CHARACTERISATION OF BACTERIAL CAUSES OF DIARRHOEA IN AN UNDER-FIVE POPULATION IN SOUTH AFRICA

Zwiitavhathu Makhari

(307037)

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

Johannesburg, 2012

Declaration

I, MAKHARI ZWIITAVHATHU, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature: .....

.....day of.....NOVEMBER.....2012

Dedication

To my mom

Phumudzo Glorious Makhari

Ethics

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Zwiitavhathu Makhari

CLEARANCE CERTIFICATE	**M10341**
PROJECT	Characterization of Bacterial Causes of Diarrhoea in an Under-Five Population in South Africa
INVESTIGATORS	Miss Zwiitavhathu Makhari.
DEPARTMENT	School of Pathology
DATE CONSIDERED	26/03/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 04/05/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr A Smith

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Makhari..

Presentations

Poster presentations

- **Zwiitavhathu Makhari**, Anthony M. Smith, Karen H. Keddy, Michelle Groome, Cheryl Cohen, Jocelyn Moyes, Shabir A. Madhi. Characterization of Bacterial Causes of Diarrhoea in an Under-five Population in South Africa: April 2009 to April 2010. Wits Research Day: September 2010, Medical campus, University of Witwatersrand, South Africa.
- Nicola Page, Karen Keddy, Bhavani Poonsamy, **Zwiitavhathu Makhari**, Anthony Smith, Benjamin Mogoye, Michelle Groome; Desiree du Plessis, Sandrama Nadan, Tersia Kruger, Jocelyn Moyes, Veerle Msimang, Kathleen Kahn, Rhian Twine, Meera Chhagan, Cheryl Cohen; Shabir A. Madhi. Aetiology of diarrhoeal infections in children under five years of age enrolled in Rotavirus surveillance in South Africa in 2009/2010: Frequency of co-infections with other diarrhoeal pathogens. 9th International Rotavirus Symposium: August 2010, Sandton, South Africa.

Abstract

Introduction: Diarrhoea is a major cause of mortality and morbidity amongst children under five years of age worldwide.

Aim: To characterise the bacterial aetiologies and molecular characterises the pathogens associated with hospitalization for diarrhoeal disease among South African children aged less than 5 years

Methods: Children aged < 5 years hospitalized with diarrhoea were enrolled. Standard microbiological methods (culture, biochemical tests, serotyping) and molecular methods (PCR) were used, targeting bacterial pathogens such as diarrhoeagenic *Escherichia coli* (DEC)*, Salmonella* species, *Shigella* species, *Vibrio cholerae* and *Campylobacter* species.

Results: A total of 1816 stool specimens were processed, of which 633 (35%) were positive for enteric bacterial pathogens. Isolates in order of frequency included 562 DEC, 49 *Shigella* spp., 20 *Salmonella* spp., 2 *Campylobacter* spp. There were 48 (8%) enteric bacterial infections identified with more than one pathogen. Co-infections of bacterial pathogens with other organisms include 52 bacterial agents concurrent with *Cryptosporidium* co-infection, 128 with rotavirus co-infection and 9 episodes which included *Cryptosporidium* and rotavirus co-infections.

Conclusion: The overall recovered bacterial pathogens from stool specimens was 35% with DEC being the most commonly identified.

Acknowledgements

The fulfilment of the research arose in part out of years of research that has been done since I joined EDRU and I have worked with a great number of people who contributed to the research and they deserve special mention.

Firstly I would like to record my gratitude to Dr AM Smith for his supervision; advice and guidance from the early stage of this research, as well as giving me extraordinary experience throughout the work, his scientific intuition has made him as a constant oasis of ideas and passion in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist. I gratefully thank Dr KH Keddy the HOD of EDRU for her advice, supervision and crucial contributions, involvement with her originality has triggered and nourished my intellectual maturity that I will benefit from in future. Many thanks to Professor SA Madhi for his supervision, valuable advice and I have benefited from his guidance. The Department of Science and Technology/ National Research Foundation (DST/NRF) South African Research Chair Initiative (SARCHI) in Vaccine Preventable Diseases is acknowledged for providing financial support to my study.

I would like to thank the National Institute for Communicable Diseases a division of National Health Laboratory Services (NHLS) for providing me the opportunity to undertake my masters.

Collective and individual acknowledgements are also owed to my colleagues at EDRU, M Dickmolo; T Gonose; H Ismail; V Moonalall; T Mazibuko; I Mtambo; M Ngomane and A Sooka for their support and laboratory assistance. I would like to thank F Mnyameni for being the first person who taught me to work in ultra high vacuum system.

It is a pleasure to pay tribute also to Rotavirus Surveillance members, C Cohen; L Mlambo; B Malope-Kgokong; J Moyes; V Msimang; S Ntuli; S Walaza; N Page; M Groome; P Bos; J Mphahlele; I Peenze, M Sauermann; M Seheri; M Chhagan; H Dawood; S Haffejee; D Wilson; B Zychska; K Kahn; S Tollman; R Twine; D Steele; U Chetty; B Dooka; M Hlobo; S Kashe; A Koena; M Letyane; T Makgoba; D Makinita; J Malapane; W Malinga; P Moche; N Mofokeng; W Ngubane; M Nkosi; A Sambo; G Senne; N Sigasa and K Shangase for their high quality in sample collection and data management and to all participants who provided stool specimens.

Thanks to the great Lord for the strength to carry on in good and in bad times, but words fail me to express my appreciation to my fiancé G Magadzu whose dedication, love and persistent confidence in me has taken the load off my shoulders.

Where would I be without my family? My parents deserve a special mention for their inseparable support and prayers, my mother Phumudzo Glorious and my dad Nkhumeleni Ulrich Makhari. Thanks to Azwiandi, Nyadzani, Mbilummbi, Mulaifa, Dembe, Ipfi and Gudani for being supportive and caring siblings. I also want to thank my aunts Ndanduleni, Mukondeleli and Ndidzulafhi for their encouragements and support throughout.

A lot of appreciation to the support, advice, encouragement and patience to my friend N Tau with V Magomani, thanks for always being there for me and for not judging me. Finally I want to thank everyone who was important to the successful realization of this dissertation, as well as expressing my apology that I couldn't mention personally one by one.

Contents

List of figures Page

List of tables Page

List of abbreviations

BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
CDC	Centre for Disease Control
CI	confidence interval
cfu	colony-forming unit
CHBH	Chris Hani Baragwanath Hospital
DAEC	diffusely adherent *Escherichia coli*
DCA	deoxycholate citrate agar
DEC	diarrhoeagenic *Escherichia coli*
DGMH	Dr George Mukhari Hospital
DMP	diagnostic media products
DNA	deoxyribonucleic acid
EAggEC	enteroaggregative *Escherichia coli*
EAST	*E. coli* heat-stable-like enterotoxin
EDRU	Enteric Diseases Reference Unit
EDTA	ethylenediaminetetraacetic acid

EHEC	enterohaemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
g	gram
Gas	gas production
GERMS-SA	The Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa
H_2O	water
M	molar
mg	milligram
ml	millilitre
mM	millimolar
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	4-nitroblue tetrazolium chloride
NHLS	National Health Laboratory Services

NICD	National Institute for Communicable Diseases
OR	odds ratio
PCR	polymerase chain reaction
rpm	resolution per minute
SDS	sodium dodecyl sulphate
spp.	species
SSC buffer	saline-sodium citrate
STEC	shiga-toxin producing *Escherichia coli*
TAE buffer	tris-acetate- ethylenediaminetetraacetic acid
TBA	trypticase blood agar
TBE buffer	tris-borate- ethylenediaminetetraacetic acid
TCBS	thiosulfate citrate bile-salt sucrose
TE buffer	tris- ethylenediaminetetraacetic acid
USA	United States of America
XLD	xylose-lysine deoxycholate
%	percent
^{O}C	degree celsius

µl	microlitre
µm	micrometre
µM	micromolar
U	units
~	approximately

Chapter 1: Introduction

1.1 Aetiology of diarrhoeal diseases

Studies conducted between 1980 to 2000 showed a gradual decline worldwide in diarrhoeal mortality and morbidity among children under 5 years of age. Diarrhoea, nevertheless, remains a major cause of mortality and morbidity among children under five years of age, especially in developing countries (1-3).

Acute diarrhoeal disease has significant impact of public health globally, with pathogenic agents such as bacteria (*Salmonella, Shigella,* diarrhoeagenic *Escherichia coli, Vibrio cholerae* [serogroups O1 and O139] and *Campylobacter)*, parasites (*Cryptosporidium, Giardia* and *Entamoeba histolytica*) and viruses (Rotavirus, adenovirus, norovirus and astrovirus), recognized as leading aetiologic agents (4-7). Rotavirus in particular is the leading cause of severe diarrhoea in children under five years of age worldwide and involves significant medical and societal costs (8,9).

There is a paucity of information on the aetiologic agents causing diarrhoeal disease in most African countries, including South Africa (10,11).

Knowledge of the enteropathogens responsible for diarrhoeal diseases is essential for implementation of appropriate public health measures which include effective control or prevention programmes and treatment strategies. South Africa is amongst a few countries in sub-Saharan Africa which has statistical data on the cause of human deaths; however, the cases might be under-registered or misclassified, with 21572 estimated deaths annually amongst children 0-4 years of age (12). Several studies describing childhood diarrhoea have been

published in developing and developed countries, but only a few studies covering a broad range of newly discovered diarrhoeal agents among children have been undertaken in South Africa (2,13,14).

The aim of the study was to determine the bacterial pathogens associated with diarrhoea requiring hospitalization in children under-5 years of age and to characterize circulating serotypes.

1.2Bacterial agents

1.2.1 *Escherichia coli*

E. coli plays a role in the maintenance of normal gut physiology, but certain strains possess virulence factors that enable them to cause disease in the intestinal tract of immuno-compromised individuals or damage intestinal mucosal integrity. Strains of *E. coli* are associated with colonization of gastrointestinal tract of children under five years of age and *E. coli* may cause life-threatening diarrhoea which may be fatal (15). The infection may be associated with contaminated drinking water or food, malnutrition or poor sanitation or poor personal hygiene (16). There are six different pathotypes of diarrhoeagenic *E. coli* (DEC) that are now recognized, which represent a leading bacterial cause of paediatric diarrhoea, however, data regarding their role in community-acquired diarrhoea in developing countries are scarce (15,17).

Classification and taxonomy

E. coli falls under genus of the family Enterobacteriaceae and tribe *Escherichiae.* They are facultative anaerobes and gram-negative bacillus which does not produce spores (18). Most strains of *E. coli* are motile but there are some which are non-motile.

Biochemical characteristics

E. coli is capable of fermenting a wide variety of carbohydrates. Has a characteristic feature of the ability to ferment lactose, even though there are some strains which are slow fermenters of lactose. The biochemical characteristics are summarized in the table 1.1 below.

Table 1.1: Biochemical characteristics of *E. coli* (19)

Test	Reaction
***Ortho*-nitrohenyl-β-D-galactopyranosid (ONPG)**	**+**
Indole	**+**
Methyl red	**+**
Citrate	**-**
Lysine decarboxylase (LDC)	**+**
Arginine dihydrolase	**-**
Ornithine decarboxylase (ODC)	**V**
Motility	**+**
D-Glucose with gas	**+**
Lactose	**+**
Sucrose	**V**
D-manitol	**+**
Adonitol	**-**
D-sorbitol	**+**

(-)-not reactive; (+)-reactive; (V)-variable

Pathogenesis

Some strains of *E. coli* are capable of causing infection in humans. The most important feature of DEC is their capability of colonizing the intestinal mucosal surface and competition for nutrients with other intestinal bacteria. *E. coli* strains possess the surface adherence fimbriae. DEC strains possess specific fimbrial antigens which enhance their ability to colonize and adhere to the small intestinal mucosa. The *E. coli* genome has two genetic components which are virulence related plasmids and chromosomal pathogenicity islands. DEC are categorized into six different pathotypes which are enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and shiga-toxin producing *E. coli* (STEC)[which includes enterohaemorrhagic *E. coli* (EHEC)]. These pathotypes have different virulence properties and have different ways of interacting with the intestinal mucosa. They cause different clinical syndromes, differ in their epidemiologic distributions and falls into distinctive O and H serotypes (15,20).

Enterotoxigenic *Escherichia coli:*

Strains of ETEC initiate infection by attaching to specific receptors on the surface of enterocytes in the small intestine. The virulence determinants involved includes two types of enterotoxin which include LT (oligomeric heat-labile enterotoxin) and ST (monomeric heat-stable enterotoxin). Some strains of ETEC may have only LT or only ST but some have both LT and ST. The LT resembles cholera toxin (CT) from *V. cholerae.* LT is characterized into type I (LTI) and type II (LTII). LTI is a plasmid encoded periplasmic protein and LTII is chromosomally encoded.

LTI and LTII have similar structure and mode of action. In contrast, ST is a peptide which stimulates guanylate cyclase, resulting in intracellular accumulation of cyclic guanylate monophosphate (cGMP), in the intestinal epithelial cells which leads to diarrhoea. ST is classified into two groups (STa and STb) which are found on plasmid with STa associated with human infections. ETEC adherence ability to the intestinal epithelium is mediated by adhesive fimbriae also called *coli surface antigens* (CSAs) (15,21).

Enteroinvasive *Escherichia coli:*

EIEC strain has a preference for colonic mucosal site and it has a close resemblance to *Shigella* species. The virulence determinants involves genes specific for invasiveness which are found on 140-MDa plasmid (pINV). These genes encode invasion plasmid antigens (IpaA, IpaB, IpaC and IpaD) which are required for strains pathogenicity. EIEC are classified by the existence of virulence plasmid (VP) and by their capability to stimulate entry into epithelial cells. The bacterium crosses the epithelial layer in the colonic mucosa by invading M-cells. This leads to membrane ruffling and engulfment of the bacterium within the vacuole where the bacteria lyse the membrane, gaining access to the cell cytoplasm. Infected epithelial cells by EIEC strains may secrete IL-8 which facilitates invasion by bacteria (15,22).

Diffusely adherent *Escherichia coli:*

Initiation of infection due to DAEC strains is due to its ability to adhere to HEp-2 cells and the presence of the Afa/Dr family or adhesins involved in diffuse adherent (AIDA). The ability to cause diarrhoea remains controversial for DAEC (22,23). Virulence factors involved include the Afa/Dr operon which consists of the *afa* gene which is responsible for biosynthesis of Afa

adhesins and Afa invasins. The Afa is involved in internalization of adherent bacteria into epithelial cells which is mediated by the fimbrial structures with one surface fimbria (F1845) encoded via the *daaC* gene (24). DAEC strains may also be associated with the ability to induce inter-leukin-8 (IL-8) secretion in the intestinal epithelial cells (25).

Enteroaggregative *Escherichia coli:*

The ability of EAggEC to initiate diarrhoeal infection involves adherence to intestinal mucosa by aggregative adherence fimbriae (AAF) and fimbriae factor (26). Most strains of EAggEC carry the AAF subtypes which are encoded by the 65-MDa virulence plasmid (pAA) which include *aggA* (AAF/I); *aafA* (AAF/II) and *aag-3* (AAF/III). The AAF/I are associated with HEp-2 adherence and erythrocyte haemagglutination. AAF/III functions as an adhesin. EAggEC also produces plasmid encoded proteins (Pet) which causes cell elongation and exfoliation due to its ability to cleave alpha III spectrin within the cytoskeleton of epithelium cells (25,27). EAggEC strains also produce *E. coli* heat-stable-like enterotoxin (EAST) which interacts with epithelial cells (26,28).

Enteropathogenic *Escherichia coli:*

Important characteristics of EPEC involved in initiation of infection include attaching and effacing (A/E) mediated by local effacement of the microvillus, reorganization of actin filaments in the intestinal cells and intimate adherence between the bacterium and the host's epithelial cell membrane. The genes associated with A/E lesions formation are found on the locus of enterocytes effacement (LEE) on a pathogenicity island of *E. coli* chromosome and it is also considered as a major virulence protein shared by all EPEC strains (15,29). Pathogenesis of EPEC include localized non-intimate attachment of the organism to the intestinal epithelium via the

inducible bundle-forming pili (BFP) encoded by the *bfp* operon. EPEC strains are considered as typical or atypical. They are referred as typical when they posses *E. coli* adherence plasmid (EAF) with *bfp* gene encoding the bundle-forming pilli (BFP) (30). EPEC possesses a chromosomal locus which has type III protein secretion system (TTSS) that transports proteins across the cytoplasmic and outer membrane of the bacterial pathogens and the host cell membrane where they interfere with host cell signalling cascades. The adhesion intimin which is an outer membrane protein encoded by *E. coli* attaching and effacing (*eaeA)* gene is required for intimate adherence of EPEC strains to host cell at the site of A/E lesions and translocated intimin receptor (Tir) (15,31). EPEC strains may also possess genes for the production of EAST and cytolethal distending toxin (CDT). Not all strains of EPECs encode these toxins (15).

Shiga-toxin producing *Escherichia coli* and Enterohaemorrhagic *Escherichia coli:*

STEC/EHEC strains initiate infection by attaching to the intestinal mucosa which is mediated by a plasmid-encoded pilus. These strains also posses the pathogenicity island (LEE), which is the same pathogenicity island found within EPEC strains and they also posses the enterohaemolysin gene (22,32,33). The major virulence genes in the STEC/EHEC strains which contribute to its ability to cause infection include genes encoding Shiga-toxin *(stx*), intimin (*eae*) and haemolysin *(hly).* These genes are responsible for the principal manifestation of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). The production of Shiga-toxin is one of the defining characteristics and it contains two major groups which are Shiga-toxin 1 (encoded by s*tx1*) and Shiga-toxin 2 (encoded by s*tx2*). STEC/EHEC may express s*tx1* only, s*tx2* only or both (34). Shiga-toxin from STEC/EHEC is identical to Shiga-toxin from *S. dysenteriae* type 1 (22) The structural

genes for s*tx1* and s*tx2* are found on lysogenic lambdoid bacteriophage and the genes for s*tx2* are chromosomally encoded (35). Production of Shiga-toxin from *E. coli* is repressed by iron and reduced temperature but expression of s*tx2* is unaffected by these factors. Fluid secretion in response to Shiga-toxin involves killing of absorptive villus tip intestinal epithelial cells and it does not increase active secretion of chloride ions. Shiga-toxin can induce the expression of cytokines such as tumour necrosis factor alpha (TNF-α) and IL-6 from the macrophage. STEC/EHEC possesses the haemolysin plasmid and there are two genetically distinct phage encoded haemolysins (*Ehly1* and *Ehly2*) (15,36). The *eae* gene encodes for intimin, an outer membrane protein required for intimate attachment, allowing the bacteria to adhere to the intestinal mucosa for initiation of infection. Some strains of EHEC has the chromosomal genes eae, same as EPEC which makes EHEC strains to cause more severe illness than STEC because of their ability to attach to the host intestinal cells (33).

Epidemiology

Transmission of DEC is via contaminated food or water with human or animal faeces containing the bacteria, poor sanitation, poor personal hygiene, and rarely person-to-person spread (15,37).

DEC is the most commonly isolated bacteria in children under-5 years of age with diarrhoea, contributing to approximately 40% of diarrhoeal episodes in children globally (38). Many strains of DEC primarily affect children in developing countries due to inadequate sanitary conditions. The prevalence of DEC infection varies by location. It is considered as the most important among bacteria causing childhood diarrhoea in developing countries (20); EPEC is responsible

for approximately 10% of diarrhoeal episodes amongst infants and children under five years of age (39).

STEC/EHEC is considered as the most virulent DEC pathotypes because of their ability to cause outbreaks, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). It is estimated to cause 73000 diarrheal episodes USA annually, 85% of which is attributed to foodborne transmission (40-42). The reservoir for STEC/EHEC is mostly cattle (35). Many outbreaks are associated with beef products and unpasteurized milk. A wide range of other food products implicated to outbreaks include cheese, yoghurt, fermented sausage, apple juice, lettuce etc. Contaminated water is also considered as another route of transmission. It is mostly associated with illness in children aged 6 months to five years of age (43).

In South Africa, DEC is the most commonly detected bacteria amongst children under-5 years of age with diarrhoea, with EPEC and DAEC being the most predominant pathotypes (14,44). In South Africa, EPEC was described as the most commonly isolated DEC pathotype among children with diarrhoea, with a 26.5% isolation rate (14). A study in Tanzania showed high detection of DEC with 35.7% from 451 cases which had diarrhoea, EAggEC being the most commonly isolated with 63% among DEC pathotypes and EIEC with only one case (45). In Mozambique, a study showed a 44.7% recovery of DEC from 520 diarrhoeal cases investigated (46). A study done from Botswana showed low isolation of DEC with 0.5% recovery of the bacterial pathogen from diarrhoeal cases investigated (47).

A recent outbreak which occurred in Germany during May 2011 was caused by an EAggEC pathotype of serotype O104 which produced Shiga-toxin 2 (48). There were more than 3000 cases with 54 fatalities due to the outbreak. More than 800 cases experienced HUS (49).

Clinical features

Several clinical syndromes that occur from infection with pathogenic *E. coli* strains are due to bacteria that acquired genetic elements encoding for virulence factors. The syndromes include enteric/diarrhoeal diseases, urinary tract infection (UTI), sepsis/ meningitis and haemolytic uraemic syndrome (HUS) (26). Different pathotypes are capable of causing various infections in children. All DEC pathotypes are associated with fever and it can be mild for some pathotypes, also vomiting, watery or acute diarrhoea with or without blood and mucus can occur (15). DAEC strains are associated with UTI, cystitis and asymptomatic bacterial infection. UTIs can be found among healthy individuals, persons with compromised urinary tract due to anatomic abnormalities and also with premature death (24). EHEC/ STEC are associated with HUS which is a common cause of acute renal failure in children and chronic kidney disease; bloody diarrhoea, fever and haemorrhagic colitis (35). The incubation period varies from 3 to 4 days after ingestion of contaminated food or water (50).

EAggEC is mostly associated with watery diarrhoea due to host susceptibility, immune response and heterogeneity of virulence among the strains. It can also be due to the amount of bacteria ingested by the infected host (51). EPEC may be associated with acute diarrhoea and severe diarrhoea may also occur during neonatal period. It can also be associated with travellers'

diarrhoea (52). ETEC may cause diarrhoea which might be mild, self-limiting or may result in severe purging similar to diarrhoea caused by *V. cholerae* (15).

Diagnosis

Definitive diagnosis of infection is based on isolation of organisms from food products, stool culture or rectal swab. Diagnosis requires special isolation techniques and specialized media such as MacConkey agar, deoxycholate citrate agar and xylose-lysine deoxycholate agar. If processing is delayed, a rectal swab sample can be placed in Cary and Blair transport media. The STEC/ EHEC O157 strains do not ferment sorbitol; therefore specimens are also cultured onto MacConkey agar with sorbitol which is considered a convenient method for screening of STEC/ EHEC O157 (20,53).

Identification of diarrhoeagenic *E. coli* strains requires that these organisms be differentiated from non-pathogenic strains of *E. coli*. The practical and rapid way of characterizing DEC pathotypes depends on detecting the virulence factors. This can be done by using PCR which detects for virulence genes. PCR methods have shown to be highly specific and sensitive in identification of human DEC. Routine diagnostic laboratories lack the capacity to detect DEC pathotypes because they characterise DEC by biochemical characteristics (54-56). Confirmation of serotypes of DEC strains requires identification of presumptive isolates with O and H antiserum (57).

Phenotypic variability among strains belonging to the same species results in some bacterial isolates presenting characteristics that are atypical for candidate identification. In this situation, 16S rRNA gene sequence analysis can be used for determining identity of these organisms

(58,59). During outbreak investigations, molecular typing methods such as pulsed-field gel electrophoresis (PFGE) is used for investigating of the relationship among strains of the same DEC pathotypes and also to trace back the source of infection (20,60).

1.2.2 Salmonella

Salmonella are one of the leading causes of community acquired foodborne bacterial gastroenteritis worldwide among children under-5 years (61), with high incidence in infants (62). The bacterium is characterized by its flagella (H) antigen, somatic (O) antigen and the polysaccharide (Vi) antigen. The Vi antigen is associated with *Salmonella* Typhi; *Salmonella* Paratyphi C may occasionally have the Vi antigen (63). The infection is mostly associated with contaminated food or water, poor sanitation and poor personal hygiene (64,65).

Classification and taxonomy

Salmonella falls under genus of the family Enterobacteriaceae. *Salmonella* are gram-negative, facultative anaerobic bacilli which do not produce spores (66). The genus of *Salmonella* are separated into two species which includes *Salmonella enterica* containing 6 subspecies (I, II, IIIa, IIIb, IV, and VI) and *Salmonella bongori*. Members of the seven subspecies can be serotyped into one of 2500 serotypes according to antigenically diverse surface structure: somatic (O) antigen and flagella (H) antigen (67).

Biochemical characteristics

The species of *Salmonella* differ in their reaction to certain carbohydrates and the biochemical characteristics are summarized in table 1.2 below.

Table 1.2: Biochemical characteristics of *Salmonella* species (19)

Test	Ssp I	Ssp II	Ssp IIIa	Ssp IIIb	Ssp IV	Ssp VI	*S. bongori*
ONPG	-	-	+	+	-	V	+
Indole	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
LDC	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+
ODC	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+
D-Glucose with gas	+	+	+	+	+	+	+
Lactose	-	-	-	+	-	V	-
Sucrose	-	-	-	-	-	-	-
D-manitol	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-
D-sorbitol	+	+	+	+	+	+	+

(-)-not reactive; (+)-reactive; (v)-variable; (Ssp)-subspecies

Pathogenesis

Salmonella infection begins with ingestion of the organisms. The bacterium crosses the small intestine by translocating through the M-cells and the adjacent epithelium, whereby binding of M-cell is characterized by specialized fimbriae (*Ipf).* Most of the genes controlling entry into M-cells are located on *Salmonella* Pathogenicity Island which contains multiple functionally related genes necessary for specific virulence phenotype. They carry major pathogenicity island 1 and 2 (SPI1 and SPI2), which encode specialized devices for the delivery of virulence proteins into the host (68,69). The SPI1 encodes genes which are necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory response (70,71). In contrast, SPI2 encodes genes important for intracellular replication (72,73). Once inside the M-cells they tend to initiate cytoskeletal rearrangement leading to membrane ruffling and bacterial internalization (74-76). *Salmonella* consist of genes necessary for its survival in the macrophage which includes PhoP/ PhoQ which regulates over 40 genes inducing the expression of PhoP-activated genes (77). These genes are expressed within the macrophage phagosome (78). They also promote antimicrobial resistance (79).

Salmonella associated with intestinal invasion possess plasmids which has the *Salmonella* plasmid virulence (*spv)* gene. The *spv* gene enhances growth of *Salmonella* strains within the macrophages and phagocytic cells. These genes are stimulated by *Rpos,* which is a sigma factor produced due to low concentrations of nutrients (80,81).

Epidemiology

Human infections are acquired via contact with animals or humans shedding S*almonella* or via contaminated environments. The majority of human infections are foodborne. A large number of human outbreaks have been linked to foods of animal origin, with beef products representing a wide range as a source of human infection (64). The faecal-oral route may also be associated with the spread of *Salmonella* infections. Typhoid fever and other enteric fevers are spread mainly from person to person via faecal oral route. Contaminated food and water with human faeces is another route of transmission (65).

Salmonella causes an estimated 1.4 million human cases with 15 thousand hospitalizations and more than 400 deaths annually from the United States (64,82). Globally, human health impact due to non-typhoidal *Salmonella* (NTS) is at an estimate of 93.8 million illnesses, of which 80.3 million illnesses are due to foodborne with 155 thousand deaths annually (83). In developing countries, the mortality rate of invasive NTS in HIV infection may be as high as 23-47% and HIV infection is considered as a risk factor for invasive NTS (84,85).

Diarrhoeal infections due to *Salmonella* infections from Mozambique was 15.8% in 520 diarrhoeal cases investigated (46). Hospitalizations due to invasive NTS infection are estimated at approximately 27% of episodes among African children (86). In sub-Saharan Africa, the occurrence of invasive NTS is higher than the occurrence of typhoidal fever among children (87). A study in Mozambique showed a 15.8% recovery of *Salmonella* species from 520 investigated cases (46).

