Assessment of the incidence of *E.coli* in Tyume and Bufallo rivers in the Eastern Cape Province of South Africa

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A thesis submitted in fulfilment of the requirements for the award of a degree of

Doctor of Philosophy (PhD)
(Microbiology)

In the Department of Biochemistry and Microbiology
Faculty of Science and Agriculture
University of Fort Hare

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DECLARATION

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture, School of Science, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other university, either in part or in its entirety, for the award of any degree.

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Date: _____May 2013_____

ACKNOWLEDGEMENTS

I would like to thank God, if it was not for Him and His Grace, none of this would have been possible. All I can say is 'Ebenezer, Hitherto hath the LORD helped ME.'

I would also like to thank my supervisor Prof Okoh, but knowing him, he will say "Dont thank me o, thank God', so, I'd like to thank God for my supervisor Prof Okoh. This thesis would not have been possible without his help, support and patience throughout my studies. He believed and had faith in me when I didn't believe in myself. Words fail me; I can ever thank him enough.

I'd also like to thank Govan Mbeki Research and Development Centre, University of Fort Hare, Alice and its staff, particularly in the award of Prestigious Doctoral Bursary. I'd also thank the staff members of Department of Microbiology and Biochemistry, University of Fort Hare, for their support and assistance since the start of my postgraduate work. Dr Green thank you for all your help, you were always there to help me, not once did you shut your door on me.May the good Lord bless you abundantly.

To all the members of AEMREG, thank you all for the support and good laughs. I'm very grateful to Doctor I Omodele and Thulani Sibanda for the friendship and support. Thanks to all my friends, new and old, especially, Fredrick Matongo who was always there for me whenever I needed him .Thanks to the love of my life Tresor Kalombo, I don't even know where to start, thank u so much for always being there for me when I needed you the most, knowing that I can call on you whenever things got hectic, you mean the world to me.

I would also like to thank all the members of City of Light Christian Ministries especially my neighbors Pastor David and his wife for all their prayers and the teachings.

Lastly I would like to thank my loving parents (Sipho and Nomvuyo Koba) for the sacrifices they made for me, my sister (Nolonwabo Koba) and my brothers (Mthetheleli and Simphiwe). Their prayers, love, encouragement kept me going. My prayer is for God to keep you all to witness that your sufferings were not in vain. To my niece Kanyi, aunty loves you like her own; you are one of the best things that has ever happened in my life.

DEDICATION

This Thesis is dedicated to:

My late grandfather: Mr S.Matshoba

(*His last words to me were 'above all things make sure you get an education', I know he is smiling down on me*)

My grandmother: Mrs N Matshoba

My parents: Sipho and Nomvuyo Koba

My sister: Nolonwabo Koba

My brothers: Simphiwe and Mthetheleli Koba

If I was given another chance to choose my family, I would definitely choose all of you.

Thank you for all the love and prayers. Ndiyilento ndiyiyo yimithandazo yenu.

LIST OF ABBREVIATIONS

A/A	Aggregative adherence
AE	Adhesion-effacing
Afa	afimbrial adhesin
AID	Acute Infectious Diarrhea
APHA	American Public Health Association
ATCC	American Type Culture Collection,
BFP	Bundle-forming pilus
Bp	Base pair
cAMP	cyclic Adenosine monophosphate
CCA	Chromocult Coliform Agar
CDC	Centre for Disease Control and Prevention
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusively adherent *E. coli*
DAF	Decay-accelerating factor
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEC	Diarrheagenic *Escherichia coli*
dGTP	deoxyguanosine triphosphate
DHEC	Diarrhea associated *E.coli*
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOH	Department of Health
Dr	Drori antigen

dTTP	deoxythymidine triphosphate
DWAF	Department of Water Affairs and Forestry
E.coli	*Escherichia coli*
e.g.	*Exempli gratia*, for example
Eae	Attaching and effacing gene
EAEC	Enteroaggregative *Escherichia coli*
EC	Eastern Cape
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EIA	Enzyme immune assay
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EPEC	Enteropathogenic *E. coli*
ESBL	extended spectrum β-lactamase
et al	(*et alii*) and others
EtBr	Ethidium bromide
ETEC	Enterotoxigenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
Fig	Figure
g	Gram
H	Flagella antigen
h	Hour
HIV	Human Immunodeficiency Virus
HUS	haemolytic uremic syndrome
Ial	Invasion-associated locus

IMS	immunomagnetic separation
IntI	Integron-encoded site-specific recombinase
K	Capsular antigen
L	Litre
LEE	Locus of enterocytes effacement
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
MAPK	Mitogen-activated Protein Kinase
mg	Milligram
$MgCl_2$	Magnesium Chloride
MH	Mueller-Hinton
Min	Minutes
ml	Millilitre
mM	Millimole
MRSA	multi-drug resistant *Staphylococcus aureus*
NA	Nutrient Agar
NaCl	Sodium Chloride
NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standards
NIH	National Institutes of Health
NMEC	Neonatal Meningitis *Escherichia coli*
NO_2	Nitrogen Dioxide
NO_3	Nitrate
No.	Number
O	Somatic antigen
O_2	Oxygen

^{o}C	Degrees Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
Per	Plasmid encoded regulator
Pet	Plasmid-encoded toxin
pH	potential Hydrogen
PKA	Protein kinase A
PMN	Poly morphonuclear leukocyte
RNA	Ribonucleic acid
SEPEC	Sepsis *E.coli*
SLT	Shiga-like toxin
SPATE	Serine Protease Auto Transporter
SPSS	Statistical Analysis System
ST	Heat-stable enterotoxin
STEC	Shiga Toxigenic *Escherichia coli*
Stx	Shiga toxin
TAE	Tris-acetate
Taq	*Thermus aquaticus*
TBE	Tris boric acid EDTA buffer
tEPEC	typical EPEC
Tir	Translocated intimin receptor
TJs	Tight junctions
Tris-HCl	Trishydroxymethylaminomethane-Hydrochloric acid
UK	United Kingdom
UPEC	Uropathogenic *Escherichia coli*
US	United States

USA	United States of America
USEPA	United States Environmental Protection Agency
UTI	Urinary tract infections
UV	Ultra Violet
V	Voltage
VT	Verotoxin
WHO	World Health Organization
B	Beta
µg	Microgram
µℓ	Microlitre
µm	Micrometre

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GENERAL ABSTRACT

Waterborne diseases are among the leading causes of morbidity and mortality in developing countries and every year around 2.2 million people die due to basic hygiene related diseases like coliform diarrhoea. Universal access to safe drinking water and sanitation has been promoted as an essential step in reducing these preventable diseases (Tambekar and Banginwar, 2005; Patil, 2004; Charan, 2004). Diarrheagenic *Escherichia coli* are one of the most important etiologic agents of acute diarrhea and represent a major public health problem in developing countries like South Africa

The present study was conducted between August 2010 and July 2011 to investigate the prevalence and distribution of virulent *E. coli* strains from water samples collected from Tyume and Bufallo River, located in Eastern Cape Province of South Africa using conventional microbiological methods and PCR analysis. Twelve Water samples were collected from three different sites of the rivers, viz; upstream, middle stream and the downstream of the dam. *E.coli* was isolated by the membrane filtration method on mFC.
A total of 374 isolates from both rivers were identified by convenctional microbiological techniques. For both Bufallo and Tyume River, A large proportion (87 and 114, respectively) of the isolates from the mid stream samples satisfied the identification characteristics for *E. coli (*blue colonies on MFC agar and violet/purple colonies on Chromocult agar*)* and thus revealing high levels contamination when compared to isolates from the downstream (55 and 47) and the upstream (30 and 31)

All the isolates that satisfied the primary identification stage were subjected to PCR. DNA was extracted using the boiling method and then the DNA was used as a template for PCR.

Specific PCR analysis was performed on all *E. coli* isolates to amplify the *alr* gene that codes for alanine racemase Out and of the 202 isolates amplified for Tyume river, 70 (35%) were positively identified as *E. coli* since they possessed the *alr* gene fragment. and out of the 172 isolates amplified from Buffalo River, 80(47 %) were also positively identified as *E. coli*. For both Tyume and Bufallo River, the highest prevalence was observed midstream (39% and 56% respectively).

The identified *E. coli* were further characterized into different pathotypes. Amplification of the *shig* gene, *LT* gene, *EaeA* gene, *Eagg* gene and the *ST* gene were used to detect pathogenic *E.coli*. In Tyume River, Genes of ETEC (*lt* or *st*) were detected in 21/70 (30%); Gene of EPEC (*eae)* was detected in 14/70 specimens (35%); Genes of EAEC (*Eagg*) was detected in 14/70(35%) and genes of EIEC (*shig*) were detected in 11/70(16%). In Bufallo River, no DEC was recovered upstream and downstream of the river. EAEC (8%) was the only pathotypes recovered midstream of the river.

Strains of all five *E. coli* categories showed high-level resistance to ampicillin, tetracycline, cotrimoxazole, and chloramphenicol but were highly susceptible to quinolones, aminoglycosides, and novobiocin. The highest resistance (100%) amongst the isolates was observed to ampicillin by EAEC, Heat Labile (ETEC) and EIEC, followed by 87.5% by EAEC to carbenicillin. The highest susceptibility was to quinolones (100%) by all the four categories of *E.coli*. The screening for antibiotic resistance genes revealed the absence of *SHV*, *CTMX* and *TetC* genes as they were not detected in any of the *E.coli* isolates. However, *TEM* genes were observed in 80% of the isolates. Integron conserved segment was detected in these same organisms in the same proportion as TEM

CHAPTER 1

GENERAL INTRODUCTION

In almost all South African urban areas, the inhabitants are provided with adequate supply of drinking water, but in many rural settings, the situation is not the same. A report released in 1994 estimated that 14 million people had no access to clean water. Although improvement measures implemented to rectify this situation, 7 million of the 14 million people in rural areas still lack safe and clean water (Duse *et al.*, 2003). More than one million people in the developing countries have no safe drinking water. To overcome the issue of not having adequate water supply, people in rural communities use untreated water directly from rivers, streams and boreholes (Fig 1.1). These sources are often contaminated with all sorts of material including faecal material which may be deposited either by wild/domestic animals as well as by human beings.

Fig 1. 1: Villagers using river water for their domestic purposes (Source: DWAF, 2003)

AMREF (2012) reported that 2.4 billion people have no adequate sanitation. According to the WHO (1996) inadequate sanitation adversely affects the water quality of these sources because open defecation in the environment is often the only option for households with no proper sanitation. It is also conceivable that livestock can, through defecation create faecally-polluted domestic environments (Wright *et al*, 2004). The livestock of most villagers feed on the evergreen grasses on the river bed; excretion is deposited directly into the water (Jeng *et al*, 2004). The feacal material contains harmful organisms like bacteria, viruses, protozoa, and fungi. These micro-organisms may lead to significant health risks in humans, especially infants, the elderly and immunocompromised. In more severe cases may result in chronic illness and even death (Theron and Cloete, 2002).

Fig 1. 2: A picture depicting the lack of sanitary facilities in developing countries (Source: West Africa Wash Journalist Network, 2012)

Waterborne diseases have become a major public health problem, in both developing and developed world (WHO, 2002). A number of viruses, bacteria and protozoa associated with more severe health outcomes may plausibly be transmitted through use of contaminated recreational water. Bacteria may cause life-threatening diseases such as typhoid, cholera and leptospirosis. Bacteria and protozoa may induce illnesses with a wide range of severity. As that may be the case; the true extent of waterborne diseases in the developing world cannot be fully known as the data is not readily available (Cronin *et al*, 2006). The first reviews of the occurrence of disease associated with the use of recreational waters were undertaken by the American Public Health Association (APHA) in the early 1920s. Simons *et al.* (1922) carried out a study to determine the prevalence of infectious diseases which may be transmitted by recreational water contact. From then on, major epidemiological studies have been conducted to investigate the incidences and the causes of waterborne diseases. In 2008, 134 recreational water illness outbreaks were reported by 38 states and Puerto Rico, with at least 13,966 cases of illness (CDC, 2011).

In the Limpopo Province, South Africa (SA), 187 cholera cases were treated and 3 deaths (DOH, 2008). Cholera cases have also been reported in Zambia and Mozambique where people travelled through the Beitbridge area in Zimbabwe (DOH, 2008). A cholera outbreak led to 1 298 deaths in Angola thousands of people infected due to contaminated drinking water, poor sanitation and dense urban living were regarded as the main cause of the outbreak in the capital city of Luanda (Timberg, 2006). In 1993 the largest water outbreak ever documented in the United States of America (USA) occurred in Wisconsin caused by Cryptosporidium contamination, with over 400,000 cases and 54 deaths (Hoxie *et al.*, 1997). In 1994, thousands of Rwandese refugees died in the border limits of Rwanda-Zaire due mainly to spread of cholera instigated by untreated water. Contaminated water continues to

be the main source of concern and sporadic outbreak of diseases. This study focused on pathogenic *E.coli.*

Escherichia coli was first described by Theodor Escherich in 1885 as *Bacterium coli commune*, which he isolated from the feaces of newborns (Todar, 2008). *E. coli* is a consistent inhabitant of the human intestinal tract and is known as commensal *E.coli*. The commensal *E.coli* and its host organisms co-exist in accord for long periods of time with mutual benefits for each other (Kaper *et al.*, 2004). *Escherichia coli,* a member of the *Enterobacteriaceae* family, is a Gram-negative, rod-shaped (1.1-1.5 X 2-6 μm), motile organism that are oxidase-negative, glucose, lactose and sucrose fermenting, with an optimum growth pH of 6.0-7.0 and temperature of 37°C but there are certain laboratory strains can multiply at temperatures up to 49°C (Fotadar *et al*, 2005). The bacterium can grow in the presence or absence of O_2. Under anaerobic conditions, it grows by means of fermentation, producing "mixed acids and gas" as end products. It can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes (Todar, 1998). Cells are positive in the Methyl-Red test, but negative in the Voges-Proskauer assay. Cells do not use citrate, do not produce H_2S or lipase, and do not hydrolyze urea (Bergeys manual, 1994). Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. *E. coli* can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a unicellular organism. It is the member of the faecal coliform group, unable to breakdown urease (Todar, 1998).

In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination. This was based on the ground that *E. coli* is found abundantly in human and animal feces at a concentration of approximately 10^9 per gram (Edberg *et al.*, 2000) and comprises about 1% of the total biomass in the large intestine (Leclerc *et al.*, 2001), furthermore it is not usually found in other niches. Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it is easier to isolate than known gastrointestinal pathogens. Regrowth of *E. coli* in water distribution systems is not a concern, since *E. coli* rarely grows outside the human or animal gut (Geldreich, 1996). The inability of *E. coli* to grow in water, combined with its short survival time in water environments, suggests that the detection of *E. coli* in a water system is a good indicating of recent faecal contamination and the possible indicator of the presence of pathogenic organisms such as *Salmonella, Campylobacter, Giardia Cryptosporidium* or Norovirus (Todar, 1998).

Although most *E. coli* are harmless commensal organisms that commonly inhabit the lower intestine of warm-blooded organisms, however some strains are capable of causing life threatening diseases to humans. The pathogenecity of *E.coli* was first demonstrated in 1935 when the strain of *E. coli* was shown to be the causative agent in an outbreak of diarrhea in infants (Todar, 2008). Ever since then, pathogenic *E. coli* have been divided into numerous categories based on their possession of virulence factors cclinical symptoms and sites of pathogenesis of the host (Fig 1.3) (Gyles and Fairbrother, 2004; Milon *et al.*, 1999; Nataro and Kaper, 1998). These pathogenic strains are broadly categorized as either diarrheagenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (Kaper *et al.*, 2004; Russo and Johnson, 2000). Within each of these broad groups are sets of strains known as pathotypes that share common virulence factors and elicit similar pathogenic outcomes (Marrs *et al.*, 2005). Several pathotypes of diarrheagenic *E. coli* give rise to gastroenteritis, but rarely cause

disease outside of the intestinal tract. ExPEC, on the other hand, have maintained their impeccable ability to exist in the gut without consequence, but have the capacity to disseminate and colonize other host niches including the blood, central nervous system, and urinary tract, resulting in disease. DEC strains are foodborne and waterborne pathogens that cause a wide spectrum of symptoms, ranging from mild gastroenteritis to severe diseases such as hemorrhagic colitis, thrombotic thrombocytopenic purpura, and haemolytic uremic syndrome (HUS) (Karmali *et al.*, 1985). Diarrheagenic *Escherichia coli* (DEC) is an important agent of childhood diarrhea which represents a major public health problem in developing countries (Nataro and Kaper, 1998; Soltan Dallal., 2001; Mitchell *et al.*, 2005; Akinjogunla *et al.*, 2009).

In South Africa it has been estimated that diarrheal diseases are the primary causes of death in infants that are younger than 5 years, leading to about 160-200 deaths per day (Nemarude *et al.*, 2008). Diarrhoea due to infection is widespread throughout the developing world. In Southeast Asia and it is responsible for 8.5% all deaths. In 1998 it was estimated to have killed 2.2 million people, most of whom where under 5 years old (WHO, 2000). Poor sanitation, personal hygiene and environmental conditions are some of the factors that facilitate the transmission of the disease. (Galane *et al.*, 2001; Campos *et al.*, 2004; Kalantar *et al.*, 2011).

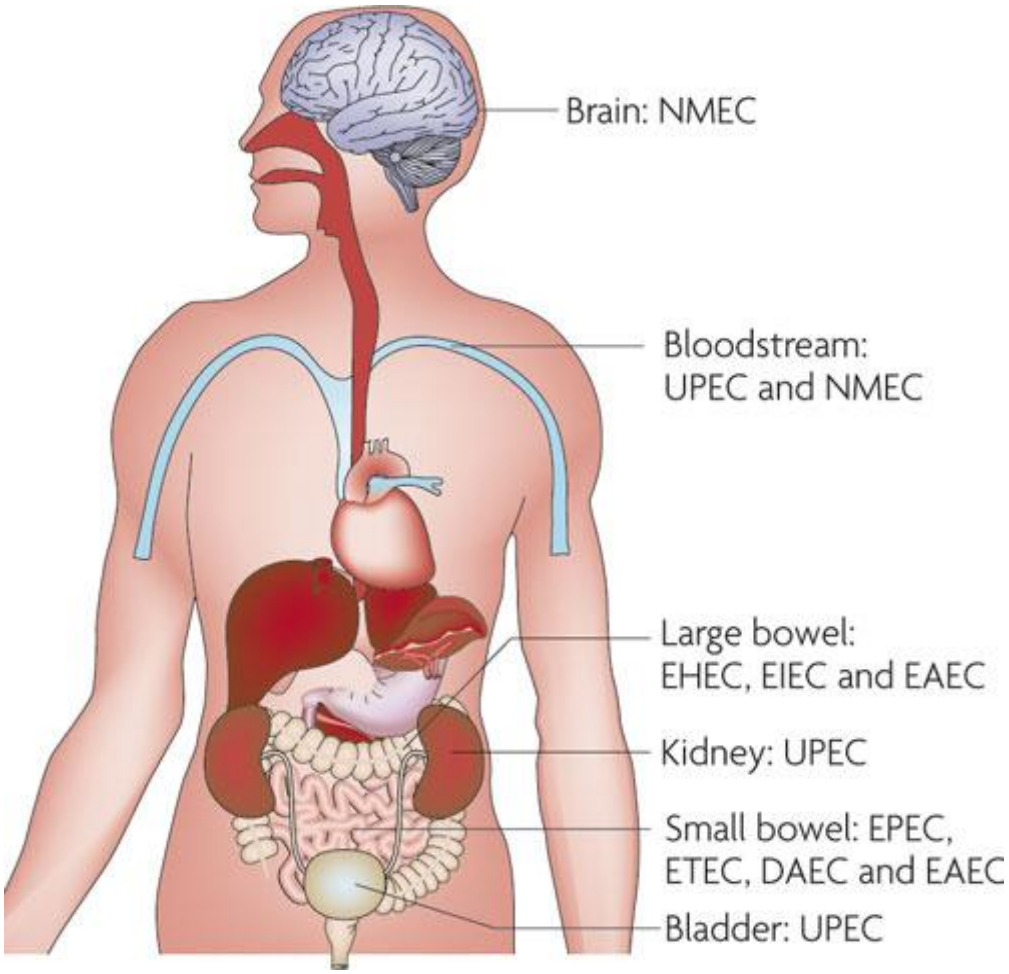

Fig 1. 3: Sites of pathogenic Escherichia coli colonization (Source: Croxen and Finlay, 2010).

