent diarrhoea*: is an episode of diarrhoea, with or without blood, which lasts at least 14 days. Undernourished children and those with other illnesses, such as AIDS, are more likely to develop persistent diarrhoea, and their condition is worsened by malabsorption.

University of Ghana http://ugspace.ug.edu.gh

## 2.2 Effect of pathogen-specific diarrhoeas on nutritional status

Diarrhoea resulting from enteric infection may share co-morbidity to several clinical symptoms (Alam and Ashraf, 2003; Aramayo *et al.*, 2009; Crum *et al.*, 2005; Tormo *et al.*, 2008). The current review focuses on the growth impairment that may result from enteric infection. It is estimated that by the time most children living in imporverished countries attain their second birthday, they would have had about 13 diarrhoea episodes, mainly due to enteric infections (Schorling and Guerrant, 1990; Wardlaw *et al.*, 2010). The vulnerability of these children to enteric infections is increased soon after weaning, particularly when receiving a protein-poor diet (Woodward, 2001). The diarrhoeas may result in both transient growth deficits and in delayed and cumulative effects resulting in permanent growth faltering later in life (Checkley *et al.*, 2003; Molbak, 2000). Although malnourished children tend to "catch up" if given a chance, those with repeated diarrhoeal episodes as a result of repeated infections with enteric pathogens have this catch-up growth linearly ablated (Checkley *et al.*, 2002; Guerrant *et al.*, 1992b; Petri *et al.*, 2008; Schorling and Guerrant, 1990) [Figure 4].

It is challenging to design human experiments to establish the relationship between pathogen-specific diarrhoeas and nutritional status. There is therefore only limited knowledge associating pathogen-specific diarrhoea to growth deficits, and available data are mainly observational studies (Checkley *et al.*, 2008).

University of Ghana http://ugspace.ug.edu.gh

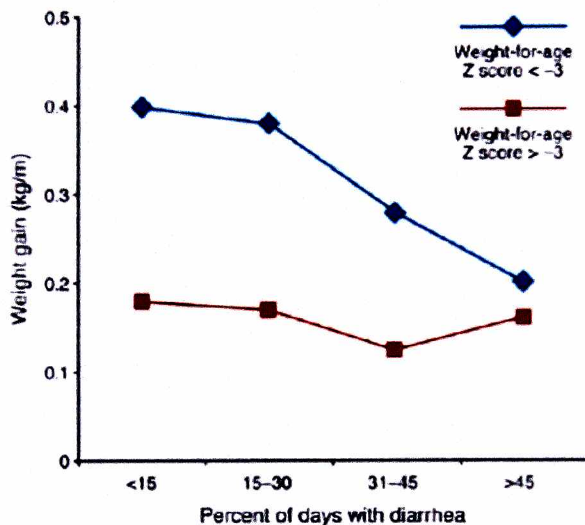



**Figure 4: Diarrhoea linearly ablates 'catch-up' growth.** A plot of weight gained against percentage days with diarrhoea in an observational study of humans. Adapted from (Petri *et al.*, 2008; Schorling and Guerrant, 1990).

UNIVERSITY OF GHANA
BALME LIBRARY

University of Ghana http://ugspace.ug.edu.gh

A recent murine model however, demonstrated the bi-directional nature of diarrhoea and malnutrition in C57BL/6 mice. The EAEC strains used, EAEC 042 and JM221 can also infect humans (Roche *et al.*, 2010). Infected mice fed on a low-protein (2%) diet were observed to have minimal weight gain and, in some cases, even weight loss. On the other hand, in infected mice fed a regular diet (20% protein), a progressive weight gain was observed, with no slowing of growth [Figure 5] (Roche *et al.*, 2010). Additional data from their study revealed important findings which would be usefull in future studies of disease pathophysiology and for preclinical testing of new therapeutics for EAEC. Notably, they found that; i) weaned mice fed a protein-poor diet can be infected with EAEC ii) growth shortfalls occur in neonatal mice challenged with EAEC, iii) stool shedding of organisms accompanies growth shortfalls (Roche *et al.*, 2010). A similar murine model demonstrated that undernutrition with cryptosporidiosis causes mucosal disruption, reduced absorptive surface, and increased proinflammatory cytokine responses, leading to growth impairment (Coutinho *et al.*, 2008). Furthermore, the intensity of the inflammatory response and mucosal disruption parallelled the burden of the *C. parvum* infection (Coutinho *et al.*, 2008). It is postulated that the separate murine models mirror what happens in real human situation under a similar condition of malnutrition and infection (mal-ed, 2010).

University of Ghana http://ugspace.ug.edu.gh

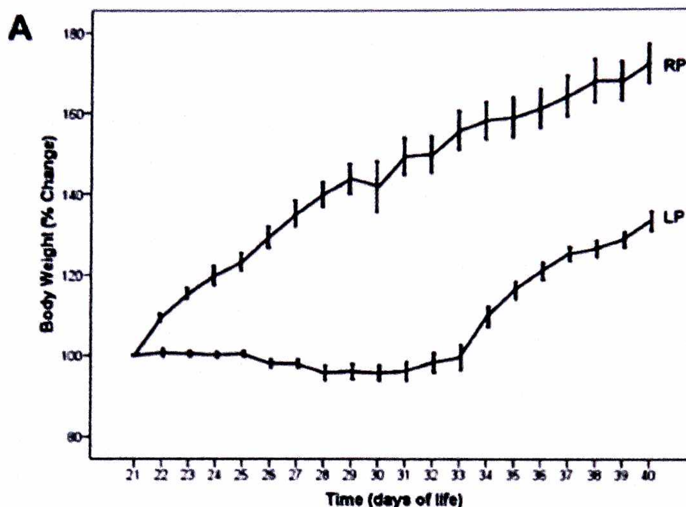



**Figure 5: Effect of undernutrition on EAEC-challenged weaned C57BL/6 mice.** A) Weight was gained progressively in the mice receiving chow having regular amount of protein (RP). The set of mice receiving eucaloric chow (LP) showed minimal weight gain and, in some cases, even weight loss. Growth velocities of nourished and undernourished mice were significantly different on days 23–40 ($P < 0.001$). Adapted from (Roche *et al.*, 2010).

University of Ghana http://ugspace.ug.edu.gh

## 2.3 Protein-Energy Malnutrition (PEM)

Nutritional disorders stem from imbalance between supply of protein-energy and the body's demand for them to ensure optimal growth and function. This imbalance includes both inadequate and excessive nutrient intake; the former leading to malnutrition in the form of wasting, stunting and underweight whilst the latter results in overweight and obesity (Antwi, 2008). Protein-energy malnutrition (PEM) in young children is currently the most important nutritional problem in most countries in Asia, Latin America, the Near East and Africa (Hamer *et al.*, 2004; Joosten and Hulst, 2008; Medhin *et al.*, 2010).

The term PEM is used to describe a broad array of clinical conditions ranging from mild to serious malnutrition (WHO, 1995b). At one end of the spectrum, mild PEM manifests itself mainly as poor physical growth in children; at the other end of the spectrum is kwashiorkor and nutritional marasmus. Marasmus represents an adaptive response to starvation whilst kwashiorkor represents a maladaptive response to starvation (Lin *et al.*, 2007; WHO, 2000b). Kwashiorkor and nutritional marasmus are characterized by the presence of oedema and severe wasting, respectively. It was not until the 1930s that Cicely Williams, working in Ghana, described in detail the condition she termed "kwashiorkor" (using the local Ga word meaning "the disease of the displaced child"). In the 1950s kwashiorkor began to get a great deal of attention. It was often described as the most important form of malnutrition, and it was believed to be caused mainly by protein deficiency (Golden, 1988).

University of Ghana http://ugspace.ug.edu.gh

The current view is that most PEM is the result of inadequate intake or poor utilization of food and energy, not a deficiency of one nutrient and not usually simply a lack of dietary protein. It has also been increasingly realized that infections contribute importantly to PEM (Checkley *et al.*, 2008). In most populations studied in poor countries, the point prevalence rate for kwashiorkor and nutritional marasmus combined is 1 to 5 percent, whereas 30 to 70 percent of children up to five years of age manifest mild or moderate PEM, diagnosed mainly on the basis of anthropometric measurements. Failure to grow adequately is the first and most important manifestation of PEM. It often results from consuming too little food, especially energy, and is frequently aggravated by infections. A child who manifests growth failure may be shorter in length or height or lighter in weight than expected for a child of his or her age, or may be thinner than expected for height. Three conditions necessary to prevent malnutrition or growth failure include: adequate food availability and consumption; good health and access to medical care; and adequate care and feeding practices. If any one of these is absent, PEM is a likely outcome (FAO, 2010; WHO, 2000b).

PEM or undernutrition remains a major cause of disability and mortality, and is ranked as the top cause of global burden of disease (Ezzati *et al.*, 2002). Fifty three percent of deaths in children less than five years of age are attributable to PEM (Bryce *et al.*, 2005; Muller and Becher, 2006). The potential negative

University of Ghana http://ugspace.ug.edu.gh

impact of child undernutrition goes beyond the individual, affecting society and future generations (Grantham-McGregor *et al.*, 2007; Victora *et al.*, 2008).

### 2.3.1 Mild and moderate PEM

The prevalence of serious PEM (kwashiorkor, marasmic kwashiorkor and nutritional marasmus) is usually between about 1 and 5 percent, except in famine areas. In contrast, the prevalence of moderate and mild malnutrition in many countries of sub-Saharan Africa and South Asia add up to 30 to 70 percent [Figure 6] (FAO, 2010). PEM may be seen in as many as 15 to 50 percent of young children. Figure 6 illustrates that both energy deficiency and protein deficiency play a part, but that energy deficiency is more important. It suggests that protein deficiency plays a greater part in kwashiorkor and energy deficiency in nutritional marasmus. A method was suggested that distinguished three categories of mild to moderate PEM based on weight and height measurements of children. Subsequently these categories came to be known as follows:

a) ***wasting***: acute current, short-duration malnutrition, where weight for age and weight for height are low but height for age is normal;
b) ***stunting***: past chronic malnutrition, where weight for age and height for age are low but weight for height is normal;
c) ***wasting and stunting***: acute and chronic or current long-duration malnutrition, where weight for age, height for age and weight for height are all low.

University of Ghana http://ugspace.ug.edu.gh

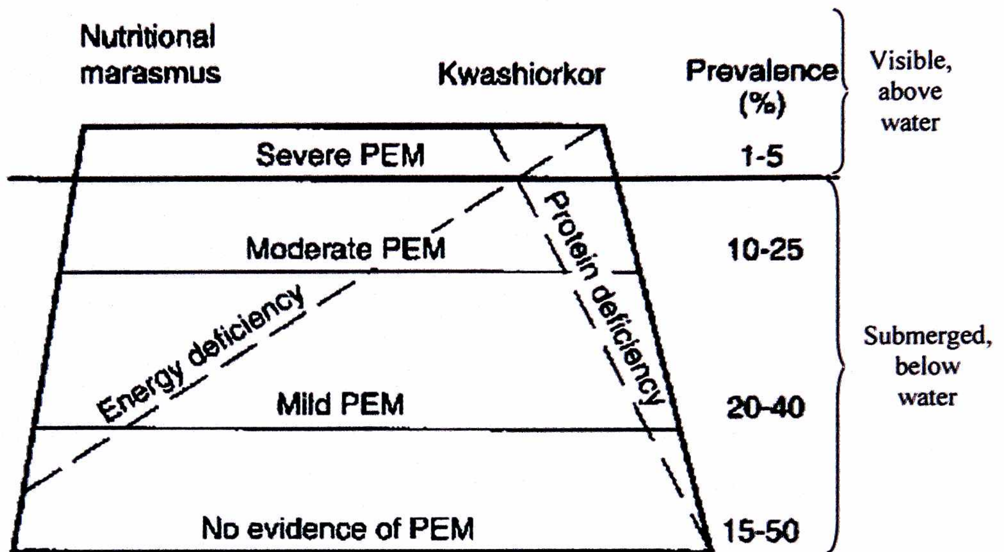



**Figure 6: An iceberg of Protein Energy Malnutrition.** Only about 20% of severe Protein Energy Malnutrition (kwashiorkor, nutritional marasmus and marasmic kwashiorkor) constitute the top, exposed part of the iceberg: they are easy to be diagnosed from the clinical manifestation. The other 80% is submerged, and can only be diagnosed by the use of anthropometric measurements. Adapted from (FAO, 2010)

UNIVERSITY OF GHANA

University of Ghana http://ugspace.ug.edu.gh

### 2.3.2 Epidemiology and causes of PEM

Estimates from WHO suggest that the number of underweight children worldwide rose from 195 million in 1975 to an estimated 200 million at the end of 1994 (de Onis *et al.*, 1993; FAO, 2010). Between 2000 and 2002, it was estimated that 852 million people were undernourished worldwide, with most (about 96%) living in developing countries (FAO, 2004). PEM, unlike the other important nutritional deficiency diseases, is a macronutrient deficiency, not a micronutrient deficiency. Although termed PEM, it is now generally accepted to stem in most cases from energy deficiency, often caused by insufficient food intake. Energy deficiency is more important and more common than protein deficiency. It is very often associated with infections and with micronutrient deficiencies (Dickson *et al.*, 2000; Stoltzfus *et al.*, 2004). Inadequate care, for example infrequent feeding, may play a part. For satisfactory nutrition, foods and the nutrients they contain must be available to the family in adequate quantity; the correct balance of foods and nutrients must be fed at the right intervals; the individual must have an appetite to consume the food; there must be proper digestion and absorption of the nutrients in the food; the metabolism of the person must be reasonably normal; and there should be no conditions that prevent body cells from utilizing the nutrients or that result in abnormal losses of nutrients. Factors that adversely influence any of these requisites can be causes of malnutrition, particularly PEM. The aetiology, therefore, can be complex. Certain factors that contribute to PEM, particularly in the young child, are related to the host, the agent (the diet) and the environment. The underlying causes could also be categorized as those related to

University of Ghana http://ugspace.ug.edu.gh

the child's food security, health (including protection from infections and appropriate treatment of illness) and care, including maternal and family practices such as those related to frequency of feeding, breastfeeding and complementary feeding. Some factors enumerated by FAO as involved in the aetiology of PEM include (FAO, 2010):

a) the young child's high need for both energy and protein per kilogram relative to those of older family members;
b) inappropriate complementary feeding practices;
c) staple diets that are often of low energy density (not infrequently bulky and unappetizing), low in protein and fat content and not fed frequently enough to children;
d) inadequate or inappropriate child care because of, for example, time constraints for the mother or lack of knowledge regarding the importance of exclusive breastfeeding;
e) inadequate availability of food for the family because of poverty, inequity or lack of sufficient arable land, and problems related to intrafamily food distribution;
f) infections (viral, bacterial and parasitic) which may cause anorexia, reduce food intake, hinder nutrient absorption and utilization or result in nutrient losses;
g) famine resulting from droughts, natural disasters, wars, civil disturbances, etc.

University of Ghana http://ugspace.ug.edu.gh

## 2.4 Aetiological Agents of Infectious Diarrhoea

A wide array of microbes causes diarrhoea in children, and includes viruses, bacteria and parasites (Brooks *et al.*, 2006; Gomez-Duarte *et al.*, 2009; Haque *et al.*, 2003b; Ramani and Kang, 2009). Even in the best of studies no enteric pathogen is identified in one-third of cases, and infections with multiple putative enteric pathogens are observed frequently (Petri *et al.*, 2008).

It is most important to ascertain the etiologic agents of diarrhoea in children in developing countries, as this is the predominant group that die from diarrhoea and are at risk for the vicious cycle of diarrhoea and malnutrition (Figure 1). Enteric pathogens that are the cause of most severe acute diarrhoea, as assessed by mortality, include rotavirus, *Vibrio cholerae*, *Shigella* spp., *Salmonella* spp., enteropathogenic *E. coli* (EPEC), and EAEC. Studies linking specific microbes with malnutrition are limited, but currently there is data linking malnutrition and attendant loss of cognitive function to infection with EAEC, enterotoxigenic *E. coli* (ETEC), *Shigella* spp., *Ascaris lumbricoides*, *Cryptosporidium* spp., *Entamoeba histolytica*, *Giardia lamblia*, and *Trichuris trichiura* (Chang *et al.*, 2002; Checkley *et al.*, 2003; Mata, 1992; Steiner *et al.*, 1998). A better understanding of which enteric pathogens are responsible for how much of the burden of diarrhoea morbidity and mortality is required. Although such elucidation would be challenging, it would permit a more informed allocation of resources for the development of treatments and vaccines and should be a research priority (Petri *et al.*, 2008).

University of Ghana http://ugspace.ug.edu.gh

## 2.5 Diarrhoeagenic *Escherichia coli* (DEC)

*Escherichia coli* are the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life, and thereafter *E. coli* and the host derive mutual benefit for decades (Kaper *et al.*, 2004). As long as these bacteria do not acquire genetic elements encoding virulence factors, they remain commensals in the gut. However, for some reasons that are not so clear, some *E coli* clones acquire specific virulence factors, which increase their ability to adapt to new niches and allow them to cause a broad spectrum of diseases. *E. coli* strains that acquire 'foreign' DNA encoding enterotoxins, adhesions, or invasion factors become virulent and can cause either a plain, watery diarrhoea or inflammatory dysentery are collectively termed DEC (Weintraub, 2007).

DEC have been recognized as intestinal pathogens since the 1940s when Bray hypothesized that *E. coli* subtypes might account for common infantile diarrhoea of unknown etiology (Bray, 1945). Conclusively, using the Kauffman White scheme of O:H serotyping, Neter tested the Bray hypothesis and reported that certain 'enteropathogenic' serovarieties of *E. coli* showed close association with infantile diarrhoea (Neter *et al.*, 1955). Their etiologic role in diarrhoea was verified by Levine *et al.* in volunteer challenge experiments reported in 1978 (Levine *et al.*, 1978). Half a century of microbiology research revealed that most 'enteropathogenic' serotypes harboured virulence genes that are absent in non-pathogens so that by 1998, five categories of DEC that had been unequivocally

University of Ghana http://ugspace.ug.edu.gh

associated with diarrhoea were known and at least three more categories were under evaluation (Nataro *et al.*, 1998).

There are six well-described categories or pathotypes of DEC. Each of these categories has virulence attributes that help them to cause diseases by different mechanisms. The categories include enterotoxigenic *E. coli* (ETEC), which are characterized by producing heat-stable or heat-labile enterotoxins or both; enterohaemorrhagic *E. coli* (EHEC), which are characterized by attaching-and-effacing (A/E) lesions and produce shiga-like toxin or verotoxins; enteropathogenic *E. coli* (EPEC), which elicit characteristic attaching and effacing lesions on the intestinal mucosa; enteroinvasive *E. coli* (EIEC), which has the ability to invade epithelial cells similar to *Shigella* and is characterized by the presence of a large invasiveness plasmid; diffusely adherent *E. coli* (DAEC) demonstrates pattern of diffuse adherence, and enteroaggregative *E. coli* (EAEC), which demonstrate a characteristic "stacked-brick" aggregative adherence when cultured with HEp-2 cells (Nataro and Kaper, 1998; Nataro *et al.*, 1998). Other categories of DEC have been proposed, such as cell detaching *E. coli* (CDEC). However, their significance remains uncertain (Abduch Fabrega *et al.*, 2002).

University of Ghana http://ugspace.ug.edu.gh

## 2.6 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC), is a category of the DEC, and was discovered only in the last two decades (Nataro *et al.*, 1987). EAEC is associated with diarrhoea in several contexts: traveler's diarrhoea (Adachi *et al.*, 2001; Cabada and White, 2008), paediatric diarrhoea (Paul *et al.*, 1994), food borne out-breaks (Scavia *et al.*, 2008) and human immunodeficiency virus (Samie *et al.*, 2007b; Wanke *et al.*, 1998). The diarrhoea may be symptomatic or asymptomatic (Nataro *et al.*, 1987), acute or persistent in nature (Lima *et al.*, 2000; Okeke *et al.*, 2003). EAEC is the second most common cause of traveler's diarrhoea, and is a common cause of acute and persistent diarrhoeal illness in children and adults presenting to emergency departments and inpatient units in the USA (Nataro *et al.*, 2006). A recent study in Ghana indicates that, asymptomatic carriage of EAEC by adults may place infants and younger children at risk (Opintan *et al.*, 2010).

The current review gives priority to EAEC, as an emerging pathogen with new challenges in diagnosis and epidemiology. By definition, EAEC are *E. coli* lacking the heat-stable (ST) and heat-labile (SL) toxins of ETEC, and adhere to HEp-2 cells in an aggregative or 'stacked brick' pattern (Nataro, 2005). Within the EAEC pathotypes (category), two sub-categories have also been described based on the presence or absence of the master (*aggR*) regulon (Elias *et al.*, 2002a; Gioppo *et al.*, 2000; Itoh *et al.*, 1997). Typical- and atypical-EAEC refers to EAEC which possess the master regulon, or do not, respectively.

University of Ghana http://ugspace.ug.edu.gh

### 2.6.1 Virulence profile of EAEC

A few dozens of putative EAEC virulence genes and virulence factors have been identified to-date. They include the master regulon (Jenkins *et al.*, 2005; Nataro *et al.*, 1995; Nataro *et al.*, 1994; Sheikh *et al.*, 2002), fimbriae and enterotoxins (Bernier *et al.*, 2002; Czeczulin *et al.*, 1997; Nataro *et al.*, 1993; Nataro *et al.*, 1994), outer membrane protein (Monteiro-Neto *et al.*, 2003), dispersin transporter (Iwashita *et al.*, 2006; Nishi *et al.*, 2003), yersiniabactin iron-scavenging system (Schubert *et al.*, 1998) and lectin (Basu *et al.*, 2004). The clinical implication of these genes and factors however remain elusive (Nataro, 2005). Table 1 shows selected EAEC genes and their description.

University of Ghana http://ugspace.ug.edu.gh

**Table 1. Selected EAEC virulence factor and their description**

| Gene | Description/reference |
|---|---|
| **Master regulator** | |
| *aggR* | Regulates a package of EAEC plasmid virulence genes, including genes encoding aggregative adherence factors, fimbriae AAF/I and AAF/II, a dispersin protein (aap), and a large cluster of genes inserted on a pathogenicity island at the *PheU* locus (Jenkins *et al.*, 2005; Nataro, 2005; Nataro *et al.*, 1994; Sheikh *et al.*, 2002). |
| **Secreted proteins** | |
| *aap* | Is a secreted low-molecular-mass protein (10.2 kDa) that coats the bacterial surface and promotes dispersal of EAEC on the intestinal mucosa. *aap* lies immediately upstream of *aggR* in EAEC strain 042 (Grover *et al.*, 2001; Sheikh *et al.*, 2002). |
| *aaiC* | Is a chromosomal gene which is regulated by *aggR*. It encodes some 25 contiguous genes *(aaiA-Y)*, which are localized to a 117 kb pathogenicity island (PAI) inserted at pheU. Many of these genes have homologues in other Gram-negative bacteria and were recently proposed to constitute a type VI secretion system (T6SS). AaiC is a secreted protein that has no apparent homologues within the GenBank (Dudley *et al.*, 2006). |
| *pic* | The Pic protein has mucinase activity and is capable of causing haemagglutination of erythrocytes (Behrens *et al.*, 2002; Harrington *et al.*, 2009). |
| **Dispersin transporter** | *aatA* is one of an autotransport system comprising a |

University of Ghana http://ugspace.ug.edu.gh

| | |
|---|---|
| *aatA* | cluster of five genes (designated *aat-PABCD*) on the AA probe. *aatA* localizes to the outer membrane and facilitates the export of the dispersin (aap) across the outer membrane. It has a homolog with *E. coli* TolC (Imuta *et al.*, 2008; Iwashita *et al.*, 2006). Recent gene analyses by some authors suggest that *aatA* is strongly associated with virulence of *E. coli* from avian sources (Dai *et al.*, 2010; Li *et al.*, 2010). |
| **Enterotoxins** | |
| *Pet* | The plasmid-encoded toxin (Pet) of EAEC is a 104 kDa serine protease autotransporter, produced by the Enterobacteriaceae and other Gram-negative pathogens. After Pet is released into the extracellular medium, it enters intestinal epithelial cells by clathrin-dependent endocytosis. The internalized toxin then escapes the endomembrane system and cleaves the cytosolic actin-binding protein α-fodrin. The resulting disruption to actin architecture and cellular morphology contributes to the pathogen induced mucosal damage (Dautin and Bernstein, 2007; Navarro-Garcia *et al.*, 2007; Navarro-Garcia *et al.*, 2001; Nemec *et al.*, 2010; Scaglione *et al.*, 2008). |
| *astA*/EAST1 | Encodes the enteroaggregative heat-stable toxin, which has physical and mechanistic similarities to *E. coli* STa enterotoxin (Harrington *et al.*, 2006; Menard and Dubreuil, 2002). |
| **Fimbriae** | |
| *aggA* | Encodes AAF/I haemagglutination of erythrocytes and adherence adherence to colonic mucosa (Nataro *et al.*, 1993; Nataro *et al.*, 1994). |
| *aafA* | Encodes AAF/II, which mediates adherence to colonic mucosa and haemagglutination of erythrocytes (Czeczulin *et al.*, 1997). |
| *agg3A* | Encodes AAF/III haemagglutination of erythrocytes (Bernier *et al.*, 2002). |

University of Ghana http://ugspace.ug.edu.gh

## 2.7 Diagnostic Challenges in the Identification of EAEC

EAEC was originally identified by the characteristic "stacked-brick" aggregative adherence (Figure 7), when cultured in static Luria broth at 37°C and incubated for 3 hours in HEp-2 cells (Nataro and Kaper, 1998; Nataro *et al.*, 1987). The Hep-2 assay has remained a research tool, and its application has not been translated into the clinical diagnosis of EAEC.

Molecular diagnostics have been developed for detection of EAEC as alternatives to the adherence assay to epithelial cells, which is expensive and demands cell culture facilities (Nataro and Kaper, 1998). Among them, a multi-plex PCR detection of three EAEC plasmid borne loci has been proposed (Cerna *et al.*, 2003). This assay detects *aggR*, encoding a transcriptional activator of several EAEC virulence genes (Harrington *et al.*, 2006; Nataro *et al.*, 1994); *aatA* (formerly known as EAEC or CVD432 probe fragment), encoding an outer membrane protein that is part of a protein transporter system (Baudry *et al.*, 1990; Nishi *et al.*, 2003); and *aap*, which encodes the anti-aggregation protein or dispersin (Sheikh *et al.*, 2002). These gene markers are present in the high-molecular-weight plasmid (pAA2) of the EAEC prototype strain 042 (Czeczulin *et al.*, 1999).

Majority of HEp-2 positive strains are also positive for the anti-aggregation protein transporter gene (*aatA*) by PCR (Schmidt *et al.*, 1995). However, in several studies, it has been found that about 10% of EAEC strains verified by the

University of Ghana http://ugspace.ug.edu.gh

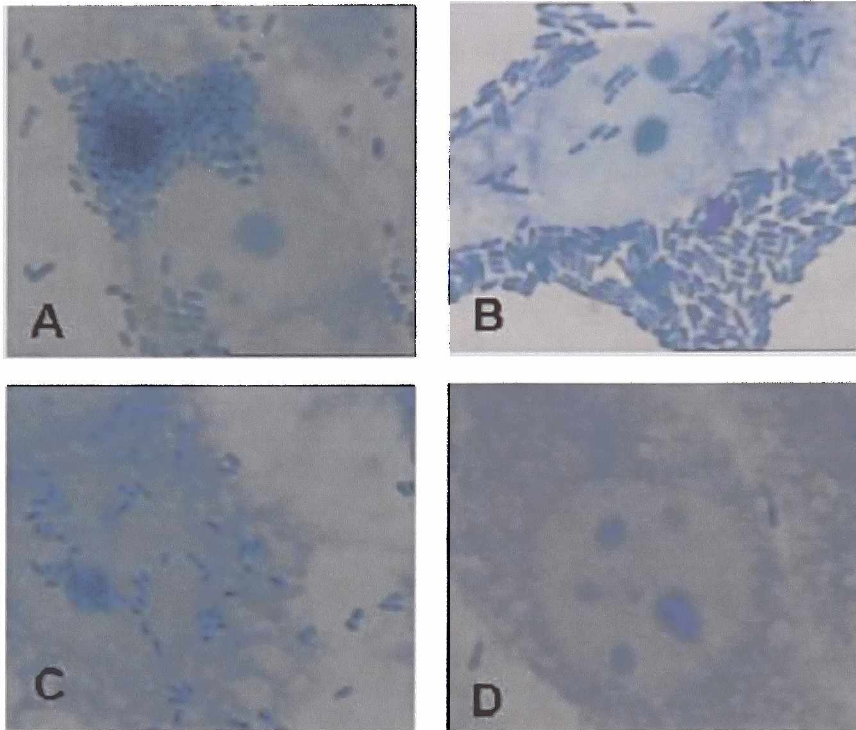



**Figure 7: Adherence pattern of diarrhoeagenic *E. coli* (DEC) to cultured epithelial cell.** A, Localized adherence by enteropathogenic *E. coli* (EPEC); B, aggregative adherence by enteroaggregative *E. coli* (EAEC); C, diffuse adherence by Diffusely adherent *E. coli* (DAEC); and D, non-adherent control strain. Adapted from (Okeke, 2009)

University of Ghana http://ugspace.ug.edu.gh

HEp-2 assay were negative in the PCR assay (Jenkins *et al.*, 2006; Menard and Dubreuil, 2002; Weintraub, 2007). This clearly provided evidence to show that it is difficult to provide a genotypic definition for EAEC and to design specific molecular biological assays for detection. The HEp-2 cell adherence assay is currently performed only in research settings, and is labour intensive.

Several attempts have been made to develop a molecular biological assay for the identification of EAEC. A cryptic DNA fragment sequence known as "CVD432" or aggregative adherence (AA), from the pAA has been used as an EAEC molecular marker in epidemiological studies and comprises the locus *att* that encodes an ABC (ATP-binding cassette) transporter system (Baudry *et al.*, 1990; Okeke *et al.*, 2000a). A transcription activator known as "*aggR*," the gene of which lies on pAAs, has been described as the major EAEC virulence regulator for diverse virulence genes (Nataro, 2005). Recently, some epidemiological studies have suggested that CVD432-positive strains, which are predicted to carry the aggR regulon, are the true EAEC pathogens termed "typical EAEC" (Harrington *et al.*, 2006; Jenkins *et al.*, 2006). However, AA probe-negative strains share virulence factors with AA probe positive strains, which clearly indicate that additional factors are involved in the AA phenotype in these EAEC strains (Bouzari *et al.*, 2001). A problem with using DNA probes for EAEC demonstrates heterogeneity and no single study has been able to demonstrate a 100% correlation with the HEp-2 cell assay (Sarantuya *et al.*, 2004).

University of Ghana http://ugspace.ug.edu.gh

Several assays such as autoagglutination or clump test (Albert *et al.*, 1993; Iwanaga *et al.*, 2002) and quantitative biofilm test (Wakimoto *et al.*, 2004) have been devised and at least, evaluated for the screening of EAEC. The clump test may be less useful in the diagnosis of EAEC since several bacteria strains can autoagglutinate. However, the quantitative biofilm assay may be a useful screening tool when a large number of strains are examined in clinical and epidemiologic studies. In the study by Wakimoto *et al.*, all EAEC strains confirmed by the HEp-2 assay demonstrated an $OD_{570} > 0.2$, and the incidence of EAEC among the strains with an $OD_{570} > 0.2$ was 89.2% (Wakimoto *et al.*, 2004). Furthermore, the test may be available without the need for a spectrophotometer, since a biofilm demonstrating an $OD_{570} > 0.2$ is clearly visible. In addition, this assay may contribute to demonstrating the true incidence of EAEC with and without *aggR* among clinically isolates of *E. coli*. Of the 28 PCR-positive (*aggR* and EAST) strains screened for biofilm, 25 (89.2%) demonstrated positive results by microtiter plate method (Wakimoto *et al.*, 2004).

Pet is a protease encoded on the pAA plasmid of strain 042 and other EAEC strains (Eslava *et al.*, 1998). Although this toxin is unique to EAEC, the prevalence of Pet among EAEC isolates varies between 18-44 % (Vila *et al.*, 2000; Yamazaki *et al.*, 2000). The emergence of EAEC infection in Brazil (Zamboni *et al.*, 2004) and the detection complexity of Pet expressing EAEC isolates led to the development of a methodology for Pet detection directly from

University of Ghana http://ugspace.ug.edu.gh

supernatants of bacterial isolates using a slot blot immunoassay (Taddei *et al.*, 2005).

Other proposed diagnostic tests include an enzyme-linked immunosorbent assay (ELISA) for quantitative detection of secretory immunoglobulin A to EAEC (Sutjita *et al.*, 2000) and cytokine response patterns to enteropathogens in which a specific pattern may become a distinguishing pathogen signature (Greenberg *et al.*, 2002). More studies and better diagnostic tools are needed to allow for a better understanding of the true epidemiology of EAEC in children.

Serotyping of EAEC is a problem due to their aggregative phenotype, many of the strains auto-agglutinate and is often described in the literature as nontypable or as O-rough. EAEC from German children demonstrated 14 typable isolates and all belonged to different serotypes (Huppertz *et al.*, 1997). In another study in UK, 97 EAEC strains were serotyped and found to belong to 40 different O-types. In one of the studies, 93 out of 143 EAEC strains could be serotyped and belonged to as many as 47 different serotypes (Jenkins *et al.*, 2006).

University of Ghana http://ugspace.ug.edu.gh

## 2.8 Pathogenesis of EAEC infections

The pathogenesis of EAEC is complex (Elias *et al.*, 2002b; Harrington *et al.*, 2005; Nataro, 2005). EAEC is able to bind to jejunal, ileal and colonic epithelium (Huang *et al.*, 2006a). Electron microscopy of infected small and large-intestinal mucosa, from children between 3 and 190 months, infected with several different EAEC strains, reveals bacteria in a thick mucus layer above the intact enterocyte brush border (Hicks *et al.*, 1996). In the colon, EAEC elicits inflammatory mediators and produces cytotoxic effects such as microvillus vesiculation, enlarged crypt openings, and increased epithelial cell extrusion (Harrington *et al.*, 2005).

As reviewed by Haung and others, EAEC pathogenesis involves three stages: **(1)** Adherence to the intestinal mucosa by aggregative adherence fimbriae (AAF) or other adherence factors (Hicks *et al.*, 1996); **(2)** Production of mucus by bacteria and the host cell forming a biofilm on the surface of the enterocytes; and **(3)** Release of toxins and elicitation of an inflammatory response, mucosal toxicity and intestinal secretion (Harrington *et al.*, 2005; Huang *et al.*, 2004; Nataro, 2005).

EAEC adherence to the intestinal mucosa requires AAF and adherence factors (Moreira *et al.*, 2003). Three AAF structural subunits encoded by *aggA* (AAF/I), aafA (AAF/ II) and agg-3 (AAF/III) on the 60–65 MDa pAA plasmid have been described, each EAEC isolate carries only one AAF subtype. *aggA* encodes

University of Ghana http://ugspace.ug.edu.gh

AAF/I, and is responsible for the aggregative phenotype and human erythrocyte haemagglutination of some EAEC strains (Nataro *et al.*, 1993). *aafA* encodes AAF/II, which allows EAEC to adhere to the intestinal mucosa (Czeczulin *et al.*, 1997). Both *aggA* and *aafA* are regulated by the transcriptional activator *aggR*. AAF/III functions as an adhesin, and is encoded by agg-3, which has a sequence closely related to that of the agg and aaf operon of DAEC (Bernier *et al.*, 2002). Other adherence factors have also been described. Three membrane-associated proteins (MAPs), of 18, 20 and 58 kDa, are believed to play an important role in EAEC adherence to and haemagglutination of animal cells (Spencer *et al.*, 1998). One study has characterized the outer-membrane protein (OMP) profiles of EAEC strains from children with diarrhoea from Sao Paulo, Brazil, and has observed a heterogeneity in OMP profiles, suggesting that EAEC strains are very heterogeneous (Monteiro-Neto *et al.*, 2003). AggR regulates the expression of a secreted low-molecular weight protein known as dispersin (aap) (Sheikh *et al.*, 2002). Aap lies immediately upstream of aggR in EAEC strain 042. Dispersin is a 10.2 kDa protein that has been identified in 80% of EAEC isolates from one laboratory (Sheikh *et al.*, 2002). This protein is exported by an ATPbinding cassette (ABC) transporter complex which is encoded by a genetic locus on the EAEC virulence plasmid pAA2 (Nishi *et al.*, 2003). The locus consists of a cluster of five genes (designated aat-PABCD), including homologues of an inner-membrane permease (AatP), an ABC protein (AatC) and an OMP TolC (AatA). AatA localizes to the outer membrane independently of its ABC partner. Dispersin appears to require the Aat complex for outermembrane translocation,

University of Ghana http://ugspace.ug.edu.gh

but not for secretion across the inner membrane. In a similar manner to the dispersin gene (*aap*), transcription of the aat cluster is dependent on AggR, a regulator of a package of virulence genes in EAEC (Jenkins *et al.*, 2005). Dispersin is responsible for mediating dispersal of EAEC across the intestinal mucosa to allow for efficient adherence and aggregation. This protein neutralizes the negatively charged LPS of the EAEC surface, allowing the positively charged AAF to splay out from the bacterium. In a volunteer challenge study, dispersin has been shown to be highly immunogenic, suggesting that it is a potential vaccine candidate (Nataro *et al.*, 1995). Undoubtedly, other potential AAF and adherence factors exist, and they are currently being investigated.

The second stage of EAEC pathogenesis involves production of a mucus layer by the bacteria and the intestinal mucosa. Animal and *in vitro* culture studies show that EAEC survives within the mucus layer, explaining why individuals infected with EAEC, especially children in developing countries with pre-existent malnutrion, may develop mucoid stools, malnutrition, and persistent colonization with prolonged diarrhoea. One study has identified biofilm production in 48 of 62 (77 %) EAEC strains from Japanese children with diarrhoea, using a quantitative biofilm assay, suggesting that this assay may be a useful and convenient screening tool for EAEC (Wakimoto *et al.*, 2004). Transposon mutagenesis studies suggest that biofilm production by EAEC strain 042 is dependent on two genes. *Fis* is a chromosomal gene encoding a DNA-binding protein involved in growth-phase-dependent regulation, and *yafK* encodes a secreted 28 kDa protein (Sheikh *et al.*,

University of Ghana http://ugspace.ug.edu.gh

2001). Both genes are mediated by AAF and likely reflect its interaction with the intestinal mucosa. Molecular epidemiologic studies are ongoing to determine the clinical impact of infection with EAEC strains that produce biofilm, and to investigate the genetic markers that identify biofilm-producing EAEC.

The third stage of EAEC pathogenesis involves release of EAEC toxins, and elicitation of an inflammatory response, mucosal toxicity and intestinal secretion. Numerous EAEC toxins have been described. Both animal and human studies show that EAEC toxins are destructive to the tips and sides of intestinal villi and enterocytes. Several toxins are part of this process. The three toxins best studied are plasmid encoded toxin (Pet) (Navarro-Garcia *et al.*, 2001), EAEC heat-stable enterotoxin (EAST1) (Menard and Dubreuil, 2002), and Shigella enterotoxin 1 (ShET1) (Behrens *et al.*, 2002).

Pet is a cytopathic serine protease autotransporter that functions as an enterotoxin and a cytotoxin (Dutta *et al.*, 2002). Intracellular expression of Pet is accompanied by cleavage of spectrin within the cytoskeleton of intestinal microvilli. *In vitro* studies show that purified toxin induces cell elongation and rounding, followed by exfoliation of cells from the substratum. These effects are accompanied by loss of actin stress fibres and electrophysiologic changes (Sui *et al.*, 2003). EAST1 is encoded by astA and is a heat-stable protein similar to the heat-stable toxin of ETEC. EAST1 was originally detected in EAEC strains.

University of Ghana http://ugspace.ug.edu.gh

However, EAST1 has subsequently been identified in ETEC, EHEC, EPEC and DAEC (Menard and Dubreuil, 2002).

The host inflammatory response to EAEC infection is dependent on the host innate immune system and the EAEC strain. The role of putative virulence genes and clinical outcomes is unclear. EAEC carrying 'virulence' genes are not always associated with disease; however, virulence factors are associated with increased levels of faecal cytokines and inflammatory markers, such as interleukin 8 (IL-8), interferon (INF)-γ, lactoferrin, faecal leukocytes, and occult blood (Huang *et al.*, 2004; Jiang *et al.*, 2002). IL-8 is an important pro-inflammatory chemokine involved in EAEC pathogenesis. IL-8 is responsible for recruiting neutrophils to the epithelial mucosa without mucosal injury, and facilitates intestinal fluid secretion (Kucharzik *et al.*, 2005; Sansonetti *et al.*, 1999).

Travellers to Mexico who developed symptomatic illness due to EAEC infection excreted high concentrations of faecal IL-8 compared to travellers who did not (Jiang *et al.*, 2003). In addition to IL-8, intestinal epithelial cells infected with EAEC 042, the prototype strain, have been shown to upregulate several genes (Huang *et al.*, 2006a). These cellular responses are primarily mediated by flagellin (fliC), a major bacterial surface protein of EAEC (Harrington *et al.*, 2005). Flagellin causes IL-8 release from several epithelial cell lines by binding to Toll-like receptor 5 (TLR5). TLR5 induce transcription of pro-inflammatory cytokines from epithelial and monocytic cells (Khan *et al.*, 2004).

University of Ghana http://ugspace.ug.edu.gh

## 2.9 Inflammatory Diarrhoea and its Assessment

Diarrhoea can range from a self-limiting, benign condtion to severe, life-threatening illness, the complications of which are often related to infection with pathogens that invade the mucosa to cause inflammation (Guerrant *et al.*, 1999b; Venkataraman *et al.*, 2003). In clinical settings, it is often required to distinguish between inflammatory and irritable bowel syndrome (IBS), a non-inflammatory disease which represents a large part of gastroenterological practice (Tibble *et al.*, 2000). Usually, clinicians rely on several biochemical studies to help support a diagnosis of either IBD or gauge a patient's disease activity during a particular office visit (Walker *et al.*, 2007).

Intestinal inflammation can be assessed by the use of $^{111}$ indium neutrophil technique (Saverymuttu *et al.*, 1986). In addition to the high cost (about £300/patient) of this procedure, there are practical problems with obtaining complete faecal collections over 4 days. For these reasons, the use of labeled indium has been limited to only research centers. An alternative non-invasive method to quantify intestinal inflammation is to analyse protease resistant neutrophil derived proteins such as elastase (Adeyemi and Hodgson, 1992) or lactoferrin (Guerrant *et al.*, 1992a; Uchida *et al.*, 1994) in stool specimens. Calprotectin is one such protein, which has also been evaluated in paediatric infections (Bunn *et al.*, 2001a; Bunn *et al.*, 2001b).

University of Ghana http://ugspace.ug.edu.gh

Conventional stool culture in all patients presenting with acute diarrhoea is impractical, expensive and time-consuming, and results in a very low yield of positive cultures (Guerrant *et al.*, 1985; Venkataraman *et al.*, 2003). In the investigation of infectious gastro-intestinal disorders and IBD, several authors used lactoferrin as a marker for neutrophil infiltration (Choi *et al.*, 1996; Fine *et al.*, 1998; Guerrant *et al.*, 1992a; Kane *et al.*, 2003). In several of these studies however, faecal lactoferin was semi-quantified by the use of latex agglutination (Fine *et al.*, 1998; Samie *et al.*, 2007b). As both lactoferrin and calprotectin can be measured with ease in faeces, they have the potential to be used in a routine screening procedure as an aid to discriminate between normal and inflammed intestines (Lundberg *et al.*, 2005; Roseth *et al.*, 1997). Currently, most of these assays are not used in routine screening of IBD because they are expensive. A 96-well ELISA plate of calprotectin and lactoferrin costs $ 870 and $ 835, respectively.

University of Ghana http://ugspace.ug.edu.gh

### 2.9.1 Pathophysiology of Lactoferrin

Lactoferrin is a glycoprotein consisting of a single polypeptide chain of about 80 kDa with two globular lobes each containing an iron-binding site (Steijns and van Hooijdonk, 2000). Lactoferrin is the second most abundant protein in human milk, and also found in most exocrine secretions including tears, saliva, intestinal mucus and genital secretions (Farnaud and Evans, 2003). Because lactoferrin is found in the specific granules of neutrophils, it serves as a surrogate marker for neutrophil infiltration (Baynes *et al.*, 1988; Farnaud and Evans, 2003). Multiple activities, including anti-microbial, anti-inflammatory, immunomodulatory activity have been described for lactoferrin (Baveye *et al.*, 1999; Brock, 2002; Choi *et al.*, 1996; Kirkpatrick *et al.*, 2002; Levay and Viljoen, 1995). However, the relevance of each of these putative mechanisms in humans remains to be proven (Brock, 2002; Farnaud and Evans, 2003).

Human and bovine lactoferrin are well characterized. They consist of 691 and 689 amino acids, respectively; the sequence identity is 69% (Pierce *et al.*, 1991). The 3-D structures of bovine and human lactoferrin are very similar (Steijns and van Hooijdonk, 2000). The concentration of lactoferrin in human milk is $5.3 \pm 1.9$ mg/mL in colostrum, and approximately 1 mg/mL after the first month of lactation (Hamosh, 2001). In contrast, the concentration of lactoferrin in bovine milk is very low (1.5 mg/mL in colostral whey and 20-200 μg/mL in milk) (Steijns and van Hooijdonk, 2000).

University of Ghana http://ugspace.ug.edu.gh

Several *in vitro* studies have demonstrated some inhibitory effects of lactoferrin on enteric pathogens, including bacteria (Bessler *et al.*, 2006), parasites (Leon-Sicairos *et al.*, 2005) and viruses (Seganti *et al.*, 2004). Both recombinant and purified human lactoferrin have been reported to inhibit the AA phenotype of EAEC in tissue culture cells (de Araujo and Giugliano, 2000; Ochoa *et al.*, 2006 ).