In South Africa, *Salmonella* infection is commonly reported from Gauteng, Eastern Cape and KwaZulu-Natal provinces and occurs predominantly among children under-5 years of age. *Salmonella* enterica serotype Typhimurium with prevalence of 45% and *Salmonella* Enteritidis with prevalence of 60% were the most commonly isolated non-typhoidal *Salmonella* (44).

Clinical features

Incubation period develops after 6 to 72 hours of ingestion of contaminated food or water. Infection may be prolonged for 10 to 14 days by a low grade fever in rare cases (88). Specific *Salmonella* serotypes produce characteristic syndromes which include gastroenteritis, enteric fever and bacteraemia (89-91).

NTS may occur without gastrointestinal symptoms in children (90). Children infected with NTS primarily suffer from acute diarrhoea which can be self limiting. Children may have complications such of extra-intestinal *Salmonella* infections such as bacteraemia, meningitis or arthritis (92). Enteric (typhoid) fever is a systemic illness which results from infection with the human pathogen, *Salmonella* Typhi and *Salmonella* Paratyphi. The clinical manifestations include high fever, abdominal pain, transient diarrhoea or constipation, severe head ache, loss of appetite and nausea (93).

Diagnosis

Definitive diagnosis of infection is based on isolation of organisms from food products, stool culture or rectal swab. It requires special isolation techniques and specialized media such as MacConkey agar, deoxycholate citrate agar, xylose-lysine deoxycholate agar and selenite broth. If processing of samples is delayed, a rectal swab can be placed in Cary and Blair transport

media. The most commonly used serotyping method is based on the basis of O and H antigenic properties. The O antigen denotes the serogroup and H antigen denotes the serotype. The Vi antigen is specific to *Salmonella* Typhi (94,95). There are molecular sub-typing methods which complement traditional serotyping methods which can be sensitive, reproducible and cost effective which include multiplex PCR (96), real time PCR (97), PFGE and multilocus sequence typing (MLST), multilocus variable number of tandem repeat analysis (MLVA) (64). These methods are also useful during outbreak for comparing patterns to previous patterns of the same serotype.

1.2.3 Shigella

Shigella is amongst one of the gut bacteria which can be pathogenic. It is commonly isolated from patients with diarrhoea. Most of the episodes are seen within children under-5 years and has been recognized as a major cause of epidemics (98-100). High morbidity and mortality associated with *Shigella* infections are due to dysentery (101,102). Infections due to S*higella* species is of global public health concern in developed and developing countries (103).

Classification and taxonomy

Shigella is a genus which falls under the family of Enterobacteriaceae, tribe *Escherichiae.* They are gram-negative rods, facultative anaerobes, non motile bacteria which does not produce spores (66,104,105). *Shigella* is grouped into four species containing 47 serotypes which are classified on the bases of their O antigen. The species include *Shigella dysenteriae* with 13 serotypes*, Shigella flexneri* with 6 serotypes*, Shigella boydii* with 18 serotypes and *Shigella sonnei* with 1 serotype (105-107).

Biochemical characteristics

Shigella species differ in their reaction to certain carbohydrates and their biochemical characteristics are summarized in table 3 below.

Table 1.3: Biochemical characteristics of *Shigella* species (19,108)

Test	*Shigella sonnei*	*Shigella dysenteriae*	*Shigella boydii*	*Shigella flexneri*
Indole	-	V	V	V
LCD	-	-	-	-
ODC	+	-	-	-
Motility	-	-	-	-
Glucose with gas	-	-	-	-
ONPG	+	-	-	-
D-manitol	+	-	+	+

(-)-not reactive; (+)-reactive; (V)-variable

Pathogenesis

Shigella initiates infection after gaining access into the intestinal mucosa. After ingestion of the organism by the host, it crosses the intestinal epithelium by transcytosis via M-cells. It invades the colonic epithelial cells through a type III secretion system which is encoded by a pathogenicity island located on a plasmid (108-110). As the bacterium enters the epithelial cell it causes rearrangement of the cell cytoskeleton resulting in bacterial membrane ruffling and

345 engulfment within the vacuole (111). The bacterium then escapes from the vacuole which
346 results in induction of apoptosis amongst infected cells. This leads to a release of IL-8 pro-
347 inflammatory cytokine which attracts the polymorphonuclear cells (PMN) within the site of
348 infection stimulating invasion by bacteria (112-114). *Shigella* consists of two loci encoded
349 virulence plasmid which include Ipa locus and mxi-spa locus that are required for invasion (115).
350 The Ipa locus is encoded by invasion plasmid antigen which includes IpaA, IpaB, IpaC and IpaD.
351 The mxi-spa locus encodes the components of a type III secretory system involved in the
352 delivery of the Ipa proteins from bacterial cytoplasm to host cell (116,117).

353 *S. dysenteriae* type 1 produces a potent exotoxin (Stx) which causes vascular damage and which
354 also resembles the Shiga-like toxin produced by *E. coli* (118).

355 Epidemiology

356 *Shigella* infection is spread via ingestion of contaminated food and water. Person-to-person and
357 faecal oral route are considered as another mode of transmission (107,119).

358 *Shigella* species are estimated to cause more than 150 million human infections annually, with
359 1.1 million deaths amongst children who are under five years of age living in developing
360 countries (99,120). In developing countries the most prevalent species of *Shigella* causing
361 infections is *S. flexneri*. Outbreaks involving *S. dysenteriae* have also been described. In
362 developed countries, the most prevalent species is *S. sonnei* (99,121,122). A study conducted
363 from Central African Republic in Bangui showed isolation of *S. flexneri* with 10% from 156
364 investigated cases(123) and a study in Kenya also showed a 16% isolation of *Shigella* with *S.*

flexneri as the most commonly isolate with 54% (124). A study in Mozambique showed a 15.4% recover of *Shigella* species from 520 investigated cases (46).

In South Africa, invasive and non-invasive *Shigella* infections are mostly described from Gauteng and Western Cape provinces amongst children under five years of age. *S. flexneri* and *S. sonnei* are the most prevalent species (44). Outbreaks due to *S. flexneri* and *S. sonnei* have been reported from Mpumalanga, Limpopo and Northern Cape provinces of South Africa (125). *S. dysenteriae* type 1 was once described to be associated with an outbreak in KwaZulu-Natal province (126). In South Africa, the incidence of *Shigella* in children under five are not known. In sub-Saharan Africa there is high incidence of *S. flexneri* and *S. dysenteriae* occurrence compared to the occurrence of *S. boydii* and *S. sonnei* (99).

Clinical features

The incubation period after ingestion of the bacterium might be from 12 hours to 2 days before symptoms starts to show (127). *Shigella* has a low infective dose which can range from 10 to 100 organisms. This makes it important for public health point of view (128).

Shigella species causes Shigellosis which can be characterized by dysentery and anorexia (128,129). Dysentery amongst children results in loss of serum protein into faeces. It may also cause complications in infants and young children such as dehydration, rectal prolapsed and intestinal perforation (130). Bacteraemia is rarely associated with Shigellosis although it might be seen amongst malnourished infants and immune-compromised children (121,131). Complications amongst neonates with Shigellosis include septicaemia, meningitis and dehydration (132).

Diagnosis

Definitive diagnosis of infection is based on isolation of organisms from food products, stool culture or rectal swab. Diagnosis requires special isolation techniques and specialized media such as MacConkey agar, deoxycholate citrate agar, xylose-lysine deoxycholate agar and selenite broth. The commonly used serotyping method is based on the basis of O antigenic properties (118,133).

More rapid approach on identification of *Shigella* serotypes can be done using PCR assays based on specific genes, also DNA microarrays which allow detection of *Shigella.* It is considered as more specific, sensitive and reproducible method (134).

1.2.4 Campylobacter

Campylobacter in the human gastrointestinal tract presents both as commensals or pathogen and also in wild or domesticated animals. Infections due to *Campylobacter* are considered zoonotic with poultry as a major source of infection (135-137). C*ampylobacter* is amongst major causes of bacterial diarrhoea within humans worldwide (138). The dominant source of infection includes contact and consumption of poultry, unpasteurised milk and contaminated drinking water (139,140).

Several species of *Campylobacter* are associated with human infections. *Campylobacter jejuni* and *Campylobacter coli* are considered as the most common species infecting humans (141,142), with majority of cases occurring in children under five years (143,144).

Classification and taxonomy

Campylobacter is a genus which falls under the family of Campylobacteraceae (145). *Campylobacter* species are microaerophilic, curved or small spiral-shaped cells, gram negative rods which does not produce spores (146,147). The genus *Campylobacter* has 18 species and not all species are associated with extra and enteric intestinal illness in humans. The *Campylobacter* species which are pathogenic to humans includes *Campylobacter jejuni, Campylobacter coli, Campylobacter upsaliensis, Campylobacter lari, Campylobacter fetus, Campylobacter hyointestinalis, Campylobacter concisus*, *Campylobacter sputorum, Campylobacter curvus* and *Campylobacter rectus* (148,149).

Biochemical characteristics

Campylobacter species have a typical biochemical characteristic and their reaction to certain carbohydrates are summarised in table 1.4 below.

Table 1.4: Biochemical characteristics of enteropathogenic species of *Campylobacter* (145,150)

Test	*Campylobacter coli*	*Campylobacter jejuni*	*Campylobacter lari*	*Campylobacter fetus*
Catalase	**+**	**V**	**+**	**+**
Nitrate	**+**	**+**	**+**	**+**
Hippurate	**-**	**+**	**-**	**-**
Indoxyl acetate	**+**	**+**	**-**	**-**
Nalidixic acid	**S**	**S**	**V**	**R**
Cephalothin	**R**	**S**	**S**	**S**

(S)-susceptible; (R)-resistant; (-)-not reactive; (+)-reactive; (V)-variable

Pathogenesis

Factors responsible for *Campylobacter* species to initiate infection are not well known. *Campylobacter* jejuni is the most studied species. Based on clinical manifestations, mechanisms of infection include adherence and production of toxins; invasion and proliferation within the intestine (151,152). The organism crosses the mucosal layer, whereby the bacteria adhere and invade through the epithelial cell resulting in the damage and inflammation of the mucosal layer (153-155). The adherence and invasion of the epithelial cell by the bacteria is dependent on both motility and flagella expression (156-158). *Campylobacter* have several virulence properties which are involved in initiation of infection. The *cadF* gene which encodes for an outer-membrane protein responsible for invasion and which also acts as an adhesin (159). The bacteria are also associated with cytotoxin production encoded by cytolethal distending toxin

(*cdtA, cdtB* and *cdtC* genes) (160,161). These toxins are responsible for slow distending resulting in cell death (162,163).

Epidemiology

Campylobacter species are fastidious organisms which have a broad range of environmental existence. They can be recovered from river water, vegetables and shellfish but mostly recovered from chicken, sheep, cattle and swine (164).

The infections due to *Campylobacter* are sporadic. They have been associated with contaminated water, unpasteurised milk, poultry, beef and also contact with pets and farm animals (138,165). Infections due to *Campylobacter* species are commonly induced by *C. jejuni,* with *C. coli*, C. *lari*, *C. fetus* and *C. upsaliensis* are less isolated (141,166,167).

Some cases due to *Campylobacter* maybe unreported because infections may be asymptomatic, mild or self limiting. *Campylobacter* infections are estimated to cause 2.5 million cases of diarrhoea annually, with 13 000 hospitalizations and 124 deaths worldwide with infants being the most affected (143,144).

In Africa *Campylobacter* is one of the commonly isolated bacteria amongst children under-5 years, with *C. jejuni* the being the most frequent species of *Campylobacter* (11,168,169). A study conducted in from Kampala-Uganda showed *Campylobacter* species as a frequent cause of acute childhood diarrhoea (170). There was low isolation of *Campylobacter* species from Mozambique with 1.8% detection from 520 investigated diarrhoeal cases (46). A study done from Botswana showed low isolation of *Campylobacter* species with 0.5% recovery of the bacterial pathogen from diarrhoeal cases investigated (47).

458 Clinical features

459 The incubation period after ingestion of *Campylobacter* organisms ranges from 24-72 hours. It
460 can also take longer than a week until symptoms starts to show (171).

461 *Campylobacter* species causes infections which are characterised by an inflammatory diarrhoea,
462 bloody stool and even fever. Some cases cause non-inflammatory diarrhoea with watery stool in
463 the absence of blood may occur, mostly in young children (152,172).

464 There are complications that are associated with *Campylobacter* infection which include
465 Guillain-Barré syndrome (GBS), septic abortion, bacteraemia, cystitis and nephritis (135,173-
466 175). Most infections due to *Campylobacter* result in acute, self limited gastrointestinal illness
467 which may be characterised by diarrhoea, fever and abdominal pains. Such illnesses are usually
468 seen amongst young children (172).

469 Diagnosis

470 Species of *Campylobacter* are culturally fastidious organisms and recovering the organism from
471 specimens is quiet challenging. Isolation, identification and confirmation of *Campylobacter*
472 bacteria require the use of enrichment both after incubation from selective media.
473 *Campylobacter* species are sensitive to dry conditions and they grow best in atmosphere
474 containing 5 – 10% oxygen at 37^{o}C even though some may grow at 42^{o}C. From culture on
475 selective media, the suspected colonies were microscopically examined for Gram stain
476 morphological characteristic such as gull-wing gram negative bacilli (176,177). Culture of
477 *Campylobacter* species requires special isolation techniques and specialized media such as
478 trypticase soya agar with 5% sheep blood or media which contains antibiotics which reduces the

emergence of other enteric organisms and *Campylobacter* are considered as microaerophilic (178).

Other techniques which are also recommended that can be used include filtration method, PCR and enzyme-linked immuno-sorbent assay (179,180). There are commercially available kits such as latex agglutination which can be used for confirmation of *Campylobacter* species (181,182).

Strain differentiation can be analysed with genotyping methods such as flagellin typing, PFGE of chromosomal DNA, ribotyping and multiplex PCR-RFLP (179,183).

1.2.5 *Vibrio cholerae*

Diarrhoeal illness due to *V. cholerae* is considered amongst leading cause of morbidity and mortality within children under-5 years globally (1,2,184). The majority of cases are seen in Africa and Asia (185). The epidemics of diarrhoeal illnesses caused by *V. cholerae* O1 and O139 are associated with poor economic conditions, poor sanitary systems and poor public hygiene (186). The infection is spread via direct faecal oral contamination or contaminated food (184,187). Aquatic environment plays an important ecological and epidemiological role; contaminated water is considered the most common route of transmission (188).

Classification and taxonomy

V. cholerae is a genus which falls under the family Vibrionaceae. *V. cholerae* is a facultative anaerobe, curved and motile gram negative rod which does not produce spores (189,190). There are more than 200 serogroups of *V. cholerae,* which are classified based on variation in the heat-stable somatic (O) antigen of the strain (192). The O antigen classifies *V. cholerae* into serogroup O1 and non-O1. Serogroup O1 is further classified into two biotypes which include

500 classical and El Tor and also into three major serotypes which are Inaba, Ogawa and Hikojima
501 (192-194).

502 Biochemical characteristics

503 *V. cholerae* bacteria is characterised by distinctive reactions with certain carbohydrates; the
504 biochemical characteristics are summarized in table 1.5 below.

505 **Table 1.5:** Biochemical characteristics of *V. cholerae* (195)

Test	Reaction
D-Glucose acid	**+**
D-Glucose gas	**-**
Arginine	**-**
LDC	**+**
ODC	**+**
Sucrose	**+**
Nitrate	**+**
Oxidase	**+**

506 (-)-not reactive; (+)-reactive

507 Pathogenesis of *Vibrio cholerae* O1

508 As *V. cholerae* O1 bacteria are ingested by the host, the bacteria passes through the acid barrier
509 of the stomach, colonizing the epithelial cells via the toxin co-regulated pilus (TCP) (196). The

bacterium has adherence ability to M-cells, producing an enterotoxin secreted from the bacterial outer membrane into the extracellular environment. This results in transport disruption of ion which leads to loss of water and electrolytes (197).

V. cholerae O1 produces cholera toxin (CT) which consists of subunit A and subunit B. Subunit A possesses two components (A_1 and A_2) which are linked by two disulfide bond. Activation of A_1 component by adenylate cyclase leads to an increase in cyclic adenosine monophosphate, blocking the absorption of sodium and chloride by microvillus (198).

V. cholerae have several virulence properties involved in initiation of infection which includes the CT, *zot* (199), *cep* (200), *ace*, *orfU* (201), *tcpA* (202,203) and *toxR* (204).

Epidemiology

Infection caused by *V. cholerae* O1 can be spread via direct faecal oral route and ingestion of contaminated food. Water is the most important source of transmission of cholera (184,205). Cholera outbreak causes more than 100 thousand deaths worldwide with majority being children. It is difficult to control Cholera; some people may not show symptoms due to Cholera (206).

Infections due to *V. cholerae* are endemic in Southern Africa and outbreaks have been reported from countries including Zimbabwe. Cholera outbreaks cause alarm, disruption and a slowdown in development within affected places (207,208). A Zimbabwean outbreak caused 93592 cases with a fatality rate of 4.3%. The outbreak was caused by *V. cholerae* serogroup O1 of either Ogawa or Inaba (209). This outbreak was linked to a South African outbreak which caused approximately 12 705 cases, with 65 deaths in November 2008 to April 2009 (210). *V. cholerae*

serogroups which are associated with pandemic cholera outbreaks include serogroup O1 and more rarely, serogroup O139. Non-O1 serogroups associated with localised outbreaks may also occur. The El Tor biotype is responsible for the current pandemic (186,211).

In Haiti, during October 2010, a cholera outbreak started which was caused by a *V. cholerae* O1 biotype El Tor strain; the outbreak is still ongoing; 121518 cases and 2591 deaths were reported in December 2010 (212).

Clinical features

After ingestion of *V. cholerae* organisms, the incubation period commonly ranges from 12-72 hours before symptoms starts to show. The clinical signs associated with loss of fluid due to cholera include extensive thirst, hypotension, weakness, fatigue and rice watery stool (213,214).

There are complications associated within cholera that is observed amongst patients which may result from improper treatment or diagnosis such as acute renal failure, hypoglycaemia and premature delivery (1,185,215).

Diagnosis

V. cholerae survives well in faecal specimens if kept moist. However if there is a delay in specimens processing due to transportation, the specimen may be kept in a transport media such as Cary and Blair. The recovery is done through plating on thiosulphate citrate bile salts sucrose (TCBS) agar medium which inhibits growth of most gastrointestinal organisms and supports the growth of *V. cholerae* bacteria. From culture on selective media, the suspected colonies were microscopically examined for Gram stain morphological characteristic such as

551 comma shape gram negative bacilli. The stool specimen can be examined with a microscope for
552 appearance of the organism (203,216,217).

553 There are molecular typing methods which include PFGE (218), amplified fragment length
554 polymorphism (219), ribotyping (220), and multilocus sequence typing (221,222) which are used
555 to characterise the molecular epidemiology of the organism. PCR can also be used as a quick
556 and useful molecular method for detection of the bacteria (223,224).

557 **1.3 Viral agents**

558 Viral diarrhoea among children under five years of age is considered as a major cause of
559 mortality and morbidity worldwide. Several groups of enteric viruses are responsible for acute
560 viral diarrhoea amongst children during their first years of life. Three major categories of viruses
561 are recognized by their clinical importance and they include reoviruses (Rotavirus), astrovirus
562 and adenovirus (4,225).

563 Rotavirus is one of the important etiological agents in severe dehydrating diarrhoea within
564 children. Serotypes such as G1P[8], G2P[4], G3P[8], G4P[8] AND G9P[8] are considered as
565 important cause of diarrhoea among children under-5 years worldwide (226). Astrovirus have
566 eight serotypes responsible for diarrhoea (227) and a limited number of adenovirus strains have
567 been associated with childhood diarrhoea which includes type 40 and type 41strains (228).

568 Viral gastroenteritis is transmitted via person-to-person by faecal oral route, faecally
569 contaminated water and infected fomites or surfaces (229). Globally approximately 40% of
570 hospitalizations and death amongst children under-5 years due to diarrhoea are attributed to
571 rotavirus infection. It occurs in a sporadic seasonal form. It can also occur as a severe

gastroenteritis of infants and younger children of 2-3 years with a peak at the age of 6-24 months (230).

Control measures include disinfecting environmental surfaces with effective agents such as chlorhexidine gluconate, quaternary ammonium and 95% ethanol. There are vaccines such as RotaRix and RotaTeq vaccines, their trials were effective against severe Rotavirus gastroenteritis. Vaccines are now administered to children in countries as part of their immunization schedule (231). These vaccines were introduced in South Africa and Malawi during the year 2009 to look at the effectiveness of Rotavirus vaccine once it was introduced into the immunisation programme. As part of the surveiilance, the bacterial aetiology of diarrhoeal disease was also explored in era of Rotavirus vaccine(232).

1.4 Parasitic agents

Gastrointestinal parasitic infections are globally endemic. They have been described continuously as the cause of illness and disease. They are linked to lack of sanitation, lack of access to safe drinking water and improper hygiene. They are also associated with poverty. People of all age group are affected by the prevalence of parasitic infections and children are the worst affected. Soil-transmitted helminths are the most common intestinal parasites and they include *Ascaris lumbricoids, Trichuris trichiura* and hookworms. They are not commonly known to cause diarrhoea (233).

In Sub-Saharan Africa it is estimated that approximately a quarter of the total population is infected with one or more helminths parasites but nematode worms are also among prevalent gastrointestinal parasites (234,235).

Common intestinal protozoan's which cause significant morbidity in children and immuno-compromised patients include *Entamoeba histolytica, Giardia intestinalis* and *Cryptosporidium parvum. Giardia intestinalis* is the most prevalent protozoan worldwide with approximately 200 million people being affected (236-238).

Public health interventions such as provision of clean drinking water, community health education; observation of food hygiene and maintenance of functioning sanitation systems are essential to long term control in communities (239).

1.5 Aim and specific objectives

Aim

To characterise the bacterial aetiologies and molecular characterises the pathogens associated with hospitalization for diarrhoeal disease among South African children aged less than 5 years

Specific objectives

- To describe the prevalence of bacterial pathogens in children under-5 years hospitalized for diarrhoea.
- To describe the prevalence of co-infections between bacteria and other bacteria, rotavirus and parasitic infections.
- To describe geographical distributions of bacterial pathogens within areas such as rural and urban areas.
- To investigate the usefulness of bacterial colony blots and DNA probing, as a method for separating and identifying individual strains of co-infections of DEC.

Chapter 2: Methods and Materials

This project was a descriptive epidemiological surveillance study.

2.1 Study sites

The study was conducted from four surveillance sites which included Gauteng Province (Chris Hani Baragwanath Hospital); Mpumalanga Province (Mapulaneng Hospital and Matikwana Hospital) and North-West Province (Dr George Mukhari Hospital). Since different geographical sites tend to differ in the detection rates of diarrhoeal bacterial pathogens, the surveillance sites were divided according to their geographic variations which included urban area (Gauteng Province and North-West Province) and rural area (Mpumalanga Province) (240).The hospitals are situated as indicated on figure 2.1 below.

Figure 2.1: Rotavirus Surveillance sites (Dr GMH: Dr George Mukhari Hospital, CHBH: Chris Hani Baragwanath Hospital, Agincourt: Mapulaneng and Matikwana Hospitals) (241)

2.2 Participant enrolment and specimen collection

Stool specimens were collected by Surveillance Officers from children under-5 years admitted to the study site hospital (at least slept overnight in hospital), who presented with 3 or more episodes of loose stool over the period of 24 hours (240).

2.2.1 Inclusion criteria

All children under five years of age who slept overnight in a sentinel surveillance hospital or who presented to a sentinel clinic, who meet the case definition for diarrhoea and resided in the geographical area under surveillance were eligible for inclusion. Two separate severe gastroenteritis episodes were separated at least seven days between date of discharge and date of onset of the new episode. Residence in the surveillance area were defined as sleeping in the area for at least one week prior to onset of illness (240).

2.2.2 Exclusion criteria

All children who resided outside the defined geographical area and above the age of five years old were excluded. Children with onset of diarrhoea >14 days before admission were not enrolled. Children re-submitted within seven days of a previous episode of diarrhoea were not considered as a new case. Patients who refused consent were included in the surveillance programme (240).

2.2.3 Sampling

All children who meet the inclusion criteria were enrolled. At sentinel clinics systemic sampling (e.g. every 2nd case) was used if numbers of cases was too great for the surveillance officer to capture (240).

2.3 Laboratory procedures

2.3.1 Processing of stool swab specimens

The study was undertaken from April 2009 to June 2011. The swabs of stool were transported to EDRU in Cary and Blair transport media [Diagnostic Media Products (DMP), NHLS, Sandringham, Johannesburg, South Africa] (242). There was a hierachy to testing of samples for different pathogens based on stool availability. It was Rotavirus, then bacteria and then parasites if the stool was still enough. The length of time it took for specimens to reach the reference laboratory from the time the specimen was collected from the sentinel site had a mean time of five days. The stool swabs were inoculated into 2ml of saline (DMP) and were vortex mixed. For isolation of diarrhoeagenic *E. coli* (DEC), *Shigella* species, *Salmonella* species, *V. cholerae,* the suspension was swabbed onto MacConkey agar (DMP), MacConkey agar with sorbitol (DMP), xylose-lysine deoxycholate (XLD) agar (DMP), deoxycholate citrate agar (DCA) (DMP), thiosulfate citrate bile-salt sucrose (TCBS) agar (DMP) and inoculated into SeleniteF medium (DMP). The media was incubated aerobically at 37^{o}C for 18-24 hours (207,243).

For isolation of *Campylobacter* species two methods were used. Firstly the suspension was swabbed onto *Campylobacter* selective media (DMP), which was incubated in microaerophilic conditions at 42^{o}C for 48-96 hours (207,244). The agar plates which had growth after 48 hours were further processed. However the agar plates which had no growth were further incubated for the maximum of 96 hours. Secondly the filtration method was used for isolation of *Campylobacter* species, using a cellulose nitrate filter membrane (0.65µm pore size) (Sartorius-Stedim Biotech GmbH, Germany, USA) laid onto tryptose agar with 5 % sheep blood (TBA) (DMP). Four to 6 drops of suspension were inoculated onto the filter membrane. The

membrane was dried at room temperature for 10 to 15 minutes. Then filter was removed from agar with forceps and agar was incubated in microaerophilic conditions at 42^{o}C for 48-96 hours (180).

The detection of both *Cryptosporidium* and rotavirus was done by the NICD as part of the larger study. For identification of parasites such as *Cryptosporidium; Ascaris lumbricoids*; *Giardia intestinalis; Entamoeba histolytica* etc, stool specimens were analysed at the Parasitology Reference Unit, of the NICD with methods such as direct smear, Ziehl-Neelsen stain and formal ether sedimentation techniques. Identification of rotavirus was undertaken at the Viral Gastrointestinal Unit, of the NICD, with methods such as enzyme immunoassay and RT-PCR (240).

2.3.2 Detection and picking of suspected bacterial colonies

2.3.2.1 *Escherichia coli*

Suspected colonies of *E. coli* on MacConkey agar (DMP) were either dry pink with a surrounding pink area of precipitated bile salt; mucoid or colourless; on MacConkey agar with Sorbitol (DMP) colonies were colourless for suspected shiga-toxin producing *E. coli* (STEC) [which includes the enterohaemorrhagic *E. coli* (EHEC)] serotype O157; on XLD agar (DMP) colonies were colourless or yellow and on DCA (DMP) colonies were colourless or pink (207).

2.3.2.2 Salmonella species

Suspected colonies of *Salmonella* species on MacConkey agar (DMP) were colourless; on XLD agar (DMP) and DCA (DMP) colonies were colourless with a black spot (H_2S) in the middle (207).

689 *2.3.2.3 Shigella* species

690 Suspected colonies of *Shigella* species on MacConkey agar (DMP) were colourless; on XLD agar
691 (DMP) and DCA (DMP) colonies were colourless and small (207,243).