NMEC: Neonatal Meningitis *Escherichia coli*; UPEC: Uropathogenic *Escherichia coli;* EHEC: Enterohaemorrhagic *Escherichia coli*; DAEC: Diffusely adherent *Escherichia coli;* EACE: Enteroaggregative *Escherichia coli,* ETEC: Enterotoxigenic *Escherichia coli*

Currently, six *E. coli* pathotypes recognized to cause diarrhoea in humans are: Enteropathogenic *E. coli*, enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) or shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli*, enterotoxigenic *E. coli*(ETEC) and diffusely adhering *E. coli* (DAEC*)* (Turner et al., 2006; (Clarke, 2001; Abduch-Fabrega *et al.*, 2002), however, certain pathotypes of *E. coli* including The Shiga toxin–producing *E. coli (*STEC) and ETEC are potent pathogens associated with waterborne

disease outbreaks and mortality in humans (Ram *et al.*, 2007). Diarrheagenic *E. coli* strains possess specific fimbrial antigens that enhance their Intestine-colonizing ability and allow adherence to the small mucosa bowel. Once having colonized, the strains use very different pathogenic strategies to cause changes in the arrangement of the bowel's mucosa (Donnenberg, 1999) (**Fig 1.4)**. Detection techniques include bioassays (e.g. cell culture), immunologic assays (e.g. immunoblotting or EIA), and DNA assays (e.g. PCR, probing) (Thompson *et al.*, 2003).

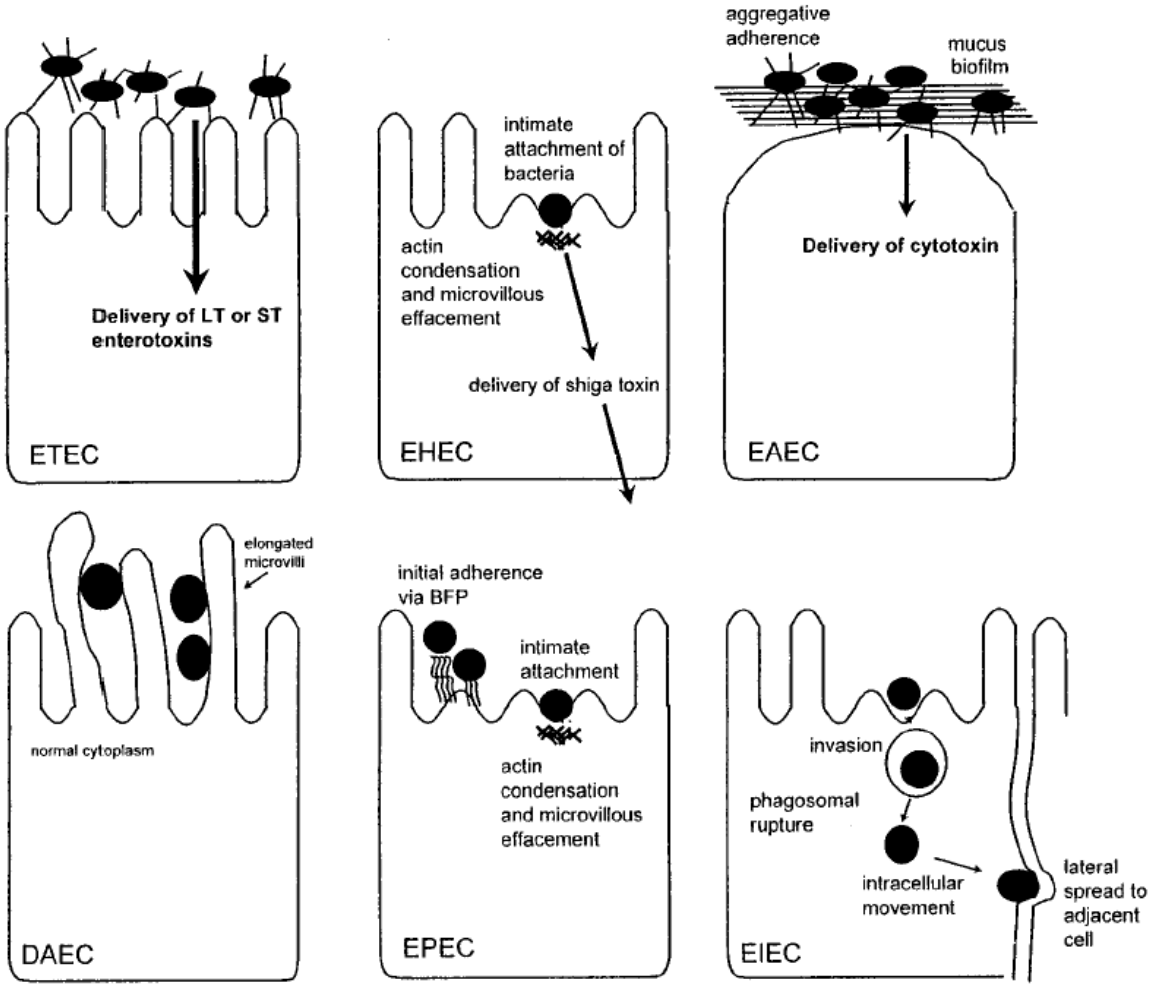

Fig 1. 4: Pathogenic schemes of the diarrheagenic *E. coli*, each with a unique feature in its interaction with eukaryotic cells (Source: Nataro and Kaper, 1998).

Outbreaks caused by pathogenic *E.coli* have been well documented worldwide1990 (Browning *et al,* 1990; Isaacson, 1993; Germanii *et al,* 1996). The first reported case of EHEC in Africa was a sporadic case of hemorrhagic uremic syndrome caused by EHEC O157:H7 and reported from South Africa in 1990 (Browning *et al,* 1990). A large outbreak of bloody diarrhea affecting thousands of individuals, some of whom died, occurred in South Africa and Swaziland (Isaacson, 1993). *E. coli* O157 was isolated from 22.5% of 89 stool samples, and epidemiological investigations implicated waterborne spread. In 1996, Germanii *et al* described an EHEC outbreak in Central African Republic. Outbreak of pathogenic *E. coli* have been reported in Canada, in occurred in Canada where an estimated 2300 people became ill and 7 died from exposure to EHEC contaminated drinking-water (Hrudey *et al,* 2003) and a recreational water outbreak in 2001 at a beach in Montreal, Quebec, resulted in the hospitalization of 4 children (Bruneau, 2004).Baba et al, 2005 reported an outbreak of gastroenteritis in Japan where 176 people were affected A more recent outbreak due to pathogenic *E.coli* occurred during May–June 2011, an outbreak of bloody diarrhea and haemolytic uremic syndrome occurred in Germany and other parts of Europe (Struelens *et al* 2011). The Shiga toxin–producing *E. coli* (STEC) serotype O104 strain was the cause of this outbreak that caused >4,000 cases and 50 deaths. This outbreak strain showed an unusual combination of virulence factor of STEC and enteroaggregative *E. coli* (EAggEC) (WHO, 2011).

The discovery of penicillin in the 1940s and several other antibiotics in consequent years led to great improvements in the management of infectious diseases for the most part in the developed world. Since this discovery, there was a certainty in the medical fraternity that this would lead to the abolition of infectious diseases. Despite this great success, the increased use of antibiotics led to the inevitable development of resistance (Norrby *et al.,* 2005), with

diseases and disease agents that were once thought to have been controlled by antibiotics re-emerging in new forms resistant to antibiotic therapies (Levy and Marshall, 2004). This worldwide emergence of multi-drug resistant bacterial strains has rendered the current drugs used for treatment useless, causing treatment failures (Hancock, 2005). Resistance to antimicrobials is as a result of three main strategies namely enzymatic inactivation of the drug (Davies, 1994), modification of target sites (Spratt, 1994) and extrusion by efflux (Nikaido, 1994).

The health consequences associated with *E. coli* infection have been greatly worsened by the emergence of multidrug-resistant *E. coli*. This mounting phenomenon, which has been deemed to be worse that the multi-drug resistant *Staphylococcus aureus* (MRSA) ,whose restraint is considered as one of the biggest challenges in the twenty-first century in the field of science and medicine, already has some established consequences regarding bacteria-host relationships (Santos *et al.*, 2007). From the late 1990s, multidrug-resistant Enterobacteriaceae (mostly *Escherichia coli*) that produce extended-spectrum β lactamases (ESBLs), such as the CTX-M enzymes emerged within the community setting as an important cause of UTIs. Recent reports have also described ESBL-producing *E coli* as a cause of bloodstream infections associated with these community-onset urinary tract infections (UTIs). The Shiga toxin–producing *E. coli* (STEC) serotype O104 that was responsible for an outbreak in Germany showed extended spectrum β-lactamase (ESBL) activity (Struelens *et al*, 2011; Rubino *et al*, 2011).

Researches in United States (US) found that the strain designated "ST131" was a major cause of serious antimicrobial-resistant *E. coli* infections in the US in 2007, with reported incidences in multiple countries exhibiting fluoroquinolone or extended-spectrum

cephalosporin resistance (Anonymous, 2010). Due to the resistance of ESBL-producing *E. coli* and other bacteria of the same group to a variety of drugs tested, the carbapenems are used. However, these drugs are expensive and have not been tested in carefully controlled trials against other drugs for the treatment of ESBL bacteria (Pitout and Laupland, 2008) It is believed that resistance of these strains results from the improper use of antimicrobials both in humans and in animals (Padungtod *et al.*, 2006). The containment of this drug resistance requires that, new potent antimicrobial compounds be identified as alternatives to existing antibiotics (Overbye and Barrett, 2005).

The spread of resistant genes is made worse when they form part of a mobile gene cassette, which provides for horizontal transfer by several mechanisms including: (i) mobilization of individual cassettes by the integron-encoded integrase (Collis and Hall, 1995), (ii) movement when the integron containing the cassette relocates by targeted transposition (Brown *et al*, 1996; Craig, 1996, Minakhina *et al*, 1999), (iii) dissemination of larger transposons such as Tn*21* carrying integrons (Liebert *et al*, 1999), and (iv) movement of conjugative plasmids containing integrons among different bacterial species. Gene cassettes consist of a gene flanked by a recombination site, known as a 59-base element, which is recognized by the integron-encoded site-specific recombinase (IntI). Gene cassettes are known to exist as free circular molecules (Colis and Hall, 1995) and are transcribed only when captured and inserted into an integron, usually at the *attI* recombination site 104 bp upstream of the *intI1* gene (Hall *et al.*, 1995). New cassettes are continually being discovered, and now over 60 cassettes that confer resistance to a range of antimicrobial agents have been identified (Laraki *et al*, 1999, White *et al*, 2000).

Integrons are elements that participate in a powerful site-specific recombination system and play a major role in the spread antibiotic resistance genes. Many antibiotic resistance genes found in Gram-negative bacteria are part of a gene cassette inserted into an integron (Reechia and Hall, 1956). Since many gene cassettes of integron contain antimicrobial resistance genes in Gram-negative bacteria, the horizontal transfer of integron through plasmids and transposons has been found to play an important role in the dissemination of antimicrobial resistance genes and the development of multiresistance. Few studies have reported the prevalence of integron and gene cassettes in the enteric faecal flora of humans and animals (Leverstein *et al*, 2001; Collis and Hall, 1995).

1.2 Aim and Objectives

The broad aim of the study was to evaluate the incidence of *E. coli* in two surface water bodies (Tyume and Buffalo River) in the Eastern Cape Province of South Africa. The specific objectives include**:**

- To isolate presumptive *E. coli* from Tyume and Buffalo River water.
- To confirm the identities of the *E. coli* isolates by the polymerase chain reaction.
- To carry out molecular characterization of the identified *E. coli* isolates into its different pathotypes.
- To determine the antibiogram properties of the identified *E. coli* pathotypes.
- To evaluate antibiotic resistance gene patterns amongst the antibiotic resistant *E.coli* pathotypes.

CHAPTER 2

LITERATURE REVIEW

2. Introduction

E.coli, a rather harmless commensal organism that is confined to the intestinal lumen can cause infection in the debilitated or immunosuppressed host, or when the gastrointestinal barriers are violated. The worldwide burden of these diseases is staggering, with hundreds of millions of people affected annually. Diarrheagenic *Escherichia coli* (DEC) is an important agent of childhood diarrhea which represents a major public health problem in developing countries and is now being recognized as emerging entero-pathogens in the developed countries (Nataro and Kaper, 1998; Soltan Dallal, 2001; Mitchell *et al*., 2005; Akinjogunla *et al.,* 2009). The diversity among the diarrheagenic *E. coli* pathogenic types and antigens means that children may be subject to repeated infection by different subtypes without immune protection (Okeke, 2009). Travelers from lower burden countries commonly contract infectious diarrhea when they visit high-burden areas (Okeke, 2009). Moreover, HIV-infected patients have emerged as another hyper-susceptible sub-population from whom diarrheagenic *E. coli* pathotypes are often recovered (Okeke, 2009; Samie *et al.,* 2007).

Diarrheagenic *E. coli* have been recognized as intestinal pathogens since the 1940s when Bray hypothesized that that *E. coli* subtypes might account for common infantile diarrhea of unknown etiology and identified a strain of *Bacterium coli var neapolitanum* (Bray *et al.,* 1940). DEC was usually transmitted through food or water contaminated with human or animal faeces. Person-to-person transmission might also take place, but is probably less

common (Wood *et al.*, 1983; Harris *et al.*, 1985; Nataro *et al.*, 1998). Poor sanitation, personal hygiene and environmental conditions are some of the factors that facilitate the transmission of the disease. Thus, DEC is more prevalent in the developing countries (Galane *et al.*, 2001; Campos *et al.*, 2004; Kalantar *et al.*, 2011).

Identification of diarrheagenic *Escherichia coli* cannot be done only by culture and biochemical tests, since they are indistinguishable from the non pathogenic *Escherichia coli* commonly found in human faeces. Similarly, specific serotyping is not always correlated with their pathogenecity. Hence, the discrimination of different diarrheagenic *E.coli* strains requires DNA target based screening by molecular techniques (Rappelli *et al.*, 2005).

DEC strains were among the first pathogens for which molecular diagnostic methods were developed. Major strides have been taken in the development of nucleic acid-based probe technologies as well as PCR methods. PCR has become a versatile, rapid, and sensitive molecular genetics typing method of pathogenic microbes ((Nataro and Kaper, 1998). differentiating diarrheagenic strains from non-pathogenic members for distinguishing one category from another.

The emergence antibiotic resistant *E. coli* strains have rendered first choice antibiotics as useless. Although some pathotypes show promise for vaccine development, not enough is known about predominant subtypes to assure that vaccines will be effective in the places where they are most needed (Okeke, 2009).

2.1 Enteroaggregative *E.coli* (EAEC)

EAEC was first described in 1985, recognized by its distinctive adherence to HEp-2 cells in an aggregative, "stacked brick-like" pattern (Nataro *et al.*, 1998; Pereira *et al.*, 2008). This adherence pattern, distinguishable from the adherence patterns manifested by EPEC and DAEC, was first significantly associated with diarrhea among Chilean children in 1987 (Nataro *et al.*, 1987). This pathotypes is the most recently identified diarrheagenic *E coli* and is the second most common cause of travellers' diarrhea after ETEC in both developed and developing countries (Nataro *et al.*, 1998). EAEC are commonly being recognized as a cause of endemic and epidemic diarrhea worldwide and recently, has been shown to cause acute diarrheal illness in newborns and children in industrialized countries HIV-infected persons living in developed countries. EAEC is also considered as a 'potential' bioterrorism agent by the USA National Institutes of Health (Huang & DuPont, 2004). EAEC exert a complex pathogen-host immune interaction where the host inflammatory response to EAEC infection id dependent on the host innate immune system and the EAEC strain (Kaur *et al.*, 2010).

2.1.1 Clinical manifestations of EAEC infection

Not all EAEC infections result in symptomatic illness (Adachi *et al.*, 2002); however, most studies suggest that EAEC infection results in gastrointestinal disease. The most commonly reported symptoms are watery diarrhoea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever. EAEC can cause both an acute and a chronic diarrhoea illness the incubation period of EAEC diarrhoeal illness ranges from 8 to 18 h.

Malnourished hosts, especially children living in developing countries, may be unable to repair mucosal damage and thus may become prone to persistent or chronic diarrhoea. The clinical manifestations of EAEC diarrhoea vary from individual to individual, depending upon the genetic composition of the host, host immune response, heterogeneity of virulence among EAEC strains, and the amount of bacteria ingested by the infected host (Jiang *et al.*, 2003). Persistent diarrhea (lasting more than 14 days) may occur in select populations, including HIV/AIDS patients and malnourished children in developing countries. Risk factors for EAEC include travel to developing countries, ingestion of contaminated food and water, poor hygiene, host susceptibility, and possibly immunosuppressant HIV infection (Huang & DuPont, 2004; Huang *et al.*, 2004).

Several studies have suggested that patients infected with EAEC manifest intestinal inflammation (Steiner *et al.*, 1998). In a study in Poland, acute episode of bacterial diarrhea by EAEC was showed to result in the development of post infectious irritable bowel syndrome (IBS) (Sobieszanska *et al.*, 2007)

2.1.2 Pathogenesis of EAEC

The pathogenesis of EAEC is very complex as strains are heterogeneous. The best studied virulence factor is the master regulator *AggR*. EAEC pathogenesis involves three stages (1) adherence to the intestinal mucosa by aggregative adherence fimbriae (AAF) and adherence factors; (2) increased production of mucus by bacteria and the host cell forming a biofilm on the surface of the enterocytes; and (3) release of toxins and elicitation of an inflammatory response, mucosal toxicity and intestinal secretion (Harrington *et al.*, 2005; Nataro, 2005;

Huang *et al.*, 2004; Harrington *et al.*, 2005). EAEC adherence to the intestinal mucosa requires aggregative adherence fimbriae (AAF) and adherence factors (Moreira *et al.*, 2003).

Enteroaggregative *E. coli* (EAEC) gains entry to the host by attaching to the enterocytes in both the small and large bowels by means of the aggregative adherence fimbriae (AAF) that causes interleukin-8 (IL-8) to respond to the stimuli, thus allowing the formation of biofilm on the surface of cells. Plasmid-encoded toxin (Pet), a serine protease auto transporter of the Enterobacteriaceae (SPATE) targets α-fodrin (also known as SPTAN1), which disrupts the actin cytoskeleton and induces exfoliation (Fig 2.1) (Crowen and Finlay, 2010).

