ANTIBIOGRAM PROFILING OF *ESCHERICHIA COLI* PATHOTYPES ISOLATED FROM KAT RIVER AND FORT BEAUFORT ABSTRACTION WATER

University of Fort Hare
Together in Excellence

Dissertation submitted in fulfilment of the requirements for award of Master of Science Degree in Microbiology

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DECLARATION

I, the undersigned, declare that this dissertation submitted to the University of Fort Hare for the degree of Masters of Science in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exception of 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.

Name: ____

Signature: ____

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DEDICATION

I dedicate this dissertation to:

My parents, Ms L. Nontongana and Mr S. Soci

My grandmother Mrs N.D. Nontongana

My late grandfather Mr E. Nontongana

My late uncle Mthobhana and his beloved family

My second parents Ta Phum, Ngelwa, Scakaa and Zakhe

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And all my aunts, uncles and cousins OoThangane namaTshawe amahle

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ABSTRACT

Escherichia coli (*E. coli*) is a widespread species that includes a broad variety of strains, ranging from highly pathogenic strains causing worldwide outbreaks of severe disease to virulent isolates which are part of the normal intestinal flora or which are well characterized and safe laboratory strains. The pathogenicity of a given *E. coli* strain is mainly determined by specific virulence factors which include adhesins, invasins, toxins and capsule. The aim of this study was to evaluate the prevalence and antibiogram profiles of *E. coli* pathotypes previously isolated from Kat River and Fort Beaufort abstraction water. A total of 171 *E. coli* isolates showed at least one pathogenic determinant among the isolated 278 *E. coli*. The other 107 isolates were negative for the tested virulence genes.

All 278 presumptive isolates tested positive for the *UidA* gene, and were therefore classified as non-categorized pathogenic *E. coli*. The 171 pathogenic isolates had at least one characteristic gene of pathogenic *E. coli* and were identified and classified as enteropathogenic *E. coli* (6%), enterotoxigenic*E. coli* (131), uropathogenic *E. coli* (6), neonatal meningitis *E. coli* (14), diffusely adherent *E. coli* (1) and enterohaemrrhagic *E. coli* (1). Interestingly, no virulence genes were detected for the enteroinvasive *E. coli* and the enteroaggregative *E. coli* .The antibiotic resistance profiles for all isolates that were identified as *E. coli* showed 100% resistance to penicillin G, 98% resistance to ampicillin, 38% resistance to trimethoprim-sulphamethoxazole and 8% resistance to streptomycin. Multiple antibacterial resistance (MAR) was also observed, where 44% of the isolates were resistant to three antibiotics and 8% resistant to four antibiotics. The results of this study showed the Kat River and Fort Beaufort abstraction water are reservoirs of pathogenic strains of *E. coli* which harbour antibiotic resistance determinants that can cause serious health risks to the people in the surrounding communities.

CHAPTER ONE

1.1 General Introduction

Antibiotic-resistant bacteria have been found in a surprisingly diverse range of environments, including clinics, animal pens, orchards, aquaculture, food, sewage as well as chlorinated and unchlorinated water supplies (Chopra and Robert, 2001). Antimicrobial resistance has become a major medical and public health problem as it has direct links with disease management (Ramamurthy, 2008). At the turn of the century, the World Health Organisation (WHO) estimated that infectious diseases accounted for 45% of deaths in Africa and that these diseases were responsible for 48% of premature deaths worldwide (Okeke and Sosa, 2009). Bacteria cause a significant proportion of infections in Africa. Unfortunately, in a remarkably short time, resistance to antibiotics has undermined the idealistic hope that bacterial infections would cease to be an important cause of Disease and death (Fasehun, 1997).

Reports from different parts of Nigeria have observed temporal trends in the prevalence of resistance among enteric organisms, such as *E. coli* and *Shiegella* (Okeke *et al.*, 2000; Iwalokun, 2001). In multiple studies, resistance to commonly used antimicrobials, including trimethoprim-sulphamethoxazole (TMP-SMX, also known as cotrimoxazole), ampicillin, tetracycline and chloramphenicol has shown increasing prevalence in the last 15 years (Okeke *et al.*, 2000; Iwalokun, 2001). Historically, resistance to antimicrobials has been seen for all agents, soon after their discovery. Not long after the discovery of penicillin did Alexander Fleming identify *Staphylococci* that were resistant to the first 'wonder drug'. He correctly predicted that careless use of antibiotics could lead to clinical failures with these drugs (Davis, 1997).

Antibiograms lend information that can be used to raise awareness of resistance problems, support the use of optimal empiric treatment, and identify opportunities to reduce inappropriate antibiotic usage and to discover success of such efforts (Fridkin *et al.,* 2001; Chin *et al.*, 1999; Van Beneden *et al.,* 2003). A typical antibiogram displays the total number of bacterial isolates tested against a range of antimicrobials and includes the percentage of bacterial isolates susceptible or resistant to each antimicrobial agent tested (Fridkin *et al.*, 2001).

A variety of laboratory methods can be used to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. The most common methods include the disk diffusion method, agar dilution, broth micro-dilution, and testing by antimicrobial gradient agar strips (E-test method). Disk diffusion is a qualitative test, and if performed appropriately, the diameter of the zone of growth inhibition around the antibiotic disk will reliably predict the *in vivo* effectiveness of many antimicrobial agents. In many clinical microbiology laboratories, the antimicrobial gradient method is used routinely for testing common, rapidly growing, and certain fastidious bacterial pathogens (CLSI, 2006). The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility.

1.2 Justification of this research

Waterborne diseases are caused by pathogenic microorganisms that most commonly are transmitted through contaminated freshwater. Infection commonly results during bathing, washing, drinking, in the preparation of food, or the consumption of food thus infected (WHO, 2008). Various forms of waterborne diarrheal disease probably are the most noticeable examples, and affect mainly children in developing countries; and according to the World Health Organization, such disease account for an estimated 4.1% of the total global burden of disease, and cause about 1.8 million human deaths annually. The World Health Organization estimates that 88% of that burden is attributable to unsafe water supply, sanitation and hygiene (WHO, 2008).