692 2.3.2.4 *Campylobacter* species

693 Suspected colonies of *Campylobacter* species on *Campylobacter* selective media and TBA (DMP)
694 were flat, grey, irregular and some colonies would spread along the streak line; raised and
695 round with mucoid appearance (244).

696 3.3.2.5 *Vibrio cholerae*

697 Suspected colonies of *V. cholerae* on MacConkey agar (DMP) were colourless; on XLD agar
698 (DMP) and DCA (DMP) colonies were colourless and on TCBS agar (DMP) colonies were shiny
699 yellow (243).

700 **2.3.3 Biochemical identification**

701 The Vitek- 2 Compact identification system (bioMérieux, Marcy l'Etoile, France) was used. All
702 suspected colonies of *E. coli*, *Shigella* species, *Salmonella* species, *V. cholerae* were further
703 analyzed biochemically using the Vitek- 2 Compact gram negative identification cards (GN)
704 (bioMérieux) (245,246). All suspected colonies of *Campylobacter* species were analyzed by the
705 Gram staining technique (247) and suspected curved rods were further analyzed biochemically
706 using the Vitek- 2 Compact *Neisseria-Haemophilus* identification cards (NH) (bioMérieux) (245).

707 The Vitek identification procedure was performed as described by the manufacturer's manual
708 and the method is as follows: the suspected colonies are inoculated onto Columbia agar (DMP)

and incubated at 37^{o}C for 18-24 hours. Following incubation the culture was emulsified in a plastic tube which contained 3ml saline (DMP) to a turbidity of 0.5-0.63 McFarland. The detection card was inserted into the tube that contained the suspension. The cards were scanned and the cassette was placed in a Vitek 2 compact machine. From the Vitek 2 Compact system, the cards were filled with the suspension and then the cassette was transferred to an incubator which determines the biochemical reaction (245).

2.3.4 Serotyping

2.3.4.1 *Salmonella* species

O-Phase determination

When biochemical reactions confirmed an isolate as *Salmonella* species, the isolate was inoculated onto tryptose slope media (DMP) and incubated at 37^{o}C for 18- 24 hours. Three drops of saline (DMP) was added to tryptose slope agar (DMP) which contained the culture and emulsified to obtain a milky suspension. A loop full of bacterial suspension was placed on a microscope slide and mixed with saline (DMP) by tilting the slide back and forth then agglutination was inspected. If agglutination was not observed, serotyping was continued with a loop full of bacterial suspension mixed with O-polyvalent antisera (Mast Assure, Mast Group Ltd., Merseyside, United Kingdom) on a microscopic slide by tilting the slide back and forth. As agglutination was observed with specific polyvalent antisera (Mast Assure) serotyping was continued. A loop full of bacterial suspension was added with a drop of specific O-monovalent antisera (Mast Assure) on a microscope slide and mixed by tilting the slide back and forth. If agglutination was observed in saline (DMP), the isolate was sub-cultured onto blood agar and

re-serotyped. If the isolate remained auto-agglutinating in saline (DMP), a heavy suspension was made in normal saline and was boiled (95^{o}C) for 10 seconds. Then the isolate was re-serotyped (248).

H-Phase determination

Swarm agar (DMP) was autoclaved, poured into a sterile Petri-dish plate and was allowed to set at room temperature. The isolate was inoculated onto the centre of swarm agar (DMP) and was incubated at 37^{o}C for 18- 24 hours in an upright position. Swarm agar promotes improved formation of the H antigen and the bacteria are able to swarm well over the medium. Small amount of growth was placed onto a microscope slide and mixed with a drop of H-polyvalent antisera (Mast Assure) with a sterile loop and agglutination was inspected. As agglutination was observed with specific polyvalent antisera (Mast Assure), serotyping was continued. A small amount of bacterial growth was mixed with a drop of specific monovalent antisera (Mast Assure) on a microscope slide and mixed by tilting the slide back and forth (248). The used positive control strains included *Salmonella* Typhi (NCTC8382) and *Salmonella* Isangi (WHO 578).

2.3.4.2 *Shigella* species

When biochemical reactions confirmed an isolate as *Shigella* species, the isolate was inoculated onto Columbia agar (DMP) and incubated at 37^{o}C for 18-24 hours. A small amount of bacterial growth was placed on a microscope slide and mixed with saline (DMP) by tilting the slide back and forth and agglutination was inspected. If agglutination was not observed, the bacterial

751 growth was emulsified with a drop of *S. flexneri* polyvalent/ *S. boydii* polyvalent/ *S. sonnei*
752 polyvalent/ *S. dysenteriae* polyvalent-O antisera (Mast Assure) and mixed by tilting the slide
753 back and forth. If agglutination was observed with specific polyvalent antisera (Mast Assure),
754 serotyping was continued with appropriate monovalent antisera (Mast Assure). If the isolate
755 auto-agglutinated in saline, the isolate was sub-cultured onto Columbia agar and was incubated
756 at 37^{o}C for 18-24 hours and serotyping was continued. If the isolate remained auto-
757 agglutinating in saline, a heavy suspension was made in saline (DMP) and was boiled for 10
758 seconds. Then the isolate was re-serotyped (243). The used positive control strains included *S.*
759 *dysenteriae* type 1 (ATCC49546) and *S. sonnei* phase2 (ATCC9290).

760 2.3.4.3 Diarrhoeagenic *Escherichia coli*

761 An isolate considered as *E. coli* biochemically and confirmed as DEC by multiplex PCR, was
762 incubated onto Columbia agar (DMP) at 37^{o}C for 18- 24 hours. A single colony was inoculated
763 into 15ml nutrient beef broth (DMP) and was incubated at 37^{o}C for 18- 24 hours. The lid of the
764 bottle was removed and replaced with a non-absorbent cotton wool and the broth was
765 incubated in a boiling (95^{o}C) water bath for 1 hour. The boiled culture was allowed to cool at
766 room temperature and the cotton wool was discarded and the bottle top was replaced. The
767 broth was placed in the fridge. The broth was removed from the fridge and placed on the bench
768 top to reach room temperature while being inverted for 3 to 6 times every 30 minutes. Sixty
769 microlitre of the boiled suspension was added to 60µl of saline (DMP) in glass tube and was
770 inspected for auto-agglutination. Sixty microlitre of the boiled suspension was added to 60µl of
771 O-polyvalent antisera (Mast Assure) in a glass tube. The tubes were gently shaken and were
772 covered with aluminium foil and incubated at 52^{o}C water bath for 18- 24 hours. The tubes were

773 removed and read against a black background. As agglutination was observed, serotyping was
774 continued with appropriate monovalent antisera (Mast Assure) (57).

775 **2.3.5 Preparation of crude genomic DNA from bacteria**

776 Isolates were incubated onto Columbia agar (DMP) at 37^{o}C for 18-24 hours. A sterilized wire
777 loop was used to collect a half loop (1µl) full of bacterial culture. The culture was inoculated into
778 1.5ml tube which contained 400µl of autoclaved TE buffer (appendices 6.1). The tube was
779 vortex mixed for 5 seconds and was incubated in a heating block at 95^{o}C for 25 minutes. The
780 tube was vortex mixed for 10 seconds and centrifuged at 3000-12000rpm for 3 minutes. As the
781 pellet had settled, 50µl of the supernatant was transferred to a 1.5ml tube and was stored at 2-
782 8^{o}C fridge (249). The stored supernatant was further used as a template for PCR.

783 **2.3.6 Identification of diarrhoeagenic *Escherichia coli* using PCR**

784 PCR was comprised of three separate multiplex PCR reactions (Reactions A, B and C); these PCRs
785 were used to detect for virulence genes associated with different pathotypes of DEC. PCR
786 primers (appendices 6.2), interpretation of results (appendices 6.3, table 6.1) and PCR product
787 sizes (appendices 6.3, table 6.2) are described in the appendices sections. The positive controls
788 were used for confirmation of PCR results (appendices 6.2). The PCR reactions were prepared in
789 0.2ml tubes and the reaction mixture of 25µl contained 7 components as listed below. The
790 negative control was also prepared in a 0.2ml tube with a mixture of 25µl containing 6
791 components with no DNA template.

(i) Autoclaved de-ionized water 15µl

(ii) PCR Gold buffer with no $MgCl_2$ (10X) 2.5µl

(iii)	$MgCl_2$ (25mM)	1.5µl (1.35µl for reaction A)
(iv)	dNTPs mix (2.5 mM)	2µl
(v)	Primer mix (20 µM) (appendices 6.2)	3µl
(vi)	Amplitaq Gold DNA Polymerase (5 U/µl)	0.3µl
(vii)	DNA template (2.3.5)	1µl

Cycling conditions were as follows: one starting cycle at 95°C for 7 minutes, 35 cycles of denaturation at 94°C for 75 seconds, primer annealing at 60°C for 90 seconds and primer extension at 72°C for 90 seconds; one final cycle at 72°C for 5 minutes. Following PCR, reactions were stored at 4^oC (55,249,250).

2.3.7 Agarose gel electrophoresis for analysis of PCR products

Twenty-five microlitre of PCR products was mixed with 3µl of loading dye (appendices 6.1). From the mixture, 6µl of the mixture was loaded to the wells of a prepared 1.5% agarose gel (appendices 6.3) and also 3µl of HyperLadder IV (Bioline, Boston, USA) was loaded as a DNA marker at separate wells of the gel. Electrophoresis was performed (appendices 6.3). The gel image was captured using the Molecular Image® Gel Doc™ XR System (Bio-Rad Laboratories Inc, Hercules, USA) (appendices 6.3) (249).

2.3.8 Colony blotting and DNA probing

The isolates were plated onto Columbia agar (DMP), and incubated at 37^oC for 18-24 hours. Growth from the agar plate was swabbed with a sterile swab (DMP) and inoculated into saline (DMP). Turbidity of 0.90-1.30 ($6X10^6$ to $8X10^6$ cfu/ml) was measured with a Vitek Densichek

(bioMérieux) and from the suspension; 1ml of suspension was added to 9ml of saline (DMP) for 10^{-6} target dilution. The serial 10-fold dilutions in saline were performed until a final dilution of 10^{-6} was obtained. From the 10^{-6} dilution, 300µl was dispensed onto Mueller Hinton agar (DMP) and a glass rod was used to spread the suspension over the agar plate. The glass rod was dipped into absolute ethanol (Merck) and flamed in a Bunsen burner for sterilization. The Mueller Hinton agar (DMP) was incubated at 37^{o}C for 18- 24 hours. Mueller Hinton agar (DMP) plate that contained colonies was pre-cooled for 30 minutes and nylon membrane (82mm diameter) (Roche Diagnostics GmbH) was laid onto the surface of the pre-cooled plate for 2 minutes. The membrane was removed with forceps, laid onto a new plate of Columbia agar (DMP) and was incubated for four hours at 37^{o}C incubator. The membrane was removed with forceps and was laid onto a plastic film which contained 1ml of denaturation solution (appendices 6.4.1) and was incubated for 15 minutes. The membrane was air dried on a new plastic film for 1 minute and it was transferred onto a plastic film which contained 1ml of neutralization solution (appendices 6.4.1) and was incubated for 15 minutes. The membrane was air dried on a new plastic film for 1 minute and it was transferred onto a plastic film which contained 1ml of 2X SSC buffer (appendices 6.4.1) and was incubated for 10 minutes. The membrane was then incubated at 80^{o}C for 30 minutes. The membrane was incubated at 37^{o}C for 1 hour with 0.5ml Proteinase K solution (appendices 6.4.1). The membrane was pre-hybridized using the Hybridiser HB-1D (Techne Ltd, Duxford Cambridge, UK) at 42^{o}C with 15ml of DIG easy hybrid (Roche Diagnostics GmbH) for 1 hour. The prepared DIG-labelled DNA probe (appendices 6.4.2, table 6.4) (Roche Diagnostics GmbH) was denatured at 95^{o}C for 5 minutes and was rapidly placed in ice. Then the membrane was hybridized with the denatured DIG- labelled DNA probe at 42^{o}C for 2 hours. The

membrane was washed twice with low stringency buffer (appendices 6.4.1) at 25^{o}C for 5 minutes and the membrane was washed again twice with high stringency buffer (appendices 6.4.1) at 68^{o}C for 15 minutes. The membrane was washed briefly with washing buffer (Roche Diagnostics GmbH) for 3 minutes. The membrane was blocked with 40ml blocking solution (Roche Diagnostics GmbH) for 30 minutes. The membrane was hybridized with 15ml Antibody solution (Roche Diagnostics GmbH) at 25^{o}C for 30 minutes. The membrane was washed twice with 15ml of washing buffer (Roche Diagnostics GmbH) for 15 minutes. The membrane was equilibrated with 15ml detection buffer (Roche Diagnostics GmbH) for 3 minutes. The membrane was covered with chromogenic AP substrate solution (appendices 6.4.1) and was incubated in the dark for 18 hours. The reaction was stopped with 50ml TE buffer (appendices 6.1) for 5 minutes and the membranes was visually inspected for colonies which showed colour signals. The positive controls were used against all tested samples for confirmation of signals on the blots (251).

2.3.9 Storage of enteric pathogens

The isolates were plated onto Columbia agar (DMP), and incubated at 37^{o}C for 18- 24 hours. The bacterial culture was swabbed from Columbia agar into a 1.5 ml cryo-tube which contained 1ml tryptic soy broth with 10% glycerol (DMP). The cryo-tube was labelled with the reference number, isolate name and date of storage. The cryo-tube was stored in a freezer at -70^{o}C (242).

2.3.10 Statistical analysis

Epi Info software version 6 (CDC, Atlanta, GA, USA) was used for all statistical analysis. Mantel-Haenzel chi-squared test was used for determining proportion of bacterial pathogens positivity.

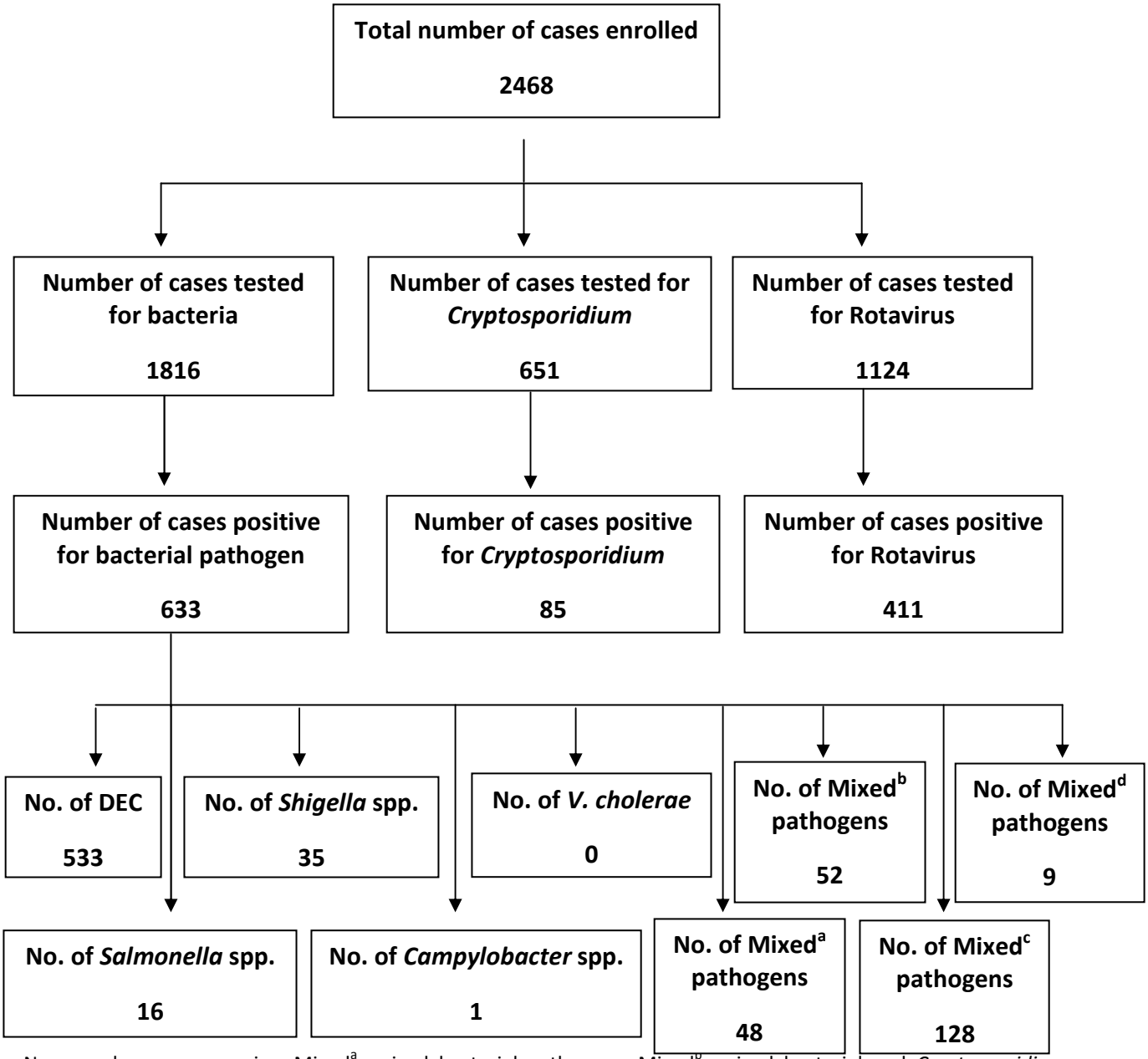
The test was also used to compare the gender distribution amongst all the tested candidates. For all statistical analysis, $p < 0.05$ was regarded as statistically significant.

2.4 Limitations

- The stool specimens were not collected during weekends and public holidays.
- A stringent case definition was used to include children under five years of age with symptoms of three or more loose stool in the past 24 hours who slept overnight at a sentinel hospital. Children under five years of age who were not admitted were therefore excluded and these children may have been more representative of bacterial causes of diarrhoea.
- The use of unmodified Cary and Blair transport media caused a low recovery of *Campylobacter* spp. among collected stool specimens of children.
- To maximise identification of bacteria, a sweep of stool was planted onto different specialised media for culture of bacteria, which might cause suppression of other bacterial pathogens.

Chapter 3: Results

The study was undertaken from April 2009 to June 2011. A total of 1816 specimens were processed for bacterial pathogens of which 633 (35%) cases were positive for a bacterial pathogen. Of the 633 cases of bacterial positives, 562 (89%) cases were positive for DEC, 49 (8%) cases were positive for *Shigella* species, 20 (3%) cases were positive for *Salmonella* species and 2 (0.3%) cases were positive for *Campylobacter* species. There were 48 (8%) cases of enteric bacterial infections identified with more than one bacterial pathogen. There were 52 cases of bacterial agents concurrent with *Cryptosporidium* co-infection, 128 cases with rotavirus co-infection and 9 cases episodes which included *Cryptosporidium* and rotavirus co-infections (Figure 3.1).



No.- number, spp.- species, Mixed[a]- mixed bacterial pathogens, Mixed[b]- mixed bacterial and *Cryptosporidium*, Mixed[c]- mixed bacterial and Rotavirus, Mixed[d]- mixed bacterial pathogen with *Cryptosporidium* and Rotavirus

Figure 3.1: Number of Diarrhoeal cases (April 2009 to June 2011), including number of cases tested and number of positives for bacterial aetiological agents.

The highest number of monthly specimens received occurred over the period of February 2010 to July 2010, ranging from 90 specimens to 170 specimens. The bacterial positivity rate was low ranging from 20% to 45% compared to other months with bacterial positivity rate ranging from 55% to 65% (Figure 3.2). From the collected data, when investigated by month, there was a bacterial positivity rate peak during summer season from November 2009 to January 2010 (Figure 3.2).

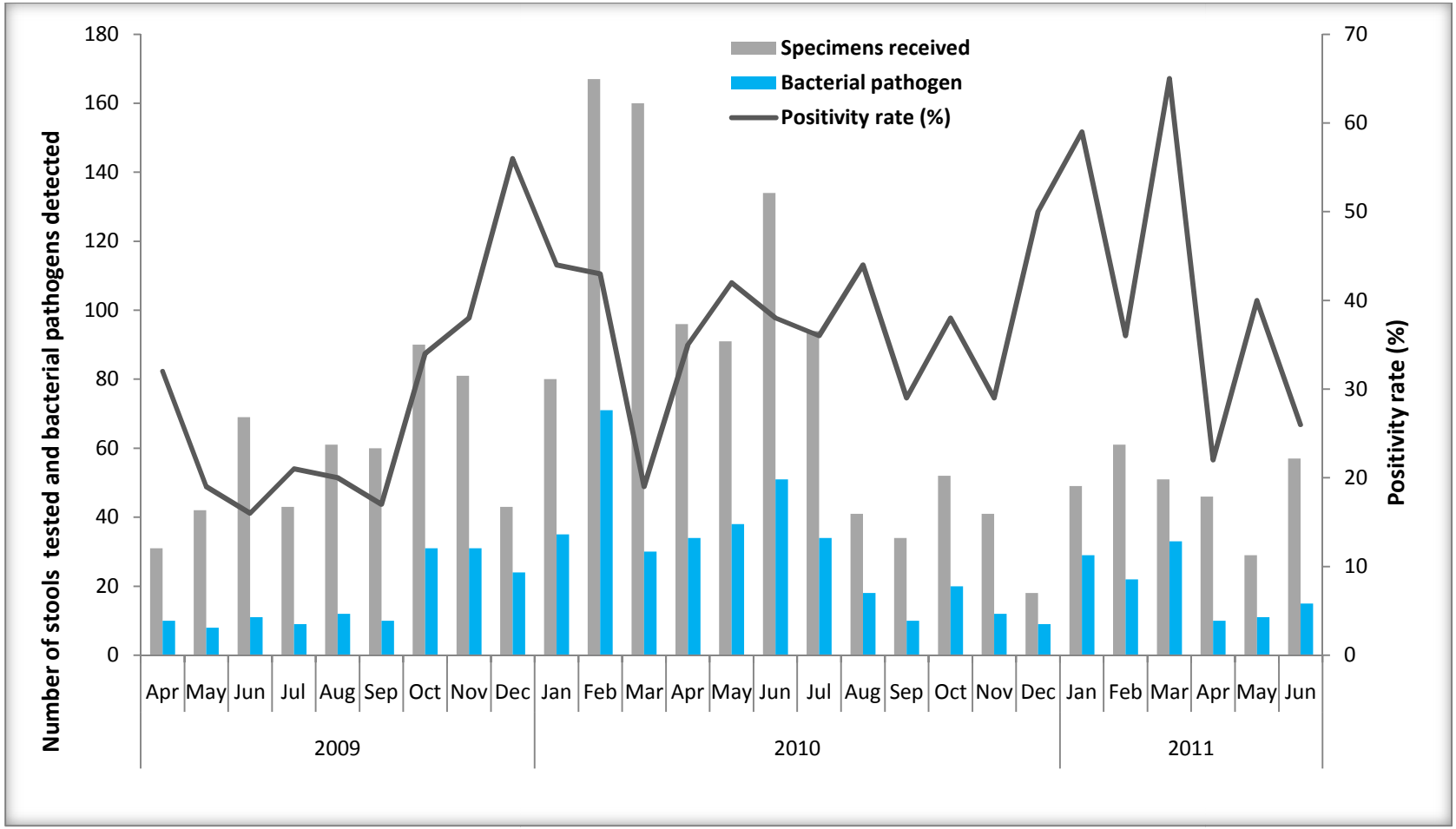


Figure 3.2: Number of diarrhoeal cases, number of enteric bacterial pathogens identified from cases and the bacterial positivity rate identified from all study sites during April 2009 to June 2011

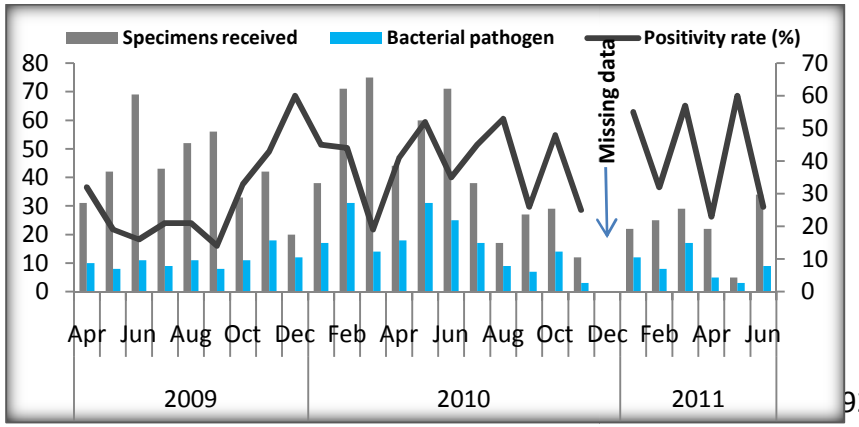
CHBH was the first surveillance hospital to start with the study and it started during April 2009. From CHBH, occurrence of diarrhoeal cases was more or less stable with the number of specimens received ranging from 5-75 cases over a month (Figure 3.3).

Matikwana Hospital commenced in August 2009 (Figure 3.3). As the hospital began the project, the number of received specimens from the hospital was low. Then, from the beginning of January 2010, the total number of received specimens increased ranging from 5-45 cases over a month, with a bacterial positivity rate high during summer season (November 2009 to January 2010). For the period of December 2010 to June 2011, the bacterial positivity rate was high with low number of specimens received (Figure 3.3).

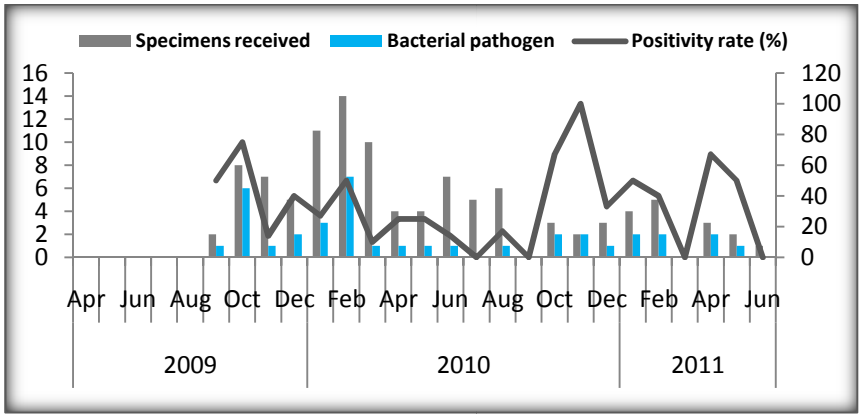
Mapulaneng Hospital only commenced with the project a month later than Matikwana Hospital from September 2009. The number of specimens received from Mapulaneng was low ranging from 2 cases to 16 cases over a month. There was no bacterial positivity rate peak seen during summer season.

DGMH commenced with the project during October 2009. The number of specimens received ranged from 0-55 over a month. When DGMH started with the study there, were high number of specimens received. During the summer season (November 2010 to January 2011) there was a drop in the number of specimens (Figure 3.3). From February to July of 2010, there was an increase in the number of specimens received. However the bacterial positivity rate was low, ranging from 5% to 40% compared to other months with bacterial positivity rates ranging from 60%-70% (Figure 3.3).