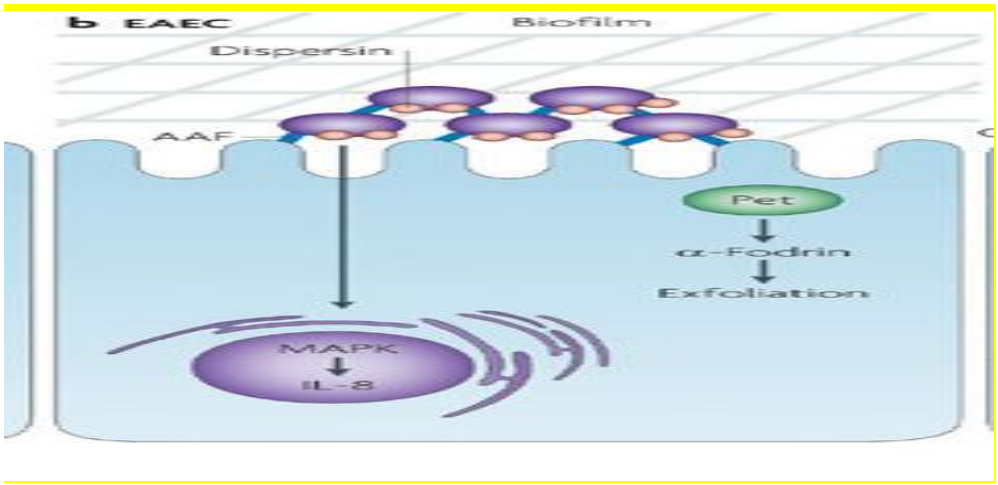

Fig 2. 1 : Molecular mechanisms of EAEC pathogenecity (Source: Crowen and Finlay, 2010)

2.1.3 Epidemiology

The populations best studied for infection by Enteroaggregative *E.coli* (EAEC) include children, adults and HIV-infected persons, living in developing and developed regions, and international travellers to developing countries (Vernacchio *et al.*, 2006). Outbreaks of

diarrheal illness due to EAEC have been reported. North American and European travellers to developing regions such Guadalajara, Mexico, Ocho Rios, Jamaica; and Goa, India, frequently experience diarrheal illness caused by EAEC infection (Adachi *et al.*, 2001). Travellers are at particular risk because of limited exposure to the pathogen at home and, thus, have fragile immunity to EAEC infection. (Mathewson *et al.*, 1985; Adachi *et al.*, 2001).

EAEC is also a common bacterial cause of diarrhoea among children and HIV-infected persons residing in developing and developed regions of the world (Ruttler *et al.*, 2002; Nataro *et al.*, 2006). EAEC is a cause of persistent diarrhoea and malnutrition in children and HIV-infected persons living in developed countries is the second most common cause of travellers' diarrhoea. In another study, Cobeljic et al. (1996) described an outbreak affecting 19 infants in the nursery of a hospital in Serbia. Infants with diarrhea typically manifested liquid green stools; in three, mucus was visibly apparent. There was no gross blood and the source of infection was unclear. In reported two outbreaks of severe lethal diarrheic malnutrition in Mexico City hospitals persistent diarrhea developed in affected infants, and five patients died despite aggressive support. The Autopsy taken revealed that the infants who died illustrated severe necrotic lesions of the ileal mucosa (Eslava, 1993). In 1993, a massive outbreak affected 2697 children in Japan, representing an attack rate of 40 %(Itoh *et al.*, 1997).

Between 60-90% of HIV-infected patients in Africa without access to antiretroviral experience bouts of diarrhea and EAEC strains have repeatedly been isolated from HIV-positive patients with diarrhea (Mwachari *et al.*, 1998, Gassama *et al.*, 1998. In a study that was carried out in South Africa by Samii et al (2010), among the *E. coli* strains from clinical

samples (including stool, sputum and urine) from HIV and AIDS patients studied, EAEC was the most prevalent with 22.3%. In one Central African Republic study, EAEC was isolated from 12.7% of 110 HIV-positive patients with diarrhea and none of 73 asymptomatic controls. In Senegal, EAEC were recovered from in 42 (27%) HIV-positive patients with diarrhea and was only rarely recovered in HIV-negative individuals (Gassama-Sow, 2004). %). Patients who are infected with HIV frequently develop persistent diarrheal disease for which the etiology remains enigmatic (Bartlett *et al.*, 1992; Lambl *et al.*, 1999).

EAEC has also been a culprit in diarrhoeal outbreaks in Europe, the UK, Switzerland and Japan. The association of EAEC with diarrheal disease appears to be geographic. On the Indian subcontinent, five separate studies have been published which demonstrate the importance of EAEC in paediatric diarrhea. These studies include hospitalized patients with persistent diarrhea (Bhan *et al.,* 1989), outpatients visiting health clinics (Bhatnagar *et al.*, 1993), and cases of sporadic diarrhea detected during household surveillance (Bhan *et al.*, 1989). In a study conducted in Brazil, Guerrant and fellow researchers demonstrated a consistent association between EAEC and the persistent diarrhea syndrome. In this area, EAEC had been implicated over 50% of persistent diarrhea cases (Fang *et a*l., 1995., Lima *et al.*, 1992).

2.1.4 Detection and Diagnosis

The 'gold' standard for detection of EAEC is the HEp-2 cell adherence assay. This assay identifies EAEC by its 'stacked brick' aggregating configuration on the surface of the host cells. Although variations in the AA pattern can be discerned, the presence of bacterial clusters in a stacked-brick configuration should be used to identify EAEC strains. Essential components of this adherence pattern include binding of the bacteria to the glass surface, to

the human epithelial cell borders, and to one another Further characterization of this aggregative pattern has been recently described as typical honeycomb formation, lack of honeycombing, and aggregative adherence with lined-up cells(Nataro & Kaper, 1998).

Variations in the assay have been observed. A formalin-fixed HEp-2 cell-adherence assay shows sensitivity of (98 %) and specificity of (100 %) when compared to the traditional assay. Reduced risk of contamination has also been observed (Miqdady *et al.*, 2002). The HEp-2 cell adherence assay is currently performed only in research settings; however, it has with a disadvantage of labour intensity (Huang *et al.*, 2004). Comparative studies (Vial *et al.*, 1990) suggest that the technique was first described by Cravioto et al. (1970). This involved incubation of bacteria with cells, without applying any changes in medium during the course of the assay and is best method to discriminate the three patterns (AA, DA, and LA). It should also be stressed that AAF adhesions are maximally expressed in static Luria broth cultures at 37°C (Nataro *et al.*, 1994). As it may, it has some minor limitations which include time requirements and limited availability in reference laboratories. These limitations have led to the search for other diagnostic methods, including polymerase chain reaction (PCR) assays, DNA probes, and quantitative biofilm assays.

PCR detection of *aggR* may not prove to be an appropriate initial screening test for EAEC, but it is informative because it can identify typical EAEC, which is postulated to have a more pathogenic role than EAEC lacking *aggR*. Multiplex PCR assays for multiple EAEC genes may help overcome the diverse genetic composition of EAEC strains with much more improved sensitivity, but problems with specificity continue to necessitate confirmation with the HEp-2 cell assay (Jenkino *et al.*, 2006., Bouzari *et al.*, 2005) Real-time PCR has been

demonstrated to be at least as sensitive as conventional PCR for the detection of EAEC (Bischoff *et al.*, 2005)

A DNA probe from the pAA plasmid of EAEC is specific for EAEC strains, but has variable sensitivity (Scaletsky *et al.*, 2002). Several lines of evidence suggest that the large plasmids present in most EAEC strains have a high degree of DNA homology. From strain 17-2, Baudry et al. (1990) selected a 1.0-kb plasmid-derived *Sau*3a fragment that hybridized with a fragment of similar size from the 65-MDa plasmid of strain 042. Evaluation studies of this fragment as a diagnostic some studies revealed 89% sensitivity (Baudry *et al.*, 1990; Kang *et al.*, 1995), while in other studies, the sensitivity was substantially low. A problem with using DNA probes and PCR assays to identify EAEC is that EAEC strains are extremely heterogeneous, and this may account for the varying sensitivity of these techniques (Sarantuya *et a*l., 2004).

The biofilm assay using a microtiter plate has been said to have potential as a preliminary screening tool in developing countries because it is quick. However, the test's sensitivity (77%) and specificity (100%) require the HEp-2 cell assay for more accurate detection of EAEC (Wakimoto *et al.*, 2004)

2.1.5 Treatment

Some studies were embarked upon to bring about effective treatment of cases. In this regard, three clinical trials for evaluating treatment of EAEC diarrhoea in travellers were conducted. Two were conducted among travellers to developing countries and 1 was conducted among HIV-infected patients in a developing (Glandt *et al.*, 1999). The first trial evaluated the clinical response of among 29 US travellers to Jamaica and Mexico suffering from EAEC

diarrhoea compared the clinical responses to ciprofloxacin treatment with responses to placebo. A dosage of 500 mg twice a day for 3 days was administered and a significant reduction in diarrhea was observed (Glandt *et al.*, 1999).

In another trial that included US travellers to trial Guatemala, Kenya, Guadalajara and Mexico evaluated the clinical response to rifaximin with responses to placebo among 43 patients with EAEC diarrhea. Thirty of the patients were treated with rifaximin and 13 with placebo. The patients treated with rifaximin had a significant reduction in the duration of post-treatment diarrhoea compared to placebo (Infante *et al.*, 2004). In the third trial, a double blind, placebo-controlled, crossover treatment trial involving 24 HIV infected patients with EAEC diarrhea, those treated with ciprofloxacin reported 50% fewer bowel movements and a 42% decrease in other enteric symptoms compared with those who received placebo (Wanke *et al.*, 1998). These 3 clinical studies indicate that the fluoroquinolone, especially ciprofloxacin, and rifaximin may be the antimicrobial treatments of choice for symptomatic EAEC infections.

Susceptibility patterns of EAEC strains appear to vary by geographic region. Some studies have reported EAEC to have moderate- to high-level resistance to ampicillin, tetracycline, trimethoprim, sulfamethoxazole and Chloramphenicol (Sobieszczanska *et al.*, 2003). In most regions of the world, EAEC strains are susceptible to fluoroquinolone, azithromycin, rifaximin, amoxicillin/clavulanic acid, and nalidixic acid (Glandt *et al.*, 1999; Infante *et al.*, 2004). A recent study by (Aslani *et al.*, 2011) showed that EAEC isolates exhibited resistance to ampicillin, erythromycin cephalothin, co-trimoxazole, tetracycline and nalidixic acid (57.1%) and reduced resistance to ciprofloxacin and norfloxacin. In India EAEC, resistance

to trimethoprim sulfamethoxazole, ampicillin and nalidixic acid (Raju and Ballal, 2009). Over 80% of EAEC isolates are resistant to TMP-SMX.

2.2 Enteroinvasive *E. Coli* (EIEC)

The discovery of strains which could cause dysentery and were intermediate between *Shigella* and *E. coli* in biochemical characteristic in 1944 caused the separation of the two genera to be questioned (van Der Beld and Reubsaet, 2012). EIEC strains were first shown to be capable of causing diarrhea in volunteer studies conducted by DuPont et al. (1971). EIEC strains are biochemically, genetically, and pathogenetically related closely to *Shigella* spp, so much so that it has been proposed that they should be classified as one species in genus *Escherichia*. Like *Shigella* spp, EIEC strains are generally lysine decarboxylase negative, non motile and 70% are unable to ferment lactose. In fatty acid analysis, *Shigella* and *E. coli* form one cluster with other genera of the family Enterobacteriaceae (Brenner *et al*., 1982), therefore it is difficult to distinguish from *Shigella* species *(*Benner *et al*., 1992; van Der Beld and Reubsaet, 2012*)*.

EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that, as in *Shigella*, is thought to be an outer membrane protein. Also, like *Shigella*, EIEC are invasive organisms. They do not produce LT or ST toxin. There are no known animal reservoirs of EIEC; hence the primary source for EIEC appears to be infected humans. EIEC are transmitted through the faecal-oral route. Although the infective dose of *Shigella* is

low (in the range of 10 to few hundred cells), volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Following the ingestion of EIEC the organisms invade the epithelial cells of the intestine resulting in a mild form of dysentery often mistaken for dysentery caused by *Shigella* species (Todar, 1998).

2.2.1 Clinical Manifestation

The clinical syndrome of EIEC is identical to that of *Shigella* dysentery with. It includes a dysentery-like diarrhea with fever (Todar, 1998). EIEC can also cause a broad spectrum of human diseases including: an invasive inflammatory colitis; may also educe a watery diarrhea syndrome indistinguishable from the secretory diarrhea seen with ETEC. Only a minority of patients experience the appearance of blood and mucus in the stools and leukocytes in the stool, tenesmus; and fever of infected individuals. (Nataro and Kaper, 1998).

2.2.2 Pathogenecity of EIEC

Shigella gain access to the submucosa through microfold (M) cells and, following replication in macrophages, invade the basolateral side of colonocyte; all of these processes are achieved by effectors that are secreted into host cells by the type III secretion system. Once in the colonocyte cytoplasm, more effectors are injected to hijack host machinery, prevent detection by the host immune system and promote cell-to-cell dissemination of the bacterium (Fig 2.2) (Crowen and Finlay, 2010).

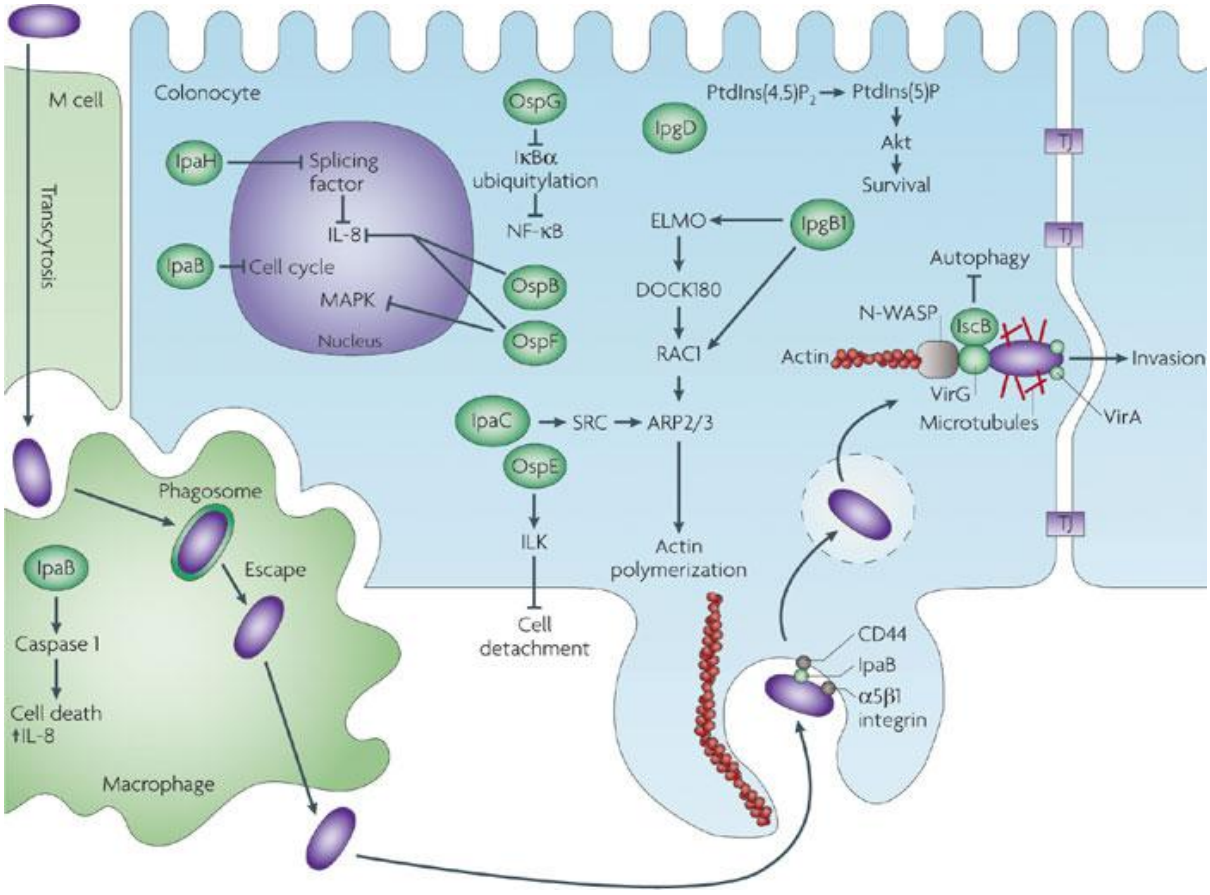

Fig 2. 2 : Molecular mechanisms of EIEC pathogenicity (Source: Crowen and Finlay, 2010).

2.2.3 Epidemiology

The available literature on documented EIEC outbreaks are usually foodborne or waterborne. The data dates back as early as early as 1973 (Tulloch *et al.*, 1973). In another recorded EIEC outbreak, Twenty-eight of 37 people developed acute dysentery in from 24 to 48 hours after eating contaminated, imported French Camembert cheese. An invasive strain *of Escherichia coli* (ONT: NM) was isolated from stool specimens from 7 of 10 ill passengers who developed diarrhea during a 5-day ocean cruise (Snyder *et al.*, 1984). A large diarrhea outbreak due to enteroinvasive *Escherichia coli* (EIEC) serogroup O143 occurred in Houston,

Texas (Gordillo *et al*., 1992). In Africa, the epidemiology of EIEC is not well studied (Okeke, 2009). No epidemics and no recent reports of outbreaks caused by EIEC are found in the literature.

2.2.4 Detection and diagnosis

There are very few biochemical characteristics that differentiate *Shigella* and EIEC from each other and the two most convenient are mucate and acetate tests. EIEC may be positive for either or both, whereas, with rare exceptions *Shigella* strains are negative for both (Lan *et al*., 2004). Salicin fermentation and esculin hydrolysis have also been used to differentiate the two groups. EIEC strains, like *Shigella* and unlike most other *E. coli*, will also often bind Congo red, ferment sugars anearogenically, fail to ferment lactose or to decarboxylate lysine, however, although biochemical tests are valuable pre-screens, none show sufficient sensitivity or specificity to be considered diagnostic for EIEC (Van Der Beld and Reubsaet, 2012).

In order to distinguish EIEC from other *E. coli,* the invasive potential of EIEC may be tested using the classical phenotypic "Sereny Assay" also known as "guinea pig "keratoconjunctivitis" which correlates with the ability of the strain to invade epithelial cells and spread from cell to cell (Kopecko, 1984); the other method that can be used is the HeLa cell tissue culture" method where the ability of EIEC to form plaques correlates with virulence characteristics of these bacteria (Menard and Sonsonetti, 1994). However, molecular methods are preferred over these assays (Knutton *et al*., 1987).

Efforts have been intensified to develop molecular methods to discriminate between the two microorganisms (*Shigella* and EIEC), resulting in development of conventional, multiplex as well as real-time PCR methods for this purpose (Yamazaki *et al* ., 2011). PCR for detection of EIEC is based on the detection of the *ial* and *ipaH* genes (Frankel *et al.*, 1989). This assay distinguishes EIEC and *Shigella* from other diarrheal pathogens. In Senegal, *ipaH*-positive *E. coli*) were recovered from 16 of 279 people with diarrhea and 8 of 276 controls. In South Africa, *ial* positive EIEC were recovered from a wastewater treatment plant (Omar and Barnard, 2010).

Probes can also be used for the detection of EIEC and *Shigella* spp. The probes that have been described are: (i) "Probe pMR17" (a 17-kb *Eco*RI fragment derived from pInv of a *Shigella flexneri* serotype 5 strains) (Gomes *et al.*, 1987; wood *et al.*, 1986) and (ii) "*ial"* (a 2.5-kb *Hin*dIII fragment isolated from pInv of an EIEC strain). Both these probes show100% sensitivity and specificity for EIEC strains that have retained their virulence (Gomes *et al.*, 1987). Pal et al. (1997) have developed an ELISA to detect the *ipaC* gene, which is contained on the Inv plasmid of EIEC and *Shigella* (Pal *et al.*, 1997). Using this assay, these investigators identified EIEC and *Shigella* strains isolated from the stools of children in Kuwait. The added advantage of this assay over other described methods is that cost effective.