Escherichia coli infections usually result when water contaminated with the bacteria is consumed. The general symptom of the infection is mostly diarrhea which can cause death in immuno-compromised individual such as the very young and the elderly, due to dehydration from prolonged illness (Kinge *et al.*, 2010). *E. coli* O157:H7 is now a well-recognized cause of human illness. Although outbreaks of *E. coli* O157:H7 infections are frequently associated with food or milk derived from cattle, other sources, including fresh fruits and vegetables and water, have been implicated (Mead and Griffin, 1998). In the United States, the first reported drinking water outbreak of *E. coli* O157:H7 infections occurred in 1989 in rural Missouri (Swerdlow *et al.*, 1992). Since this outbreak, many others have been associated with drinking water. Three were small and occurred in a camp, a recreational vehicle park, and a well (Centre for Disease Control and Prevention [CDC], 2004). Three highly publicized drinking water outbreaks of *E. coli* O157:H7 infections (one each in Wyoming, New York, and Canada), have focused increased attention on the safety of drinking water (CDC, 2005). More recently, the European

Food Safety Authority (2011) reported a novel strain of *Escherichia coli* O104:H4 bacteria that caused a serious outbreak of foodborne illness in northern Germany in May through June 2011. The illness was characterized by bloody diarrhea, with a high frequency of serious complications, including hemolytic-uremic syndrome (HUS), a condition that requires urgent treatment. The outbreak was originally thought to have been caused by an enterohemorrhagic (EHEC) strain of *E. coli*, but it was later shown to have been caused by an enteroaggregative *E. coli* (EAEC) strain that had acquired the genes to produce Shiga toxins.

The use of antimicrobials in medicine and their applications in animal husbandry has brought about phenotypic changes, often due to chromosomal mutations, and antibiotic resistance in *E. coli* has been globally identified in isolates from environmental, animal and human sources (Wash *et al.*, 2005). Kinge *et al.* (2010) reported that *E. coli* had been linked to well-known antibiotic-resistant gene pools and that these genes are transferred into the normal flora of humans and animals, where they exert a strong selective pressure for the emergence and the spread of resistance in *E. coli* strains. Eventually they find their way into the environment via wastewater, manure and sewage sludge. In much of the developing world without access to good quality medicines, infections continue to be the major killers, and in all countries infections with resistant microorganisms are a major cause of death. Antimicrobial resistance is a public health threat and characteristic of many pathogens causing different diseases. It is generally not a problem of disease pathology but one of limited therapy options (Public Health Importance, 2012; WHO, 2013), thus containment strategies must be adapted to the needs of specific disease control and treatment programs.

1.3 Hypothesis

This research was premised on the hypothesis that *Escherichia coli* pathotypes in the Kat River are reservoirs of antibiotic resistance determinants against commonly prescribed and routinely tested antibiotics in the Nkonkobe Local Municipality.

1.4 Research questions

In this study the following questions will be answered:

1. What is the prevalence of *E. coli* pathotypes in the Kat River and Fort Beaufort abstraction water?
2. What are the antibiogram characteristics of *E. coli* isolates obtained from the Fort Beaufort abstraction water and the Kat River?
3. What is the prevalence of antibiotic resistance determinants in the pathotypes?
4. What measures should be taken to prevent the spread of resistant strains of bacteria within our communities due to the consumption of contaminated water?

1.5 Aim and objectives of this study

In order to answer the above research questions, the following aim and objectives were set:

Aim: This study was aimed at evaluating the prevalence and antibiogram profiles of *E. coli* pathotypes isolated from the Kat River and Fort Beaufort abstraction water in the Nkonkobe local municipality in the Eastern Cape Province.

The specific objectives included the following:

- To conduct molecular confirmative identification of presumptive *E. coli* isolates recovered from the Kat River and the Fort Beaufort abstraction water.
- To characterize the recovered isolates into the respective pathotypes.
- To investigate the antibiotic susceptibility patterns of the *E. coli* pathotypes.
- To assess the prevalence of relevant antibiotic resistance determinants in the pathotypes.

CHAPTER TWO : LITERATURE REVIEW

Pathogenic variants of *Escherichia coli* are amongst some of the leading resistant pathogens (Grabow, 1996). Many types of microorganisms cause infection in humans and animals, so disease prevention and treatment strategies must be improved to reflect infection risk factors and available treatment options. Over the past decades, most pathogenic microbes have acquired resistance to one or more antimicrobials. These enteric bacteria are reportedly causative agents of various diseases and their complications (Grabow, 1996). Such diseases include dysentery, haemolytic uraemic syndrome which is a sequel of *Escherichia coli,* cholera and its associated manifestations such as hypo-valaemic shock, acidosis sand haemo-concentration which may be due to *Vibrio cholera* (Obi, 2004). Enteropathogenic forms of *E. coli* have recently become more well-known than the "ordinary" forms. Obi (2004) further reported these forms to cause severe stomach cramps, diarrhoea (often bloody), and vomiting and perhaps slight fever. They produce a toxin that can attack the body in several areas: the gut (causing bloody diarrhoea), the kidneys (causing kidney failure), and sometimes the nervous system.

The increasing use of antimicrobial agents in both human medicine and animal agriculture has resulted in many pathogens developing drug resistance. *E. coli* also plays an important role in many existing surveillance systems for antibiotic resistance in the bacterial flora of livestock and food of animal origin (Riou *et al.*, 1985; Wright *et al.*, 1999). But recently, a decline in the effectiveness of antibiotics has raised concerns in veterinary and human medicine (Philippa and Trnobransk, 1998; Mateu and Martin, 2001; Steinke *et al.*, 2001; Catry *et al.*, 2003).

2.1 *E. coli* pathotypes

Escherichia coli is an organism that occurs universally in sewage and, because it is a faecal coliform, it plays an important role in the sanitary analysis of water (Zamxaka *et al.,* 2004). Its presence in water indicates the presence of faecal contamination and the likelihood of other pathogenic microbes. Eight pathogenic strains of *E. coli* are frequently isolated from humans and animals (Wasteson *et al.,* 2001). These differ from other commensals in that they express virulence factors, which are molecules directly involved in pathogenesis, but which are also important for normal metabolic functions (Donnenberg *et al.,* 2001). These pathogenic strains include:

- The enterophathogenic *E. coli* strain (EPEC)
- The enterotoxigenic *E. coli* strain (ETEC)
- The enteroaggregative *E. coli* strain (EAEC)
- The enteroinvasive *E. coli* strain (EIEC)
- The diffusely adherent *E. coli* (DAEC)
- The enterohemorrhagic *E. coli* strain (EHEC)
- Uropathogenic *E. coli* (UPEC)
- Neonatal meningitis *E. coli* (NMEC)

E. coli is an important member of the normal intestinal micro flora of humans and other mammals which has also been widely exploited as a cloning host in recombinant DNA technology. But *E. coli* is more than just a laboratory workhorse or harmless intestinal inhabitant; it can also be a highly versatile, and frequently deadly, pathogen (James *et al.,* 2004). Due to their distinct virulence properties and the clinical symptoms of the host, pathogenic *E. coli* strains have been broadly classified into two major categories: the enteric (also known as diarrheoagenic) pathogens and the extra-intestinal pathogens. Diarrheoagenic *E. coli* (Levine, 1987; Nataro and Kaper, 1998) have been broadly divided into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). Extra-intestinal *E. coli* include Uropathogenic *E. coli* (UPEC) and Neonatal meningitis *E. coli* (NMEC), these strains cause infections outside the gastrointestinal tract.