### 2.9.2 Lactoferrin (LF) assays for enteric pathogens

Commercially prepared latex beads coated with antibodies against lactoferrin (*LEUKO*-TEST, TechLab, Blacksburg, VA) provide a semi-quantitative agglutination method for the detection of faecal LF (Guerrant *et al.*, 1992a). It has been used as a screening test for inflammatory diarrhoea (Choi *et al.*, 1996; Huicho *et al.*, 1996; Huicho *et al.*, 1997). This latex agglutination test has been evaluated and found to be useful in the screening of invasive enteropathogens in travellers' diarrhoea (Scerpella *et al.*, 1994). The latex agglutination test for LF is positive in infection with DEC, *Clostridium difficile*, *Shigella*, *Campylobacter*, *E. histolytica* and *Cryptosporidium* spp (Alcantara *et al.*, 2003; Ashraf *et al.*, 2007; Bouckenooghe *et al.*, 2000; Fried *et al.*, 2002; Kohli *et al.*, 2008; McIver *et al.*, 2001; Miller *et al.*, 1994; Venkataraman *et al.*, 2003).

The IBD-SCAN (TechLab, Blacksburg, VA), which is an ELISA method, is able to quantify faecal LF in a gram of stool. Some authors have used the IBD-Scan in

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

**Table 2: Summaries of LF assays in relation to pathogen-specific diarrhoeas**

| Country/year | Assay type | Summaries and conclusions/references |
|---|---|---|
| Brazil 2003 | semi-quantitative | Stool samples of Brazilian children with/without *Cryptosporidium* associated diarrhoea were analyzed. Only 1/14 volunteers challenged with *Cryptosporidium* had increased LF. However 12/17 in previous study had mild to moderately elevated LF. The authors concluded that cryptosporidiosis is associated with mild inflammation, especially in children in an endemic area (Alcantara *et al.*, 2003). |
| US 1996 | semi-quantitative | 55 faecal samples, 46 with and 9 without diarrhoea were tested. Of 28 samples with salmonellosis, shigellosis or campylobacteriosis, 93% had detectable LF. In 83% of samples with rotavirus or no detectable pathogen, LF was negative at a titre of 1:50. All 9 controls without diarrhoea were negative at 1:50. The authors concluded that the use of LF to screen for inflammatory diarrhoea selects specimens for which stool culture is 5-fold more likely to yield an invasive bacterial pathogen (Choi *et al.*, 1996). |
| Bangladesh 2007 | semi-quantitative | 594 patients enrolled under the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) were evaluated. Faecal occult blood test (FOBT) and LF were done in 448/594 patients from whom either a single enteropathogen (53%) or no pathogen (22%) were identified and 146 were excluded for multiple pathogens. Invasive and non-invasive pathogens were isolated from 24% and 76% of the patients, respectively. They concluded that FOBT and LF are not useful in differentiating inflammatory and non-inflammatory diarrhoea in patients in Dhaka (Ashraf *et al.*, 2007). |

University of Ghana http://ugspace.ug.edu.gh

**Table 2 (Continuation): Summaries of LF assays in relation to pathogen-specific diarrhoeas**

| | | |
|---|---|---|
| Mexico and India 2000 | semi-quantitative | 45 cases of EAEC diarrhoea were compared to 56 controls with ETEC diarrhoea, and 126 controls without identifiable pathogens. For EAEC, ETEC and the controls without identifiable pathogens, LF was found in 60%, 26.8% and 21.4%, respectively. The authors concluded that EAEC infection is associated with an intestinal inflammatory response (Bouckenooghe *et al.*, 2000). |
| South Africa 2006 | semi-quantitative | 244 Stool samples were tested and 44 *Cryptosporidium* found. 57% of *Cryptosporidium* positive samples were diarrhoeic and 26 (59.1%) had elevated LF content (Samie *et al.*, 2006a). |
| South Africa 2007 | semi-quantitative | Stool samples from 255 patients and 67 primary school children were analyzed. Of all pathogens detected, only *C. jejuni* was significantly associated with diarrhoea and elevated FL (Samie *et al.*, 2007a). |
| South Africa 2006 | semi-quantitative | *E. histolytica* was significantly associated with diarrhoea and with the presence of LF (Samie *et al.*, 2006b). |
| US 2010 | quantitative | 26/46 stools collected had elevated quantitative levels, with only 5 subjects having diarrhoea. Of 2 samples with *C. difficle* infection, both were liquid and, when compared with all other liquid stools (n=22), the mean LF was statistically higher (134.1 vs 28.8 µg/ml, $p$=0.008) (Archbald-Pannone *et al.*, 2010). |
| Korea 2006 | semi-quantitative | Intestinal inflammation associated with increased faecal LF, important in bacterial enteric infection, was not found in Norovirus-associated gastroenteritis (Ko *et al.*, 2006). |

University of Ghana http://ugspace.ug.edu.gh

## 2.10 Cryptosporidiosis: Background and Epidemiology

Cryptosporidiosis is a notifiable disease in several countries yet its incidence in humans is probably underestimated in most settings. This is partly because testing for *Cryptosporidium* in many countries is not included in routine examination of human stool samples, and partly because of insufficient reporting systems (Valentiner-Branth *et al.*, 2003). Nonetheless, *Cryptosporidium* species are recognized globally as important causes of diarrhoea in children and adults. Currently, there are at least 20 valid *Cryptosporidium* species and over 40 unnamed species in mammals, birds and reptiles which only have genotype names (Coupe *et al.*, 2005; Xiao and Ryan, 2004).

Cryptosporidiosis is associated with: a) sporadic, often water-borne, outbreaks of self-limiting diarrhoea in otherwise healthy persons; b) chronic, life-threatening illness in immune-compromised patients, most notably those with human immunodeficiency virus/Acquired immune deficiency syndrome (HIV/AIDS); and c) diarrhoea and malnutrition in young children in developing countries (Mor and Tzipori, 2008). In industrialized nations, improved water-management practices have resulted in a decline in cryptosporidiosis in the general population (Lake, 2007); and antiretrovirals have curbed the incidence and severity in patients with HIV/ AIDS (Maggi *et al.*, 2000). In the absence of these interventions, the burden of cryptosporidiosis continues to fall heavily on developing regions, where infection is both more ubiquitous and clinically consequential (Mor and Tzipori, 2008).

University of Ghana http://ugspace.ug.edu.gh

Cryptosporidiosis occurs worldwide and is responsible for significant morbidity and mortality, especially in HIV-infected patients (Caccio and Pozio, 2006). Most cases of cryptosporidiosis are due to sporadic rather than outbreak-associated infections (Hunter *et al.*, 2004). However, waterborne and food-borne outbreaks are reported frequently and represent around 10% of all cases of *Cryptosporidium* infection (Hunter *et al.*, 2004). There is still no curative treatment (Abubakar *et al.*, 2007), making cryptosporidiosis a major public health issue and an economic problem (Corso et al., 2003; Jex et al., 2011; Kothavade, 2011).

The prevalence of cryptosporidiosis in children varies considerably across sub-Saharan Africa and within certain subsets of the population. In Ghana for example, Adjei et al reported cryptosporidiosis prevalence of about 29 % in Accra and in Kumasi, Addy et al reported about 12 % (Addy and Aikins-Bekoe, 1986; Adjei *et al.*, 2004). In South Africa, Berkowitz et al reported a prevalence of about 20 % in Johannesburg and Geyer reported 27% in Pretoria (Berkowitz, 1988; Geyer *et al.*, 1993). Although rarely diagnosed in this setting, coinfection with other enteric pathogens occurs frequently, because of common exposure through poor sanitation and hygiene and because of immune predisposition due to HIV infection (Mor and Tzipori, 2008). In most sub-Saharan countries, cryptosporidiosis prevalence peaks among children aged 6–12 months and decreases thereafter. Breast-feeding is speculated to afford some protection, either through conferment of immunoglobin or avoidance of contaminated water (Mor and Tzipori, 2008). This may explain why infection is delayed until after the age

University of Ghana http://ugspace.ug.edu.gh

of 6 months, an age that is commonly marked by the introduction of complementary foods. Children are likely to experience infection throughout childhood and adolescence, although the clinical significance becomes less apparent with age. This is probably attributable to development of immunity following frequent exposure to oocysts in the contaminated environment. Experimental studies reveal that repeated exposure to *Cryptosporidium parvum* promotes an IgG response that imparts partial protection against subsequent infection and illness (Chappell *et al.*, 1999).

Cryptosporidiosis was recognised in human beings in 1976, and was prominent as a cause of severe diarrhoeal illness in patients with HIV/AIDS in the 1980s and 1990s. Cryptosporidiosis is now additionally recognised as a major cause of waterborne diarrhoeal illness in developed and developing regions, and as a pathogen with long-term effect on childhood growth and development in impoverished areas (Kosek *et al.*, 2001). Infection and illness caused by *Cryptosporidium* species is ubiquitous and has been reported in more than 40 countries on six continents (Ethelberg *et al.*, 2009; Fall, 2003; Gay-Andrieu *et al.*, 2007; Zu *et al.*, 1994).

Critical determinants of the epidemiology of cryptosporidium infections include its small size, its low infectious dose, its high chlorine resistance, and its zoonotic potential. Cryptosporidia are spread by the ingestion of oocysts excreted by

University of Ghana http://ugspace.ug.edu.gh

infected people or animals. Infection can be transmitted through the consumption of faecally contaminated water or food, via direct person-to-person or animal-to-person contact, and contact with contaminated environmental sources (Kosek *et al.*, 2001).

### 2.10.1 Diagnosis and genotyping of *Cryptosporidium* spp

The pathology and diagnosis of cryptosporidiosis in humans is widely documented for *C. hominis* and *C. parvum* (Warren, 2008). Microscopy, employing dyes which stain the oocyst in stool specimens has generally been used in resource poor facilities for the diagnosis of cryptosporidiosis (Addy and Aikins-Bekoe, 1986; Adjei *et al.*, 2004; Morse *et al.*, 2007). There are commercially available enzyme immunoassays (EIAs) for the diagnosis of cryptosporidiosis. These have not only eliminated the rather cumbersome and the time required to perform direct microscopy, but additionally show greater sensitivities and specificities (Garcia et al., 2000; Sharp et al., 2001). Usually, however, the direct microscopy and the EIA methods offer no information on the infecting species and are unhelpful in epidemiological investigations (Fall, 2003).

The development of genetic tools has now made possible the detection of *Cryptosporidium* oocysts by PCR and species identification by sequencing, restriction fragment length polymorphism (RFLP), or the use of species-specific

University of Ghana http://ugspace.ug.edu.gh

probes (Caccio and Pozio, 2006; Coupe *et al.*, 2005; Stroup *et al.*, 2006). The molecular probes often target *Cryptosporidium* oocysts wall protein (COWP) or the 18s rRNA. In several cases, both *C. parvum* and *C. hominis* have a relatively conserved target region and further molecular differentiation such as RFLP is required. However, genotyping for specific identification remains restricted to reference or specialized laboratories. An emerging method for characterizing DNA samples is by the use of High Resolution Melting (HRM) (Reja *et al.*, 2010).

University of Ghana http://ugspace.ug.edu.gh

## 2.11 Dysentery: Background and Epidemiology

Bacillary dysentery is most commonly caused by microorganisms belonging to the genus *Shigella*, whereas amebic dysentery is caused by the protozoan parasite *Entamoeba histolytica*. Approximately, 164.7 million cases of shigellosis are reported worldwide, of which 163.2 million are in developing countries and 1.5 million in industrialized countries (Kotloff *et al.*, 1999). Each year 1.1 million people are estimated to die from *Shigella* infection and 580,000 cases of shigellosis are reported among travelers from industrialized countries (Kotloff *et al.*, 1999; WHO, 1999). Estimates of *E. histolytica* infections have primarily been based on examinations of stool for ova and parasites, but these tests are insensitive and cannot differentiate *E. histolytica* from morphologically identical species that are nonpathogenic, such as *E. dispar* and *E. moshkovskii* (Haque *et al.*, 2003a). Specific and sensitive means to detect *E. histolytica* in stool are now available and include antigen detection and PCR (Haque *et al.*, 2000). The disease is more severe in the very young and old and in patients receiving corticosteroids (Haque *et al.*, 2003a).

University of Ghana http://ugspace.ug.edu.gh

### 2.11.1 Diagnosis and genotyping of dysentery

There are four serogroups of *Shigella* with varying global distributions. *S. flexneri* is mostly seen in developing countries (median 60% of isolates), followed by *S. sonnei* (median 15%), *S. dysenteriae* and *S. boydii* with equal frequency (median 6%). In industrialized countries *S. sonnei* (median 77%) is mostly seen, followed by *S. flexneri* (median 16%), *S. boydii* (median 2%) and *S. dysenteriae* (median 1%) (Kotloff et al., 1999). Whilst *S. sonnei* have no serotype within its serogroup, the others have several serotypes based on their somatic O- antigen.

Rate of isolation of *Shigella* by routine stool culture is traditionally low, when the specimens are not cultured promptly or when the specimens are not transported on Cary- Blair transport media. The fastidiousness of the organism may account for low recovery rates (Opintan and Newman, 2007). Molecular methods of diagnosing shigellosis usually target genes coding for the invasive plasmid antigen H (*ipaH* gene) found in both *Shigella* spp and EIEC. In addition to the *ipaH* gene, EIEC also has *eae*, coding for the protein intimine located on the Locus of enterocyte effacement (LEE). Technically, *Shigella*, have only *ipaH* whilst EIEC have both *ipaH* and *eae*.

Molecular methods such as PCR and pyrosequencing reveal details needed for the differentiation of the Entamoeba complex (Stensvold *et al.*, 2010). Traditionally, *E. histolytica* is considered the pathogenic form and *E. dispar* as a commensal.

University of Ghana http://ugspace.ug.edu.gh

However, current findings employing molecular methods suggest that certain clones of *E. dispar* can be associated or be potentially responsible for intestinal or liver tissue damage, similar to that observed with *E. histolytica* (Ximenez *et al.*, 2010).

## 2.12 Giardiasis: Background and Epidemiology

Giardiasis is caused by *Giardia duodenalis* (synonymous *G. lamblia* and *G. intestinalis*) which is a flagellated protozoan parasite that reproduces in the small intestine. It is a cosmopolitan pathogen with a very wide host range, including domestic and wild animal species, as well as human beings (Plutzer *et al.*, 2010). Giardiasis is found worldwide and is especially common in areas where poor sanitary conditions and insufficient water treatment facilities prevail (Ortega and Adam, 1997). Human-derived *Giardia* were earlier assigned to a separate species (*G. lamblia*) and the major lineages have been defined by analysis of human-derived *Giardia* isolates, designated assemblages A and B (Mayrhofer *et al.*, 1995). *Giardia* cysts are transmitted by the faecal–oral route, either direct or indirect. Potential mechanisms of transmission include: person to person, animal to animal, zoonotic (animal to human), waterborne from humans or animals through drinking water or recreational contact such as swimming and food borne from contamination of water used in food preparation and manufacture or from food handlers (Karanis *et al.*, 2007; Plutzer *et al.*, 2010; Shields *et al.*, 2008).

University of Ghana http://ugspace.ug.edu.gh

### 2.12.1 Diagnosis and genotyping of giardiasis

Microscopically, giardiasis is diagnosed by the identification of cysts or trophozoites in the feces, using direct mounts as well as concentration procedures. In addition, samples of duodenal fluid (e.g., Enterotest) or duodenal biopsy may demonstrate trophozoites. Despite the value of duodenal biopsy or aspiration for the diagnosis of giardiasis, biopsy only supplements stool examination. Biopsy is less sensitive than stool examination but will identify patients for whom the diagnosis cannot be ascertained by stool examination alone (Ortega and Adam, 1997). Commercial kits for the assay of *Giardia* spp include antigen detection tests by enzyme immunoassays (EIAs) and detection of parasites by immunofluorescence (Boone et al., 1999; Rosoff et al., 1989; Sharp et al., 2001). Molecular diagnosis of giardiasis has mainly focused on genes coding for the beta giardin of the oocyst or the trophozoite (Calderaro *et al.*, 2010; Guy *et al.*, 2003; Haque *et al.*, 2007).

University of Ghana http://ugspace.ug.edu.gh

# CHAPTER THREE

## MATERIALS AND METHODS

### 3.1 Ethical consideration, Study design, Population and settings

The study was reviewed and approved by the Institutional Review Board of the University of Ghana Medical School, Ghana. Participation was voluntary and enrollment was subject to parents/guardians' approval, through signature or by thumb-printing their names after the purpose of the study was explained to them.

This was a prospective cross sectional study, and enrollment was done between August 2007 and May 2008 and involved children ≤ 5 years consulting at the Princess Marie Louise Children's Hospital (PML), Accra, Ghana. Consecutive children from whom consent was given by their caregivers were included. The diarrhoea sub-population was composed of outpatients brought to the hospital for treatment; and the control children without diarrhoea, were visiting for routine child welfare care. No follow-up was done after the initial recruitment as a part of this study.

Sample size for the current study was earlier determined based on achieving a 95% power of detecting major causes or sources of diarrhoea, with significance level set at d= 0.05. The equation $n = \frac{z^2pq}{d^2}$; where n= sample size, z= confidence interval, d= significance level, p= proportion in the population with diarrhoea and q= (1-p) = proportion in the population without diarrhoea was used. No reliable

University of Ghana http://ugspace.ug.edu.gh

record of p was known but based on an earlier study in Korle-Bu (Namboodiri, 2008); p was not expected to exceed 23%. Putting this into the equation above the minimum sample size was 272:

$$n = \frac{(1.96)^2 0.23 * 0.77}{(0.05)^2} = 2.72.1 \approx 272$$

### 3.1.1 Interviews and diarrhoea definition

A structured questionnaire was used to obtain information on the children from the parents/guardians. The questionnaire for the two sub-populations was similar. The only difference being that, the questionnaire for the children without diarrhoea had no questions on diarrhoea history. Information that was sought included bio-data, duration of diarrhoea, residence/location, breast feeding status and medication taken before visiting hospital [Appendix I: i, ii and iii]. Diarrhoea was defined as the passage of three or more unformed stools within a 24 h period, according to the WHO's guidelines on Integrated Management of Childhood Illness (WHO, 1995b). Diarrhoea lasting < 14 days was defined as acute and those lasting ≥ 14 days, persistent. The control (non-diarrhoea) group consisted of children who did not have diarrhoea at least within the 24 h period prior to enrollment.

University of Ghana http://ugspace.ug.edu.gh

### 3.1.2 Anthropometric data and nutritional status assessment

Height or length measurements in centimeter to the nearest one decimal were performed for children above or below two years of age respectively. Weight measurements in kilogram to the nearest one decimal were performed using a 25 kg Salter hanging scale (CMS Weighing equipment, High Holborn, London, UK). The Z-score, weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ) were calculated by use of soft-ware designed for nutrition studies (EPINUT, World Health Organization, Geneva; Epi Info version 6.0, Centers for Disease Control and Prevention, Atlanta). These anthropometric Z-scores are a measure of SD above or below the median for the international reference population. Z-score values were used to determine the nutritional status of children based on the following definition: WAZ; well nourished (> -1), mild (-2 to -1), moderate (-3 to -2) and severe (< -3) malnutrition; HAZ, normal height ( ≥ -2), moderate stunting (-3 < -2) and severe stunting (< -3), WHZ; normal weight ( ≥ -2), moderate wasting (-3 < -2) and severe wasting (< -3) (Waterlow *et al.*, 1977).

Epi Info graphics was used to draw the growth curves for males and females, for all children less than 2 years of age.

University of Ghana http://ugspace.ug.edu.gh

## 3.2 Specimen Processing and Microbiological Analysis

A stool specimen from each participating child was collected into a sterile container and processed within 4 h of collection. A bit of a fresh stool specimen from each child was kept frozen at - 20°C in cryo-vials (deidentified) until it was being sent to the Center for Global Health, University of Virginia, USA, for further analysis. A flow chart of how a stool specimen was processed at the Microbiology Department, UGMS, Ghana and the Centre for Global Health, UVa, USA is shown in Figure 8.

### 3.2.1 Routine stool culture

Bacteria were cultured on MacConkey (MAC), Salmonella-Shigella (SS) and deoxychocolate (DCA) agars (Oxoid, Maryland, USA), using standard techniques (Barrow and Feltham, 1993; WHO, 1987). Selenite F broth was used as enrichment for *Salmonella* before sub-culturing onto MAC, SS and DCA.

A stool specimen was plated onto full plate DCA and SS as primary plates and a loopfull also inoculated into selenite F broth [Figure 9]. To obtain discrete colonies, loops were flamed in between plating and aseptic conditions maintained in all cases. Agar plates together with broths were both incubated overnight at 37°C (DAY 1). After 24 hr incubation, primary plates were read and a loop full of broth culture subcultured onto fresh half plate DCA and incubated overnight at 37°C.

University of Ghana http://ugspace.ug.edu.gh

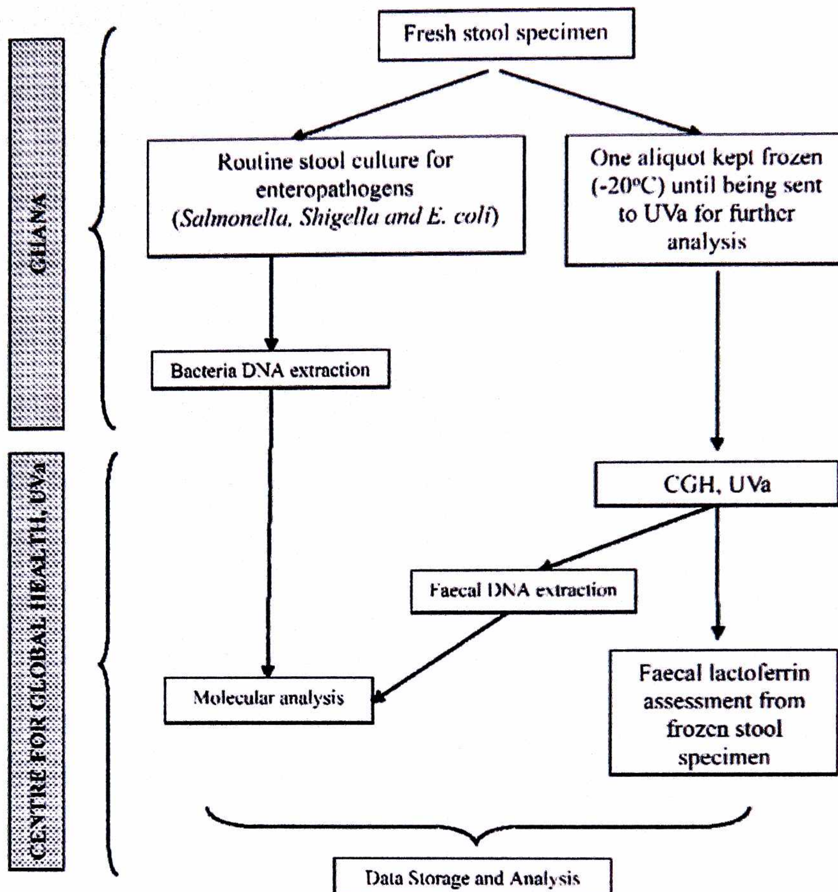



**Figure 8: A flowchart on processing of a fresh stool specimen.** A stool specimen was processed within 4 h of collection, and was cultured for routine enteric bacteria. Genomic DNA was extracted from bacterial isolates (*E. coli*) and frozen stool specimens by the use of the Wizard genomic kit (Promega, Madison, USA) and the QIAamp stool kit (Qiagen, Valencia, CA), respectively. Faecal lactoferrin was quantified by the use of the IBD-SCAN (TechLab, Blacksburg, VA).

University of Ghana http://ugspace.ug.edu.gh

The primary plates were also reincubated overnight at 37°C. After 48 hr incubation the primary plates were further examined, and those without growth were discarded. The half plates were similarly read at 24 hr and 48 hr interval of incubation at 37°C. Latose and non-lactose fermenting colonies were biochemically characterized.

### 3.2.2 Biochemical and serological identification of isolates

The biochemical tests carried out were urea, Triple Sugar Iron (TSI), oxidase, indole and citrate utilization test. A pure colony of suspected isolates on MacConkey plate was used for the biochemical tests, and reactions were read and recorded after a 24 h incubation period. For each test, a suspected isolate was suspended in peptone water and by the use of a straight loop; agar slants of citrate, urea and TSI were inoculated and incubated at 37°C. Indole test was performed by the addition of few drops of Kovac's reagent to an overnight peptone broth culture. A positive control, indicated by the formation of a pink interphase in the overnight's culture was set up using *E. coli* (ATCC 25922).

Serogrouping of *Shigella* was done by the slide agglutination tests with *Shigella* polyvalent grouping anti-sera (Mast Group Ltd., Merseyside, U.K). Fresh clean glass slide was used, and a positive and a negative control test were performed alongside each experiment, using test organism and sterile distilled water respectively.

University of Ghana http://ugspace.ug.edu.gh

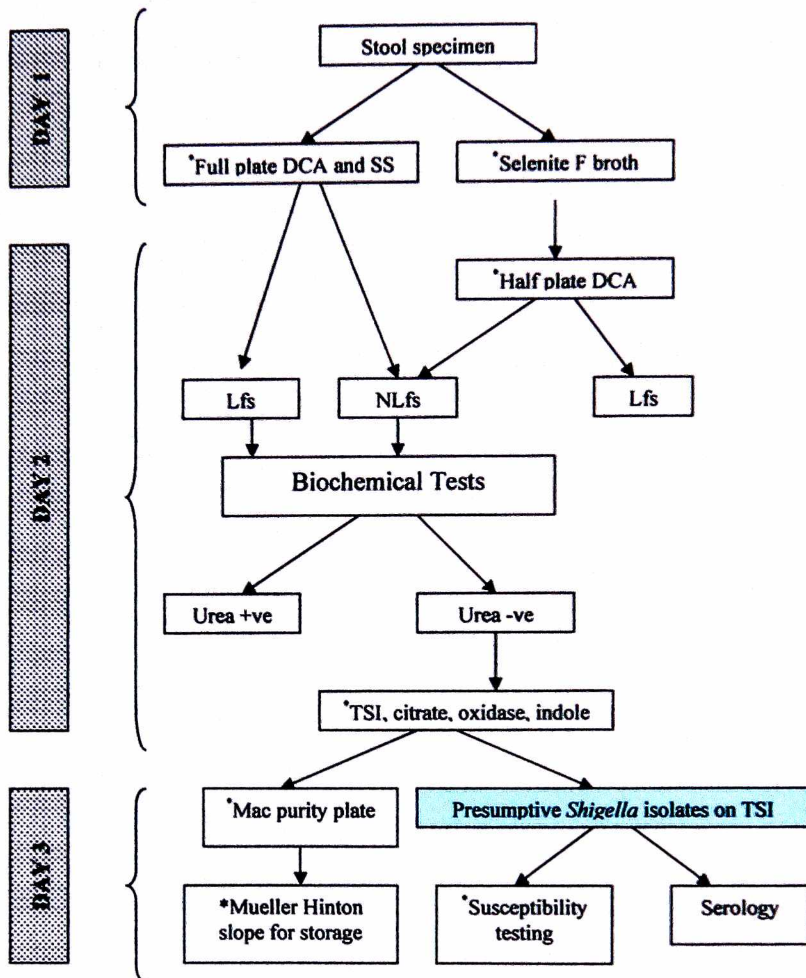



**Figure 9: A flowchart on culture of stool specimens for bacteria.** In addition to *Salmonella* and *Shigella*, other enteropathogens were characterized.

* Lfs – lactose fermentors, NLfs – non-lactose fermentors

University of Ghana http://ugspace.ug.edu.gh

### 3.2.3 Archiving of bacterial strains

Isolated *E. coli* and typed *Shigella* strains were sub-cultured from MacConkey purity plates onto Mueller Hinton (MH) slopes and labeled appropriately. The stoppers of the slopes were sealed with paraffin material to make them air-tight after overnight's incubation at 37°C and stored in a dark room for further studies. All information of an identified bacteria strain was recorded on the reverse sheet of the questionnaire and also entered into Microsoft Access data files.

University of Ghana http://ugspace.ug.edu.gh

### 3.3 Bacterial DNA Extraction (*E. coli* Isolates)

A genomic bacterium DNA was extracted from all the *E. coli* isolates, at the Microbiology Department of the University of Ghana Medical School. Over-night pure growth of bacteria on MacConkey plates were sub-cultured into 3 ml Trypticase Soy Broth (TSB) and incubated aerobically without shaking at 37°C. Bacterial cells were harvested from the over-night culture by centrifugation at 13,000 rpm for 8 min and DNA extracted by the alkaline lyses method using the Wizard Genomic kit (Promega, Madison, USA) with some minor modifications. To lyse cells, bacteria were uniformly suspended in 600 µl Nuclei Lysis solution and incubated at 80°C for 5 min over a water bath [Figure 10]. After cooling to room temperature, 3 µl RNase was added to the suspension, and mixed by inverting the tube 3-5 times. Suspension was then incubated for 1 h at 37°C. Proteins were precipitated by cold treatment after 200 µl of precipitation solution was added, vortexed and centrifuged at 13,000 rpm for 3 min. 600 µl were transferred into new Eppendolf tubes and DNA precipitated by cold treatment after the addition of 600 µl ethanol. The DNA pellet was purified by ethanol purification and centrifugation and air-dried for about 30 min and kept dry in a cool cupboard for further analysis. DNA pellet was rehydrated in 100 µl of Rehydration solution overnight at 4°C when needed for further analysis and thereafter, rehydrated bacterial DNA was kept frozen at -80 freezer.

For purposes of analysis, DNA extracted from the *E. coli* isolates are referred to as bacterial DNA.

University of Ghana http://ugspace.ug.edu.gh

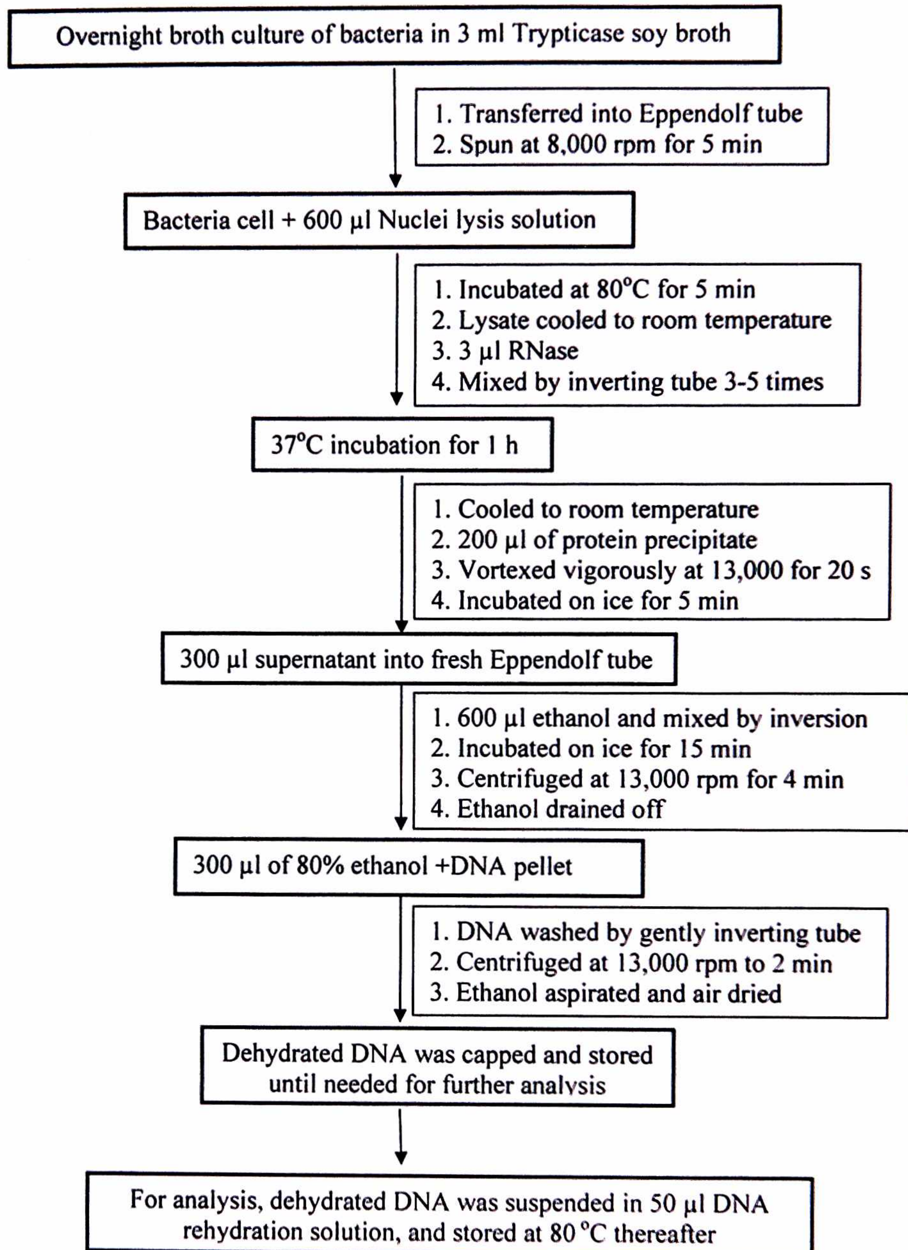



**Figure 10: A flowchart showing bacterial DNA extraction.** DNA was extracted by the alkaline method, using the Wizard Genomic kit (Promega, Madison, USA)

University of Ghana http://ugspace.ug.edu.gh

### 3.4 Stool DNA Extraction

The QIAamp stool kit (Qiagen, Valencia, CA) was used to extract genomic DNA from frozen stool specimens with some minor modifications. The modifications included the addition of small and big beads (MO BIO Laboratory Inc., Carlsbad, CA) to weighed stool specimen before the addition of lysis buffer (ASL). The stool-bead-mixture was bead-beated for 2 minutes in a Mini Beadbeater (Biospec Prdt., Bartlesville, USA) to make a uniform homogeneous mixture with the lysis buffer. Additionally, mixtures were incubated at 80 °C instead of the 70 °C recommended by the manufacture to lyse enteric pathogens, and eluted DNA was further purified in Zymogen columns (MO BIO Laboratory Inc., Carlsbad, CA). Figure 11 shows a flow chart of how stool DNA was extracted and the modifications made in the Qiagen stool kit (modifications highlighted). For each stool aliquot, between 15 – 20 µg or µl of stool was used depending on stool consistency.

DNAs were also extracted from pure control strains obtained from the Centre of Global Health, University of Virginia (EAEC 042 and 17-2, *Shigella*, *Cryptosporidium* and *Giardia* oocyst, *E. histolytica*. The initial steps in the DNA extraction process, which principally removes impurities from stool specimen was avoided, when dealing with pure cells. All DNAs were kept frozen at - 80°C until needed for analysis.

University of Ghana http://ugspace.ug.edu.gh

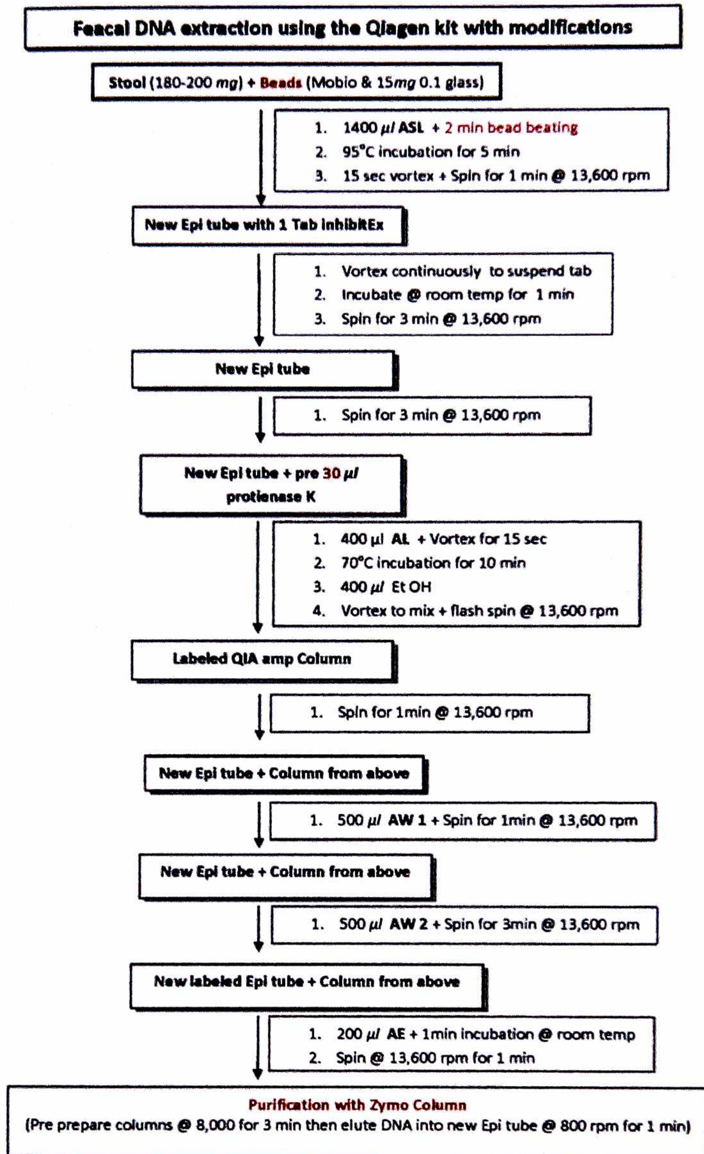



**Figure 11: A flowchart of stool DNA extraction**. The QIAamp stool kit (Qiagen, Valencia, CA) was used and modifications are highlighted (red). ASL, AL, AW1, AW2 and AE are stool diluents, lysis, ethanol washing and DNA eluting buffers, respectively. * Epi tube – Eppendolf tube.

University of Ghana http://ugspace.ug.edu.gh

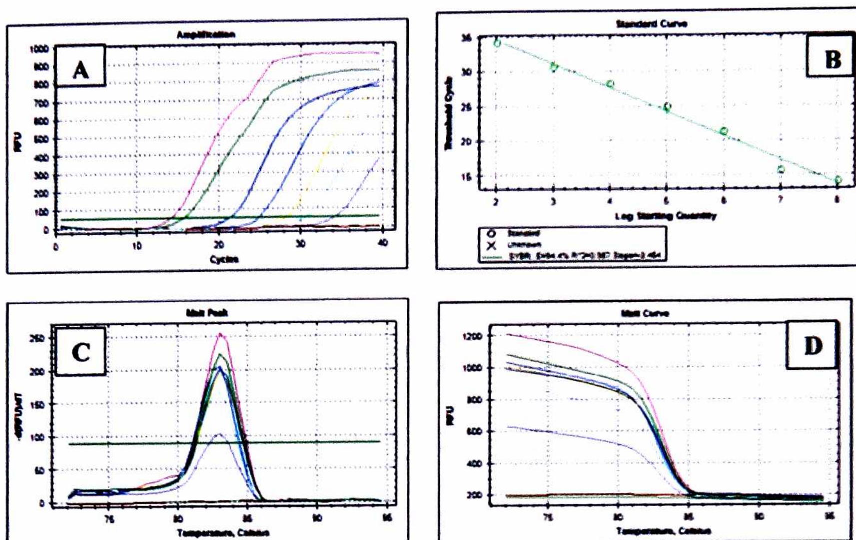
### 3.5 Optimization, Sensitivity and Specificity of Real-Time PCR (qPCR)

Real-Time PCR reaction was first optimized using DNA obtained from pure control strains as templates, and annealing temperatures and primer sets described elsewhere (Cerna *et al.*, 2003; Czeczulin *et al.*, 1999; Gene ID, 2009; Guy *et al.*, 2003; Haque *et al.*, 2007; Sethabutr *et al.*, 1993). Where necessary, the annealing temperature was modified. A 25 µl reaction volume consisted of 12.5 µl of SYBR-Green -490 (Bio-Rad Laboratories, Madison, USA), 1 µl of each 6.2 µM primer, 5 µl template and PCR grade water. For the sensitivity of the PCR reactions, serial dilutions of known quantities (cfu/ml) of the individual control organisms (cells) were separately suspended and homogenized in 180 µl of phosphate buffer solution (PBS). DNAs were then extracted from each set of the cell-PBS-mixture, using the Qiagen kit as described earlier. The initial steps in DNA extraction process, which principally removes impurities from stool specimen was avoided, when dealing with pure cells. The eluted DNAs were used to template qPCR to determine the least number of organisms that can reproducibly be detected.

For specificity, known quantity (cfu/ml) of each of the control strains (*E. coli* 042, *Cryptosporidium*, *E. histolytica* and *Giardia*) was used to spike 180 µg of enteric-pathogen-free stool specimen, and again faecal DNA extracted using the methods described above. The faecal DNA obtained was then used to template qPCR for each of the organisms in single-plex reactions to check whether there would be interferences. In large cohorts of experiments, known quantities of all four

University of Ghana http://ugspace.ug.edu.gh

organisms were also used to spike 180 μg enteric-pathogen-free stool specimen as above, and again faecal DNA extracted and used to template single-plex reactions. Reproducibly, the least amount of organism that was detected in a gram of stool specimen was $10^3$ per a gram of stool. All the qPCR reactions were carried out in a 25 μl final volume as described above. Figures 12 (i-vi) show curves (amplification, standard, negative differentiation of fluorescence unit over temperature) and quantification data table of some of the genes screened.

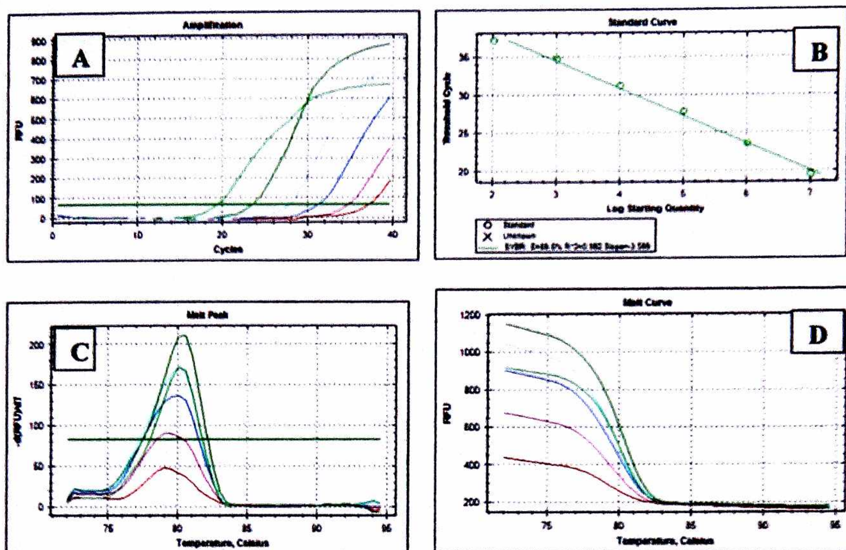



**Quantification Data**

| Well | Fluor | Content | Threshold Cycle ( C(t) ) | C(t) Mean | C(t) Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev | Well Note |
|---|---|---|---|---|---|---|---|---|---|---|
| B07 | SYBR | Std-2 | 15.73 | 15.73 | 0.000 | 1.000E+07 | 7.000 | 1.00E+07 | 0.00E+00 | aggR_7 |
| B11 | SYBR | Std-6 | 30.61 | 30.61 | 0.000 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 | aggR_3 |
| C09 | SYBR | Std-4 | 24.93 | 24.93 | 0.000 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 | aggR_5 |
| D05 | SYBR | Std-1 | 14.10 | 14.10 | 0.000 | 1.000E+08 | 8.000 | 1.00E+08 | 0.00E+00 | aggR_8 |
| F07 | SYBR | Std-3 | 21.39 | 21.39 | 0.000 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 | aggR_6 |
| F11 | SYBR | Std-7 | 34.19 | 34.19 | 0.000 | 1.000E+02 | 2.000 | 1.00E+02 | 0.00E+00 | aggR_2 |

**Figure 12 (*i*): EAEC's *aggR* gene optimized curves and quantification data.** SYBER- Green fluorescence for A) PCR amplification, B) standard curve, C) melt peaks and D) melt curve just before data was captured.

University of Ghana http://ugspace.ug.edu.gh

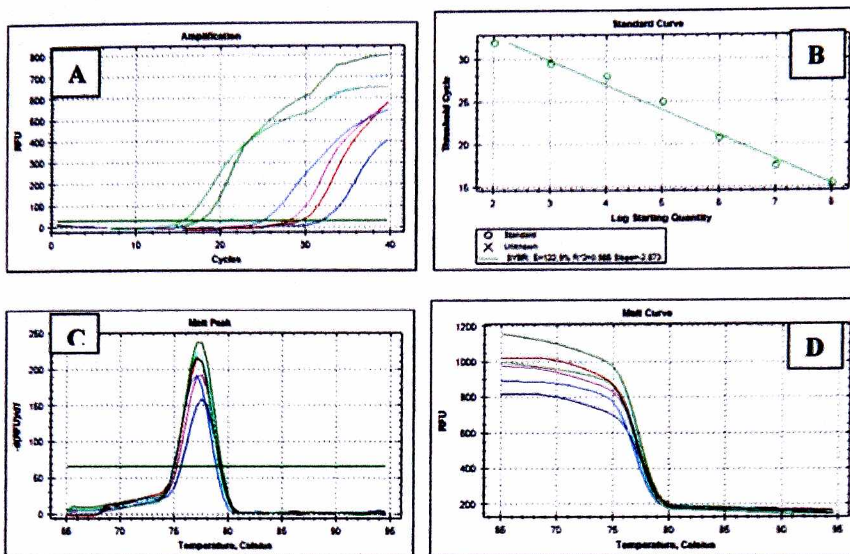



**Quantification Data**

| Well | Fluor | Content | Threshold Cycle ( C(t) ) | C(t) Mean | C(t) Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev | Well Note |
|---|---|---|---|---|---|---|---|---|---|---|
| D02 | SYBR | Std-1 | 19.45 | 19.45 | 0.000 | 1.000E+07 | 7.000 | 1.00E+07 | 0.00E+00 | aatA 10_7 |
| D05 | SYBR | Std-2 | 23.60 | 23.60 | 0.000 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 | aatA 10 _6 |
| D08 | SYBR | Std-3 | 27.89 | 27.89 | 0.000 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 | aatA 10_5 |
| D11 | SYBR | Std-4 | 31.28 | 31.28 | 0.000 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 | aatA 10_4 |
| F03 | SYBR | Std-5 | 34.81 | 34.81 | 0.000 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 | aatA 10 _3 |
| F10 | SYBR | Std-6 | 37.23 | 37.23 | 0.000 | 1.000E+02 | 2.000 | 1.00E+02 | 0.00E+00 | aatA 10_2 |

**Figure 12 (*ii*): EAEC's *aatA* gene optimized curves and quantification data.** SYBER- Green fluorescence for A) PCR amplification, B) standard curve, C) melt peaks and D) melt curve just before data was captured.