2.2.5 Treatment

Quinolones are the drugs of choice for the treatment of EIEC (Gassama-Sow et *al.*, 2004). In a study conducted by Prado and colleagues, TMP-SMX was found to be effective in the diarrhea caused by *Shigella* and enteroinvasive *E. coli* in children (Prado *et al.*, 1992).

In another study, EIEC O164 strain designated "RIMD05091045" was isolated from a travelling patient in Japan. The strain showed multidrug resistance against streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole) and ampicillin, and reduced susceptibility to ciprofloxacin (Ahmed *et al.*, 2004).

2.3 Diffusely Adherent *E. coli* (DAEC)

The term "diffusely adherent *E. coli*" was initially used to refer to any HEp-2-adherent *E. coli* strain that did not form EPEC-like micro colonies. With the discovery of EAEC, most authors now recognize DAEC as an independent category of potentially DEC. DAEC are comprised of heterogeneous groups of organisms with variable virulence. DAEC are divided into two classes, those which harbour afimbrial adhesins (Afa)/Drori antigen (Dr) adhesions and those that express an adhesin involved in diffuse adherence, which is a potential cause of infantile diarrhoea (Nataro and Kaper, 1998). These *E. coli* strains have been found to be associated with urinary tract infections (UTIs) (pyelonephritis and cystitis) and with various enteric infections *(*Archambaud *et al.*, 2003*)*.

2.3.1 Clinical Features

DAEC is Associated with the watery diarrhea that can become persistent in young children in both developing and developed countries as well as recurring urinary tract infections (Croxen and Finlay, 2010). Few clinical studies permit adequate description of the clinical syndrome associated with DAEC infection. In one study, the majority of patients infected with DAEC had watery diarrhea without blood or faecal leukocytes (Poitrineau, 1995). The intestinal carriage of these strains has also been reported to be widespread in older children and adults.

The consequences of this persistence are unknown, but several observations have suggested a potential role in the development of chronic inflammatory intestinal disease (Servin, 2005). DAEC may cause diarrhea in very young children (Scaletsky *et al.*, 2002).

2.3.2 Pathogenesis

Diffusely adherent *E. coli* (DAEC) forms a diffuse attaching pattern on enterocytes of the small bowel, which is mediated through afimbrial (Afa) and fimbrial adhesins, which are collectively known as Afa–Dr fimbriae. Most Afa–Dr fimbriae bind to complement decay-accelerating factor (DAF); a subset of Afa–Dr fimbriae bind to receptors in the carcinoembryonic-antigen-related cell-adhesion molecule (CEACAM) family. The autotransported toxin Sat has been implicated in lesions of tight junctions (TJs) in Afa–Dr-expressing DAEC, as well as in increased permeability. (Fig 2. 3) (Crowen and Finlay, 2010).

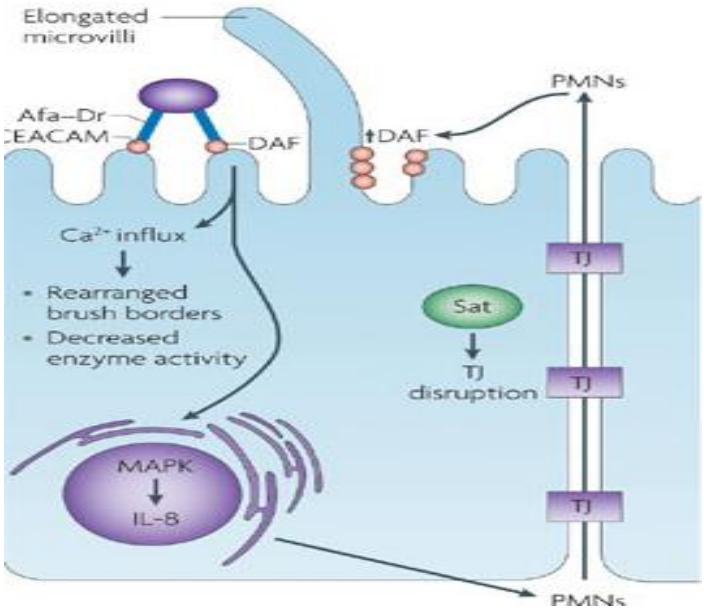

Fig 2. 3: Molecular Mechanism of DAEC pathogenecity (Source: Crown and Finlay, 2010)

2.3.3 Epidemiology

The epidemiology and pathogenesis of the diffusely adherent *E. coli* (DAEC) are not well understood. Several studies have implicated DAEC strains as agents of diarrhea, while other studies have not recovered DAEC strains more frequently from diarrheal patients than from asymptomatic controls. Levine et al. (1997**)** showed that the relative risk of DAEC in association with diarrhea increased with age from 1 year to 4–5 years in Santiago, Chile (1993). Other epidemiologic features, such as the mode of acquisition of DAEC infection, are also as yet undetermined. (Jallat *et al.*, 1998) showed that DAEC strains account for a large proportion of diarrheal cases among hospitalized patients in France who have no other identified. This report suggests that DAEC strains may be important diarrheal pathogens in the developed world. In Maputo, Mozambique, HEp-2 adherent DAEC were identified in 125 (22.8%) patients and 42 (11%) controls ($P < 0.0001$) (Rapelli *et al.*, 2005). In that study, DAEC was more frequently detected than EPEC, ETEC, EIEC (identified by PCR) or EAEC (identified by HEp-2 adherence) (Rapelli *et al.*, 2005). In the Central African Republic, one study found DAEC in 8.2% of HIV-positive patients with diarrhea and only 1.4% of symptomless controls (Germani *et al.*, 1998). In 49 children with diarrhea (20.7%) and 40 children without diarrhea (17.3%), diffusely adherent *E. coli* (DAEC) isolates were detected and were not found to be associated with diarrhea (Scaletsky *et al.,* 2002).

2.3.4 Detection

DAEC strains are defined by the presence of the DA pattern in the HEp-2 adherence assay .A 700-bp polynucleotide fragment derived from the *daaC* gene (Bilge *et al.*, 1993) has been used as a DAEC DNA probe; *daaC* encodes a molecular usher necessary for expression of the F1845 fimbriae. Approximately 75% of DAEC strains from around the world are positive

with this F1845 gene probe (Nataro *et al.*, 1996). However, due to the genetic relatedness of F1845 to other members of the Dr Family of adhesins, false-positive reactions with the DA probe may occur, albeit with unknown frequency. No PCR assay has yet been described to identify DAEC

Two types of adhesins mediating the DA pattern have so far been described dividing the DAEC strains into AIDA-I-dependent group and those that their adhesins is encoded by a family of related operons, which include both fimbrial and afimbrial adhesins. These groups of proteins are collectively designated Afa-Dr adhesins (Labigne-Roussel, 1984). The first afimbrial adhesin (*afa*) operon belonging to this group was characterized and sequenced in 1984 (Nowick *et al.*, 1987), and subsequently another operon in this family as well as the adhesins receptor were described (Benz and Schmidt, 1989). AIDA-I is a 100 kDa outer membrane protein which is associated with DA phenotype and was described by Benz *et al.* (Benz and Schmidt, 1992) who also showed that this adhesin was not commonly encountered among DEAC isolates (Nataro and Kaper, 1998). The *afa/dr/daa* operons are genes that arise and are expressed in a variety of genetic backgrounds (Servin, 2005) and the pathogenesis of DAEC seems to be predominantly mediated through Afa/Dr adhesin interactions with host cells. In addition a secreted auto transporter toxin (Sat) has also been implicated in pathogenesis, but nevertheless, the implication of Afa/Dr DAEC strains in diarrhea remains controversial.

Phenotypic detection of DEAC is based on the mannose-resistant diffuse adhesion of these strains to cultured epithelial HEp-2 or HeLa cells (Nataro and Kaper, 1983.). The adhesion assay however, is not specific for Afa/Dr DAEC detection, since other pathogenic *E. coli* including EPEC strains may show this pattern of adhesion. Other phenotypic assays have also

been developed, but none has proved convenient and universal to be used for identification of all Afa/Dr DAEC isolates (Le Bouguenec *et al.*, 1992). Colony hybridization using various probes have also been developed and used in epidemiological studies (Stapleton *et al*., 1991, Le Bouguenec *et al*., 1992), but this technique is laborious and time consuming and not suitable for use on individual strains. Design of PCR methods that allow identification of all known Afa/Dr adhesins has been achieved (Le Bouguenec

, 2001)

Colony hybridization using various probes have also been developed and used in epidemiological studies, but this technique is laborious and time consuming and not suitable for use on individual strains. Design of PCR methods that allow identification of all known Afa/Dr adhesins has been achieved (Le Bouguenec *et al*., 2001).

2.3.5 Treatment

The frequencies of resistance to ampicillin, cephalothin, cotrimoxazole, streptomycin, sulfonamide, and tetracycline were each >50% in a study conducted in Brazil. Resistance to chloramphenicol (20%) was less frequent. All the isolates were susceptible to ceftazidime, gentamicin, lomefloxacin, ofloxacin, and nalidixic acid, and two strains were susceptible to all antibiotics tested (Lopes *et al*., 2005).

2.4 Enteropathogenic *E. coli* (EPEC)

Until the 1970s serotyping was the only means of distinguishing EPEC strains from those of normal flora, since no biochemical, microbiological or animal tests were available for their differentiation (Levine, 1989). The 12 serogroups originally recognized by the World Health Organization as EPEC or the classical EPEC were; O26, O55¸ O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Hernandez *et al.*, 2009). Once defined solely on the basis of O and H serotypes, EPEC is now defined on the basis of pathogenetic characteristics. EPEC strains are said to be "moderately-invasive", meaning they are not as invasive as *Shigella,* and unlike ETEC or EAEC, they cause an inflammatory response (Nataro and Kaper, 2004). Typical EPEC strains also carry a virulence plasmid, which bears genes encoding bundle-forming pili, the plasmid encoded regulator and other putative virulence genes (Todar, 1998).

Enteropathogenic *Escherichia coli* (EPEC) is the most extensively studied member of the attaching and effacing (A/E) family of pathogens which, like its close relative enterohaemorrhagic *E. coli* (EHEC), is human specific whilst other members have adapted to species including rabbits, dogs, pigs, cattle and sheep. In most cases, it is the young and old those are most susceptible to infection, via the oral–feacal route, resulting in a severe watery diarrhea that can be fatal if untreated (Nataro and Kaper, 1998).

In the 1970s and 1980s, classical EPEC serotypes were associated with disease in many parts of Africa, suggesting that EPEC was a predominant cause of diarrhea at that time. Notwithstanding, in South Africa, one study identified Rotavirus and EPEC as the most

common cause of childhood diarrhea and throughout the 1970s and 80s (Robins-Brown, 1984).

2.4.1 Clinical Manifestations

EPEC cause a watery diarrhea that may contain mucus but typically does not have blood in it. Vomiting, fever, malaise and dehydration are also associated. The symptoms may last for a brief period of several days, although instances of long, chronic EPEC disease have been noted. EPEC induce a profuse watery, sometimes bloody, diarrhea. They are a leading cause of infantile diarrhea in developing countries (Todar, 1998). Fecal leukocytes are seen only occasionally, but more sensitive tests for inflammatory diarrhea such as an anti-lactoferrin latex bead agglutination test are frequently positive with EPEC infection (Miller *et al.*, 1994).

2.4.2 Pathogenesis

EPEC attaches to the small bowel through the bundle-forming pilus (BFP), forming localized adhesions (LA). Intimate attachment is mediated by the interaction between intimin and the translocated intimin receptor (Tir). Tir is phosphorylated by host tyrosine kinases, and phosphorylated Tir recruits Nck, which activates neural Wiskott–Aldrich syndrome protein (N-WASP) and the actin-related protein complex to mediate actin rearrangements and pedestal formation. Using the locus of the enterocyte effacement-encoded type III secretion system, a large repertoire of effector proteins is injected into the host cell, subverting host cell pathways (Crowen and Finlay**,** 2010).

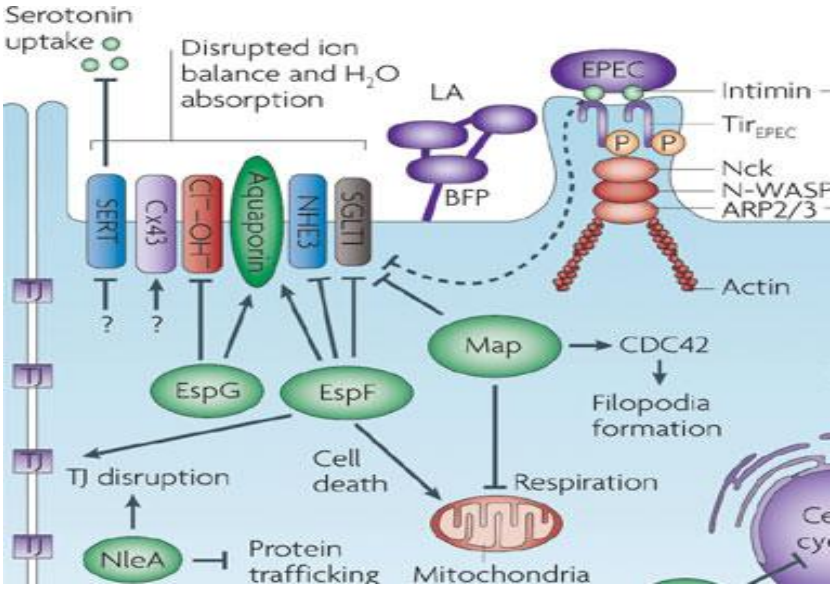

Fig 2. 4: Pathogenesis of EPEC (Source: Crowen and Finlay, 2010).

2.4.3 Epidemiology

In Brazil, for Example, EPEC can be isolated from stools of over 40% of infants with acute diarrhea and was associated with a mortality of 7% (Fagundes-Neto & Scaletsky, 2000). The geographic distribution of all EPEC is generally the same as for the ETEC, with a more severe disease in infants and young children, and so EPEC are much less important in traveler's diarrhea. Common-source community outbreaks are rare in geographic areas with satisfactory sanitation. However, sporadic cases are seen in the United States, Canada, and Europe, and outbreaks occur in these areas, but most commonly in close-contact institutions such as hospital nurseries, day-care centers, and nursing homes.

EPEC once caused frequent outbreaks of infant diarrhea in the US and the United Kingdom (UK) Robins-Brown, 1987). These community-acquired and nosocomial outbreaks were

often explosive, with up to 50% mortality (Giles *et al.*, 1949; Levine and Edelman, 1984, Robins-Brown *et al.*, 1982). EPEC strains are no longer as important a cause of diarrhea in developed countries as they were in the 1940s and 1950s; However, several outbreaks of diarrhea due to EPEC have been reported in the last two decades in the US, the UK, Finland, and other developed countries. These outbreaks frequently occur in day care centers ((Bower *et al.*, 1989; Paulozi *et al.*, 1989) and occasionally occur in paediatric wards (Bower *et al.*, 1989., Rethbaun *et al.*, 1982). An outbreak due to atypical EPEC was recently reported among adults who ate at a gourmet buffet in Minnesota (Hedberg *et al.*, 1994). However, EPEC strains are also associated with sporadic cases of diarrhea in the United States and other developed countries (Bokete *et al*, 1997., Levine and Edelman, 1984., Sherman *et al.*, 1989).

Numerous case-control studies on six continents found EPEC to be more frequently isolated from infants with diarrhea than from matched healthy controls particularly in the 0- to 6-month age group. Studies in Brazil (Gomes *et al.*, 1989; Toledo *et al.*, 1983), Mexico (Cravioto *et al.*, 1988), and South Africa have shown that 30 to 40% of infant diarrhea can be attributed to EPEC, and in some studies, EPEC infection exceeds rotavirus infection in incidence (Cravioto *et al.*, 1988; Gomes *et al.*, 1989). EPEC strains are an important cause of disease in all settings: nosocomial outbreaks, outpatient clinics, patients admitted to hospitals, community-based longitudinal studies, and urban and rural settings (Okeke, 2009).

2.4.4 Detection

Originally, HEp-2 cell-adherence assay performed with serologically defined EPEC strains showed that 80% of these strains adhere to HEp-2 cells in vitro (Cravioto *et al.*, 1979). The

HEp-2 assay has been modified often since its first description, including such variations as extending the incubation time to 6 h or changing the growth medium during the incubation. However, collaborative studies have shown that the assay performed essentially as first described provides the best ability to differentiate among EPEC, EAEC, and DAEC isolates. After the introduction of the term "attaching and effacing" actin accumulation under the attached bacteria was demonstrated using *in vivo* culturing of human intestinal biopsies (Knutton *et al.*, 1987). Staining this electron dense material produced the actin fluorescent assay (FAS) which enabled researchers to detect the ability of a strain to produce A/E lesions in vitro (Knutton *et al.*, 1989). It should however be noted that a negative FAS result may depend on the cell type used and the bacteria should be confirmed as non-pathogenic by alternative methods (Hernandes *et al.*, 1999). The localized adherence pattern of EPEC strains was shown to be associated with the presence of a 60 MDa plasmid called pMAR2 from which a DNA fragment of 1 kb was isolated which has been used extensively in epidemiological studies. The presence of the *E. coli* adherence factor (EAF) plasmid carrying *bfp* operon, encoding the type IV bundle-forming pilus (BFP), and *per* operons, a transcriptional activator called plasmid encoded regulator (Per) is the basis of typical and atypical classification of EPEC strains (Trabulsi *et al.*, 1992).

All EPEC strains lack genes encoding Shiga toxin (*stx*) although they share A/E phenotype with some other strains of *E. coli*, therefore, strains that are *eae*+ *bfpA*+ *stx*- are classified as typical EPEC (tEPEC). Production of BFP protein induces the localized adherence pattern (LA) and most of tEPEC strains belong to classic O: H serotypes (Trabulsi *et al.*, 2002). Atypical EPEC (aEPEC), on the other hand, are of *eae*+ *bfpA*- *stx*- genetic background and display localized-like (LLA), diffuse (DA), or aggregative adherence patterns which is associated with the *E. coli* common pilus and other known adhesins (Scaletsky *et al.*, 2010).

Most of the over 200 O-serogroups that have been identified among aEPEC strains, do not belong to classical EPEC serogroups and many have been designated nontypeable (Schmidt, 2010).

2.4.5 Treatment

Broad-spectrum antibiotics are recommended in chronic and/or life-threatening cases. Fortunately, EPEC diarrhoea is usually self-limited and rehydration is the most effective treatment (Kandakai-Olukemi *et al.*, 2009)

2.5 Enterotoxigenic *E. coli* (ETEC)

ETEC is estimated to cause 280-400 million diarrheal episodes per year in children under 5 years of age, resulting in 300,000 to 500,000 deaths, ETEC is the second leading cause of death in children less than 5 years of age, Often ETEC is the first enteric infection experienced by infants in low resource countries, and in endemic areas almost all children will have had one ETEC diarrhea episode in their first year of life. One out of every six travelers to endemic areas has been observed to be infected with ETEC (Steffen *et al.*, 2005; CDC, 2004).

The history of ETEC began in 1956 in Calcutta. De and his colleagues injected live strains of *E. coli*, isolated from children and adults with a cholera-like illness. Upon realization that some *E. coli* isolates from patients with diarrhea could induce fluid accumulation in a ligated rabbit ileal loop model, it was found that some ETEC elaborated toxin very similar to

cholera-toxin (De *et al.*, 1956; Sack *et al.*, 1973). These findings were not followed up until 1968, based on Sack et al (1973) reports. It was also reported in Calcutta that adults and children with a cholera-like illness, had almost pure growth of *E. coli* in both stool and the small intestine. These *E. coli* isolates were found to produce a strong cholera-like secretory response in rabbit ileal loops, both as live cultures and as culture filtrates. The patients were also found to have antitoxin responses to the heat-labile enterotoxin produced by these organisms. In the 1970s and early 1980s, strains producing this heat labile enterotoxins and/or heat stable enterotoxins. ETEC is the most important but under recognized bacterial cause of diarrhea or cholera like disease in all age groups in areas with poor sanitation and inadequate clean water (Nataro and Kaper, 2004). The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower numbers (Todar, 1998).