ETEC (Nataro and Kaper, 1998) are a major cause of diarrhoea in infants from developing countries and in adults from developed countries traveling to these regions (traveller's diarrhoea). The major route of infection of these pathogens for humans is faecal contamination of food and water (Kuhnert *et al*., 2000). EPEC represent another major cause of diarrhoea in third world countries, they are usually spread by contaminated food and inhabit the small intestine where they attach tightly to the epithelial cells of the villus tips and cause typical lesions called attaching and effacing lesions (Kaper, 1994). STEC (Shiga-like toxin-producing *E. coli* or verotoxin-producing *E. coli*) cause a wide range of symptoms in humans (Griffin, 1999) including uncomplicated diarrhoea, but also more severe diseases like haemorrhagic colitis and the often deadly haemorrhagic uremic syndrome. Cattle form the main reservoir of STEC and faecal contamination of food represents the usual source of infection for humans but due to a seemingly low infectious dose, human to human transmission has also been detected in outbreaks (Kuhnert *et al*., 2000). EIEC closely resemble Shigella and cause mainly watery diarrhoea and dysentery in severe cases. Transmission seems to be mainly through contaminated food and water. EAEC present a typical adherence pattern on cell cultures, with bacteria aligning in parallel clusters like bricks in a wall (Nataro *et al*., 1998). Typically, the persistence of diarrhoea is often for more than 14 days. The epidemiology and the exact pathogenic potential of DAEC are less well understood than those of EAEC.

Extra-intestinal pathogenic *E. coli* have been categorized mainly into uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). UPEC represent by far the most frequent cause of urinary tract infections in humans while NMEC cause neonatal meningitis and sepsis in babies (Johnson, 1991). NMEC most probably enter the body through the intestinal mucosa and subsequently cause sepsis or localize in the central nervous system.

2.2 Evolution of pathogenic *E. coli*

Pathogenic *E. coli* strains differ from those that prevail in the enteric flora of healthy individuals in that they are more likely to express virulence factors. Studies have shown that the expression of these virulence factors interrupts the normal host physiology and causes disease. In addition to their role in disease processes, virulence factors enable the pathogens to abuse their hosts in ways inaccessible to commensal strains, and thus to spread and persist in the bacterial community (Whittam, 1996; Pupo *et al.*, 1997).

2.2.1 Enteropathogenic *E. coli*

a) Pathogenesis

The distinctive histopathology brought by this group of *E. coli* is termed attaching and effacing (A/E) lesions and is caused by the intimate attachment of bacteria to the intestinal epithelial cells and effacement of enterocyte microvilli (Chen and Frankel, 2005). Formation of the micro ulcers and exfoliation of the cells at the site of EPEC attachment was first described in experimentally infected pigs (Staley *et al.*, 1969) and subsequently biopsies from infected infants (Chen and Frankel, 2005). A protein called intimin facilitates the bacterial attachment to the outer cell membrane and is encoded by the *eae* gene which along with all other genetic elements required for this phenomenon are found on the locus of enterocyte effacement (LEE), a large genomic pathogenicity island which was discovered in 1995 (McDaniel *et al.*, 1995). The *eae* is one of the genes currently used for the molecular diagnosis of EPEC. Pathogenesis of these bacteria however is many faceted which has not been fully unravelled as yet and may involve factors other than those directly responsible for A/E lesions as well as more specialized intestinal cells (Cravioto *et al.,* 2001; Nataro and Kaper, 2001).

b) Epidemiology

Although EPEC are among the most important pathogens infecting children less than 2 years of age in the developing world, their prevalence may differ depending on differences in study population, age group, diagnostic criteria and diagnostic tools used (Ochoa *et al.,* 2008). Over the last several decades, the significance of EPEC infection has declined in published literature. The deterioration might be due to interventions, mainly breast-feeding promotion, or to the overestimation of these organisms in earlier studies that used O- or O:H typing compared to the recent ones, in which molecular methods and/or adherence assays were used for EPEC diagnosis (Okeke, 2009; Ochoa, 2011; Katouli *et al.,* 1988). A study conducted in the south of Iran (Katouli *et al.*, 1988) using serological test alone for identification of EPEC reported an isolation rate of 30.7% for this pathotypes in children of less than 3 years old with diarrhoea. The isolates belonged to 12 different serogroups, of which O128 and O126 were the most prevalent. A similar study published 2 years later reported EPEC as the most common pathogen among patients admitted to 4 children's hospitals in Tehran and those attending an outpatient clinic in Sanandaj with an isolation rate of 26.7% and 20.1% respectively (Katouli *et al.*, 1990). Recent studies dealing with diarrhoea in children under 5 years of age have reported varying rates of isolation for EPEC ranging from 12.6-44.9% showing a significant association between EPEC isolation and diarrhoea (Alikhani *et al.,* 2006; Alikhani *et al.,* 2007; Aslani *et al.,* 2008).

Carriage of enteric pathogens is shown to be common in developing countries and has been attributed to the interaction of many factors including host susceptibility (related to the child's age, breastfeeding, nutritional and immunological status), bacterial virulence factors (different virulence genes), and environmental factors (poor hygiene, and high faecal contamination) (Jafari, 2012). However, data from a recently developed quantitative real-time PCR (qRT-PCR) has shown a significantly higher bacterial load in EPEC associated diarrhoeal samples compared to controls (Ochoa and Contreas, 2011). Prolonged and persistent episodes of diarrhoeal disease (diarrhoea> 14 days) constitute a significant portion of the global burden of diseases in children (Ochoa and Contreas, 2011).