University of Ghana http://ugspace.ug.edu.gh

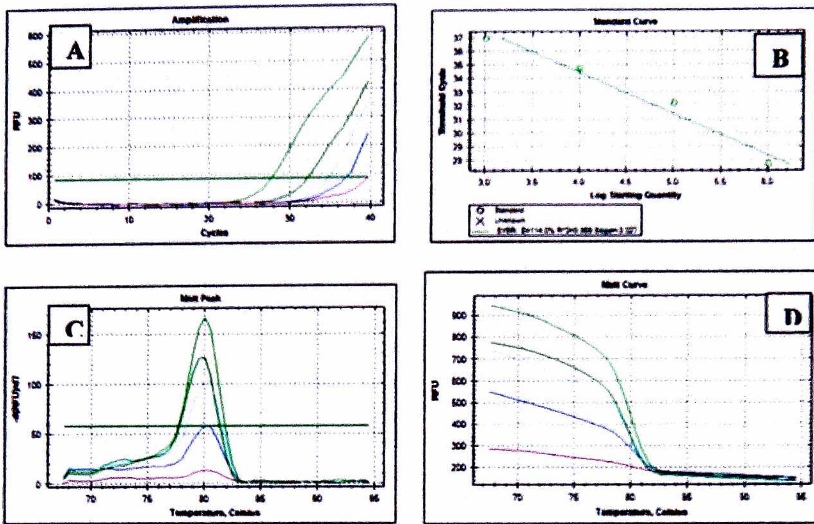



**Quantification Data**

| Well | Fluor | Content | Threshold Cycle ( C(t) ) | C(t) Mean | C(t) Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev | Well Note |
|---|---|---|---|---|---|---|---|---|---|---|
| B02 | SYBR | Std-1 | 15.45 | 15.45 | 0.000 | 1.000E+08 | 8.000 | 1.00E+08 | 0.00E+00 | aap 10_8 |
| B06 | SYBR | Std-2 | 17.54 | 17.54 | 0.000 | 1.000E+07 | 7.000 | 1.00E+07 | 0.00E+00 | aap 10_7 |
| B10 | SYBR | Std-3 | 20.78 | 20.78 | 0.000 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 | aap 10_6 |
| F02 | SYBR | Std-4 | 24.98 | 24.98 | 0.000 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 | aap 10_5 |
| F06 | SYBR | Std-5 | 27.97 | 27.97 | 0.000 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 | aap 10_4 |
| F10 | SYBR | Std-6 | 29.45 | 29.45 | 0.000 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 | aap 10_3 |
| H04 | SYBR | Std-7 | 31.93 | 31.93 | 0.000 | 1.000E+02 | 2.000 | 1.00E+02 | 0.00E+00 | aap 10_2 |

**Figure 12 (*iii*): EAEC's *aap* gene optimized curves and quantification data.** SYBER- Green fluorescence for A) PCR amplification, B) standard curve, C) melt peaks and D) melt curve just before data was captured.

University of Ghana http://ugspace.ug.edu.gh

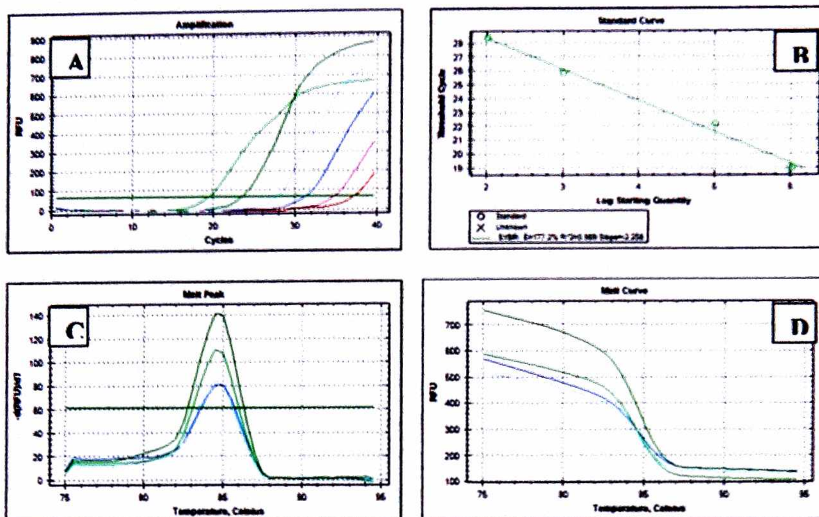



**Quantification Data**

| Well | Fluor | Content | Threshold Cycle ( C(t) ) | C(t) Mean | C(t) Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev | Well Note |
|---|---|---|---|---|---|---|---|---|---|---|
| B06 | SYBR | Std-1 | 27.73 | 27.73 | 0.000 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 | aaiC 10_6 |
| C06 | SYBR | Std-2 | 32.24 | 32.24 | 0.000 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 | aaiC 10_5 |
| D06 | SYBR | Std-3 | 34.77 | 34.77 | 0.000 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 | aaiC 10_4 |
| E06 | SYBR | Std-4 | 36.98 | 36.98 | 0.000 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 | aaiC 10_3 |
| F06 | SYBR | Std-5 | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 | aaiC 10_2 |

**Figure 12 (*iv*): EAEC's *aaiC* gene optimized curves and quantification data.** SYBER- Green fluorescence for A) PCR amplification, B) standard curve, C) melt peaks and D) melt curve just before data was captured.

University of Ghana http://ugspace.ug.edu.gh

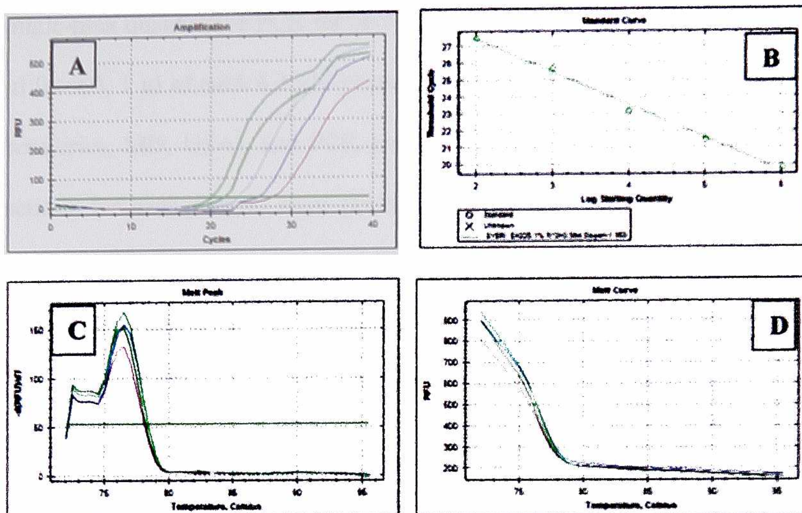



**Quantification Data**

| Well | Fluor | Content | Threshold Cycle ( C(t) ) | C(t) Mean | C(t) Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev | Well Note |
|---|---|---|---|---|---|---|---|---|---|---|
| B06 | SYBR | Std-1 | 18.95 | 18.95 | 0.000 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 | Crypt 10_6 |
| D06 | SYBR | Std-2 | 22.20 | 22.20 | 0.000 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 | Crpt 10_5 |
| F06 | SYBR | Std-3 | 25.89 | 25.89 | 0.000 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 | Crypt 10_3 |
| H06 | SYBR | Std-4 | 28.39 | 28.39 | 0.000 | 1.000E+02 | 2.000 | 1.00E+02 | 0.00E+00 | Crypt 10_2 |

**Figure 12 ($\nu$): Cryptosporidium's 18s rRNA gene optimized curves and quantification data.** SYBER- Green fluorescence for A) PCR amplification, B) standard curve, C) melt peaks and D) melt curve just before data was captured.

University of Ghana http://ugspace.ug.edu.gh



**Quantification Data**

| Well | Fluor | Content | Threshold Cycle ( C(t) ) | C(t) Mean | C(t) Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev | Well Note |
|---|---|---|---|---|---|---|---|---|---|---|
| B06 | SYBR | Std-1 | 19.83 | 19.83 | 0.000 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 | *Eh* 10_6 |
| C06 | SYBR | Std-2 | 21.50 | 21.50 | 0.000 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 | *Eh* 10_5 |
| D06 | SYBR | Std-3 | 23.17 | 23.17 | 0.000 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 | *Eh* 10_4 |
| E06 | SYBR | Std-4 | 25.71 | 25.71 | 0.000 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 | *Eh* 10_3 |
| F06 | SYBR | Std-5 | 27.50 | 27.50 | 0.000 | 1.000E+02 | 2.000 | 1.00E+02 | 0.00E+00 | *Eh* 10_2 |

**Figure 12 (*vi*): *E. histolytica*'s *Eh* gene optimized curves and quantification data**. SYBER- Green fluorescence for A) PCR amplification, B) standard curve, C) melt peaks and D) melt curve just before data was captured.

University of Ghana http://ugspace.ug.edu.gh

## 3.6 Detection of Enteric Pathogens from Stool DNA

A single-plex quantitative PCR for each gene pair [Table 3] consisted of template (5 µl faecal), 1 µl of each 6.2 µM primer, 12.5 µl of SYBR-Green -490 (Bio-Rad Laboratories, MD, USA), and PCR grade water to a reaction volume of 25 µl. Reactions for each sample were performed using the Bio-Rad iQCycler Real-Time Detection System in Bio-Rad iCycler 96-well plates, where positive and negative controls were included with each reaction set. The results were analyzed with a user-defined threshold of 200 PCR baseline-subtracted curve-fit relative fluorescence units. Melt curve (ct) data collection and analysis was enabled at cycles 3 and 4, with an increase in set point temperatures after cycle 2 by 0.5°C. Appendix III shows the cycling protocol of the real-time PCR of each of the target genes screened.

Multiple loci for EAEC (*aap, aatA, aggR* and *aaiC*) and single loci for *Shigella*, *Cryptosporidium* and *Giardia* species from faecal DNA were sought. Table 3 shows the target genes, annealing temperatures and the amplification protocol used. Standard cultures with known numbers of *E. coli* 042 and 17-2, *Shigella*, *Cryptosporidium* and *Giardia* oocysts were used as reference and positive controls. Water and *E. coli* K-12 were used as negative controls. Melt curve analysis was used to determine positivity of samples using a user defined threshold.

UNIVERSITY OF GHANA
BALME LIBRARY

University of Ghana http://ugspace.ug.edu.gh

**Table 3: Target genes screened from stool DNA**

| Strain | Gene target | Location | Primer sequence (5' – 3') | PCR size (bp) | Annealing tempera-ture (°C) | Source/ reference |
|---|---|---|---|---|---|---|
| EAEC | *aaiC* | Chromosome | CTTCTGCTCTTAGCAGGGAGTTTG<br>AAGCGTGAAATGCCTGAGGA | 123 | 47.5 | Nataro's Lab |
| | *aatA* | Plasmid | CCTRTGTTGATGCTCGAGAGA<br>CKTTCCTCCTCCTCAAGGACAT | 118 | 55 | Nataro's Lab |
| | *aap* | Plasmid | CTTGGGTATCAGCCTGAATG<br>AACCCATTCGGTTAGAGCAC | 310 | 45 | (Cerna *et al.*, 2003) |
| | *aggR* | Plasmid | CTAATTGTACAATCGATGTA<br>ATGAAGTAATTCTTGAAT | 308 | 45 | (Czeczulin *et al.*, 1999) |
| *Shigella*/EIEC | *ipaH* | Plasmid | GTTCCTTGACCGCCTTTCCGATACCGTC<br>GCCGGTCAGCCACCCTCTGAGAGTAC | 619 | 60.5 | (Sethabutr *et al.*, 1993) |

University of Ghana http://ugspace.ug.edu.gh

**Table 3 (Continuation): Target genes screened from stool DNA**

| | | | | | | |
|---|---|---|---|---|---|---|
| *Crypto-sporidium* | 18s rRNA | Chromosome | CTCCACCAACTAAGAACGGCC<br>TAGAGATTGGAGGTTGTTCCT | 213 | 60 | Gene ID cgd7_230 (Gene ID, 2009) |
| *E. histolytica* | Eh | Chromosome | AACAGTAATAGTTTCTTTGGTTAGTAAAA<br>CTTAGAATGTCATTTCTCAATTCAT | 134 | 60 | (Haque *et al.*, 2007) |
| *Giardia lamblia* | P241 | Chromosome | CATCCGCGAGGAGGTCAA<br>GCAGCCATGGTGTCGATCT | 74 | 60 | (Guy *et al.*, 2003) |

University of Ghana http://ugspace.ug.edu.gh

### 3.7 Intestinal Inflammation Assessment

Intestinal inflammations were quantitatively assessed from frozen stool specimens using the IBD SCAN (TechLab, Blacksburg, VA) according to the manufacturer's instructions. Stool specimens were allowed to thaw and were serially diluted 10-fold with a diluents supplied by the manufacturer. These were then analyzed by a polyclonal antibody-based ELISA method. Detailed procedure is described elsewhere (Kane *et al.*, 2003) , and absorbance of each assay well was measured spectrophotometrically at 450 and 620 nm ($A_{450/620}$). Faecal lactoferrin concentrations in µg/ml were determined by comparison with a standard curve using purified human lactoferrin and analyzed by linear regression in Microsoft Excel. The lowest dilution of a specimen with an absorbance at 450/620 nm within the linear portion of the curve was used to determine the lactoferrin concentration. The final lactoferrin concentration was obtained by multiplying the dilution factor by the concentration. A positive control (purified human lactoferrin) and a negative control (washing buffer) was included in each batch of stools analyzed, and linear regression was performed separately for each batch using standard controls supplied by the manufacturer. This assessment was performed only on subjects with adequate (> 60 ml or mg, depending on stool consistency) stool specimens and where necessary, the experiment was repeated.

University of Ghana http://ugspace.ug.edu.gh

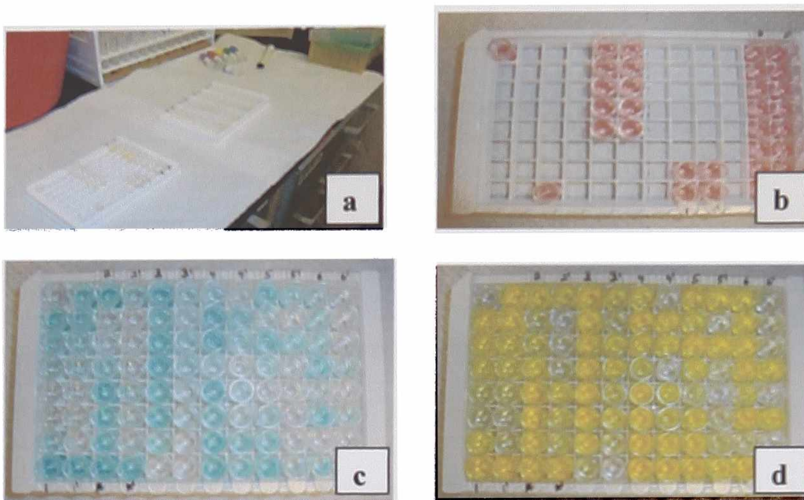


**Figure 13a: Photographs showing some reactions of the lactoferrin assay (LF).** The IBD-SCAN was used according to the manufacturer's instruction. a) 200 µl of a stool-diluent suspension was inoculated into a microtitre well, which is coated with immobilized polyclonal antibodies to human LF. b) LF, if present, binds to the antibodies during 30 min incubation at 37°C. After the incubation, polyclonal antibodies are coupled to horseradish peroxidase (conjugate) and allowed to bind to captured LF during 30 min incubation. c) Unbound conjugate was washed, and a substrate (tetra-methyl-benzidene and hydrogen peroxide) is added for colour development. d) After a 15-min substrate incubation, 0.6-N sulfuric acid solution is added to quench the reaction, and the absorbance of each assay well was measured spectrophotometrically at 450/620 nm ($A_{450/620}$).

University of Ghana http://ugspace.ug.edu.gh

| Lactoferrin (µg/ml) Std. | OD 1 | OD 2 | Ave. OD |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 6.25 | 0.03 | 0.041 | 0.0355 |
| 12.5 | 0.082 | 0.11 | 0.096 |
| 25 | 0.215 | 0.188 | 0.2015 |
| 50 | 0.388 | 0.361 | 0.3745 |
| e 100 | 0.836 | 0.766 | 0.801 |

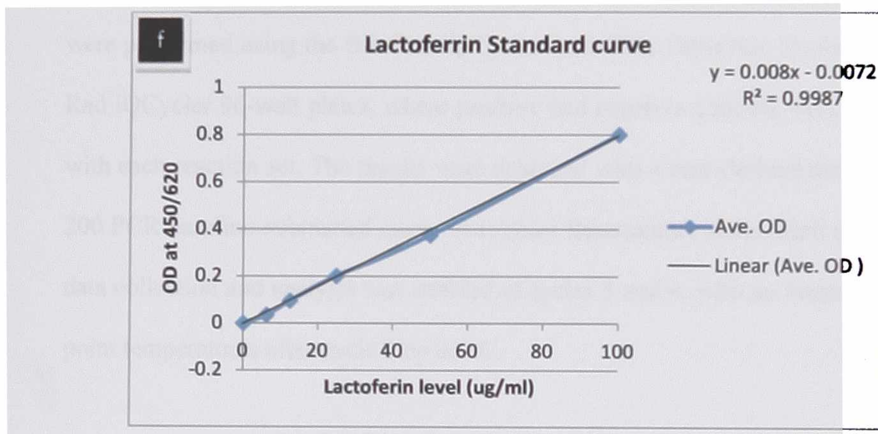



**Figure 13b: Standard curve fit of lactoferrin (LF) using purified human controls.** Each batch of assay included purified human lactoferrin of known concentrations, a positive and a negative control. e) Averages of the ELISA readings of each of the known concentrations were calculated. f) A standard curve was generated using the purified human lactoferrin and analyzed by linear regression using Microsoft Excel. The lowest dilution of specimen giving a reading for absorbance at $A_{450/620}$ within the linear portion of the curve was used to determine the lactoferrin concentration. The final concentration was obtained by multiplying the concentration by the dilution factor.

University of Ghana http://ugspace.ug.edu.gh

### 3.8 Real-Time PCR Detection of EAEC from Bacterial DNA

The same set of the EAEC genes screened earlier from stool DNA were analyzed in the bacterial DNA, under similar conditions. A single-plex quantitative PCR for the EAEC virulence genes [Table 3] consisted of 1 µl template, 1 µl of each 6.2 µM primer, 12.5 µl of SYBR-Green -490 (Bio-Rad Laboratories, MD, USA), and PCR grade water to a reaction volume of 25 µl. Reactions for each sample were performed using the Bio-Rad iQCycler Real-Time Detection System in Bio-Rad iQCycler 96-well plates, where positive and negative controls were included with each reaction set. The results were analyzed with a user-defined threshold of 200 PCR baseline-subtracted curve-fit relative fluorescence units. Melt curve (ct) data collection and analysis was enabled at cycles 3 and 4, with an increase in set point temperatures after cycle 2 by 0.5°C.

University of Ghana http://ugspace.ug.edu.gh

### 3.9 Genotyping of Amplified *Cryptosporidium*-positive stools

PCR-products from stool DNA that were *Cryptosporidium*-positive were further analyzed. The amplicons were purified using the PureLink™ PCR Purification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A minimum volume of 50 µl PCR-product was purified. Basically, the amplified PCR-product was bound onto Spin Columns and washed with buffers, and finally eluted with nuclease free water. Total DNA concentration after purification was measured in µg/mL by a spectrophotometer.

Purified PCR-products were then sequenced in both directions using forward and reverse primer sets of *Cryptosporidium parvum* 18S rRNA (Table 3) at the DNA Sequencing Facility at the Cancer Unit of the University of Virginia. The forward and reverse sequences (Applied Biosystem) were manually joined and analyzed using BLAST (Basic Local Alignment Search Tool), a public available database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Electrogrames and the sequences are documented in Appendix III.

Because the above method was not so helpful in deciphering the genotypes of *Cryptosporidium*, high resolution melting (HRM) analysis was employed (Herrmann *et al.*, 2006; Reja *et al.*, 2010). The procedure was highly automated using the Rotor-Gene Q 5 plex HRM instrument (QIAGEN, Hilden, Germany). The run was done using Eva Green fluorescence dye (Biotium, Hayward, USA), in a 25 µl total volume. The only human involvement was the loading of the

University of Ghana http://ugspace.ug.edu.gh

master-mix (300 nM each primer), and the amplified DNA templates onto the racks of the machine. Additionally, the concentrations of the amplified DNAs together with a control (*C. parvum*), previously determined by photospectometry (µg/L) were fed into the software for the purposes of analysis. The system was programmed to compare the melt curves of the positive control (*C. parvum*) to those of the unknowns and a blank without any DNA. The Qiagen Type-it HRM PCR kit was used in preparing the master-mix according to the manufacturer's instructions. The basic principle in HRM is that as the PCR products dissociate with increasing temperature, dye is progressively released and fluorescence diminishes. The fluorescence measurements are collected at corresponding temperature increaments and plotted as a 'melt curve'. Melt curve shape and position are characteristic of each sample allowing them to be compared or discriminated. Even, a single base change between samples can be readily detected and identified (Herrmann *et al.*, 2006; Reja *et al.*, 2010).

University of Ghana http://ugspace.ug.edu.gh

### 3.10 Data Storage and Analysis

Data from the questionnaires were stored in Microsoft Access files and later exported to various statistical packages for further analyses. To avoid any experimental biases, stool specimens were coded before testing and only decoded for purposes of analysis. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Chicago) and Epi-Info. Z-score calculation and weight for-age percentile plots were done by the nutritional soft-ware in Epi-Info.

Statistical tests included $\chi^2$ for associations of pathogens with age groups, diarrhoea and non-diarrhoea and the paired *t* test for associations with lactoferrin level. Odds ratio and 95% confidence intervals are reported for all 2 X 2 comparisons. Two-tailed tests were used and $p < 0.05$ was considered statistically significant.

University of Ghana http://ugspace.ug.edu.gh

# CHAPTER FOUR

## RESULTS

### 4.1 Study Population and Base-line Characteristics

Within the nine-month study period, 287 children ≤ 5 years were recruited with only 13 excluded from analysis because of insufficient data. Figure 14 shows an insert map of Ghana with plots of the locations of the sampled children. Majority of the children enrolled resided in the Greater Accra Metropolis, and a few of them were from the Central (Kasoa) and Eastern (Nsawam) regions of Ghana. Of the 274 children included in analysis, 170 (62%) were with and 104 (38%) without diarrhoea; there were more males 156 (56.9%) than females. Acute and persistent diarrhoea comprised 85.3% (145) and 7.6% (13) of total cases respectively. Duration of symptoms in the remaining children with diarrhoea (7.1%) was not recorded. Table 4 shows the baseline characteristics of the study population.

University of Ghana http://ugspace.ug.edu.gh

**Figure 14: Settlements of the enrolled children in the study.** Majority of the enrolled children were from the Greater Accra Metropolis. A few of them however came from the Central and the Eastern regions of Ghana. The only *Shigella flexneri* isolated from stool culture came from a child from the Central region. Sampled children with and without diarrhoea are represented as green dots and red triangles, respectively. The sizes of the shapes are proportional to the number of children enrolled from each locality.

University of Ghana http://ugspace.ug.edu.gh

**Table 4: Baseline characteristics of study population**

| Characteristic | Diarrhoea *N* (%) | Non-diarrhoea *N* (%) |
|---|---|---|
| Age/months | *N* =170 | *N* = 104 |
| 0 – 6 | 26 (15.3) | 35 (33.7) |
| 7 – 12 | 54 (31.8) | 30 (28.8) |
| 13 – 24 | 77 (45.3) | 24 (23.1) |
| 25 - 60 | 13(7.6) | 15 (14.4) |
| Sex | *N* =170 | *N* =104 |
| Male | 97 (57.1) | 73 (42.9) |
| Female | 59 (56.7) | 45 (43.3) |
| Weight/kg | *N* =170 | *N* =104 |
| 2.5 – 4.9 | 10 (5.9) | 4 (3.8) |
| 5.0 – 9.9 | 121 (71.2) | 75 (72.1) |
| 10 – 19.9 | 34 (20.0) | 22 (21.2) |
| 20 – 86.0 | 5 (2.9) | 3 (2.9) |
| WAZ | *N* =168 | *N* =101 |
| Normal (> -1) | 75 (44.6) | 60 (59.4) |
| Mild (-1 to -2) | 33 (19.6) | 19 (18.8) |
| Moderate (-2 to -3) | 32(19.0) | 12 (11.9) |
| Severe (<-3) | 28 (16.7) | 10 (9.9) |
| HAZ | *N* = 86 | *N* = 84 |
| Normal (≥ -2) | 48 (55.8) | 61 (72.6) |
| Moderate (-2 to -3) | 6 (7.0) | 11 (13.1) |
| Severe (<-3) | 32 (37.2) | 12 (14.3) |
| WHZ | *N* = 82 | *N* = 79 |
| Normal (≥ -2) | 58 (70.3) | 66 (83.5) |
| Moderate (-2 to -3) | 6 (7.3) | 8 (10.1) |
| Severe (<-3) | 18 (22.0) | 5 (6.3) |

WAZ, HAZ and WHZ are Z-scores for weight-for-age, height-for-age and weight-for-height, respectively.

University of Ghana http://ugspace.ug.edu.gh

### 4.2 Nutritional Status and Growth Faltering

At least one anthropometric measurement was taken for 269 out of the 274 children analyzed. Of 269 children from whom weight measurements were recorded, 134 (49.8%) showed mild to severe malnutrition (WAZ < -1) [93/168 (55.4%) in children with and 41/101 (40.6%) without diarrhoea, OR=1.82 (95% CI, 1.102-2.988), *p*=0.023]. Of 170 children from whom height or length measurements were recorded, 61 (35%) showed moderate to severe stunting (HAZ < -2) [38/86 (44.2%) in children with diarrhoea, and 23/84 (27.4%) without OR=2.10 (95% CI, 1.110-3.972), *p*=0.026]. Of 161 children from whom both weight and height measurements were recorded, 37 (22.9%) showed moderate to severe wasting (WHZ < -2) [24/82 (29.3%) in children with diarrhoea, and 13/79 (16.5%) without OR=2.101 (95% CI, 0.989-4.454), *p*=0.062]. The mean age, weight and height were; 15.1/14.6 months, 9.8/9.5 kg and 88.0/84.0 cm (diarrhoea/non-diarrhoea, respectively).

In the diarrhoea sub-population, growth faltering started as early as 3 months of age in the male sub-group and by the seventh month several (>4) of the children were observed to fall below the 5% percentile category [Figure 15a, 15b]. In the non-diarrhoea sub-population fewer (<9) children were observed to fall below the 5% percentile compared to the diarrhoea sub-population [Figure 16a, 16b]. In the separate sub-populations of diarrhoea and non-diarrhoea, there was no significant statistical association in gender among the children who fell below the 5% percentile curve (p>0.05).

University of Ghana http://ugspace.ug.edu.gh

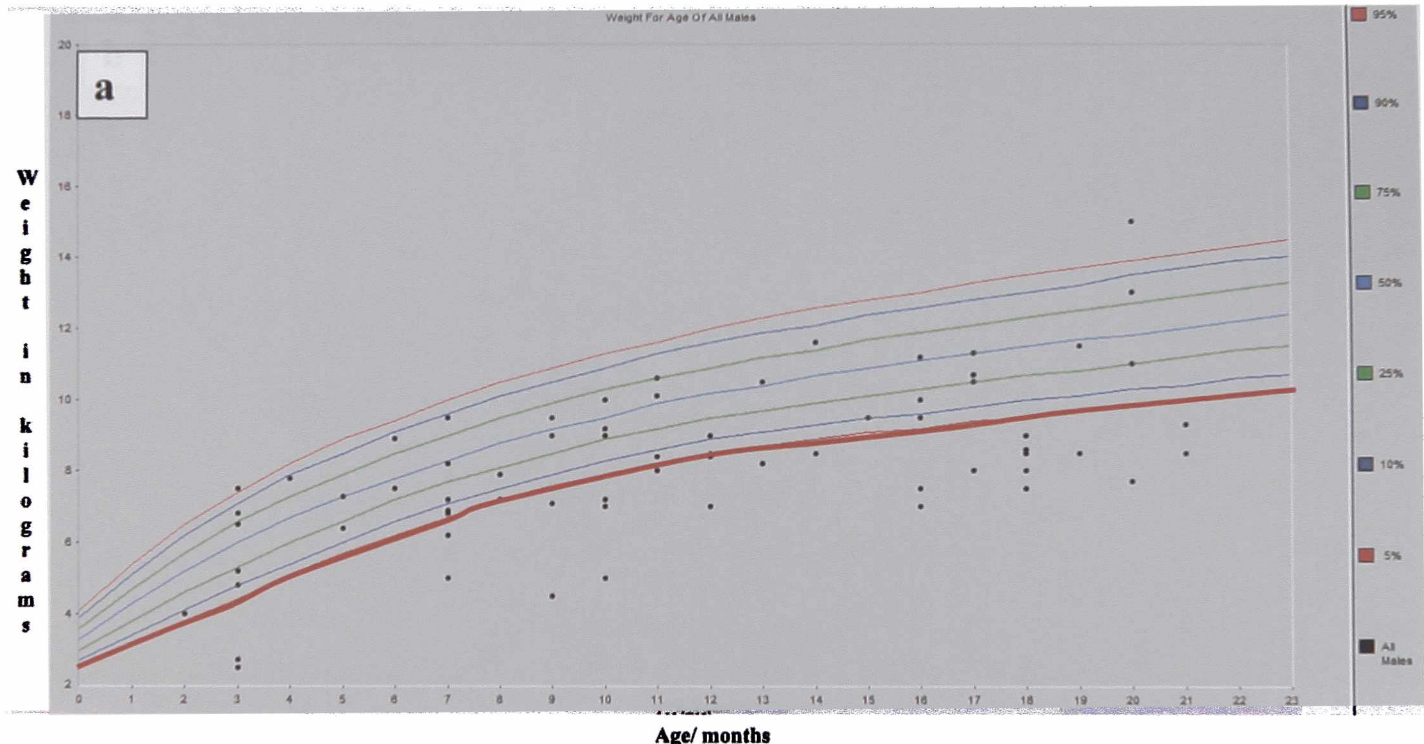



**Figure 15a: Weight-for-age plot of male infants and young children (< 2 years of age) in the diarrhoea sub-population.** In this sub-category, growth shortfall was observed as early as 3 months, and 37.9% (25/66) of the males fell below the 5% percentile curve. The percentiles (curves) are measurements that show where a child is compared with a reference population (de Onis and Blossner, 2003). A child with normal growth characteristics is expected to fit above the 5% percentils curve (heavy red curve).

University of Ghana http://ugspace.ug.edu.gh

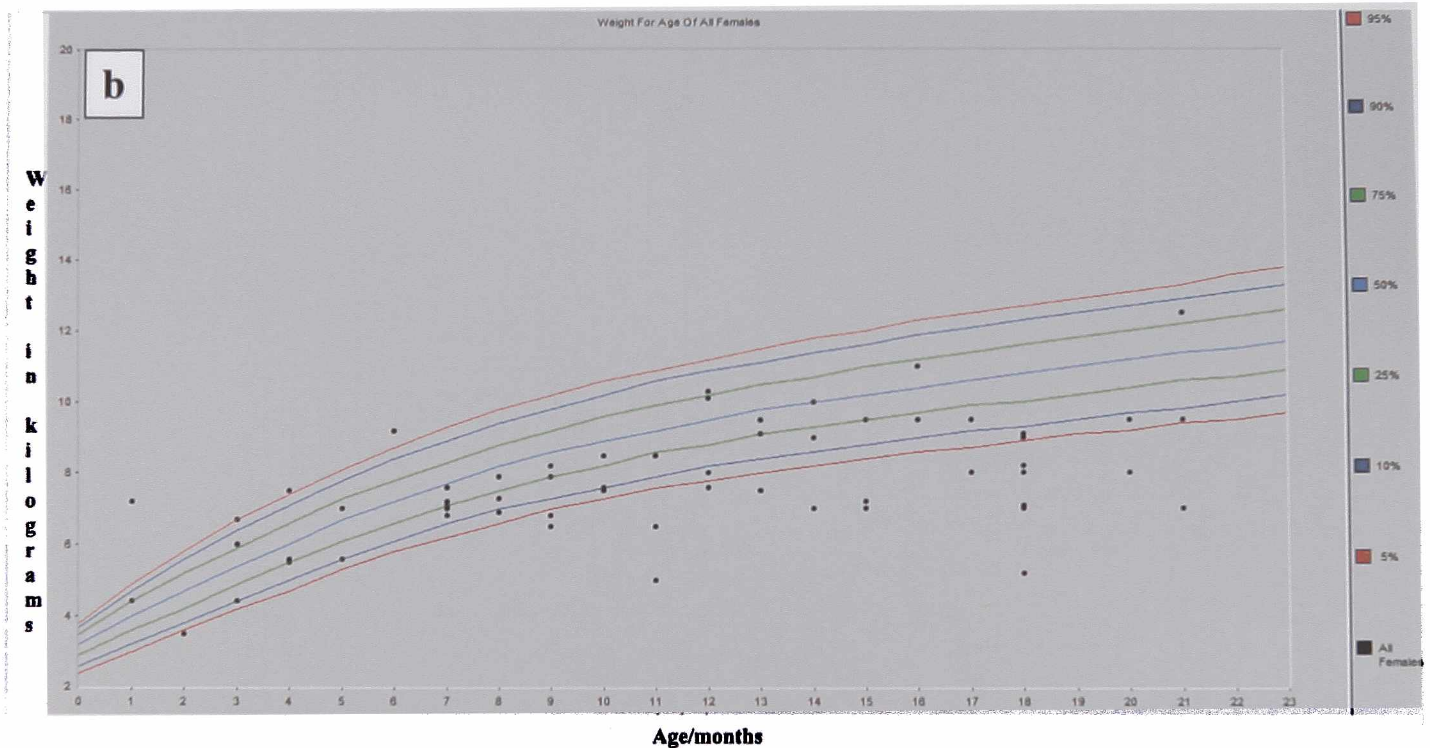



**Figure 15b: Weight-for-age plot of female infants and young children (< 2 years of age) in the diarrhoea sub-population.** In this sub-category, growth shortfall was observed around 9 months, and 31.1% (18/58) of the females fell below the 5% percentile curve. The percentiles (curves) are measurements that show where a child is compared with a reference population (de Onis and Blossner, 2003). A child with normal growth characteristics is expected to fit above the 5% percentils curve (heavy red curve).

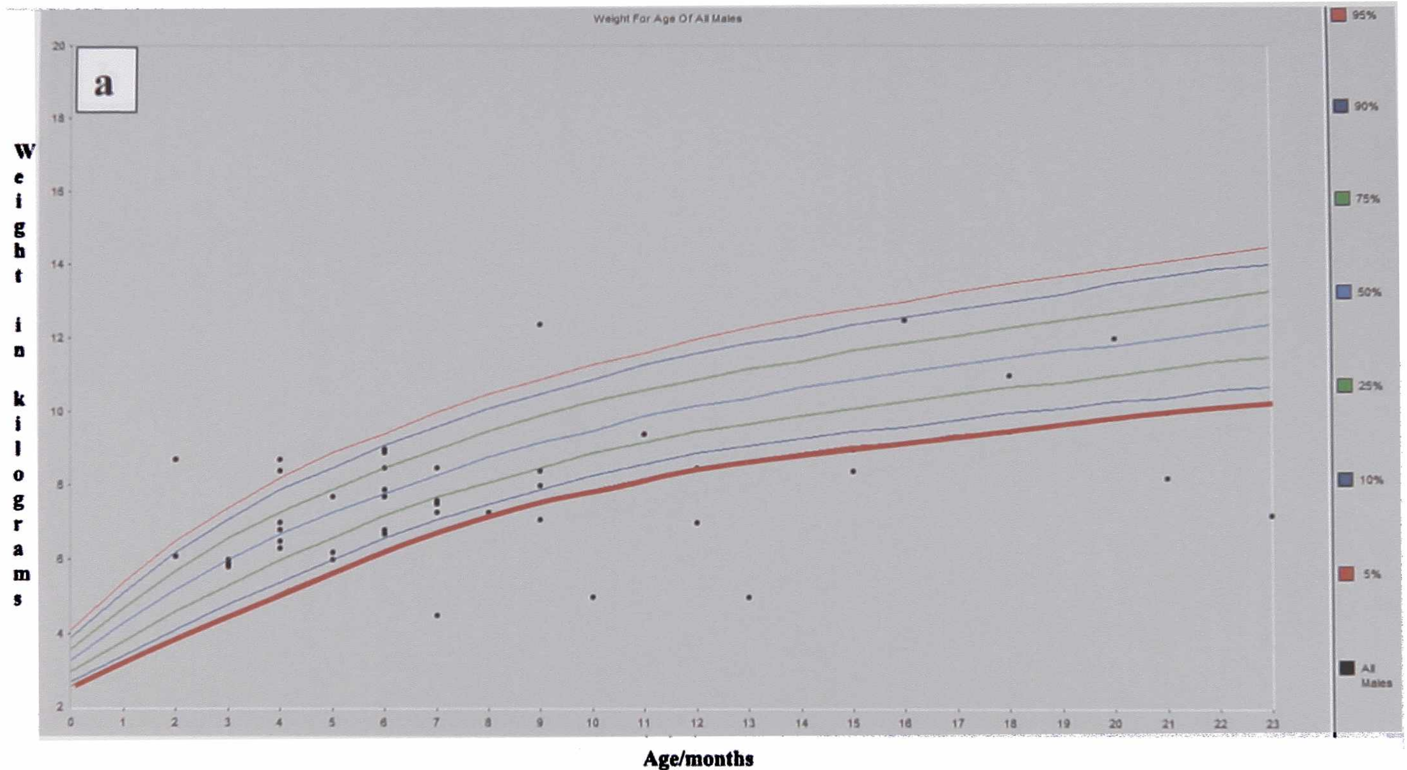



**Figure 16a: Weight-for-age plot of infants and young children (< 2 years of age) in the non-diarrhoea sub-population.** In this sub-category, fewer male children 20.5% (9/44) fell below the 5% percentile curve. The percentiles (curves) are measurements that show where a child is compared with a reference population (de Onis and Blossner, 2003). A child with normal growth characteristics is expected to fit above the 5% percentils curve (heavy red curve).

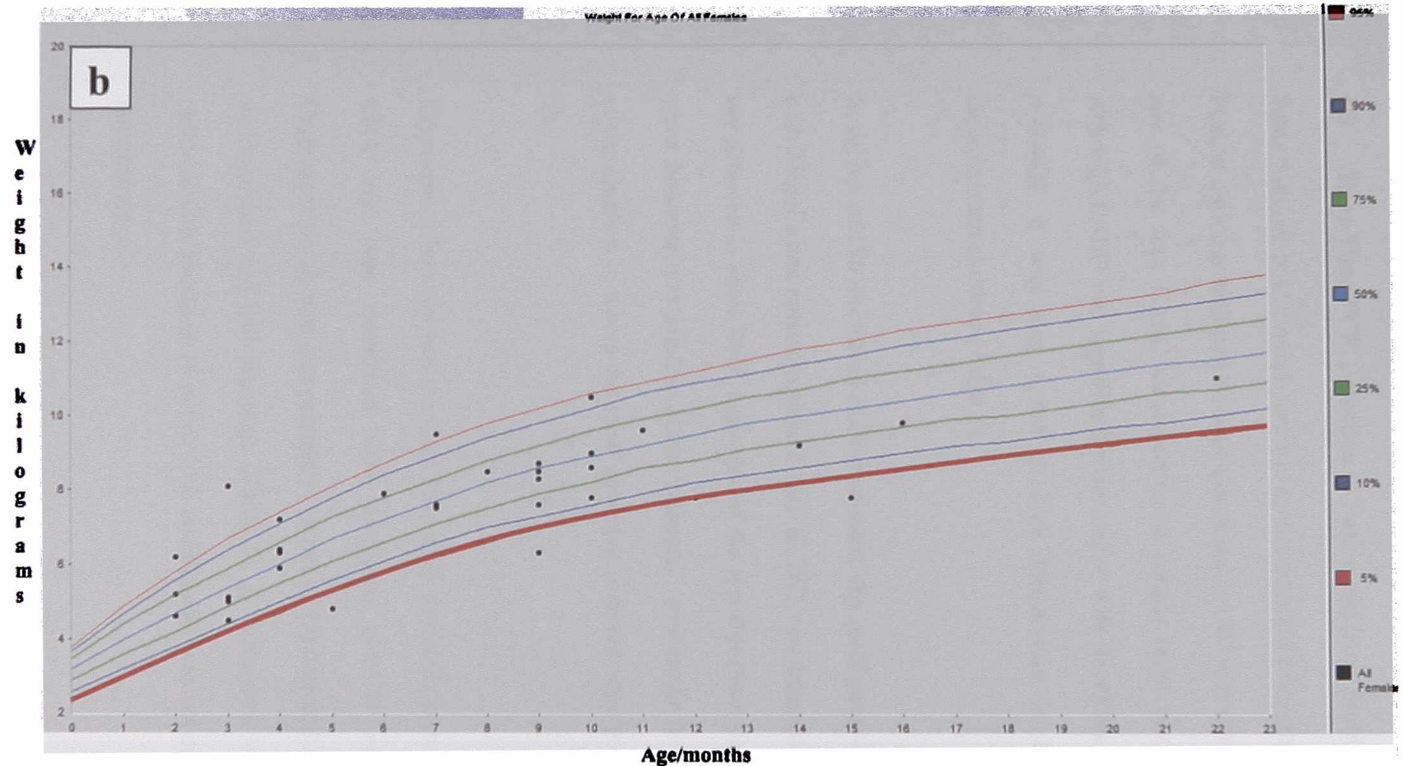



**Figure 16b: Weight-for-age plot of infants and young children (< 2 years of age) in the non-diarrhoea sub-population.** In this sub-category, far fewer female children 9.1% (3/33) fell below the 5% percentile curve. The percentiles (curves) are measurements that show where a child is compared with a reference population (de Onis and Blossner, 2003). A child with normal growth characteristics is expected to fit above the 5% percentils curve (heavy red curve).

University of Ghana http://ugspace.ug.edu.gh

## 4.3 Real-Time PCR Detection of Pathogens from Stool DNA

Table 5 shows bacteria and parasitic agents detected from stool DNA by real-time PCR in children with and without diarrhoea. EAEC was defined as positivity for any of the four EAEC virulence genes sought (*aap*, *aatA*, *aggR* and *aaiC*). Although EAEC was significantly associated with diarrhoea (147/170 vs 80/104, $p$=0.042), it was also found in high frequencies in both nourished and malnourished children [Table 5].

In six out of 170 diarrhoeal stool specimens, the *ipaH* gene which is expressed by both *Shigella* and enteroinvasive *E. coli* (EIEC) was detected. The *ipaH* gene was not detected in stool DNA from any of the non-diarrhoea children. The numbers were, however, too small to attain statistical significance (p>0.05). Five out of 6 of the children in whom the *ipaH* gene was detected were well- nourished (Table 5).

*Cryptosporidium* spp was the most frequently detected protozoan parasite in stool DNA and was associated with diarrhoea (14/170 vs 1/104, $p$=0.011). Cryptosporidiosis was also primarily (10 out of 14) detected in children who were malnourished and had diarrhoea (Table 5). *E. histolytica* was only detected in children with diarrhoea (5 out of 170) and *Giardia* was not detected in either sub-populations.

University of Ghana http://ugspace.ug.edu.gh

**Table 5 : Organisms detected by real-time PCR from stool DNA**

| | Diarrhoea (n=170) | | | Non-diarrhoea (n=104) | | | | |
|---|---|---|---|---|---|---|---|---|
| | No. (%) | WN (n=75) | MN (n=95) | No. (%) | WN (n=60) | MN (n=41) | Odds Ratio [95%CI] | p-value |
| Any infection | | | | | | | | |
| EAEC | 147 (86.5) | 66 | 79 | 80 (76.9) | 49 | 30 | 1.917 (1.018-3.612) | 0.048 |
| *Shigella*/EIEC | 6 (3.5) | 5 | 1 | 0 (0) | - | - | n/a | |
| *Cryptosporidium* spp | 14 (8.7) | 4 | 10 | 1 (1.0) | 1 | 0 | 9.244 (1.197-71.371) | 0.011 |
| *E. histolytica* | 5 (3.0) | 2 | 3 | 0 (0) | - | - | n/a | |
| *Giardia* spp | 0 (0) | - | - | 0 (0) | - | - | - | |

*p-value is between diarrhoeal and non-diarrhoeal stool specimen

WN - well nourished (WAZ > 1) MN – malnourished (WAZ < 1)

n/a – not applicable

University of Ghana http://ugspace.ug.edu.gh

## 4.4 EAEC Virulence Genes Distribution (Stool DNA)

Of the four genes associated with EAEC, *aatA* was the most frequently detected (67.2%) of all stool DNA, followed by *aap* (59.9%), *aggR* (42.7%) and *aaiC* (33.6%) [Table 6]. EAEC's plasmid gene *aap* was significantly associated with diarrhoea [OR=2.506 (95% CI, 1.516-4.144), $p$ <0.001] and the chromosomal gene *aaiC* was not [OR=1.639 (95% CI, 0.0962-2.792), $p$=0.086] [Table 6]. Multiple gene combinations were also observed in EAEC infections in the study population, and the presence of any three genes was associated with diarrhoea [OR=2.101 (95% CI, 1.261-3.502), $p$=0.006] [Table 6].

None of the EAEC virulence genes was associated with malnutrition (WAZ < -1) (p>0.05) [Table 7].