ETEC may produce: heat-labile enterotoxin (LT) that is similar in molecular size, sequence, antigenicity, and function to the cholera toxin (Ctx). ETEC may also produce a heat stable toxin (ST) that is of low molecular size and resistant to boiling for 30 minutes. There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or STh is predominant in human isolates only (Todar, 1998).

2.5.1 Clinical Manifestation

The diarrheal disease caused by ETEC that was first recognized consisted of a cholera-like illness in both adults and children in Calcutta. Since then, many studies around the world have shown that ETEC-induced diarrhea may range from very mild to very severe. There are,

however, short-term, asymptomatic carriers of the organisms (Black, 1993). Diarrhea produced by ETEC is of the secretory type: the disease begins with a sudden onset of watery stool (without blood or inflammatory cells) and often vomiting, which leads to dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool (Black *et al.*, 1981; Sack, 1975). The loss of fluids progressively results in a dry mouth, rapid pulse, lethargy, decreased skin turgor; decreased blood pressure, muscle cramps, and eventually shock in the most severe forms. The illness is typically abrupt in onset with a short incubation period (14 to 50 h). The diarrhea is watery, usually without blood, mucus, or pus; fever and vomiting are present in a minority of patients. ETEC diarrhea may be mild, brief, and self-limiting or may result in severe purging similar to that seen in *V. cholerae* infection. Most life-threatening cases of ETEC diarrhea occur in weanling infants in the developing world (Todar, 1998).

2.5.2 Pathogenesis

Enterotoxigenic *Escherichia coli* (ETEC) become anchored to enterocytes of the small bowel through colonization factors (CFs) and an adhesin that is found at the tip of the flagella (EtpA). Tighter adherence is mediated through Tia and TibA. Two toxins, heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST) that cause intestinal epithelial cells to secrete excess fluid through cyclic AMP (cAMP) and cyclic GMP (cGMP)-mediated activation of cystic fibrosis transmembrane conductance regulator (CFTR). Some strains produce only one of the toxins while others produce both (Crowen and Finlay, 2010).

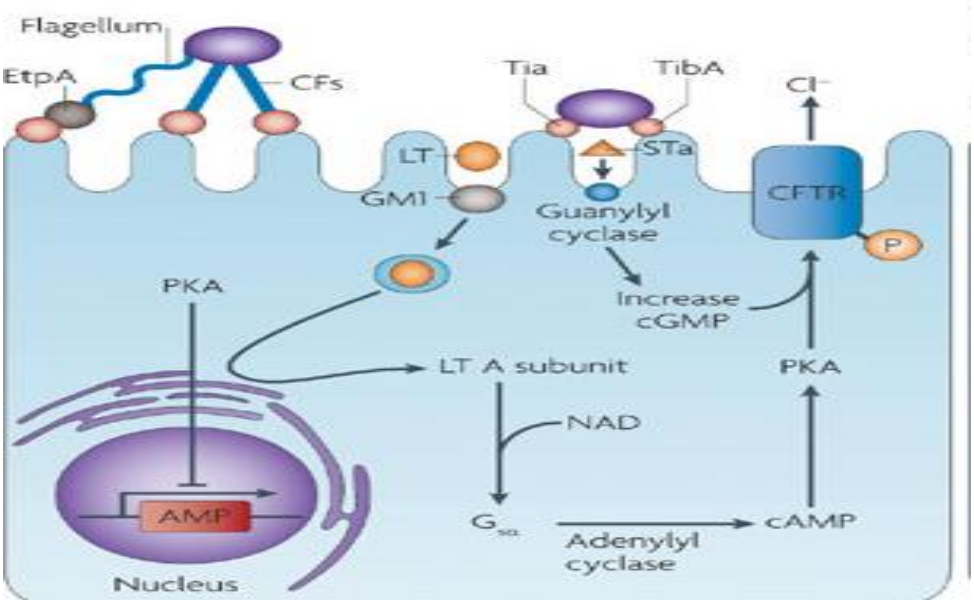

Fig 2. 5: Pathogenesis of ETEC (Source: Crowen and Finlay, 2010).

2.5.3 Epidemiology

A large US nursery outbreak in 1974-75 was attributed to ST-producing ETEC (Ryder *et al.*, 1970) and shortly after, Levine et al. (1977) demonstrated that strains producing ST alone were capable of eliciting diarrhea, nausea, vomiting and abdominal cramps in adult volunteers. This finding is backed by epidemiological evidence, including controlled studies performed in different parts of Africa, which suggest that ST-producing ETEC are more strongly associated with childhood diarrhea than are LT-producing strains, even though the latter may be more common overall (Shukry *et al.*, 1986; Okeke, 2000; Waiyaki *et al.*, 1986, Steinsland *et al.*, 2002). The same may also be true for travelers' diarrhea (Shaheen *et al.*, 2003).

Early studies identified ETEC by screening for toxigenic activity of isolates or with immunologic reagents that agglutinated the heat-stable or heat-labile enterotoxins. ETEC is

one of the best documented and predominant causes of diarrhea in travelers visiting African countries from Europe and North America (Schults *et al*., 2000; Black, 1990 and Qadri *et al*., 2005).

From 1996 to 2003, 16 outbreaks of Enterotoxigenic *Escherichia coli* (ETEC) infections in the United States and on cruise ships were confirmed (Beaty *et al*., 2004). The most common symptoms reported by ill passengers were diarrhea (99%), abdominal cramps (78%), nausea (63%), headache (47%), myalgia (39%), fever (27%), and vomiting (22%). A multifocal outbreak of diarrhea caused by ETEC, involving 175 Israel soldiers and at least 54 civilians, occurred in the Golan Heights (Huerta *et al*., 2000). Similar reports implicated ETEC in infantile diarrheas in Kivu Province, Zaire, and Lagos, Nigeria (de Mol *et al*, 1983; Stintzing, 1982; Yala *et al*., 1985).

In countries like Bangladesh and Egypt, the majority of cases of ETEC occur in children less than 2 years of age, and between 15 and 18% of children 3 years and younger experience ETEC-associated diarrhea episodes. ETEC is less prevalent in children 5 years and older, as well as in adults, because of natural immunity that develops following several episodes of the disease (Qadri *et al*., 2010). In Japan, large-scale outbreaks involving more than 500 patients per incident from 1997 to 2000 occurred. Two big outbreaks occurred again in 2010 and 2011 caused by ETEC O148:H28 (NIID, 2012)

2.5.4 Detection

Traditionally ETEC detection was reliant on detection of the enterotoxins LT and/or ST. Detection of ST was originally done using the rabbit ligated ileal loop but the expense and lack of standardization caused this test to be replaced by the suckling mouse assay (Gianella,

1976), which became the standard test for the presence of STa for many years. The suckling-mouse assay entails the measurement of intestinal fluid in CD4 infant mice after percutaneous injection of culture supernatants. LT is detected using cell culture assays, e.g. the Y1 adrenal cell assay and Chinese hamster ovary cell assay. However, detection of both ST and LT by bioassays has been replaced by PCR and immunological assays.

Immunologic assays are easier to implement in clinical laboratories and include the traditional Biken test (Honda *et al.*, 1981), ELISA (Yolken *et al.*, 1977), latex agglutination (Ito *et al.*, 1983), and two commercially available tests, the reversed passive latex agglutination test (Scotland *et al.*, 1983) and the staphylococcal coagglutination test (Chapman and Daly, 1982). Both of the commercially available tests are reliable and easy to perform (Speirs *et al.*, 1991).

ETEC strains were among the first pathogenic microorganisms for which molecular diagnostic techniques were developed. Several PCR assays exist for ETEC that are quite sensitive and specific when used directly on clinical. As early as 1982 (Mosley *et al.*, 1982), DNA probes were found to be useful in the detection of LT- and ST-encoding genes in stool and environmental samples. Since that time, several advances in ETEC detection have been made, but genetic techniques continue to attract the most attention and use. It should be stressed that there is no perfect test for ETEC: detection of colonization factors is impractical because of their great number and heterogeneity.

2.5.5 Treatment

Fluoroquinolone (e.g., ciprofloxacin, norfloxacin, and ofloxacin) are the most commonly recommended agents, since increasing antimicrobial resistance to traditional agents has been documented in several areas (Du Pont, 1995). Travellers to developing areas are often

concerned with the development of traveller's diarrhea and may seek a means of preventing it. Antimicrobials that have been used in effective treatment include doxycycline, trimethoprim-sulfamethoxazole, erythromycin, norfloxacin, ciprofloxacin, ofloxacin, azithromycin, and rifamycin (Ericsson, 2003., Suck, 1990). The non absorbable antimicrobial rifaximin (Du Pont, 1991) was effective in treating traveller's diarrhea in adults, using 200 mg two times a day for 3 days. Resistance rates to previously effective drugs such as the tetracyclines, trimethoprim-suphamethoxazole and ampicillin have risen to 30-90% in some parts of the world (Mikhail *et al*., 1990; Vila *et al*., 1999, Sharp *et al*., 1995; Shaheen *et al*, 2003; Lamikanra *et al*, 1990).

2.6 Enterohaemorrhagic *E.coli* (EHEC)

EHEC bacteria were first discovered in 1977 by the production of cytotoxin, verotoxin (VT), lethal to Vero (African green monkey) cells, which led to these pathogens being called verocytotoxigenic *E. coli* (VTEC) (Konowalchuk *et al*.,1977). EHEC produces toxins, known as verotoxins or Shiga-like toxins because of their similarity to the toxins produced by *Shigella dysenteriae* EHEC can grow in temperatures ranging from 7°C to 50°C, with an optimum temperature of 37°C. Some EHEC can grow in acidic foods, down to a pH of 4.4, and in foods with a minimum water activity (Aw) of 0.95. It is destroyed by thorough cooking of foods until all parts reach a temperature of 70°C or higher. *E. coli* O157:H7 is the most important EHEC serotype in relation to public health; however, other serotypes have frequently been involved in sporadic cases and outbreaks. Globally, the most significant serotype has been EHEC O157:H7 but infections sustained by EHEC strains belonging to serogroups other than O157. These strains are also referred to as Shiga-toxin producing E. coli, or STEC.

These strains are now usually referred to as non–O157 EHEC (Nataro and Kaper, 1998, Tozzi *et al*., 2003). Among 940 human non-O157 STEC isolates submitted to the Centers for Disease Control (CDC) between 1983 and 2002, the most common serogroups were O26, O111, O103, O121, O45, and O145 (Brooks *et al*., 2005). The prevalence of human EHEC non-O157 infections has increased in several countries. The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes (Todar, 1998). Cattle are generally regarded as the main natural reservoir of EHEC (Nataro and Kaper, 1998).

2.6.1 Clinical Manifestation

EHEC typically cause an afebrile bloody colitis and, in about 10% of patients, this infection can be followed by HUS (Pickering *et al*., 1994). They are involved in episodes of diarrhea with complications. Serotype O157:H7 is the prototype of increasing importance and is associated with hemorrhagic colitis, bloody diarrhea and the haemolytic uremic syndrome (HUS). HUS involves a triad of haemolytic anaemia, thrombocytopenia and renal failure. EHEC cause disease of the large intestine that may present as simple watery diarrhea and then progress to bloody stools with ulcerations of the bowel.) The incubation period of EHEC diarrhea is usually 3 to 4 days, although incubation times as long as 5 to 8 days or as short as 1 to 2 days have been described in some outbreaks. The initial complaint is usually non bloody diarrhea, although this is preceded by abdominal pain and a short-lived fever in many patients. Vomiting occurs in about half of the patients during the period of nobody diarrhea and/or at other times in the illness. Within 1 or 2 days, the diarrhea becomes bloody and the patient experiences increased abdominal pain. This stage usually lasts between 4 and 10 days. In severe cases, fecal specimens are described as “all blood and no stool (Todar, 1998, WHO,

2011). While the kidney is the organ most commonly affected in HUS, evidence of central nervous system, pancreatic, skeletal and myocardial involvement may also be present (Richardson *et al.*, 1988; Sebbag *et al.*, 1999; Siegler, 1994).

2.6.2 Pathogenesis of EHEC

EHEC injects effector proteins such as Tir and EspFu into the host cytoplasm through the T3SS (1). Tir localizes to the host membrane and binds to intimin to intimately attach the bacteria to the cell. Tir and EspFu recruit host factors (2) to subvert host cytoskeleton and actin polymerization (3) (Fig 2. 6) (FICIM, 2012).

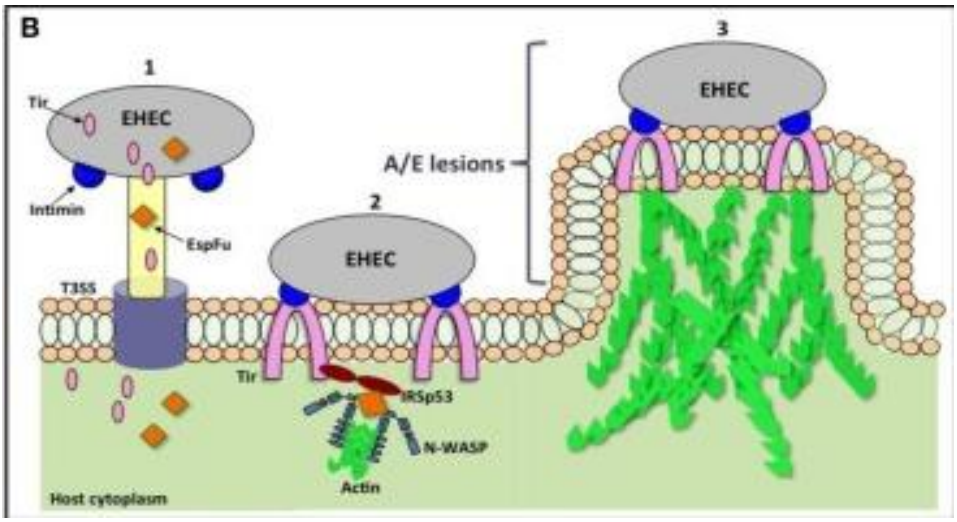

Fig 2. 6: Pathogenesis of EHEC (Source: Frontiers in Cellular and Infection Microbiology, 2012).

2.6.3 Epidemiology

Since the first EHEC O157:H7 outbreaks in the USA in the early 1980s, there has been a dramatic increase in the number of reported infections caused by this O group in the developed countries (Nataro and Kaper 1998), Also, outbreaks with hundreds of cases of EHEC infection have been reported from the United Kingdom (Gillespie *et al.*, 2005). In

United States, 2006: More than 200 people in 26 states were infected with *E coli* O157:H7. Thirty-one cases of HUS with three fatalities occurred (CDC, 2004). In the U.S., the Centers for Disease Control and Prevention (CDC) estimates that EHEC O157:H7 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths each year. In 1983,

Outbreaks affecting several thousands of individuals have also occurred in Japan, where over 9,000 children were infected (Michino *et al.*, 1998). In addition, in Scotland severe, fatal outbreaks with 21 deaths among over 400 individuals infected with the *E. coli* O157:H7 have occurred (Ahmed and Donaghy, 1998). Moreover, an international outbreak caused by EHEC O157 occurred among tourists belonging to five nationalities visiting Spain (Pebody *et al.*, 1999).

The first documentation of EHEC in Africa was a sporadic case of hemorrhagic uremic syndrome caused by EHEC O157:H7 and reported from South Africa in 1990 (Browning *et al.*, 1990). Three years later, a South African laboratory described one of the largest EHEC outbreaks in the world, which began on a sugar plantation in Swaziland, and resulted in approximately 2,000 deaths (Isaacson *et al.*, 1993; Effler *et al.*, 1993). A 1998 outbreak of O111 EHEC occurred in Nigeria (Okeke *et al.*, 2003).

Early May 2011, an unusually high number of HUS cases were reported in Germany. The outbreak strain was characterized very thoroughly at the Robert Koch Institute. The strain is serotype O104:H4 and evolves from a progenitor that belongs to the enteroaggregative pathotypes. This combination is very rare and has previously been described in strains of serotype O111:H2 involved in a small outbreak of HUS in children in France (Morabito *et al.*, 1998) where 782 cases of HUS and 46 deaths (Joris *et al.*, 2012). Another recent outbreak

due to *E. coli* O157:H7 occurred in Japan, five people were killed and more than 100 were sickened in the Hokkaido area outbreak (Japan Times, 2010).

2.6.4 Detection and Diagnosis

Because humans do not normally carry EHEC, clinical cases can be diagnosed by finding these organisms in fecal samples. Food and environmental samples may also be tested to determine the source of the infection. EHEC are sometimes difficult to identify. They are a minor population in the fecal flora or food. They also closely resemble commensal *E. coli* except in verocytotoxin production. However, the verocytotoxin alone does not necessarily identify an organism as EHEC; additional virulence factors must also be present (WHO, 2010).

For the detection of EHEC O157:H7, selective and differential media have been developed for based on its lack of β-glucuronidase activity and the inability of most strains to rapidly ferment sorbitol (Todar, 1998, Nataro and Kaper, 2004). Other media, including commercial chromogenic agars (e.g., rainbow agar), are also available, but because other strains of *E. coli*, as well as other bacteria, can grow on these media, prior enrichment for *E. coli* O157 helps with detection, particularly in samples from food and the environment. For enrichment, samples may be cultured in liquid enrichment medium, or immunomagnetic separation (IMS) can be used to concentrate the members of serogroup O157 before plating. In IMS, magnetic beads coated with an antibody to the O157 antigen are used to bind these organisms. Colonies suspected to be EHEC O157:H7 are confirmed to be *E. coli* with biochemical tests, and shown to have the O157 somatic antigen and H7 flagella antigen with immunoassays. A variety of tests including enzyme-linked immunosorbent assays (ELISAs), agglutination,

PCR, immunoblotting or Vero cell assay can be used to detect the verocytotoxin or its genes. Phage typing and pulsed field gel electrophoresis can subtype EHEC O157:H7 for epidemiological studies. Subtyping is important in finding the source of an outbreak and tracing transmission.

Detection of non-O157 EHEC, selective media and isolation techniques have also been developed. IMS beads are commercially available for concentrating some common EHEC serogroups including O26, O103, O111 and O145. A selective rhamnose MacConkey medium containing cefixime and telluride (CT-RMAC) is used to isolate and identify EHEC O26. Isolation of most non-O157 EHEC relies on screening colonies for verocytotoxin, the genes that produce this toxin and/or other virulence genes associated with EHEC (Nataro and Kaper, 1998). MacConkey agar or other media normally used to culture *E. coli* can be used to grow these organisms. Some prescreening techniques target specific serogroups or serotypes known to be associated with human EHEC disease. Techniques to identify most non-O157 EHEC are very labor-intensive, and these tests are not available at most laboratories. Probe and PCR techniques for additional EHEC virulence factors can often provide crucial information. (Vora *et a*l., 2004).

2.6.5 Treatment

While there is controversy about the use of antibiotics, the use of antimotility (antidiarrheal) such as loperamide agents in hemorrhagic colitis also seems to increase the risk for developing HUS is definitely not indicated in the management of disease due to EHEC; there is evidence that the use of such agents can increase the risk for development of HUS,

possibly by delaying intestinal clearance of the organism and thereby increasing toxin absorption (Imolai *et al.*, 1994). Treatment of renal disease due to EHEC is primarily supportive, except for some experimental therapies currently being evaluated in clinical trials. Patients with complications may require intensive care including dialysis, transfusion and/or platelet infusion. Patients who develop irreversible kidney failure may need a renal transplant (Armstrong *et al.*, 1995).