A regular review of the illness in developing countries has shown that EPEC, ETEC and EAEC are the main pathogens associated with this complication, and are responsible for 30-40% of all persistent diarrhoeal episodes in children (Abba *et al.,* 2009). In developed countries, EPEC is the most common pathogen isolated from children with persistent diarrhoea accounting for more than half of the episodes (Nugen *et al.*, 2006).

2.2.2 Enterotoxigenic *E. coli*

a) Pathogenesis

ETEC is the most important but under recognized bacterial cause of diarrhoea or cholera-like disease in all age groups in areas with poor sanitation and inadequate clean water (Qadri, 2005). ETEC strains stick to intestinal epithelial cells via a diverse group of proteinacious surface structures known as colonization factors (CFs) which can be fimbrial, non-fimbrial (Croxen *et al.,* 2010). The more recent nomenclature (Jafari, 2012) refer to these structures as coli surface (CS) antigen, but some of the old names still persist such as colonization factor antigen I (CFA/I). Despite the fact that more than 25 CFs have been identified thus far, on many strains no CFs are detected which might be attributed to the technique(s) used for detection, true lack of CFs or as yet, unidentified ones (Isidean *et al.,*2011).

Following the initial adhesion and colonization, ETEC strains cause diarrhoea by producing heat-labile (LT) and/or heat-stable (ST) enterotoxins, which are plasmid-encoded (Johnson and Nolan, 2005). ETEC bacteria produces the small STs as a 72-amino acids preprotoxin which is processed into an 18-19 amino acid active toxin called STa and a 42 amino acid toxin referred to as STb. STa is produced by both human and animal strains, whereas STb is mainly detected in strains of veterinary origins (Nagy and Fekete, 2005). LT like the closely related cholera toxin is a member of AB5 family of toxins which are heterohexameric molecules consisting of five B subunits and a single A subunit (Beddoe *et al.*, 2010). The A1 domain constitutes the active toxin and is linked to the A2 domain via a disulfide bond (Fleckenstein *et al.*, 2010). The A2 fragment is the helical portion of the molecule and anchors the A subunit to the B pentamer which binds irreversibly to GM1 ganglioside as receptors on cell surface (Nagy and Fekete, 2005). The toxin is then internalized and guanine nucleotide- binding protein is stimulated by the A subunit, the levels of intracellular cyclic AMP are increased resulting in diarrhoea.

b) Epidemiology

Among travel-related diseases, travelers' diarrhea is the most frequent illness reported by travellers from developed countries visiting developing countries (Health Protection Agency, 2010). The exact prevalence of travellers' diarrhea is difficult to determine as only a small number of patients seek medical advice in their country of origin (as travellers' diarrhea occurs during travel) and studies are limited by resources in high-risk, developing countries. However, it is known that the destination of travel is the most important risk factor for getting travellers' diarrhea. Travellers visiting high-risk areas are more likely to suffer from this illness compared to those travelling to a low-risk region (Dupont, 2008).

A number of studies have investigated which bacteria cause travellers' diarrhoea in different areas of the world (Nataro, 1998; Diemert, 2006; CDC, 2013). Strains of enterotoxigenic *Escherichia coli* (ETEC) are the most common cause of travellers' diarrhoea in most regions except in South East Asia where *Campylobacter* are the most common cause (Ouyang-Latimer *et al*., 2011). In a surprisingly large proportion of travellers' diarrhoea cases (~20–40%), the cause remains unidentified, even after extensive microbiological examination (DuPont, 2009). However, the majority of these cases with an unidentified causative agent are likely to be of bacterial origin, which explains the effectiveness of antibiotics in the treatment of these patients (DuPont, 2009). Another risk-factor for travellers' diarrhoea is the season in which a traveller visits a country. For example, among British travellers to Tunisia, rates of travellers' diarrhoea have been reported to vary from 16–18% between May and July and 20–23% from August to October (Steffen, 2005). Similarly, in Jamaica and elsewhere, rates of travellers' diarrhoea have been reported to be highest between June and October (Steffen, 2005). High rates of travellers' diarrhoea occur in the hot pre-monsoon months (Lopez-Gigosos *et al*., 2009) and lower rates occur during the monsoon season in areas of South and Southeast Asia (Steffen *et al*., 2004)

2.2.3 Enteroaggregative *E. coli*

a) Pathogenesis

Enteroaggregative *E. coli* (EAEC) surfaced in 1985 and is recognized by its characteristic adherence to HEp-2 cells in an aggregative, stacked brick-like pattern (Harrington *et al*., 2006). It causes both severe and persistent diarrhoea among children, adults and HIV positive persons, in both developing and developed countries (Huang *et al*., 2006). EAEC is the second most common cause of traveller's diarrhoea, and is a common cause of acute diarrhoeal illness in children and adults (4.5%). The basic line of attack of EAEC include colonization of the intestinal mucosa, probably predominantly that of the colon, followed by secretion of enterotoxins and cytotoxins (Weintraub, 2007). The USA National Institutes of Health has categorized EAEC as a category B potential bioterrorism agent (Kaur *et al*., 2010). A study from Brazil identified EAEC infection as the most common cause of diarrhoea in young children, and was found to be more commonly associated with diarrhoea in children less than 2 years of age (Araujo *et al*., 2007; Kaur *et al*., 2010). EAEC pathogenesis involves three steps: (1) adherence to the intestinal mucosa by aggregative adherence fimbriae (AAF) and adherence factors, (2) increased production of mucus that encrusts EAEC on the surface of enterocytes; and (3) release of toxins and elicitation of an inflammatory response, mucosal toxicity, and intestinal secretion (Nataro, 2005; Kaur *et al*., 2010). The most commonly reported symptoms of EAEC infections are watery diarrhoea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever (Kaur *et al*., 2010; Adachi *et al*., 2002), and these clinical manifestations vary from individual to individual, depending upon genetic composition of the host (Jiang *et al*., 2003).