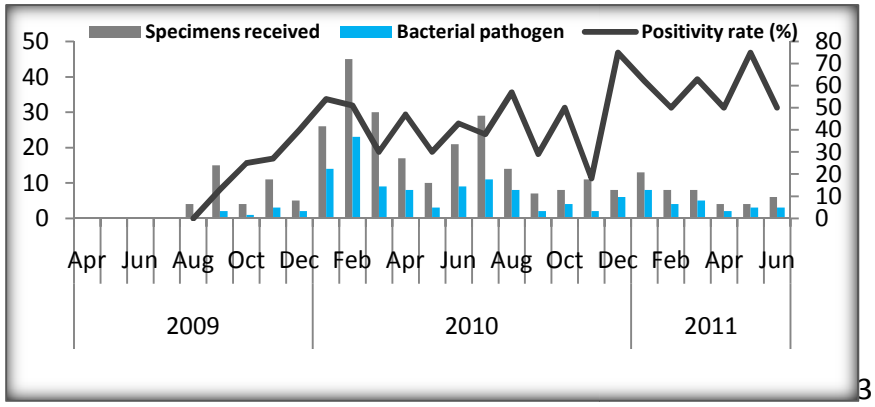
Chris Hani Baragwanath Hospital



Mapulaneng Hospital



Matikwana Hospital



Dr George Mukhari Hospital

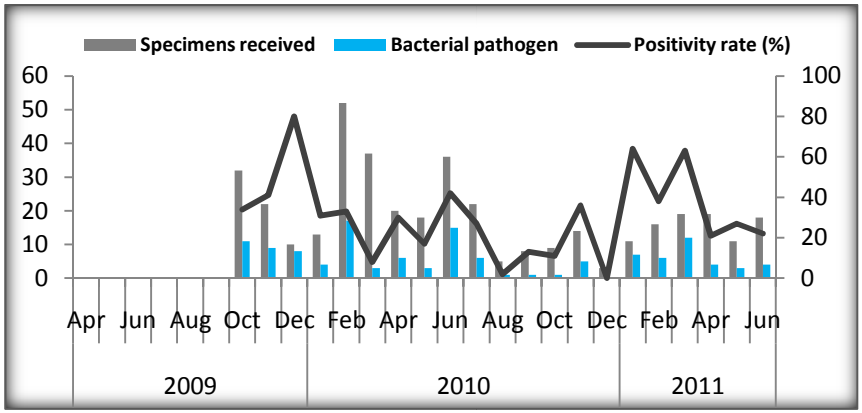


Figure 3.3: Number of diarrhoeal cases, number of enteric bacterial pathogens identified from cases and the bacterial positivity rate separated by study site during April 2009 to June 2011

The highest number of specimens was received from CHBH with an overall total number of 1007, followed by DGMH with a total number of 395 cases, then Matikwana Hospital with a total of 308 cases and Mapulaneng Hospital with a total of 106 cases (Table 3.1). Occurrence of diarrhoeal infections from urban areas (CHBH and DGMH) was higher than the occurrence of diarrhoeal infections from rural areas (Mapulaneng and Matikwana hospitals) with a p-value of 0.004 which was considered statistically significant (Table 3.1). High bacterial positivity rate of bacterial pathogens was seen from Matikwana Hospital with 43%. Other hospitals bacterial positivity rate ranged from 32% to 35% (Table 3.1). DEC was the most prevalent pathogen isolated from all the surveillance sites (Table 3.1). There were few cases of *Salmonella* species (16 cases) and *Shigella* species (35 cases) compared to the cases of DEC. There were no cases due to *Salmonella* from Matikwana Hospital (Table 3.1). A single isolate of *Campylobacter* was recovered from CHBH (Table 3.1). With cases of co-infections of bacterial pathogens (48 cases), co-infections of bacterial pathogens with *Cryptosporidium* (52 cases) and co-infections of bacterial pathogen with rotavirus (128 cases), all the hospitals had occurrence of co-infections, even though the highest numbers were seen from CHBH (Table 3.1). For co-infections of bacterial pathogens with both *Cryptosporidium* and Rotavirus, only 9 cases were observed from CHBH and Matikwana Hospital (Table 3.1). The incidence rate estimates of diarrhoeal disease per sentinel site ranged from 0.1% to 0.03% (table 3.1). The incidence rates were calculated using the mid-year population estimates for 2010 from statistics South Africa document number P0302. All calculated incidence were expressed as new cases per size of provincial population at risk multiplied by 100.

954 **Table 3.1:** Frequency of bacterial pathogens and mixed bacterial pathogens with *Cryptosporidium* and Rotavirus isolated from
955 children with diarrhoea during April 2009 to June 2011 per surveillance hospitals

Surveillance hospitals	Received specimens	Positives (n/N)	DEC (n/N)	*Salmonella* spp. (n/N)	*Shigella* spp. (n/N)	*Campylobacter* spp. (n/N)	Mixed[a] (n/N)	Mixed[b] (n/N)	Mixed[c] (n/N)	Mixed[d] (n/N)	Incidence rate (%)
CHBH	**1007**	**338 (34%)**	**275 (81%)**	**13 (4%)**	**25 (7%)**	**1 (0.3%)**	**24 (7%)**	**25 (7%)**	**91 (27%)**	**7 (2%)**	**0.1**
Mapulaneng	**106**	**37 (35%)**	**32 (86%)**	**1 (3%)**	**1 (3%)**	**0**	**3 (8%)**	**2 (5%)**	**1 (3%)**	**0**	**0.03**
Matikwana	**308**	**132 (43%)**	**120 (91%)**	**0**	**4 (3%)**	**0**	**8 (6%)**	**11 (8%)**	**18 (14%)**	**2 (2%)**	**0.1**
DGMH	**395**	**126 (32%)**	**106 (84%)**	**2 (2%)**	**5 (4%)**	**0**	**13 (10%)**	**14 (11%)**	**18 (14%)**	**0**	**0.1**
Total	**1816**	**633 (35%)**	**533 (84%)**	**16 (3%)**	**35 (6%)**	**1 (0.2%)**	**48 (6%)**	**52 (8%)**	**128 (20%)**	**9 (1%)**	

956 DEC- diarrhoeagenic *Escherichia coli*, spp.- species, Mixed[a]- mixed bacterial pathogens, Mixed[b]- mixed bacterial and *Cryptosporidium*, Mixed[c]- mixed bacterial
957 and Rotavirus, Mixed[d]- mixed bacterial pathogen with *Cryptosporidium* and Rotavirus, N- received specimens, n- positives, CHBH- Chris Hani Baragwanath
958 Hospital, DGMH- Dr George Mukhari Hospital, Incidence rate= number of new cases over a particular period X100/ size of population at risk.

For all age groups, there were always more male children compared to female children enrolled (Figure 3.4). The highest bacterial positivity rate was observed among ages ranging from 13-18 months with a total bacterial positivity rate of 67%. However the probability was not statistically significant (p: 0.2) with ages ranging from 0-6 months being considered as a base line (Table 3.2). For cases of co-infection of bacterial pathogens, more cases were seen from age group of 43-48 months (p: 0.09). For cases of co-infections of bacterial pathogens with both *Cryptosporidium* and Rotavirus, these were only identified in the age groups of 7-18 months and 25-30 months (Table 3.2). The age ranging from 49-54 months had a high rate of bacterial positivity (Table 3.2).

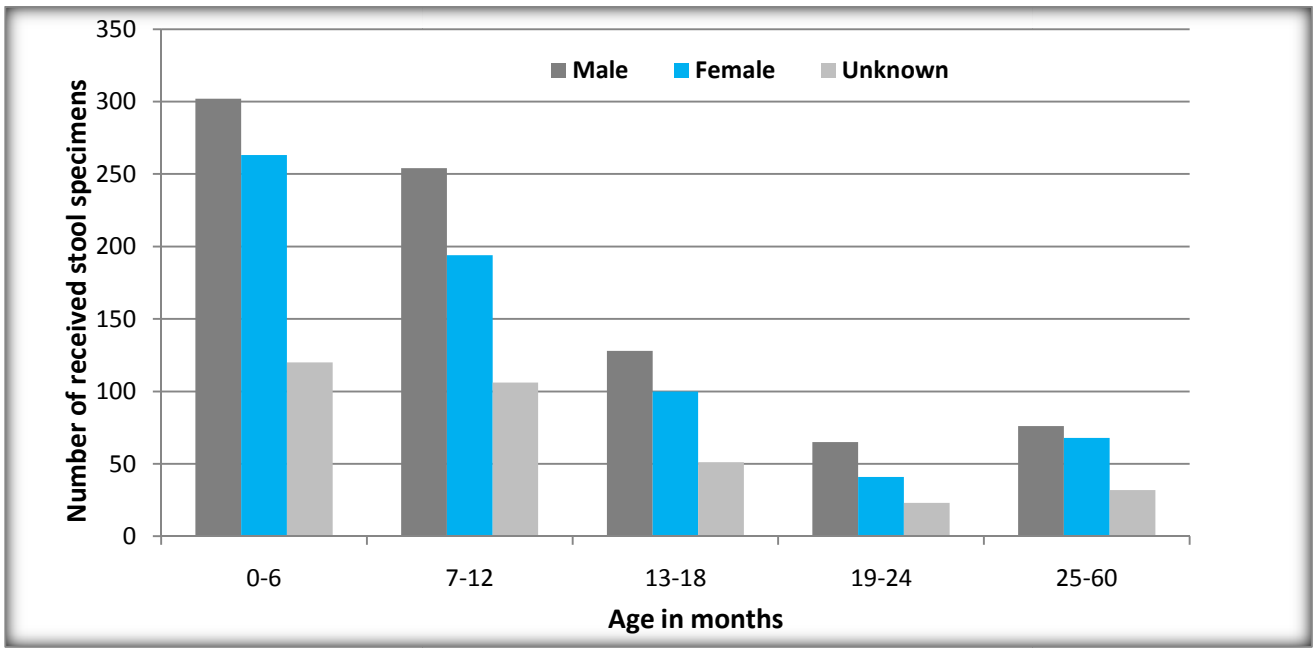


Figure 3.4: Gender distribution of the children who were enrolled at the surveillance hospitals (Median age: 9 months; Mean age: 11 months)

Table 3.2: Frequency and age distribution of bacterial pathogens and mixed bacterial pathogens with *Cryptosporidium* and Rotavirus isolated from children with diarrhoea during April 2009 to June 2011 per age in month (Median age: 9; Mean age: 11)

Age in months	Received specimens	Positives (n/N)	DEC (n/N)	*Salmonella* spp. (n/N)	*Shigella* spp. (n/N)	*Campylobacter* spp. (n/N)	Mixed[a] (n/N)	Mixed[b] (n/N)	Mixed[c] (n/N)	Mixed[d] (n/N)	P-values (95%CI; OR)	DEC P-values (95%CI; OR)	Mixed[a] P-values (95%CI; OR)
0-6	684	222 (32%)	198 (89%)	5 (2%)	5 (2%)	1 (0.5%)	13 (6%)	10 (5%)	43 (19%)	0	1	1	1
7-12	554	188 (34%)	160 (85%)	7 (4%)	8 (4%)	0	13 (7%)	15 (8%)	41 (22%)	3 (2%)	0.6 (1.4; 1.1)	0.2 (1.2; 0.7)	0.7 (2.6; 1.2)
13-18	279	102 (67%)	85 (83%)	1 (1%)	10 (10%)	0	6 (6%)	14 (14%)	24 (24%)	5 (5%)	0.2 (1.6; 1.2)	0.2 (1.2; 0.6)	1 (2.7; 1.0)
19-24	129	50 (39%)	41 (82%)	1 (2%)	2 (4%)	0	6 (12%)	3 (6%)	12 (24%)	0	0.2 (1.9; 1.3)	0.2 (1.3; 0.6)	0.1 (6.1; 2.2)
25-30	75	32 (43%)	23 (72%)	0	5 (16%)	0	4 (13%)	4 (13%)	6 (19%)	1 (3%)	0.07 (2.5; 1.5)	0.02 (0.7; 0.3)	0.2 (7.5; 2.3)
31-36	31	14 (45%)	9 (64%)	0	2 (14%)	0	3 (21%)	1 (7%)	1 (7%)	0	0.1 (3.5; 1.7)	0.02 (0.7; 0.2)	0.06 (17.7; 4.4)
37-42	22	7 (32%)	5 (71%)	0	2 (29%)	0	0	1 (14%)	1 (14%)	0	0.9 (2.4; 0.9)	0.2 (1.6; 0.3)	
43-48	18	8 (44%)	5 (63%)	1 (13%)	0	0	2 (25%)	2 (25%)	2 (25%)	0	0.3 (4.3; 1.7)	0.05 (0.9; 0.2)	0.09 (29.2; 5.4)
49-54	9	5 (56%)	4 (80%)	0	1 (20%)	0	0	1 (20%)	1 (20%)	0	0.1 (6.3; 1.7)	0.4 (4.5; 0.4)	
55-60	15	5 (33%)	3 (60%)	1 (20%)	0	0	1 (20%)	1 (20%)	0	0	0.9 (3.1; 1.0)	0.1 (1.4; 0.2)	0.3 (38.6; 4.0)
Total	1816	633 (35%)	533 (84%)	16 (3%)	35 (6%)	1 (0.2)	48 (6%)	52 (8%)	128 (20%)	9 (1%)	0.3 (1.3; 1.1)	0.1 (1.0; 0.6)	0.6 (2.4; 1.3)

DEC- diarrhoeagenic *Escherichia coli*, spp.- species, Mixed[a]- mixed bacterial pathogens, Mixed[b]- mixed bacterial pathogens and *Cryptosporidium*, Mixed[c]- mixed bacterial pathogens and Rotavirus, Mixed[d]- mixed bacterial pathogen with *Cryptosporidium* and Rotavirus, N- received specimens, n- positives; P-values – base line was considered as age 0-6 months, Positives column- only number of bacterial positives and Mixed[a] without Mixed[b], Mixed[c] and Mixed[d], OR- odds ratio.

Isolated Diarrhoeagenic *Escherichia coli*

Of all bacteria isolated from specimens, more than half were *Escherichia coli* (1083) and 550 was normal flora of the gut. The occurrence of DEC was found to be the highest among all recovered bacterial pathogens and the difference in pathogen distribution was statistically significant (p: 0.01), even though there were no cases of STEC/ EHEC recovered from all the cases, which is the most virulent pathotype because of its capability to cause outbreaks (Table 3.3). From all hospitals, the most common pathotypes were EPEC (31%), DAEC (34%) and EAggEC (24%). Results indicate EPEC as a leading cause of diarrhoea among children from the other sites. However from Matikwana there was a shift in the common pathotype with DAEC as the most common pathotype recovered (p: 0.02) (Table 3.3).

Table 3.3: Frequency of diarrhoeagenic *E. coli* pathotypes detected from children with diarrhoea per surveillance hospitals

Surveillance hospitals	DAEC (n/N)	EAggEC (n/N)	EIEC (n/N)	EPEC (n/N)	ETEC (n/N)	STEC&EHEC (n/N)	Total
CHBH	**89 (32%)**	**66 (24%)**	**10 (4%)**	**92 (33%)**	**18 (7%)**	**0**	**275**
Mapulaneng	**12 (38%)**	**7 (27%)**	**0**	**11 (34%)**	**2 (6%)**	**0**	**32**
Matikwana	**45 (38%)**	**32 (22%)**	**1 (0.8%)**	**25 (21%)**	**17 (14%)**	**0**	**120**
DGMH	**34 (32%)**	**23 (22%)**	**1 (0.9%)**	**38 (36%)**	**10 (9%)**	**0**	**106**
Total	**180 (34%)**	**128 (24%)**	**12 (2%)**	**166 (31%)**	**47 (9%)**	**0**	**533**

CHBH- Chris Hani Baragwanath Hospital, DGMH- Dr George Mukhari Hospital, DAEC- diffusely adherent *E. coli,* EAggEC- enteroaggregative *E. coli,* EIEC- enteroinvasive *E. coli,* EPEC-enteropathogenic *E. coli,* ETEC-enterotoxigenic *E. coli* and STEC-shiga-toxin producing *E. coli* & EHEC- enterohaemorrhagic *E. coli*

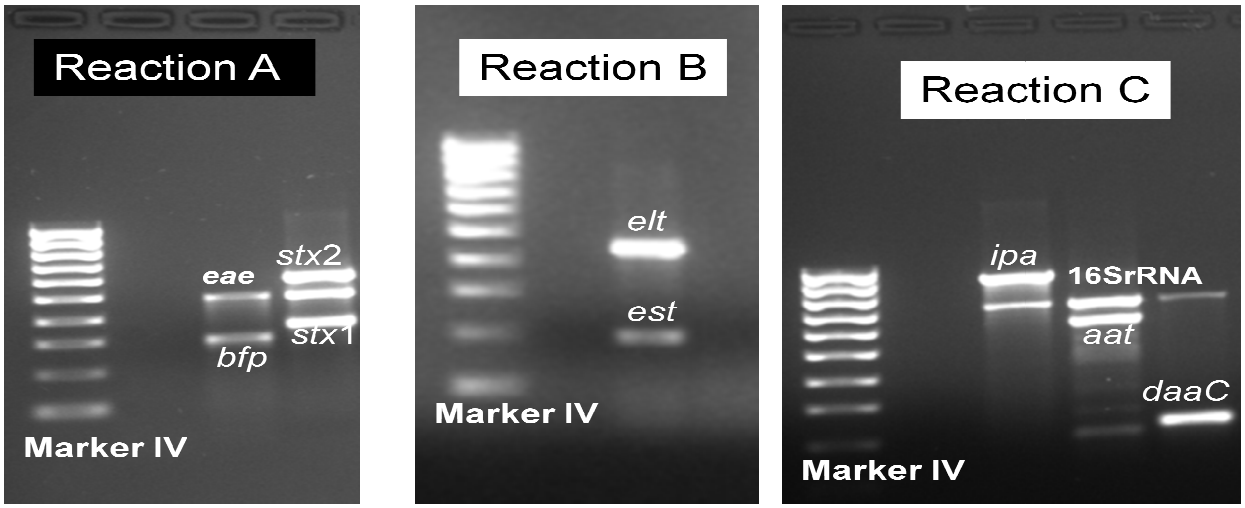


Figure 3.5: Representative picture of an agarose gel with all the detected genes for DEC.

As the occurrence of DEC was investigated by age group, most numbers were seen among ages ranging from 0-18 months of age (Table 3.4). For children 0-6 months of age, the EPEC pathotype was the most common. For age ranging from 7-18 months, the DAEC pathotype were the most common (Table 3.4). DAEC was the most common pathotype recovered with a total of 180 cases, with EPEC being the second most recovered pathotype with 166 cases (Table 3.4). The representative pictures of the PCR products on a gel are included to show how the genes are presented (Figure 3.5). The interpretation of the genes are summarised under appendices in Table 6.1.

Table 3.4: Frequency and age distribution of diarrhoeagenic *E. coli* pathotypes detected (Median age: 9 months; Mean age: 11 months)

Age in months	DAEC (n/N)	EAggEC (n/N)	EIEC (n/N)	EPEC (n/N)	ETEC (n/N)	STEC&EHEC (n/N)	Total
0-6	**51 (26%)**	**51 (26%)**	**2 (1%)**	**85 (43%)**	**9 (5%)**	**0**	**198**
7-12	**57 (36%)**	**41 (26%)**	**4 (3%)**	**43 (27%)**	**15 (9%)**	**0**	**160**
13-18	**37 (44%)**	**13 (15%)**	**1 (1%)**	**20 (24%)**	**14 (16%)**	**0**	**85**
19-24	**15 (37%)**	**12 (29%)**	**2 (5%)**	**8 (29%)**	**4 (10%)**	**0**	**41**
25-30	**7 (30%)**	**6 (26%)**	**2 (9%)**	**6 (26%)**	**2 (9%)**	**0**	**23**
31-36	**4 (44%)**	**4 (44%)**	**0**	**1 (11%)**	**0**	**0**	**9**
37-42	**4 (80%)**	**0**	**0**	**1 (20%)**	**0**	**0**	**5**
43-48	**2 (40%)**	**0**	**1 (20%)**	**1 (20%)**	**1 (20%)**	**0**	**5**
49-54	**1 (25%)**	**0**	**0**	**1 (25%)**	**2 (50%)**	**0**	**4**
55-60	**2 (67%)**	**0**	**0**	**0**	**0**	**0**	**3**
Total	**180 (34%)**	**128 (24%)**	**12 (2%)**	**166 (31%)**	**47 (9%)**	**0**	**533**

DAEC- diffusely adherent *E. coli,* EAggEC- enteroaggregative *E. coli,* EIEC- enteroinvasive *E. coli,* EPEC-enteropathogenic *E. coli,* ETEC-enterotoxigenic *E. coli* and STEC-shiga-toxin producing *E. coli &* EHEC-enterohaemorrhagic *E. coli*

There are different O serogroups which can be related to different pathotypes of DEC. The O serogroups of DEC which were mostly isolated include serogroup O15 (8%), O86 (8%), O119 (7%), O153 (7%) and the most common O serogroup was O127 (11%) (Table 3.5).

Table 3.5: Common serogroups of diarrhoeagenic *E. coli,* i.e. only serogroups which were found represented by >3 isolates are listed below

Serogroups	Number of isolates (n/N)	Serogroups	Number of isolates (n/N)
O5	4 (1%)	O109	4 (1%)
O6	11 (3%)	O111	8 (2%)
O8	10 (3%)	O117	17 (4%)
O11	8 (2%)	O118	4 (1%)
O15	31 (8%)	O119	26 (7%)
O16	14 (4%)	O125abc	4 (1%)
O17	4 (1%)	O127	44 (11%)
O20	8 (2%)	O128abc	15 (4%)
O21	21 (5%)	O137	8 (2%)
O25	16 (4%)	O141ac	5 (1%)
O27	4 (1%)	O142	5 (1%)
O44	9 (2%)	O153	26 (7%)
O46	9 (2%)	O167	7 (2%)
O55	18 (5%)	O175	6 (2%)
O85	9 (5%)	O176	12 (3%)
O86	30 (8%)		

n- number of serogroup; N- total number of serogroups included.

Table 3.5.1: Top ten serogroups of different pathotypes of diarrhoeagenic *E. coli.*

	Pathotypes				
	DAEC	**EAggEC**	**EIEC**	**EPEC**	**ETEC**
Serogroups	**O15 (7%)**	**O11 (5%)**	**O5 (8%)**	**O6 (2%)**	**O6 (6%)**
	O21 (8%)	**O15 (9%)**	**O7 (8%)**	**O8 (2%)**	**O8 (6%)**
	O25 (3%)	**O21 (2%)**	**O25 (8%)**	**O15 (2%)**	**O128abc (23%)**
	O55 (0.6%)	**O25 (3%)**	**O28ac (8%)**	**O25 (2%)**	-
	O86 (14%)	**O46 (5%)**	**O38 (8%)**	**O55 (8%)**	-
	O117 (7%)	**O85 5%7)**	**O119 (8%)**	**O86 (2%)**	-
	O119 (3%)	**O119 (4%)**	**O127 (8%)**	**O111 (4%)**	-
	O127 (8%)	**O128abc (3%)**	**O141ac (8%)**	**O119 (9%)**	-
	O153 (6%)	**O153 (9%)**	**O143 (8%)**	**O127 (22%)**	-
	O176 (3%)	**O176 (3%)**	**O153 (8%)**	**O153 (2%)**	-

DAEC- diffusely adherent *E. coli,* EAggEC- enteroaggregative *E. coli,* EIEC- enteroinvasive *E. coli,* EPEC-enteropathogenic *E. coli,* ETEC-enterotoxigenic *E. coli;* n- number of serogroup per pathotype; N- total number of DEC per pathotype.

Isolated *Salmonella* species

There were 16 (3%) cases of *Salmonella* species detected from the study and most cases were seen from CHBH, with a total of 13 cases (Table 3.6). The most common serotype was *Salmonella* Typhimurium with a total of 7 cases (Table 3.6). Other *Salmonella* serotypes which were recovered included *Salmonella* Isangi (2 cases); *Salmonella* Enteritidis (2 cases); *Salmonella* Newport (1 case) and *Salmonella* Kentucky (1 case). Two *Salmonella* could not be serotyped and one of the two could not be serotyped due to auto-agglutination (Table 3.6).

Table 3.6: Occurrence of *Salmonella* serotypes per surveillance hospitals

Salmonella serotypes	CHBH	Mapulaneng Hospital	Matikwana Hospital	DGMH	Total
Typhimurium	6	1	0	0	7
Isangi	2	0	0	0	2
Enteritidis	0	0	0	2	2
Typhi	1	0	0	0	1
Newport	1	0	0	0	1
Kentucky	1	0	0	0	1
Auto-agglutination	1	0	0	0	1
Species	1	0	0	0	1
Total	13	1	0	2	16

CHBH- Chris Hani Baragwanath Hospital, DGMH- Dr George Mukhari Hospital

Isolated *Shigella* species

There were 35 (6%) cases of *Shigella* species identified from the study and most cases were seen from CHBH (Table 3.7). The most common serotype was *S. flexneri* type 2a from CHBH (Table 3.7), however, *S. sonnei* phase I with a total of 7 cases was the second most common serotype isolated. Other serotypes which were recovered include *S. boydii* type 2 (2 cases); *S. flexneri* type 1b (2 cases); *S. flexneri* type 3a (1 cases); *S. flexneri* type 6 (3 cases) and *S. sonnei* phase II (1 cases). There was one *Shigella* which was untypeable (Table 3.7).

Table 3.7: Occurrence of *Shigella* serotypes per surveillance hospitals

Shigella serotypes	CHBH	Mapulaneng Hospital	Matikwana Hospital	DGMH	Total
***Boydii* type 2**	**2**	**0**	**0**	**0**	**2**
***Flexneri* type 1b**	**2**	**0**	**0**	**0**	**2**
***Flexneri* type 2a**	**9**	**1**	**3**	**5**	**18**
***Flexneri* type 3a**	**1**	**0**	**0**	**0**	**1**
***Flexneri* type 6**	**3**	**0**	**0**	**0**	**3**
***Sonnei* phase I**	**6**	**0**	**1**	**0**	**7**
***Sonnei* phase II**	**1**	**0**	**0**	**0**	**1**
Untypeable	**1**	**0**	**0**	**0**	**1**
Total	**25**	**1**	**4**	**5**	**35**

CHBH- Chris Hani Baragwanath Hospital, DGMH- Dr George Mukhari Hospital

Isolated co-infections

Cases which had co-infections included mixed bacterial infections of different pathotypes of DEC [58% (28/48)], co-infections of DEC with *Salmonella* species [10% (5/48)], co-infections of DEC with *Shigella* species (29%) and a single case of co-infection of DEC with *Campylobacter* species [2% (1/48)] (Table 3.8). As co-infections of bacterial pathogens are investigated by age group, the age groups which were seen with most cases of mixed bacterial pathogens were children from 0-12 months (Table 3.8). DEC was mostly seen mixed with *Shigella* species, with a

total of 14 cases (Table 3.8). There was only one case of DEC mixed with *Campylobacter* species seen (Table 3.8). Most commonly isolated co-infections of bacterial pathogen were EPEC mixed with other DEC pathotypes which include DAEC, EAggEC, EIEC and ETEC with a total of 19 cases (Table 3.9).

Table 3.8: Age distribution of co-infections caused by diarrhoeagenic *E. coli* pathotypes with other bacterial pathogens (Median age: 9 months; Mean age: 11 months)

Age in months	DEC&DEC	DEC& *Salmonella* spp.	DEC& *Shigella* spp.	DEC& *Campylobacter* spp.	Total
0-6	9	2	2	0	13
7-12	9	1	3	0	13
13-18	4	1	1	0	6
19-24	1	1	3	1	6
25-30	2	0	2	0	4
31-36	1	0	2	0	3
37-42	0	0	0	0	0
43-48	2	0	0	0	2
49-54	0	0	0	0	0
55-60	0	0	1	0	1
Total	28	5	14	1	48

DEC- diarrhoeagenic *Escherichia coli,* spp. - species

Table 3.9: Number of cases of mixed bacterial infection

	DAEC	EAggEC	EIEC	EPEC	ETEC	EPEC& EIEC	Total
DAEC	**-**	**8**	**0**	**0**	**1**	**0**	**9**
EAggEC	**0**	**-**	**0**	**0**	**1**	**0**	**1**
EPEC	**8**	**7**	**2**	**-**	**1**	**0**	**19**
***Salmonella* Eastbourne**	**0**	**1**	**0**	**0**	**0**	**0**	**1**
***Salmonella* Heidelberg**	**0**	**0**	**0**	**1**	**0**	**0**	**1**
***Salmonella* Typhimurium**	**1**	**0**	**0**	**1**	**0**	**0**	**1**
***Salmonella* Virchow**	**0**	**0**	**0**	**1**	**0**	**0**	**1**
***Shigella boydii* type 2**	**0**	**2**	**0**	**0**	**0**	**0**	**2**
***Shigella flexneri* type 2a**	**2**	**2**	**1**	**1**	**0**	**1**	**7**
***Shigella flexneri* type 6**	**0**	**2**	**0**	**2**	**0**	**0**	**4**
***Shigella sonnei* phase II**	**0**	**1**	**0**	**0**	**0**	**0**	**1**
Campylobacter jejuni	**0**	**1**	**0**	**0**	**0**	**0**	**1**
Total	**11**	**24**	**3**	**6**	**3**	**1**	**48**

DAEC- diffusely adherent *E. coli,* EAggEC- enteroaggregative *E. coli,* EIEC- enteroinvasive *E. coli,* EPEC-enteropathogenic *E. coli,* ETEC-enterotoxigenic *E. Coli*

There were co-infection cases which involved bacterial pathogen with *Cryptosporidium* [8% (52/633)], bacterial pathogen with Rotavirus [20% (128/633)] and bacterial pathogen with both *Cryptosporidium* and Rotavirus [1% (9/633)] (Table 3.10).