An *E coli* O157:H7 vaccine produced at the National Institutes of Health (NIH) was tested in a phase 2 clinical trial (Ahmed, 2006). The vaccine tested consisted of an *E coli* O157:H7 O-specific polysaccharide covalently linked to recombinant exoprotein A of *Pseudomonas aeruginosa*. The trial demonstrated that patients who received one or two doses had increased titers of serum IgG LPS antibodies after one week and an eightfold increase in six weeks. A second dose did not educe a booster response. At 26 weeks after the first dose, the geometric mean titer of the serum IgG LPS antibodies was about 20-fold higher than the prevaccination titer. Serum samples had high titers of bactericidal activity that correlated roughly with serum IgG LPS antibody titers. Patients had no serious adverse reactions after either dose. Human serum amyloid P component has been shown to protect against *E coli* O157:H7 Shiga toxin 2 in a mouse model. Mice that received 50-mg/kg doses of human SAP twice a day were completely protected against twice the LD50 of Shiga toxin 2. Administration of exogenous SAP to patients who have EHEC and HUS may offer potential benefit, but efficacy in humans must be tested in clinical trials (Armstrong, 2006).

Chapter 3

Materials and Methods

3.1 Description of the study sites

3.1.1 The Tyume River Catchment

The Tyume River is located in the Nkonkobe Local Municipality, under the Amatole District Municipality of the Eastern Cape Province, South Africa. The population of the Eastern Cape is largely non-urban and poor, with an inadequate of water supply infrastructure. The rural communities of this municipality comprise both scattered villages and subsistence farmers, and formalized towns. The inhabitants of these villages mostly rely on river water for agriculture, recreation, livelihood, fishing, and domestic purposes including bathing and laundry (Amatole Water, 2012). A total of six sampling points (Table 3.1) were selected along the Tyume River, with 2 points each located upstream, midstream and downstream of the river.

3.1.2 The Buffalo River Catchment

The Buffalo River originates from the seeps of the Amatola Mountains and flows for 126 km in a general south-eastern direction before emptying into the Indian Ocean at the East London Harbour. Its major tributaries are the Mgqakwebe, Ngqokweni and Yellowoods. It has four dams, the Maden, Rooikrantz, Laing and Bridle Drift dams. Population density is highest in the middle and lower reaches of the catchment. The catchment's big cities are King Williams Town and East London while the townships include Zwelitsha, Pakamisa and Mdantsane. All townships continue to experience population growth and housing development (formal and informal) with no corresponding improvements to the sewage treatments works (Amatole Water, 2012), thus putting a lot of pressure to water and wastewater systems. As in

the Tyume catchment study, six sampling points were also selected for the Buffalo River sampling with 2 points each located upstream, midstream and downstream of the river (Table 3.2).

Table 3. 1: Sampling sites selected in the Tyume River Catchment

Sample site	Uses	Geographic Coordinates
Hala	Livestock Watering	32º36'39"S and 26 º54'34"E
Khayalethu	Livestock and domestic purposes	32 º38'22"S and 26 º56'10"E
Sinakanaka	Recreation and Irrigation and domestic purposes	32 º45'37"S and 26 º51'27E
Alice	Irrigation, Fishing and domestic purposes	32 º45'17"S and 26 º50'31"E
Drayini	Domestic animals purposes	32 º48'37"S and 26 º52'20"E
Manqulweni	Domestic purposes	32 º54'50"S and 26 º56'13"E

Table 3. 2: Sampling sites in the Buffalo River Catchment

Sample site	Uses	Geographic Coordinates
Madden Dam	Livestock watering	32 º44'22"S 27 º17'54"E
Rooikrantz Dam	Livestock watering	32 º45'19"S 27 º19'35E
King Williams	Recreation and domestic purposes	32 º56'19"S 27 º27'56"E
Eluxozweni	Irrigation, Fishing and domestic purposes	32 º53'23"S 27 º23'17"E
Briddle Drift Dam	Fishing and swimming	32 º58'30"S 27 º42'22"E
Parkside	Fishing and swimming	33 º01'23"S 27 º42'22"E

3.2 Sample Collection

Water samples from both rivers were collected directly and aseptically in sterile 1.75 L plastic bottles. All the bottles were sealed and properly labeled. The samples were then placed in ice boxes and transported to the laboratory at the University of Fort Hare for analysis.

3.3 Presumptive Isolation of *E. coli*

Water samples were analysed following standard methods for feacal coliforms (APHA, 1998). Hundred millilitres (100 ml) of serially diluted water samples were filtered through a 0.45µm nitrocellulose filter membrane. Membrane filters were removed aseptically with sterile forceps and placed on m-FC agar in triplicates for each sample. The plates were incubated at 44.5ºC for 24 hours. The distinct blue colonies that grew on m-FC agar were aseptically transferred to Chromocult Coliform Agar (CCA) (Merck, South Africa) for presumptive identification of *E. coli* isolates. When cultured on Chromocult agar, *E. coli* exhibits a dark-blue to violet color. To ensure purity the isolates were subcultured several times on CCA and once on nutrient agar (NA) (Merck, South Africa). A loopful of the isolate from NA was then aseptically inoculated into nutrient broth (NB) (Merck, South Africa), incubated with vigorous shaking at 37ºC for 6 h and preserved in 20% sterile glycerol at -80ºC. Nutrient agar slants of the isolates were also prepared and refrigerated for routine use.

3.4 Confirmatory Identification of *E. coli* Isolate by PCR

3.4.1 DNA Extraction.

DNA was extracted using the boiling method as described by Alzahrani et al. (2010). Briefly, 24 h old cultures were suspended in 200 µl distilled water. The suspension was homogenized by vortexing for 5 s and boiled at 94 - 95°C for 15 min. After centrifugation at 15000 rpm for 15 min at 4°C, the supernatant was removed and stored at -80°C for further assays.

3.4.2 DNA Amplification

PCR was performed on a total of 203 presumptive *E. coli* isolates. Primers specific for a conserved region situated within the *E. coli* alanine racemase gene were selected (Table 3.3) (Yokoigawa *et al.*, 1999; Daly *et al.*, 2002). The primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The standard reaction mixture contained 1.25 units of thermostable DNA polymerase, 1× Ex Taq buffer, 2 mM $MgCl_2$, 10 pmol of each oligonucleotide primer, 10 nmol of dNTP (deoxyribonucleoside triphosphate), and 2 µL of template DNA suspension in a final volume of 50 µL. The PCR reaction consisted of 35 cycles of 20s denaturation at 95°C, primer annealing/extension at 72°C for 90 s, and a final extension for 5 min at 72°C. *E. coli* ATCC 25922 was used as a positive control.

Table 3. 3: Primer sequences and expected size of PCR-amplified gene targets of the pathogenic strains of Escherichia coli.

Target strain	**Gene target**	**Primer sequence 5'–3'**	**Amplicon size (bp)**
E. coli	*Alr*	CTGGAAGAGGCTAGCCTGGACGAG	366
		AAAATCGGCACCGGTGGAGCGATC	

3.4.3 Gel Electrophoresis

The amplicons (5 µl aliquots) were resolved in a 1% (w/v) agarose gel (Merck, SA). The gel was stained with Ethidium bromide (0.001µg/ml) and visualized under the UV trans-illuminator (Alliance 4.7). A 100-bp DNA ladder (Promega, USA) was included on each gel as a molecular size standard. The electrophoresis was carried out at 84 V for 1 h 30 min.

3.5 Molecular characterization of the isolates by singleplex PCR

For molecular characterization of *E. coli* pathotypes, the PCR assays were done in 50 µl reaction volumes containing 25 µl of PCR master mix, 1 µl each of the forward and reverse primer, 5 µl of template DNA, and 18 µl of nuclease free water. The thermal cycling conditions for EAEC, EPEC, and ETEC (Heat Labile) were as follows: initial activation step at 95°C for 15 min, followed by 35 cycles consisting of denaturing at 94°C for 45 s, annealing at 55°C for 45 s, extension at 68°C for 2 min and final elongation at 72°C for 5 min (Omar, 2007). The cycling conditions for EIEC were: 96 °C for 4 min; 35 cycles of 94 °C for 30 s, 58°C for 30 s and 72°C for 1 min; and a final 7 min extension at 72°C (Vilchez *et al*., 2009) while those for ETEC (Heat Stable) were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 final extension (Matar *et al*., 2002)

The primer sequences, target genes and expected amplification products are listed in Table 3.4. All PCR reactions were performed in a MyCycler^{TM} Thermal Cycler PCR system (BioRad, USA). Positive controls (Table 3.4) and negative controls (nuclease free water) were included in all PCR reactions.

Table 3. 4: Primer sequences and expected amplicon sizes of the pathogenic Strains of *Escherichia coli.*

Target strain	Gene Target	Primer Sequence (5'–3')	Amplicon Size (bp)
EAEC	Eagg	AGA CTC TGG CGA AAG ACT GTA TC	194
		ATG GCT GTC TGT AAT AGA TGA GAA C	
EPEC	*EaeA*	CTG AAC GGC GAT TAC GCG AA	917
		GAC GAT ACG ATC CAG	
ETEC	*LT*	GGC GAC AGA TTA TAC CGT GC	450
		CGG TCT CTA TAT TCC CTG TT	
	ST	ATTTTTCTTTCTGTATTGTCTT	190
		CACCCGGTACAAGCAGGATT	
EIEC	*shig*	CTGGTAGGTATGGTGAGG	320
		CCAGGCCAACAATTATTTCC	

Table 3. 5: Bacterial strains used in molecular characterization

Reference nr	Bacterial Culture
DSM 10819	NMEC
DSM 8695	EPEC
DSM 4816	UPEC
DSM 10973	ETEC
DSM 10974	EAEC
DSM 9025	EIEC

The positive control *E. coli* strains were purchased from DSMZ (Germany)

3.6. Antimicrobial susceptibility test

Antimicrobial susceptibility testing was done on Mueller-Hinton (MH) agar (Merck, South Africa) using the standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Cultures (18-22 h old) were transferred into test tubes containing 5 ml of sterile normal saline. The turbidity of the suspension was adjusted to 0.5 McFarland standards. Sterile swabs were then used to inoculate the MH agar plates by spreading the suspension uniformly on the surface of the agar, after which the antibiotic discs were placed on the bacterial lawn. Disks were purchased from Mast Diagnostics (Mast Group, Merseyside, UK). The plates were incubated at 35 ± 2°C for 18 to 24 h. After incubation the plates were examined for zones of inhibition which were then measured and interpreted using the minimal inhibitory concentration (MIC) breakpoints for Enterobacteriaceae (CLSI, 2006) as shown in Table 3.6.

Table 3. 6: Zone diameter interpretative standards and equivalent minimal inhibitory concentration (MIC) breakpoints for Enterobacteriaceae

Report/Test Group	Antimicrobial Agent	Disc content	Zone Diameter nearest whole (mm)			Equivalent MIC Breakpoints (µg/ml)	
			R	I	S	R	S
O	Streptomycin (S)	10 µg	≤ 11	14-20	≥ 15	-	-
B	Ciprofloxacin (CIP)	5 µg	≤ 15	16-20	≥ 21	≥ 4	≤ 1
A	Ampicillin (AP)	25 µg	≤ 13	14-16	≥ 17	≥ 32	≤ 8
	Erythromycin (E)	15 µg	≤ 13	14-22	≥ 23	≤ 8	≤ 0.5
C	Chloramphenicol (C)	30 µg	≤ 12	13-17	≥ 18	≥ 32	≤ 8
B	Meropenem (MEM)	10 µg	≤ 13	14-15	≥ 16	≥ 16	≤ 4
B	Cefuroxime (CXM)	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8
U	Norfloxacin (NOR)	10 µg	≤ 12	13-16	≥ 17	≥ 16	≤ 4
C	Kanamycin (K)	30 µg	≤ 13	14-17	≥ 18	≥ 25	≤ 6
A	Gentamycin (GM)	10 µg	≤ 12	13-14	≥ 15	≥ 8	≤ 4
O	Doxycycline (DXT)	30 µg	≤ 12	13-15	≥ 16	≥ 16	≤ 4
B	Imipenem (IMI)	10 µg	≤ 13	14-15	≥ 16	≥ 16	≤ 4
B	Amikacin (AK)	30 µg	≤ 14	15-16	≥ 17	≥ 32	≤ 16
C	Tetracycline (T)	10 µg	≤ 14	15-18	≥ 19	≥ 16	≤ 4
B	Cefotaxime (CTX)	30 µg	≤ 14	15-17	≥ 23	≥ 64	≤ 8

3.7. Detection of antibiotic resistant genes

The commonly resistant genes including *TEM, SHV, CTXM,* and *TetC* were used as target genes. The primers for this PCR detection were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, South Africa and the sequences are shown in Table 3.7. The reaction mixture (25 µl) for PCR assay included 12.5 µl of PCR master mix, 0.5 µl of each primer, 2.5 µl of template DNA and 9 µl of PCR grade water. The conditions for PCR assay for *TEM, SHV,CTMX* were as follows: pre-denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 50 s and a final extension at 72°C for 10 min (Cai et al., 2012). For *TetC* gene the PCR conditions were as follows: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C followed by 10 min at 72°C) (Agerso et al., 2005). The products were then subjected to 2% agarose gel electrophoresis.

Table 3. 7: Primer sequences and expected size of PCR-amplified gene targets of antibiotic resistant genes

Gene	Primer sequence (5'–3')	Expected amplicon size (bp)
TEM	GTCGCCGCATACACTATTCTCA	258
	CGCTCGTCGTTTGGTATGG	
CTX-M	CGGGAGGCAGACTGGGTGT	381
	TCGGCTCGGTACGGTCGA	
SHV	GCCTTGACCGCTGGGAAAC	319
	GGCGTATCCCGCAGATAAAT	
***TetC* gene**	GGTTGAAGGCTCTCAAGGGC	505
	GGTTGAAGGCTCTCAAGGGC	

3.8. Detection of Integron gene cassette

In order to verify the presence of gene cassettes within the Integrons conserved segment, PCR reactions were performed using primers targeting the conserved sequences flanking the variable region (Table 3.8). The PCR reaction was done in a total volume of 25 µl under the following conditions: initial denaturation at 94°C for 12 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 5 min of extension at 72°C.

Table 3. 8: Primers for the integron conserved segment

Target Gene	Primer Sequences 5'–3'	Amplicon size (bp)
Integrons conserved Segment	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable

Chapter 4:

RESULTS

4.1 Distribution of *E. coli* in Tyume and Bufallo rivers

A total of 374 presumptive *E. coli* isolates from both rivers were isolated by convenctional microbiological techniques. For both the Bufallo and Tyume rivers, a large proportion (87 and 114, respectively) of the isolates from the mid-stream samples satisfied the identification characteristics for *E. coli* (blue colonies on MFC agar and violet/purple colonies on Chromocult agar) and thus revealing high levels of pollution when compared to the downstream samples where 55 and 47 isolates were obtained and the upstream samples where 30 and 31 isolates were obtained for the Buffalo and Tyume rivers respectively. (Fig 4.1 and 4.2).

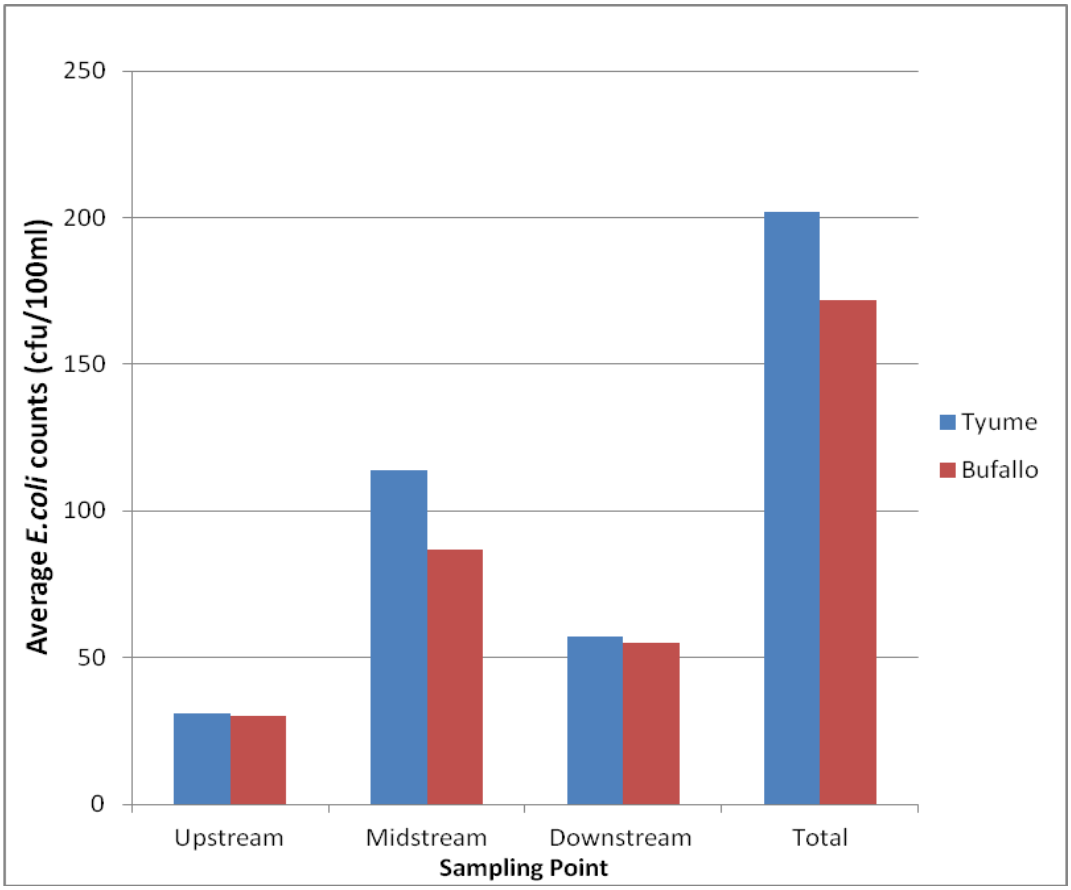

Fig 4. 1: **Distribution of E.coli in Bufallo and Tyume rivers over the period August 2010 to July 2011**

4.2 Confirmation of the identities of *E. coli* isolates using the *alr* specific PCR

Out of the 202 isolates from Tyume River that were subjected to screening by conventional PCR, (35% were positively identified as *E. coli* since they possessed the *alr* gene fragment and, out of the 172 isolates screened from Buffalo River, (47% were also positively identified as *E. coli*. The proportion of *E. coli* isolates obtained from water samples collected at different points in the Tyume and Bufallo rivers is shown in Table 4.1. For both Tyume and Bufallo rivers, the highest prevalence was observed in samples from the midstream sampling sites, which stood at 39% and 56% respectively.

Table 4. 1: Proportion of E. coli isolates obtained from water samples collected at different sampling points in the Tyume and Bufallo rivers using conventional PCR.

Location	**Upstream**	**Midstream**	**Downstream**
Tyume River	4 (13%)	44 (37%)	22 (39%)
Buffalo River	15 (50%)	49 (56%)	16 (29%)

4.3 Molecular characterisation of *E.coli* pathotypes

The confirmed *E. coli* isolates were further characterised into different pathotypes. Amplification of the *shig* gene, *LT* gene, *EaeA* gene, *Eagg* gene and the *ST* gene were used to detect pathogenic *E.coli* strains. Occurrence of DEC isolates from Tyume River is shown in Fig 4.2. Enterotoxigenic *E.coli* (ETEC) from midstream of the river was the most prevalent DEC (54%), followed by Enteropathogenic *E.coli* (EPEC) (44%), from downstream. There

was no recovery of DEC in samples from the upstream sampling site of the river. Also in Bufallo River, DEC was not recovered in both upstream and downstream samples. Enteroaggregative *E.coli* (EAEC) (8%) was the only pathotypes recovered from the midstream samples of Buffalo River. Representative gel electrophoresis profiles of the amplified products for the target genes of pathogenic *E. coli* strains are shown from Fig 4.3-4.7.