2.2.4 Enteroinvasive *E. coli*

a) Pathogenesis

Enteroinvasive *E. coli* (EIEC) is an intestinal pathogen causing enteritis, with a similar pathogenic mechanism to that of Shigella, which causes an epithelial invasion of the large bowel leading to inflammation and ulceration of the mucosa (Prats, 1995). The patients often develop the symptoms of bacillary dysentery (Mathew *et al.*, 2010). EIEC strains are different in their biochemical reactions and may ferment lactose or not at all, they are lysine decarboxylase negative, and non-motile. In addition, most EIEC strains express somatic antigens which are either strongly related or identical to Shigella antigens (Patton, 2007). EIEC invasion is mediated by a large plasmid (140 MDa) coding for the production of several outer membrane proteins involved in invasiveness (Mathew *et al.,* 2010).

b) Epidemiology

Despite the acknowledgment of EIEC as a human pathogen, very little research has been conducted on its epidemiology in South Africa as compared to the scientific attention other pathotypes have received. A potential contributor to this is that it is often observed to be an infrequent cause of diarrhoea relative to other diarrhoea-causing *E. coli* (Vieira *et al.*, 2007). In a Medline search of studies testing for the presence of EIEC, Vieira *et al*, (2007) identified 42 articles. Thirty five percent of these studies found no EIEC and 40% found EIEC to be a minor strain i.e., representing less than 4% and fewer than 10 cases of all collected samples (Okeke *et al.*, 2004; Pabst *et al.*, 2003; Shehabi, 2003 Rappelli *et al.*, 2005). There were, however, noticeable exceptions where 15 EIEC samples were identified out of 221 cases of childhood diarrhoea in a Beijing hospital (Kain *et al.*, 1999). In the late 1990's, 16 EIEC-positive isolates were identified from 279 Senegalese individuals (Gassama *et al.*, 2004), and EIEC was the predominant enteropathogen during a 2-month period of increased diarrhoea episodes in the Jordan Valley (Meqdam *et al.*, 2000). Therefore, EIEC is seldom identified; when it is, it tends to be in small numbers.

2.2.5 Diffusely adherent *E. coli*

a) Pathogenesis

Diffusely adherent *Escherichia coli* strains are identified by their diffuse adherence (DA) pattern, in which the bacteria uniformly cover the entire cell surface (Scaletsky *et al*., 2002), on cultured epithelial cells *in vitro* and have been recognized as the sixth class of diarrhoeagenic *E. coli* (Lopes *et al*., 2005). DAEC is most commonly associated with age-dependent diarrhoea and in children less than 12 months of age (Scaletsky *et al.*, 2002), however, most case-control studies have demonstrated that the association of DAEC as a diarrhoea-causing agent remains controversial. DAEC is believed to comprise a diverse group of organisms of variable enteropathogenicity (Arikawa *et al*., 2005).

DAEC strains can be classified into two large groups of *Afa/Dr* and non-*Afa/Dr* DAEC before considering their etiological role (Meraz *et al*., 2008). The DAEC strains possess afimbrial adhesions called afimbrial adhesive sheaths (*Afa*s) that are encoded by *afa* gene clusters which consists of *afaA, afaB, afaC, afaDand afaE* genes (Hussain*et al*., 2010). It has been found that DAEC strains possessing *af /dr* adhesins may not only be adherent but invasive to epithelial cells and capable of inducing an inflammatory response (Betis *et al*., 2003; Arikawa *et al*., 2005; Meraz *et al*., 2006; Meraz *et al*., 2008).

There are a limited number of studies regarding the virulence and pathogenic mechanisms of DAEC. It is presumed that the virulence factor of the organism is diffuse attachment to the intestinal lining of the infected host (Sarkar, 2008). In a study carried out by Meraz *et al*. (2008), the major clinical symptoms of the patients carrying DAEC strains were found to include watery diarrhoea, in some instances with mucus and blood, and vomiting; with vomiting being more prominent as compared to diarrhoea among children.

2.2.6 Enterohaemorrhagic *E. coli*

Enterohemorrhagic *E. coli*, also known as *E. coli* O157:H7, appeared in the last decade as an important food-borne pathogen with 73 000 cases of annual infection in the United States (Rangel *et al*., 2005; Zhao *et al*., 2006). Several studies have shown cattle to be the major reservoir of *E. coli* O157:H7, thus the cattle drinking water troughs are an important source of this pathogen on farms (Bach *et al*., 2004; Bach *et al*., 2005; Al-Saigh *et al*., 2004; Bosilevac *et al*., 2004; Low *et al*., 2005; Sheng *et al*., 2004). Most studies indicate that *E. coli* O157:H7 can survive in cattle drinking water up to 12 months and that the pathogen can be easily disseminated to other cattle (Faith *et al*., 1996; Le Jeune *et al*., 2005; Shere *et al*., 2002) and subsequently to the environmental source waters used for human consumption.

2.2.7 Uropathogenic *E. coli*

a) Pathogenesis

The urinary tract is among the most common sites of bacterial infection and *E. coli* is by far the most common infecting agent at this site. The subset of *E. coli* that causes uncomplicated cystitis and acute pyelonephritis is distinct from the commensal *E. coli* strains that comprise most of the *E. coli* populating the lower colon of humans (Philips, 1988; Manges, 2001). Although many UTI isolates seem to be clonal, there is no single phenotypic profile that causes UTIs. Specific adhesins, including P (Pap), type 1 and other fimbriae (such as F1C, S, M and Dr), seem to aid in colonization (Nowicki *et al*., 1989; Jonhsin, 2000). Several toxins are produced, including haemolysin, cytotoxic necrotizing factor and an auto-transported protease known as Sat. These virulence factors are found in differing percentages among various subgroups of UPEC (Jonhson and Stell, 2000). Uropathogenic strains possess large and small pathogenicity islands containing blocks of genes that are not found in the chromosome of faecal strains. This strain, by virtue of factors that are encoded in pathogenicity islands, is capable of infecting an immune-competent host, as it colonizes the peri-urethral area and ascends the urethra to the bladder (Guyer, 2001). Between 4 and 24 hours after infection, the new environment in the bladder selects for the expression of type 1 fimbriae (Gurther *et al*., 2001) which have an important role early in the development of a UTI (Cornel, 1996). Type 1fimbriated *E. coli* attach to mannose moieties of the uroplakin receptors that coat transitional epithelial cells (Mulvey, 1999). Attachment triggers apoptosis and exfoliation; for at least one strain, invasion of the bladder epithelium is accompanied with formation of pod-like bulges on the bladder surface that contain bacteria enclosed in a polysaccharide-rich matrix surrounded by a shell of uroplakin (Anderson, 2003). It is argued that invaded epithelial cells containing a tightly packed bacterial ‘biofilm’ could act as a reservoir for recurrent infection (Mulvey, 1999; Anderson, 2003), and indeed, in some cases of recurrent infection, the same serotype is encountered.