University of Ghana http://ugspace.ug.edu.gh

**Table 6: Enteroaggregative *E. coli* (EAEC) virulence factor-positive in stool DNA**

| Characteristic | Diarrhea (n=170) n (%) | Non-diarrhea (n=104) n (%) | Total n=274) n (%) | Odds Ratio [95% CI] | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|---|
| EAEC virulence related gene | | | | | | |
| *aaiC* | 64 (37.6) | 2 (26.9) | 92 (33.6) | 1.639 [0.962 – 2.792] | 3.327 | 0.086 |
| *aggR* | 79 (46.5) | 8 36.5 | 117 (42.7) | 1.508 [0.914 – 2.486] | 2.602 | 0.131 |
| *aatA* | 118 (69.4) | 66 (63.5) | 84 (67.2) | 1.307 [0.780 – 2.188] | 1.036 | 0.354 |
| *aap* | 116 (68.2) | 48 (46.2) | 164 (59.9) | 2.506 [1.516 – 4.144] | 13.093 | <0.001 |
| EAEC gene combination | | | | | | |
| Any 1 gene | 147 (86.5) | 80 (76.9) | 227 (82.8) | 1.917 [1.018 – 3.612] | 4.139 | 0.048 |
| Any 2 genes | 130 (76.5) | 73 (70.2) | 203 (74.1) | 1.380 [0.797 – 2.391] | 1.325 | 0.259 |
| Any 3 genes | 84 (49.4) | 33 (31.7) | 117 (42.7) | 2.101 [1.261 – 3.502] | 8.224 | 0.006 |
| All 4 genes | 34 (20.0) | 13 (12.5) | 47 (17.2) | 1.750 [0.876-3.496] | 2.554 | 0.110 |

EAEC – Enteroaggregative *Escherichia coli*, OR – odds ratio, 95%CI – confidence interval, $\chi^2$- chi-square, $P < 0.05$ is significant

University of Ghana http://ugspace.ug.edu.gh

**Table 7 : EAEC's genes in stool in association with and without malnutrition**

| | Diarrhoea (n=168) | | Non-diarrhoea (n=101) | | |
|---|---|---|---|---|---|
| | WN (n=75) | MN (n=93) | WN (n=60) | MN (n=41) | *p-value |
| EAEC genes | | | | | |
| *aaiC* | 27 (36.0) | 36 (38.7) | 21(35.0) | 7 (17.1) | 0.548 |
| *aatA* | 54 (72.0) | 62 (66.7) | 37 (61.7) | 28 (68.3) | 0.966 |
| *aggR* | 39 (52.0) | 38 (40.9) | 25 (41.7) | 13 (31.7) | 0.121 |
| *aap* | 53 (70.7) | 63 (67.7) | 31 (51.7) | 16 (39.0) | 0.584 |

*p-value is between diarrhoeal and non-diarrhoeal stool specimen

WN - well nourished (WAZ > 1) MN – malnourished (WAZ < 1)

University of Ghana http://ugspace.ug.edu.gh

## 4.5 Multiple Infection (Stool DNA)

Co-infections, with two or more pathogens detected in the stool DNA, was found predominantly in children who had diarrhoea. EAEC-*Cryptosporidium* was the most prevalent (7.6%, 13/170), followed by EAEC-*Shigella*/EIEC (2.9%, 5/170) and EAEC-*E. histolytica* (2.4%, 4/170). *Cryptosporidium-E. histolytica* and *Cryptosporidium-Shigella*/EIEC co-infection each formed 0.6% (1/170), and one child who had diarrhoea was co-infected with EAEC- *Cryptosporidium-Shigella*/EIEC (0.6%, 1/170). There was no obvious trend in the distribution of pathogens by age in the two sub-populations, especially for EAEC (Table 8).

University of Ghana http://ugspace.ug.edu.gh

**Table 8: Distribution of pathogens by age from stool DNA**

| | Diarrhoea (n=170) | | | | Non-diarrhoea (n=104) | | | |
|---|---|---|---|---|---|---|---|---|
| | Age category/months | | | | Age category/months | | | |
| **Any infection** | **0-6** (n=26) | **7-12** (n=54) | **13-24** (n=77) | **25-60** (n=13) | **0-6** (n=35) | **7-12** (n=30) | **13-24** (n=24) | **25-60** (n=15) |
| EAEC | 24 (92.3) | 42 (77.7) | 69 (89.6) | 12 (92.3) | 25 (71.4) | 24 (80) | 18 (75) | 13 (86.7) |
| *Cryptosporidium* | 3 (11.5) | 4 (7.4) | 6 (7.8) | 1 (7.7) | - | - | 1 (4.2) | - |
| *E. histolytica* | - | 2 (3.7) | 3 (3.9) | - | - | - | - | - |
| *Shigella*/EIEC | 1 (3.8) | 1 (1.8) | 3 (3.9) | 1 (7.7) | - | - | - | - |
| *Giardia spp* | - | - | - | - | - | - | - | - |

University of Ghana http://ugspace.ug.edu.gh

## 4.6 Conventional Stool Culture Results

In only 1 of 170 diarrhoea stool specimens was *Shigella* isolated as an enteric bacterial pathogen from culture. This strain was serotyped with *Shigella* polyvalent anti-sera (Mast Group Ltd., Merseyside, U.K.) and was *S. flexneri*. In the entire study population, *E. coli* was the predominant commensal recovered from culture (79.6%).

University of Ghana http://ugspace.ug.edu.gh

## 4.7 Faecal Lactoferrin Levels

Figure 17 shows enteric pathogens detected in children with/without diarrhoea and the distribution of faecal lactoferrin levels. Irrespective of the enteric pathogen detected, faecal lactoferrin levels were relatively high (manufacturer's cut-off value=7.24 µg/ml). Especially for EAEC infection, both controls and patients had a wide range of lactoferrin levels, irrespective of whether they were nourished or malnourished (Figure 17).

Children with diarrhoea had significantly higher faecal lactoferrin levels (n=143; 1658.9± 204.2 µg/ml) compared to those without diarrhoea (n=84; 935.5± 194.4 µg/ml) ($p$=0.019). The *aatA* gene and the presence of any one or two genes of EAEC were also significantly ($p<0.05$) associated with elevated faecal lactoferrin levels (Table 9). In comparing diarrhoea with non-diarrhoea stool specimens, EAEC's chromosomal gene *aaiC* showed the highest fold-increase in faecal lactoferrin level (2.7) followed by the *aap* gene (2.5). Additionally, detection of multiple virulence gene of EAEC in a stool was associated with an increased fold-rise in mean faecal lactoferrin level between children with and without diarrhoea (Table 9).

Among EAEC-infected malnourished children, two stool specimens (out of greater than 200 specimens analyzed) showed lactoferrin values that were 30 – 50 times more than the mean value for their subject group; they were therefore designated as outliers, and not included in the statistical analysis.

University of Ghana http://ugspace.ug.edu.gh

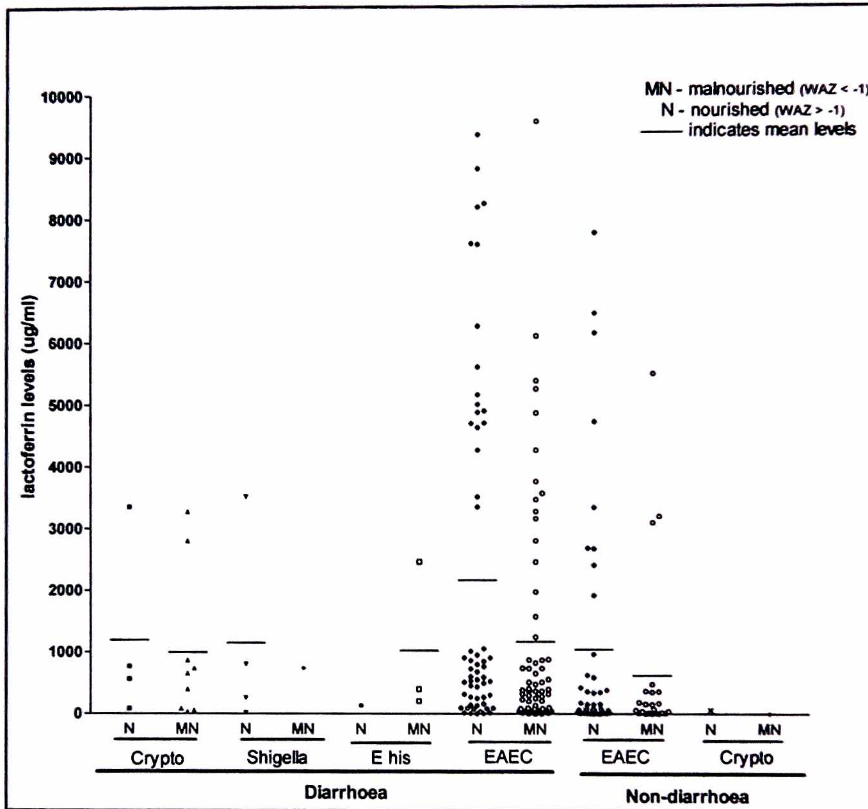



**Figure 17: Enteric pathogens detected and faecal lactoferrin (LF) levels**

Crypto - *Cryptosporidium* spp, E his - *E. histolytica*, EAEC - enteroaggregative *Escherichia coli*

*Breast feeding may cause moderately (15 ≤ 120 μg/ml) increased LF (Lima et al - unpublished observation)

University of Ghana http://ugspace.ug.edu.gh

**Table 9: EAEC genes detected and faecal lactoferrin levels**

| Characteristic | Diarrhoeal stool n (mean± S.E) μg/ml | Non-diarrhoeal stool n (mean± S.E) μg/ml | *Fold-rise | *p*-value |
|---|---|---|---|---|
| EAEC gene | | | | |
| *aaiC* | 56 (1021.0±251.6) | 25 (821.2±402.1) | *1.2* | 0.667 |
| *aggR* | 66 (1713.9±292.5) | 33 (934.1±329.2) | *1.8* | 0.104 |
| *aatA* | 98 (1706.1±244.1) | 54 (927.1±259.1) | *1.8* | 0.044 |
| *aap* | 101 (1656.7±245.9) | 39 (901.2±261.6) | *1.8* | 0.080 |
| EAEC gene combinations | | | | |
| Any 1 gene | 123(1633.5±215.4) | 66 (1175.4±356.8) | *1.4* | 0.029 |
| Any 2 gene | 107 (1679.8±231.1) | 60 (892.6±234.3) | *1.9* | 0.028 |
| Any 3 gene | 73 (1504.9±269.7) | 30 (1132.8±394.8) | *1.3* | 0 .451 |
| Any 4 gene | 30 (1319.5±400.4) | 11 (370.5±285.5) | *3.6* | 0.175 |
| All specimens | 143 (1658.9±204.2) | 84 (935.5±194.4) | *1.8* | 0.019 |

*Ratio of diarrhoea and non-diarrhoea mean lactoferrin levels

University of Ghana http://ugspace.ug.edu.gh

In the subset of the children in which bacteria was isolated, faecal lactoferrin levels were further analyzed. In this subset, children with diarrhoea had significantly higher faecal lactoferrin levels (n=105; 671.9±341.7 μg/ml) compared to those without diarrhoea (n=75; 946.2±213.1 μg/ml) [95% CI, - 423.1 to 1261.9, $p$=0.051]. However, there was no statistical significance between the children who screened positive for EAEC and the non-EAEC [EAEC (n=130); 95% CI, 14706 ± 217.7 vs non-EAEC (n=38); 95% CI 1051.3 ±258.6, $p$=0.327]

UNIVERSITY OF G
BALME LIBRA

University of Ghana http://ugspace.ug.edu.gh

### 4.8 EAEC's Dispersin Gene Associates Better with Diarrhoea ($p<0.05$)

Of the 218 *Escherichia coli* isolated from culture and DNA extracted, 203 (93.1%) were available for screening by real-time PCR. The *aatA* was the most frequently detected gene (63.5%) in bacterial DNA, followed by *aap* (49.3%), *aggR* (30.5%) and *aaiC* (20.7%) [Table 10].

Figure 18 (*i*) and (*ii*) analyzed the entire stool and bacterial DNAs, and bacterial DNA and their matching stool DNAs, respectively. The dispersin gene *aap* was significantly associated with diarrhoea in the two separate analyzes ($p<0.05$). The chromosomal *aaiC* gene attained boarder-line significance in the bacterial DNA and their corresponding matching stool DNAs (p=0.049) [Figure 18 (*ii*)]. In general, the percentage proportion of EAEC genes detected in stool DNA were higher in all cases, compared with those detected from bacterial DNA. Within the diarrhoea sub-populations in the two separate analyses, EAEC's *aaiC* and *aggR* genes were significantly detected in stool DNA ($p<0.05$) [Figure 18(*i*) and (*ii*)].

EAEC, defined as positivity for any of the four genes screened was not statistically associated with diarrhoea in the bacterial DNA ($p=0.244$) [Table 10, Figure 19].

University of Ghana http://ugspace.ug.edu.gh

**Table 10: Enteroaggregative *E. coli* (EAEC) virulence factor-positive in bacterial DNA (*E. coli*)**

| Characteristic | Diarrhoea (n=115) n (%) | Non-diarrhoea (n=88) n (%) | Total n=(203) n (%) | Risk estimate: OR [95% CI] | $\chi^2$ | *p-value* |
|---|---|---|---|---|---|---|
| EAEC virulence related gene | | | | | | |
| *aaiC* | 25 (21.7) | 17(19.3) | 42 (20.7) | 1.160 [0.582 – 2.313] | 0.178 | 0.673 |
| *aggR* | 32 (27.8) | 30 (34.1) | 62 (30.5) | 0.745 [0.409 – 1.359] | 0.922 | 0.359 |
| *aatA* | 77 (67.0) | 52 (59.1) | 129 (63.5) | 1.403 [0.789 – 2.495] | 1.331 | 0.249 |
| *aap* | 65 (56.5) | 35 (39.8) | 100 (49.3) | 1.969 [1.120 – 3.460] | 5.595 | 0.018 |
| EAEC gene combination | | | | | | |
| Any 1 gene | 94 (81.7) | 66 (75.0) | 160 (78.8) | 1.492 [0.759 – 2.933] | 1.356 | 0.244 |
| Any 2 genes | 63 (54.8) | 44 (50.0) | 107 (52.7) | 1.212 [0.695 – 2.113] | 0.457 | 0.499 |
| Any 3 genes | 33(28.7) | 21 (23.9) | 54 (26.6) | 1.841 [0.680 – 2.423] | 0.596 | 0.440 |
| All 4 genes | 9 (7.8) | 3 (3.4) | 12 (5.9) | 2.406 [0.632 – 9.164] | 1.749 | 0.186 |

EAEC – Enteroaggregative *Escherichia coli*, OR – odds ratio, 95%CI – confidence interval, $\chi^2$- chi-square, $P < 0.05$ is significant

University of Ghana http://ugspace.ug.edu.gh

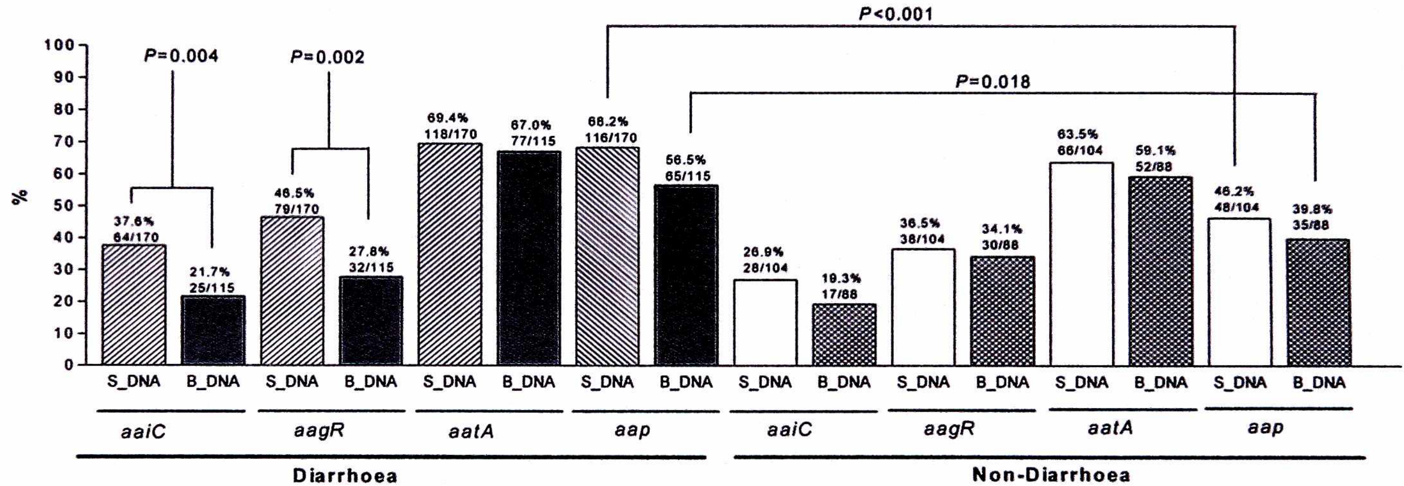



**Figure 18(*i*): Stool DNA versus bacterial DNA in the molecular diagnosis of EAEC (all stool and bacterial DNAs).** Within the diarrhoea sub-population, the detection of EAEC's chromosomal gene (aaiC), and the master plasmid regulon (aggR) were significantly detected in stool DNA, compared with bacteria DNA ($p<0.05$). The anti-aggregation gene (aap) was significantly associated with diarrhoea in both the stool and bacterial DNAs. S_DNA - stool DNA, B_DNA - bacterial DNA

University of Ghana http://ugspace.ug.edu.gh

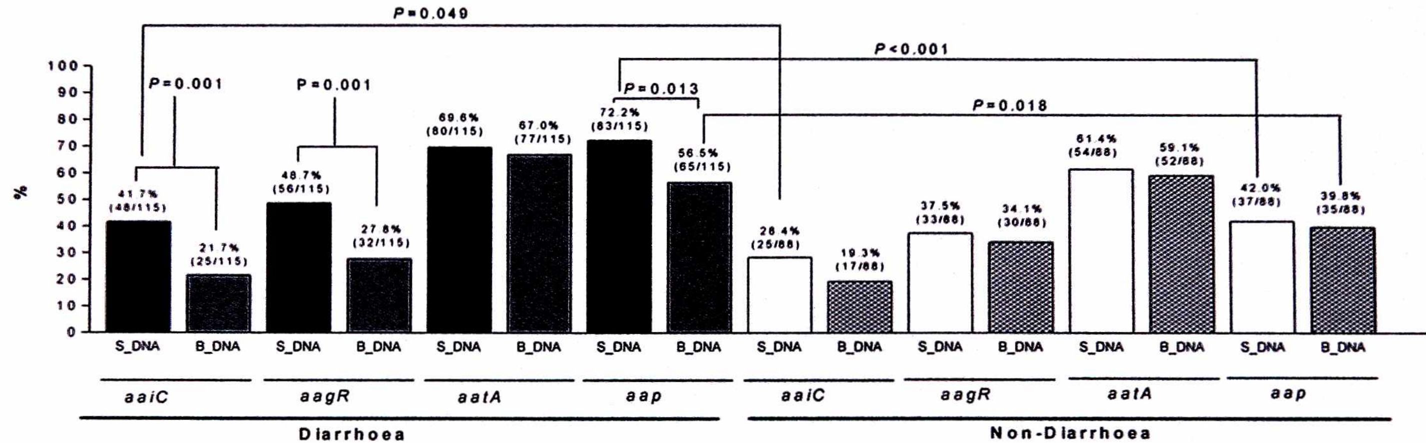



**Figure 18 (*ii*): Stool DNA versus bacteria DNA in the molecular diagnosis of EAEC (bacteria DNA and their corresponding stool DNAs).** The anti-aggregation gene (*aap*) was significantly associated with diarrhoea in both the stool and bacteria DNAs. Within the diarrhoea sub-population, the detection of EAEC's chromosomal gene (*aaiC*), the master plasmid regulon (*aggR*) and the *aap* were significantly detected in stool DNA, compared with bacteria DNA ($p<0.05$).

S_DNA - stool DNA, B_DNA - bacteria DNA

University of Ghana http://ugspace.ug.edu.gh

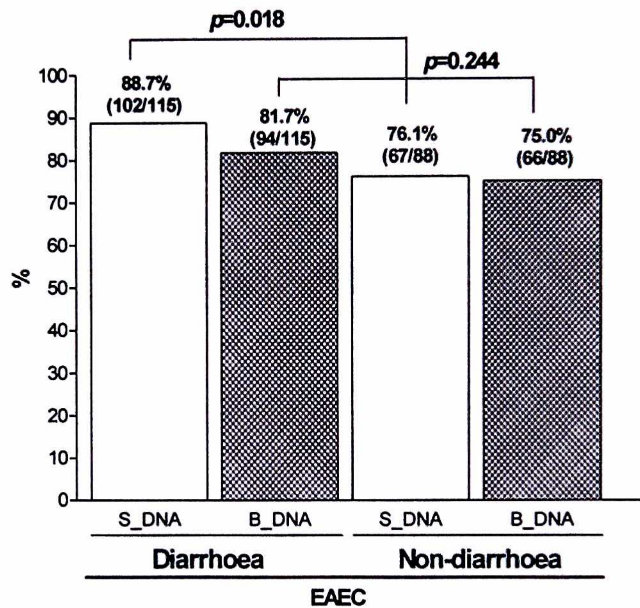



**Figure 19: EAEC is better diagnosed in stool DNA as opposed to bacterial DNA.** EAEC, defined as positivity to any of the four genes screened failed to attain statistical significance in bacterial DNA. In the cohort of children from whom E. coli was isolated by culture, EAEC was analysised in both their bacterial and stool DNAs. The association of EAEC to diarrhoea was lost in the bacterial DNA (p=0.244).

University of Ghana http://ugspace.ug.edu.gh

### 4.9 *Cryptosporidium* Genotypes: Preliminary results

The amplified region was much conserved, and therefore the BLAST analysis was not helpful in determining the circulating genotypes amplified by PCR. However, the HRM (High Resolution Melting) analysis, interrogating the melt curves of a known *C. parvum* genotype to the PCR positive unknowns indicated that 26.7% (4/15) of the *Cryptosporidium*-positive DNAs were *C. parvum*. The rest were broadly categorized as non-*C. parvum* [Figure 20].

University of Ghana http://ugspace.ug.edu.gh

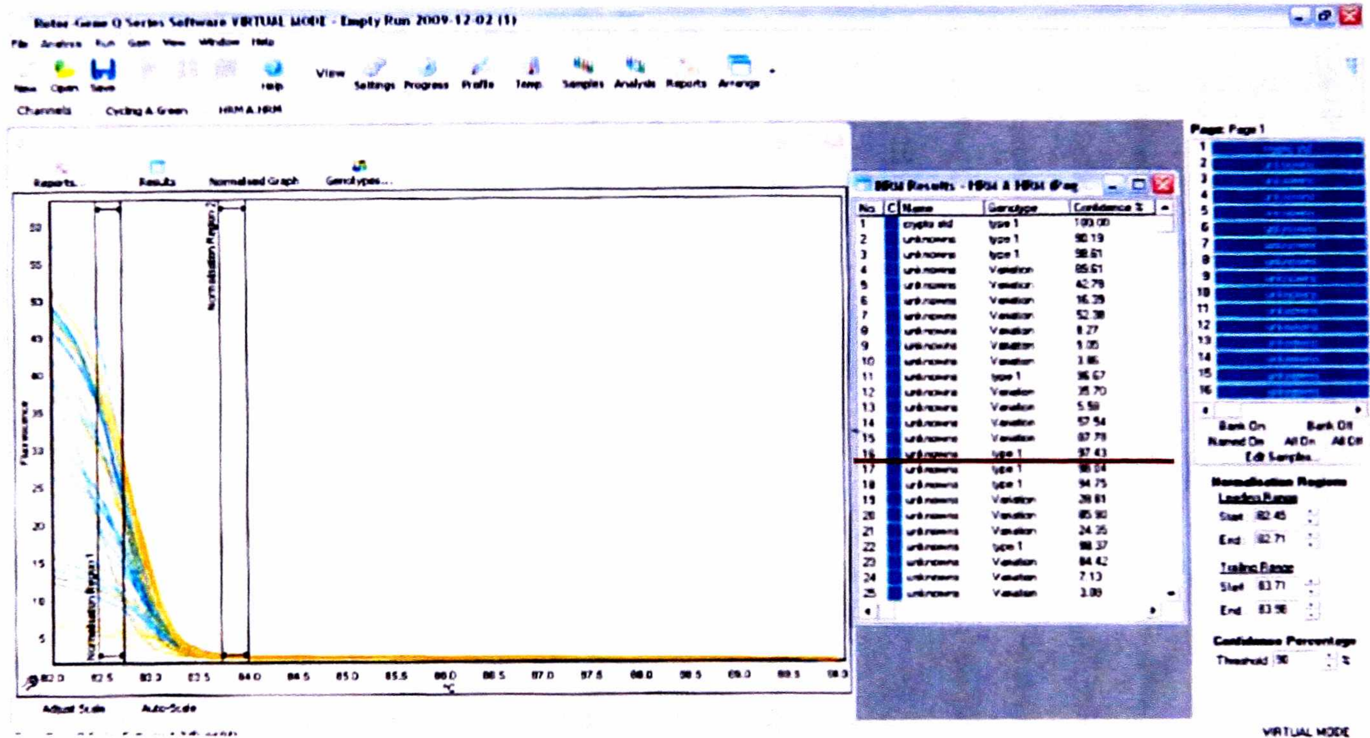


**Figure 20: HRM data analysis of Cryptosporidium-positive DNAs.** Sample # 1 (type 1) is a positive *C. parvum* control. The HRM analysis 'interogated' the melt curve of this control with the unknowns as well as a blank, containing only a buffer. 'Type 1' and 'variation' results generated by the Rotor-gene data file (Qiagen, Germany), represents *C. parvum* and non- *C. parvum* genotypes, respectively. Samples analyzed and included in the current study are samples # 2 to 16 (above red horizontal mark).

University of Ghana http://ugspace.ug.edu.gh

## 4.10 Summaries of Additional Data from Questionnaires

Educational backgrounds of the caregivers of 268 of the children were recorded, ranked and analyzed. No formal education, up to primary, elementary and vocational training was ranked as 'low'; and middle school leavers, up to Junior and Senior Secondary, and tertiary education, as 'high'. Low educational background of caregivers was a risk factor for a child presenting with diarrhoea 68.9% (115/167 in the children with, and 49.5% (50/101) without diarrhoea [OR=2.256 95% CI 1.355-3.754, ($p$=0.002)].

It was difficult to judge whether caregivers provided true response to the question on how long they exclusively breastfed. Breast feeding is widely promoted in Ghana. It is therefore speculated that caregivers wanted to appear strictly following the message, and it was difficult to include this in analysis.

About 43 % (73/170) of the caregivers of the children with diarrhoea responded 'Yes' to having taken some form of medication prior to attending hospital, and 50% (85/170) responded 'No'. No data was recorded for the rest.

University of Ghana http://ugspace.ug.edu.gh

# CHAPTER FIVE

# DISCUSSION

## 5.1 Nutritional Shortfalls among the Study Population

The prevalence of early childhood stunting and the number of people living in absolute poverty are good indicators of poor development (Bhutta *et al.*, 2008). These indicators are closely associated with poor cognitive and educational performance in children, and over 200 million children under 5 years, mostly living in south Asia and sub-Saharan Africa are not fulfilling their developmental potential (Muller and Becher, 2006; Pelletier and Frongillo, 2003; Smith, 2000; Victora *et al.*, 2010; Young and Jaspars, 2006). These disadvantaged children are likely to do poorly in school and subsequently have low incomes and provide poor care for their children, thus contributing to the intergenerational transmission of poverty (Grantham-McGregor *et al.*, 2007). In the current study, moderate to severe stunting (HAZ <-2) was observed in 35.9% of the study population; 44.2% and 27.4% in the children with and without diarrhoea, respectively. In Butajira, a rural setting in Ethiopia, the prevalence of stunting increased steadily throughout the first year of life in a cohort of children studied. At ages 2, 6 and 12 months, the prevalence of stunting was 14.6%, 26.7% and 48.1%, respectively (Medhin *et al.*, 2010). Breastfeeding has already been shown to significantly reduce mortality in infants and young children (Black *et al.*, 2008; Jones *et al.*, 2003; WHO, 2000a), but breast feeding promotions and campaigns only have a small effect on the reduction of stunting (Bhutta *et al.*, 2008). In the current study it was unclear whether the responses the caregivers provided in the

University of Ghana http://ugspace.ug.edu.gh

questionnaire reflected their true breast feeding practice, since almost everybody responded with either breastfeeding or having exclusively breast fed for at least six month. Elsewhere, exclusive breast feeding practice significantly correlated with factors such as infant birth order, caregivers' education, occupation and parity (Aghaji, 2002).

The current study identified wasting (WHZ <-2) in 21.7% of the children studied, 26.8% and 16.5% in the children with and without diarrhoea, respectively. In surveys conducted in Latin America, Asia and Africa, low birth weight was noted to be a good predictor for wasting in children (Fernandez *et al.*, 2002). The children in the current study were out-patients, and there was no data on their birth weights.

Low educational background of caregivers was a risk factor for children presenting with diarrhoea in the current study ($p$=0.002). This association may be indirectly related to the purchasing powers of these caregivers, in the provision of basic needs for themselves as well as their children. If this argument holds, it was expected that, malnutrition would also be statistically associated with low educational background of the caregivers of the children. However, no significant statistical association was detected between caregivers' educational background and stunting, wasting and under-weight status of children ($p > 0.05$). Elsewhere, maternal education and intelligence directly correlated with offspring diet and nutritional status (Hasan *et al.*, 1991; Wachs *et al.*, 2005).

University of Ghana http://ugspace.ug.edu.gh

Mild to severe malnutrition (WAZ < -1, which reflects both acute and chronic types of malnutrition) was identified in 49.8% of the study population. Severe growth shortfalls occurred in both children with and without diarrhoea in the entire study population. Growth faltering in infants from developing countries has been reported to occur as early as 2-3 months of age (Davies-Adetugbo, 1997; Dewey *et al.*, 1992; Sathian *et al.*, 1983) in contrast to developed countries (Dewey *et al.*, 1992), a fact attributable to timing of complementary feeding. For example, in the DARLING (Davis Area Research on Lactation, Infant Nutrition and Growth) study, though breast milk intakes were similar, the amount and nutrient density of food consumed after 6 months were lower in Peru than in the United States (Dewey *et al.*, 1992). In 2005, Antwi noted a 21.2% incidence of wasting (acute malnutrition) among children seen in Kumasi, Ghana (Antwi, 2008) similar to an overall incidence of 22.9% (37/161) in the current study - both consistent with the 22.1% estimate for Ghana by the World Food Programme (WFP, 2006).

In the present study, there were more children who were malnourished (WAZ < -1) and had diarrhoea than those who were malnourished without diarrhoea (93/168 vs 41/101, $p$=0.019). Additionally, approximately fourteen percent (38/269) of the children studied were severely malnourished (WAZ < -3), and, out of this number, 73.7% had diarrhoea. A self-perpetuating vicious cycle in which malnutrition and diarrhoea are synergistic is suggested, and may explain their effect on cognitive development of children, especially their semantic fluency as documented by others (Guerrant *et al.*, 2008). Data in the present study support this link between

University of Ghana http://ugspace.ug.edu.gh

malnutrition and diarrhoea, placing these groups of children at a higher risk of morbidity over time.

Considerable number of children less than two years of age fell below the 5% percentile curve, and nutritional status appeared to be worsened with increasing age within this sub-population. Elsewhere in India, nutritional status decreased with increasing age, and unhygienic weaning practices were assigned to this (Hasan *et al.*, 1991).

University of Ghana http://ugspace.ug.edu.gh

## 5.2 PCR for Detection of Pathogens

PCR methodology is more sensitive in screening for pathogens. In sub-Saharan Africa as in many other similar settings however, the cost involved cannot be passed on to patients and therefore this tool is employed mainly in research facilities. In the present study, routine bacterial culture detected only one stool culture positive for *S. flexneri* and none for *Salmonella* spp. PCR however detected six *ipaH* genes in diarrhoeal stool specimens. Although, the *ipaH* gene is expressed by both *Shigella* and enteroinvasive (EIEC), EIEC is not often detected in Ghana (Opintan *et al.*, 2010), and it is speculated that the *ipaH* gene detected most likely reflects the presence of *Shigella* spp that may have been missed on culture. The sero-group identified by culture and serology is predominant in Ghana (Opintan and Newman, 2007) as it is in many developing countries (Malakooti *et al.*, 1997; Subekti *et al.*, 1993 ).

*Cryptosporidium* spp was not only the most prevalent parasite detected, but it is also significantly associated with diarrhoea (8.7% vs 1.0%, $p$=0.011). Malnourished children with diarrhoea often had cryptosporidiosis. Prevalence rates of *Cryptosporidium* spp using presumably less sensitive methods like microscopy and ELISA in Ghana (Addy and Aikins-Bekoe, 1986; Adjei *et al.*, 2004), Liberia (Hojlyng *et al.*, 1986), Mexico (Miller *et al.*, 1994) and Guinea Bissau (Perch *et al.*, 2001) reported ranges between 7.7 - 29% in symptomatic children. The current study recorded 8.2% in symptomatic and 1.0% in non-symptomatic patients.

University of Ghana http://ugspace.ug.edu.gh

Modern diagnostic tools like PCR are able to discriminate *E. histolytica* from the non-pathogenic *E. dispar* (Haque *et al.*, 2007). The present study detected *E. histolytica* in 2.9 % (5/170) of the children who had diarrhoea. No visible blood was observed in any of the stools from these children. All the *E. histolytica* detected in the current study were from children who had diarrhoea. In Bangladesh and elsewhere, relatively few numbers of people infected with *E. histolytica* develop symptomatic disease (Ayeh-Kumi *et al.*, 2001; Haque *et al.*, 2003a; Verweij *et al.*, 2003).

The present study did not detect any *Giardia* in the stool specimens screened for the p241 gene of *Giardia* spp. The p241 gene target used is well validated (Guy *et al.*, 2003) and a positive control which was included in each PCR run was amplified whilst the negative control was not. Addy et al in 2004, found a 3.7% prevalence rate of *Giardia* in Kumasi, Ghana (Addy *et al.*, 2004). Personal communications from senior scientists indicated that *Giardia* species used to be the most frequently identified intestinal flagellate seen in routine stool examination in Accra, Ghana. Diarrhoea surviellance screening for major known pathogens and *Giardia* may indicate whether there is a decline in the incidence of giardiasis in Accra or otherwise. In a 7-year study of diarrhoea caused by parasites in Guinea Bissau, the most prevalent parasite was *Giardia lamblia* (14.8%) followed by *Cryptosporidium* (7.7%) (Perch *et al.*, 2001). Though seasonality, study duration or geographical location may influence parasite prevalence, the current study cannot pinpoint additional reasons for the zero prevalence of *Giardia*.

University of Ghana http://ugspace.ug.edu.gh

## 5.3 Co-infection

Children who had diarrhoea were often co-infected, with two or more pathogens detected in the stool, and EAEC-*Cryptosporidium* was the most prevalent (13/170) in the current study. Among HIV infected children in South Africa, Samie et al identified children who were co-infected with as many as six different species of pathogens (Samie *et al.*, 2007b). The present study detected a child who had diarrhoea with EAEC-*Cryptosporidium-Shigella*/EIEC co-infection. The HIV status of this child is however not known. Relatively, fewer children who are older than 2 years of age were sampled in the present study. However, cryptosporidiosis seemed to be more common in children < 2 years of age, and this is in agreement with an earlier study in Ghana (Adjei *et al.*, 2004).

Dozens of *Cryptosporidium* species have been characterized, and primarily, *C. parvum* and *C. hominis* have been associated with cattle and human infections, respectively. Because of the strong bond between humans and animals, *C. parvum* is also known to be associated with human infection (Samie *et al.*, 2006a; Warren, 2008). Earlier studies in Ghana employed microscopy for the diagnosis of cryptosporidiosis, and therefore did not genotype *Cryptosporidium* species (Addy and Aikins-Bekoe, 1986; Adjei *et al.*, 2004). Preliminary results in the current study showed that *C. parvum* genotypes accounts for less than 30% of cryptosporidiosis among young children with diarrhoea in Accra. Though the non-*C. parvum* could be *C. hominis*, the lack of an additional control (*C. hominis*) in the HRM analysis failed to reveal further details. There is little or no data on the epidemiology and genotypes

University of Ghana http://ugspace.ug.edu.gh

of cryptosporidiosis in sub-Saharan Africa. Samie et al found more *C. hominis* (82%) compared to *C. parvum* (18%) among school children and hospital patients in Venda, South Africa (Samie *et al.*, 2006a).

University of Ghana http://ugspace.ug.edu.gh

## 5.4 Prevalence of EAEC Virulence-associated Genes in Stool DNA

Among the EAEC plasmid genes tested (*aap*, *aatA* and *aggR*), only *aap* was significantly associated with diarrhoea ($p$=0.0003). A recent publication, which compared molecular probes to the 'gold standard' (brick-like aggregation in cultured epithelial cells), however, suggested that the *aap* gene is not restricted to EAEC, but is also detected in diffusely adherent *E coli* (DAEC) as well as in non pathogenic *E. coli* (Monteiro *et al.*, 2009). There was no significant statistical association between any individual EAEC gene (whether plasmid or chromosome borne) and malnutrition (WAZ < -1).

In the present study, EAEC virulence genes occurred in both sub-populations (diarrhoea/control, respectively): *aap* 68.2/46.2%, *aatA* 69.4/63.5%, *aggR* 46.5/36.5% and *aaiC* 37.6/26.9%. Information on the presence of EAEC virulence-associated genes in body fluids of persons in Africa is limited, as is the prevalence and distribution of this organism in Ghana. The *aggR* is known to regulate its own expression as well as that of several plasmid genes and chromosomal genes of EAEC (Dudley *et al.*, 2006), including the aggregative adherence fimbriae, a dispersin (aap), a dispersin translocator apparatus called aat and several chromosomal loci including the *aaiC* (Nataro, 2005). In a different population, Huang *et al.* failed to show any association between four EAEC virulence-associated genes (*aggA*, *aspU*, *aafA* and *aggR*) and clinical illness in travelers from the United States to Mexico (Huang *et al.*, 2003). Further, in that study, *aspU* (now designated *aatA*) was the least prevalent gene among the four EAEC virulence genes studied (Huang *et al.*, 2003), whilst this

University of Ghana http://ugspace.ug.edu.gh

same gene (*aatA*) was the most prevalent (67.2%) in the current study. In South Africa, Samie *et al.* found the *aap* gene to be the predominant among others and also associated with diarrhoea (Samie *et al.*, 2007b). These disparate findings on the relative distribution and importance of EAEC genes in diarrhoeal illnesses suggest that geographical location, type of exposure, and/or host factors may dictate the nature of EAEC infection.

Although *aatA* was the most prevalent gene observed in the present study, it was not associated with diarrhoea ($p>0.05$). Some studies have shown that the novel protein aatA, which is encoded on EAEC virulence plasmid pAA2, localizes to the outer membrane of this bacterium and facilitates export of the dispersin *aap* across the outer membrane (Imuta *et al.*, 2008; Sheikh *et al.*, 2002). Results from the current study may support this notion since *aatA* and *aap* were detected in greater than 68% of patients with diarrhoea. Further study is required for a firm conclusion. The chromosomal gene *aaiC* did not show any significant differential association between the diarrhoea and the non-diarrhoea group in the current study ($p=0.068$). About 12% (13/104) of children without diarrhoea in the current study were positive for all four EAEC genes tested.

One explanation for the high frequency of EAEC-associated virulence genes in symptomatic as well as asymptomatic patients and the heterogeneity of the different gene assortments found is that EAEC is endemic in the current study population. In support of this notion, almost all prior studies recover EAEC from controls (Albert *et*

University of Ghana http://ugspace.ug.edu.gh

*al.*, 1999; Gascon *et al.*, 2000; Valentiner-Branth *et al.*, 2003) as well as from individuals with diarrhoea (Okeke *et al.*, 2003; Rappelli *et al.*, 2005). Pathogenic and host factors may influence the initiation of a symptomatic phase, determine by and include distinct mechanisms as reviewed by Kaper (Kaper *et al.*, 2004). For example, Huang *et al.* suggested that a first exposure to EAEC infection 'primes' the immune system to prevent a second infection (Huang *et al.*, 2003). Further, in their study of traveler's diarrhoea, after the initial EAEC infection, only 4 (11%) of these students had a subsequent symptomatic EAEC infection (Huang *et al.*, 2003).

University of Ghana http://ugspace.ug.edu.gh

## 5.5 Lactoferrin levels

Lactoferrin is bactericidal to enteric pathogens, modulates the intestinal immune response, and is released by neutrophils into stool in response to infection (Walker *et al.*, 2007). In the current study, the mean lactoferrin levels in the children without diarrhoea were high compared to studies done in the developed world (Archbald-Pannone *et al.*, 2010; Kane *et al.*, 2003). Several of the available data on enteric infection and intestinal inflammation from developing countries used the latex agglutination method, and therefore lactoferrin levels were only semi-quantified (Alcantara *et al.*, 2003; Ashraf *et al.*, 2007; Bouckenooghe *et al.*, 2000; Samie *et al.*, 2007a; Samie *et al.*, 2006b). It is therefore difficult to judge whether mean lactoferrin levels in endemic populations like Ghana are generally high. The current study however observed an association between elevated faecal lactoferrin levels and diarrhoea ($p<0.05$). In Bangladesh, faecal lactoferrin levels was found not to be useful in differentiating between inflammatory and non-inflammatory diarrhoea, among patients enrolled under the International Center for Diarrhoea Disease Research (ICDDR,B) (Ashraf *et al.*, 2007).

In the current study, the mean lactoferrin levels were lower in children who were malnourished and had diarrhoea compared to those without malnutrition who had diarrhoea. It is speculated that the lactoferrin assay may be marginally less sensitive in the setting of malnutrition. Perhaps, the enterocytes are less able to synthesize lactoferrin in children who are malnourished and have diarrhoea. This observation needs further investigation for a firm conclusion. In earlier studies, the mean

University of Ghana http://ugspace.ug.edu.gh

lactoferrin levels for healthy controls from the US were less than 12.8 μg/ml (Kane *et al.*, 2003; Walker *et al.*, 2007) but healthy controls in the current study had 298.8 μg/ml. Unpublished observations by Lima *et al.* suggest that breast milk may contribute to moderately (15 to 120 μg/ml) increased faecal lactoferrin levels. Some studies have demonstrated an association of EAEC infection with inflammatory cytokines (Cennimo *et al.*, 2009; Greenberg *et al.*, 2002), and several others have associated EAEC with elevated lactoferrin levels (Bouckenooghe *et al.*, 2000; Samie *et al.*, 2007b).

In children with diarrhoea, the current study found a significant statistical association of elevated faecal lactoferrin with the *aatA* gene, and with the detection of any one or two of the EAEC genes tested ($p<0.05$). Between diarrhoea and non-diarrhoea stool specimens, EAEC's chromosomal gene *aaiC* was associated with the highest fold-rise in faecal lactoferrin level (2.7 fold-rise) followed by the *aap* gene (2.5 fold-rise). Additionally, an increased number of virulence EAEC genes detected corresponded to a rise in faecal lactoferrin level. The protective function of lactoferrin in infections with enteropathogens have been acknowledged, (Shashiraj *et al.*, 2006) and colonization/infection, particularly by EAEC in the current study probably contributed to the raised lactoferrin levels.

University of Ghana http://ugspace.ug.edu.gh

## 5.6 Molecular Diagnosis of EAEC: Stool DNA versus Bacterial DNA

Historically, EAEC was diagnosed by the AA phenotype, when cultured together with HEp-2 cells (Nataro and Kaper, 1998). This procedure requires specialized facilities, and high technical competence. Additionally, the adherence assay requires an initial stool culture – an added cost and takes about three days for results to be ready. Molecular methods eliminate the expensive $CO_2$ facility required for the adherence assay, and has given more scientists in both developing and developed countries the opportunity to investigate EAEC (Aranda *et al.*, 2004; Dedeic-Ljubovic *et al.*, 2009; Huang *et al.*, 2003; Opintan *et al.*, 2010). However, as yet, there is no single-all-encompassing molecular probe for the detection of EAEC and the molecular diagnosis of this pathogen is perplexing (Okeke, 2009). A few dozen investigators have used genomic bacterial DNA or supernatants of boiled bacteria cells to template these PCR reactions (Al-Gallas *et al.*, 2007; Antikainen *et al.*, 2009). Relatively fewer investigators have however, used stool DNAs to template these PCR reactions, and where DNA stool was used, the purpose was generally to screen for multiple enteric pathogens (Antikainen *et al.*, 2009; Samie *et al.*, 2007b).

The current study compared the molecular diagnosis of EAEC using stool DNA as opposed to bacterial DNA. Overall, the detection rates of the genes sought (*aaiC*, *aap*, *aatA* and *aggR*) were found to be comparable, in both the stool and the bacterial DNAs. In both methods, the *aatA* was the most detected gene, followed by *aap*, *aggR* and *aaiC* in that order. Though the *aatA* gene was the most frequently detected, it was not associated with diarrhoea ($p>0.05$). A recent publication suggests that the *aatA* is

University of Ghana http://ugspace.ug.edu.gh

responsible for virulence in pathogenic *E. coli* from avian sources (Li *et al.*, 2010). Perhaps some avian clones of *E. coli* can colonize humans, but are unable to initiate virulence. Only the *aap* gene was significantly associated with diarrhoea in the two screening methods; stool DNA vs bacterial DNA, respectively ($p$= <0.001 vs 0.018). The *aap* gene therefore appeared to be a 'unique' gene to screen for EAEC. A recent publication however suggests that the *aap* is not restricted to EAEC, but could be found in some non-EAECs (Monteiro *et al.*, 2009). With the exception of the chromosomal gene (*aaiC*), Monteiro et al sought for all the three plasmid genes (*aggR*, *aap* and *aatA* ) investigated in the current study from 252 DEC and non-DEC strains. In that study, 52% (26/50) of the non-DEC strains screened positive by PCR for the *aap* gene (Monteiro *et al.*, 2009). It is unclear why in the diarrhoea sub-population, EAEC's chromosomal (*aaiC*), the master regulator (*aggR*), and the dispersin (*aap*) genes were statistically associated with EAEC's detection in stool DNA in the current study. This observation requires further investigation.