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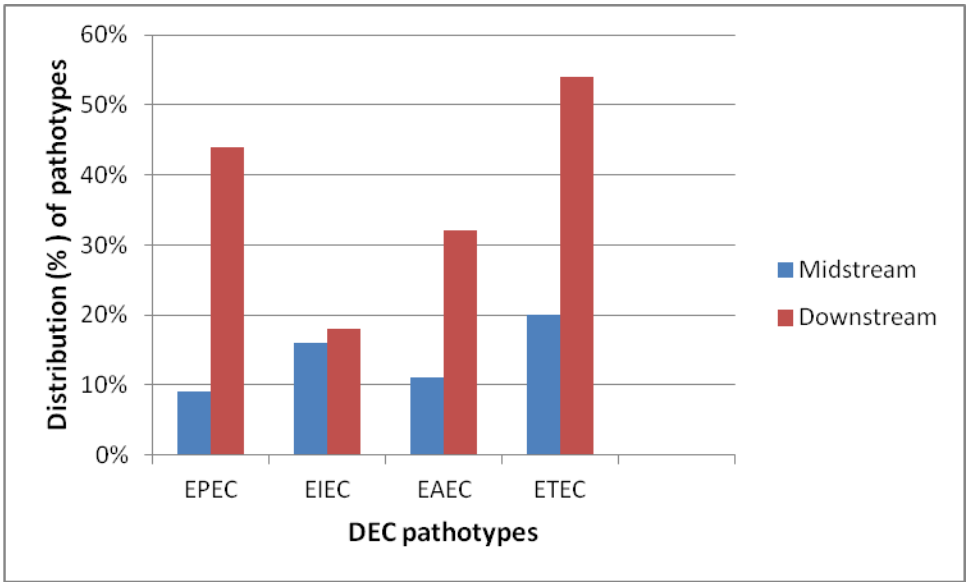

Fig 4. 2: Overall distribution of the diarrheagenic E. coli isolates in Tyume River. EPEC=Enteropathogenic E.coli; EIEC= Enteroinvasive E.coli; EAEC=Enteroaggregative E.coli; ETEC= Enterotoxigenic E.coli.

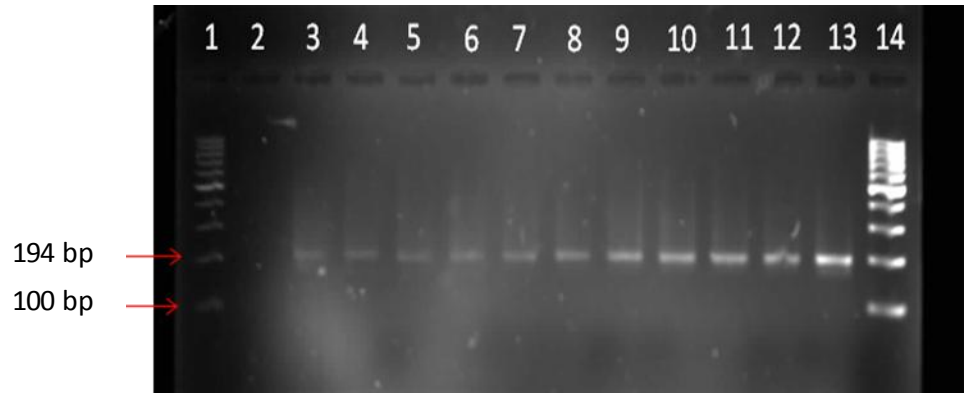

Fig 4. 3: Gel picture showing the amplification of the Eagg gene for the differentiation of EAEC.

Lane 1 and 14: 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 2: negative control (PCR grade water); Lane 3: positive control (DSM 10974); Lane 4-13. The expected molecular size of *Eagg* fragments was 194 bp.

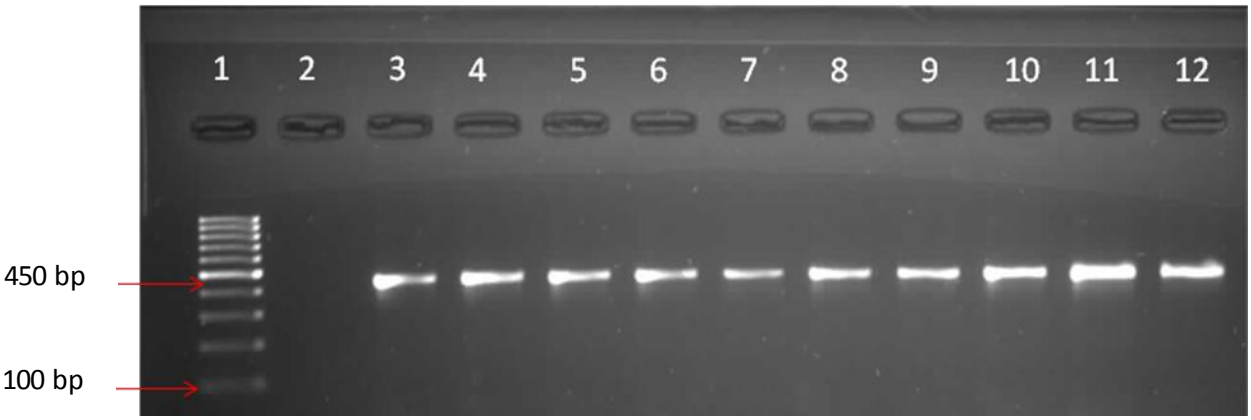

Fig 4. 4: Gel picture showing the amplification of the LT gene for the differentiation of ETEC.

Lane 1: 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 2: negative control (PCR grade water); Lane 3: positive control (DSM 10973); Lane 4-12: expected molecular size of *LT* fragments was 450 bp.

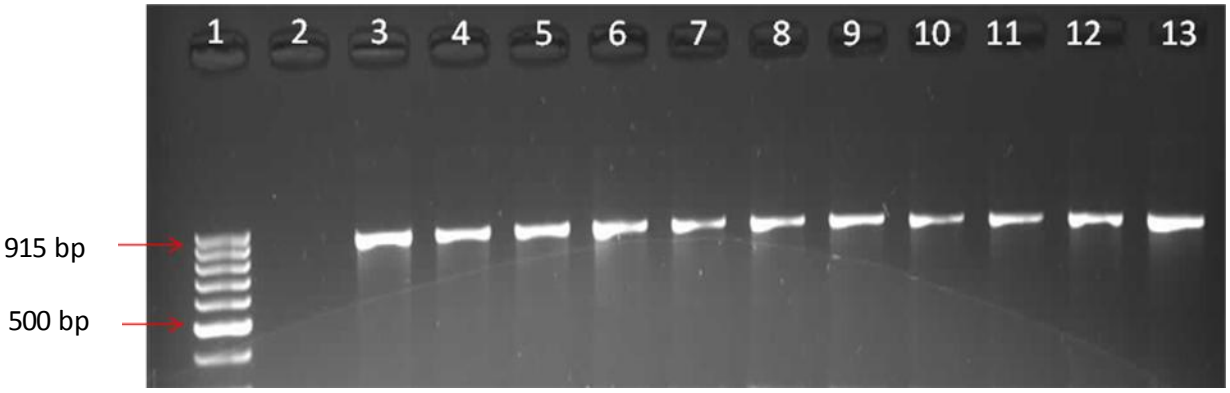

Fig 4. 5: Gel picture showing the amplification of the EaeA gene for the differentiation of EPEC.

Lane 1: 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 2: negative control; Lane 3: positive control (DSM 8695), Lane 4-9 isolates from Tyume River, Lane 10-13: isolates from Buffalo River: The expected molecular size of *EaeA* fragments was 917 bp.

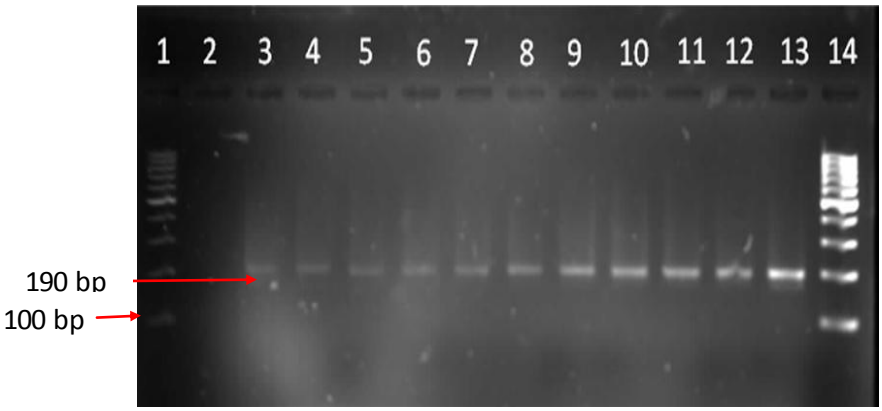

Fig 4. 6: Gel picture showing the amplification of the ST gene for the differentiation of ETEC.

Lane M and 14: 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 2: negative control (PCR grade water); Lane 3: positive control (DSM 10974); Lane 4-13. The expected molecular size of *ST* fragments was 190 bp.

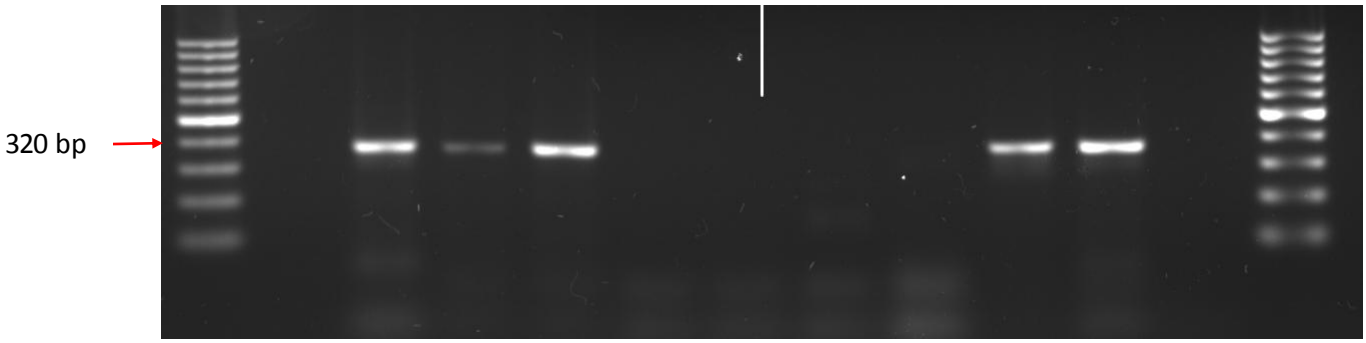

Fig 4. 7: Gel picture showing the amplification of the shig gene for the differentiation of EIEC.

Lane M and 14: 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 2: negative control (PCR grade water); Lane 3: positive control (DSM 9025); Lane 4-13. The expected molecular size of *shig* fragments was 320 bp.

4.4 Antimicrobial Susceptibility Testing

The antibiotic susceptibility patterns of the isolates are presented in Table 4.2 and 4.3. Strains of all the five *E. coli* categories that were characterised in this study showed high-level resistance to ampicillin, tetracycline, cotrimoxazole, and chloramphenicol but were highly susceptible to quinolones, aminoglycosides, cefotaxime and novobiocin. The highest resistance (100%) amongst the isolates was observed to ampicillin by EAEC, Heat Labile (ETEC) and EIEC, followed by 87.5% shown by EAEC to carbenicillin. The highest susceptibility was to quinolones (100%) by all the four categories of *E.coli*.

Table 4. 2: Antimicrobial susceptibilities patterns of different diarrheagenic E. coli isolates from Tyume River

Antimicrobial Agent	Disc content	Zone Diameter nearest whole (mm)				
		EAEC (n=14)	EPEC (n=14)	EIEC (n=11)	Heat Labile (n=8)	Heat Stable (n=13)
		No of resistant strains (100%)	No of resistant strains (100%)	No of resistant strains (100%)	No of resistant strains (100%)	No of resistant strains (100%)
Streptomycin	10 µg	2 (14%)	0 (0)	0(36%)	0(0)	0(0)
Ciprofloxacin	5 µg	0 (0)	0 (0)	0(0)	0(0)	(0)
Ampicillin	25 µg	14 (100%)	12 (85%)	11(100)	8(100%)	11(85%)
Erythromycin	15 µg	10 (71%)	11 (78.5%)	8 (72%)	6(75%)	13(100%)
Penicillin G	10 µg	12 (86%)	14 (100%)	11(100%)	7(57.5%)	13(100%)
Novobiocin	30 µg	0(0)	0(0)	0(0)	0(0)	0(00
Nalidixic Acid	30 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Carbenicillin	100 µg	14 (100%)	14 (100%)	11(100%)	8(100%)	11(100 %)
Chloramphenicol	30 µg	5 (36%)	4 (28.5%)	2(18%)	3(37.5%)	5(38%)
Meropenem	10 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Cefuroxime	30 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Sulphamethoxazole	25 µg	7 (50%)	11 (78.5)	7(63%)	4(50%)	13(100 %)
Norfloxacin	10 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Kanamycin	30 µg	0(0)	0 (0)	0(0)	0(0)	0(0)
Gentamycin	10 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Doxycycline	30 µg	10 (71%)	11(78.5)	11(100%)	6(75%)	10(76%)
Polymyxin B	300 µg	2 (14%)	0 (0)	3(27%)	0(0)	0(0)
Imipenem	10 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Amikacin	30 µg	0 (0)	0 (0)	0(0)	0(0)	0(0)
Tetracycline	10 µg	10 (71%)	12(85%)	9(81%)	8(100%)	13(100%)
Cefotaxime	30 µg	0 (0)	0(0)	0(0)	0(0)	0(0)

Table 4. 3: Antimicrobial susceptibility profiles of EAEC isolated in Bufallo River.

Antimicrobial Agent	Disc content	EAEC(n=4) No of resistant strains (100%)
Streptomycin	10 µg	0(100)
Ciprofloxacin	5 µg	0 (0)
Ampicillin	25 µg	4 (100%)
Erythromycin	15 µg	2 (50%)
Penicillin G	10 µg	3 (75%)
Novobiocin	30 µg	0(0)
Nalidixic Acid	30 µg	0 (0)
Carbenicillin	100 µg	3 (75%)
Chloramphenicol	30 µg	1 (25%)
Meropenem	10 µg	0 (0)
Cefuroxime	30 µg	0 (0)
Sulphamethoxazole	25 µg	2 (50%)
Norfloxacin	10 µg	0 (0)
Kanamycin	30 µg	0(0)
Gentamycin	10 µg	0 (0)
Doxycycline	30 µg	3 (75%)
Polymyxin B	300 µg	0 (0)
Imipenem	10 µg	0 (0)
Amikacin	30 µg	0 (0)
Tetracycline	10 µg	2 (50%)
Cefotaxime	30 µg	0 (0)

4.5 Detection of antibiotic resistant genes

The screening for antibiotic resistance genes revealed the absence of *SHV*, *CTMX* and *TetC* genes as they were not detected in any of the *E.coli* isolates. However, *TEM* genes were observed in 80% of the isolates (Fig 4.8). Integron conserved segment was detected in these same organisms in the same proportion as TEM (Fig 4.9).

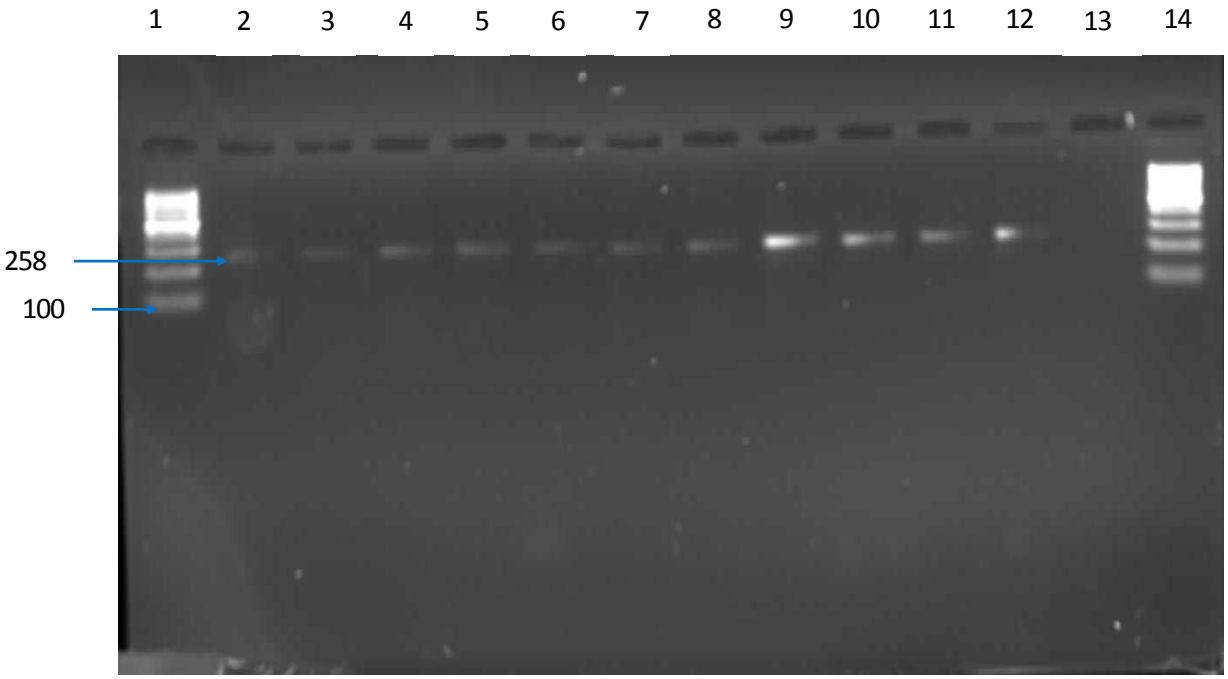

Fig 4. 8: Gel picture showing the amplification product for the TEM gene.