2.2.8 Neonatal meningitis *E. coli*

a) Pathogenesis

This *E. coli* pathotype is the most common cause of neonatal meningitis, with a case fatality rate of 15–40% and severe neurological defects in many of the survivors (Unhanad *et al.*, 1993; Dawson *et al.*, 1999). The incidence of infants with early onset sepsis due to *E. coli* infection seems to be increasing, while infection by Gram-positive organisms decreases (Stoll, 2002). *E. coli* that cause meningitis are spread haematogenously. Levels of bacteraemia correlate with the development of meningitis (Dietzman, 1974); for example, bacteraemia as of >103 CFU/mL of blood are much more likely to lead to the development of meningitis than in individuals with lower CFU/ml in their blood. These bacteria translocate from the blood to the central nervous system without apparent damage to the blood–brain barrier, which indicates a trans-cytosis process. Electron micrographs imply entry by a zippering mechanism in a process that does not affect trans-endothelial electrical resistance (Stins *et al.*, 2001). This indicates that the host-cell membrane is not significantly disrupted during entry of the bacterium.

Some insights into the mechanism of pathogenesis of these strains have been obtained. K1 strains use S fimbriae to bind to the lumenal surfaces of brain microvascular endothelium in neonatal rats (Parkkinen *et al.*, 1988). Invasion requires the outer membrane protein OmpA to bind to the GlcNAcβ1-4GlcNAc epitope of the brain microvascular endothelial cell receptor glycoprotein. Other membrane proteins — for example, IbeA, IbeB, IbeC and AslA — are also required for invasion (Kim, 2001). Invasion correlates with micro-aerobic growth and iron supplementation (Badger and Kim, 1998). CNF1 is required for invasion (Badger, 2000), as is the K1 capsule, which elicits serum resistance and has anti-phagocytic properties. In an experimental model, strains that express K1 capsule proteins and those that do not were able to cross the blood–brain barrier, but only the K1-expressing strains survived (Hoffman *et al*., 1999). As a consequence of invasion, actin cytoskeletal rearrangement occurs and tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin is induced (Reddy *at al*., 2000)

2.3 Diagnosis

For diagnosis, PCR is the most reliable and dependable method for the proper identification of all pathogenic *E. coli* (Guion *et al.*, 2009, Muller *et al.*, 2006). However, molecular methods are still not easily available in clinical laboratories worldwide. It is unrealistic and unnecessary for every diarrhoeal specimen to be screened by molecular methods, since most infection resolve spontaneously. Nevertheless, for clinical purposes it would be ideal if severe cases, bloody diarrhoea cases, hospital-acquired or suspected outbreaks were investigated for all the pathogenic *E. coli* (Ochoa and Contreas, 2011). O-serogroup identification should not be used in clinical laboratories, except as part of outbreak investigations. In relation to treatment, few studies have evaluated in a systematic manner the value on antimicrobials for the management of infection in children (Leekha *et al*., 2011; Ciccarelli, 2013). In order to design suitable clinical trials, fast, easy and low-cost diagnostic methods are needed.

2.4 Epidemiology of pathogenic *E. coli*

Transmission of pathogenic *E. coli* occurs via faecal-oral transmission (Gehlbach *et al.*, 1973). Common routes of transmission include: unhygienic food preparation, farm contamination due to manure fertilization, irrigation of crops with contaminated water or raw water, or direct consumption of sewage-contaminated water (Heaton and Jones, 2008). Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7 and they can carry it asymptomatically and shed it in their faeces (Erikson, 2010; Ferens, 2011).

2.5 Antibiotic Resistance

Antibiotic resistance in *E. coli* has been globally identified in isolates from environmental, animal and human sources (Heike and Reinhard, 2010). Studies have shown that pathogenic organisms have developed some degree of resistance to antimicrobials and they confer resistance through different mechanisms, with a negative impact on veterinary and human medicine (Zamxaka *et al.*, 2004; Walsh *et al.*, 2005; Murray, 1991). These mechanisms of resistance include alteration of receptor-binding sites of the drug, a decreased intake of drugs by altering the entry or active efflux of the drug, the destruction or inactivation of the drug, and development of resistant metabolic pathways (Levy, 1992).

Barbosa and Levy (2002) reported that the surfacing of antibiotic resistance usually results from the misuse of antibiotics as growth-promoters in animal production, for therapy and prophylaxis which have brought about phenotypic changes, often due to chromosomal mutations (Walsh *et al.*, 2005). Because humans consume these animal products, there is a probability of the spread of antibiotic resistant strains from animals to humans and thus healthy individuals can become carrier hosts for multiple antibiotic-resistant bacteria (Reinthaler *et al.*, 2003). This may enhance the risk of developing haemolytic-uraemic syndrome, a disease more severe in children infected with *E. coli* O157:H7 (Bass *et al.,* 1999). Several studies have revealed that *E. coli* is resistant to a number of antibiotics (Bass *et al.,* 1999; Lindgren *et al.,* 2003; Roberts, 2003; Kaye *et al.,* 2004). Adding to the consequences of microbial resistance to antibiotics on human health, contaminated surface water bodies with resistant bacterial strains from human activities and livestock operations have also been reported (Harakeh *et al.,* 2006; Current study). The isolates used in this study were isolated from the Kat River and surface abstraction water near the Fort Beaufort water purification plant.

2.6 *E. coli* resistance to new generation antibiotics

Escherichia coli is a frequent cause of life threatening infections (Kennedy *et al.*, 2008) and other common infections, such as urinary tract infections. Antibiotic resistance rates in *E. coli* are rapidly rising especially with regard to fluoroquinolones and third- and fourth- generation cephalosporins (Colligon, 2009). Surprisingly, most of these multi-drug resistant strains are acquired in the community rather than in health care settings (Laupland *et al.*, 2008; Mesa *et al.*, 2006). Drug resistant *E. coli* are readily acquired via the diet (food and water), and there is a major turnover of drug-resistant *E. coli* each day. When people eat sterile food, there is a rapid and substantial fall in the numbers of drug-resistant *E. coli* these people carry (Corpet, 1988).

Antibiotics which may be used to treat *E. coli* infection include amoxicillin, as well as other semi-synthetic penicillin, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides (Laupland *et al.*, 2008).