Table 3.10: Frequency of bacterial pathogens mixed with *Cryptosporidium* and Rotavirus

	Cryptosporidium	**Rotavirus**	***Cryptosporidium* & Rotavirus co-infection**
DAEC	**20**	**50**	**3**
EAggEC	**8**	**30**	**0**
EIEC	**2**	**4**	**2**
EPEC	**17**	**34**	**4**
ETEC	**2**	**2**	**0**
***Salmonella* Typhimurium**	**1**	**0**	**0**
***Salmonella* Isangi**	**0**	**1**	**0**
***Salmonella* Kentucky**	**0**	**1**	**0**
***Shigella flexneri* type 2a**	**1**	**1**	**0**
***Salmonella* species**	**0**	**1**	**0**
***Shigella flexneri* type 6**	**1**	**0**	**0**
***Shigella sonnei* phase I**	**0**	**3**	**0**
***Shigella sonnei* phase II**	**0**	**1**	**0**
Total	**52**	**128**	**9**

DAEC- diffusely adherent *E. coli,* EAggEC- enteroaggregative *E. coli,* EIEC- enteroinvasive *E. coli,* EPEC-enteropathogenic *E. coli,* ETEC-enterotoxigenic *E. coli*

Experimental colony blotting and DNA probing

We introduced colony blotting and DNA probing as an experimental method to assist in differentiating mixed DEC pathotypes. Currently at the EDRU, PCR is routinely used to screen a swab of *E. coli* culture to identify whether it is a DEC. Occasionally the PCR may indicate that there is a mixed infection of DEC pathotypes present. Then, the current routine practice to differentiate these mixed infections of DEC is to streak-out a sub-culture for individual colonies; ~10 single colonies are then selected/picked and then individually screened by PCR to identify the individual pathotypes of DEC. However, this methodology often fails to identify all individual pathotypes; often we are only able to identify a single pathotype. Therefore, a colony blotting and DNA probing method was then introduced as an experimental technique to investigate the methodology's ability to more effectively screen, separate and identify all pathotypes associated with a mixed infection of DEC pathotypes. A single colony blot/DNA probe test has the ability to screen 60-80 colonies from an agar plate. After screening the colonies, the aim was to identify all the pathotypes. Selected cases of mixed infections of DEC pathotypes were investigated by colony blotting and DNA probing (and PCR) in an attempt to separate out the individual pathotypes and identify them by colony. The agar plate containing colonies was blotted twice with two different membranes. The first blot was made by overlaying a membrane over the agar plate containing colonies and then removed. Sufficient bacterial culture remained which allowed a second blot on the same agar plate by repeating the membrane overlay procedure. Each membrane was probed with different gene probes specific for different DEC pathotypes (Figure 3.6). However, for all co-infections investigated, we were always only able to identify a single pathotype (Figure 3.6). Figure 3.6 show how EPEC colonies

could be identified following analysis of co-infection of ETEC and EPEC. There were a total of 25 cases of mixed infections of DEC pathotypes investigated (Table 3.11). Eight cases of EPEC mixed with DAEC, with EPEC [75% (6/8)] being the one pathotype mostly identified ; 6 cases of EPEC and ETEC, with EPEC [83% (5/6)] being the one pathotype mostly identified; 5 cases of EPEC and EAggEC, with EAggEC [60% (3/6)] being the one pathotype mostly identified and 4 cases of DAEC and EAggEC, with DAEC [75% (3/4)] being the one pathotype mostly identified (Table 3.11). For all the co-infections investigated, we were unable to identify both DEC pathotypes.

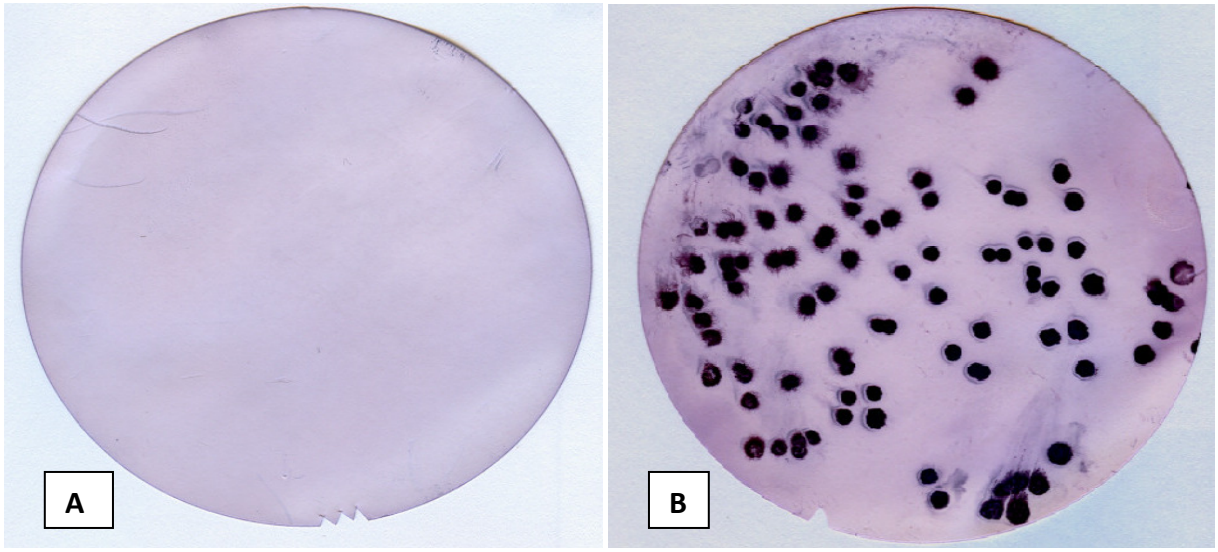


Figure 3.6: Nylon membranes which were blotted from the same agar plate and hybridised with different labelled probes. (**A**) Membrane hybridised with ETEC labelled probe. (**B**) Membrane hybridised with EPEC labelled probe

Table 3.11: Mixed diarrhoeagenic *E. coli* pathotypes investigated by colony blotting and DNA probing

No. of Co-infections investigated (EPEC and DAEC)	EPEC identified in co-infections	DAEC identified in co-infections	Both EPEC AND DAEC identified in co-infections
8	6	2	0
No. of Co-infections investigated (EPEC and ETEC)	**EPEC identified in co-infections**	**ETEC identified in co-infections**	**Both EPEC and ETEC identified in co-infections**
6	5	1	0
No. of Co-infections investigated (EPEC and EAggEC)	**EPEC identified in co-infections**	**EAggEC identified in co-infections**	**Both EPEC and EAggEC identified in co-infections**
5	2	3	0
No. of Co-infections investigated (DAEC and EAggEC)	**DAEC identified in co-infections**	**EAggEC identified in co-infections**	**Both DAEC and EAggEC identified in co-infections**
4	3	1	0
No. of Co-infections investigated (DAEC and ETEC)	**DAEC identified in co-infections**	**ETEC identified in co-infections**	**Both DAEC and ETEC identified in co-infections**
1	1	0	0
No. of Co-infections investigated (EAggEC and ETEC)	**ETEC identified in co-infections**	**EAggEC identified in co-infections**	**Both EAggEC and ETEC identified in co-infections**
1	0	1	0

No.- number, DAEC- diffusely adherent *E. coli,* EAggEC- enteroaggregative *E. coli,* EIEC- enteroinvasive *E. coli,* EPEC- enteropathogenic *E. coli,* ETEC-enterotoxigenic *E. coli*

Chapter 4: Discussion

A total of 1816 cases were processed for bacterial pathogens, with 633 (35%) positive for a bacterial pathogen with high recovery of DEC at 84% (533/633). These data are in agreement with GERMS-SA study data which showed 86% of DEC in children under five years. Other studies in developing countries also showed DEC as the most commonly recovered bacteria by 20% to 30% amongst children with diarrhoea, which is similar to our detection of DEC (29%) from the overall received specimens (20,44,252,253). There were no cases of *V. cholerae* detected since *V. cholerae* O1 is usually seen during an epidemic and in the absence of an epidemic, the organism is not frequently isolated. In Botswana there was a study which documented low isolation of DEC with recovery of 0.5% (47).

DEC pathotypes which included DAEC (34%), EPEC (31%) and EAggEC (24%), were the most common pathotypes recovered. These data are in agreement with GERMS-SA studies data which shows EPEC (64%), DAEC (10%) and EAggEC (8%) as the most commonly recovered pathotypes among children under 5 years; while other studies done in developed and developing countries also show DAEC (18%) from Brazil and EAggEC (10%) from Southern Mozambique as the most commonly recovered pathotypes of DEC. This might be because the feature of diarrhoeal infections varies from place to place depending on the geography and socioeconomic variables (14,44,253,254). Feeding habits can also play a role in infection and could be the reason why DEC tends to be frequently isolated from children younger than 12 months. There were no STEC/ EHEC detected. These data shows an agreement with a study from Northern Jordan which also had no isolation of STEC/ EHEC and GERMS-SA study data

which shows low isolation by 1%. Other studies show high recovery of STEC, such as Tehran study which showed 10% recovery (44,255-257). Other studies have documented the presents of DEC virulence genes from stools of healthy individuals (14,46,258), which might mean that maybe not all strains of DEC are pathogens or they might be less virulent than the other. There are different O serogroups which can be related to different pathotypes of DEC. The most common O serogroups of DEC which were found include serogroup O15 (8%), O86 (8%), O119 (7%), O153 (7%) and O127 (11%). These data are in agreement with other reports from developed and developing countries which also showed different serogroups of DEC which include O119 (78%), O111 (60%) and O126 (60%) from Japan; from Tehran O111, O86 and O55 were also shown to be common by 60% (44,259-263). Our study has described a wide range of DEC serogroups compared to other studies which tend to report and discuss serogroups that has outbreak potential.

The mostly seen serotype of *Salmonella* detected was *Salmonella* Typhimurium (44%). These data are in agreement with GERMS-SA studies data with 46% and other developing countries which also show *Salmonella* Typhimurium as the most recovered serotype such as Tanzania (57%), South Africa (67%) and Malawi (75%) (44,263,264). There was only one *Salmonella* Typhi recovered and other reports from GERMS-SA studies data and developing countries also show low isolation of *Salmonella* Typhi (44,87,264).

The most common serotype of *Shigella* species identified was *S. flexneri* type 2a (26%), while *S. sonnei* (17%) was the second most common serotype identified; these data are in agreement

with GERMS-SA studies data with 42% *S. flexneri* type 2a; 33% *S. sonnei* and other developed and developing countries which also show *S. flexneri* type 2a ranging from 50% to 70% and *S. sonnei* with 45% as a common serotype, even though *S. sonnei* is mostly seen in developed countries (44,99,122,124,263). *Shigella* species were more commonly isolated compared to *Salmonella* species at CHBH. The isolation of bacterial pathogen might be resulting from poor sanitation and overcrowding which promote the survival of the pathogen in the environment resulting in diarrhoeal infections. Lack of control strategies in human behaviour leaves medical authorities with the need to emphasize on the therapeutic aspect of diarrhoeal diseases and encourages basic studies for development of vaccines as a way to reduce or eradicate diarrhoea. Future studies must then focus on disclosing and identifying the risk factors and transmission routes for these emerging pathogens within different geographical areas.

There were co-infections of bacterial pathogens with other bacterial pathogens, or with *Cryptosporidium* or Rotavirus. The co-infections of DEC with DEC was mostly seen (58%) than DEC with *Campylobacter* (2%), *Salmonella* (10%) and *Shigella* (29%). These data are in agreement with studies from other developing countries which also showed co-infections of DEC pathotypes with other bacterial pathogens such as *Campylobacter* (23%), *Salmonella* (33%) and *Shigella* (43%) (94,255,265). Previous studies on DEC from developing countries have not reported on cases of mixed infections of DEC compared to our current study which often identified mixed infections of DEC. However, there were no cases of different pathotypes of DEC mixed from other studies; which was what our study had mostly recovered among bacterial co-infections. Parasitic pathogens (*Cryptosporidium* and *Giardia*) mixed with different pathotypes

of DEC and viral pathogens (Rotavirus) mixed with different bacterial pathogens are co-infections documented in other studies (94,255,265). *Cryptosporidium* is a common cause of parasitic infection in children which results in diarrhoeal illness. It is commonly documented in most co-infections with viral and bacterial infections (255,265). It might be common due to the fact that it requires special attention for it to be eliminated from water. Contact with domestic livestock might also result in co-infection of different pathogens resulting in diarrhoeal illness. There are not enough data on the correlation of HIV serostatus and enteric diarrhoeal infection (27,266).

Only one *Campylobacter* species was recovered which might be partially due to the fact that *Campylobacter* is considered as a fastidious organism and difficult to recover. The delay of transportation of specimens ''even though transport media was used'' for stool specimens may have contributed to the low isolation of *Campylobacter*. Some studies from developing countries such as Southern Mozambique and Northern Jordan also showed low recovery of *Campylobacter* ranging from 1% to 1.7% *,* due to difficulty in isolation (46,164,253,256)*,* but other African countries find *Campylobacter* as the most common pathogen recovered ranging from 15% in South Africa to 60% in Egypt, possibly due to climate differences (11,267).

The highest bacterial positivity rate was observed among ages ranging from 13-18 months, even though the probability was not statistically significant (p: 0.2). This might be due to high exposure to unhygienic environmental factors typical of children of this age. Some studies from developed and developing countries show similar patterns, with high bacterial positivity rate

amongst 13-18 months of age (253,254). Other studies in developing countries had ages ranging from 7-12 months of age with high bacterial positivity rate (257,263).

Children under the age of 2 years were the most enrolled from the beginning of the study with high bacterial positivity rate compared to children older than 2 years. There was a decrease in the number of specimens by 45% (172-311/311) in 2010 (August to December 2010) when compared to 2009 (August to December 2009) and the number of specimens positive for bacterial pathogens also decreased by 31% (68-99/99) for the same period. This period was from the beginning of the Rotavirus vaccine introduction, which might be associated with the reduction; however there are other factors such as availability of safe drinking water and availability of educational facilities on prevention of diarrhoea which must also be in consideration. The decrease in the number of specimens and the bacterial positivity rate in 2010 might be due to the effectiveness of the vaccine; maybe children were not sick enough to be admitted so that they could not be included in this study, as they did not fulfil the case definition. There were few enrolments of children older than 2 years (n=182) compared to children younger than 2 years (n=1634). This might be due to the fact that younger children are more likely to be hospitalised: children can easily be dehydrated, then they are hospitalised so that they can be administered with rehydration solutions. Detection rate of bacterial pathogens was high compared to detection rate of parasites and low compared to detection rate of Rotavirus. These might be due to pathogen interaction: children may have had diarrhoea due to bacterial pathogens. As they are recovering they encountered viral infection commonly severe enough for them to be admitted to the hospital, keeping Rotavirus as the most identified

pathogen among children with diarrhoea. Recurrent of diarrhoeal episodes have been described in South Africa among young children (269).

Not much is known about the causative agent of diarrhoeal diseases other than Rotavirus (226,229,230). This justifies the lack of detection facilities in the routine laboratories for clinical purposes. Most diagnostic laboratories only detect specific bacterial pathogens from stool specimens (14). This might underestimate other pathogens. Molecular characterization of bacterial pathogens such as DEC must be introduced in routine laboratories. Availability of such diagnostic resources could enhance identification of outbreaks and common pathogens causing diarrhoea. Introducing new methodologies would bring about challenges which need to be taken in consideration, such as costs associated with purchasing; installing and training employees to use new techniques.

There was a decrease in the number of specimens received during December 2010 and the same drop in the number of specimens received was also seen during December 2009. This might be due to fewer patients being enrolled as a result of the following: people go on vacation during this month, staff take leave during this period, and due to none of the cases meeting the case study definition, since only children admitted to the hospital and who at least sleep overnight are considered for specimens. Since this study had limitations, we cannot be certain of the proportion of bacterial infections amongst children from whom we failed to receive stool specimens because of our study definition. The definition of diarrhoeal episode might be the

same as from those whom we did not received stool specimens and this might affect the interpretation of results.

CHBH was the first surveillance hospital to start with the study and it started from April 2009. For all the sites, CHBH showed the most enrolments, possibly due to the fact that CHBH is situated at an urban area, with easy community access and covers a larger area than all other surveillance sites. Parents would be more likely to evade clinics and traditional doctors and rather go straight to the hospitals where they can obtain a better quality of medical attention (11). Mapulaneng Hospital only commenced with the project from September 2009. There was a 100% bacterial positivity rate from Mapulaneng Hospital during November 2010, which might be due to low number of specimens received. For sites such as Mapulaneng Hospital and Matikwana Hospital, they had low number of enrolments. This is due to the fact that these hospitals are small and situated in rural areas, with low population access to the hospitals, so parents would be more likely to first visit the traditional doctors and clinics, and then eventually be referred to the hospitals if their children's conditions did not improve. Matikwana Hospital commenced a month earlier than Mapulaneng Hospital in August 2009. DGMH commenced with the project during October 2009. DGMH had a low bacterial positivity rate from February to July 2010 (Autumn to Winter) (p: 0.02). These data are in agreement with other studies in developing countries showing summer season with high recovery of bacterial pathogens. However, features of diarrhoeal infection vary from place to place (255,267). The occurrence of a diarrhoeal episode from urban areas was higher than the occurrence from rural areas which was statistical significant with a p-value of 0.004. As expected, it is obvious that there are

important regional differences in the occurrence of diarrhoea in children. Comprehensive information of unusual situations in each geographical area is required, since the movement of individuals from one place to another complicates the data analysis and implementation of control strategies. The incidence rate estimates per site was very low. The mid-year estimates from stats SA only gives provincial population statistics and not per region (268). The calculated incidence rates might have been over estimated because we were focusing on a portion of the province.

As data were investigated by month, there was a high bacterial positivity rate seen during November 2009, December 2009 and January 2010 which covers the countries summer months and this data agrees with other studies done from Egyptian children and Iranian children which also showed high positivity during summer seasons. These is due to climate difference since features of diarrhoeal infections varies from place to place (255,267). High detection rate during the summer seasons may be due to high temperatures during such time of the year. High temperature promotes growth of organisms in the environment.

There were a high number of male (825) patients compared to female (666) patients, which suggests that males might be at higher risk of having diarrhoea compared to females or because there are more males among the communities. These results are in agreement with other studies in developing countries such as Cameroon and Tanzania which also showed most number among male patients ranging from 60% to 80% of males (252,256,263). There were

some for who gender was unknown which might mean that the difference may not be considered as statistically significant because of the missing data.

Antimicrobial susceptibility was not investigated for bacterial pathogens detected in our study. It is important to have knowledge of the antimicrobial susceptibility of these bacterial pathogens, as this provides helpful data to assist in epidemiological (outbreak) investigations and to assist with the correct choice of antimicrobial treatment for patients infected with these pathogens. Antimicrobial susceptibility data for enteric bacterial pathogens is well documented and reported by GERMS-SA; they recently published a paper describing some susceptibility patterns of bacterial pathogens in South Africa (270).

To date, the technique of colony blotting and DNA probing has not been used extensively for bacterial identification. As a result there are very little data published on the use of colony blotting and DNA probing. Some studies have shown that colony blot techniques can be used for detection of EIEC and *Shigella* from water samples and also for detection of shiga like toxin-producing *E. coli* from faecal samples (271,272). The lack of a simple, inexpensive technique limits investigation of the incidence of DEC co-infection. In our study the possibility of using colony blotting and DNA probing was explored. We introduced colony blotting and DNA probing to assist in differentiating mixed DEC pathotypes. Currently at the EDRU, PCR is routinely used to screen a swab of *E. coli* culture to identify whether it is a DEC. Occasionally the PCR may indicate that there is a co-infection of DEC pathotypes present. To differentiate these co-infections of DEC, the culture then needs to be subcultured and streaked-out for single colonies.

The single colonies (~10 colonies) are then selected and screened by PCR to identify the individual pathotypes of DEC. Colony blotting was introduced to separate co-infections of DEC pathotypes, by screening 60-80 colonies from one agar plate (membrane). We chose to use the flood-inoculation and spreading of an agar plate with a liquid sample of bacteria, containing an appropriately calculated concentration of bacteria; because this technique results in a more even and well separated spread of colonies (required for blotting) compared to a direct application and spreading of culture (using a loop or swab) across an agar plate. For flood-inoculation, it is important to experiment with multiple dilutions of liquid bacterial culture, to find the perfect/exact concentration of bacteria which when spread on an agar plate results in an even and well separated spread of colonies. After screening the colony blot, the aim was to identify all the pathotypes. However, our attempts to differentiate and identify all pathotypes associated with a co-infection were unsuccessful. For all co-infections investigated, we were always only able to identify a single pathotype. This might be due to the proportion of bacterial numbers in the mixed cultures. A 1:1 ratio of one pathotype to another in a mixed culture would be optimal to identify both pathotypes. However in clinical specimens, the ratio of numbers of one pathotype to another appears to be very different. Let's hypothesise ratios of 1:50 or 1:100: with this ratios and a technique only able to screen 60-80 colonies on a single blot, it becomes clear why chances are that only the predominant pathotype will be identified. Maybe, the ratios are even higher at 1:1000 or 1:10000; this will drastically (and almost definitely) exclude the chances of identifying the lesser pathotype. Even though we blotted twice on the same agar plate containing the investigated colonies (with different membranes and hybridisation with different probes), only one membrane was always shown to have a positive result, i.e., we were

always only able to identify a single pathotype. The membranes were blotted twice on the same agar plate so that we can investigate the specificity of the labelled probes which were used. The labelled probes were found to be specific. The blotted agar plate was re-incubated for three to four hours for regeneration of colonies in case of future use such as PCR. The plate was then re-frigerated. So, in summary, the technique works well for identification of a single pathotype, but fails to differentiate a culture of mixed pathotypes. The data we found compared to other similar studies, further development of the methodology may be helpful (273-275). Furthermore the sensitivity of colony blotting may be increased by working with a great range of DEC co-infections with different pathotypes. We only worked with 25 cases of different co-infections. At present, PCR is still an appropriate method for separation and identification of co-infections of DEC pathotypes.

Limitations

At certain times, there was a reduction in the number of specimens from the surveillance sites due to reasons listed below:

1. A nurses' strike occurred in August-September 2010, therefore there was no staff to collect specimens.
2. Only children who presented with 3 or more episodes of loose stool over the period of 24 hours (at least slept overnight in hospital) were included and cases of children who were treated and discharged were not enrolled.
3. Clinical criteria may have differed among the sites for which cases needed overnight admission, resulting in differences in which pathogens were identified and the data for each site would not have been affected. Sites were selected to represent differing

populations (rural versus urban), so that the data may be better to generalised to South Africa, where practices may differ across the country, so these differences would have been important.

4. Clinical criteria may differ from site to site; some sites may admit patients more easily than others.

Chapter 5: Conclusions

From the data recovered from the study, there is a suggestion of an ongoing surveillance needed in all the different provinces of the country in order to further our understanding of the bacterial pathogens circulating, especially DEC pathotypes and to monitor the occurrence of mixed bacterial pathogens. There is no evidence that any particular DEC pathotype is associated with diarrhoea in children under five years. Even in cases where diagnostic facilities are routinely available, pathogens detected in children with diarrhoea may not be the cause of the illness.

The aetiologic causes of diarrhoea among children under five years of age remain unknown for most parts of the country. The data available on the frequency of different enteric pathogens is limited, especially in the range of pathogens being tested for and the location of the study. This study opens doors to further studies addressed to analyse the specific epidemiology and virulence of diarrhoea causing pathogens per province in South Africa, so that local control strategies may be improved. Data collected from this study could inform health staff in the sentinel sites of the types of bacterial pathogens they should be alert to and also inform future control strategies locally. In health planning, the knowledge of geographical variation can be useful for setting suitable and effective strategies for disease and health interventions which are able to address local health determinants. In the regions with high diarrhoeal diseases, sanitation and community health education can have an impact on childhood morbidity.

Co-infections due to several pathogens raise the question as to whether a single pathogen is responsible for illness or whether several pathogens act in synergy, even though not all cases were found to be mixed. The occurrence of co-infections warrants further investigation.

Even though this study did not look at antimicrobial susceptibility, more data are required in order to establish empiric therapy guidelines. Documentation of more data on the environmental risk factors: e.g. water sources and potential reservoirs, e.g. contact with domestic livestock requires information - these data can help in understanding the association/relationship between environmental factors and the occurrence of diarrhoeal illness. In order to improve the strategies of disease control, the knowledge of correlation of HIV serostatus and diarrhoeal infections with enteric pathogens needs to be documented.

The colony blotting/probing technique may not be useful in South African situations since we were unable to reach our aim which was to separate mixed pathotypes of DEC in a single case. However there's a need for new development of methods that could be used for separation of mixed pathogens.