Lane 1: Low Range DNA Ladder (Fermentas Life Sciences, SA); Lane 2-13: isolates

Lane 14: negative control (PCR grade water). The expected molecular size of *TEM* fragments was 258 bp**.**

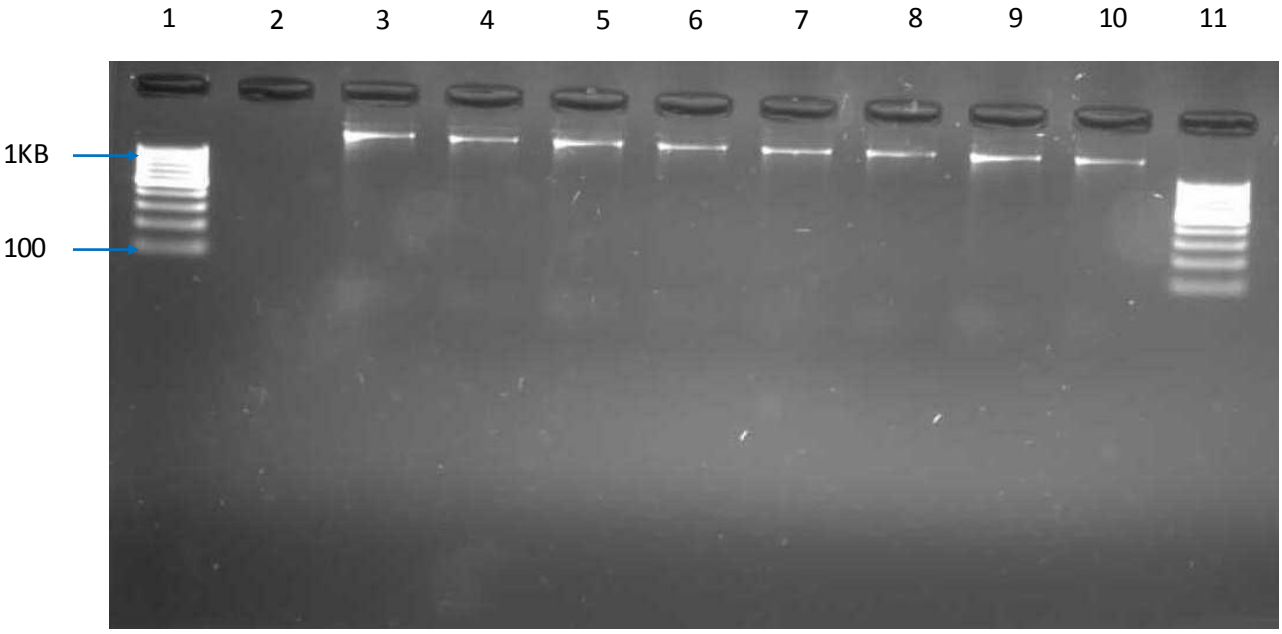

Fig 4. 9: Gel picture showing the amplification product for the Integrons conserved Segment. Lane 1 and 11: 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 2 negative control (PCR grade water); Lane 3: -10: isolates The expected molecular size of Integrons conserved Segment fragments varied from 1kb to 3kb.

Chapter 5:

Discussion

Water resources in South Africa have been under increasing threat of pollution in recent years (Fatoki *et al.* 2001; Igbinosa and Okoh, 2009). Informal settlements that lack appropriate sanitary infrastructure and proper water supply are likely to have serious consequences for health care management and disease prevention within the local communities (Lo Presti *et al.* 2000). Almost 30% of the population lack access to an adequate supply of potable water, which implies that most communities, especially in rural areas, rely mainly on river, stream, well and pond water sources for their daily water needs (Venter, 2001). These water sources are frequently exposed to microbial contamination from humans, animals and the environment (Nevondo and Cloete, 1999; Lehloesa and Muyima, 2000). The potential outbreaks of water-borne diseases therefore, continue to grow with the increasing demands for potable water (White *et al.* 2000).

Isolation of pathogens from water sources represents a serious public health risk for consumers. The presence of enteric bacterial pathogens in water sources may spell health hazards such as diarrheal diseases, which accounts for a substantial degree of morbidity and mortality in adults and children (Black, 1993; Du Pont, 1995). Previous reports on the microbial quality of river water in some rural communities of South Africa showed that water sources were unsafe for human consumption and, *E. coli* was one of the predominant potential pathogens isolated (Obi *et al.*, 2002, Zamxaka *et al.*, 2004; Momba *et al.*, 2006). The purpose of this study was to determine whether pathogenic *E. coli* are present in water sources used by Nkonkobe and Buffalo Municipalities rural communities for their daily water

needs. Such information may allow us to determine to what extent the water sources may influence infection and disease in the community.

Isolation of *E.coli* from three points (viz. upstream, midstream and downstream) in Tyume and Bufallo rivers was achieved by using the USEPA approved method for the detection of faecal indicator bacteria (FIBs) in recreational waters; the membrane filtration method. This approach is based on passing water through a filter that is placed on a medium selective (in this case, Chromocult) for the bacterial group of interest. These culture-based methods are widely accepted because of their relative ease of use, low cost, and demonstrated relationship to health risk (WHO, 2004). The results revealed that for both Bufallo and Tyume rivers, the mid stream was highly contamination when compared to the downstream and the upstream.

Culturing methods are often associated with disadvantages like of lack of selectivity, sensitivity and specificity. Consequently, the detection of the presence of presumptive *E. coli* in water sources by culturing methods alone does not definitely indicate the presence of the strains capable of causing infections and diseases in human. Also, the inability of these methods to detect *E.coli* in viable but non-culturable (VNBC) state has prompted interest in alternative techniques to monitor the microbiological quality of water (Lleo *et al.*, 2005, Omar and Bernard, 2010). The Polymerase chain reaction (PCR) has led to rapid and sensitive detection of *E. coli* from clinical and environmental samples (Nataro and Kaper, 1998; Omar and Bernard, 2010, Momba *et al.*, 2006).

PCR is a powerful molecular biology technique for the detection of target DNA in various clinical specimens, food, and water and for the detection of many kinds of pathogens. It is not only highly sensitive and specific, but it also provides rapid and reliable results. In this study,

PCR analysis of the isolates targeting the alanine racemase gene (*alr*) was carried out. Amplification was optimized to obtain the desired 366 base pair fragment. A large proportion of the isolates were from the mid-stream samples for both river. The restricted accessibility by both villagers and animals to the upstream of both rivers could be the reason why low levels of contamination were observed in the upstream.

In rivers both rivers, the midstream and the downstream is easily accessible to humans and animal as it is in close proximity to the villages and is the main water supply for the villagers. Animals who drink from the river banks, also defecate around it and when rains come this waste is washed into the river. The sanitary system in the surrounding villages and townships is also very poor. It is therefore suspected that the high level of contamination in the midstream of the rivers can also be as a result of human and animal activities. Another factor for the high level of contamination in Tyume River may be that the Alice wastewater treatment plant is situated on the banks of the river, which is also used as the receiving water body for the final effluent from the plant. Several studies have also revealed that the discharge of wastes into dams or rivers is a potential source for transmitting microbial contaminants to crops as well as fish (Tyrrel, 1999; Momba *et al.*, 2010).

The presence of E. *coli* indicates an increased likelihood of pathogens being present (U.S. Environmental Protection Agency, 1986). The points midstream and downstream of Bufallo river are often used by inhabitants for swimming. A person swimming in highly contaminated water has a greater chance of getting sick from swallowing disease-causing organisms, or from pathogens entering the body through cuts in the skin, the nose, mouth, or the ears. Therefore, the presence of possible pathogens poses a risk to people who utilize the river for recreational activities.

When using the MF, a total of 374 presumptive *E.coli* isolates were detected in both rivers. The number greatly decreased to 150 after using PCR. Several factors may have contributed to the apparently high *E. coli* frequency using culturing methods and their low frequency or absence using the PCR method.

Diarrhea caused by multidrug-resistant bacteria has been recognized as an important public health problem among children in developing countries and is a research priority of the diarrheal disease control program of the World Health Organization **(**WHO, 2004**)**. Among these bacteria, strains of the different diarrheagenic categories of *E. coli*, such as Enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli*, and enteroaggregative *E. coli* (EAggEC), are among the most important causes of acute enteritis and subsequent morbidity and mortality in children in developing countries. Although most *E. coli* strains are harmless commensals, others are pathogenic. Virulent strains were first classified according to their serotypes in the late 1940's (Wilshaw et al., 2000, Nataro and Kaper, 1998; Todar, 1998).

Infection with diarrheagenic *E. coli* (DEC) results in a spectrum of illnesses which includes infantile enteritis caused by EPEC; bacillary diarrhea caused by EIEC; traveller's diarrhoea caused by ETEC; persistent watery diarrhea caused by Eagg; mucus containing watery diarrhoea caused by DAEC; hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) caused by VTEC (Kaper and Nataro, 1998). Some VTEC cases are fatal (Doyle *et al.*, 1997; Garbutt, 1997). Diarrheagenic *E. coli* are transmitted via person-to person contact, person-to-animal contact, contaminated water and

food. Their major route of transmission is oral through the consumption of ground beef products (Bell, 2002; Meng and Doyle, 1998).

The 150 *E. coli* isolates (80 from Bufallo and 70 from Tyume) from identified by PCR that possessed the *alr* genes were characterized into different pathotypes. The following virulence associated genes of DEC were targets for PCR amplification: *shig* gene, *lt* gene, *EaeA* gene, *Eagg* gene and the *st* gene. All tested *E.coli* pathotypes were present in water samples from Tyume River (86%), however, EAEC was the only pathotypes recovered in Bufallo River (5%). These findings corroborate results from previous investigations (Garcia *et al.*, 2010; Munshi *et al.*, 2012). In Tyume River, pathogenic *E.coli* were more prevalent downstream (47%), followed by midstream (36%) and no pathotypes were detected upstream.

ETEC (st and lt) (30%) was the most prevalent pathotypes. These findings are different from those obtained by Aslani *et al.* (2008) where ETEC was the least isolated DEC pathotypes. The ETEC toxins that were detected are: (i) heat labile toxin (LT), a high-molecular weight, immunogenic toxin; and (ii) heat stable toxin (ST) a low-molecular weight, non-immunogenic (Greenberg and Guerrant, 1986; Robertson *et al.*, 1986). The *st* genes of ETEC were found in 16% of isolates compared to the *lt* genes in 11%. Other studies also showed predominance of *st* producing ETEC (Rao *et al.*, 2003; Shaheen *et al.*, 2004). ETEC are an under-recognised but extremely important cause of diarrhoea in the developing world where there is no adequate clean water and poor sanitation (Qadri *et al.*, 2005). ETEC was thought to account for approximately 200 million diarrhoea episodes and 380 000 deaths annually (Wanneras and Erling, 2004). The ETEC has been attributed as the common cause of infections among the tourists visiting Asia, Africa and South America; and also as a common

diarrhoeal pathogen in children in many developing countries of Asia, Africa and South America (Nessa *et al.*, 2007).

The second most frequently isolated pathotypes in this study was EPEC whose prevalence was20%. A study by Shetty *et al.* (2012) showed EPEC to be the more prevalent than DEC. Amplification of the *eaeA* gene was used to detect EPEC because the presence of the *eae* gene is more highly linked to EPEC than is the presence of either the *bfp* gene or EAF, as has been previously suggested (Vila *et al.*, 1999; Rosa *et al.*, 1998). EPEC and EHEC share *eaeA*, the intimin structural gene which mediates actin aggregation (Ohno *et al*, 1997; Baldwin, 1998; Murray, 2002). All the EPEC strains isolated were *eaeA* PCR positive i.e., they were all atypical EPEC isolates. Studies in other countries indicate similar results. Araujo *et al.* (2002) in Brazil isolated EPEC from 21.1% of the samples while Najand and Ghanbarpour (2006) isolated EPEC in 19.48% of their samples in India. EPEC are one of the leading causes of diarrheal morbidity and mortality among children in developing countries (Clarke *et al.*, 2002). Decades ago EPEC was also frequently seen among children in industrialized countries (Levine and Edelman, 1984).

Enteroaggregative *E. coli* was the also the second frequently identified pathotypes EAEC with a 20% prevalence. The *Eagg* gene that encodes for bundle forming fimbriae was used to detect EAEC. This is similar to a study by Garcia *et al.* (2010) where 21% of the isolates were EAEC. Contrary to this study, Nguyen *et al.* (2005) reported that EAEC (11.6%) was more prevalent than any other diarrheagenic *E.coli* investigated. EAEC produce a haemolysin related to the haemolysin produced by *E. coli* strains involved in urinary tract infections (Todar, 2008). EAEC are a heterogeneous emerging pathogen affecting all ages, prevalent in resource-rich and resource-limited settings, and are associated with acute and persistent

watery diarrhea in children, travellers and in individuals infected with HIV/AIDS (Kaper and Nataro, 1998).

The least prevalent pathotypes was EIEC which had a prevalence of 16% in this study. Despite being recognized as a human pathogen, little research has been conducted to identify the risk factors for infection. The lack of epidemiological attention to EIEC is related to the low incidence of this pathogen as a cause of diarrhea in relation to other strains of diarrheal *E. coli*, and, in most studies, the researchers did not report the occurrence of these microorganisms (Vieira *et al.*, 2007). In a study by Garcia *et al.* (2010), no enteroinvasive *E. coli* strains were recovered in their study; similar results were obtained for the Bufallo River samples. The low prevalence of EIEC in our study confirms the reports in the literature, which showed EIEC as having the lowest incidence of the diarrheal *E. coli*. Occurrence of pathogenic *E. coli* isolates shows that Tyume and Bufallo rivers therefore pose a potential risk for the transmission of pathogens to the consumers and users of raw water from these rivers.

Recently, the health consequences associated with diarrheagenic *E. coli* infection have been worsened by the emergence of multidrug-resistant *E. coli*. This growing phenomenon, whose restraint is considered as being one of the greatest challenges of the twenty-first century for science and for medicine (Santos *et al.*, 2007). Selective antibiotic pressure, associated with their inappropriate use in antimicrobial chemotherapy, as well as their use in the food industry and agropecuary are the key factors in the evolution of resistant strain phenotypes (Hawkey and Jones, 2009). The bacteria can defend themselves from the action of antibiotics by producing various metabolites that either degrade antibiotics or help the bacteria to survive by various mechanisms (Hawkey and Jones, 2009).

River water is the main receptacle reservoir of antibiotics and antibiotic resistant bacteria in the environment. They are directly introduced into surface water through fisheries, animal farms and agricultural practices (Wiggins *et al.*, 1999). A large volume of sewage and effluent containing antibiotic resistant bacteria is discharged into rivers, streams, lakes and sea water (Kolpin *et al.*, 2002). The antibiotic resistance bacteria in drinking water are a prime concern to public health (El-zanfaly, 1991). In the United States, several rivers were reported to be reservoirs of antibiotic resistant bacteria (Ash *et al.*, 2002). Although antibiotic resistance is common, antibiotics are still indicated in the management of diarrhoea. Antibiotics shorten the duration of diarrhoea, decrease stool output and may mitigate complications (Black, 1993).

The antibiotic susceptibility profiles of the isolated *E. coli* strains were elucidated. Susceptibility of *E. coli* isolates to different antimicrobial agent was measured in vitro by employing the modified Kirby-Bauer (Bauser *et al.*, 1966) method. It is frequently used to determine the drug sensitivity of microorganisms isolated from infectious process and to interpret their disease potential. This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. Commercially available antimicrobial discs (Mast Diagnostics, Merseyside, U.K.) were used for the test.

All pathotypes showed up to 94% resistance to ampicillin. Resistance in *E. coli* to ampicillin is due to the production of TEM which is also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. Although TEM-type beta-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also

found in other species of Gram-negative bacteria with increasing frequency (Maynard *et al.*, 2003). A considerable percentage of all the pathotypes in this study also showed resistance to tetracyclines. Data in the literature demonstrate high rates of tetracycline resistance in strains of enteric *E. coli* are probably related to the indiscriminate use of such antibiotics (Usein *et al.*, 2009).

Resistance to chloramphenicol was relatively low, the highest being 38% by ETEC (*st*). Chloramphenicol acts by inhibiting translation during protein synthesis and causes aplastic anaemia in a small percentage of patients, and its use is very minimal in non life-threatening situations. The observed rare bacterial resistance to Chloramphenicol has been attributed to the restricted use of the drug (Goni-Urriza *et al.*, 2000). Membrane-active agents, such as Polymyxin-B, have lowest resistance potentials because they have no specific routes of entry and it is difficult to alter fundamental bacterial membrane composition by mutation (Hancock and Falla, 1997; Ge *et al.*, 1999).

All DEC were found to be 100% susceptible to the aminoglycosides (Gentamycin, Amikacin and Kanamycin), corroborating data in the literature which suggest a good activity of these antimicrobials against enteric Gram-negative band cells. Moreover, such drugs are considered as antimicrobials used in hospitals, and resistant bacteria originating from the community are not expected (Usein *et al.*, 2009). All the pathotypes were highly susceptible to Carbapenems (100%). Carbapenems are a class of β-lactam antibiotics with a broad spectrum of antibacterial activity. They have a structure that renders them highly resistant to most β-lactamases (Livemore and Woodford, 2000), hence the observed high susceptibility rate. Carbapenems are dubbed as one of the antibiotics of last resort for many bacterial infections, such as *E. coli* (Smith, 2010). The observed 100% susceptibility to novobiocin in this study

corroborates the findings of Kobori et al. (2004) and also agrees with the findings of Garcia et al. (2010) who have reported that no antimicrobial resistance was observed against Imipenem.

The quinolones are a family of synthetic broad-spectrum antibacterial drugs (Anderson *et al.*, 2003). No resistance to the quinoles was observed in this study. The first generation of the quinolones begins with the introduction of nalidixic acid in 1962 for treatment of urinary tract infections in humans (Sanofi-Aventis, 2008). Nalidixic acid was discovered by George Lesher and coworkers in a distillate during an attempt at chloroquine synthesis (Wetland, 1993). Nalidixic acid is a synthetic chemotherapeutic agent effective against Gram-negative bacteria by binding to DNA gyrase enzyme (topoisomerase), thus inhibits DNA duplication. It is mainly used in the treatment of urinary tract infections. The extremely low toxicity of the antibiotics in these classes has resulted in their overuse in the medical community, hence the observed increased resistance.

A class, the newer fluoroquinolone possess many characteristics that make them useful antimicrobial agents, including a broad spectrum of activity against Gram-negative and gram-positive organisms, good oral absorption and tissue penetration, relatively long serum elimination half-lives that allow once or twice daily dosing, predictable drug-drug interactions, and a relatively low incidence of serious side effects. However, not all fluoroquinolone show all of these characteristics. In addition, several of the fluoroquinolone continue to be expensive alternatives to other regimens (CDC, 2012).

Chapter 6:

Conclusion and Recommendations

The bacteriological quality of the water from the two rivers was investigated in this study. *E.coli* was detected in both rivers. This emphasizes the importance of safe water supply and provision of proper sanitation facilities for the inhabitants of the informal settlements who use the river water for domestic, recreational and agricultural purposes. Some of the *E. coli* isolates obtained from the two river water samples harbored virulence markers eg; *shig, st, lt, Eagg* and *eaeA* genes, showing the potential pathogenecity of the *E. coli* strains resident in these rivers. This could also lead to the emergence of strains with new combinations of virulence genes, since most virulence factors are encoded on mobile genetic elements. Therefore, a continued surveillance of *E. coli* isolates from the river waters used for recreational or domestic purposes is required.

The *E. coli* isolates showed a high level of resistance to antimicrobial agents and multidrug resistance was extremely common. This is of serious concern and calls for caution in the indiscriminate and inappropriate use of antibiotics, and related compounds on animals and humans. Although aminoglycosides, carbapenems and fluoroquinoles were demonstrated to be effective against the isolates, periodic monitoring of antibiogram is necessary to detect any change in patterns and in characterizing the isolates. The presence of *TEM* gene and integron conserved segment in some of the isolates is worrisome and suggest *E.coli* species as important reservoirs of multidrug resistance genes in the Eastern Cape Province environment.

Recommendations

1. Strict implementation of the existing laws that serve to protect the rivers and monitoring to sustain the richness of the river is encouraged. Particular attention must be given to improve sanitation and sewerage as these are among the social factors that contribute to the degree of faecal contamination in surface waters. A regular and continuous monitoring scheme shall be developed for the two River systems.

2. Education of the public especially those families living along the rivers regarding the health implications of faecal contamination of the rivers must be done as people become aware of the conditions of the environment, they become conscious of their actions toward the environment.

3. The changing patterns of resistance to common antimicrobial agents indicates that designing a surveillance system for antimicrobial resistance and the introduction of integrated guidelines for the appropriate use of antibiotics are urgently needed.

4. A large proportion of *E. coli* pathotypes showed MAR, and this should be considered a cause for concern for veterinary health authorities. This also suggests the need for changes in the use of antibiotics in both veterinary and human medicines

5. This data will be useful in the empiric management of patients with diarrhea in the Eastern Cape region because antibiogram vary with time and geographical region.

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