Table 2.1: Surveillance data on the trend of emergence of resistance in *E. coli* worldwide

Period	Trend of emergence of resistance to antimicrobials
Past 2 decades	Major increase in resistance to new compounds i.e., Fluoroquinolones and cephalosporins
1971-1982	Major change in resistance to most antibiotic classes
1997-2007	Increased resistance to ciprofloxacin, TMP-SMP, Amoxicillin
1979-2009	Increased resistance to sulphonamide and gentamicin

(Daniel *et al.*, 2010)

CHAPTER THREE : METHODOLOGY

3.1 Description of study site

Figure 3.1 below shows the Kat River catchment area. The Kat River (Afrikaans: Katrivier) is a river in the Eastern Cape Province of South Africa. It supplies the Kat River Valley with irrigation water for large citrus orchards, as well as Seymour and Fort Beaufort of domestic water. The source of the 150 km long Kat River is in the Katberg Mountains.

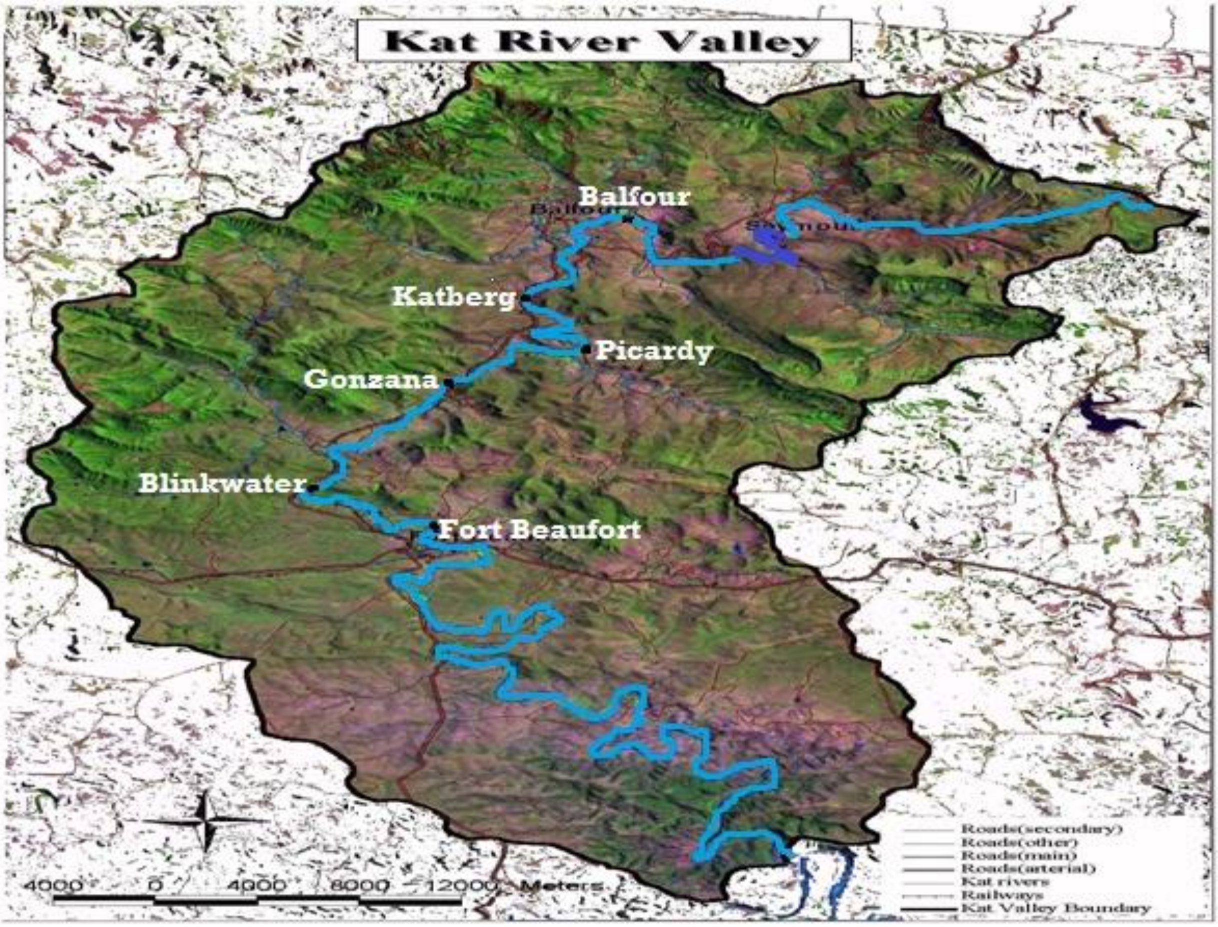

Figure 3.1: Kat River flowing through the Kat River valley.

The mouth of the river is at 32°59′44″S, 26°47′5″E where it joins the left bank of the Great Fish River on its way to the Indian Ocean south of Hamburg, Eastern Cape.

Fort Beaufort is a town situated in the Eastern Cape Province of South Africa and lies on geographical co-ordinates 32º47'0"S, 26º38'0"E. Figure 3.2 shows the Fort Beaufort abstraction water used as source of raw water at the Fort Beaufort municipal water plant. Currently raw water is extracted from the Kat River at a barrage and transferred to this raw water storage dam situated near the water treatment works.

Figure 3.2: Fort Beaufort abstraction water sampling points.

3.2 Recovery of stored isolates

A total of 400 presumptive *E. coli* isolates previously recovered from the Kat River and preserved in glycerol at -80°C as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare were used in this study. Reactivation of the bacterial stocks was done by inoculating a loopful of the glycerol stock on freshly prepared nutrient agar plates. The agar plates were then incubated at 37 °C for 24 hours and repeated twice to ensure purity before subsequent molecular analysis.

3.3Molecular identification of the isolates

a) DNA extraction

A loopful of each purified bacterial isolate was suspended in 200 µl of sterile distilled water followed by centrifugation for 5 min at 13400 rpm in an Eppendorf microcentrifuge. The supernatant was decanted and the cells re-suspended in 200 µl of sterile nuclease free water, vortexed briefly and boiled at 95 ^{o}C for 15 min followed by high speed centrifugation at 12000 rpm for 10 minutes (Samie *et al.*, 2012). The supernatant which contained the DNA was transferred into a new tube and kept at -20°C until further use.

b) Polymerase chain reaction (PCR) identification of isolates

The isolates were further confirmed as *E. coli* by PCR with the use of the *E. coli* specific primers and cycling conditions listed in Table 3.1. The amplicons were then resolved on 1.5% agarose gel at a voltage of 100V for 1 hour. The primer set listed below is designed from the 16S rRNA gene sequence, and targets the *uid*A gene. This gene codes for the β-glucuronidase enzyme of *E. coli* and functions by catalysing the cleavage of a whole range of β-glucuronides.