Chapter 6: Appendices

6.1 Buffers and Solutions

1M Tris buffer (pH 8)

- 121.1g Tris (Merck KGaA, Darmstadt, Germany, USA)
- 800ml dH_2O
- 70ml HCl (Merck)
- Autoclave for 15 minutes to sterilize

0.5M EDTA (pH 8)

- 186.1g EDTA (Sigma-Aldrich chemie GmbH, Steinheim, USA)
- 800ml dH_2O
- ~20g NAOH (Merck)
- Autoclave for 15 minutes to sterilize

Tris–EDTA (TE) buffer (pH 8)

- 200µl of 0.5M EDTA (Sigma)
- 1000µl of 1M Tris (Merck)
- 100ml dH_2O
- Autoclave for 15 minutes to sterilize

10X TAE buffer

- 48g Tris (Merck)

- 7.5g EDTA (Sigma)
- 11ml glacial acetic acid (Merck)
- 1000ml dH_2O

1X TAE buffer

- 100ml of 10X TAE buffer
- 900ml dH_2O

Loading buffer

- 0.25g Bromophenol blue (BDH Ltd, Pooled, BHIS 1TD, England, USA)
- 40g sucrose (Merck)
- 100ml dH_2O

Ethidium bromide (EtBr) solution (10 mg/ml)

- 500mg ethidium bromide (Merck)
- 50ml dH_2O

Ethidium bromide (EtBr)

- 250ml of 0.5X TBE buffer (Merck)
- 25µl of 10mg/ml Ethidium bromide

6.2 Identification of diarrhoeagenic *E. coli* using PCR

PCR reactions primer mix

PCR reaction (A) primer mix

The mixture contained all primers at 1µM and was stored at -20°C

- 120 µl deionised autoclaved water
- 10 µl eae-f primer (20µM)
- 10 µl eae-r primer (20µM)
- 10 µl bfp-f primer (20µM)
- 10 µl bfp-r primer (20µM)
- 10 µl stx1-f primer (20µM)
- 10 µl stx1-r primer (20µM)
- 10 µl stx2-f primer (20µM)
- 10 µl stx2-r primer (20µM)

PCR reaction (B) primer mix

The mixture contained ST primers at 3µM, the LT primers at 1µM and was stored at -20°C

- 120 µl deionised autoclaved water
- 30 µl ST-f primer (20µM)
- 30 µl ST-r primer (20µM)
- 10 µl LT-f primer (20µM)
- 10 µl LT-r primer (20µM)

PCR reaction (C) primer mix

The mixture contained 16SrRNA primers at 0.5µM, all other primers at 1µM and was stored at -20°C

- 130 µl deionised autoclaved water
- 10 µl ipaH-f primer (20µM)
- 10 µl ipaH-r primer (20µM)
- 10 µl pCVD432-f primer (20µM)
- 10 µl pCVD432-r primer (20µM)
- 10 µl daaC-f primer (20µM)
- 10 µl daaC-r primer (20µM)
- 5 µl 16SrRNA-primerB (20µM)
- 5 µl 16SrRNA-r primer (20µM)

Table 6.1: Interpretation of PCR reactions results for DEC

	Result of PCR Reactions								
	eae	***bfp***	***stx1***	***stx2***	***est***	***elt***	***ipa***	***aat***	***daaC***
EPEC	+								
EPEC	+	+							
EHEC	+		+						
EHEC	+			+					
EHEC	+		+	+					
STEC			+						
STEC				+					
STEC			+	+					
ETEC					+				
ETEC						+			
ETEC					+	+			
EIEC							+		
EAggEC								+	
DAEC									+

(-)-negative; (+)-positive

Table 6.2: Details of PCR primers and product sizes of DEC pathotypes

Target gene	PCR primer	Primer sequence	PCR product size	Reference
Reaction A				
eae	eae-f	TCAATGCAGTTCCGTTATCAGTT	482bp	276
	eae-r	GTAAAGTCCGTTACCCCAACCTG		
bfp	bfp-f	GGAAGTCAAATTCATGGGGGTAT	298bp	276
	bfp-r	GGAATCAGACGCAGACTGGTAGT		
stx1	stx1-f	CAGTTAATGTGGTGGCGAAGG	348bp	277
	stx1-r	CACCAGACAATGTAACCGCTG		
stx2	stx2-f	ATCCTATTCCCGGGAGTTTACG	584bp	277
	stx2-r	GCGTCATCGTATACACAGGAGC		
Reaction B				
est	ST-f	ATTTTTCTTTCTGTATTGTCTT	190bp	278
	ST-r	CACCCGGTACAAGCAGGATT		
elt	LT-f	GGCGACAGATTATACCGTGC	440bp	278
	LT-r	CGGTCTCTATATTCCCTGTT		
Reaction C				
ipa	ipaH-f	CTCGGCACGTTTTAATAGTCTGG	933bp	55
	ipaH-r	GTGGAGAGCTGAAGTTTCTCTGC		
aat	pCVD432-f	CTGGCGAAAGACTGTATCAT	630bp	279
	pCVD432-r	CAATGTATAGAAATCCGCTGTT		
daaC	daaC-f	CAGGTCATCCGGTCAGTCGG	212bp	278
	daaC-r	CAATGCCACGTACAACCGGC		
16SrRNA	16SrRNA-primerB	GATTAGATACCCTGGTAGTCC	726bp	58
	16SrRNA-r	ACGGCTACCTTGTTACGACTT		

Positive control strains for PCR reactions and serotyping of DEC included: *E. coli* (ATCC43887), positive for *eae* and *bfp* genes; *E. coli* (C4193-1), positive for *eae*, *stx1* and*stx2* genes; *E. coli* (H10407), positive for *est* and *elt* genes; *E. coli* (ATCC43893), positive for the *ipa* gene; *E. coli* (3591-87), positive for the *aat* gene and *E. coli* (D2190), positive for the *daaC* gene.

6.3 Agarose gel electrophoresis for analysis of PCR products

Step 1: A 1.5% agarose gel was prepared by dissolving 1.5g of SeaKem LE agarose powder (Whitehead Scientific (Pty) Ltd, Lonza, Rockland, ME USA) in 100ml of 1X TAE buffer (appendices 6.1) in a microwave.

Step 2: Six microlitre of 10mg / ml of ethidium bromide (appendices 6.1) was added to PCR a cooled (55^{o}C) gel and was mixed by swirling.

Step 3: Three microlitre of loading buffer (appendices 6.1) was added to the PCR product and mixed by pippetting.

Step 4: From the mixture, 6µl was loaded into the wells of 1.5% agarose gel and also 3µl of Bioline hyper-ladder IV was loaded as a DNA marker at separate wells of the gel.

Step 5: Electrophoresis was performed at ~130v for 50min.

Step 6: The gel which contained PCR products was placed into a UV transilluminator and was visualized by UV illumination.

Step 7: The gel image was captured using the BIORAD gel documentation system.

Step 8: Agarose gel was disposed into a Sanumed Solid waste container.

6.4 Colony blotting and DNA probing

6.4.1 Buffers and Solutions

Denaturation solution

To disrupt the cell membrane and uncoils protein into a random shape, releasing DNA out of the cell.

- 87.66g NaCl (Merck)
- 20g NaOH (Merck)
- 1000ml dH_2O
- Autoclave for 15 minutes to sterilize

Neutralisation solution

For adjusting and neutralising the pH contents of the membrane.

- 87.66g NaCl (Merck)
- 121g Tris (pH 7.5) (Merck)
- 1000ml dH_2O
- Autoclave for 15 minutes to sterilize

2X SSC

For binding of the DNA to the membrane through ion exchange interaction, whereby the negatively charged DNA binds to the positively charged membrane.

- 100ml 20X SSC (Roche Diagnostics GmbH, Mannheim, Germany, USA)
- 900ml dH_2O

0.5X SSC

- 25ml 20X SSC (Roche)
- 900ml dH_2O

1% SDS

For disruption of cell wall dissociation of nucleic acid: protein complexes.

- 1g Sodium dodecyl sulphate (BDH Ltd)
- 100ml dH_2O
- Boil in microwave to dissolve

Low stringency buffer

For sensitivity, but it can give non-specific hybridisation signals and high backgrounds.

- 500ml 2X SSC
- 500µl 1% SDS

High stringency buffer

To increase sensitivity, so that only specific hybridisation signals remain and non-specific background signals are reduced.

- 500ml 0.5X SSC buffer
- 500µl 1% SDS

Proteinase-K

To destruct proteins which are in the cell lysate.

- 20mg Proteinase-K (Roche Diagnostics GmbH)
- 1ml 2X SSC

Chromogenic AP substrate

To produce the indigo dye for visualisation of positive signal.

- 35µl 4-nitroblue tetrazolium chloride (NBT) (Roche Diagnostics GmbH)
- 35µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics GmbH)
- 10ml detection buffer (Roche Diagnostics GmbH)

6.4.2. PCR Labelling of DNA probe

PCR reactions contained different genes for different gene targets which included *eae* gene for EPEC probe, *elt* gene for ETEC probe, *ipa* gene for EIEC probe, *aat* gene for EAggEC probe and *daaC* for DAEC probe were used. PCR primers (appendices 6.4.2), interpretation of results (appendices 6.3, table 6.1) and PCR product sizes (appendices 6.6.2, table 6.4) are described. The control strains were used for probe labelling (appendices 6.2). PCR reactions were prepared in 0.2ml tubes and the reaction mixture of 50µl contained 6 components as listed below:

(i)	Autoclaved de-ionized water	31.25µl
(ii)	PCR buffer with $MgCl_2$(10X) (Vial 3)	5µl

(iii)	PCR DIG mix (Vial 2)	5µl
(iv)	Primer mix (20 µM)	6µl
(v)	Enzyme mix (Vial 1)	0.75µl
(vi)	Crude DNA template (2.3.6)	2µl

Cycling conditions were as follows: one starting cycle at 95°C for 7 minutes, 35 cycles of denaturation at 94°C for 75 seconds, primer annealing at 60°C for 90 seconds and extension at 72°C for 90 seconds; one final cycle at 72°C for 5 minutes. Following PCR, reactions were stored at 4^{o}C.

PCR reaction primer mix

PCR reaction A primer mix

The mixture contained *eae* gene primers at 1µM and was stored at -20°C

- 180µl deionised autoclaved water
- 10µl eae-f primer (20µM)
- 10µl eae-r primer (20µM)

PCR reaction B primer mix

The mixture contained *elt* gene primers at 1µM and was stored at -20°C

- 180µl deionised autoclaved water
- 10µl LT-f primer (20µM)
- 10µl LT-r primer (20µM)

PCR reaction C-1 primer mix

The mixture contained *ipaH* gene primers at 1µM and was stored at -20°C

- 180 µl deionised autoclaved water
- 10 µl ipaH-f primer (20µM)
- 10 µl ipaH-r primer (20µM)

PCR reaction C-2 primer mix

The mixture contained *aat* gene primers at 1µM and was stored at -20°C

- 180 µl deionised autoclaved water
- 10 µl pCVD432-f primer (20µM)
- 10 µl pCVD432-r primer (20µM)

PCR reaction C-3 primer mix

The mixture contained *daaC* gene primers at 1µM and was stored at -20°C

- 180 µl deionised autoclaved water
- 10 µl daaC-f primer (20µM)
- 10 µl daaC-r primer (20µM)

Table 6.3: Details of PCR primers and product size for PCR labelling of DNA probe

Target gene	PCR primer	Primer sequence	PCR product size	Reference
Reaction A				
Eae	eae-f	TCAATGCAGTTCCGTTATCAGTT	482bp	276
	eae-r	GTAAAGTCCGTTACCCCAACCTG		
Reaction B				
Elt	LT-f	GGCGACAGATTATACCGTGC	440bp	278
	LT-r	CGGTCTCTATATTCCCTGTT		
Reaction C-1				
Ipa	ipaH-f	CTCGGCACGTTTTAATAGTCTGG	933bp	55
	ipaH-r	GTGGAGAGCTGAAGTTTCTCTGC		
Reaction C-2				
Aat	pCVD432-f	CTGGCGAAAGACTGTATCAT	630bp	279
	pCVD432-r	CAATGTATAGAAATCCGCTGTT		
Reaction C-3				
daaC	daaC-f	CAGGTCATCCGGTCAGTCGG	212bp	278
	daaC-r	CAATGCCACGTACAACCGGC		

6.5 Informed consent form

Informed Consent Form for study participation (for parent or legal guardian)
(Version 1.0 Rotavirus Surveillance Protocal final 050109).

One copy will stay with the surveillance officer, and be filed with the clinical case report form. The other copy will be given to the parent or legal guardian.

Information section

Introduction to participant

Hello. My name is _____ (*name of surveillance officer*) _____and I would like to ask you for some of your time to explain the work that we do and to ask you and your child to please assist us in a research study that we are doing. As we discuss the information below, please feel free to ask any questions.

Purpose of the research study

In South Africa (like elsewhere in the world), laboratories and government health departments look at the germs that cause disease and where these germs occur. For this study we are interested in the germ called rotavirus. This germ infects young children and causes diarrhoea. Many children in South Africa become sick with this germ every year and many of these children will need to be in hospital on an intravenous drip. Some children may die from the severe dehydration that may be caused by this diarrhoea. Because of this potential severe complication South Africa would like to count the number of children who get sick from this germ. The Department of Health is introducing a vaccine that will protect children against this disease. It is important to know the numbers of children who are infected so that we can tell how well the vaccine is working. We are talking with you about this project because your child has been admitted to the hospital with diarrhoea which may be caused by this germ. This information will be used to try to stop these germs causing illness in other children.

Description of study procedures

If you choose to have your child join the project, we will ask you a number of questions as part of the study. During the study, we will ask about your child's present and past history, including questions about vaccines s/he received. We will also ask questions about the child's home and other children living with your child. You may choose not to answer any question. We will also look at your child's hospital records and his/ her records in the laboratory to complete the form.

In addition, you will be asked if you will allow us to take a stool specimen from your child to test for the germs which cause diarrhoea, as part of the study. The stool specimen will be taken from the child's nappy or bed pan. If the stool is very watery a piece of the nappy will be cut or the whole nappy will be taken and sent to the laboratory for detection of the germs. If there is no stool available a small tube will be inserted into the child's bottom and some stool will be sucked out.

Also, as part of the study, you will be asked if you are willing for us to take a specimen from your child to be tested for HIV infection. This will be done by a finger, heel or earlobe prick where we will collect a few drops of blood on a piece of blotting paper. The results of your child's HIV test will not be known by me or the other study nurses. If you want to know your child's HIV result we can refer you to your local VCT clinic for testing and follow up.

If you agree to the questionnaire but not to take a stool or blood specimen, this is also okay.

Your rights as a participant

It is your right to choose to have your child be part or not be a part of the project. If your child does not join, s/he will continue to receive the treatment needed for this infection and s/he will not lose any health care services. You may choose to leave the study at anytime you like. If you decide this, your child will be treated in the same way in hospital. He/ she will not lose any benefits to which he/ she may be entitled to if you stop participation in the study.

Expected duration of participation

Completing the form with questions should take about 15-20 minutes. Taking the stool specimen will take about 2 minutes. Taking the drops of blood should take only about a minute. If the child is not having diarrhoea right now we may need to come back to collect the stool specimen. We will also visit your child regularly while s/he stays in hospital to collect information about his/ her hospital stay. S/he will be visited only while in hospital.

Reasonably foreseeable risks or discomforts to the participant

The questions will be asked are general and not personal. There should be no risks to you or your child if you agree to take part in the study.

Benefits to the participant or others

Your relative will not directly benefit from this study. His/ her treatment in the hospital will not change if you agree that he/ she may take part in the study. The information we collect will be used to try to stop the germ that caused his/ her illness from causing illness in other children. If we learn that your child is due for rotavirus vaccine or any other vaccine we can refer you to the local clinic/health centre to get the vaccine. You may ask the study nurse questions about your child's health and progress in hospital which may make you feel more informed.

Alternative procedures that may be beneficial to the participant

This study does not change the way your child will be treated in hospital or after he/ she is discharged from hospital.

Confidentiality of participant information

We will keep your information confidential. The forms with your personal information will be posted in sealed envelopes to other people who work on the study and will always be kept in locked cabinets or offices. All your information from this study will be put under a special number (study number) and not your name. Therefore, none of the study personnel will know which information/ HIV result belongs to you.

Costs

You or your child will not have to pay any costs, if s/he takes part in the study.

Storage of samples for future testing

Once we have completed the testing for this study we may store your child's sample for testing in the future. These tests would be done if new viruses (germs) are identified or if new test become available. The sample will not have a name on it so that it cannot be linked to you or your child.

Contact details for Rotavirus surveillance study investigators

If you have any questions about the study, about your child's rights while taking part in the study or about any injury that may have happened because of the study, you may contact any of the people/ groups listed below:

Surveillance officer name and contact details

Dr Cheryl Cohen: 011 5550543

Dr Shabir Madhi: 011 989 9885

Dr Jocelyn Moyes: 011 346 6410

This study has received ethical approval from the Human Research Ethics committee of the University of the Witwatersrand, Johannesburg: 011 717 1234. If you have any questions about your rights as a participant in this project please do contact the office on the above number.

Parent or legal guardian declaration section

I, _____ (name of parent/guardian), acknowledge that the study questionnaire and specimen collection have all been explained to me and that I agree for my child to participate in the following study procedures:

☐ Y☐ N I agree to be interviewed and allow data from my child hospital records to be collected to answer the questions from the study questionnaire.

☐ Y☐ N I agree to the test for germs from his/her stools

☐ Y☐ N I agree to my stool sample being stored for future testing.

☐ Y☐ N I agree to the test for HIV

The signature of the parent or legal guardian below means that the study has been explained to the parent or legal guardian and that he/ she agrees that his/ her child or ward may participate.

Name of parent or legal guardian:

Signature of parent or legal guardian:

Date:

The signature of the witness below means that another person has observed the consenting of the parent or legal guardian. The witness must be impartial and not part of the study staff.

***Name of witness**:*

***Signature of witness**:*

***Date**:*

Name of surveillance officer:

FINAL OUTCOME OF PATIENT:

HOSPITAL PATIENT:	**CLINIC PATIENT:**
☐ Died	Died in clinic: ☐Y ☐N Date of death (dd/mm/yyy)___/___/_____
☐ Discharged	☐ Discharged
☐ Absconded /RHT	☐ Referred to HOSPITAL, if so name of facility;_____
☐ Referred to step down facility, if so name of facility_____	
☐ Unknown	
Date of discharge/death/RHT /absconded/referred: (dd/mm/yyyy) ___ / ___ / ____	
Discharge weight _____ kg(##.#kg)	

DATA ON PAST HIV STATUS:

1. Prior diagnosis of HIV infection: ☐ Y ☐N

1.1. If yes, date diagnosed (dd/mm/yyyy) ___ / ___ / _____

1.2. How was diagnosis made?

1.2.1.PCR ☐ Positive ☐Negative ☐Not done

1.2.2.ELISA ☐ Positive ☐Negative ☐Not done

2. Current antiretroviral use for HIV treatment: ☐ Y ☐N ☐ Unknown

If yes, date initiated (dd/mm/yyyy) ___ / ___ / _____

3. Current Bactrim prophylaxis: Y N

If yes date started: (dd/mm/yyyy) ___ / ___ / _____

Chapter 7: Reference List

1. **Kosek, M., C. Bern, and R. L. Guerrant**. 2003. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bulletin of the World Health Organization **81**:197-204.
2. **Bryce, J., C. Boschi-Pinto, K. Shibuya, and R. E. Black**. 2005. WHO estimates of the causes of death children. Lancet **365**:1147-1152.
3. **Bern, C., J. Martines, I. de Zoysa, and R. I. Glass**. 1992. The magnitude of the global problem of diarrhoeal disease: a ten-year update. Bulletin of the World Health Organization **70**:705-714.
4. **Wilhelmi, I., E. Roman, and A. Sanchez-Fauquier**. 2003. Viruses causing gastroenteritis. Clinical Microbiology and Infection **9**:247-262.
5. **Albert, M. J., A. S. G. Faruque, S. M. Faruque, R. B. Sack, and D. Mahalanabis**. 1999. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. Journal of Clinical Microbiology **37**:3458-3464.
6. **Germani, Y., M. Morillon, E. Begaud, H. Dubourdieu, R. Costa, and J. Thevenon**. 1994. Two year study of endemic enteric pathogens associated with acute diarrhea in New Caledonia. Journal of Clinical Microbiology **32**:1532-1536.
7. **Na'awas, T. E. and M. N. Abo-Shehada**. 1991. A study of the bacterial and parasitic causes of acute diarrhoea in Northern Jordan. Journal of Diarrheal Diseases Research **9**:305-309.

8. **Abba, K., R. Sinfield, C. A. Hart, and P. Garner**. 2009. Pathogens associated with persistent diarrhoea in children in low and middle income countries: systematic review. BioMed Central Infectious Diseases **9**.

9. **World Health Organizationsia**. 2007. Rotavirus vaccine. The Weekly Epidemiological Records **82**:285-296.

10. **Thapar, N. and I. R. Sanderson**. 2004. Diarrhoea in children: an interface between developing and developed countries. Lancet **363**:641-653.

11. **Robins-Browne, R. M., C. S. Still, M. D. Miliotis, N. J. Richardson, H. J. Koornhof, I. Freiman, B. D. Schoub, G. Lecatsas, and E. Hartman**. 1980. Summer diarrhoea in African infants and children. Archives of Disease in Childhood **55**:923-928.

12. **Bradshaw, D., P. Groenewald, R. Laubscher, N. Nannan, B. Nojilana, R. Norman, D. Pieterse, M. Schneider, D. E. Bourne, I. M. Timaeus, R. Dorrington, and L. Johnson**. 2003. Initial burden of disease estimates for South Africa, 2000. South African Medical Journal **93**:688.

13. **Boschi-Pinto, C., L. Velebit, and K. Shibuya**. 2008. Estimating child mortality due to diarrhoea in developing countries. Bulletin of the World Health Organization **86**:710-717.

14. **Galane, P. M. and M. Le Roux**. 2001. Molecular epidemiology of *Escherichia coli* isolated from young South African children with diarrhoeal diseases. Journal of Health, Population and Nutrition **19**:31-38.

15. **Nataro, J. P. and J. B. Kaper**. 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews **11**:142-201.

16. **Guerrant, R. L., R. Oria, O. Y. Bushen, P. D. Patrick, E. Houpt, and A. A. M. Lima**. 2005. Global impact of diarrheal diseases that are sampled by travelers: The rest of the hippopotamus. Clinical Infectious Diseases **41**:S524-S530.

17. **Estrada-Garcia, T., C. Lopez-Saucedo, R. Thompson-Bonilla, M. Abonce, D. Lopez-Hernandez, J. I. Santos, J. L. Rosado, H. L. DuPont, and K. Z. Long**. 2009. Association of diarrheagenic *Escherichia coli* pathotypes with infection and diarrhea among Mexican children and association of atypical enteropathogenic *E. coli* with acute diarrhea. Journal of Clinical Microbiology. **47**:93-98.

18. **Edwards, P. R.** 1972. Identification of Enterobacteriaceae [by] P. R. Edwards [and] W. H. Ewing. Burgess Pub. Co, Minneapolis.

19. **Farmer III, J. J., B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, M. A. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steingerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner**. 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. Journal of Clinical Microbiology **21**:46-76.

20. **Moyo, S., S. Maselle, M. Matee, N. Langeland, and H. Mylvaganam**. 2007. Identification of diarrheagenic *Escherichia coli* isolated from infants and children in Dar es Salaam, Tanzania. BioMed Central Infectious Diseases **7**:92.

21. **Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine**. 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infection Immunity **60**:2297-2304.

22. **Okeke, I. N.** 2009. Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status, uncertainties and necessities. The Journal of Infection in Developing Countries **3**:817-842.

23. **Scaletsky, I. C. A., S. H. Fabbricotti, R. L. B. Carvalho, C. R. Nunes, H. S. Maranhao, M. B. Morais, and U. Fagundes-Neto**. 2002. Diffusely adherent *Escherichia coli* as a cause of acute diarrhea in young children in Northeast Brazil: a case-control study. Journal of Clinical Microbiology **40**:645-648.

24. **Servin, A. L.** 2005. Pathogenesis of Afa/Dr diffusely adhering *Escherichia coli*. Clinical Microbiology Reviews **18**:264-292.

25. **Arikawa, K. and Y. Nishikawa**. 2010. Interleukin-8 induction due to diffusely adherent *Escherichia coli* possessing Afa/Dr genes depends on flagella and epithelial Toll-like receptor 5. Microbiology and Immunology **54**:491-501.

26. **Weintraub, A.** 2007. Enteroaggregative *Escherichia coli:* epidemiology, virulence and detection. Journal of Medical Microbiology **56**:4-8.

27. **Samie, A., C. L. Obi, R. Dillingham, R. C. Pinkerton, and R. L. Guerrant**. 2007. 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. American Journal of Tropical Medicine and Hygiene **77**:142-150.

28. **Law and Chart**. 1998. Enteroaggregative *Escherichia coli*. Journal of Applied Microbiology **84**:685-697.

29. **Afset, J. E., G. Bruant, R. Brousseau, J. Harel, E. Anderssen, L. Bevanger, and K. Bergh**. 2006. Identification of virulence genes linked with diarrhea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR.Journal of Clinical Microbiology **44**:3703-3711.

30. **Hernandes, R. T., W. P. Elias, M. A. M. Vieira, and T. A. T. Gomes**. 2009. An overview of atypical enteropathogenic *Escherichia coli*. Fedration of European Microbiology Societies Microbiology Letters **297**:137-149.

31. **Frohlicher, E., G. Krause, C. Zweifel, L. Beutin, and R. Stephan**. 2008. Characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from pigs and sheep. BioMed Central Microbiology **8**:144.

32. **Navaneethan, U. and R. A. Giannella**. 2008. Mechanisms of infectious diarrhea. Nature Clinical Practice Gastroenterologyand Hepatology **5**:637-647.

33. **Paton, J. C. and A. W. Paton**. 1998. Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* Infections. Clinical Microbiology Reviews **11**:450-479.

34. **Ding, H., L. Huang, X. Mao, and Q. Zou**. 2011. Characterization of *stx2* and its variants in *Escherichia coli* O157:H7 isolated fron patients and animals. African Journal of Biotechnology **10**:2991-2998.

35. **Alfredo, C., M. Stefano, B. Hubert, and O. Eric**. 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Veterinary Research **36**:289-311.

36. **Bekal, S., R. Brousseau, L. Masson, G. Prefontaine, J. Fairbrother, and J. Harel**. 2003. Rapid identification of *Escherichia coli* pathotypes by virulence gene detection with DNA microarrays. Journal of Clinical Microbiology **41**:2113-2125.

37. **Sharifi Yazdi, M. K., A. Akbari, and M. M. Soltan Dallal**. 2011. Multiplex polymerase chain reaction (PCR) assay for simultaneous detection of shiga-like toxin (*stx1* and *stx2),* intimin (*eae)* and invasive plasmid antigen H (i*paH)* genes in diarhoeagenic *Escherichia coli*. African Journal of Biotechnology **10**:1522-1526.

38. **Mercado, E. H., T. J. Ochoa, L. Ecker, M. Cabello, D. Durand, F. Barletta, M. Molina, A. I. Gil, L. Huicho, C. F. Lanata, and T. G. Cleary**. 2011. Fecal leukocytes in children infected with diarrheagenic *Escherichia coli*. Journal of Clinical Microbiology **49**:1376-1381.

39. **Ochoa, T. J., F. Barletta, C. Contreras, and E. Mercado**. 2008. New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. Transactions of the Royal Society of Tropical Medicine and Hygiene **102**:852-856.

40. **Hermos, C. R., M. Janineh, L. L. Han, and A. J. McAdam**. 2011. Shiga toxin-producing *Escherichia coli* in children: diagnosis and clinical manifestations of O157:H7 and non-O157:H7 infection. Journal of Clinical Microbiology **49**:955-959.

41. **Navaneethan, U. and R. A. Giannella**. 2011. Infectious colitis. Current Opinion in Gastroenterology **27**.

42. **Sodha, S. V., M. Lynch, K. Wannemuehler, M. M. Leeper, M. Malavet, J. Schaffzin, T. Chen, A. Langer, M. Glenshaw, D. Hoefer, N. Dumas, L. Lind, M. Iwamoto, T. Ayers, T. Nguyen, M. Biggerstaff, C. Olson, A. Sheth, and C. Braden**. 2010. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with a national fast-food chain, 2006: a

study incorporating epidemiological and food source traceback results. Journal of Epidemiology and Infection :1-8.

43. **Ogundele, M. O. and H. F. Ayyash**. 2010. Hemolytic uremic syndrome in children-a concise review. Journal of Pediatric Infectious Diseases **5**:309-320.

44. **Keddy, K. H., The Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA)**. 2011. Non-typhoidal *Salmonella enterica* (NTS), *Shigella* species and Diarrhoeagenic *Escherichia coli*. Communicable Diseases Surveillance Bulletin **9**:27-58.

45. **Vargas M, J. Gascon, C. Casals, D. Schellenberg, H. Urassa, E. Kahigwa, J. Ruiz and J. Vila**. 2004. Etiology of diarrhoea in children less than five years of age in Ifakara, Tanzania. American Journal of Tropical Medicine and Hygiene **70:**536-539.

46. **Nweze, E. I.** 2009. Virulence properties of diarrheagenic *E. coli* and etiology of diarrhea in infants, young children and other age groups in Southeast, Nigeria. American-Eurasian Journal of Scientific Research **4 (3):** 173-179.

47. **Rowe J. S, Shah SS, Motlhagodi S, Bafana M, Tawanana E,** et al. (2010) An Epidemiologic Review of Enteropathogens in Gaborone, Botswana: Shifting Patterns of Resistance in an HIV Endemic Region. PLoS ONE **5(6):** e10924.

48. **Chattaway, M. A., T. Dallman, I. N. Okeke, and J. Wain**. 2011. Enteroaggregative *E. coli* O104 from an outbreak of HUS in Germany 2011, could it happen again. Journal of Infection in Developing Countries **5**:425-436.

49. **Harendza, S**. 2011. “HUS diary” of a German neprologist during the current EHEC outbreak in Europe. PubMed **80:**687-689.

50. **Levine, M. M and J.P. Nataro**. 2000. Diarrhea caused by *Escherichia coli*. In: **Strickland, G. T.** (Ed.) Hunter's Tropical Medicine and Emerging Infectious Diseases, 8th Ed. WB Saunders Company, Philadelphia, USA: 334-338.

51. **Kaur, P., A. Chakraborti, and A. Asea**. 2010. Enteroaggregative *Escherichia coli*: an emerging enteric foodborne pathogen. Interdisciplinary Perspective on Infectious Diseases :1-10.