In the literature, association of EAEC with symptomatic and asymptomatic infection is not clear. Factors such as age category, heterogeneity of the strain, inoculum size and polymorphism in the IL8 promoter region have been implicated as factors determining disease course (Flores and Okhuysen, 2009; Huang *et al.*, 2006b; Jiang *et al.*, 2003; Kaur *et al.*, 2010; Okeke *et al.*, 2000b; Usein *et al.*, 2009). Four EAEC's virulence genes were sought in the current study. For the same set of children, EAEC was only associated with diarrhoea in the stool DNA and not in the bacterial DNA. EAEC was defined as positivity for one or more of the four genes screened in both

University of Ghana http://ugspace.ug.edu.gh

methods. Using bacterial DNA to template PCRs, some authors have found EAEC to be statistically associated with diarrhoea (Ochoa *et al.*, 2009; Okeke *et al.*, 2003; Opintan *et al.*, 2010). In the current study, EAEC's association with diarrhoea was lost when bacterial DNA was analyzed from the same children ($p>0.05$). It is impossible to delineate commensals from pathogenic *E. coli* on agar plates. Therefore, a possible explanation to the disparity in the current finding may be issues to do with the probability of missing out some pathotypes of *E. coli*, when fewer bacteria colonies are picked for DEC screening. The current study used the recommendations of Nataro, and picked a minimum of 3 colonies (Nataro *et al.*, 1998). To further eliminate issues with subjectivity as to the number and which bacteria colonies to pick, stool DNA may be a better sample, as demonstrated in the current study. However, in situations where susceptibility may be required to guide therapy, the use of stool DNA may not be too helpful. Secondly, one may be dealing with live as well as dead bacteria when stool DNA is used.

University of Ghana http://ugspace.ug.edu.gh

## 5.7 Study Limitations

The current study had some limitations. Accurate height measurements were the most difficult to obtain on an out-patient basis, and this limited two of the three z-scores of malnutrition (WHZ and HAZ). Knowledge of history of antimicrobial exposure is important; however this information was obtainable in only few of the patients. Some mothers/caregivers do not remember the types of drugs given to their children. Additionally, only selected pathogens were assayed using PCR and thus, other less common pathogens may have been missed. Tests for intestinal parasites other than *Cryptosporidium*, *Giardia* and *E. histolytica* were not carried out. No follow-up of the children with/without diarrhoea was done as part of the current study. Such a follow-up, especially, for the children without diarrhoea who screened positive for all the four EAEC's virulence genes may reveal whether they later had diarrhoea or otherwise.

University of Ghana http://ugspace.ug.edu.gh

## 5.8 Conclusions, Recommendations and Future Research Direction

The current study identified high rates of undernutrition, wasting and stunting, among children from southern Ghana, with the worst forms of these growth shortfalls occurring among children > 6 months. Considerable number of infants with diarrhoea fell below the 5% percentile mark (weight-for-age) when compared with international reference curves. Through the use of specific DNA-biomarkers, the study was able to determine that EAEC and *Cryptosporidium* were common intestinal pathogens associated with diarrhoea in southern Ghana. HRM analysis indicated that less than 30% of the *Cryptosporidium* associated diarrhoea were *C. parvum* genotypes, and none of the four EAEC's virulence genes investigated was associated with malnutrition ($p$>0.5). Elevated faecal lactoferrin levels were associated with diarrhoea in this group of children from southern Ghana. A relatively high level of lactoferrin was observed in EAEC colonization/infection among our study population. Of the individual EAEC's virulence genes screened in the children with/without diarrhoea, the percentage proportion of each gene found in stool DNA were higher compared to bacterial DNA. The dispersin gene (*aap*) was significantly associated with diarrhoea in both stool and bacterial DNAs ($p$<0.5).

The use of anthropometric measurements for early identification of at risk children attending health care facilities in Ghana is recommended. Mean faecal lactoferrin levels in the children with/without diarrhoea were relatively high compared to those found in the developed world. Further studies to explore or improve existing diagnostic tools in endemic populations are recommended. Majority of the diarrhoea

University of Ghana http://ugspace.ug.edu.gh

sub-population in the current study were children who had acute diarrhoea. A prospective study enrolling more children with persistent diarrhoea (duration ≥14 days) is recommended to help understand EAEC's colonization/infection. Stool DNA may offer the opportunity for the screening of multiple pathogens. Initial stool culture and identification of *E. coli* may still be required for the molecular diagnosis of EAEC, and for purposes of antimicrobial susceptibility. In such a scenario a 'bacteria sweep' from a culture plate is recommended for the molecular diagnosis of EAEC.

In future, it may be necessary to compare EAEC in HEp-2 assays with markers of inflammation such as lactoferrin levels in stool samples. This will further enhance the knowledge and pathophysiology of diarrhoea caused by EAEC. The ability of the enterocytes to secret proinflammatory cytokines and lactoferrin, need further investigation, especially, under the setting of undernutrition and diarrhoea.

University of Ghana http://ugspace.ug.edu.gh

# REFERENCES


1. **Abba, K., Sinfield, R., Hart, C.A., and Garner, P.** (2009). Pathogens associated with persistent diarrhoea in children in low and middle income countries: systematic review. BMC Infect Dis *9*, 88. Available at: doi: 10.1186/1471-2334-1189-1188.

2. **Abduch Fabrega, V.L., Piantino Ferreira, A.J., Reis da Silva Patricio, F., Brinkley, C., and Affonso Scaletsky, I.C.** (2002). Cell-detaching *Escherichia coli* (CDEC) strains from children with diarrhea: Identification of a protein with toxigenic activity. FEMS Microbiol Lett *217*, 191-197.

3. **Abubakar, I., Aliyu, S.H., Arumugam, C., Usman, N.K., and Hunter, P.R. (2007).** Treatment of cryptosporidiosis in immunocompromised individuals: systematic review and meta-analysis. Br J Clin Pharmacol *63*, 387-393.

4. **Adachi, J.A., Ericsson, C.D., Jiang, Z.D., DuPont, M.W., Pallegar, S.R., and DuPont, H.L.** (2002). Natural history of enteroaggregative and enterotoxigenic *Escherichia coli* infection among US travelers to Guadalajara, Mexico. J Infect Dis *185*, 1681-1683.

5. **Adachi, J.A., Jiang, Z.D., Mathewson, J.J., Verenkar, M.P., Thompson, S., Martinez-Sandoval, F., Steffen, R., Ericsson, C.D., and DuPont, H.L.** (2001). Enteroaggregative *Escherichia coli* as a major etiologic agent in traveler's diarrhea in 3 regions of the world. Clin Infect Dis *32*, 1706-1709.

6. **Addy, P., Antepim, G., and Frimpong, E.** (2004). Prevalence of pathogenic *Escherichia coli* and parasites in infants with diarrhoea in Kumasi, Ghana. East African Medical Journal *81*, 353 - 357

University of Ghana http://ugspace.ug.edu.gh

7. **Addy, P.A., and Aikins-Bekoe, P.** (1986). Cryptosporidiosis in diarrhoeal children in Kumasi, Ghana. Lancet *1*, 735.

8. **Adeyemi, E.O., and Hodgson, H.J.** (1992). Faecal elastase reflects disease activity in active ulcerative colitis. Scand J Gastroenterol *27*, 139-142.

9. **Adjei, A.A., Armah, H., Rodrigues, O., Renner, L., Borketey, P., Ayeh-Kumi, P., Adiku, T., Sifah, E., and Lartey, M.** (2004). *Cryptosporidium* spp., a frequent cause of diarrhea among children at the Korle-Bu Teaching Hospital, Accra, Ghana. Jpn J Infect Dis *57*, 216-219.

10. **Agbodaze, D., Abrahams, C.A., and Arai, S.** (1988). Enteropathogenic and enterotoxigenic *Escherichia coli* as aetiological factors of infantile diarrhoea in rural and urban Ghana. Trans R Soc Trop Med Hyg *82*, 489-491.

11. **Aghaji, M.N.** (2002). Exclusive breast-feeding practice and associated factors in Enugu, Nigeria. West Afr J Med *21*, 66-69.

12. **Ajjampur, S.S., Rajendran, P., Ramani, S., Banerjee, I., Monica, B., Sankaran, P., Rosario, V., Arumugam, R., Sarkar, R., Ward, H., and Kang, G.** (2008). Closing the diarrhoea diagnostic gap in Indian children by the application of molecular techniques. J Med Microbiol *57*, 1364-1368.

13. **Al-Gallas, N., Bahri, O., Bouratbeen, A., Ben Haasen, A., and Ben Aissa, R.** (2007). Etiology of acute diarrhea in children and adults in Tunis, Tunisia, with emphasis on diarrheagenic *Escherichia coli*: prevalence, phenotyping, and molecular epidemiology. Am J Trop Med Hyg *77*, 571-582.

14. **Alam, N.H., and Ashraf, H.** (2003). Treatment of infectious diarrhea in children. Paediatr Drugs *5*, 151-165.

University of Ghana http://ugspace.ug.edu.gh


15. **Albert, M.J., Faruque, A.S., Faruque, S.M., Sack, R.B., and Mahalanabis, D.** (1999). Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. J Clin Microbiol *37*, 3458-3464.

16. **Albert, M.J., Qadri, F., Haque, A., and Bhuiyan, N.A.** (1993). Bacterial clump formation at the surface of liquid culture as a rapid test for identification of enteroaggregative *Escherichia coli*. J Clin Microbiol *31*, 1397-1399.

17. **Alcantara, C.S., Yang, C.H., Steiner, T.S., Barrett, L.J., Lima, A.A., Chappell, C.L., Okhuysen, P.C., White, A.C., Jr., and Guerrant, R.L.** (2003). Interleukin-8, tumor necrosis factor-alpha, and lactoferrin in immunocompetent hosts with experimental and Brazilian children with acquired cryptosporidiosis. Am J Trop Med Hyg *68*, 325-328.

18. **Antikainen, J., Tarkka, E., Haukka, K., Siitonen, A., Vaara, M., and Kirveskari, J.** (2009). New 16-plex PCR method for rapid detection of diarrheagenic *Escherichia coli* directly from stool samples. Eur J Clin Microbiol Infect Dis *28*, 899-908.

19. **Antwi, S.** (2008). Malnutrition: missed opportunities for diagnosis. Ghana Med J *42*, 101-104.

20. **Aramayo, C.F., Gil, J.F., Cruz, M.C., Poma, H.R., Last, M.S., and Rajal, V.B.** (2009). Diarrhea and parasitosis in Salta, Argentina. J Infect Dev Ctries *3*, 105-111.

21. **Aranda, K.R., Fagundes-Neto, U., and Scaletsky, I.C.** (2004). Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic *Escherichia coli* and *Shigella* spp. J Clin Microbiol *42*, 5849-5853.

University of Ghana http://ugspace.ug.edu.gh


22. **Archbald-Pannone, L., Sevilleja, J.E., and Guerrant, R.** (2010). Diarrhea, *Clostridium difficile*, and intestinal inflammation in residents of a long-term care facility. J Am Med Dir Assoc *11*, 263-267.

23. **Ashraf, H., Beltinger, J., Alam, N.H., Bardhan, P.K., Faruque, A.S., Akter, J., Salam, M.A., and Gyr, N.** (2007). Evaluation of faecal occult blood test and lactoferrin latex agglutination test in screening hospitalized patients for diagnosing inflammatory and non-inflammatory diarrhoea in Dhaka, Bangladesh. Digestion *76*, 256-261.

24. **Ayeh-Kumi, P.F., Ali, I.M., Lockhart, L.A., Gilchrist, C.A., Petri, W.A., Jr., and Haque, R.** (2001). *Entamoeba histolytica*: genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. Exp Parasitol *99*, 80-88.

25. **Bangar, R., and Mamatha, B.** (2008). Identification of enteroaggregative *Escherichia coli* in infants with acute diarrhea based on biofilm production in Manipal, south India. Indian J Med Sci *62*, 8-12.

26. **Barrow, G.I., and Feltham, R.K.A.** (1993). Cowan and Steel's Manual for the identification of Medical Bacteria. 3rd Edition. Cambridge University Press, New York, USA. . pg 128 – 150.

27. **Basu, S., Ghosh, S., Ganguly, N.K., and Majumdar, S.** (2004). A biologically active lectin of enteroaggregative *Escherichia coli*. Biochimie *86*, 657-666.

28. **Baudry, B., Savarino, S.J., Vial, P., Kaper, J.B., and Levine, M.M.** (1990). A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. J Infect Dis *161*, 1249-1251.

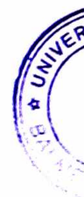
University of Ghana http://ugspace.ug.edu.gh


29. **Baveye, S., Elass, E., Mazurier, J., Spik, G., and Legrand, D.** (1999). Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. Clin Chem Lab Med *37*, 281-286.

30. **Baynes, R.D., Bezwoda, W.R., and Mansoor, N.** (1988). Neutrophil lactoferrin content in viral infections. Am J Clin Pathol *89*, 225-228.

31. **Behrens, M., Sheikh, J., and Nataro, J.P.** (2002). Regulation of the overlapping pic/set locus in Shigella flexneri and enteroaggregative *Escherichia coli*. Infect Immun *70*, 2915-2925.

32. **Berkowitz, F.V., W. Buqwana, A. Heney, C.** (1988). Cryptosporidiosis in black South African children. S Afr Med J *74*, 272-273.


33. **Bern, C., Martines, J., de Zoysa, I., and Glass, R.I.** (1992). The magnitude of the global problem of diarrhoeal disease: a ten-year update. Bull World Health Organ *70*, 705-714.

34. **Bernier, C., Gounon, P., and Le Bouguenec, C.** (2002). Identification of an aggregative adhesion fimbria (AAF) type III-encoding operon in enteroaggregative *Escherichia coli* as a sensitive probe for detecting the AAF-encoding operon family. Infect Immun *70*, 4302-4311.

35. **Bessler, H.C., de Oliveira, I.R., and Giugliano, L.G.** (2006). Human milk glycoproteins inhibit the adherence of *Salmonella* Typhimurium to HeLa cells. Microbiol Immunol *50* 877- 882.

36. **Bhutta, Z.A., Ahmed, T., Black, R.E., Cousens, S., Dewey, K., Giugliani, E., Haider, B.A., Kirkwood, B., Morris, S.S., Sachdev, H.P., and Meera, S.** (2008). What works? Interventions for maternal and child undernutrition and survival. Lancet *371*, 417-440.

University of Ghana http://ugspace.ug.edu.gh

37. **Black, R.E., Allen, L.H., Bhutta, Z.A., Caulfield, L.E., de Onis, M., Ezzati, M., Mathers, C., and Rivera, J.** (2008). Maternal and child undernutrition: global and regional exposures and health consequences. Lancet *371*, 243-260.

38. **Boeke, C.E., Mora-Plazas, M., Forero, Y., and Villamor, E.** (2010). Intestinal protozoan infections in relation to nutritional status and gastrointestinal morbidity in Colombian school children. J Trop Pediatr *56*, 299-306.

39. **Boisen, N., Ruiz-Perez, F., Scheutz, F., Krogfelt, K.A., and Nataro, J.P.** (2009). Short report: high prevalence of serine protease autotransporter cytotoxins among strains of enteroaggregative *Escherichia coli*. Am J Trop Med Hyg *80*, 294-301.

40. **Boone, J.H., Wilkins, T.D., Nash, T.E., Brandon, J.E., Macias, E.A., Jerris, R.C., and Lyerly, D.M.** (1999). TechLab and alexon Giardia enzyme-linked immunosorbent assay kits detect cyst wall protein 1. J Clin Microbiol *37*, 611-614.

41. **Bouckenooghe, A.R., Dupont, H.L., Jiang, Z.D., Adachi, J., Mathewson, J.J., Verenkar, M.P., Rodrigues, S., and Steffen, R.** (2000). Markers of enteric inflammation in enteroaggregative *Escherichia coli* diarrhea in travelers. Am J Trop Med Hyg *62*, 711-713.

42. **Bouzari, S., Jafari, A., Azizi, A., Oloomi, M., and Nataro, J.P.** (2001). Short report: characterization of enteroaggregative *Escherichia coli* isolates from Iranian children. Am J Trop Med Hyg *65*, 13-14.

University of Ghana http://ugspace.ug.edu.gh

43. **Bray, J.** (1945). Isolation of antigenically homogeneous strains of *Bact. coli neapolitanum* from summer diarrhoea of infants. . J Pathol Bacteriol *57*, 239-247.

44. **Brock, J.H.** (2002). The physiology of lactoferrin. Biochem Cell Biol *80*, 1-6.

45. **Brooks, J.T., Ochieng, J.B., Kumar, L., Okoth, G., Shapiro, R.L., Wells, J.G., Bird, M., Bopp, C., Chege, W., Beatty, M.E., Chiller, T., Vulule, J.M., Mintz, E., and Slutsker, I.** (2006). Surveillance for bacterial diarrhea and antimicrobial resistance in rural western Kenya, 1997-2003. Clin Infect Dis *43*, 393-401.

46. **Bryce, J., Boschi-Pinto, C., Shibuya, K., and Black, R.E.** (2005). WHO estimates of the causes of death in children. Lancet *365*, 1147-1152.

47. **Bunn, S.K., Bisset, W.M., Main, M.J., and Golden, B.E.** (2001a). Fecal calprotectin as a measure of disease activity in childhood inflammatory bowel disease. J Pediatr Gastroenterol Nutr *32*, 171-177.

48. **Bunn, S.K., Bisset, W.M., Main, M.J., Gray, E.S., Olson, S., and Golden, B.E.** (2001b). Fecal calprotectin: validation as a noninvasive measure of bowel inflammation in childhood inflammatory bowel disease. J Pediatr Gastroenterol Nutr *33*, 14-22.

49. **Cabada, M.M., and White, A.C., Jr.** (2008). Travelers' diarrhea: an update on susceptibility, prevention, and treatment. Curr Gastroenterol Rep *10*, 473-479.

50. **Caccio, S.M., and Pozio, E.** (2006). Advances in the epidemiology, diagnosis and treatment of cryptosporidiosis. Expert Rev Anti Infect Ther *4*, 429-443.

University of Ghana http://ugspace.ug.edu.gh

51. **Calderaro, A., Gorrini, C., Montecchini, S., Peruzzi, S., Piccolo, G., Rossi, S., Gargiulo, F., Manca, N., Dettori, G., and Chezzi, C.** (2010). Evaluation of a real-time polymerase chain reaction assay for the laboratory diagnosis of giardiasis. Diagn Microbiol Infect Dis *66*, 261-267.

52. **Campos, L.C., Franzolin, M.R., and Trabulsi, L.R.** (2004). Diarrheagenic *Escherichia coli* categories among the traditional enteropathogenic E. coli O serogroups--a review. Mem Inst Oswaldo Cruz *99*, 545-552.

53. **Casburn-Jones, A.C., and Farthing, M.J.** (2004). Management of infectious diarrhoea. Gut *53*, 296-305.

54. **Caulfield, L.E., de Onis, M., Blossner, M., and Black, R.E.** (2004). Undernutrition as an underlying cause of child deaths associated with diarrhea, pneumonia, malaria, and measles. Am J Clin Nutr *80*, 193-198.

55. **Cennimo, D., Abbas, A., Huang, D.B., and Chiang, T.** (2009). The prevalence and virulence characteristics of enteroaggregative *Escherichia coli* at an urgent-care clinic in the USA: a case-control study. J Med Microbiol *58*, 403-407.

56. **Cerna, J.F., Nataro, J.P., and Estrada-Garcia, T.** (2003). Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. J Clin Microbiol *41*, 2138-2140.

57. **Chang, S.M., Walker, S.P., Grantham-McGregor, S., and Powell, C.A.** (2002). Early childhood stunting and later behaviour and school achievement. J Child Psychol Psychiatry *43*, 775-783.

58. **Chappell, C.L., Okhuysen, P.C., Sterling, C.R., Wang, C., Jakubowski, W., and Dupont, H.L.** (1999). Infectivity of *Cryptosporidium parvum* in

University of Ghana http://ugspace.ug.edu.gh

healthy adults with pre-existing anti-C. parvum serum immunoglobulin G. Am J Trop Med Hyg *60*, 157-164.

59. **Checkley, W., Buckley, G., Gilman, R.H., Assis, A.M., Guerrant, R.L., Morris, S.S., Molbak, K., Valentiner-Branth, P., Lanata, C.F., and Black, R.E.** (2008). Multi-country analysis of the effects of diarrhoea on childhood stunting. Int J Epidemiol *37*, 816-830.

60. **Checkley, W., Epstein, L.D., Gilman, R.H., Cabrera, L., and Black, R.E.** (2003). Effects of acute diarrhea on linear growth in Peruvian children. Am J Epidemiol *157*, 166-175.

61. **Checkley, W., Gilman, R.H., Black, R.E., Lescano, A.G., Cabrera, L., Taylor, D.N., and Moulton, L.H.** (2002). Effects of nutritional status on diarrhea in Peruvian children. J Pediatr *140*, 210-218.

62. **Chen, L.C., Chowdhury, A., and Huffman, S.L.** (1980). Anthropometric assessment of energy-protein malnutrition and subsequent risk of mortality among preschool aged children. Am J Clin Nutr *33*, 1836-1845.

63. **Choi, S.W., Park, C.H., Silva, T.M., Zaenker, E.I., and Guerrant, R.L.** (1996). To culture or not to culture: fecal lactoferrin screening for inflammatory bacterial diarrhea. J Clin Microbiol *34*, 928-932.

64. **Corso, P.S., Kramer, M.H., Blair, K.A., Addiss, D.G., Davis, J.P., and Haddix, A.C.** (2003). Cost of illness in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. Emerg Infect Dis *9*, 426-431.

65. **Coupe, S., Sarfati, C., Hamane, S., and Derouin, F.** (2005). Detection of *cryptosporidium* and identification to the species level by nested PCR and restriction fragment length polymorphism. J Clin Microbiol *43*, 1017-1023.

University of Ghana http://ugspace.ug.edu.gh

66. **Coutinho, B.P., Oria, R.B., Vieira, C.M., Sevilleja, J.E., Warren, C.A., Maciel, J.G., Thompson, M.R., Pinkerton, R.C., Lima, A.A., and Guerrant, R.L.** (2008). Cryptosporidium infection causes undernutrition and, conversely, weanling undernutrition intensifies infection. J Parasitol *94*, 1225-1232.

67. **Crum, N.F., Wallace, M.R., and Oldfield, E.C.**, 3rd (2005). New issues in infectious diarrhea. Rev Gastroenterol Disord *5 Suppl 3*, S16-25.

68. **Cutting, W.A.** (1988). Mechanisms of diarrhoea and why they matter. Dialogue Diarrhoea, 4-5.

69. **Czeczulin, J.R., Balepur, S., Hicks, S., Phillips, A., Hall, R., Kothary, M.H., Navarro-Garcia, F., and Nataro, J.P.** (1997). Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli*. Infect Immun *65*, 4135-4145.

70. **Czeczulin, J.R., Whittam, T.S., Henderson, I.R., Navarro-Garcia, F., and Nataro, J.P.** (1999). Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. Infect Immun *67*, 2692-2699.

71. **Dai, J., Wang, S., Guerlebeck, D., Laturnus, C., Guenther, S., Shi, Z., Lu, C., and Ewers, C.** (2010). Suppression subtractive hybridization identifies an autotransporter adhesin gene of *E. coli* IMT5155 specifically associated with avian pathogenic *Escherichia coli* (APEC). BMC Microbiol *10*, 236.

72. **Dautin, N., and Bernstein, H.D.** (2007). Protein secretion in gram-negative bacteria via the autotransporter pathway. Annu Rev Microbiol *61*, 89-112.

University of Ghana http://ugspace.ug.edu.gh

73. **Davies-Adetugbo, A.A.** (1997). Sociocultural factors and the promotion of exclusive breastfeeding in rural Yoruba communities of Osun State, Nigeria. Soc Sci Med *45*, 113-125.

74. **de Araujo, A., and Giugliano, L.G.** (2000). Human milk fractions inhibit the adherence of diffusely adherent *Escherichia coli* (DAEC) and enteroaggregative *E. coli* (EAEC) to HeLa cells. FEMS Microbiol Lett *184* 91-94.

75. **de Onis, M., Monteiro, C., Akre, J., and Glugston, G.** (1993). The worldwide magnitude of protein-energy malnutrition: an overview from the WHO Global Database on Child Growth. Bull World Health Organ *71*, 703-712.

76. **de Onis, M., and Blossner, M.** (2003). The World Health Organization Global Database on Child growth and malnutriton: methodology and applications. Int J Epidemiol *32*, 518-526.

77. **Dedeic-Ljubovic, A., Hukic, M., Bekic, D., and Zvizdic, A.** (2009). Frequency and distribution of diarrhoeagenic *Escherichia coli* strains isolated from pediatric patients with diarrhoea in Bosnia and Herzegovina. Bosn J Basic Med Sci *9*, 148-155.

78. **Dewey, K.G., Peerson, J.M., Heinig, M.J., Nommsen, L.A., Lonnerdal, B., Lopez de Romana, G., de Kanashiro, H.C., Black, R.E., and Brown, K.H.** (1992). Growth patterns of breast-fed infants in affluent (United States) and poor (Peru) communities: implications for timing of complementary feeding. Am J Clin Nutr *56*, 1012-1018.

79. **Dickson, R., Awasthi, S., Williamson, P., Demellweek, C., and Garner, P.** (2000). Effects of treatment for intestinal helminth infection on growth and

University of Ghana http://ugspace.ug.edu.gh

cognitive performance in children: systematic review of randomised trials. BMJ *320*, 1697-1701.

80. **Dudley, E.G., Thomson, N.R., Parkhill, J., Morin, N.P., and Nataro, J.P.** (2006). Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative *Escherichia coli*. Mol Microbiol *61*, 1267-1282.

81. **Durley, A., Shenoy, A., Faruque, A.S., Suskind, R., and Ahmed, T.** (2004). Impact of a standardized management protocol on mortality of children with diarrhoea: an update of risk factors for childhood death. J Trop Pediatr *50*, 271-275.

82. **Dutta, P.R., Cappello, R., Navarro-Garcia, F., and Nataro, J.P.** (2002). Functional comparison of serine protease autotransporters of enterobacteriaceae. Infect Immun *70*, 7105-7113.

83. **Elias, W.P., Barros, S.F., Moreira, C.G., Trabulsi, L.R., and Gomes, T.A.** (2002a). Enteroaggregative *Escherichia coli* strains among classical enteropathogenic *Escherichia coli* O serogroups. J Clin Microbiol *40*, 3540-3541.

84. **Elias, W.P., Uber, A.P., Tomita, S.K., Trabulsi, L.R., and Gomes, T.A.** (2002b). Combinations of putative virulence markers in typical and variant enteroaggregative *Escherichia coli* strains from children with and without diarrhoea. Epidemiol Infect *129*, 49-55.

85. **Eslava, C., Navarro-Garcia, F., Czeczulin, J.R., Henderson, I.R., Cravioto, A., and Nataro, J.P.** (1998). Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli*. Infect Immun *66*, 3155-3163.

University of Ghana http://ugspace.ug.edu.gh

86. **Ethelberg, S., Lisby, M., Vestergaard, L.S., Enemark, H.L., Olsen, K.E., Stensvold, C.R., Nielsen, H.V., Porsbo, L.J., Plesner, A.M., and Molbak, K.** (2009). A foodborne outbreak of *Cryptosporidium hominis* infection. Epidemiol Infect *137*, 348-356.

87. **Ezzati, M., Lopez, A.D., Rodgers, A., Vander Hoorn, S., and Murray, C.J.** (2002). Selected major risk factors and global and regional burden of disease. Lancet *360*, 1347-1360.

88. **Fall, A., R. C. Thompson, R. P. Hobbs, and U. Morgan-Ryan.** ( 2003). Morphology is not a reliable tool for delineating species within *Cryptosporidium*. J Parasitol *89*, 399-402.

89. **FAO** (2004). Food and Agriculture Organization of the United Nations. Undernourishment around the world. In: The state of food insecurity in the world 2004. Rome: The Organization, pg 6 - 16.

90. **FAO** (2010). Food and Agriculture Organization (FAO). Human nutrition in developing world. FAO corporate document repository. Disorders of malnutrition. Part III. www.fao.org/docrep/w0073e/w007e05.htm. Accessed, January 2010.

91. **Farnaud, S., and Evans, R.W.** (2003). Lactoferrin--a multifunctional protein with antimicrobial properties. Mol Immunol *40*, 395-405.

92. **Fernandez, I.D., Himes, J.H., and de Onis, M.** (2002). Prevalence of nutritional wasting in populations: building explanatory models using secondary data. Bull World Health Organ *80*, 282-291.

93. **Fine, K.D., Ogunji, F., George, J., Niehaus, M.D., and Guerrant, R.L.** (1998). Utility of a rapid fecal latex agglutination test detecting the neutrophil

University of Ghana http://ugspace.ug.edu.gh

protein, lactoferrin, for diagnosing inflammatory causes of chronic diarrhea. Am J Gastroenterol *93*, 1300-1305.

94. **Flores, J., and Okhuysen, P.C.** (2009). Enteroaggregative *Escherichia coli* infection. Curr Opin Gastroenterol *25*, 8-11.

95. **Fried, K., Tarkanyi, K., Prinz, G., and Ban, E.** (2002). Detection of lactoferrin in feces for differential diagnosis in diarrhea. Orv Hetil *143*, 2141-2144.

96. **Garcia, L.S., Shimizu, R.Y., and Bernard, C.N.** (2000). Detection of Giardia lamblia, Entamoeba histolytica/Entamoeba dispar, and *Cryptosporidium parvum* antigens in human fecal specimens using the triage parasite panel enzyme immunoassay. J Clin Microbiol *38*, 3337-3340.

97. **Gascon, J., Vargas, M., Schellenberg, D., Urassa, H., Casals, C., Kahigwa, E., Aponte, J.J., Mshinda, H., and Vila, J.** (2000). Diarrhea in children under 5 years of age from Ifakara, Tanzania: a case-control study. J Clin Microbiol *38*, 4459-4462.

98. **Gay-Andrieu, E., Adehossi, E., Illa, H., Garba Ben, A., Kourna, H., and Boureima, H.** (2007). Prevalence of cryptosporidiosis in pediatric hospital patients in Niamey, Niger. Bull Soc Pathol Exot *100*, 193-196.

99. **Gene ID** (2009). Gene ID cgd7_230 (2009). Cryptosporidium Genomic Resources. 18s ribosomal RNA. Cryptosporidium DB (version 4.2).

100. **GEP** (1995). Goma Epidemiology Group. Public health impact of Rwandan refugee crisis: what happened in Goma, Zaire, in July, 1994? Goma Epidemiology Group. Lancet *345*, 339-344.

University of Ghana http://ugspace.ug.edu.gh

101. **Geyer, A., Crewe-Brown, H.H., Greeff, A.S., Fripp, P.J., Steele, A.D., Van Schalkwyk, T.V., and Clay, C.G.** (1993). The microbial aetiology of summer paediatric gastroenteritis at Ga-Rankuwa Hospital in South Africa. East Afr Med J *70*, 78-81.

102. **Gioppo, N.M., Elias, W.P., Jr., Vidotto, M.C., Linhares, R.E., Saridakis, H.O., Gomes, T.A., Trabulsi, L.R., and Pelayo, J.S.** ( 2000). Prevalence of HEp-2 cell-adherent *Escherichia coli* and characterisation of enteroaggregative *E. coli* and chain-like adherent *E. coli* isolated from children with and without diarrhoea, in Londrina, Brazil. FEMS Microbiol Lett *190*, 293-298.

103. **Golden, M.H.N.** (1988). The consequence of protein deficiency in man and its relationship to the features of kwashiokor. In: Nutritional adaptation in man. Blaxier K & Waterlow JC London. John Libbey, 1988, pg 169-189.

104. **Gomez-Duarte, O.G., Bai, J., and Newell, E.** (2009). Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagn Microbiol Infect Dis *63*, 1-9.

105. **Grantham-McGregor, S., Cheung, Y.B., Cueto, S., Glewwe, P., Richter, L., and Strupp, B.** (2007). Developmental potential in the first 5 years for children in developing countries. Lancet *369*, 60-70.

106. **Greenberg, D.E., Jiang, Z.D., Steffen, R., Verenker, M.P., and DuPont, H.L.** (2002). Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative *Escherichia coli* pathogenicity. J Infect Dis *185*, 944-949.

University of Ghana http://ugspace.ug.edu.gh

107. **Grotto, I., Mimouni, M., Gdalevich, M., and Mimouni, D.** (2003). Vitamin A supplementation and childhood morbidity from diarrhea and respiratory infections: a meta-analysis. J Pediatr *142*, 297-304.

108. **Grover, V., Ghosh, S., Sharma, N., Chakraborti, A., Majumdar, S., and Ganguly, N.K.** (2001). Characterization of a galactose specific adhesin of enteroaggregative *Escherichia coli*. Arch Biochem Biophys *390*, 109-118.

109. **Guerrant, D.I., Moore, S.R., Lima, A.A., Patrick, P.D., Schorling, J.B., and Guerrant, R.L.** (1999a). Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. Am J Trop Med Hyg *61*, 707-713.

110. **Guerrant, R.L., Araujo, V., Soares, E., Kotloff, K., Lima, A.A., Cooper, W.H., and Lee, A.G.** (1992a). Measurement of fecal lactoferrin as a marker of fecal leukocytes. J Clin Microbiol *30*, 1238-1242.

111. **Guerrant, R.L., Lima, A.A., Barboza, M., Young, S., Silva, T., Barrett, L., Bao, Y., Fox, J., and Moore, S.** (1999b). Mechanisms and impact of enteric infections. Adv Exp Med Biol *473*, 103-112.

112. **Guerrant, R.L., Oria, R.B., Moore, S.R., Oria, M.O., and Lima, A.A.** (2008). Malnutrition as an enteric infectious disease with long-term effects on child development. Nutr Rev *66*, 487-505.

113. **Guerrant, R.L., Schorling, J.B., McAuliffe, J.F., and de Souza, M.A.** (1992b). Diarrhea as a cause and an effect of malnutrition: diarrhea prevents catch-up growth and malnutrition increases diarrhea frequency and duration. Am J Trop Med Hyg *47*, 28-35.

University of Ghana http://ugspace.ug.edu.gh

114. **Guerrant, R.L., Shields, D.S., Thorson, S.M., Schorling, J.B., and Groschel, D.H.** (1985). Evaluation and diagnosis of acute infectious diarrhea. Am J Med *78*, 91-98.

115. **Gutierrez-Cisneros, M.J., Cogollos, R., Lopez-Velez, R., Martin-Rabadan, P., Martinez-Ruiz, R., Subirats, M., Merino, F.J., and Fuentes, I.** (2010). Application of real-time PCR for the differentiation of *Entamoeba histolytica* and *E. dispar* in cyst-positive faecal samples from 130 immigrants living in Spain. Ann Trop Med Parasitol *104*, 145-149.

116. **Guy, R.A., Payment, P., Krull, U.J., and Horgen, P.A.** (2003). Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl Environ Microbiol *69*, 5178-5185.

117. **Hamer, C., Kvatum, K., Jeffries, D., and Allen, S.** (2004). Detection of severe protein-energy malnutrition by nurses in The Gambia. Arch Dis Child *89*, 181-184.

118. **Hamosh, M.** (2001). Bioactive factors in human milk. Pediatr Clin North Am *48*, 69-86.

119. **Haque, R., Huston, C.D., Hughes, M., Houpt, E., and Petri, W.A., Jr.** (2003a). Amebiasis. N Engl J Med *348*, 1565-1573.

120. **Haque, R., Mollah, N.U., Ali, I.K., Alam, K., Eubanks, A., Lyerly, D., and Petri, W.A., Jr.** (2000). Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. J Clin Microbiol *38*, 3235-3239.

121. **Haque, R., Mondal, D., Kirkpatrick, B.D., Akther, S., Farr, B.M., Sack, R.B., and Petri, W.A., Jr.** (2003b). Epidemiologic and clinical characteristics

University of Ghana http://ugspace.ug.edu.gh

of acute diarrhea with emphasis on *Entamoeba histolytica* infections in preschool children in an urban slum of Dhaka, Bangladesh. Am J Trop Med Hyg *69*, 398-405.

122. **Haque, R., Roy, S., Siddique, A., Mondal, U., Rahman, S.M., Mondal, D., Houpt, E., and Petri, W.A., Jr.** (2007). Multiplex real-time PCR assay for detection of *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp. Am J Trop Med Hyg *76*, 713-717.

123. **Harrington, S.M., Dudley, E.G., and Nataro, J.P.** (2006). Pathogenesis of enteroaggregative *Escherichia coli* infection. FEMS Microbiol Lett *254*, 12-18.

124. **Harrington, S.M., Sheikh, J., Henderson, I.R., Ruiz-Perez, F., Cohen, P.S., and Nataro, J.P.** (2009). The Pic protease of enteroaggregative *E. coli* promotes intestinal colonization and growth in the presence of mucin. Infect Immun *67*, 5587-5596.

125. **Harrington, S.M., Strauman, M.C., Abe, C.M., and Nataro, J.P.** (2005). Aggregative adherence fimbriae contribute to the inflammatory response of epithelial cells infected with enteroaggregative *Escherichia coli*. Cell Microbiol *7*, 1565-1578.

126. **Hasan, J., Khan, Z., and Sinha, S.N.** (1991). Socio-cultural factors influencing nutritional status of infants -- a longitudinal study. Indian J Matern Child Health *2*, 84-86.

127. **Herrmann, M.G., Durtschi, J.D., Bromley, L.K., Wittwer, C.T., and Voelkerding, K.V.** (2006). Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clin Chem *52*, 494-503.

University of Ghana http://ugspace.ug.edu.gh

128. **Hicks, S., Candy, D.C., and Phillips, A.D.** (1996). Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. Infect Immun *64*, 4751-4760.

129. **Hojlyng, N., Molbak, K., and Jepsen, S.** (1986). *Cryptosporidium* spp., a frequent cause of diarrhea in Liberian children. J Clin Microbiol *23*, 1109-1113.

130. **Huang, D.B., and Dupont, H.L.** (2004). Enteroaggregative *Escherichia coli:* an emerging pathogen in children. Semin Pediatr Infect Dis *15*, 266-271.

131. **Huang, D.B., Jiang, Z.D., and Dupont, H.L.** (2003). Association of virulence factor-positive and -negative enteroaggregative *Escherichia coli* and occurrence of clinical illness in travelers from the United States to Mexico. Am J Trop Med Hyg *69*, 506-508.

132. **Huang, D.B., Mohamed, J.A., Nataro, J.P., DuPont, H.L., Jiang, Z.D., and Okhuysen, P.C.** (2007). Virulence characteristics and the molecular epidemiology of enteroaggregative *Escherichia coli* isolates from travellers to developing countries. J Med Microbiol *56*, 1386-1392.

133. **Huang, D.B., Mohanty, A., DuPont, H.L., Okhuysen, P.C., and Chiang, T.** (2006a). A review of an emerging enteric pathogen: enteroaggregative *Escherichia coli.* J Med Microbiol *55*, 1303-1311.

134. **Huang, D.B., Nataro, J.P., DuPont, H.L., Kamat, P.P., Mhatre, A.D., Okhuysen, P.C., and Chiang, T.** (2006b). Enteroaggregative *Escherichia coli* is a cause of acute diarrheal illness: a meta-analysis. Clin Infect Dis *43*, 556-563.

University of Ghana http://ugspace.ug.edu.gh

135. **Huang, D.B., Okhuysen, P.C., Jiang, Z.D., and DuPont, H.L.** (2004). Enteroaggregative *Escherichia coli*: an emerging enteric pathogen. Am J Gastroenterol *99*, 383-389.

136. **Huicho, L., Campos, M., Rivera, J., and Guerrant, R.L.** (1996). Fecal screening tests in the approach to acute infectious diarrhea: a scientific overview. Pediatr Infect Dis J *15*, 486-494.

137. **Huicho, L., Garaycochea, V., Uchima, N., Zerpa, R., and Guerrant, R.L.** (1997). Fecal lactoferrin, fecal leukocytes and occult blood in the diagnostic approach to childhood invasive diarrhea. Pediatr Infect Dis J *16*, 644-647.

138. **Hunter, P.R., Hughes, S., Woodhouse, S., Syed, Q., Verlander, N.Q., Chalmers, R.M., Morgan, K., Nichols, G., Beeching, N., and Osborn, K.** (2004). Sporadic cryptosporidiosis case-control study with genotyping. Emerg Infect Dis *10*, 1241-1249.

139. **Huppertz, H.I., Rutkowski, S., Aleksic, S., and Karch, H.** (1997). Acute and chronic diarrhoea and abdominal colic associated with enteroaggregative *Escherichia coli* in young children living in western Europe. Lancet *349*, 1660-1662.

140. **Imuta, N., Nishi, J., Tokuda, K., Fujiyama, R., Manago, K., Iwashita, M., Sarantuya, J., and Kawano, Y.** (2008). The *Escherichia coli* efflux pump TolC promotes aggregation of enteroaggregative *E. coli* 042. Infect Immun *76*, 1247-1256.

141. **Itoh, Y., Nagano, I., Kunishima, M., and Ezaki, T.** (1997). Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable : H10 associated with a massive outbreak of gastrointestinal illness. J Clin Microbiol *35*, 2546-2550.

University of Ghana http://ugspace.ug.edu.gh

142. **Iwanaga, M., Song, T., Higa, N., Kakinohana, S., Toma, C., and Nakasone, N.** (2002). Enteroaggregative *Escherichia coli*: incidence in Japan and usefulness of the clump-formation test. J Infect Chemother *8*, 345-348.

143. **Iwashita, M., Nishi, J., Wakimoto, N., Fujiyama, R., Yamamoto, K., Tokuda, K., Manago, K., and Kawano, Y.** (2006). Role of the carboxy-terminal region of the outer membrane protein AatA in the export of dispersin from enteroaggregative *Escherichia coli*. FEMS Microbiol Lett *256*, 266-272.

144. **Jenkins, C., Chart, H., Willshaw, G.A., Cheasty, T., and Smith, H.R.** (2006). Genotyping of enteroaggregative *Escherichia coli* and identification of target genes for the detection of both typical and atypical strains. Diagn Microbiol Infect Dis *55*, 13-19.

145. **Jenkins, C., van Ijperen, C., Dudley, E.G., Chart, H., Willshaw, G.A., Cheasty, T., Smith, H.R., and Nataro, J.P.** (2005). Use of a microarray to assess the distribution of plasmid and chromosomal virulence genes in strains of enteroaggregative *Escherichia coli*. FEMS Microbiol Lett *253*, 119-124.

146. **Jex, A.R., Smith, H.V., Nolan, M.J., Campbell, B.E., Young, N.D., Cantacessi, C., and Gasser, R.B.** (2011). Cryptic parasite revealed improved prospects for treatment and control of human cryptosporidiosis through advanced technologies. Adv Parasitol *77*, 141-173.

147. **Jiang, Z.D., Greenberg, D., Nataro, J.P., Steffen, R., and DuPont, H.L.** (2002). Rate of occurrence and pathogenic effect of enteroaggregative *Escherichia coli* virulence factors in international travelers. J Clin Microbiol *40*, 4185-4190.

University of Ghana http://ugspace.ug.edu.gh

148. **Jiang, Z.D., Okhuysen, P.C., Guo, D.C., He, R., King, T.M., DuPont, H.L., and Milewicz, D.M.** (2003). Genetic susceptibility to enteroaggregative *Escherichia coli* diarrhea: polymorphism in the interleukin-8 promotor region. J Infect Dis *188*, 506-511.

149. **Jones, G., Steketee, R.W., Black, R.E., Bhutta, Z.A., and Morris, S.S.** (2003). How many child deaths can we prevent this year? Lancet *362*, 65-71.

150. **Joosten, K.F., and Hulst, J.M.** (2008). Prevalence of malnutrition in pediatric hospital patients. Curr Opin Pediatr *20*, 590-596.

151. **Kane, S.V., Sandborn, W.J., Rufo, P.A., Zholudev, A., Boone, J., Lyerly, D., Camilleri, M., and Hanauer, S.B.** (2003). Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. Am J Gastroenterol *98*, 1309-1314.

152. **Kaper, J.B., Nataro, J.P., and Mobley, H.L.** (2004). Pathogenic *Escherichia coli*. Nat Rev Microbiol *2*, 123-140.

153. **Karanis, P., Kourenti, C., and Smith, H.** (2007). Water-borne transmission of protozoan parasites: a review of world-wide outbreaks and lessons learnt J Wat Health *5*, 1-38.

154. **Kaur, P., Chakraborti, A., and Asea, A.** (2010). Enteroaggregative *Escherichia coli*: An Emerging Enteric Food Borne Pathogen. Interdiscip Perspect Infect Dis *2010*, 254159. Available at: doi: 254110.251155/252010/254159.

155. **Kirkpatrick, B.D., Daniels, M.M., Jean, S.S., Pape, J.W., Karp, C., Littenberg, B., Fitzgerald, D.W., Lederman, H.M., Nataro, J.P., and**

University of Ghana http://ugspace.ug.edu.gh

**Sears, C.L.** (2002). Cryptosporidiosis stimulates an inflammatory intestinal response in malnourished Haitian children. J Infect Dis *186*, 94-101.

156. **Ko, G., Jiang, Z.D., Okhuysen, P.C., and DuPont, H.L.** (2006). Fecal cytokines and markers of intestinal inflammation in international travelers with diarrhea due to Noroviruses. J Med Virol *78*, 825-828.

157. **Kohli, A., Bushen, O.Y., Pinkerton, R.C., Houpt, E., Newman, R.D., Sears, C.L., Lima, A.A., and Guerrant, R.L.** (2008). *Giardia duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg *102*, 718-725.

158. **Kosek, M., Alcantara, C., Lima, A.A., and Guerrant, R.L.** (2001). Cryptosporidiosis: an update. Lancet Infect Dis *1*, 262-269.

159. **Kosek, M., Bern, C., and Guerrant, R.L.** (2003). The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull World Health Organ *81*, 197-204.

160. **Kothavade, R.J.** (2011). Challenges in understanding the immunopathogenesis of Cryptosporidium infections in humans. Eur J Clin Microbiol Infect Dis *30*, 1461-1472.

161. **Kotloff, K.L., Winickoff, J.P., Ivanoff, B., Clemens, J.D., Swerdlow, D.L., Sansonetti, P.J., Adak, G.K., and Levine, M.M.** (1999). Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull World Health Organ *77*, 651-666.

162. **Kucharzik, T., Hudson, J.T., 3rd, Lugering, A., Abbas, J.A., Bettini, M., Lake, J.G., Evans, M.E., Ziegler, T.R., Merlin, D., Madara, J.L., and William, I.R.** (2005). Acute induction of human IL-8 production by intestinal

University of Ghana http://ugspace.ug.edu.gh

epithelium triggers neutrophil infiltration without mucosal injury. Gut *54*, 1565-1572.

163. **Lake, I.R.N., G. Bentham, G. Harrison, F.C. Hunter, P.R. and Kovats, SR.** ( 2007). Cryptosporidiosis decline after regulation, England and Wales, 1989–2005. Emerg Infect Dis *13*, 623-625.

164. **Lauer, J.A., Betran, A.P., Barros, A.J., and de Onis, M.** (2006). Deaths and years of life lost due to suboptimal breast-feeding among children in the developing world: a global ecological risk assessment. Public Health Nutr *9*, 673-685.

165. **Leon-Sicairos, N., Reyes-Lopez, M., Canizalez-Roman, A., Bermudez-Cruz, R.M., Serrano-Luna, J., Arroyo, R., and de la Garza, M.** (2005). Human hololactoferrin: endocytosis and use as an iron source by the parasite *Entamoeba histolytica*, . Microbiology *151*, 3859-3871.

166. **Levay, P.F., and Viljoen, M.** (1995). Lactoferrin: a general review. Haematologica *80*, 252-267.

167. **Levine, M.M., Bergquist, E.J., Nalin, D.R., Waterman, D.H., Hornick, R.B., Young, C.R., and Sotman, S.** (1978). *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet *1*, 1119-1122.

168. **Li, G., Feng, Y., Kariyawasam, S., Tivendale, K.A., Wannemuehler, Y., Zhou, F., Logue, C.M., Miller, C.L., and Nolan, L.K.** (2010). AatA is a novel autotransporter and virulence factor of avian pathogenic *Escherichia coli*. Infect Immun *78*, 898-906.

169. **Lima, A.A., Moore, S.R., Barboza, M.S., Jr., Soares, A.M., Schleupner, M.A., Newman, R.D., Sears, C.L., Nataro, J.P., Fedorko, D.P., Wuhib, T.,**

University of Ghana http://ugspace.ug.edu.gh

**Schorling, J.B, and Guerrant, R.L.** (2000). Persistent diarrhea signals a critical period of increased diarrhea burdens and nutritional shortfalls: a prospective cohort study among children in northeastern Brazil. J Infect Dis *181*, 1643-1651.

170. **Lin, C.A., Boslaugh, S., Ciliberto, H.M., Maleta, K., Ashorn, P., Briend, A., and Manary, M.J.** (2007). A prospective assessment of food and nutrient intake in a population of Malawian children at risk for kwashiorkor. J Pediatr Gastroenterol Nutr *44*, 487-493.

171. **Lorntz, B., Soares, A.M., Moore, S.R., Pinkerton, R., Gansneder, B., Bovbjerg, V.E., Guyatt, H., Lima, A.M., and Guerrant, R.L.** (2006). Early childhood diarrhea predicts impaired school performance. Pediatr Infect Dis J *25*, 513-520.

172. **Lundberg, J.O., Hellstrom, P.M., Fagerhol, M.K., Weitzberg, E., and Roseth, A.G.** (2005). Technology insight: calprotectin, lactoferrin and nitric oxide as novel markers of inflammatory bowel disease. Nat Clin Pract Gastroenterol Hepatol *2*, 96-102.

173. **Maggi, P., Larocca, A.M., Quarto, M., Serio, G., Brandonisio, O., Angarano, G., and Pastore, G.** (2000). Effect of antiretroviral therapy on cryptosporidiosis and microsporidiosis in patients infected with human immunodeficiency virus type 1. Eur J Clin Microbiol Infect Dis *19*, 213-217.

174. **mal-ed** (2010). http://mal-ed.fnih.org/: The interactions of Malnutrition and Enteric Infections: Consequences for child health and development.

175. **Malakooti, M.A., Alaii, J., Shanks, G.D., and Phillips-Howard, P.A.** (1997). Epidemic dysentery in western Kenya. Trans R Soc Trop Med Hyg *91*, 541 - 543.

University of Ghana http://ugspace.ug.edu.gh

176. **Mata, L.** (1992). Diarrheal disease as a cause of malnutrition. Am J Trop Med Hyg *47*, 16-27.

177. **Mayrhofer, G., Andrews, R.H., Ey, P.L., and Chilton, N.B.** (1995). Division of *Giardia* isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with *Giardia muris*. Parasitology *111 ( Pt 1)*, 11-17.

178. **McIver, C.J., Hansman, G., White, P., Doultree, J.C., Catton, M., and Rawlinson, W.D.** (2001). Diagnosis of enteric pathogens in children with gastroenteritis. Pathology *33*, 353-358.

179. **Medhin, G., Hanlon, C., Dewey, M., Alem, A., Tesfaye, F., Worku, B., Tomlinson, M., and Prince, M.** (2010). Prevalence and predictors of undernutrition among infants aged six and twelve months in Butajira, Ethiopia: the P-MaMiE Birth Cohort. BMC Public Health *10*, 27.

180. **Menard, L.P., and Dubreuil, J.D.** (2002). Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1): a new toxin with an old twist. Crit Rev Microbiol *28*, 43-60.

181. **Mensah, P., Yeboah-Manu, D., Owusu-Darko, K., and Ablordey, A.** (2002). Street foods in Accra, Ghana: how safe are they? Bull World Health Organ *80*, 546-554.

182. **Miller, K., Duran-Pinales, C., Cruz-Lopez, A., Morales-Lechuga, L., Taren, D., and Enriquez, F.J.** (1994). *Cryptosporidium parvum* in children with diarrhea in Mexico. Am J Trop Med Hyg *51*, 322-325.

University of Ghana http://ugspace.ug.edu.gh

183. **Molbak, K.** (2000). The epidemiology of diarrhoeal diseases in early childhood. A review of community studies in Guinea-Bissau. Dan Med Bull *47*, 340-358.

184. **Monteiro-Neto, V., Bando, S.Y., Moreira-Filho, C.A., and Giron, J.A.** (2003). Characterization of an outer membrane protein associated with haemagglutination and adhesive properties of enteroaggregative *Escherichia coli* O111:H12. Cell Microbiol *5*, 533-547.

185. **Monteiro, B.T., Campos, L.C., Sircili, M.P., Franzolin, M.R., Bevilacqua, L.F., Nataro, J.P., and Elias, W.P.** (2009). The dispersin-encoding gene (aap) is not restricted to enteroaggregative *Escherichia coli*. Diagn Microbiol Infect Dis *65*, 81-84.

186. **Mor, S.M., and Tzipori, S.** (2008). Cryptosporidiosis in children in Sub-Saharan Africa: a lingering challenge. In Clin Infect Dis, pp. 915-921.

187. **Moreira, C.G., Carneiro, S.M., Nataro, J.P., Trabulsi, L.R., and Elias, W.P.** (2003). Role of type I fimbriae in the aggregative adhesion pattern of enteroaggregative *Escherichia coli*. FEMS Microbiol Lett *226*, 79-85.

188. **Morse, T.D., Nichols, R.A., Grimason, A.M., Campbell, B.M., Tembo, K.C., and Smith, H.V.** (2007). Incidence of cryptosporidiosis species in paediatric patients in Malawi. Epidemiol Infect *135*, 1307-1315.

189. **Muller, O., and Becher, H.** (2006). Malnutrition and childhood mortality in developing countries. Lancet *367*, 1978.

190. **Namboodiri, S.** (2008). Genetic Basis of Quinolone Resistance in *Escherichia coli* Isolates from Ghana. BSc Thesis submitted to the Department of Biology, Haverford College, Haverford, PA, USA, 1-28.

University of Ghana http://ugspace.ug.edu.gh

191. **Nataro, J.P.** (2005). Enteroaggregative *Escherichia coli* pathogenesis. Curr Opin Gastroenterol *21*, 4-8.

192. **Nataro, J.P., Deng, Y., Cookson, S., Cravioto, A., Savarino, S.J., Guers, L.D., Levine, M.M., and Tacket, C.O.** (1995). Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. J Infect Dis *171*, 465-468.

193. **Nataro, J.P., and Kaper, J.B.** (1998). Diarrheagenic *Escherichia coli*. Clin Microbiol Rev *11*, 142-201.

194. **Nataro, J.P., Kaper, J.B., Robins-Browne, R., Prado, V., Vial, P., and Levine, M.M.** (1987). Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr Infect Dis J *6*, 829-831.

195. **Nataro, J.P., Mai, V., Johnson, J., Blackwelder, W.C., Heimer, R., Tirrell, S., Edberg, S.C., Braden, C.R., Glenn Morris, J., Jr., and Hirshon, J.M.** (2006). Diarrheagenic *Escherichia coli* infection in Baltimore, Maryland, and New Haven, Connecticut. Clin Infect Dis *43*, 402-407.

196. **Nataro, J.P., Steiner, T., and Guerrant, R.L.** (1998). Enteroaggregative *Escherichia coli*. Emerg Infect Dis *4*, 251-261.

197. **Nataro, J.P., Yikang, D., Giron, J.A., Savarino, S.J., Kothary, M.H., and Hall, R.** (1993). Aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli* requires two unlinked plasmid regions. Infect Immun *61*, 1126-1131.

University of Ghana http://ugspace.ug.edu.gh

198. **Nataro, J.P., Yikang, D., Yingkang, D., and Walker, K.** (1994). AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli*. J Bacteriol *176*, 4691-4699.

199. **Navarro-Garcia, F., Canizalez-Roman, A., Burlingame, K.E., Teter, K., and Vidal, J.E.** (2007). Pet, a non-AB toxin, is transported and translocated into epithelial cells by a retrograde trafficking pathway. Infect Immun *75*, 2101-2109.

200. **Navarro-Garcia, F., Canizalez-Roman, A., Luna, J., Sears, C., and Nataro, J.P.** (2001). Plasmid-encoded toxin of enteroaggregative *Escherichia coli* is internalized by epithelial cells. Infect Immun *69*, 1053-1060.

201. **Nemec, K.N., Scaglione, P., Navarro-Garcia, F., Huerta, J., Tatulian, S.A., and Teter, K.** (2010). A host-specific factor is necessary for efficient folding of the autotransporter plasmid-encoded toxin. Biochimie *92*, 171-177.

202. **Neter, E., Westphal, O., Luderitz, O., Gino, R.M., and Gorzynski, E.A.** (1955). Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. Pediatrics *16*, 801-808.

203. **Nishi, J., Sheikh, J., Mizuguchi, K., Luisi, B., Burland, V., Boutin, A., Rose, D.J., Blattner, F.R., and Nataro, J.P.** (2003). The export of coat protein from enteroaggregative *Escherichia coli* by a specific ATP-binding cassette transporter system. J Biol Chem *278*, 45680-45689.

204. **Ochoa, T.J., Brown, E.L., Guion, C.E., Chen, J.Z., McMahon, R.J., and Cleary, T.G.** (2006 ). Effect of lactoferrin on Enteroaggregative *E. coli* (EAEC) Biochem Cell Biol *84*, 369-376.

University of Ghana http://ugspace.ug.edu.gh

205. **Ochoa, T.J., Ruiz, J., Molina, M., Del Valle, L.J., Vargas, M., Gil, A.I., Ecker, L., Barletta, F., Hall, E., Cleary, T.G., and Lanata, C.F.** (2009). High frequency of antimicrobial drug resistance of diarrheagenic *Escherichia coli* in infants in Peru. Am J Trop Med Hyg *81*, 296-301.

206. **Okeke, I.N.** (2006). Diagnostic insufficiency in Africa. Clin Infect Dis *42*, 1501-1503.

207. **Okeke, I.N.** (2009). Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status, uncertainties and necessities. J Infect Dev Ctries *3*, 817-842.

208. **Okeke, I.N., Lamikanra, A., Czeczulin, J., Dubovsky, F., Kaper, J.B., and Nataro, J.P.** (2000a). Heterogeneous virulence of enteroaggregative *Escherichia coli* strains isolated from children in Southwest Nigeria. J Infect Dis *181*, 252-260.

209. **Okeke, I.N., Lamikanra, A., Steinruck, H., and Kaper, J.B.** (2000b). Characterization of *Escherichia coli* strains from cases of childhood diarrhea in provincial southwestern Nigeria. J Clin Microbiol *38*, 7-12.

210. **Okeke, I.N., Ojo, O., Lamikanra, A., and Kaper, J.B.** (2003). Etiology of acute diarrhea in adults in southwestern Nigeria. J Clin Microbiol *41*, 4525-4530.

211. **Olesen, B., Neimann, J., Bottiger, B., Ethelberg, S., Schiellerup, P., Jensen, C., Helms, M., Scheutz, F., Olsen, K.E., Krogfelt, K., Peteren, E., Molbak, K., and Gerner-Smidt, P.** (2005). Etiology of diarrhea in young children in Denmark: a case-control study. J Clin Microbiol *43*, 3636-3641.

212. **Opintan, J., and Newman, M.J.** (2007). Distribution of serogroups and serotypes of multiple drug resistant *Shigella* isolates. Ghana Med J *41*, 4-8.

University of Ghana http://ugspace.ug.edu.gh


213. **Opintan, J.A., Bishar, R.A., Newman, M.J., and Okeke, I.N.** (2010). Carriage of diarrhoeagenic *Escherichia coli* by older children and adults in Accra, Ghana. Trans R Soc Trop Med Hyg *104*, 504-506.

214. **Ortega, Y.R., and Adam, R.D.** (1997). *Giardia*: overview and update. Clin Infect Dis *25*, 545-549.

215. **Osumanu, I.K.** (2008). Reducing childhood diarrhea morbidity: does behaviour change matter? A case study from Northern Ghana. World Health Popul *10*, 53-63.

216. **Paul, M., Tsukamoto, T., Ghosh, A.R., Bhattacharya, S.K., Manna, B., Chakrabarti, S., Nair, G.B., Sack, D.A., Sen, D., and Takeda, Y.** (1994). The significance of enteroaggregative *Escherichia coli* in the etiology of hospitalized diarrhoea in Calcutta, India and the demonstration of a new honey-combed pattern of aggregative adherence. FEMS Microbiol Lett *117*, 319-325.

217. **Pelletier, D.L., and Frongillo, E.A.** (2003). Changes in child survival are strongly associated with changes in malnutrition in developing countries. J Nutr *133*, 107-119.

218. **Perch, M., Sodemann, M., Jakobsen, M.S., Valentiner-Branth, P., Steinsland, H., Fischer, T.K., Lopes, D.D., Aaby, P., and Molbak, K.** (2001). Seven years' experience with *Cryptosporidium parvum* in Guinea-Bissau, West Africa. Ann Trop Paediatr *21*, 313-318.

219. **Petri, W.A., Jr., Miller, M., Binder, H.J., Levine, M.M., Dillingham, R., and Guerrant, R.L.** (2008). Enteric infections, diarrhea, and their impact on function and development. J Clin Invest *118*, 1277-1290.

University of Ghana http://ugspace.ug.edu.gh

220. **Pierce, A., Colavizza, D., Benaissa, M., Maes, P., Tartar, A., Montreuil, J., and Spik, G.** (1991). Molecular cloning and sequence analysis of bovine lactotransferrin. Eur J Biochem *196*, 177-184.

221. **Plutzer, J., Ongerth, J., and Karanis, P.** (2010). *Giardia* taxonomy, phylogeny and epidemiology: Facts and open questions. Int J Hyg Environ Health *213*, 321-333.

222. **Ramani, S., and Kang, G.** (2009). Viruses causing childhood diarrhoea in the developing world. Curr Opin Infect Dis *22*, 477-482.

223. **Rappelli, P., Folgosa, E., Solinas, M.L., Dacosta, J.L., Pisanu, C., Sidat, M., Melo, J., Cappuccinelli, P., and Colombo, M.M.** (2005). Pathogenic enteric *Escherichia coli* in children with and without diarrhea in Maputo, Mozambique. FEMS Immunol Med Microbiol *43*, 67-72.

224. **Regua-Mangia, A.H., Gomes, T.A., Vieira, M.A., Irino, K., and Teixeira, L.M.** (2009). Molecular typing and virulence of enteroaggregative *Escherichia coli* strains isolated from children with and without diarrhoea in Rio de Janeiro city, Brazil. J Med Microbiol *58*, 414-422.

225. **Reja, V., Kwok, A., Stone, G., Yang, L., Missel, A., Menzel, C., and Bassam, B.** (2010). ScreenClust: Advanced statistical software for supervised and unsupervised high resolution melting (HRM) analysis. Methods *50*, S10-14.

226. **Roche, J.K., Cabel, A., Sevilleja, J., Nataro, J., and Guerrant, R.L.** (2010). Enteroaggregative *Escherichia coli* (EAEC) impairs growth while malnutrition worsens EAEC infection: a novel murine model of the infection malnutrition cycle. J Infect Dis *202*, 506-514.

University of Ghana http://ugspace.ug.edu.gh

227. **Rohde, J.R., and Northrup, R.S.** (1976). Taking science where the diarrhoea is. In: Ciba Foundation Symposium. Acute diarrhoea in children. Amsterdam: Elsevier; 1976 . 339-365.

228. **Roseth, A.G., Aadland, E., Jahnsen, J., and Raknerud, N.** (1997). Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. Digestion *58*, 176-180.

229. **Rosoff, J.D., Sanders, C.A., Sonnad, S.S., De Lay, P.R., Hadley, W.K., Vincenzi, F.F., Yajko, D.M., and O'Hanley, P.D.** (1989). Stool diagnosis of giardiasis using a commercially available enzyme immunoassay to detect Giardia-specific antigen 65 (GSA 65). J Clin Microbiol *27*, 1997-2002.

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

231. **Samie, A., Obi, C.L., Barrett, L.J., Powell, S.M., and Guerrant, R.L.** (2007a). Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: studies using molecular diagnostic methods. J Infect *54*, 558-566.

232. **Samie, A., Obi, C.L., Dillingham, R., Pinkerton, R.C., and Guerrant, R.L.** (2007b). Enteroaggregative *Escherichia coli* in Venda, South Africa: distribution of virulence-related genes by multiplex polymerase chain reaction in stool samples of human immunodeficiency virus (HIV)-positive and HIV-negative individuals and primary school children. Am J Trop Med Hyg *77*, 142-150.

University of Ghana http://ugspace.ug.edu.gh

233. **Samie, A., Obi, L.C., Bessong, P.O., Stroup, S., Houpt, E., and Guerrant, R.L.** (2006b). Prevalence and species distribution of *E. Histolytica* and *E. dispar* in the Venda region, Limpopo, South Africa. Am J Trop Med Hyg *75*, 565-571.

234. **Sansonetti, P.J., Arondel, J., Huerre, M., Harada, A., and Matsushima, K.** (1999). Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. Infect Immun *67*, 1471-1480.

235. **Sarantuya, J., Nishi, J., Wakimoto, N., Erdene, S., Nataro, J.P., Sheikh, J., Iwashita, M., Manago, K., Tokuda, K., Yoshinaga, M., Miyata., K., and Kawano., Y.** (2004). Typical enteroaggregative Escherichia coli is the most prevalent pathotype among E. coli strains causing diarrhea in Mongolian children. J Clin Microbiol *42*, 133-139.

236. **Sathian, U., Joseph, A., and Waterlow, J.C.** (1983). Exclusive breast feeding versus supplementation: a prospective study in a rural south Indian community. Ann Trop Paediatr *3*, 157-161.

237. **Saverymuttu, S.H., Camilleri, M., Rees, H., Lavender, J.P., Hodgson, H.J., and Chadwick, V.S.** (1986). Indium 111-granulocyte scanning in the assessment of disease extent and disease activity in inflammatory bowel disease. A comparison with colonoscopy, histology, and fecal indium 111-granulocyte excretion. Gastroenterology *90*, 1121-1128.

238. **Scaglione, P., Nemec, K.N., Burlingame, K.E., Grabon, A., Huerta, J., Navarro-Garcia, F., Tatulian, S.A., and Teter, K.** (2008). Structural characteristics of the plasmid-encoded toxin from enteroaggregative *Escherichia coli*. Biochemistry *47*, 9582-9591.

University of Ghana http://ugspace.ug.edu.gh


239. **Scavia, G., Staffolani, M., Fisichella, S., Striano, G., Colletta, S., Ferri, G., Escher, M., Minelli, F., and Caprioli, A.** (2008). Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. J Med Microbiol *57*, 1141-1146.

240. **Scerpella, E.G., Okhuysen, P.C., Mathewson, J.J., Guerrant, R.L., Latimer, E., Lyerly, D., and Ericsson, C.D.** (1994). Evaluation of a New Latex Agglutination Test for Fecal Lactoferrin in Travelers' Diarrhea. J Travel Med *1*, 68-71.

241. **Schmidt, H., Knop, C., Franke, S., Aleksic, S., Heesemann, J., and Karch, H.** (1995). Development of PCR for screening of enteroaggregative *Escherichia coli*. J Clin Microbiol *33*, 701-705.

242. **Schmidt, W.P., Cairncross, S., Barreto, M.L., Clasen, T., and Genser, B.** (2009). Recent diarrhoeal illness and risk of lower respiratory infections in children under the age of 5 years. Int J Epidemiol *38*, 766-772.

243. **Schorling, J.B., and Guerrant, R.L.** (1990). Diarrhoea and catch-up growth. Lancet *335*, 599-600.

244. **Schubert, S., Rakin, A., Karch, H., Carniel, E., and Heesemann, J.** (1998). Prevalence of the "high-pathogenicity island" of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. Infect Immun *66*, 480-485.

245. **Seganti, L., DiBiase, A.M., Marchetti, M., Pietrantoni, A., Tinari, A., and Superti, F.** (2004). Biometals antiviral activity of lactoferrin towards naked viruses. Biometals *17*, 295-299.

University of Ghana http://ugspace.ug.edu.gh


246. **Sethabutr, O., Venkatesan, M., Murphy, G.S., Eampokalap, B., Hoge, C.W., and Echeverria, P.** (1993). Detection of Shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. J Infect Dis *167*, 458-461.

247. **Sharp, S.E., Suarez, C.A., Duran, Y., and Poppiti, R.J.** (2001). Evaluation of the Triage Micro Parasite Panel for detection of *Giardia lamblia*, *Entamoeba histolytica/Entamoeba dispar*, and *Cryptosporidium parvum* in patient stool specimens. J Clin Microbiol *39*, 332-334.

248. **Shashiraj, Faridi, M.M., Singh, O., and Rusia, U.** (2006). Mother's iron status, breastmilk iron and lactoferrin--are they related? Eur J Clin Nutr *60*, 903-908.

249. **Sheikh, J., Czeczulin, J.R., Harrington, S., Hicks, S., Henderson, I.R., Le Bouguenec, C., Gounon, P., Phillips, A., and Nataro, J.P.** (2002). A novel dispersin protein in enteroaggregative Escherichia coli. J Clin Invest *110*, 1329-1337.

250. **Sheikh, J., Hicks, S., Dall'Agnol, M., Phillips, A.D., and Nataro, J.P.** (2001). Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. Mol Microbiol *41*, 983-997.

251. **Shields, J.M., Gleim, E.R., and Beach, M.J.** (2008). Prevalence of *Cryptosporidium* spp. and *Giardia intestinalis* in swimming pools, Atlanta, Georgia Emerg Infect Dis *14*, 948-950.

252. **Smith, L.H., L** (2000). Explaining Child Malnutrition in Developing Countries: A Cross-Country Analysis. International Food Policy Institute, Washington, DC (Washington).

University of Ghana http://ugspace.ug.edu.gh

253. **Snyder, J.D., and Merson, M.H.** (1982). The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. Bull World Health Organ *60*, 605-613.

254. **Spencer, J., Chart, H., Smith, H.R., and Rowe, B.** (1998). Expression of membrane-associated proteins by strains of enteroaggregative *Escherichia coli.* FEMS Microbiol Lett *161*, 325-330.

255. **Steijns, J.M., and van Hooijdonk, A.C.** (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr *84 Suppl 1*, S11-17.

256. **Steiner, T.S., Lima, A.A., Nataro, J.P., and Guerrant, R.L.** (1998). Enteroaggregative *Escherichia coli* produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. J Infect Dis *177*, 88-96.

257. **Stensvold, C.R., Lebbad, M., Verweij, J.J., Jespersgaard, C., von Samson-Himmelstjerna, G., Nielsen, S.S., and Nielsen, H.V.** (2010). Identification and delineation of members of the *Entamoeba* complex by pyrosequencing. Mol Cell Probes *24*, 403 - 406.

258. **Stoltzfus, R.J., Chway, H.M., Montresor, A., Tielsch, J.M., Jape, J.K., Albonico, M., and Savioli, L. (2004).** Low dose daily iron supplementation improves iron status and appetite but not anemia, whereas quarterly anthelminthic treatment improves growth, appetite and anemia in Zanzibari preschool children. J Nutr *134*, 348-356.

259. **Stroup., S.E., Roy, S., McHele, J., Maro, V., Ntabaguzi, S., Siddique, A., Kang, G., Guerrant, R.L., Kirkpatrick, B.D., Fayer, R., Herbein, J., Ward, H., R., Haque, R., and Houpt, E.R.** ( 2006). Real-time PCR detection

University of Ghana http://ugspace.ug.edu.gh

and speciation of *Cryptosporidium* infection using Scorpion probes. J Med Microbiol *55*, 1217-1222.

260. **Subekti, D., Lesmana, M., Komalarini, S., Tjaniadi, P., Burr, D., and Pazzaglia, G.** (1993 ). Enterotoxigenic *Escherichia coli* and other causes of infectious pediatric diarrheas in Jakarta. Indonesia Southeast Asia J Trop Med Public Health *24*, 420 - 424.

261. **Sugi, K., Saitoh, O., Hirata, I., and Katsu, K.** (1996). Fecal lactoferrin as a marker for disease activity in inflammatory bowel disease: comparison with other neutrophil-derived proteins. Am J Gastroenterol *91*, 927-934.

262. **Sui, B.Q., Dutta, P.R., and Nataro, J.P.** (2003). Intracellular expression of the plasmid-encoded toxin from enteroaggregative *Escherichia coli.* Infect Immun *71*, 5364-5370.

263. **Sutjita, M., Bouckenooghe, A.R., Adachi, J.A., Jiang, Z.D., Mathewson, J.J., Ericsson, C.D., and DuPont, H.L.** (2000). Intestinal secretory immunoglobulin A response to enteroaggregative *Escherichia coli* in travelers with diarrhea. Clin Diagn Lab Immunol *7*, 501-503.

264. **Taddei, C.R., Fasano, A., Ferreira, A.J., Trabulsi, L.R., and Martinez, M.B.** (2005). Secreted autotransporter toxin produced by a diffusely adhering *Escherichia coli* strain causes intestinal damage in animal model assays. FEMS Microbiol Lett *250*, 263-269.

265. **Tibble, J., Teahon, K., Thjodleifsson, B., Roseth, A., Sigthorsson, G., Bridger, S., Foster, R., Sherwood, R., Fagerhol, M., and Bjarnason, I.** (2000). A simple method for assessing intestinal inflammation in Crohn's disease. Gut *47*, 506-513.

University of Ghana http://ugspace.ug.edu.gh

266. **Tormo, R., Polanco, I., Salazar-Lindo, E., and Goulet, O.** (2008). Acute infectious diarrhoea in children: new insights in antisecretory treatment with racecadotril. Acta Paediatr *97*, 1008-1015.

267. **Uchida, K., Matsuse, R., Tomita, S., Sugi, K., Saitoh, O., and Ohshiba, S.** (1994). Immunochemical detection of human lactoferrin in feces as a new marker for inflammatory gastrointestinal disorders and colon cancer. Clin Biochem *27*, 259-264.

268. **UN** (2002). United Nations. Millennium development goals. Available at: http://www.un.org./milleniumgoals. Accessed June 8, 2008.

269. **UNICEF** (2002). United Nations Children's Fund: Oral Rehydration Therapy. Fact sheet on achievements and challenges. 2002.

270. **UNICEF** (2009). ChildInfo: Monitoring the situation of children and women. Child Nutrition. Statistics by Area/ Child Nutrition, Ghana. Available at: http://www.childinfo.org/undernutrition.html.

271. **Usein, C.R., Tatu-Chitoiu, D., Ciontea, S., Condei, M., and Damian, M.** (2009). *Escherichia coli* pathotypes associated with diarrhea in Romanian children younger than 5 years of age. Jpn J Infect Dis *62*, 289-293.

272. **Vaishnavi, C., Bhasin, D.K., and Singh, K.** (2000). Fecal lactoferrin assay as a cost-effective tool for intestinal inflammation. Am J Gastroenterol *95*, 3002-3003.

273. **Vaishnavi, C., Kochhar, R., Bhasin, D., Thennarasu, K., and Singh, K.** (2003). Simultaneous assays for *Clostridium difficile* and faecal lactoferrin in ulcerative colitis. Trop Gastroenterol *24*, 13-16.

University of Ghana http://ugspace.ug.edu.gh

274. **Vaishnavi, C., Thapa, B.R., Thennarasu, K., and Singh, K.** (2002). Faecal lactoferrin assay as an adjunct to *Clostridium difficile* diarrhoea. Indian J Pathol Microbiol *45*, 69-73.

275. **Valentiner-Branth, P., Steinsland, H., Fischer, T.K., Perch, M., Scheutz, F., Dias, F., Aaby, P., Molbak, K., and Sommerfelt, H.** (2003). Cohort study of Guinean children: incidence, pathogenicity, conferred protection, and attributable risk for enteropathogens during the first 2 years of life. J Clin Microbiol *41*, 4238-4245.

276. **Van Den Broeck, J., Eeckels, R., and Vuylsteke, J.** (1993). Influence of nutritional status on child mortality in rural Zaire. Lancet *341*, 1491-1495.

277. **van Langenberg, D.R., Gearry, R.B., Wong, H.L., Ward, M., and Gibson, P.R.** (2010). The potential value of faecal lactoferrin as a screening test in hospitalized patients with diarrhoea. Intern Med J *40*, 819-827.

278. **Velarde, J.J., Varney, K.M., Inman, K.G., Farfan, M., Dudley, E., Fletcher, J., Weber, D.J., and Nataro, J.P.** (2007). Solution structure of the novel dispersin protein of enteroaggregative *Escherichia coli*. Mol Microbiol *66*, 1123-1135.

279. **Venkataraman, S., Ramakrishna, B.S., Kang, G., Rajan, D.P., and Mathan, V.I.** (2003). Faecal lactoferrin as a predictor of positive faecal culture in south Indian children with acute diarrhoea. Ann Trop Paediatr *23*, 9-13.

280. **Verweij, J.J., Oostvogel, F., Brienen, E.A., Nang-Beifubah, A., Ziem, J., and Polderman, A.M.** (2003). Short communication: Prevalence of Entamoeba histolytica and Entamoeba dispar in northern Ghana. Trop Med Int Health *8*, 1153-1156.

University of Ghana http://ugspace.ug.edu.gh


281. **Victora, C.G., Adair, L., Fall, C., Hallal, P.C., Martorell, R., Richter, L., and Sachdev, H.S.** (2008). Maternal and child undernutrition: consequences for adult health and human capital. Lancet *371*, 340-357.

282. **Victora, C.G., de Onis, M., Hallal, P.C., Blossner, M., and Shrimpton, R.** (2010). Worldwide timing of growth faltering: revisiting implications for interventions. Pediatrics *125*, 473-480.

283. **Vila, J., Vargas, M., Henderson, I.R., Gascon, J., and Nataro, J.P.** (2000). Enteroaggregative escherichia coli virulence factors in traveler's diarrhea strains. J Infect Dis *182*, 1780-1783.

284. **Wachs, T.D., Creed-Kanashiro, H., Cueto, S., and Jacoby, E.** (2005). Maternal education and intelligence predict offspring diet and nutritional status. J Nutr *135*, 2179-2186.

285. **Wakimoto, N., Nishi, J., Sheikh, J., Nataro, J.P., Sarantuya, J., Iwashita, M., Manago, K., Tokuda, K., Yoshinaga, M., and Kawano, Y.** (2004). Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. Am J Trop Med Hyg *71*, 687-690.

286. **Walker, T.R., Land, M.L., Kartashov, A., Saslowsky, T.M., Lyerly, D.M., Boone, J.H., and Rufo, P.A.** (2007). Fecal lactoferrin is a sensitive and specific marker of disease activity in children and young adults with inflammatory bowel disease. J Pediatr Gastroenterol Nutr *44*, 414-422.

287. **Wanke, C.A., Gerrior, J., Blais, V., Mayer, H., and Acheson, D.** (1998). Successful treatment of diarrheal disease associated with enteroaggregative *Escherichia coli* in adults infected with human immunodeficiency virus. J Infect Dis *178*, 1369-1372.

University of Ghana http://ugspace.ug.edu.gh


288. **Wardlaw, T., Salama, P., Brocklehurst, C., Chopra, M., and Mason, E.** (2010). Diarrhoea: why children are still dying and what can be done. Lancet *375*, 870-872.

289. **Warren, C.A., and R. L. Guerrant. ( 2008). Clinical Disease and Pathology. In Fayer, R., Xiao, L.** (eds). *Cryptosporidium* and Cryptosporidiosis, second edition. CRC press, New York, USA 235-253.

290. **Waterlow, J.C., Buzina, R., Keller, W., Lane, J.M., Nichaman, M.Z., and Tanner, J.M.** (1977). The presentation and use of height and weight data for comparing the nutritional status of groups of children under the age of 10 years. Bull World Health Organ *55*, 489-498.

291. **Weintraub, A.** (2007). Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. J Med Microbiol *56*, 4-8.

292. **WFP** (2006). World Food Programme. World hunger - Ghana. (2006). Available at: http://www.wfp.org/country_brief/indexcountry.asp?country=288.

293. **WHO** (1987). World Health Organization. Manual for laboratory investigations of acute enteric infections. World Health Organization. Geneva.

294. **WHO** (1995a). World Health Organization. Physical status: the use and interpretation of anthropometry. World Health Organ Tech Rep Ser *854*, 1-452.

295. **WHO** (1995b). World Health Organization: The Treatment of Diarrhoea. A manual for Physicians and other Senior Health Workers. Geneva, World Health Organization. *WHO/CDR/95.3*.

University of Ghana http://ugspace.ug.edu.gh

296. **WHO** (1999). World Health Organization (1999). Generic protocol to estimate the burden of Shigella diarrhoea and dysenteric mortality. . WHO/V and B/ 99:26.

297. **WHO** (2000a). Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. WHO Collaborative Study Team on the Role of Breastfeeding on the Prevention of Infant Mortality. Lancet *355*, 451-455.

298. **WHO** (2000b). World Health Organization, Dept of Nutrition for Health and Development. Nutrition for health and development: a global agenda for combating malnutrition. World Health Organization. Available at http://whqlibdoc.who.int/hq/2000/WHO_NHD_00.6.pdf.

299. **Woodward, B.** ( 2001). The effect of protein-energy malnutrition on immune competence. In: Suskenand RM, Tontisirin K, eds. Nutrition, immunity and infection in infants and children. Philadelphia, PA: Vevey/Lippincott. Williams and Wilkins, pg 89-116.

300. **Xiao, L., and Ryan, U.M.** (2004). Cryptosporidiosis: an update in molecular epidemiology. Curr Opin Infect Dis *17*, 483-490.

301. **Ximenez, C., Cerritos, R., Rojas, L., Dolabella, S., Moran, P., Shibayama, M., Gonzalez, E., Valadez, A., Hernandez, E., Valenzuela, O., Limon, A., Partida., O., and Silva, E.F.** (2010). Human amebiasis: breaking the paradigm? Int J Environ Res Public Health *7*, 1105-1120.

302. **Yakoob, J., Abbas, Z., Beg, M.A., Naz, S., Khan, R., Islam, M., and Jafri, W.** (2010). Prevalences of Giardia lamblia and *Cryptosporidium parvum* infection in adults presenting with chronic diarrhoea. Ann Trop Med Parasitol *104*, 505-510.

University of Ghana http://ugspace.ug.edu.gh

303. **Yamazaki, M., Inuzuka, K., Matsui, H., Sakae, K., Suzuki, Y., Miyazaki, Y., and Ito, K.** (2000). Plasmid encoded enterotoxin (Pet) gene in enteroaggregative *Escherichia coli* isolated from sporadic diarrhea cases. Jpn J Infect Dis *53*, 248-249.

304. **Young, H., and Jaspars, S.** (2006). The meaning and measurement of acute malnutrition in emergencies. A primer for decision-makers. Humanitarian Practice Network at ODI *56*, 1-60.

305. **Zamboni, A., Fabbricotti, S.H., Fagundes-Neto, U., and Scaletsky, I.C.** (2004). Enteroaggregative *Escherichia coli* virulence factors are found to be associated with infantile diarrhea in Brazil. J Clin Microbiol *42*, 1058-1063.

306. **Zu, S.X., Li, J.F., Barrett, L.J., Fayer, R., Shu, S.Y., McAuliffe, J.F., Roche, J.K., and Guerrant, R.L.** (1994). Seroepidemiologic study of *Cryptosporidium* infection in children from rural communities of Anhui, China and Fortaleza, Brazil. Am J Trop Med Hyg *51*, 1-10.

University of Ghana http://ugspace.ug.edu.gh

# APPENDICES

## APPENDIX I : Questionnaires administered in this study

### i) Questionnaire for children with (A) and without (B) diarrhoea

**QUESTIONNAIRE (A)**
*DIARRHOEA STUDY*

1.0 Date: ______________ 1.1 Project Site: ____________________

**2.0 Background of patient**

2.1 Name: ________________________ 2.2 Age: ____ 2.3 Sex: ____

2.4 Area/ Residence:

2.5 Who takes care of child most of the time especially **week days**? __________

2.6 Weight of child:

2.7 Height of child:

2.8 Fever? Yes [ ] No [ ]

**3.0 History of diarrhoea**

3.1 Frequency in a day: __________ 3.2 For how long? ______________

3.2 Did you take any antibiotic drug before visiting clinic? Yes [ ] No [ ]

3.3 Antibiotic drug name ______________________________________

**4.0 How long did you exclusively breast feed?**

4.1. 6 months [ ]

4.2 6 months [ ]

4.3 others? (please state) ______________________________

4.4 In your estimation, what is the likely cause of the diarrhoea?

______________________________________________________________

**5.0 Educational background of mother/ guardian** [Please tick]

5.1 Up to primary school [ ]

5.2 Up to JSS [ ]

5.3 Up to SSS Sec. sch. [ ]

5.4 Up to Univ Poly tech. [ ]

5.5 Others (please state)

University of Ghana http://ugspace.ug.edu.gh

## QUESTIONNAIRE (B)

*CONTROL FOR DIARRHOEA STUDY*

1.0 Date: ______________ 1.1 Project Site: ______________________

**2.0 Background of patient**

2.1 Name: 2.2 Age: 2.3 Sex:

2.4 Area/ Residence: ________________________________________

2.5 Who takes care of child most of the time especially **week days**? __________

2.6 Weight of child: ____________________

2.7 Height of child: ____________________

**3.0 How long did you exclusively breast feed?**

3.1. 6 months [ ]
3.2 < 6 months [ ]
3.3 Others (please state) ________________________________________

**5.0 Educational background of mother/ guardian** [Please tick]

5.1 Up to primary school [ ]

5.2 Up to JSS [ ]

5.3 Up to SSS Sec. sch. [ ]

5.4 Up to Univ/ Poly tech. [ ]

5.5 Others (please state) ________________________________________

University of Ghana http://ugspace.ug.edu.gh

## ii) A copy of the consent form used in the study

**CONSENT FORM (A)**
*Diarrhoea Study*

**Introduction**
Diarrhoeal diseases remain a major cause of childhood death and incapacitation in developing countries. One of the main difficulties in the management of acute and persistent diarrhoea is the inability to identify the etiological agents, which could be of viral, parasitic or bacterial source. For many of the pathogens, optimal diagnostic testing is unavailable, impractical, or prohibitively expensive for developing countries like Ghana. In this study we seek to culture for diarrhoeal agents of bacterial origin especially those which are not routinely cultured for in Ghana.

**What is required from you the client**
You would be assisted to fill a structured questionnaire after which a stool specimen would be taken. You are please assured that any information you provide would be handled in confidentiality and it will only be used for research purposes.

**Benefit of study to you the client**
There is no direct monetary benefit. Indirectly, you benefit by not making any payment for the laboratory investigations. In addition, since sensitivity tests would be carried out on any significant isolate this would positively direct treatment.

**Consent**
The study has been explained adequately to me and I understand that my participation is purely voluntary. I therefore give my consent and understand that I could withdraw my participation at any time.

............................................................ Thumb print or signature

............................................................ Name

**Contact Information of Investigator**
If you have any further questions or for any reason you wish to redraw your consent you should contact me on the following address:

**JAPHETH A. OPINTAN**
DEPT. OF MICROBIOLOGY
UNIVERSITY OF GHANA MEDICAL SCHOOL
KORLE-BU, ACCRA

Email: japh_opintan@yahoo.com Tel (Office): 021-665404

University of Ghana http://ugspace.ug.edu.gh

**iii) Completed copies of administered questionnaires**

QUESTIONNAIRE (A)
DIARRHOEA STUDY

1.0 Date: 29/5/08    1.1 Project Site: Pul

2.0 Background of patient

2.1 Name: [redacted]    2.2 Age: ____    2.3 Sex: M

2.4 Area/ Residence: ____

2.5 Who takes care of child most of the time especially week days? Mother

2.6 Weight of child: 7.2 Kg

2.7 Height of child: 85 cm

2.8 Fever?    Yes [ ]    No [✓]

3.0 History of diarrhoea

3.1 Frequency in a day: 3 times    3.2 For how long? 2 days

3.2 Did you take any antibiotic/drug before visiting clinic? Yes [✓]    No [ ]

3.3 Antibiotic/drug name: Paracetamol Syrup

4.0 How long did you exclusively breast feed?

4.1 6 months [ ]

4.2 < 6 months [ ]

4.3 others? (please state) breastfeeding

4.4 In your estimation, what is the likely cause of the diarrhoea?

____

5.0 Educational background of mother/ guardian (Please tick)

5.1 Up to primary school [ ]

5.2 Up to JSS [✓]

5.3 Up to SSS/Sec. tch [ ]

5.4 Up to Univ/Poly tech [ ]

5.5 Others (please state) ____

University of Ghana http://ugspace.ug.edu.gh

63
240

## QUESTIONNAIRE (B)
### CONTROL FOR DIARRHOEA STUDY

1.0 Date: 10 - 4 - 08 1.1 Project Site: Pant

**2.0 Background of patient**

2.1 Name [redacted] 2.2 Age: 4/12 2.3 Sex: F

2.4 Area/ Residence: Nima / 0244379818

2.5 Who takes care of child most of the time especially week days? Mother

2.6 Weight of child: 10.5 kg

2.7 Height of child: 92 cm

**3.0 How long did you exclusively breast feed?**

3.1. 6 months [ ]
3.2 < 6 months [ ]
3.3 Others (please state) 1 year seven mths

**5.0 Educational background of mother/ guardian** [Please tick]

5.1 Up to primary school [ ]
5.2 Up to JSS ✓
5.3 Up to SSS/Sec. sch. [ ]
5.4 Up to Univ/Poly tech. [ ]
5.5 Others (please state)

1.88 | -2.08
0.08 | -3.45

University of Ghana http://ugspace.ug.edu.gh

**APPENDIX II:** Media and equipment used

**1.1) *Preparation of agar media and broths for culture, identification bacteria***

The following media, broths and standard solutions were prepared using sterile distilled water. Where appropriated the solutions were either autoclaved at 121°C for 15 min at 1.12 kg $cm^{-2}$ pressure or filter sterilized with 0.22 or 0.45 µm filter units (MILLEX®-HA, Molsheim, France).

For agar plates, dehydrated powders were dissolved in the appropriate volumes of distilled water according to manufacturer's instructions and autoclaved. When cooled to about 55°C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri Dishes and allowed to set.

For broths and slopes, dehydrated powders were dissolved in the appropriate volumes of distilled water and warmed to completely dissolve. Appropriate volumes were then dispensed into appropriate tubes before they were autoclaved. Autoclaved tubes were then slanted at appropriate gradients before they set for agar slopes.

Sterility and quality of media after each preparation were controlled by respectively leaving a plate on a bench for the next day and also by inoculating randomly selected media with positive and negative controlled organisms.

University of Ghana http://ugspace.ug.edu.gh

**a) Deoxycholate citrate agar (DCA) per 1 litre**

***i) Composition***

| | |
|---|---|
| 5.0 g | Beef Extract |
| 5.0 g | Peptone |
| 5.4 g | Sodium thiosulphate |
| 12.0 g | Agar agar |
| 0.02 g | Neutral red |
| 1.0 g | Ferric citrate |
| 5.0 g | Sodium desoxycholate |
| 8.5 g | Sodium citrate |
| 10.0 g | Lactose |
| | pH 7.3 ± 0.2 |

***ii) Preparation***

Prepared according to the manufacturer's (BIOTEC) instructions. When cooled to about 55°C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri Dishes and allowed to set.

**b) MacConkey agar (per 1 litre)**

***i) Composition***

| | |
|---|---|
| 20.0 g | Peptone |
| 10.0 g | Lactose |
| 5.0 g | Bile salts |
| 5.0 g | NaCl |
| 0.075 g | Neutral red |
| 12.0 g | Agar |
| | pH 7.4 ± 0.2 |

***ii) Preparation***

Prepared according to the manufacturer's (OXOID) instructions. When cooled to about 55°C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri Dishes and allowed to set.

University of Ghana http://ugspace.ug.edu.gh

**c) Mueller Hinton agar (per 1 litre)**

***i) Composition***

2.0 g Beef infusion solids
17.5 g Acid Hydrolysed Casein
1.5 g Starch
17.0 g Agar No. 1
50-100 mg/L Calcium ions
20-35 mg/L Magnesium ion
pH 7.3 ± 0.1

***ii) Preparation***

Prepared according to the manufacturer's (BIOTEC) instructions. When cooled to about 55°C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri Dishes and allowed to set.