52. **Hill, S. M., A. D. Phillips, and J. A. Walker-Smith**. 1991. Enteropathogenic *Escherichia coli* and life threatening chronic diarrhoea. Gut **32**:154-158.

53. **Nguyen, T. V., P. Le Van, C. Le Huy, and A. Weintraub**. 2004. Diarrhea caused by Rotavirus in children less than 5 years of age in Hanoi, Vietnam. Journal of Clinical Microbiology **42**:5745-5750.

54. **Nessa, K., D. Ahmed, J. Islam, F. M. L. Kabir, and M. A. Hossain**. 2007. Usefulness of a multiplex PCR for detection of diarrheagenic *Escherichia coli* in a diagnostic microbiology laboratory setting. Bangladesh Journal of Medical Microbiology **1**:38-42.

55. **Vidal, M., E. Kruger, C. Duran, R. Lagos, M. Levine, V. Prado, C. Toro, and R. Vidal**. 2005. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. Journal of Clinical Microbiology **43**:5362-5365.

56. **Wani, S. A., A. Nabi, I. Fayaz, I. Ahmad, Y. Nishikawa, K. Qureshi, M. A. Khan, and J. Chowdhary**. 2006. Investigation of diarrhoeic faecal samples for enterotoxigenic, Shiga toxin-producing and typical or atypical enteropathogenic *Escherichia coli* in Kashmir,

India. Federation of European Microbiological Societies Microbiology Letters **261**:238-244.

57. **Oskorv, I., F. Orskov, B. Jann, and K. JANN**. 1977. Serology, chemistry and genetics of O and K antigens of *Escherichia coli*. American Society for Microbiology **41**:667-710.

58. **Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J. P. Gayral, and D. Raoult**. 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. Journal of Clinical Microbiology **38**:3623-3630.

59. **Tang, Y. W., N. M. Ellis, M. K. Hopkins, D. H. Smith, D. E. Dodge, and D. H. Persing**. 1998. Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. Journal of Clinical Microbiology **36**:3674-3679.

60. **Nemoy, L. L., M. Kotetishvili, J. Tigno, A. Keefer-Norris, A. D. Harris, E. N. Perencevich, J. A. Johnson, D. Torpey, A. Sulakvelidze, Jr. J. G. Morris, and O. C. Stine**. 2005. Multilocus sequence typing versus pulsed-field gel electrophoresis for characterization of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates. Journal of Clinical Microbiology **43**:1776-1781.

61. **Majowicz, S. E., J. Musto, E. Scallan, F. J. Angulo, M. Kirk, S. J. O'Brien, T. F. Jones, A. Fazil, R. M. Hoekstra, and for the International Collaboration on Enteric Disease 'Burden of Illness' Studies**. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clinical Infectious Diseases **50**:882-889.

62. **Cui, S., J. Li, Z. Sun, C. Hu, S. Jin, F. Li, Y. Guo, L. Ran, and Y. Ma**. 2009. Characterization of *Salmonella enterica* isolates from infants and toddlers in Wuhan, China. Journal of Antimicrobial Chemotherapy **63**:87-94.

63. **Girard, M. P., D. Steele, C. L. Chaignat, and M. P. Kieny**. 2006. A review of vaccine research and development: The human immunodeficiency virus (HIV). The Open Vaccine Journal **24**:2732-2750.

64. **Hoelzer, K., Y. Soyer, L. D. Rodriguez-Rivera, K. J. Cummings, P. L. McDonough, D. J. Schoonmaker-Bopp, T. P. Root, N. B. Dumas, L. D. Warnick, Y. T. Grohn, M. Wiedmann, K. N. K. Baker, T. E. Besser, D. D. Hancock, and M. A. Davis**. 2010. The prevalence of multidrug resistance is higher among bovine than human *Salmonella enterica* serotype Newport, Typhimurium, and 4,5,12:i:- isolates in the United States but differs by serotype and geographic region. Applied Environmental and Microbiology **76**:5947-5959.

65. **Kapperud, G., H. Stenwig, and J. Lassen**. 1998. Epidemiology of *Salmonella* Typhimurium O: 4-12 infection in Norway. American Journal of Epidemiology **147**:774-782.

66. **Borman, E. K., C. A. Stuart, and K. M. Wheeler**. 1944. Taxonomy of the family Enterobacteriaceae. Journal of Bacteriology **48**:351-367.

67. **Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan**. 2000. *Salmonella* nomenclature. Journal Clinical Microbiology **38**:2465-2467.

68. **Baumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams**. 1998. Evolution of host adaptation in *Salmonellaenterica*. Infection and Immunity **66**:4579-4587.

69. **Paiva de Sousa, C. and J. D. Dubreuil**. 2001. Distribution and expression of the astA gene (EAST1 toxin) in *Escherichia coli* and *Salmonella*. International Journal of Medical Microbiology **291**:15-20.

70. **Galyov, E. E., M. W. Wood, R. Rosqvist, P. B. Mullan, P. R. Watson, S. Hedges, and T. S. Wallis**. 1997. A secreted effector protein of *Salmonella* Dublin is translocated into

eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. Molecular Microbiology **25**:903-912.

71. **Watson, P. R., S. M. Paulin, A. P. Bland, P. W. Jones, and T. S. Wallis**. 1995. Characterization of intestinal invasion by *Salmonella* Typhimurium and *Salmonella* Dublin and effect of a mutation in the invH gene. Infection and Immunity **63**:2743-2754.
72. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow**. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Molecular Microbiology **30**:175-188.
73. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden**. 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular Microbiology **30**:163-174.
74. **Galtin, J. E.** 1996. Molecular genetic bases of *Salmonella* entry into host cells. Molecular Microbiology **20**:263-271.
75. **Hobbie, S., L. M. Chen, R. J. Davis, and J. E. Galan**. 1997. Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella* Typhimurium in cultured intestinal epithelial cells. The Journal of Immunology **159**:5550-5559.
76. **Watson, P. R., E. E. Galyov, S. M. Paulin, P. W. Jones, and T. S. Wallis**. 1998. Mutation of invH, but not stn, reduces *Salmonella*-induced enteritis in cattle. Infection and Immunity **66**:1432-1438.

77. **Ohl, M. E. and S. I. Miller**. 2001. *Salmonella*: a model for bacterial pathogenesis. Anual Review of Medicine **52**:259-274.

78. **Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller**. 1995. PhoP/PhoQ transcriptional repression of *Salmonella* Typhimurium invasion genes: evidence for a role in protein secretion. Molecular Microbiology **17**:169-181.

79. **Miller, S. I. and J. J. Mekalanos**. 1990. Constitutive expression of the phoP regulon attenuates *Salmonella* virulence and survival within macrophages. Journal Bacteriology **172**:2485-2490.

80. **Dong, T. and H. E. Schellhorn**. 2010. Role of RpoS in virulence of pathogens. Infection and Immunity **78**:887-897.

81. **Gulig, P. A., H. Danbara, D. G. Guiney, A. J. Lax, F. Norel, and M. Rhen**. 1993. Molecular analysis of spv virulence genes of the *Salmonella* virulence plasmids. Molecular Microbiology **7**:825-830.

82. **Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., and Tauxe, R. V**. 1999. Food-related illness and death in the United States. Emerging Infectious Diseases Journal **5**(5):607-625

83. **Molyneux, E. M., L. A. Mankhambo, A. Phiri, S. M. Graham, H. Forsyth, A. Phiri, A. L. Walsh, and L. K. Wilson**. 2009. The outcome of non-typhoidal *Salmonella* meningitis in Malawian children, 1997-2006. Annals of Tropical Paediatrics **29**(1):13-22.

84. **Hanes, D**. 2003. Nontyphoid *Salmonella*. In: **Miliotis, M. D and J. W. Bier** (Eds). International Handbook of Foodborne Pathogens. Marcel Dekker, New York, USA: 137-149.

85. **Graham, S. M, A. L. Walsh, E. M. Molyneux, A. J. Phiri and M. E. Molyneux**. 2000. Clinical presentation of non-typhoidal salmonellosis in Malawian children. Transactions of the Royal Society of Tropical Medicine and Hygiene **94**:310-314.

86. **Gordon, M. A., S. M. Graham, A. L. Walsh, L. Wilson, A. Phiri, E. Molyneux, E. E. Zijlstra, R. S. Heyderman, C. A. Hart, and M. E. Molyneux**. 2008. Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clinical Infectious Diseases **46**:963-969.

87. **Crump, J. A., S. P. Luby, and E. D. Mintz**. 2004. The global burden of typhoid fever. Bulletin of the World Health Organization **82**:346-353.

88. **Mason, C.J and R. N. Longfield**. 2000. Nontyphoidal *Salmonella* infections. In: **Strickland, G. T**. (Ed.) Hunter's Tropical Medicine and Emerging Infectious Diseases, 8th Ed. WB Saunders Company, Philadelphia, USA: 484-491.

89. **Crump, J. A., K. Kretsinger, K. Gay, R. M. Hoekstra, D. J. Vugia, S. Hurd, S. D. Segler, M. Megginson, L. J. Luedeman, B. Shiferaw, S. S. Hanna, K. W. Joyce, E. D. Mintz, F. J. Angulo, and The emerging infections program FoodNet and NARMS working groups**. 2008. Clinical response and outcome of infection with *Salmonella enterica* serotype Typhi with decreased susceptibility to fluoroquinolones: a United States FoodNet Multicenter retrospective cohort study. Antimicrobial Agents and Chemothery **52**:1278-1284.

90. **Morpeth, S. C., H. O. Ramadhani, and J. A. Crump**. 2009. Invasive non-Typhi *Salmonella* disease in Africa. Clinical Infectious Diseases **49**:606-611.

91. **Yang, Y. J., M. C. Huang, S. M. Wang, J. J. Wu, C. P. Cheng, and C. C. Liu**. 2002. Analysis of risk factors for bacteremia in children with nontyphoidal *Salmonella* gastroenteritis. European Journal of Clinical Microbiology and Infectious Diseases **21**:290-293.

92. **Zaidi, E. I. M. A., R. I. C. H. Bachur, and M. A. R. V. Happer**. 1999. Non-typhi *Salmonella* bacteremia in children. The Pediatric Infectious Disease Journal **18**.

93. **Whitaker, J. A., C. Franco-Paredes, C. Del Rio, and S. Edupuganti**. 2009. Rethinking typhoid fever vaccines: Implications for travelers and people living in highly endemic areas. Journal of Travel Medicine **16**:46-52.

94. **Kapoor, L., H. K. Eideh, S. Ahmad, P. Lal, M. Deb, and S. S. Thukral**. 2006. Molecular typing of *Salmonella enterica* serotype Worthington isolates from infantile diarrhoea. Indian Journal of Medical Research **123**:691-696.

95. **Oracz, G., W. Feleszko, D. Golicka, J. Maksymiuk, A. Klonowska, and H. Szajewska**. 2003. Rapid diagnosis of acute *Salmonella* gastrointestinal infection. Clinical Infectious Diseases **36**:112-115.

96. **Alvarez, J., M. Sota, A. B. Vivanco, I. Perales, R. Cisterna, A. Rementeria, and J. Garaizar**. 2004. Development of a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. Journal of Clinical Microbiology **42**:1734-1738.

97. **Hoorfar, J., P. Ahrens, and P. Radstrom**. 2000. Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. Journal of Clinical Microbiology **38**:3429-3435.

98. **Angulo, F. J. and D. L. Swerdlow**. 1995. Bacterial enteric ifections in persons infected with human immunodeficiency virus. Clinical Infectious Diseases **21**:S84-S93.

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

100. .**von Seidlein, L., D. R. Kim, M. Ali, H. Lee, X. Wang, V. D. Thiem, D. G. Canh, W. Chaicumpa, M. D. Agtini, A. Hossain, Z. A. Bhutta, C. Mason, O. Sethabutr, K. Talukder, G. B. Nair, J. L. Deen, K. Kotloff, and J. Clemens**. 2006. A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS Medicine **3**:e353.

101. **Guerin, P. J., C. Brasher, E. Baron, D. Mic, F. Grimont, M. Ryan, P. Aavitsland, and D. Legros**. 2004. Case management of a multidrug-resistant *Shigella* dysenteriae serotype 1 outbreak in a crisis context in Sierra Leone, 1999-2000. Transactions of the Royal Society of Tropical Medicine and Hygiene **98**:635-643.

102. **Nair, G., T. Ramamurthy, M. Bhattacharya, T. Krishnan, S. Ganguly, D. Saha, K. Rajendran, B. Manna, M. Ghosh, K. Okamoto, and Y. Takeda**. 2010. Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. Gut Pathogens **2**:4.

103. **Guerrant, R. L., J. M. Hughes, N. L. Lima, and J. Crane**. 1990. Diarrhea in developed and developing countries: Magnitude, special settings, and etiologies. Review of Infectious Diseases **12**:S41-S50.

104. **Ewing, W. H.** 1949. *Shigella* nomenclature. Journal of Bacteriology. **57**:633-638.

105. **Penatti, M. P. A., L. M. Hollanda, G. Nakazato, T. A. Campos, M. Lancellotti, M. Angellini, M. Brocchi, M. M. M. Rocha, and W. D. d. Silveira**. 2007. Epidemiological characterization of resistance and PCR typing of *Shigella flexneri* and *Shigella sonnei* strains isolated from bacillary dysentery cases in Southeast Brazil. Brazilian Journal of Medical and Biological Research **40**:249-258.

106. **Kingombe, C. L., M. L. Cerqueira-Campos, and J. M. Faber**. 2005. Molecular strategies for the detection, identification, and differentiation betwwen enteroinvasive *Escherichia coli* and *Shigella* species. Journal of Food Protection **68**:7549-7553.

107. **Theron, J., D. Morar, M. Du Preez, V. S. Br÷zel, and S. N. Venter**. 2001. A sensitive seminested PCR method for the detection of shigella in spiked environmental water samples. Water Research **35**:869-874.

108. **Lysenko, O. and P. H. A. Sneath**. 1959. The use of models in bacterial classification. Journal of Geneneral Microbiology **20**:284-290.

109. **Cossart, P. and P. J. Sansonetti**. 2004. Bacterial invasion: The paradigms of enteroinvasive pathogens. Science **304**:242-248.

110. **Tran Van Nhieu, G., R. l. Bourdet-Sicard, G. Dumenil, A. Blocker, and P. J. Sansonetti**. 2000. Bacterial signals and cell responses during *Shigella* entry into epithelial cells. Cellular Microbiology **2**:187-193.

111. **Parsot, C.** 2005. *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. Federation of European Microbiological Societies Microbiology Letters **252**:11-18.

112. **Phalipon, A. and P. J. Sansonetti**. 2003. Shigellosis: Innate mechanisms of inflammatory destruction of the intestinal epithelium, adaptive immune response, and vaccine development. Critical Reviews in Immunology **23**:371-401.

113. **Philpott, J., E. Girardin, and J. Sansonetti**. 2001. Innate immune responses of epithelial cells following infection with bacterial pathogens. Current Opinion in Immunology **13**:410-416.

114. **Sansonetti, P. J.** 2004. War and peace at mucosal surfaces. Nature Reviews of Immunology **4**:953-964.

115. **Torres, A. G.** 2004. Current aspects of *Shigella* pathogenesis. Reviws of Latinamerican Microbiology **46**:89-97.

116. **Blocker, A., P. Gounon, E. Larquet, K. Niebuhr, V. Cabiaux, C. Parsot, and P. Sansonetti**. 1999. The tripartite type III secreton of *Shigella flexneri* inserts ipab and ipac into host membranes. The Journal of Cell Biology **147**:683-693.

117. **Venkatesan, M. M., J. M. Buysse, and E. V. Oaks**. 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the spa locus. Journal of Bacteriology **174**:1990-2001.

118. **Niyogi, S. K.** 2005. Shigellosis. Journal of Microbiology **43**:133-143.

119. **Hale, T. L.** 1991. Genetic basis of virulence in *Shigella* species. Microbiology and Molecular Biology Reviews **55**:206-224.

120. **Koterski, J. F., M. Nahvi, M. M. Venkatesan, and B. Haimovich**. 2005. Virulent *Shigella flexneri* causes damage to mitochondria and triggers necrosis in infected human monocyte-derived macrophages. Infection and Immunity **73**:504-513.

121. **Edwards, B. H.** 1999. *Salmonella* and *Shigella* species. Clinics in Laboratory Medicine **19**:469-487.

122. **Prado, V. A. L. E., R. O. S. A. Lagos, J. P. Nataro, O. S. Matin, C. A. R. O. ARELLANO, J. Y. WANG, A. A. BORCZYK, and M. M. Levine**. 1999. Population-based study of the incidence of *Shigella* diarrhea and causative serotypes in Santiago, Chile. The Pediatric Infectious Disease Journal **18**.

123. **Manirakiza, A, P. Megne-Boudjeka, G. Bobossi-Serengbe, R. Bercion and A. Le Faou.** 2010. Prevalence of shigellosis diarrhoea in a paediatrics population: hospital based survey in Bangui, Central African Republic**.** Journal of Infection in Developing Countries **4(10)**:655- 657.

124. **Brooks J. T, J. B. Ochieng, L. Kumar, G. Okoth, R. L. Shapiro, J. B. Wells, M. Bird, C. Bopp, W. Chege, M. E. Beatty, T. Chiller, J. M. Vulule, E. Mintz and L. Slutsker**. 2006. Surveillance for bacterial diarrhea and antimicrobial resistance in rural western Kenya, 1997–2003. Clinical Infectious Diseases **43:**393–401.

125. **National Institute for Communicable Diseases**. 2008. Diarrhoeal disease oubreak-school trip. Communicable Diseases Communique **7**:1-2.

126. **Pillay, D. G., A. J. Karas, and A. W. Sturm**. 1997. An outbreak of Shiga bacillus dysentery in KwaZulu-Natal, South Africa. Journal of Infection **34**:107-111.

127. **Finkelstein, Y., O. Moran, Y. Avitzur, M. Nussinovitch, L. Harel, B. Volovitz, and J. Amir**. 2002. Clinical dysentery in hospitalized children. Infection **30**:132-135.

128. **Goodgame, R. W**. 2001. Viral causes of diarrhoea. Gastroenterol Clinics of North America **30**: 779-795. 130. **Abu-Elyazeed, R. R., T. F. Wierzba, R. W. Frenck, S. D. Putnam, M.**

R. Rao, S. J. Savarino, K. A. Kamal, L. F. Peruski, I. A. Abd-El Messih, S. A. El-Alkamy, A. B. Naficy, and J. D. Clemens. 2004. Epidemiology of *Shigella*-associated diarrhea in rural Egyptian children. The American Journal of Tropical Medicine and Hygiene **71**:367-372.

129. **Ozuah, P. O. and H. M. Adam**. 1998. *Shigella* Update. Pediatrics in Review **19**:100.

130. **Bennish, M. L.** 1991. Potentially lethal complications of shigellosis. Review of Infectious Diseases **13**:S319-S324.

131. **Greenberg, D., S. Marcu, R. Melamed, and M. Lifshitz**. 2003. *Shigella* bacteremia: A retrospective study. Clinical Pediatrics **42**:411-415.

132. **Viner, Y., D. Miron, E. Gottfried, D. Segal, and A. Luder**. 2001. Neonatal shigellosis. The Israel Medical Association journal **3**:964-966.

133. **Soofi, S. B., M. A. Habib, L. von Seidlein, M. J. Khan, S. Muhammad, N. Bhutto, M. I. Khan, S. Rasool, A. Zafar, J. D. Clemens, Q. Nizami, and Z. A. Bhutta**. 2011. A comparison of disease caused by *Shigella* and *Campylobacter* species: 24 months community based surveillance in 4 slums of Karachi, Pakistan. Journal of infection and Public Health **4**:12-21.

134. **Li, Y., B. Cao, B. Liu, D. Liu, Q. Gao, X. Peng, J. Wu, D. A. Bastin, L. Feng, and L. Wang**. 2009. Molecular detection of all 34 distinct O-antigen forms of *Shigella*. Journal of Medical Microbiology **58**:69-81.

135. **Keener, K. M., M. P. Bashor, P. A. Curtis, B. W. Sheldon, and S. Kathariou**. 2004. Comprehensive review of *Campylobacter* and poultry processing. Comprehensive Reviews in Food Science and Food Safety **3**:105-116.

136. **Samuel, M. C., D. J. Vugia, S. Shallow, R. Marcus, S. Segler, T. McGivern, H. Kassenborg, K. Reilly, M. Kennedy, F. Angulo, R. V. Tauxe, and for the Emerging Infections Program FoodNet Working Group**. 2004. Epidemiology of sporadic *Campylobacter* infection in the United States and declining trend in incidence, FoodNet 1996–1999. Clinical Infectious Diseases **38**:S165-S174.

137. **van Pelt, W., W. de, W. J. Wannet, E. J. Ligtvoet, M. A. Widdowson, and D. van**. 2003. Laboratory surveillance of bacterial gastroenteric pathogens in the Netherlands, 1991-2001. Epidemiology and Infection **130**:431-441.

138. **Altekruse, S. F., N. J. Stern, P. I. Field, and D. L. Swerdlow**. 1999. *Campylobacter jejuni*: an emerging foodborne pathogen. Emerging Infectious Diseases Journal **5**:28-35.

139. **Eberhart-Phillips, J., N. Walker, N. Garrett, D. Bell, D. Sinclair, W. Rainger, and M. Bates**. 1997. Campylobacteriosis in New Zealand: results of a case-control study. Journalof Epidemiology and Community Health **51**:686-691.

140. **Neimann, J., J. Engberg, K. Molbak, and H. C. Wegener**. 2003. A case control study of risk factors for sporadic *Campylobacter* infections in Denmark. Epidemiology and Infection **130**:353-366.

141. **Acheson, D. and B. M. Allos**. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical Infectious Diseases **32**:1201-1206.

142. **Allos, B. M. and M. J. Blaser**. 1995. *Campylobacter jejuni* and the expanding spectrum of related infections. Clinical Infectious Diseases **20**:1092-1101.

143. **Ailes, E., L. Demma, S. Hurd, J. Hatch, T. F. Jones, D. Vugia, A. Cronquist, M. Tobin-D'Angelo, K. Larson, E. Laine, K. Edge, S. Zansky, and E. Scallan**. 2008. Continued decline

in the incidence of *Campylobacter* Infections, FoodNet 1996-2006. Foodborne Pathogens and Disease **5**:329-337.

144. **Fullerton, K. E., L. A. Ingram, T. F. Jones, B. J. Anderson, P. V. McCarthy, S. Hurd, B. Shiferaw, D. Vugia, N. Haubert, T. Hayes, S. Wedel, E. Scallan, O. Henao, and F. J. Angulo**. 2007. Sporadic *Campylobacter* infection in infants: A population-based surveillance case-control study. The Pediatric Infectious Disease Journal **26**:19-24.

145. **Vandamme, P**. 2000. Taxonomy of the family Campylobacteraceae. 3-26. In **Nachamkin, I; Blaser, M. J**. eds. *Campylobacter.* American Society for Microbiology, Washington, DC.

146. **Moran, A. P. and M. E. Upton**. 1987. Factors affecting production of coccoid forms by *Campylobacter jejuni* on solid media during incubation. Journal of Applied Microbiology **62**:527-537.

147. **Rollins, D. M. and R. R. Colwell**. 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Applied Environmental Microbiology **52**:531-538.

148. **Nachamkin, I**. 1996. *Campylobacter, Helicobacter* and related spiral bacteria. In: Murray, P. R, ed. Manual of Clinical Microbiology :402-409.

149. **Fennell, C. L., P. A. Totten, T. C. Quinn, D. L. Patton, K. K. Holmes, and W. E. Stamm**. 1984. Characterization of *Campylobacter*-like organisms isolated from homosexual men. Journal of Infectious Diseases **149**:58-66.

150. **Sellars, M. J., S. J. Hall, and D. J. Kelly**. 2002. Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. Journal of Bacteriology **184**:4187-4196.

151. **Konkel, M. E., M. R. Monteville, V. Rivera-Amill, and L. A. Joens**. 2001. The pathogenesis of *Campylobacter jejuni*-mediated enteritis. Current Issues in Intestinal Microbiology **2**:55-71.

152. **Wassenaar, T. M.** 1997. Toxin production by *Campylobacter* spp. Clinical Microbiology Reviews **10**:466-476.

153. **Bras, A. M. and J. M. Ketley**. 1999. Transcellular translocation of *Campylobacter jejuni* across human polarised epithelial monolayers. Federation of European Microbiological Societies Microbiology Letters **179**:209-215.

154. **Everest, P. H., H. Goossens, J. P. Butzler, D. Lloyd, S. Knutton, J. M. Ketley, and P. H. Williams**. 1992. Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. Journal of Medical Microbiology **37**:319-325.

155. **Konkel, M. E., S. F. Hayes, L. A. Joens, and W. Cieplak**. 1992. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. Microbial Pathogenesis **13**:357-370.

156. **Grant, C. C., M. E. Konkel, W. Cieplak, Jr., and L. S. Tompkins**. 1993. Role of flagella in adherence, internalization, and translocation of Campylobacter jejuni in nonpolarized and polarized epithelial cell cultures. Infection and Immunity **61**:1764-1771.

157. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, and B. A. M. Van der Zeijst**. 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. European Molecular Biology Organization Journal **10**:2055-2061.

158. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry**. 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni:* the role of motility in adherence and invasion of eukaryotic cells. Molecular Microbiology **14**:883-893.

159. **Konkel, M. E., S. A. Gray, B. J. Kim, S. G. Garvis, and J. Yoon**. 1999. Identification of the Enteropathogens Campylobacter jejuni and Campylobacter coli Based on the cadF Virulence Gene and Its Product. Journal of Clinical Microbiology **37**:510-517.

160. **Lara-Tejero, M. and J. E. Galan**. 2001. CdtA, CdtB, and CdtC Form a Tripartite Complex That Is Required for Cytolethal Distending Toxin Activity. Infection and Immunity **69**:4358-4365.

161. **Purdy, D., C. M. Buwell, A. E. Hodgson, K. McAlpine, I. Hendeeson, and S. A. Leach**. 2000. Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. Journal of Medical Microbiology **49**:473-479.

162. **Eyigor, A., K. A. Dawson, B. E. Langlois, and C. L. Pickett**. 1999. Detection of Cytolethal Distending Toxin Activity and cdt Genes in *Campylobacter* spp. Isolated from Chicken Carcasses. Applied Environmental Microbiology **65**:1501-1505.

163. **Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin**. 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* spp. cdtB gene. Infection and Immunity **64**:2070-2078.

164. **Kemp, G. K., M. L. Aldrich, M. L. Guerra, and K. R. Schneider**. 2001. Continuous online processing of fecal- and ingesta-contaminated poultry carcasses using an acidified sodium chlorite antimicrobial intervention. Journal of Food Protection **64**:807-812.

165. **Friedman, C. R., R. M. Hoekstra, M. Samuel, R. Marcus, J. Bender, B. Shiferaw, S. Reddy, S. D. Ahuja, D. L. Helfrick, F. Hardnett, M. Carter, B. Anderson, R. V. Tauxe, and for the Emerging Infections Program FoodNet Working Group**. 2004. Risk Factors for Sporadic Campylobacter Infection in the United States: A Case-Control Study in FoodNet Sites. Clinical Infectious Diseases **38**:S285-S296.

166. **Moore, J. E., C. Deborah, S. G. D. James, F. Seamus, L. Brigid, M. Motoo, A. M. David, M. Francis, B. C. Millar, O. Rebecca, O. Lisa, O. Michele, R. R. Juluri, J. R. Paul, S. Andrew, and W. Paul**. 2005. *Campylobacter*. Veterinary Research **36**:351-382.

167. **Taylor, D. N., J. A. Kiehlbauch, W. Tee, C. Pitarangsi, and P. Echeverria**. 1991. Isolation of Group 2 Aerotolerant *Campylobacter* Species from Thai Children with Diarrhea. Journal of Infectious Diseases **163**:1062-1067.

168. **Georges, M. C., I. K. Wachsmuth, D. M. Meunier, N. Nebout, F. Didier, M. R. Siopathis, and A. J. Georges**. 1984. Parasitic, bacterial, and viral enteric pathogens associated with diarrhea in the Central African Republic. Journal of Clinical Microbiology **19**:571-575.

169. **Samie, A., C. L. Obi, L. J. Barrett, S. M. Powell, and R. L. Guerrant**. 2007. Prevalence of *Campylobacter* species, Helicobacter pylori and Arcobacter species in stool samples from the Venda region, Limpopo, South Africa: Studies using molecular diagnostic methods. Journal of Infection **54**:558-566.

170. **Mshana, S. E., M. Joloba, A. Kakooza, and D. Kaddu-Mulindwa**. 2009. *Campylobacter* spp among children with acute diarrhea attending Mulago hospital in Kampala - Uganda. African Health Science **9**:201-205.

171. **Wassenaar, T. M., M. Kist, and A. de Jong**. 2007. Re-analysis of the risks attributed to ciprofloxacin-resistant *Campylobacter jejuni* infections. International Journal of Antimicrobial Agents **30**:195-201.

172. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology **143**:5-21.

173. **Allos, B. M.** 1997. Association between *Campylobacter* Infection and Guillain-Barre Syndrome. Journal of Infectious Diseases **176**:S125-S128.

174. **Pacanowski, J., V. Lalande, K. Lacombe, C. Boudraa, P. Lesprit, P. Legrand, D. Trystram, N. Kassis, G. Arlet, J. L. Mainardi, F. Doucet-Populaire, P. M. Girard, J. L. Meynard, and CAMPYL Study Group**. 2008. *Campylobacter* bacteremia: clinical features and factors associated with fatal outcome. Clinical Infectious Diseases **47**:790-796.

175. **Winer, J. B.** 2001. Guillain Barre syndrome. Molecular Pathology **54**:381-385.

176. **Taylor, D. N., P. Echeverria, C. Pitarangsi, J. Seriwatana, L. Bodhidatta, and M. J. Blaser**. 1988. Influence of strain characteristics and immunity on the epidemiology of *Campylobacter* infections in Thailand. Journal of Clinical Microbiology **26**:863-868.

177. **Wang, H. and D. R. Murdoch**. 2004. Detection of *Campylobacter* species in faecal samples by direct Gram stain microscopy. Pathology **36**:343-344.

178. **Nachamkin, I**. *Campylonacter* and *Arcobacter.* 1995. In **Murray P. R; Baron, E. J; Pfaller , M. A; Tenover, F. C; Yolken, R. H**. eds. Manual of clinical microbiology. Washington, DC. American Society for Microbiology :483-491.

179. **Bessede, E., A. Delcamp, E. Sifre, A. Buissonniere, and F. Megraud**. 2011. New methods for detection of *Campylobacters* in stool samples in comparison to culture. Journal of Clinical Microbiology **49**:941-944.

180. **Steele, T. W. and S. N. McDermott**. 1984. The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter* Jejuni from feces. Pathology **16**:263-265.

181. **Cloak, O. M., G. Duffy, J. J. Sheridan, I. S. Blair, and D. A. McDowell**. 2001. A survey on the incidence of *Campylobacter* spp. and the development of a surface adhesion polymerase chain reaction (SA-PCR) assay for the detection of *Campylobacter jejuni* in retail meat products. Food Microbiology **18**:287-298.

182. **Padungtod, P., D. L. Hanson, J. Wilson, J. Bell, E. Linz, and J. B. Kaneene**. 2002. Identification of *Campylobacter jejuni* isolates from cloacal and carcass swabs of chickens in Thailand by a 5 nuclease fluorogenic polymerase chain reaction assay. Journal of Food Protection **65**:1712-1716.

183. **Wassenaar, T. M. and D. G. Newell**. 2000. Genotyping of *Campylobacter* spp. Applied Environmental Microbiology **66**:1-9.

184. **WHO**. 2010. Cholera. http://www.who.int/mediacentre/factsheets/fs107/en/. Accessed 18/01/2011

185. **Sack, D. A., R. B. Sack, and C. L. Chaignat**. 2006. Getting serious about Cholera. New England Journal of Medicine **355**:649-651.

186. **Tauxe, R; Seminario, L; Tapia, A. R; Libel, M**. 1994. The Latin America wepidemic. In *Vibrio cholerae* and cholera ed. **Wachsmuth, I. K; Blake, P. A; Olsvic, O. Washington, DC**. American Society for Microbiology :321-344.

187. **Lipp, E. K., A. Huq, and R. R. Colwell**. 2002. Effects of global climate on infectious disease: the Cholera model. Clinical Microbiology Reviews **15**:757-770.

188. **Huq, A., C. A. Whitehouse, C. J. Grim, M. Alam, and R. R. Colwell**. 2008. Biofilms in water, its role and impact in human disease transmission. Current Opinion in Biotechnology **19**:244-247.

189. **Yamai, S., T. Okitsu, T. Shimanda, and Y. Katsube**. 1997. Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin, and additional of novel serogroups. Journal of Kansenshogaku Zashi **71**:1037-1045.

190. **Ramamurthy, T., A. Pal, S. C. Pal, and G. B. Nair**. 1992. Taxonomical implications of the emergence of high frequency of occurrence of 2,4-diamino-6,7-diisopropylpteridine-resistant strains of *Vibrio cholerae* from clinical cases of cholera in Calcutta, India. Journal of Clinical Microbiology **30**:742-743.

191. **Shimada, T., E. Arakawa, K. Itoh, T. Okitsu, A. Matsushima, Y. Asai, S. Yamai, T. Nakazato, G. B. Nair, M. J. Albert, and Y. Takeda**. 1994. Extended serotyping scheme for *Vibrio cholerae*. Current Microbiology **28**:175-178.

192. **Chattopadhyay, D. J., B. L. Sarkar, M. Q. Ansari, B. K. Chakrabarti, M. K. Roy, A. N. Ghosh, and S. C. Pal**. 1993. New phage typing scheme for *Vibrio cholerae* O1 biotype El Tor strains. Journal of Clinical Microbiology **31**:1579-1585.

193. **Morris, J. G., T. Takeda, B. D. Tall, G. A. Losonsky, S. K. Bhattacharya, B. D. Forrest, B. A. Kay, and M. Nishibuchi**. 1990. Experimental non-O group 1 *Vibrio cholerae* gastroenteritis in humans. Journal of Clinical Investigation **85**:697-705.

194. **Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik, C. Whitfield, D. Coplin, N. Kido, J. Klena, D. Maskell, C. R. H. Raetz, and P. D. Rick**. 1996. Bacterial polysaccharide synthesis and gene nomenclature. Trends in Microbiology **4**:495-503.

195. **Davis, B. R., G. R. Fanning, J. M. Madden, A. G. Steigerwalt, H. B. Bradford, Jr., H. L. Smith, Jr., and D. J. Brenner**. 1981. Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species, *Vibrio mimicus*. Journal of Clinical Microbiology **14**:631-639.

196. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos**. 1987. Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proceedings of the National Academy of Sciences **84**:2833-2837.

197. **Spangler, B. D.** 1992. Structure and function of Cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiology and Molecular Biology Reviews **56**:622-647.

198. **Finkelstein, R. A. and J. J. Lospalluto**. 1969. Pathogenesis of experimental cholera: preparation and isolation of choleragen and choleragenoid. Journal of Experimental Medicine **130**:185-880.

199. **Fasano, A., B. Baudry, D. W. Pumplin, S. S. Wasserman, B. D. Tall, J. M. Ketley, and J. B. Kaper**. 1991. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proceedings of the National Academy of Sciences **88**:5242-5246.

200. **Pearson, G. D., A. Woods, S. L. Chiang, and J. J. Mekalanos**. 1993. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proceedings of the National Academy of Sciences **90**:3750-3754.

201. **Trucksis, M., J. E. Galen, J. Michalski, A. Fasano, and J. B. Kaper**. 1993. Accessory Cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. Proceedings of the National Academy of Sciences **90**:5267-5271.

202. **Bhattacharya, T., S. Chatterjee, D. Maiti, R. K. Bhadra, Y. Takeda, G. B. Nair, and R. K. Nandy**. 2006. Molecular analysis of the rstR and orfU genes of the CTX prophages integrated in the small chromosomes of environmental *Vibrio cholerae* non-O1, non-O139 strains. Environmental Microbiology **8**:526-634.

203. **Sack, D. A., R. B. Sack, G. B. Nair, and A. K. Siddique**. 2004. Cholera. Lancet **363**:223-233.

204. **Rivera, I. N. G., J. Chun, A. Huq, R. B. Sack, and R. R. Colwell**. 2001. Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. Appllied Environmental Microbiology **67**:2421-2429.

205. **Faruque, S. M., M. J. Albert, and J. J. Mekalanos**. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiology andMolecular Biology Reviews **62**:1301-1314.

206. **Cooke, M. L.** 2010. Causes and management of diarrhoea in children in a clinical setting. South African Journal of Clinical Nutrition **23**:42-46.

207. **Mahon, C. R. and G. Manuselis**. 2000. Textbook of diagnostic microbiology. Saunders.

208. **Talavera, A. and E. Perez**. 2009. Is cholera disease associated with poverty? http://www.jidc.org/index.php/journal/article/view/19762952. Accessed 15/06/2010.

209. **Harris, J. B., R. C. LaRocque, R. C. Charles, R. N. Mazumder, A. I. Khan, and P. K. Bardhan**. 2010. Cholera's western front. Lancet **376**:1961-1965.

210. **Outbreak Response Unit, Enterics Diseases Reference Unit, SA-FELTP, National Institute for Communicable Disease, Limpopo Local and Provincial Departments of Health, Mpumalanga SD, and Communicable Disease Control**. 2009. Cholera outbreak. Communicable Diseases Communique **8**:1-7.

211. **Tarr, C. L., J. S. Patel, N. D. Puhr, E. G. Sowers, C. A. Bopp, and N. A. Strockbine**. 2007. Identification of *Vibrio* isolates by a multiplex PCR assay and rpoB sequence determination. Journal of Clinical Microbiology **45**:134-140.

212. 2010. As cholera returns to Haiti, blame is unhelpful. The Lancet Infectious Diseases **10**:813.

213. **Deen, J. L., L. von Seidlein, D. Sur, M. Agtini, M. E. S. Lucas, A. L. Lopez, D. R. Kim, M. Ali, and J. D. Clemens**. 2008. The high burden of cholera in children: comparison of incidence from endemic areas in Asia and Africa. PLoS Neglected Tropical Diseases **2**:e173.

214. **Kustner, H. G. V. and G. Du Plessis**. 1991. The cholera epidemic in South Africa, 1980-1987. South African Medical Journal **79**:539-544.

215. **Sack, R. B., A. K. Siddique, I. M. Longini, A. Nizam, M. Yunus, M. S. Islam, J. G. Morris, A. Ali, A. Huq, G. B. Nair, F. Qadri, S. M. Faruque, D. A. Sack, and R. R. Colwell**. 2003. A 4-Year study of the epidemiology of *Vibrio cholerae* in four rural areas of Bangladesh. Journal of Infectious Diseases **187**:96-101.

216. **BENENSON, A. S., M. R. Islam, and W. B. GREENOUGH**. 1964. Rapid identification of *Vibrio cholerae* by darkfield microscopy. Bullettinof the World Health Organization **30**:827-831.

217. **Harwood, V. J., J. P. Gandhi, and A. C. Wright**. 2004. Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. Journal of Microbiological Methods **59**:301-316.

218. **Cameron, D. N., F. M. Khambaty, I. K. Wachsmuth, R. V. Tauxe, and T. J. Barrett**. 1994. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed-field gel electrophoresis. Journal of Clinical Microbiology **32**:1685-1690.

219. **Jiang, S. C., V. Louis, N. Choopun, A. Sharma, A. Huq, and R. R. Colwell**. 2000. Genetic diversity of *Vibrio cholerae* in Chesapeake Bay determined by amplified fragment length polymorphism fingerprinting. Appllied Environmental Microbiology **66**:140-147.

220. **Popovic, T., C. Bopp, O. Olsvik, and K. Wachsmuth**. 1993. Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. Journal of Clinical Microbiology **31**:2474-2482.

221. **Farfan, M., D. Minana, M. C. Fuste, and J. G. Loren**. 2000. Genetic relationships between clinical and environmental *Vibrio cholerae* isolates based on multilocus enzyme electrophoresis. Microbiology **146**:2613-2626.

222. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt**. 1998. Multilocus sequence typing: A portable approach to the identification of clones within

populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences **95**:3140-3145.

223. **Fields, P. I., T. Popovic, K. Wachsmuth, and O. Olsvik**. 1992. Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae* O1 strains from the Latin American cholera epidemic. Journal of Clinical Microbiology **30**:2118-2121.

224. **Varela, P., M. Rivas, N. Binsztein, M. L. Cremona, P. Herrmann, O. Burrone, R. A. Ugalde, and A. C. C. Frasch**. 1993. Identification of toxigenic *Vibrio cholerae* from the Argentine outbreak by PCR for ctx A1 and ctx A2-B. Federation of European Biochemical Societies Letters **315**:74-76.

225. **Murray, C. J. and A. D. Lopez**. 1997. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet **349**:1436-1442.

226. **Ahmed, H. M., J. B. Coulter, O. Nakagomi, C. A. Hart, J. M. Zaki, A. A. Al-Rabaty, W. Dove, and N. A. Cunliffe**. 2006. Molecular characterization of Rotavirus gastroenteritis strains, Iraqi Kurdistan. Emerging Infectious Disesaes Journal **12**:824-826.

227. **Glass, R. I., P. E. Kilgore, R. C. Holman, S. Jin, J. C. Smith, P. A. Woods, M. J. Clarke, M. S. Ho, and J. R. Gentsch**. 1996. The epidemiology of Rotavirus diarrhea In the United States: surveillance and estimates of disease burden. Journal of Infectious Diseases **174**:S5-S11.

228. **Scott-Taylor, T. H. and G. W. Hammond**. 1995. Local succession of adenovirus strains in pediatric gastroenteritis. Journal Medical Virology **45**:331-338.

229. **Ansari, S. A., V. S. Springthorpe, and S. A. Sattar**. 1991. Survival and vehicular spread of human Rotaviruses: possible relation to seasonality of outbreaks. Review of Infectious Diseases **13**:448-461.

230. **Nafi, O.** 2010. Rotavirus gastroenteritis among children aged under 5 years in Al Karak, Jordan. Eastern Mediterranean Health Journal **16**:1064-1069.

231. **Ruiz-Palacios, G. M., I. Perez-Schael, F. R. Velazquez, H. Abate, T. Breuer, S. C. Clemens, B. Cheuvart, F. Espinoza, P. Gillard, B. L. Innis, Y. Cervantes, A. C. Linhares, P. Lopez, M. Macias-Parra, E. Ortega-Barria, V. Richardson, D. M. Rivera-Medina, L. Rivera, B. Salinas, N. Pavia-Ruz, J. Salmeron, R. Ruttimann, J. C. Tinoco, P. Rubio, E. Nunez, M. L. Guerrero, J. P. Yarzabal, S. Damaso, N. Tornieporth, X. Saez-Llorens, R. F. Vergara, T. Vesikari, A. Bouckenooghe, R. Clemens, B. De Vos, and M. O'Ryan**. 2006. Safety and efficacy of an attenuated vaccine against severe Rotavirus gastroenteritis. New England Journal of Medicine **354**:11-22.

232. **Madhi, S. A., N. A. Cunliffe, D. Steele, D. e. Witte, M. Kirsten, C. Louw, B. Ngwira, J. C. Victor, P. H. Gillard, B. B. Cheuvart, H. H. Han, and K. M. Neuzil**. 2010. Effect of human Rotavirus vaccine on severe diarrhea in African infants. New England Journal of Medicine **362**:289-298.

233. **Bethony, J., S. Brooker, M. Albonico, S. M. Geiger, A. Loukas, D. Diemert, and P. J. Hotez**. 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet **367**:1521-1532.

234. **Brooker, S., A. C. A. Clements, and D. A. P. Bundy**. 2006. Global epidemiology, ecology and control of soil-transmitted helminth infections, p. 221-261. *In*: **I. H. Simon** (ed.),

2423 Advances in Parasitology Global Mapping of Infectious Diseases: Methods, Examples and
2424 Emerging Applications. Volume 62 ed. Academic Press.
2425 235. **de Silva, N. R., S. Brooker, P. J. Hotez, A. Montresor, D. Engels, and L. Savioli**. 2003. Soil-
2426 transmitted helminth infections: updating the global picture. Trends in Parasitology
2427 **19**:547-551.
2428 236. **Leav, B. A., M. Mackay, H. D. Ward, and D. Acheson**. 2003. *Cryptosporidium* species:
2429 new insights and old challenges. Clinical Infectious Diseases **36**:903-908.
2430 237. **Minenoa, T. and M. A. Avery**. 2003. Giardiasis: recent progress in chemotherapy and
2431 drug development. Current Pharmaceutical Design **9**:841-855.
2432 238. **Pillai, D. R. and K. C. Kain**. 2003. Common intestinal parasites. Options in Infectious
2433 Disease **5**:207-217.
2434 239. **Brooker, S., N. B. Kabatereine, F. Fleming, and N. Devlin**. 2008. Cost and cost-
2435 effectiveness of nationwide school-based helminth control in Uganda: intra-country
2436 variation and effects of scaling-up. Health Policy and Planning **23**:24-35.
2437 240. **Cohen, C, Madhi, S. A, and Page, N**. 2009. Rotavirus sentinel surveillance programme.
2438 **1**(1):1-60.
2439 241. **Nations Online**. 2009. Map of South African provinces.
2440 http://www.nationsonline.org/oneworld/map/za_provinces_map2.htm. Accessed
2441 19/01/2011.
2442 242. **Wasfy, M., B. Oyofo, A. Elgindy, and A. Churilla**. 1995. Comparison of preservation
2443 media for storage of stool samples. Journal of Clinical Microbiology **33**:2176-2178.

243. **Center for Disease and Control and National Center for Infectious Diseases**. 1999. Laboratory methods for the diagnosis of epidemic dysentery and cholera. CDC.

244. **Murray, P. R. and American Society for Microbiology**. 1995. Manual of clinical microbiology. The American Society for Microbiology Press.

245. **bioMérieux-South Africa**. 2009. Vitek 2 systems: vitek 2 compact training manual. bioMeriéux Inc.

246. **O'Hara, C. M.** 2005. Manual and automated instrumentation for identification of Enterobacteriaceae and other aerobic gram-negative bacilli. Clinical Microbiology Reviews **18**:147-162.

247. **Kumari, S., R. L. Ichhpujani, and World Health Organization.Regional Office for South-East Asia**. 2000. Guidelines on standard operating procedures for microbiology. SEARO WHO.

248. **Grimont, P. A. D. and F. X. Weill**. 2007. Antigenic formulae of the *Salmonella* serovas.

249. **Gomez-Duarte, O. G., J. Bai, and E. Newell**. 2009. Detection of *Escherichia coli, Salmonella* spp., *Shigella* spp., Yersinia enterocolitica, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagnostic Microbiology and Infectious Disease **63**:1-9.

250. **Toma, C., Y. Lu, N. Higa, N. Nakasone, I. Chinen, A. Baschkier, M. Rivas, and M. Iwanaga**. 2003. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. Journal of Clinical Microbiology **41**:2669-2671.

251. **Roche Applied Science**. 2009. DIG application manual. https://www.roche-applied-science.com/prodinfo_fst.jsp?page=/PROD_INF/MANUALS/DIG_MAN/dig_toc.htm. Accessed 20/06/2009.

252. **Yongsi, H. B. N.** 2008. Pathogenic microorganisms associated with childhood diarrhea in low-and-middle income countries: case study of Yaoundé - Cameroon. International Journal of Environmental Research and Public Health **5**:213-229.

253. **Mandomando, I. M., E. V. Macete, J. O. A. Q. Ruiz, S. E. R. G. Sanz, F. A. T. I. Abacassamo, X. A. V. I. Valles, J. A. H. I. Sacarlal, M. M. Navia, J. O. R. D. Vila, P. L. Alonso, and J. O. A. Q. Gascon**. 2007. Etiology of diarrhea in children younger than 5 years of age admitted in a rural hospital of Southern Mozambique. American Journal of Tropical Medicine and Hygiene **76**:522-527.

254. **Spano, L. C., A. D. Sadovsky, P. N. Segui, K. W. Saick, S. M. S. Kitagawa, F. E. L. Pereira, U. Fagundes-Neto, and I. C. A. Scaletsky**. 2008. Age-specific prevalence of diffusely adherent *Escherichia coli* in Brazilian children with acute diarrhoea. Journal of Medical Microbiology **57**:359-363.

255. **Jafari, F., L. J. Garcia-Gil, S. Salmanzadeh-Ahrabi, L. Shokrzadeh, M. M. Aslani, M. A. Pourhoseingholi, F. Derakhshan, and M. R. Zali**. 2009. Diagnosis and prevalence of enteropathogenic bacteria in children less than 5 years of age with acute diarrhea in Tehran children's hospitals. Journal of Infection **58**:21-27.

256. **Youssef, M., A. Shurman, M. E. Bougnoux, M. Rawashdeh, S. Bretagne, and N. Strockbine**. 2000. Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan. Federation of European Microbiological Societies Immunology & Medical Microbiology **28**:257-263.

257. **Smith, A. M., N. P. Tau, A. Sooka, K. H. Keddy, and R. a. M. D. S. i. S. A. G.-S. for the Group for Enteric**. 2011. Surveillance for enterohaemorrhagic *Escherichia coli* associated with human diarrhoea in South Africa, 2006–2009. Journal of Medical Microbiology **60**:681-683.

258. **Rappelli P, E. Folgosa, M. L. Solinas, J. L. Dacosta, C. Pisanu, M. Sidat, J Melo, P. Cappuccinelli and M. M. Colombo.** 2005. Pathogenic enteric *Escherichia coli* in children with and without diarrhea in Maputo, Mozambique. FEMS Immunology and Medical Microbiology **43**:67–72. 261. **Regua-Mangia, A. H., T. A. Gomes, M. A. Vieira, J. R. Andrade, K. Irino, and L. M. Teixeira**. 2004. Frequency and characteristics od diarrhoeagenic *Escherichia coli* strains isolated from children with and without diarrhoea in Rio de Janeiro, Brazil. Journal of Infection **48**:161-167.

259. **Tamaki, Y., H. Narimatsu, T. Miyazoto, N. Nakasone, N. Higa, C. Toma, and M. Iwanga**. 2005. The relationship between O-antigens and pathogenic genes of diarrhea-associated *Escherichia coli*. Japan Journal of Infectious Diseases **58**:65-69.

260. **Blanco, J. E., M. Blanco, J. Blanco, A. Mora, L. Balaguer, M. Mourino, A. Juarez, and W. H. Jansen**. 1996. O serogroups, biotypes, and eae genes in *Escherichia coli* strains

isolated from diarrheic and healthy rabbits. Journal of Clinical Microbiology **34**:3101-3107.

261. **Bouzari, S., M. M. Aslani, M. Oloomi, A. Jafari, and A. Dashti**. 2011. Comparison of multiplex PCR with serogrouping and PCR-RFLP of fliC gene for the detection of enteropathogenic *Escherichia coli* (EPEC). Brazilian Journal of Infectious Diseases **15**:365-369.

262. **Campos, L. C., M. R. Franzolin, and L. R. Trabulsi**. 2004. Diarrheagenic *Escherichia coli* categories among the traditional enteropathogenic *E. coli* O serogroups: a review. Memorias do Instituto Oswaldo Cruz **99**:545-552.

263. **Moyo, S., N. Gro, M. Matee, J. Kitundu, H. Myrmel, H. Mylvaganam, S. Maselle, and N. Langeland**. 2011. Age specific aetiological agents of diarrhoea in hospitalized children aged less than five years in Dar es Salaam, Tanzania. BioMed Central Pediatrics **11**:19.

264. **Feasey, N. A., B. N. Archer, R. S. Heyderman, B. D. Sooka, M. A. Gordon, and K. H. Keddy**. 2010. Typhoid fever and invasive nontyphoid Salmonellosis, Malawi and South Africa. Emerging Infectious Diseases **16**:1448-1451.

265. **Nimri, L. F., Z. Elnasser, and R. Batchoun**. 2004. Polymicrobial infections in children with diarrhoea in a rural area of Jordan. Federation of European Microbiological Societies Immunology & Medical Microbiology **42**:255-259.

266. **Obi, C. L, E Green1, P. O Bessong, B de Villiers, A. A Hoosen, E. O Igumbor and N Potgieter.** 2004. Gene encoding virulence markers among *Escherichia coli* isolates from

diarrhoeic stool samples and river sources in rural Venda communities of South Africa. Water South Africa **30(1):** 37-42

267. **Wierzba, T. F., I. A. Abdel-Messih, R. E. M. O. Abu-Elyazeed, S. D. Putnam, K. A. Kamal, P. A. T. R. Rozmajzl, S. F. Ahmed, A. B. D. E. Fatah, K. H. A. L. Zabedy, H. I. Shaheen, J. O. H. N. Sanders, and R. O. B. E. French**. 2006. Clinic-based surveillance for bacterial-and Rotavirus-associated diarrhoea in Egyptian children. The American Journal of Tropical Medicine and Hygiene **74**:148-153.

268. **Statistics South Africa.** 2010. Mid-year population estimates. http://www.statssa.gov.za/publications/P0302/P03022010.pdf. Accessed 20/08/2012.

269. **Department of Health White paper.** 1998. https://www.doh.gov.za. Accessed 23/05/2005.

270. **Crowther-Gibson, P., N. Govender, D. A. Lewis, C. Bamford, A. Brink, A von Gottberg, K. Klugman, M du Plessis, B. Harris, K.H. Keddy and M. Botha.** 2011. Human infections and antibiotic resistance. South African Medical Journal **101(8):** 567-578

271. **Szakal, D., I. Gado, and T. Pal**. 2001. A colony blot immunoassay to detect enteroinvasive *Escherichia coli* and *Shigella* in water samples. Journal of Applied Microbiology **90**:229-236.

272. **Hull, A. E., D. W. Acheson, P. Echeverria, A. Donohue-Rolfe, and G. T. Keusch**. 1993. Mitomycin immunoblot colony assay for detection of Shiga-like toxin-producing

Escherichia coli in fecal samples: comparison with DNA probes. Journal of Clinical Microbiology **31**:1167-1172.

273. **Grim, C. J., Y. G. Zo, N. A. Hasan, A. Ali, W. B. Chowdhury, A. Islam, M. H. Rashid, M. Alam, J. J. Morris, A. Huq, and R. R. Colwell**. 2009. RNA Colony Blot Hybridization Method for Enumeration of Culturable *Vibrio cholerae* and *Vibrio mimicus* Bacteria . Journal of Applied Environmental Microbiology **75**:5439-5444.

274. **Sayler, G. S., M. S. Shields, E. T. Tedford, A. Breen, S. W. Hooper, K. M. Sirotkin, and J. W. Davis**. 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Journal of Applied Environmental Microbiology **49**:1295-1303.

275. **Schultsz, C., G. J. Pool, R. Ketel, B. Wever, P. Speelman, and J. Dankert**. 1994. Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. Journal of Clinical Microbiology **32**:2393-2397.

276. **Stacy-Phipps, S, J. J. Mecca and J. B. Weiss.** 1995. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during the course of infection. Journal of Clinical Microbiology **33**: 1054-1059.

277. **Cebula, T. A, W. L Payne and P. Feng**. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay- multiplex PCR. Journal of Clinical Microbiology **33**: 248-250.

278. **Lopez-Saucedo, C, J. F. Cerna, N. Villegas-Sepulveda, R. Tompson, F. R. Velazquez, J. Torres, P. I. Tarr and T. Estrada-Garcia**. 2003. Single multiplex polymerase chain reaction to detect diverse loci association with diarrhoeagenic *Escherichia coli.* Emerging Infectious Diseases Journal **9**:127-131.

279. **Schmidt, H, C. Knop, S. Franke, S. Aleksic, J. Heesemann and H. Karch.** 1995. Development of a PCR for screening of enteroaggregative *Escherichia coli*. Journal of Clinical Microbiology **33**:701-705.