**d) Triple Sugar Iron (TSI) per 1 litre**

***i) Composition***

3.0 g 'Lab-Lemco' powder
3.0 g Yeast extract
20.0 g Peptone
5.0 g NaCl
10.0 g Lactose
1.0 g Glucose
0.03 g Ferric citrate
0.3 g Sodium thiosulphate
*q.s* Phenol red
12.0 g Agar
pH 7.4 ± 0.2

***ii) Preparation***

Prepared according to the manufacturer's (OXOID) instructions. Completely dissolved mixtures were dispensed into tubes before they were autoclaved. Tubes were slanted at appropriate gradient before setting.

University of Ghana http://ugspace.ug.edu.gh

**e) Peptone water (per 1 litre)**

***i) Composition***

10.0 g Peptone
5 g NaCl
pH 7.1 ± 0.2

***ii) Preparation***

Prepared according to the manufacturer's (BIOTEC) instructions. Completely dissolved mixtures were dispensed into tubes before they were autoclaved.

**f) Urea slope (per 100 ml)**

***i) Composition***

1.0 g Peptone
1.0 g glucose
5.0 g $NaCl_2$
1.2 g Di-sodium phosphate
0.8 g Potassium dihydrogen phosphate
0.012 g Phenol red
15 g Agar
pH 6.8 ± 0.2

***ii) Preparation***

Prepared according to the manufacturer's (OXIOD) instructions. Completely dissolved mixtures was autoclaved. When cooled, 5 ml of filter sterilized 40 % urea solution (SR 20) was added aseptically to autoclaved mixtures before they were dispensed into sterile bottles and sloped.

University of Ghana http://ugspace.ug.edu.gh

**g) Selenite F broth per Litre**

***i) Composition***

5.0 g Peptone
4.0 g lactose
10.0 g Sodium phosphate
pH 7.1 ± 0.2

***ii) Preparation***

Prepared according to the manufacturer's (BIOTEC) instructions. Completely 4 g of sodium biselenite (CAT 3/175) was dissolved in 1 L of distilled water and 19 g of selenite broth base added and warmed for 10 min in a boiling water. Appropriate volumes were then dispensed into appropriate tubes.

*NB: Under no circumstances was this autoclaved*

**h) Kovac's indole reagent**

***i) Composition***

5.0 g *p*-dimethylaminobenzaldehyde
75 ml Amyl alcohol
25 ml Conc. HCl

***ii) Preparation***

The aldehyde was dissolved in the alcohol by gentle warming in a water bath (about 50 – 55 °C). When cooled, the acid was added with care and kept in a brown bottle to protect it from sun light.

University of Ghana http://ugspace.ug.edu.gh

### 1.2) *Equipment used in this study*

| Equipment | Model | Manufacturer |
|---|---|---|
| -20 Freezer | MF 304 | Unicef, Denmark |
| -80 Ultra flow freezer | MDF-392 | Sanyo Electric Co., Ltd., Japan |
| Autoclave | | Omrou Corporation, Japan |
| Centrifuge | Mini Spin | Eppendorf, Hamburg, Germany |
| Centrifuge | 5804R | Eppendorf, Hamburg, Germany |
| Commercial ice system | | Cornelius |
| Electric pipettor | | Drummond Sci., Co, Broomall, USA |
| ELISA reader | MKII | Titerkek Multiskan Plus |
| Icycler iQ | Multicolor RT-Time | BIO-RAD, USA |
| Incubator | IC-62 | Yamato Scientific Co., Ltd., Japan |
| Mini Beadbeater | 607 | Biospec Prdt., Bartlesville, USA |
| PCR/UV Work Station | CleanSpot | COY Lab. Prds., Michigan, USA |
| Rotor-Gene | Q-5 | Qiagen, Hilden, Germany |
| Spectrophotometer | RS 232C | Eppendorf., Hamburg, Germany |
| Water bath | 1225 PC | VWR Scientific Prdts,. Inc., USA |
| Weighing scale | X564 | Mettler Toledo, Switzerland |

University of Ghana http://ugspace.ug.edu.gh

**APPENDIX III: Cycling conditions of target genes screened**

| Gene target | Cycling condition/protocol |
|---|---|
| *aaiC* | 95°C (5 min, 1 cycle); 95 °C, 47.5 °C, and72 °C (20 s at each temperature, 40 cycles); and a final extension step (8 min, 72 °C). |
| *aatA* | 50°C (2 min, 1 cycle); 95 °C (5 min, 1 cycle); 95 °C, 55 °C, and 72 °C (20 s at each temperature, 40 cycles); and a final extension step (10 min, 72 °C). |
| *aap* | 50°C (2 min, 1 cycle); 95 °C (5 min, 1 cycle); 95 °C, 45 °C, and 72 °C (45 s at each temperature, 40 cycles); and a final extension step (8 min, 72 °C). |
| *aggR* | 95°C (5 min, 1 cycle); 95 °C, 45 °C, and 72 °C (10 s at each temperature, 39 cycles); 72 °C (1 min, 39 cycles); and a final extension step (6 min, 72 °C). |
| *Shigella/ipaH* | 95°C (5 min, 1 cycle); 95 °C, 60.5 °C, and72 °C (20 s at each temperature, 40 cycles); and a final extension step (10 min, 72 °C). |
| *Crypto* 18sRNA | 95°C (13.3 min, 1 cycle); 95 °C and 60 °C (15 s, 40 cycle); 72 °C (20 s, 40 cycles); and a final extension step (7 min, 72 °C). |
| Eh | 95°C (3 min, 1 cycle); 95 °C, 59 °C, and72 °C (1 min at each temperature, 40 cycles); 72°C (10 min, 1 cycle); and a final extension step (8 min, 72 °C). |
| P241 | 95°C (10 min, 1 cycle); 95 °C and (15 s , 40 cycle); 60 °C (1 min, 40 cycle); and a final extension step (7 min, 72 °C). |

University of Ghana http://ugspace.ug.edu.gh

## APPENDIX IV: Sequenced data of *Cryptosporidium* positive stool DNA

### *i) Sequence report of the positive Cryptosporidium*

```
>09-22101_012.ab1
CCAATACGTTATGAGAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAA
GGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGG
CTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTG
ACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTA
GTTGGTGGAGAGCACCCCCATTAGTATTCAGAAGAGCTATCAATCTGTCAATCC
TTCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAG
GCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGA
CCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAG
TAAGGAACAACCTCCAATCTCTAAA

>09-22103_010.ab1
TCCATAACTTATGAGATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGG
CTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCT
TAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGAC
AGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGAGGGGAACCCCCATAGCATCAGAGAGCTATCAATCTGTCAATCCTT
CCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGC
TCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACC
ATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTA
AGGAACAACCTCCAATCTCTAA

>09-22105_024.ab1
ACAGAATTATGAGATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCT
GAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTA
ATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGACAG
ATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTG
GTGGAGAGGGGATCCATAGATCAGAAGAGCTATCAATCTGTCAATCCTTCCTAT
GTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCAC
TCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCATACT
CCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAAGGAA
CAACCTCCAATCTCTAA

>09-22107_022.ab1
CGCAATGACAATCATGTAGTATCTGAGTCTATTGGGTTCTGGGGGGAGTATGGT
CGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCC
TGCGGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAG
GATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGT
TCTTAGTTGGTGGAGGGGGACATCAATAGCATCAAGATCTAGCTATCAATCTGT
CAATCCTTCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAG
CCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCC
TTGCGACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTG
```

University of Ghana http://ugspace.ug.edu.gh

AAGGAGTAAGGAACAACCTCCAATCTCTAA

>09-22109_020.ab1
AGCAATCCCACTATGAGAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGC
AAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGC
GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGAT
TGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT
TAATTGGTGGAGAGGACTTCCTTACATCAGACGAGCTATCAATCTGTCATCCTT
CCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGC
TCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACC
ATACTCCCCCCACAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTA
AGGAACAACCTCCAATCTCTA

>09-22111_018.ab1
CCAATACGTTATGAGAATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAG
GCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGC
TTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGA
CAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAG
TTGGTGGAGAGGGAACCATAGTATCAAGATGAGCTATCAATCTGTCAATCCTTC
CTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCT
CCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCA
TACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAA
GGAACAACCTCCAATCTCTAA

>09-22113_024.ab1
AACAATAACGTTATGAGAATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCA
AGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCG
GCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATT
GACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTT
AGTTGGTGGAGAGGATTACAAATACATCAGAAGAGCTATCAATCTGTCAATCCT
TCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGG
CTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGAC
CATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGT
AAGGAACAACCTCCAATCTCTAA

>09-22115_022.ab1
GCAATTTCGATTATCAGATAGAGGGTCTGTTACGTTCGTGGGGCTCTAGAGCAT
TCTAGGTCCTATTCTTCCCTTCGACTTAGTGAATTGCCGATGACGGGGTGGAAC
AAATCATTTGCCACCACTTGTAGTTATTATGACTTCTCATGGTTTTGGGGGCTG
ACTGAACTCAACCAGGCCGTTCTTCGAGGAGAGAGTGGTGCATGGCCGTTCTTA
GTTGGTGGAGAACATTACTTTCTCTCTTTATCTTCGTTGTCATCGTCACTCCTT
CTGATTGCTGTTGATTTGTTCTTTAAGTCATGTAACGTGGACGGCCCCACACTC
CAAGAATGGGCCCTGCCCTTACCTAAATCCCCACCTATTATCACATTTTGTGAC
TTGTAAGTTTCCCTTCCCATCGAGGAACAACCTCCAATCTATAAGAAGGAGTAA
GGAACAACCTCCAATCTCTAA

University of Ghana http://ugspace.ug.edu.gh

>09-22117_020.ab1
CCCCATACATTATGAGAATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAA
GGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGG
CTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTG
ACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTA
GTTGGTGGAGGGAAACCCATACATCAGAAAGAGCTATCGATCTGTCAATCCTTC
CTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCT
CCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCA
TACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAA
GGAACAACCTCCAATCTCTAA

>09-22119_018.ab1
AGGGTCGGTCATTCTGACACGGCTGAACGGGTCGCTTTAGCCGCTGGCTTAACT
CGGGAGAGCGGTTCATCCTTGAACATGGGCTCGATGCGGTTGTGTCTCAGATGG
AGACTGTGATAGACCGCTGGTCATTGATGATCAGGGAAGACAGATAAATGGCTA
TATATTGGGTATCCTAACGGCTCGTTATCGCGGGGCGAAGACAGTCGTGACGCC
GCTCAGCACGACGAGCGCTCTGGAGGAAAGCGGTTGGTTCGAGAACATTCACAG
AACACGGATTGGATCGCCGTATGTCGTAGCCGAAATGGCGCGAGCGGTGGCGCA
CCCCGTTGTTGGTTTCGAGGCGAATGGTGGCTTTCTGCTCGGCGATGATGTGAC
GTTGAAGTCCGGTTTATTGCGCCGCCTCCCAACGCGTGATTCAGTTTTGCCAGC
CGTGGCCGTTCTTAGTTGGTGGAGAGCAGGCAATCAATCACCGATGACTAGGCG
GCGCGGATGCCGGAATTGGCCTCGATCATCGCCAAGCAAAAAGCCACCATTCGA
CTCGAAACCAAGGGGGGGTGCGCCACCGCTCGCGCGATTTCGGCTACGACATAC
GGCCATCCAATCCGTGTTCTGTGAATGTTCTGCAACCAACCGCTTTCCTCAAGA
GCGCTCGTCAAGCTGAGCGGCGTCACGACTGTTTTCGCACCGAGATATCGAGCC
GTTAGGATACCCAATATATCGCCATTTATCTGTCTTCCCTGATCATCAATGACC
AGAGGTCTATCACCGTCTCCATCTGTCGACACAACTGCATCGAGCCCATGTTCT
GCGATAAATGCGCTCGCCCGAGTTAAGTCAGCGGGGTCAAGCGCTTCTGTATCA
ACCGCGACGAACTGTTCCATCCTGCGAAAAGGAACAACCTCCAATCTCTAAA

>09-22121_032.ab1
ACAGGACCTTATGAGATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGG
CTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCT
TAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGAC
AGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGAGGGACCCCAATAGATCAGAAGAGCTATCAATCTGTCAATCCTTCC
TATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCTC
CACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCAT
ACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAAG
GAACAACCTCCAATCTCTAA

>09-22123_030.ab1
AGCGGAGCAGTATTGAGCAGTCTCATGACTTTGTGGTAGCTGGGGGGAGTATGG
TCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGC
CTGCGGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAA
GGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCG
TTCTTAGTTGGTGGAGGGGCTAAACTTAGGATCGACAACGAGCTATCATCTAAG

University of Ghana http://ugspace.ug.edu.gh

>09-22117_020.ab1
CCCCATACATTATGAGAATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAA
GGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGG
CTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTG
ACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTA
GTTGGTGGAGGGAAACCCATACATCAGAAAGAGCTATCGATCTGTCAATCCTTC
CTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCT
CCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCA
TACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAA
GGAACAACCTCCAATCTCTAA

>09-22119_018.ab1
AGGGTCGGTCATTCTGACACGGCTGAACGGGTCGCTTTAGCCGCTGGCTTAACT
CGGGAGAGCGGTTCATCCTTGAACATGGGCTCGATGCGGTTGTGTCTCAGATGG
AGACTGTGATAGACCGCTGGTCATTGATGATCAGGGAAGACAGATAAATGGCTA
TATATTGGGTATCCTAACGGCTCGTTATCGCGGGGCGAAGACAGTCGTGACGCC
GCTCAGCACGACGAGCGCTCTGGAGGAAAGCGGTTGGTTCGAGAACATTCACAG
AACACGGATTGGATCGCCGTATGTCGTAGCCGAAATGGCGCGAGCGGTGGCGCA
CCCCGTTGTTGGTTTCGAGGCGAATGGTGGCTTTCTGCTCGGCGATGATGTGAC
GTTGAAGTCCGGTTTATTGCGCCGCCTCCCAACGCGTGATTCAGTTTTGCCAGC
CGTGGCCGTTCTTAGTTGGTGGAGAGCAGGCAATCAATCACCGATGACTAGGCG
GCGCGGATGCCGGAATTGGCCTCGATCATCGCCAAGCAAAAAGCCACCATTCGA
CTCGAAACCAAGGGGGGGTGCGCCACCGCTCGCGCGATTTCGGCTACGACATAC
GGCCATCCAATCCGTGTTCTGTGAATGTTCTGCAACCAACCGCTTTCCTCAAGA
GCGCTCGTCAAGCTGAGCGGCGTCACGACTGTTTTCGCACCGAGATATCGAGCC
GTTAGGATACCCAATATATCGCCATTTATCTGTCTTCCCTGATCATCAATGACC
AGAGGTCTATCACCGTCTCCATCTGTCGACACAACTGCATCGAGCCCATGTTCT
GCGATAAATGCGCTCGCCCGAGTTAAGTCAGCGGGGTCAAGCGCTTCTGTATCA
ACCGCGACGAACTGTTCCATCCTGCGAAAAGGAACAACCTCCAATCTCTAAA

>09-22121_032.ab1
ACAGGACCTTATGAGATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGG
CTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCT
TAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGAC
AGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGAGGGACCCCAATAGATCAGAAGAGCTATCAATCTGTCAATCCTTCC
TATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCTC
CACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCAT
ACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAAG
GAACAACCTCCAATCTCTAA

>09-22123_030.ab1
AGCGGAGCAGTATTGAGCAGTCTCATGACTTTGTGGTAGCTGGGGGGAGTATGG
TCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGC
CTGCGGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAA
GGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCG
TTCTTAGTTGGTGGAGGGGCTAAACTTAGGATCGACAACGAGCTATCATCTAAG

University of Ghana http://ugspace.ug.edu.gh

TCAATCCTTCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAA
GCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGC
CTTGCGACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCT
GAAGGAGTAAGGAACAACCTCCAATCTCTAA

>09-22125_028.ab1
CATCAGCGTCATTTCGTTGCGGCACCGTGACGAAGGATTAAATCTGGCAGCATC
GGACGTGCACTATCTGTGGCGGATTCTGCGCGAATACTACCATCGTGGCCTCTG
CGATCTGCTGGTGGTCAATTCGCGACTGGCGCTGGATCGGGTCTGGAAAGGGGA
ACGCTTTAGCTGGTTTATGACCCGCCTGCTGCATGATTTTCCGGATCAGAACGC
GTTCGACGCTAAAATGCAGGCCGCCGACCGCCGCTACTATCTGGGCTCGCGCGC
CGGGCTGACCACCATCGCGGAAAACTACGTGGGCTTGCCCATGGAGCGTGTCGC
CTGAATTAAGAGGAGTCATCATGCCGTTCGTTCAAACCGTGCCCGTATTTAAGC
TGTATGGCGAAGAGTGCGGCTGGCCGACGCCGGACCTGCTGCACTGTGAGTCGA
TTTTACAGCGCAGCAGCCTGTATCAGTGGCATATTCGCGTCCATCAGCATGCGG
AAATGGTCCAGCTTCTCTATCTGCATCAGGGGCGGGCGGAGATCGAAATTGAAG
GCGCCACCGCGGTGATGACGGAGTCCTGCATCCAGGTGGTTCCCGCCCTCTGTA
TTCACGGGTTTAATTTTTCACCCGGGACGCAAGGGTTTGTGCTCTCTCTGGCGC
TGCCGCTGCTGAGCCGGTTTGAAAAATCAGTTTCGGTCGCCCTCTGGATGTGCT
TTACGTGCCGCATGTGTGCCGGTGGGCCCTTTCTTATTTTGGTAGAGGTGAGGT
GTGGTAGGGGACCAAAGCAATCGAGACGGCGACCGAACTGATTTTCATACCGGC
TCAGCAGCGGCAGCGCCAGAGAGAGCACAAACCCTTGCGTCCCGGGTGAAAAAT
TAAACCCGTGAATACAGAGGGCGGGAACCACCTGGATGCAGGACTCCGTCATCA
CCGCGGTGGCGCCTTCAATTTCGATCTCCGCCCGCCCCTGATGCAGATAGAGAA
GCTGGACCATTTCCGCATGCTGATGGACGCGAATATGCCACTGATACAGGCTGC
TGCGCTGTAAAATCGACTCACAGTGCAGCAGGTCCGGCGTCGGCCAGCCGCACT
CTTCGCCATACAGCTTAAATACGGGCACGGTTTGAACGAACGGCATGATGACTC
CTCTTAATTCAGGCGACACGCTCCATGGGCAAGCCCACGTAGTTTTCCGCGATG
GTGGTCAGCCCGGCGCGCGAGCCCAGATAGTAGCGGCGGTCGGCGGCCTGCATT
TTAGCGTCGAACGCGTTCTGATCCGGAAAATCATGCAGCAGGCGGGTCATAAAC
CAGCTAAAGCGTTCCCCTTTCCAGACCCGATCCAGCGCCAGTTGCGAGTAGGCC
GCCAGCAGATCGCTGCGGCCACGATGGTAGTATTCGCGCAGAATCCGCCACAGA
TAGTTCACGTCCGATGCCGCCAGATTTAATCCTTTCGCCCCCGGTTGGCGGAA
CGATATGCCGCCGCCATCCCCCCACCGGGAAACAACCTCCAATCTCTAAACCC

>09-22127_026.ab1
CCCAATGAAGTTATGAGGAATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGC
AAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGC
GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGAT
TGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT
TAGTTGGTGGAGAGGGGAATCCCATAGCATCAGAAGAGCTATCAATCTGTCAAT
CCTTCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGC
AGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGC
GACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGG
AGTAAGGAACAACCTCCAATCTCTAAA

University of Ghana http://ugspace.ug.edu.gh

>09-22121_032.ab1
ACAGGACCTTATGAGATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGG
CTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCT
TAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGATTGAC
AGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGAGGGACCCCAATAGATCAGAAGAGCTATCAATCTGTCAATCCTTCC
TATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCTC
CACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCAT
ACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGGAGTAAG
GAACAACCTCCAATCTCTAA

>09-22123_030.ab1
AGCGGAGCAGTATTGAGCAGTCTCATGACTTTGTGGTAGCTGGGGGGAGTATGG
TCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGC
CTGCGGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAA
GGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCG
TTCTTAGTTGGTGGAGGGGCTAAACTTAGGATCGACAACGAGCTATCATCTAAG
TCAATCCTTCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAA
GCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGC
CTTGCGACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCT
GAAGGAGTAAGGAACAACCTCCAATCTCTAA

>09-22125_028.ab1
CATCAGCGTCATTTCGTTGCGGCACCGTGACGAAGGATTAAATCTGGCAGCATC
GGACGTGCACTATCTGTGGCGGATTCTGCGCGAATACTACCATCGTGGCCTCTG
CGATCTGCTGGTGGTCAATTCGCGACTGGCGCTGGATCGGGTCTGGAAAGGGGA
ACGCTTTAGCTGGTTTATGACCCGCCTGCTGCATGATTTTCCGGATCAGAACGC
GTTCGACGCTAAAATGCAGGCCGCCGACCGCCGCTACTATCTGGGCTCGCGCGC
CGGGCTGACCACCATCGCGGAAAACTACGTGGGCTTGCCCATGGAGCGTGTCGC
CTGAATTAAGAGGAGTCATCATGCCGTTCGTTCAAACCGTGCCCGTATTTAAGC
TGTATGGCGAAGAGTGCGGCTGGCCGACGCCGGACCTGCTGCACTGTGAGTCGA
TTTTACAGCGCAGCAGCCTGTATCAGTGGCATATTCGCGTCCATCAGCATGCGG
AAATGGTCCAGCTTCTCTATCTGCATCAGGGGCGGGCGGAGATCGAAATTGAAG
GCGCCACCGCGGTGATGACGGAGTCCTGCATCCAGGTGGTTCCCGCCCTCTGTA
TTCACGGGTTTAATTTTTCACCCGGGACGCAAGGGTTTGTGCTCTCTCTGGCGC
TGCCGCTGCTGAGCCGGTTTGAAAAATCAGTTTCGGTCGCCCTCTGGATGTGCT
TTACGTGCCGCATGTGTGCCGGTGGGCCCTTTCTTATTTTGGTAGAGGTGAGGT
GTGGTAGGGGACCAAAGCAATCGAGACGGCGACCGAACTGATTTTCATACCGGC
TCAGCAGCGGCAGCGCCAGAGAGAGCACAAACCCTTGCGTCCCGGGTGAAAAAT
TAAACCCGTGAATACAGAGGGCGGGAACCACCTGGATGCAGGACTCCGTCATCA
CCGCGGTGGCGCCTTCAATTTCGATCTCCGCCCGCCCCTGATGCAGATAGAGAA
GCTGGACCATTTCCGCATGCTGATGGACGCGAATATGCCACTGATACAGGCTGC
TGCGCTGTAAAATCGACTCACAGTGCAGCAGGTCCGGCGTCGGCCAGCCGCACT
CTTCGCCATACAGCTTAAATACGGGCACGGTTTGAACGAACGGCATGATGACTC
CTCTTAATTCAGGCGACACGCTCCATGGGCAAGCCCACGTAGTTTTCCGCGATG
GTGGTCAGCCCGGCGCGCGAGCCCAGATAGTAGCGGCGGTCG
GCGGCCTGCATTTTAGCGTCGAACGCGTTCTGATCCGGAAAATCATGCAGCAGG

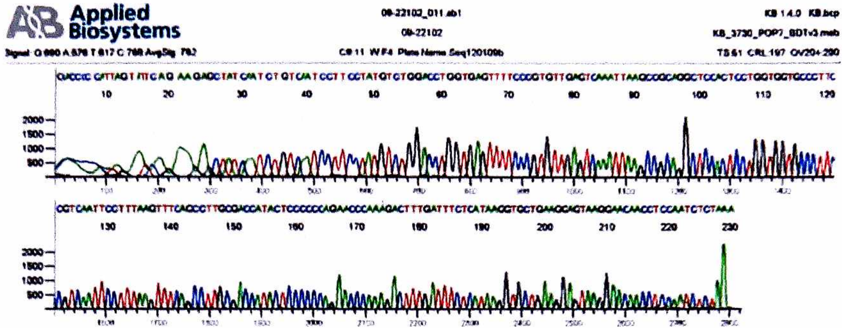
University of Ghana http://ugspace.ug.edu.gh

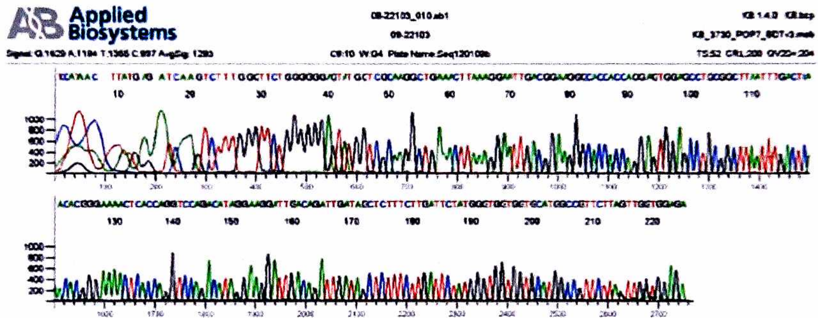
CGGGTCATAAACCAGCTAAAGCGTTCCCCTTTCCAGACCCGATCCAGCGCCAGT
TGCGAGTAGGCCGCCAGCAGATCGCTGCGGCCACGATGGTAGTATTCGCGCAGA
ATCCGCCACAGATAGTTCACGTCCGATGCCGCCAGATTTTAATCCTTTCGCCCC
CGGTTGGCGGAACGATATGCCGCCGCCATCCCCCCACCGGGAAACAACCTCCAA
TCTCTAAACCC

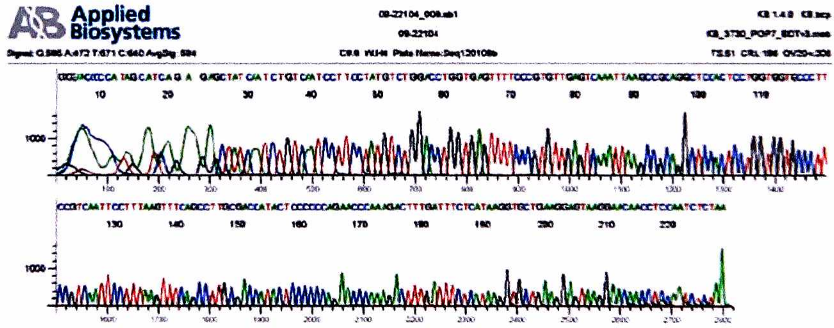
>09-22127_026.ab1
CCCAATGAAGTTATGAGGAATCAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGC
AAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGC
GGCTTAATTTGACTCAACACGGGAAAACTCACCAGGTCCAGACATAGGAAGGAT
TGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT
TAGTTGGTGGAGAGGGGAATCCCATAGCATCAGAAGAGCTATCAATCTGTCAAT
CCTTCCTATGTCTGGACCTGGTGAGTTTTCCCGTGTTGAGTCAAATTAAGCCGC
AGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGC
GACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCATAAGGTGCTGAAGG
AGTAAGGAACAACCTCCAATCTCTAAA

University of Ghana http://ugspace.ug.edu.gh

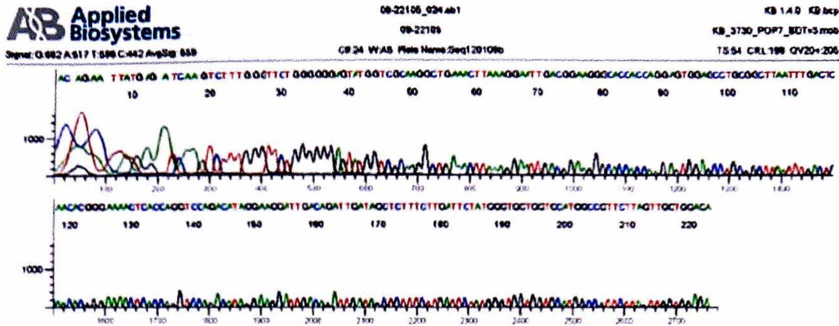
## 2) *Electrogram report of the positive Cryptosporidium*

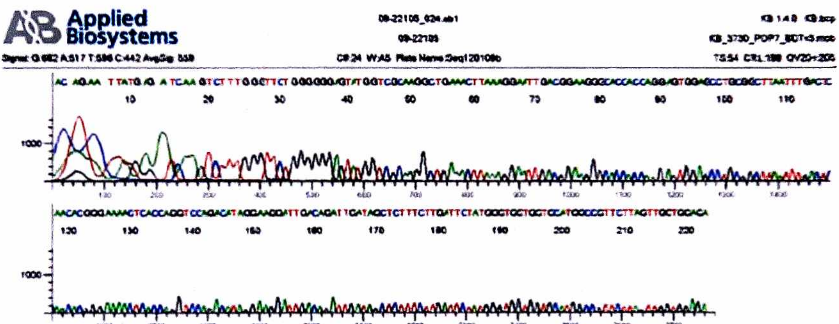


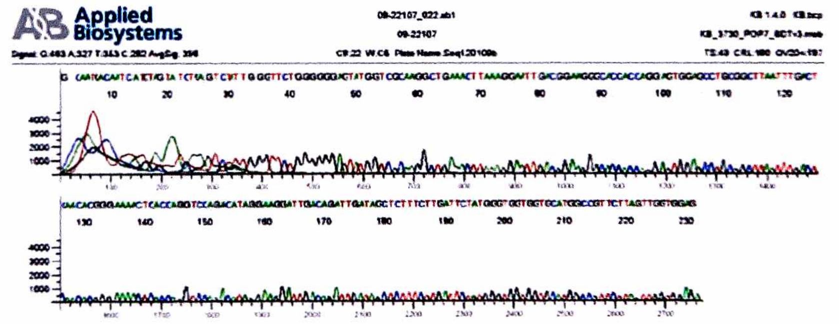



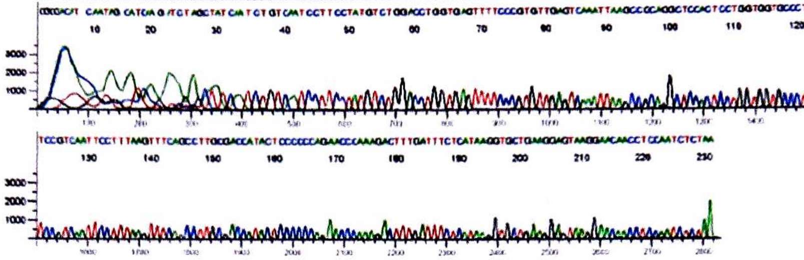
University of Ghana http://ugspace.ug.edu.gh

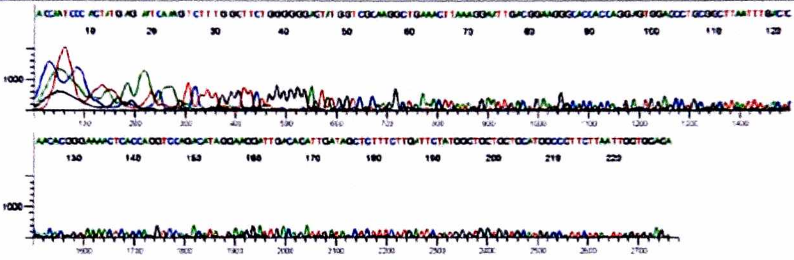


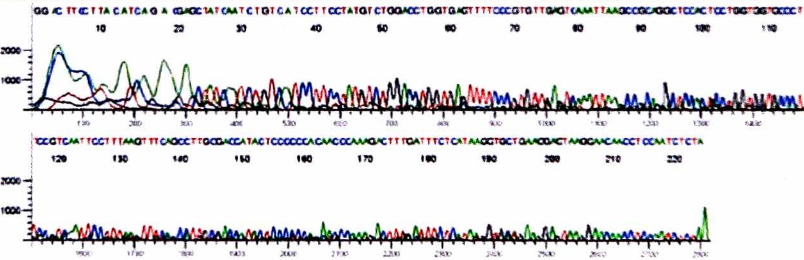



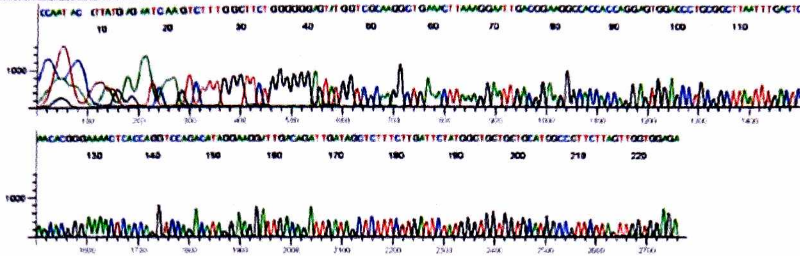
University of Ghana http://ugspace.ug.edu.gh

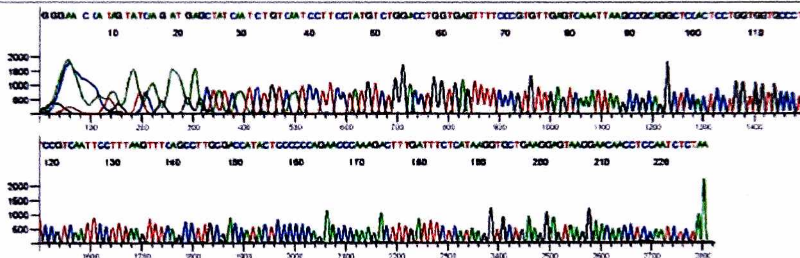


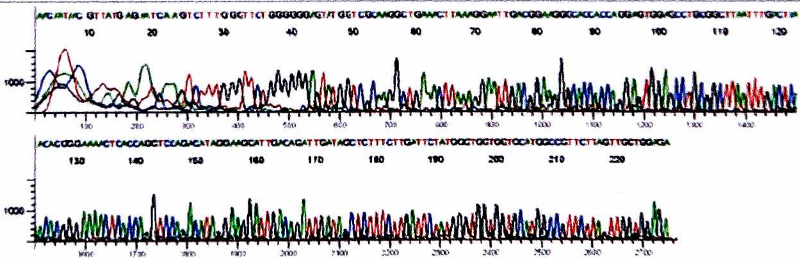




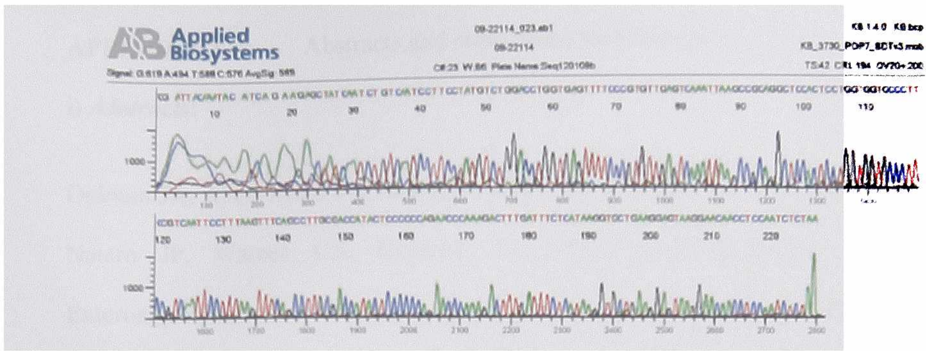
University of Ghana http://ugspace.ug.edu.gh

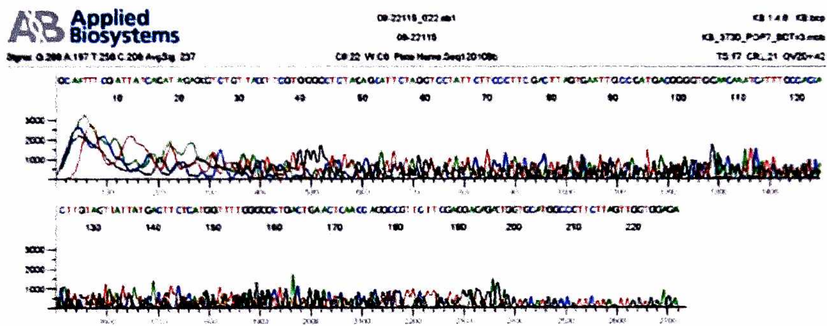


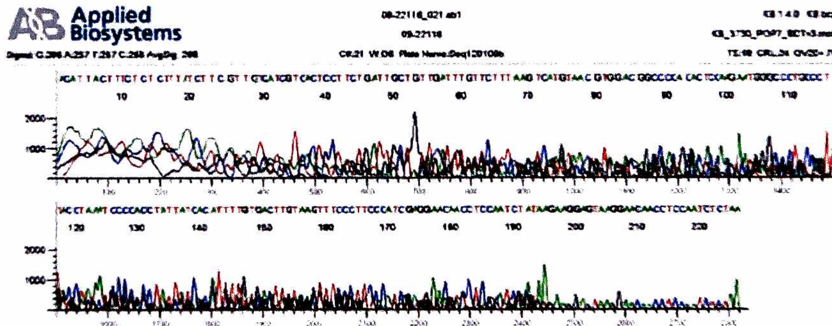




University of Ghana http://ugspace.ug.edu.gh



University of Ghana http://ugspace.ug.edu.gh

**APPENDIX V:** Abstracts and publication emanating from thesis

**i) *Abstracts*:**

Opintan JA, Ayeh-Kumi PF, Assrim R, Gepi-Attee R, Sevilleja JEAD, Roche JK, Nataro JP, Warren CA, Guerrant RL (2009) Cryptosporidium spp and Enteroaggregative *E. coli* infection and lactoferrin levels in childhood diarrhea in Accra, Ghana. 58th American Society for Tropical Medicine and Hygiene Annual General Meeting Nov 18 – 22, 2009, Washington, DC. Abstract number 84.

Opintan JA, Newman MJ, Assrim R, Gepi-Attee R, Sevilleja JEAD, Warren CA, Geurrant RL (2009) Enteroaggregative *Escherichia coli* virulence genes in association with diarrhoea duration, nutritional status and age in children with and without diarrhoea in Accra, Ghana. 4th Annual Carey, Marshall, Thorner Scholar's Day April 13 2009, University of Virginia, Charlottesville.

**ii) *Paper*:**

Opintan JA et al (2010). Pediatric Diarrhea in Southern Ghana: Etiology and Association with Intestinal Inflammation and Malnutrition. *Am J. Trop Med Hyg*. 83; 936 – 943.

University of Ghana http://ugspace.ug.edu.gh

Am. J. Trop. Med. Hyg., 83(4), 2010, pp. 936–943
doi:10.4269/ajtmh.2010.09-0792
Copyright © 2010 by The American Society of Tropical Medicine and Hygiene

# Pediatric Diarrhea in Southern Ghana: Etiology and Association with Intestinal Inflammation and Malnutrition

Japheth A. Opintan,* Mercy J. Newman, Patrick F. Ayeh-Kumi, Raymond Affrim, Rosina Gepi-Attee, Jesus E. A. D. Sevilleja, James K. Roche, James P. Nataro, Cirle A. Warren, and Richard L. Guerrant

*Department of Microbiology, University of Ghana Medical School, Accra, Ghana; Princess Marie Louise Children's Hospital, Accra, Ghana; Center for Global Health, Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, Virginia; Center for Vaccine Development, Department of Pediatrics and Medicine, University of Maryland, Baltimore, Maryland*

*Abstract.* Diarrhea is a major public health problem that affects the development of children. Anthropometric data were collected from 274 children with ($N = 170$) and without ($N = 104$) diarrhea. Stool specimens were analyzed by conventional culture, polymerase chain reaction for enteroaggregative *Escherichia coli* (EAEC), *Shigella*, *Cryptosporidium*, *Entamoeba*, and *Giardia* species, and by enzyme-linked immunosorbent assay for fecal lactoferrin levels. About 50% of the study population was mildly to severely malnourished. Fecal lactoferrin levels were higher in children with diarrhea ($P = 0.019$). Children who had EAEC infection, with or without diarrhea, had high mean lactoferrin levels regardless of nutritional status. The EAEC and *Cryptosporidium* were associated with diarrhea ($P = 0.048$ and 0.011, respectively), and malnourished children who had diarrhea were often co-infected with both *Cryptosporidium* and EAEC. In conclusion, the use of DNA-biomarkers revealed that EAEC and *Cryptosporidium* were common intestinal pathogens in Accra, and that elevated lactoferrin was associated with diarrhea in this group of children.

## INTRODUCTION

Diarrhea is a principal cause of morbidity and mortality in children < 5 years of age in developing countries, where acute watery diarrhea accounts for nearly two million diarrhea-related deaths annually in this age group.[1,2] In the last decades, however, while mortality caused by diarrhea has been decreasing worldwide mainly because of improved hygiene, morbidity attributable to diarrhea remains high.[2–4] A vicious cycle ensues between diarrhea and malnutrition, and studies have shown that malnutrition with frequent diarrheal episodes slows cognitive and physical development of children.[5,6] One mechanism for this is that diarrheagenic pathogens damage intestinal epithelium and reduce its absorptive function, leading to nutrient depletion and malnutrition.[6]

Obstacles to recognition of at-risk children are several. The plight of sub-optimally breast fed and malnourished children is often largely invisible because they are only mildly or moderately undernourished.[7] Additionally, anthropometric measurements are not routinely performed to identify malnourished children in most clinics and hospitals in Ghana, thereby missing the opportunity for diagnosis and appropriate management.[8] The most established method to identify those with malnutrition is by the use of z-scores, with the reference population defined for the study country or from the standard international reference chart of the National Center for Health and the World Health Organization (WHO).[9]

The agents capable of causing infectious diarrhea and the mechanisms responsible for disease pathogenesis are generally known, but the true prevalence of these agents in developing countries is poorly understood.[10] For example, in most sub-Saharan African countries including Ghana, microbiological methods for clinical investigation of diarrheal diseases are usually restricted to identifying conventional enteric bacteria such as *Salmonella* and *Shigella*, *Escherichia coli* (*E. coli*) isolates are often not fully characterized because of the lack of resources. Additional pathogens of potential importance include enteroaggregative *E. coli* (EAEC), which is associated with diarrhea in several contexts: traveler's diarrhea,[11,12] pediatric diarrhea,[13] foodborne outbreaks,[14] human immunodeficiency virus,[15] symptomatic and asymptomatic cases,[16] acute and persistent diarrhea,[5,17] among others; and *Cryptosporidium*, *Entamoeba histolytica*, and *Giardia*, which are parasitic causes of diarrhea.[18–20] Unfortunately, investigation of diarrhea caused by these parasites in most developing countries largely depends on expert microscopy, where technical competence is necessary.

The objective of this study was, first, to determine the prevalence of EAEC, *Shigella* spp., *Cryptosporidium* spp., *E. histolytica*, and *Giardia lamblia* in children < 5 years of age with and without diarrhea in southern Ghana. Second, this study aims to determine whether these enteropathogens were associated with intestinal inflammation in either nourished or malnourished children.

## MATERIALS AND METHODS

**Ethical clearance.** The study was reviewed and approved by the Institutional Review Board of the University of Ghana Medical School, Ghana. Participation was voluntary and enrollment was subject to parents/guardians' approval, through signature or by thumb printing their names after the purpose of the study was explained to them.

**Study design, population, and settings.** This was a prospective cross-sectional study carried out between August 2007 and May 2008, of children ≤ 5 years of age consulting at the Princess Marie Louise Children's Hospital (PML), Accra, Ghana. Consecutive children from whom consent was given by their caregivers were included in the study. The group of children with diarrhea was recruited from the outpatient clinic and these children were brought to the hospital for acute health care. The control group of children without diarrhea was also from the outpatient clinic, but these children were visiting for routine child welfare care. No follow-up was done after the initial recruitment as a part of this study.

*Address correspondence to Japheth A. Opintan, Center for Global Health, Division of Infectious Diseases and International Health, University of Virginia, MR6, Carter-Harrison Bldg, Rm 2709, 345 Crispel Drive, Charlottesville, VA 22903. E-mails: japh_opintan@yahoo.com or jyo4g@virginia.edu

University of Ghana http://ugspace.ug.edu.gh



**Interviews and diarrhea definition.** A structured questionnaire was used to obtain information on the children from their parents/guardians. Information that was obtained included demographic data, duration of diarrhea, residence/location, breast feeding status, and medication taken before visiting the hospital. Diarrhea was defined as the passage of three or more unformed stools within a 24-h period. Diarrhea duration lasting < 14 days was defined as acute and those lasting ≥ 14 days, persistent. The control (non-diarrhea) group consisted of children who have not passed three or more unformed stools at least within the 24-h period before enrollment.

**Anthropometric data and nutritional status assessment.** Height or length measurements in centimeter to the nearest one decimal were performed for children above or below 2 years of age, respectively. Weight measurements in kilogram to the nearest one decimal were performed using a 25 kg Salter hanging scale (CMS Weighing equipment, High Holborn, London, UK). The Z-score, weight-for-age (WAZ), height-for-age (HAZ), and weight-for-height (WHZ) were calculated by use of software designed for nutrition studies (EPINUT, World Health Organization, Geneva; Epi Info version 6.0, Centers for Disease Control and Prevention, Atlanta, GA). These anthropometric Z-scores are a measure of SD above or below the median for the international reference population. Z-score values were used to determine the nutritional status of children on the basis of the following definition: WAZ, well nourished (> −1), mild (−2 to −1), moderate (−3 to −2), and severe (< −3) malnutrition; HAZ, normal height (≥ −2), moderate stunting (−3 < −2), and severe stunting (< −3); WHZ, normal weight (≥ −2), moderate wasting (−3 < −2), and severe wasting (< −3).

**Specimen processing and microbiological analysis.** A stool specimen from each participating child was collected into a sterile container and processed within 2 h of collection. Routine enteric bacteria were cultured on MacConkey (MAC), *Salmonella-Shigella* (SS), and deoxychocolate (DCA) agars (Oxoid, Columbia, MD), using standard techniques. Selenite F broth was used as pre-enrichment for *Salmonella* before sub-culturing onto MAC, SS, and DCA. Bacterial colonies after an overnight incubation period at 37°C were identified by standard biochemical methods and stored on Muellar Hinton slopes for further analysis. No microscopy was performed for the detection of parasites. One aliquot of a fresh stool specimen from each child was kept frozen at −20°C in cryo-vials (deidentified) until sent to the Center for Global Health, University of Virginia for further analysis.

**Fecal DNA extraction.** We used the QIAamp stool kit (Qiagen, Valencia, CA) to extract genomic DNA from frozen stool specimens with some minor modifications. The modifications included the addition of dry beads (MO BIO Laboratory Inc., Carlsbad, CA) to weighed stool specimen before the addition of lysis buffer (ASL). The mixture was bead-beated for 2 minutes to make a uniform homogeneous mixture with the lysis buffer. Additionally, we incubated mixtures at 80°C instead of the 70°C recommended by the manufacture to lyse enteric pathogens. For each stool aliquot, between 15 and 20 µg or µL of stool was used depending on stool consistency. DNAs were also extracted from appropriate control organisms. All DNAs were kept frozen at −80°C until needed for analysis.

**Quantitative real-time polymerase chain reaction (PCR).** A single-plex quantitative PCR for each gene pair (Table 1) consisted of 5 µL template, 1 µL of each 6.2 µM primer, 12.5 µL of SYBR-Green −490 (Bio-Rad Laboratories, Beltsville, MD), and PCR grade water to a reaction volume of 25 µL. Reactions for each sample were performed using the Bio-Rad iQCycler Real-Time Detection System in Bio-Rad iCycler 96-well plates, where positive and negative controls were included with each reaction set. Table 1 shows the target genes, locations, and annealing temperature for each primer set. The results were analyzed with a user-defined threshold of 200 PCR baseline-subtracted curve-fit relative fluorescence units. Melt curve (ct) data collection and analysis was enabled at cycles 3 and 4, with an increase in set point temperatures after cycle 2 by 0.5°C.

We sought multiple loci for EAEC (*aap*, *aatA*, *aggR*, and *aaiC*) and single loci for *Shigella*, *Cryptosporidium*, and *Giardia* species. Standard cultures with known numbers of *E. coli* 042 and 17-2, *Shigella*, *Cryptosporidium*, and *Giardia* oocysts were used as reference and positive controls. Water and *E. coli* K-12 were used as negative controls. Melt curve analysis was used to determine positivity of samples using a user defined threshhold.

**Intestinal inflammation assessment.** Intestinal inflammations were quantitatively assessed from frozen stool specimens using the IBD SCAN (TechLab, Blacksburg, VA) according

TABLE 1
Target genes screened from fecal DNA*

| Strain | Gene target | Location | Primer sequence (5′–3′) | PCR size (bp) | Annealing temperature (°C) | Source/reference |
|---|---|---|---|---|---|---|
| EAEC | *aaiC* | Chromosome | CTTCTGCTCTTAGCAGGGAGTTTG<br>AAGCGTGAAATGCCTGAGGA | 123 | 47.5 | Nataro's Laboratory |
| | *aatA* | Plasmid | CCTRTGTTGATGCTCGAGAGA<br>CKTTCCTCCTCCTCAAGGACAT | 118 | 55 | Nataro's Laboratory |
| | *aap* | Plasmid | CTTGGGTATCAGCCTGAATG<br>AACCCATTCGGTTAGAGCAC | 310 | 45 | Cerna and others[21] |
| | *aggR* | Plasmid | CTAATTGTACAATCGATGTA<br>ATGAAGTAATTCTTGAAT | 308 | 45 | Czeczulin and others[22] |
| *Shigella*/EIEC | *ipaH* | Plasmid | GTTCCTTGACCGCCTTTCCGATACCGTC<br>GCCGGTCAGCCACCCTCTGAGAGTAC | 619 | 60.5 | Sethabutr and others[23] |
| *Cryptosporidium* | 18s rRNA | Chromosome | CTCCACCAACTAAGAACGGCC<br>TAGAGATTGGAGGTTGTTCCT | 213 | 60 | Gene ID cgd7_230[24] |
| *E. histolytica* | Eh | Chromosome | AACAGTAATAGTTTCTTTGGTTAGTAAAA<br>CTTAGAATGTCATTTCTCAATTCAT | 134 | 60 | Haque and others[25] |
| *Giardia lamblia* | P241 | Chromosome | CATCCGCGAGGAGGTCAA<br>GCAGCCATGGTGTCGATCT | 74 | 60 | Guy and others[26] |

*PCR = polymerase chain reaction; EAEC = enteroaggregative *Escherichia coli*; EIEC = enteroinvasive *E. coli*.

University of Ghana http://ugspace.ug.edu.gh



to the manufacturer's instructions. Stool specimens were allowed to thaw and were serially diluted 10-fold and analyzed by a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) method. The detailed procedure is described elsewhere,[27] and absorbance of each assay well was measured spectrophotometrically at 450 and 620 nm ($A_{450/620}$). Fecal lactoferrin concentrations in µg/mL were determined by comparison with a standard curve using purified human lactoferrin and analyzed by linear regression in Microsoft excel (Redmond, WA). The lowest dilution of a specimen with an absorbance at 450/620 nm within the linear portion of the curve was used to determine the lactoferrin concentration. The final lactoferrin concentration was obtained by multiplying the dilution factor by the concentration. A positive control (purified human lactoferrin) and a negative control (washing buffer) was included in each batch of stools analyzed and linear regression was performed separately for each batch using standard controls. This assessment was performed only on subjects with adequate stool specimens and where necessary, the experiment was repeated. Among EAEC-infected malnourished children, two stool specimens (out of > 200 specimens analyzed) showed lactoferrin values that were 30–50 times more than the mean value for their subject group, were therefore designated as outliers, and not included in the statistical analysis. No satisfactory explanation for results on these two specimens was evident, because no diarrhea was present in one, and breast feeding was unlikely in both.

**Statistical analysis.** To avoid any experimental biases, stool specimens were coded before testing and only decoded for purposes of analysis. Statistical analyses were performed using SPSS software (version 17.0, SPSS, Chicago, IL) and Epi-Info. Statistical tests included $\chi^2$ for associations of pathogens with age groups, diarrhea, and non-diarrhea and the paired *t* test for associations with lactoferrin level. Odds ratio (OR) and 95% confidence intervals (CI) are reported for all 2 × 2 comparisons. Two-tailed tests were used and $P < 0.05$ was considered statistically significant.

## RESULTS

**Study population and nutritional status.** Within the 9-month study period, 287 children ≤ 5 years of age were recruited with only 13 excluded from analysis because of insufficient data. Of the 274 children included for analysis, 170 (62%) were with and 104 (38%) were without diarrhea; there were more males 156 (56.9%) than females. Acute and persistent diarrhea included 85.3% (145/170) and 7.6% (13/170) of total cases, respectively. Duration of symptoms in the remaining children with diarrhea (7.1%) was not recorded.

At least one anthropometric measurement was taken for 269 out of the 274 children analyzed. Of 269 children from whom weight measurements were recorded, 134 (49.8%) showed mild to severe malnutrition (WAZ < −1) (93/168 [55.4%] in children with and 41/101 [40.6%] without diarrhea, OR = 1.82 [95% CI, 1.102–2.988], $P = 0.023$). Of 170 children from whom height or length measurements were recorded, 61 (35%) showed moderate to severe stunting (HAZ < −2) (38/86 [44.2%] in children with and 23/84 [27.4%] without diarrhea, OR = 2.10 [95% CI, 1.110–3.972], $P = 0.026$). Of 161 children from whom both weight and height measurements were recorded, 37 (22.9%) showed moderate to severe wasting (WHZ < −2) (24/82 [29.3%] in children with and 13/79 [16.5%] without diarrhea, OR = 2.101 [95% CI, 0.989–4.454], $P = 0.062$). The mean age, weight, and height were 15.1/14.6 months, 9.8/9.5 kg, and 88.0/84.0 cm (diarrhea/non-diarrhea, respectively). Table 2 shows the baseline characteristics of the study population.

**Microbiological studies.** In only 1 of 170 diarrhea stool specimens was *Shigella* recovered as an enteric bacterial pathogen from culture. This strain was serotyped with *Shigella* polyvalent anti-sera (Mast Group Ltd., Merseyside, UK) and was *Shigella flexneri*. In the entire study population, *E. coli* was the predominant bacterium obtained from culture (79.6%), followed by *Klebsiella* spp. (5.1%). Other commensals included 9.8% of the total and no bacteria grew in 5.5% of the total stool specimens cultured.

**Pathogen detection by real-time PCR.** Table 3 shows bacterial and parasitic agents detected from fecal DNA by real-time PCR in children with and without diarrhea. The EAEC was defined as positivity for any of the four EAEC virulence genes sought (*aap*, *aatA*, *aggR*, and *aaiC*). Although EAEC was significantly associated with diarrhea (147/170 versus 80/104, OR 1.917 [95% CI = 1.024–3.592], $P = 0.048$), it was also found in similar frequencies in both nourished and malnourished children.

In 6 out of 170 diarrheal stool specimens, the *ipaH* gene, which is expressed by both *Shigella* and enteroinvasive *E. coli* (EIEC) was detected. The *ipaH* gene was not detected in fecal DNA from any of the children without diarrhea. The numbers were, however, too small to assess statistical significance. Five out of 6 of the children in whom the *ipaH* gene was detected were well-nourished (Table 3).

*Cryptosporidium* spp. was the most frequently detected protozoan parasite in fecal DNA and was associated with diarrhea (14/170 versus 1/104, OR = 9.244 [95% CI 1.197–71.371], $P = 0.011$). Cryptosporidiosis was also primarily (10 out of 14)

TABLE 2
Baseline characteristics of study population*

| Characteristic | Diarrhea *N* (%) | Non-diarrhea *N* (%) |
|---|---|---|
| Age/months | *N* = 170 | *N* = 104 |
| 0–6 | 26 (15.3) | 35 (33.7) |
| 7–12 | 54 (31.8) | 30 (28.8) |
| 13–24 | 77 (45.3) | 24 (23.1) |
| 25–60 | 13 (7.6) | 15 (14.4) |
| Sex | *N* = 170 | *N* = 104 |
| Male | 97 (57.1) | 73 (42.9) |
| Female | 59 (56.7) | 45 (43.3) |
| Weight/kg | *N* = 170 | *N* = 104 |
| 2.5–4.9 | 10 (5.9) | 4 (3.8) |
| 5.0–9.9 | 121 (71.2) | 75 (72.1) |
| 10–19.9 | 34 (20.0) | 22 (21.2) |
| 20–86.0 | 5 (2.9) | 3 (2.9) |
| WAZ | *N* = 168 | *N* = 101 |
| Normal (> −1) | 75 (44.6) | 60 (59.4) |
| Mild (−1 to −2) | 33 (19.6) | 19 (18.8) |
| Moderate (−2 to −3) | 32 (19.0) | 12 (11.9) |
| Severe (< −3) | 28 (16.7) | 10 (9.9) |
| HAZ | *N* = 86 | *N* = 84 |
| Normal (≥ −2) | 48 (55.8) | 61 (72.6) |
| Moderate (−2 to −3) | 6 (7.0) | 11 (13.1) |
| Severe (< −3) | 32 (37.2) | 12 (14.3) |
| WHZ | *N* = 82 | *N* = 79 |
| Normal (≥ −2) | 58 (70.3) | 66 (83.5) |
| Moderate (−2 to −3) | 6 (7.3) | 8 (10.1) |
| Severe (< −3) | 18 (22.0) | 5 (6.3) |

*WAZ = weight-for-age; HAZ = height-for-age; WHZ = weight-for-height.

University of Ghana http://ugspace.ug.edu.gh



TABLE 3
Organisms detected by real-time PCR from fecal DNA*

| | Diarrhea ($N$ = 170) | | | Non-diarrhea ($N$ = 104) | | | |
|---|---|---|---|---|---|---|---|
| | No. (%) | WN ($N$ = 75) | MN ($N$ = 95) | No. (%) | WN ($N$ = 60) | MN ($N$ = 41) | Odds ratio [95% CI] $P$ value† |
| Any infection | | | | | | | |
| EAEC | 147 (86.5) | 66 | 79 | 80 (76.9) | 49 | 30 | 1.917 (1.018–3.612) 0.048 |
| *Shigella*/EIEC | 6 (3.5) | 5 | 1 | 0 (0) | – | – | n/a |
| *Cryptosporidium* spp. | 14 (8.7) | 4 | 10 | 1 (1.0) | 1 | 0 | 9.244 (1.197–71.371) 0.011 |
| *E. histolytica* | 5 (3.0) | 2 | 3 | 0 (0) | – | – | n/a |
| *Giardia* spp. | 0 (0) | – | – | 0 (0) | – | – | – |

*PCR = polymerase chain reaction; WN = well nourished (WAZ > 1); MN = malnourished (WAZ < 1); EAEC = enteroaggregative *Escherichia coli*; EIEC = enteroinvasive *E. coli*; n/a = not applicable.
† $P$ value is between diarrheal and non-diarrheal stool specimen.

detected in children who were malnourished and had diarrhea (Table 3). *Entamoeba histolytica* was only detected in children with diarrhea 5 out of 170 (2.9%) and *Giardia* was not detected in either sub-populations.

We observed children who were co-infected, with two or more pathogens detected in the stool, predominantly in children who had diarrhea. The EAEC-*Cryptosporidium* was the most prevalent (7.6%, 13/170), followed by EAEC-*Shigella*/EIEC (2.9%, 5/170), and EAEC-*E. histolytica* (2.4%, 4/170). *Cryptosporidium*-*E. histolytica* and *Cryptosporidium*-*Shigella*/EIEC co-infection each formed 0.6% (1/170), and one child who had diarrhea was co-infected with EAEC-*Cryptosporidium*-*Shigella*/EIEC (0.06%, 1/170). There was no obvious trend in the distribution of pathogens by age in the two sub-populations, especially for EAEC (Table 4).

**EAEC virulence genes distribution.** Of the four genes associated with EAEC, *aatA* was the most frequently detected (67.2%) of all fecal DNA, followed by *aap* (59.9%), *aggR* (42.7%), and *aaiC* (33.6%) (Table 5). The EAEC's plasmid gene *aap* was significantly associated with diarrhea (OR = 2.506 [95% CI, 1.516–4.144], $P$ < 0.001) and the chromosomal gene *aaiC* was not (OR = 1.639 [95% CI, 0.0962–2.792], $P$ = 0.086) (Table 5). Multiple gene combinations were also observed in EAEC infections in our study population, and the presence of any three genes was associated with diarrhea (OR = 2.101 [95% CI, 1.261–3.502], $P$ = 0.006). We did not find any of the EAEC virulence genes associated with malnutrition (WAZ < −1) ($P$ > 0.05).

**Fecal lactoferrin levels.** Figure 1 shows enteric pathogens detected in children with/without diarrhea and the distribution of fecal lactoferrin levels. Generally, the mean lactoferrin levels were lower in children who had diarrhea and were also malnourished compared with those with diarrhea but were nourished. Regardless of the enteric pathogen detected, fecal lactoferrin levels were relatively high (manufacturer's cut-off value = 7.24 µg/mL). Especially for EAEC infection, both controls and patients had a wide range of lactoferrin levels, regardless of whether they were nourished or malnourished (Figure 1).

Children with diarrhea had significantly higher fecal lactoferrin levels ($N$ = 143; 1658.9 ± 204.2 µg/mL) compared with those without diarrhea ($N$ = 84; 935.5 ± 194.4 µg/mL) ($P$ = 0.019). The *aatA* gene and the presence of any one or two genes of EAEC were also significantly ($P$ < 0.05) associated with elevated fecal lactoferrin levels (Table 6). In comparing diarrhea with non-diarrhea stool specimens, EAEC's chromosomal gene *aaiC* showed the highest fold-increase in fecal lactoferrin level (2.7) followed by the *aap* gene (2.5). Additionally, detection of a multiple virulence gene of EAEC in a stool was associated with an increased fold-rise in the mean fecal lactoferrin level between children with and without diarrhea (Table 6).

## DISCUSSION

**Nutritional shortfalls among our study population.** Mild to severe malnutrition (WAZ < −1, which reflects both acute and chronic types of malnutrition) was identified in 49.8% of our study population. Severe growth shortfalls occurred in both children with and without diarrhea in the entire study population. Growth faltering in infants from developing countries has been reported to occur as early as 2–3 months of age[28,29] in contrast to developed countries,[28] a fact attributable to timing of complementary feeding. For example, in the DARLING (Davis Area Research on Lactation, Infant Nutrition and Growth) study, although breast milk intakes were similar, the amount and nutrient density of food consumed after 6 months were lower in Peru than in the United States.[28] In 2005, Antwi[8] noted a 21.2% incidence of wasting (acute malnutrition) among children seen in Kumasi, Ghana, similar to an overall incidence of 22.9% (37/161) in the current study—both consistent with the 22.1% estimate for Ghana by the World Food Program.[30]

TABLE 4
Distribution of pathogens by age*

| | Diarrhea ($N$ = 170) | | | | Non-diarrhea ($N$ = 104) | | | |
|---|---|---|---|---|---|---|---|---|
| | Age category/months | | | | Age category/months | | | |
| Any infection | 0–6 ($N$ = 26) | 7–12 ($N$ = 54) | 13–24 ($N$ = 77) | 25–60 ($N$ = 13) | 0–6 ($N$ = 35) | 7–12 ($N$ = 30) | 13–24 ($N$ = 24) | 25–60 ($N$ = 15) |
| EAEC | 24 (92.3) | 42 (77.7) | 69 (89.6) | 12 (92.3) | 25 (71.4) | 24 (80) | 18 (75) | 13 (86.7) |
| *Cryptosporidium* | 3 (11.5) | 4 (7.4) | 6 (7.8) | 13 (7.7) | – | – | 1 (4.2) | – |
| *E. histolytica* | – | 2 (3.7) | 3 (3.9) | – | – | – | – | – |
| *Shigella*/EIEC | 1 (3.8) | 1 (1.8) | 3 (3.9) | 1 (7.7) | – | – | – | – |
| *Giardia* spp. | – | – | – | – | – | – | – | – |

*EAEC = enteroaggregative *Escherichia coli*; EIEC = enteroinvasive *E. coli*.

University of Ghana http://ugspace.ug.edu.gh



TABLE 5

Enteroaggregative *Escherichia coli* (EAEC) virulence factor-positive in stool samples*

| Characteristic | Diarrhea (N = 170) N (%) | Non-diarrhea (N = 104) N (%) | Total (N = 274) N (%) | OR [95% CI] | $\chi^2$ | P value |
|---|---|---|---|---|---|---|
| EAEC virulence-related gene | | | | | | |
| *aaiC* | 64 (37.6) | 28 (26.9) | 92 (33.6) | 1.639 [0.962–2.792] | 3.327 | 0.086 |
| *aggR* | 79 (46.5) | 38 (36.5) | 117 (42.7) | 1.508 [0.914–2.486] | 2.602 | 0.131 |
| *aatA* | 118 (69.4) | 66 (63.5) | 184 (67.2) | 1.307 [0.780–2.188] | 1.036 | 0.354 |
| *aap* | 116 (68.2) | 48 (46.2) | 164 (59.9) | 2.506 [1.516–4.144] | 13.093 | < 0.001 |
| EAEC gene combination | | | | | | |
| Any 1 gene | 147 (86.5) | 80 (76.9) | 227 (82.8) | 1.917 [1.018–3.612] | 4.139 | 0.048 |
| Any 2 genes | 130 (76.5) | 73 (70.2) | 203 (74.1) | 1.380 [0.797–2.391] | 1.325 | 0.259 |
| Any 3 genes | 84 (49.4) | 33 (31.7) | 117 (42.7) | 2.101 [1.261–3.502] | 8.224 | 0.006 |
| All 4 genes | 34 (20.0) | 13 (12.5) | 47 (17.2) | 1.750 [0.876–3.496] | 2.554 | 0.110 |

*OR = odds ratio; 95% CI = confidence interval; $P < 0.05$ is significant.

Approximately 14% (38/269) of the children we studied were severely malnourished (WAZ < −3), and, out of this number, 73.7% had diarrhea. A self-perpetuating vicious cycle in which malnutrition and diarrhea are synergistic is suggested, and may explain their effect on cognitive development of children, especially their semantic fluency.[6] Our data supports this link between malnutrition and diarrhea, placing these groups of children at a higher risk of morbidity over time.

**Bacterial culture versus real-time PCR for detection of pathogens.** The PCR methodology is more sensitive in screening for pathogens. In sub-Saharan Africa as in many settings, however, the cost involved cannot be passed on to patients and therefore this tool is used mainly in research facilities. In this study, routine bacterial culture detected only one stool positive for *S. flexneri* and none for *Salmonella* spp. However, PCR detected six *ipaH* genes in diarrheal stool specimens. Although, the *ipaH* gene is expressed by both *Shigella* and enteroinvasive (EIEC), EIEC is not often detected in Ghana,[31] and we speculate that the *ipaH* gene detected most likely reflects the presence of *Shigella* spp. that was missed on the culture. The sero-group identified by culture and serology is predominant in Ghana[32] as it is in many developing countries.

*Cryptosporidium* spp. was not only the most prevalent parasite detected, but it is also significantly associated with diarrhea (8.7% versus 1.0%, $P = 0.011$). Children who were malnourished and had diarrhea often had cryptosporidiosis. Prevalence rates of *Cryptosporidium* spp. using presumably less sensitive methods like microscopy and ELISA in Ghana,[33,34] Liberia,[35] Mexico,[36] and Guinea Bissau[37] reported ranges between 7.7% and 29% in symptomatic children. The current study recorded 8.2% in symptomatic and 1.0% in non-symptomatic patients.

*Entamoeba histolytica* causes amebic colitis, amebic dysentery, and liver abscess. Modern diagnostic tools like PCR are able to discriminate *E. histolytica* from the non-pathogenic

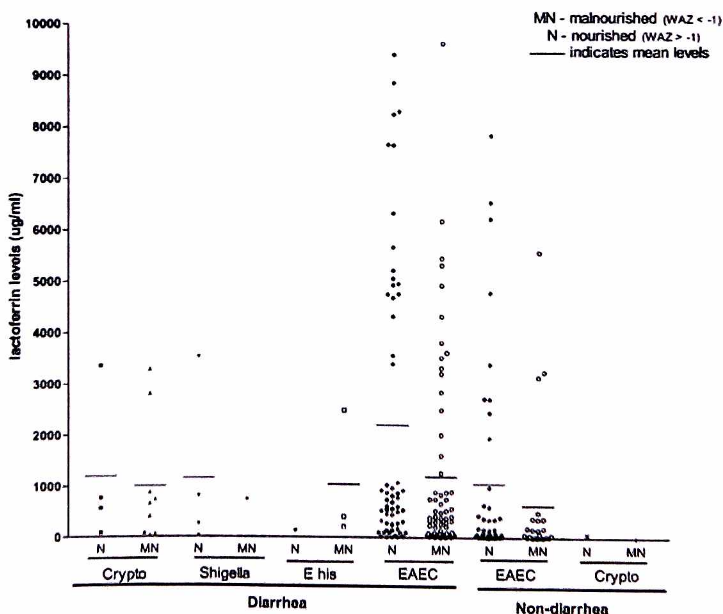



FIGURE 1. Enteric pathogens detected and fecal lactoferrin (LF) levels. Crypto = *Cryptosporidium* spp.; E his = *E. hiostolytica*; EAEC = enteroaggregative *Escherichia coli*. *Breast-feeding may cause moderately (15 ≤ 120 µg/mL) increased LF (Lima and others, unpublished observation).

University of Ghana http://ugspace.ug.edu.gh



TABLE 6
EAEC genes detected and fecal lactoferrin levels*

| Characteristic | Diarrheal stool n (mean ± SE) μg/mL | Non-diarrheal stool n (mean ± SE) μg/mL | Fold-rise† | P value |
|---|---|---|---|---|
| EAEC gene | | | | |
| *aaiC* | 56 (1021.0 ± 251.6) | 25 (821.2 ± 402.1) | 1.2 | 0.667 |
| *aggR* | 66 (1713.9 ± 292.5) | 33 (934.1 ± 329.2) | 1.8 | 0.104 |
| *aatA* | 98 (1706.1 ± 244.1) | 54 (927.1 ± 259.1) | 1.8 | 0.044 |
| *aap* | 101 (1656.7 ± 245.9) | 39 (901.2 ± 261.6) | 1.8 | 0.080 |
| EAEC gene combinations | | | | |
| Any 1 gene | 123 (1633.5 ± 215.4) | 66 (1175.4 ± 356.8) | 1.4 | 0.029 |
| Any 2 gene | 107 (1679.8 ± 231.1) | 60 (892.6 ± 234.3) | 1.9 | 0.028 |
| Any 3 gene | 73 (1504.9 ± 269.7) | 30 (1132.8 ± 394.8) | 1.3 | 0.451 |
| Any 4 gene | 30 (1319.5 ± 400.4) | 11 (370.5 ± 285.5) | 3.6 | 0.175 |
| All specimens | 143 (1658.9 ± 204.2) | 84 (935.5 ± 194.4) | 1.8 | 0.019 |

*EAEC = enteroaggregative *Escherichia coli*.
†Ratio of diarrhea and non-diarrhea mean lactoferrin levels.

*E. dispar*.[25] This study detected *E. histolytica* in 2.9% (5/170) of the children who had diarrhea. No visible blood was observed in any of the stools from these children. All the *E. histolytica* detected in the current study were from children who had diarrhea. In Bangladesh and elsewhere, relatively few numbers of people infected with *E. histolytica* develop symptomatic disease.[18,38,39]

This study did not detect any *Giardia* in the stool specimens screened for the p241 gene of *Giardia* spp. The p241 gene target we used is well validated[26] and our positive control, which was included in each PCR run was amplified while the negative control was not. Addy and others[40] in 2004, found a 3.7% prevalence rate of *Giardia* in Kumasi, Ghana. In a 7-year study of diarrhea caused by parasites in Guinea Bissau, the most prevalent parasite was *Giardia lamblia* (14.8%) followed by *Cryptosporidium* (7.7%).[37] Although seasonality, study duration, or geographical location may influence parasite prevalence, we cannot pinpoint additional reasons for the zero prevalence of *Giardia* in the current study.

Children who had diarrhea were often co-infected, with two or more pathogens detected in the stool, and EAEC-*Cryptosporidium* was the most prevalent (13/170) in the current study. Among human immunodeficiency virus (HIV)-infected children in South Africa, Samie and others[15] identified children who were co-infected with as many as six different species of pathogens. This study detected a child who had diarrhea with EAEC-*Cryptosporidium-Shigella*/EIEC co-infection. The HIV status of this child is however not known. Relatively fewer children who are > 2 years of age were sampled in this study. However, cryptosporidiosis seemed to be more common in children < 2 years of age, and this is in agreement with an earlier study in Ghana.[34]

**Prevalence of EAEC virulence-associated genes.** Among the EAEC plasmid genes (*aap*, *aatA*, and *aggR*) we tested, only *aap* was significantly associated with diarrhea ($P = 0.0003$). A recent publication, which compared molecular probes to the "gold standard" (aggregation of cultured epithelial cells), however, suggested that the *aap* gene is not restricted to EAEC, but is also detected in diffusely adherent *E. coli* (DAEC) and in non-pathogenic *E. coli*.[41] We did not observe any significant statistical association between any individual EAEC gene (whether plasmid or chromosome borne) and malnutrition (WAZ < −1).

Information on the presence of EAEC virulence-associated genes in body fluids of persons in Africa is limited, as is the prevalence and distribution of this organism in Ghana. The *aggR* is known to regulate its own expression and that of several plasmid genes and chromosomal genes of EAEC,[42] including the aggregative adherence fimbriae, a dispersin (aap), a dispersin translocator apparatus called aat, and several chromosomal loci including the *aaiC*.[43] In a different population, Huang and others failed to show any association between four EAEC virulence-associated genes (*aggA*, *aspU*, *aafA* and *aggR*) and clinical illness in travelers from the United States to Mexico.[43] Furthermore, in that study, *aspU* (now designated *aatA*) was the least prevalent gene among the four EAEC virulence genes studied,[43] whereas this same gene (*aatA*) was the most prevalent (67.2%) in the current study. In South Africa, Samie and others[15] found the *aap* gene to be the predominant among others and also associated with diarrhea. These disparate findings on the relative distribution and importance of EAEC genes in diarrheal illnesses suggest that geographical location, type of exposure, and/or host factors may dictate the nature of EAEC infection. For example, among the risks of contracting traveler's diarrhea, the country of destination was the most important determining factor as reported by Cabada and White.[12]

Although *aatA* was the most prevalent gene observed in this study, it was not associated with diarrhea ($P > 0.05$) (Table 5). Some studies have shown that the novel protein aatA, which is encoded on EAEC virulence plasmid pAA2, localizes to the outer membrane of this bacterium and facilitates export of the dispersin *aap* across the outer membrane.[45,46] Our results may support this notion because *aatA* and *aap* were detected in greater than 68% of patients with diarrhea. Further study is required for a firm conclusion. Of interest, the chromosomal gene *aaiC* did not show any significant differential association between the diarrhea and the non-diarrhea group in the current study ($P = 0.068$). About 12% (13/104) of children without diarrhea in the current study were positive for all four EAEC genes tested. The presence of 2, 3, or 4 genes in stool specimens of study children suggests a strong association. For example, the presence of three of these genes is associated with a higher OR than one gene alone (Table 5).

One explanation for the high frequency of EAEC-associated virulence genes in symptomatic and asymptomatic patients and the heterogeneity of the different gene assortments found is that EAEC is endemic in our study population. In support of this notion, almost all prior studies recover EAEC from controls[47–49] and from individuals with diarrhea.[17,50] Pathogenic factors may influence the initiation of a symptomatic phase,

University of Ghana http://ugspace.ug.edu.gh



determined by and include distinct mechanisms as reviewed by Kaper and others.[51] For example, Huang and others suggested that a first exposure to EAEC infection "primes" the immune system to prevent a second infection.[44] Furthermore, in their study of traveler's diarrhea, after the initial EAEC infection, only 4 (11%) of the subjects had a subsequent symptomatic EAEC infection.[44]

**Lactoferrin levels.** Lactoferrin is bactericidal to enteric pathogens, modulates the intestinal immune response, and is released by neutrophils into stool in response to infection.[52] Of interest in the current study, the mean lactoferrin levels were lower in children who were malnourished and had diarrhea compared with those without malnutrition who had diarrhea. We speculate that the lactoferrin assay may be marginally less sensitive in the setting of malnutrition. Perhaps, the enterocytes are less able to synthesize lactoferrin in children who are malnourished and have diarrhea. This observation needs further investigation for a firm conclusion. In earlier studies, the mean lactoferrin levels for healthy controls were less than 12.8 μg/mL[27,52] compared with healthy controls (298.8 μg/mL) in the current study. We do not have immediate explanation for this observation. However, unpublished observations by Lima and others suggest that breast milk may contribute to moderately (15 to 120 μg/mL) increased fecal lactoferrin levels. Some studies have demonstrated an association of EAEC infection with inflammatory cytokines[53,54] and several others have associated EAEC with elevated lactoferrin levels.[15,55] In children with diarrhea, we found a significant statistical association of elevated fecal lactoferrin with the *aap* gene, and with the detection of any one or two of the EAEC genes tested ($P < 0.05$). Between diarrhea and non-diarrhea stool specimens, EAEC's chromosomal gene *aaiC* was associated with the highest fold-rise in fecal lactoferrin level (2.7) followed by the *aap* gene (2.5). Additionally, more virulence EAEC genes detected corresponded to a rise in the fecal lactoferrin level (Table 6). The protective function of lactoferrin on infections with enteropathogens have been acknowledged,[56] and colonization/infection, particularly by EAEC in the current study probably contributed to the high lactoferrin levels (Table 6).

The current study had some limitations. Accurate height measurements were the most difficult to obtain on an outpatient basis, and this limited two of the three z-scores of malnutrition (WHZ and HAZ). Knowledge of history of antimicrobial exposure is important, however, this information was obtainable in only a few of the patients. Only selected pathogens were assayed using PCR and thus, we may have missed other less common pathogens. That is, no stool analyses for intestinal parasites other than *Cryptosporidium*, *Giardia*, and *E. histolytica* were performed, therefore, missing helmintic infections.

In conclusion, through the use of specific DNA-biomarkers, we were able to determine that EAEC and *Cryptosporidium* were common intestinal pathogens and that elevated fecal lactoferrin levels were associated with diarrhea in this group of children from southern Ghana.

Received December 28, 2009. Accepted for publication July 6, 2010.

Acknowledgments: We thank David Kwarteng and the nurses at the Princess Marie Louise Hospital for data collection and anthropometric measurements. Relanna Pinkerton at UVA reviewed all statistical analyses.

Financial support: This study was supported in part by the University of Ghana Medical School, College of Health Sciences, Accra, Ghana and Pfizer-supported funds to the Center for Global Health, University of Virginia, Charlottesville, VA.

Authors' addresses: Japheth A. Opintan, Mercy J. Newman, and Patrick F. Ayeh-Kumi, Department of Microbiology, University of Ghana Medical School, Korle-Bu, Accra, Ghana, E-mails: jyo4g@virginia.edu, newmerci@yahoo.co.uk, and payehkumi@yahoo.com. Raymond Affrim and Rosina Gepi-Attee, Princess Marie Louise Children's Hospital, Accra, Ghana, E-mails: raffrim@yahoo.com and rgepi_attee@yahoo.com. Jesus E. A. D. Sevilleja, James K. Roche, Cirle A. Warren, and Richard L. Guerrant, Center for Global Health, Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, VA, E-mails: emsevilleja@yahoo.com, jkr7m@virginia.edu, ca6t@virginia.edu, and rlg9a@virginia.edu. James P. Nataro, Center for Vaccine Development, Department of Pediatrics and Medicine, University of Maryland, Baltimore, MD, E-mail: jpn2r@virginia.edu.

## REFERENCES

1. Bryce J, Boschi-Pinto C, Shibuya K, Black RE, 2005. WHO estimates of the causes of death in children. *Lancet 365:* 1147–1152.
2. Kosek M, Bern C, Guerrant RL, 2003. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull World Health Organ 81:* 197–204.
3. Bern C, Martines J, de Zoysa I, Glass RI, 1992. The magnitude of the global problem of diarrhoeal disease: a ten-year update. *Bull World Health Organ 70:* 705–714.
4. Durley A, Shenoy A, Faruque AS, Suskind R, Ahmed T, 2004. Impact of a standardized management protocol on mortality of children with diarrhoea: an update of risk factors for childhood death. *J Trop Pediatr 50:* 271–275.
5. Lima AA, Moore SR, Barboza MS Jr, Soares AM, Schleupner MA, Newman RD, Sears CL, Nataro JP, Fedorko DP, Wuhib T, Schorling JB, Guerrant RL, 2000. Persistent diarrhea signals a critical period of increased diarrhea burdens and nutritional shortfalls: a prospective cohort study among children in northeastern Brazil. *J Infect Dis 181:* 1643–1651.
6. Guerrant RL, Oria RB, Moore SR, Oria MO, Lima AA, 2008. Malnutrition as an enteric infectious disease with long-term effects on child development. *Nutr Rev 66:* 487–505.
7. UNICEF, Ghana, 2009. *ChildInfo: Monitoring the Situation of Children and Women*. Child Nutrition. Statistics by Area/Child Nutrition.
8. Antwi S, 2008. Malnutrition: missed opportunities for diagnosis. *Ghana Med J 42:* 101–104.
9. Joosten KF, Hulst JM, 2008. Prevalence of malnutrition in pediatric hospital patients. *Curr Opin Pediatr 20:* 590–596.
10. Gomez-Duarte OG, Bai J, Newell E, 2009. Detection of *Escherichia coli, Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica, Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. *Diagn Microbiol Infect Dis 63:* 1–9.
11. Adachi JA, Jiang ZD, Mathewson JJ, Verenkar MP, Thompson S, Martinez-Sandoval F, Steffen R, Ericsson CD, DuPont HL, 2001. Enteroaggregative *Escherichia coli* as a major etiologic agent in traveler's diarrhea in 3 regions of the world. *Clin Infect Dis 32:* 1706–1709.
12. Cabada MM, White ACJr, 2008. Travelers' diarrhea: an update on susceptibility, prevention, and treatment. *Curr Gastroenterol Rep 10:* 473–479.
13. Paul M, Tsukamoto T, Ghosh AR, Bhattacharya SK, Manna B, Chakrabarti S, Nair GB, Sack DA, Sen D, Takeda Y, 1994. The significance of enteroaggregative *Escherichia coli* in the etiology of hospitalized diarrhoea in Calcutta, India and the demonstration of a new honey-combed pattern of aggregative adherence. *FEMS Microbiol Lett 117:* 319–325.
14. Scavia G, Staffolani M, Fisichella S, Striano G, Colletta S, Ferri G, Escher M, Minelli F, Caprioli A, 2008. Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. *J Med Microbiol 57:* 1141–1146.
15. Samie A, Obi CL, Dillingham R, Pinkerton RC, Guerrant RL, 2007. Enteroaggregative *Escherichia coli* in Venda, South

University of Ghana http://ugspace.ug.edu.gh




Africa: distribution of virulence-related genes by multiplex polymerase chain reaction in stool samples of human immunodeficiency virus (HIV)-positive and HIV-negative individuals and primary school children. *Am J Trop Med Hyg 77:* 142–150.

16. Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial P, Levine MM, 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr Infect Dis J 6:* 829–831.
17. Okeke IN, Ojo O, Lamikanra A, Kaper JB, 2003. Etiology of acute diarrhea in adults in southwestern Nigeria. *J Clin Microbiol 41:* 4525–4530.
18. Haque R, Huston CD, Hughes M, Houpt E, Petri WA Jr, 2003. Amebiasis. *N Engl J Med 348:* 1565–1573.
19. Kosek M, Alcantara C, Lima AA, Guerrant RL, 2001. Cryptosporidiosis: an update. *Lancet Infect Dis 1:* 262–269.
20. Ortega YR, Adam RD, 1997. *Giardia*: overview and update. *Clin Infect Dis 25:* 545–549, quiz 550.
21. Cerna JF, Nataro JP, Estrada-Garcia T, 2003. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. *J Clin Microbiol 41:* 2138–2140.
22. Czeczulin JR, Whittam TS, Henderson IR, Navarro-Garcia F, Nataro JP, 1999. Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. *Infect Immun 67:* 2692–2699.
23. Sethabutr O, Venkatesan M, Murphy GS, Eampokalap B, Hoge CW, Echeverria P, 1993. Detection of *Shigellae* and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. *J Infect Dis 167:* 458–461.
24. *Cryptosporidium* DB (version 4.2) 2009. *Cryptosporidium* Genomic Resources: 18s ribosomal RNA.
25. Haque R, Roy S, Siddique A, Mondal U, Rahman SM, Mondal D, Houpt E, Petri WA Jr, 2007. Multiplex real-time PCR assay for detection of *Entamoeba histolytica, Giardia intestinalis*, and *Cryptosporidium* spp. *Am J Trop Med Hyg 76:* 713–717.
26. Guy RA, Payment P, Krull UJ, Horgen PA, 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. *Appl Environ Microbiol 69:* 5178–5185.
27. Kane SV, Sandborn WJ, Rufo PA, Zholudev A, Boone J, Lyerly D, Camilleri M, Hanauer SB, 2003. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. *Am J Gastroenterol 98:* 1309–1314.
28. Dewey KG, Peerson JM, Heinig MJ, Nommsen LA, Lonnerdal B, Lopez de Romana G, de Kanashiro HC, Black RE, Brown KH, 1992. Growth patterns of breast-fed infants in affluent (United States) and poor (Peru) communities: implications for timing of complementary feeding. *Am J Clin Nutr 56:* 1012–1018.
29. Sathian U, Joseph A, Waterlow JC, 1983. Exclusive breast feeding versus supplementation: a prospective study in a rural south Indian community. *Ann Trop Paediatr 3:* 157–161.
30. World Food Programme WFP, 2006. *World hunger—Ghana*. Available at: http://www.wfp.org/country_brief/indexcountry.asp?country=288.
31. Opintan JA, Rima AB, Newman MJ, Okeke IN, 2010. Carriage of diarrhoeagenic *Escherichia coli* by older children and adults in Accra, Ghana. *Trans R Soc Trop Med Hyg 104:* 504–506.
32. Opintan J, Newman MJ, 2007. Distribution of serogroups and serotypes of multiple drug resistant *Shigella* isolates. *Ghana Med J 41:* 4–8.
33. Addy PA, Aikins-Bekoe P, 1986. Cryptosporidiosis in diarrhoeal children in Kumasi, Ghana. *Lancet 1:* 735.
34. Adjei AA, Armah H, Rodrigues O, Renner L, Borketey P, Ayeh-Kumi P, Adiku T, Sifah E, Lartey M, 2004. *Cryptosporidium* spp., a frequent cause of diarrhea among children at the Korle-Bu Teaching Hospital, Accra, Ghana. *Jpn J Infect Dis 57:* 216–219.
35. Hojlyng N, Molbak K, Jepsen S, 1986. *Cryptosporidium* spp., a frequent cause of diarrhea in Liberian children. *J Clin Microbiol 23:* 1109–1113.
36. Miller K, Duran-Pinales C, Cruz-Lopez A, Morales-Lechuga L, Taren D, Enriquez FJ, 1994. *Cryptosporidium parvum* in children with diarrhea in Mexico. *Am J Trop Med Hyg 51:* 322–325.
37. Perch M, Sodemann M, Jakobsen MS, Valentiner-Branth P, Steinsland H, Fischer TK, Lopes DD, Aaby P, Molbak K, 2001. Seven years' experience with *Cryptosporidium parvum* in Guinea-Bissau, West Africa. *Ann Trop Paediatr 21:* 313–318.
38. Ayeh-Kumi PF, Ali IM, Lockhart LA, Gilchrist CA, Petri WA Jr, Haque R, 2001. *Entamoeba histolytica*: genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. *Exp Parasitol 99:* 80–88.
39. Verweij JJ, Oostvogel F, Brienen EA, Nang-Beifubah A, Ziem J, Polderman AM, 2003. Short communication: prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in northern Ghana. *Trop Med Int Health 8:* 1153–1156.
40. Addy P, Antipim G, Frimpong E, 2004. Prevalence of pathogenic *Escherichia coli* and parasites in infants with diarrhoea in Kumasi, Ghana. *East Afr Med J 81:* 353–357.
41. Monteiro BT, Campos LC, Sircili MP, Franzolin MR, Bevilacqua LF, Nataro JP, Elias WP, 2009. The dispersin-encoding gene (*aap*) is not restricted to enteroaggregative *Escherichia coli*. *Diagn Microbiol Infect Dis 65:* 81–84.
42. Dudley EG, Thomson NR, Parkhill J, Morin NP, Nataro JP, 2006. Proteomic and microarray characterization of the *AggR* regulon identifies a *pheU* pathogenicity island in enteroaggregative *Escherichia coli*. *Mol Microbiol 61:* 1267–1282.
43. Huang DB, Jiang ZD, Dupont HL, 2003. Association of virulence factor-positive and -negative enteroaggregative *Escherichia coli* and occurrence of clinical illness in travelers from the United States to Mexico. *Am J Trop Med Hyg 69:* 506–508.
44. Nataro JP, 2005. Enteroaggregative *Escherichia coli* pathogenesis. *Curr Opin Gastroenterol 21:* 4–8.
45. Imuta N, Nishi J, Tokuda K, Fujiyama R, Manago K, Iwashita M, Sarantuya J, Kawano Y, 2008. The *Escherichia coli* efflux pump TolC promotes aggregation of enteroaggregative *E. coli* 042. *Infect Immun 76:* 1247–1256.
46. Sheikh J, Czeczulin JR, Harrington S, Hicks S, Henderson IR, Le Bouguenec C, Gounon P, Phillips A, Nataro JP, 2002. A novel dispersin protein in enteroaggregative *Escherichia coli*. *J Clin Invest 110:* 1329–1337.
47. Gascon J, Vargas M, Schellenberg D, Urassa H, Casals C, Kahigwa E, Aponte JJ, Mshinda H, Vila J, 2000. Diarrhea in children under 5 years of age from Ifakara, Tanzania: a case-control study. *J Clin Microbiol 38:* 4459–4462.
48. Valentiner-Branth P, Steinsland H, Fischer TK, Perch M, Scheutz F, Dias F, Aaby P, Molbak K, Sommerfelt H, 2003. Cohort study of Guinean children: incidence, pathogenicity, conferred protection, and attributable risk for enteropathogens during the first 2 years of life. *J Clin Microbiol 41:* 4238–4245.
49. Albert MJ, Faruque AS, Faruque SM, Sack RB, Mahalanabis D, 1999. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *J Clin Microbiol 37:* 3458–3464.
50. Rappelli P, Folgosa E, Solinas ML, Dacosta JL, Pisanu C, Sidat M, Melo J, Cappuccinelli P, Colombo MM, 2005. Pathogenic enteric *Escherichia coli* in children with and without diarrhea in Maputo, Mozambique. *FEMS Immunol Med Microbiol 43:* 67–72.
51. Kaper JB, Nataro JP, Mobley HL, 2004. Pathogenic *Escherichia coli*. *Nat Rev Microbiol 2:* 123–140.
52. Walker TR, Land ML, Kartashov A, Saslowsky TM, Lyerly DM, Boone JH, Rufo PA, 2007. Fecal lactoferrin is a sensitive and specific marker of disease activity in children and young adults with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr 44:* 414–422.
53. Cennimo D, Abbas A, Huang DB, Chiang T, 2009. The prevalence and virulence characteristics of enteroaggregative *Escherichia coli* at an urgent-care clinic in the USA: a case-control study. *J Med Microbiol 58:* 403–407.
54. Greenberg DE, Jiang ZD, Steffen R, Verenker MP, DuPont HL, 2002. Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative *Escherichia coli* pathogenicity. *J Infect Dis 185:* 944–949.
55. Bouckenooghe AR, Dupont HL, Jiang ZD, Adachi J, Mathewson JJ, Verenkar MP, Rodrigues S, Steffen R, 2000. Markers of enteric inflammation in enteroaggregative *Escherichia coli* diarrhea in travelers. *Am J Trop Med Hyg 62:* 711–713.
56. Ochoa TJ, Cleary TG, 2009. Effect of lactoferrin on enteric pathogens. *Biochimie 91:* 30–34.

University of Ghana http://ugspace.ug.edu.gh