Table 3.1: Primer sequences and expected sizes of PCR-amplified target genes of *E. coli.*

Target strains	Target genes	Primer sequence (5’→3’)	Amplicon size (bp)
E. coli	***uid*A gene**	**Forw-1 GACCTCGGTTAGTTCACAGA** **Rev-2 CACACGCTGACGCTGACCA**	**147**

Source: Rekha *et al.*, 2006

Protocol for PCR

In a single PCR reaction tube the following reagents were added for each isolate: 12.5µl of master mix, 5µl of sample DNA, 0.5µl of the forward and reverse primer, and 6.5µl of nuclease free water to make a 25µl reaction, which was amplified using the PCR conditions as described by Moyo *et al*. (2007). The amplification conditions were: one *Taq* activation cycle of 94 ºC for 15 minutes followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 58 ºC for 1 minute, and extension at 72 ºC for 3 minutes, and then one cycle of final extension at 72 ºC for 5 minutes. The PCR products were then separated by electrophoresis in 1.5% agarose gels containing ethidium bromide (1 mg/ml). The positively identified isolates were taken as *E. coli* and further characterized into the respective pathotypes.

3.4 Pathotype Identification

For the detection of pathotypes by PCR, extraction of DNA was also done following the boiling method as described by Maugeri *et al*. (2004). Targets recommended for the detection of the pathotypes (Table 3.2) include EHEC targets such as the rarely found stx1, the commonly found stx2 and intimin (*eae*), an adhesion factor responsible for the attaching and effacing (*A/E*) lesions found in EHEC and EPEC. ETEC targets included the heat-labile toxin (lt) and the heat-stable toxin (*stII*). The targets for EIEC, EAEC, DAEC, UPEC and NMEC were the invasion plasmid antigen (*ipaH*), aggregative adherent fimbriae (*aafII*), F1845 Fimbriae (*daaE*), Pyelonephritis-associated pili (*pap*), and the invasion of the brain endothelial (*IbeA*) genes respectively. The annealing temperatures for each primer set are also listed in Table 3.2 below.

3.5 Antibiotic susceptibility test

Antimicrobial susceptibility testing was done on Mueller-Hinton agar (MHA) (Merck biolab) by the standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011). Fresh cultures (about 22 h old) were transferred into test tubes containing 5 ml sterile normal saline. The turbidity of the suspension was adjusted to 0.5 McFarland standards (equivalent to 1.5×10^{8}CFU/100ml). Sterile swabs were soaked into the bacterial suspensions and used to inoculate the MH agar plates by spreading uniformly on the surface of the agar, after which five antibiotic discs were placed equidistant from each other in a circular format on the agar surface and the plates were incubated at 35 ± 2 °C for 18 to 24 h. The antibiotics used in this study are shown in Table 3.3 below. A total of 278 isolates were used for antimicrobial susceptibility testing against the panel of 10 test antibiotics. 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, 2011), Table 3.3.

Table 3.2: *E. coli* target genes and primer sequences used to identify/characterise *E. coli* pathotypes.

Target strains	*Target genes*	Primer sequence (5'→3')	Amplicon size (bp)	Annealing Temp (°C)
EHEC	*Shiga toxin 1 (stx1)*	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG	348	55
	Shiga toxin 2 (stx2)	ATCCTATTCCCGGGAGTTTAC G GCGTCATCGTATACACAGGAGC	584	55
	Intimin (Eae) gene	TCAATGCAGTTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG	482	55
EPEC	*Intimin (Eae)* gene	TCAATGCAGTTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG	482	54
	Bundle-forming pilus (Bfp)	GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTAGT	300	53
ETEC	*Heat-labile toxin (lt)*	GCACACGGAGCTCCTCAGTC TCCTTCATCCTT TCA ATG GCT TT	218	58
	Heat stable toxins (stII)	AAAGGAGAGCTTCGTCACATTTT AATGTCCGT CTT GCG TTA GGAC	129	56
EIEC	*Invasin plasmid antigen (ipaH)*	CTC GGC ACG TTT TAA TAG TCTGG GTGGAG AGC TGA AGT TTC TCTGC	933	53
EAEC	*Aggregative adherent fimbriae (aafII)*	CACAGGCAACTGAAATAAGTCTGG ATT CCC ATG ATG TCA AGC ACT TC	378	56
DAEC	*F1845 Fimbriae (daaE)*	GAACGT TGG TTA ATG TGG GGT AA TAT TCA CCG GTC GGT TAT CAG T	542	54
UPEC	*Pyelonephritis-associated pili (pap)*	AAC CTGGCTTACGCAACTGTACCC GT CTG CAA AAT CAT GGA T	556	58
NMEC	*Invasion of brain endothelial (IbeA) gene*	TGGAACCCGCTCGTAATATAC CTGCCTGTTCAAGCATTGCA	900	58

Table 3.3: Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae.

Test/ Report group	Antimicrobial agent	Disk Content (µg)	Zone diameter Breakpoints, nearest whole mm			MIC Interpretive standard (µg/mL)		
			S	I	R	S	I	R
A	Ampicillin	10	≥ 17	14-16	≤13	≤6	16	≥ 32
A	Gentamycin	10	≥ 15	13-14	≤12	≤4	8	≥ 16
B	Amikacin	30	≥ 17	15-16	≤14	≤16	32	≥ 64
O	Streptomycin	10	≥ 15	12-14	≤11	-	-	-
O	Kanamycin	30	≥ 18	14-17	≤13	≤16	32	≥ 64
C	Tetracycline	30	≥ 15	12-14	≤11	≤ 4	8	≥ 16
B	Ciproflaxacin	5	≥ 21	16-20	≤15	≤1	2	≥ 4
U	Norflaxacin	10	≥ 17	13-16	≤12	≤4	8	≥ 16
B	Trimethoprime-sulfamethoxazole	1.25/23.75	≥ 16	11-15	≤10	≤2/38	-	≥ 4/76
C	Chloramphenicol	30	≥ 18	13-17	≤12	≤8	16	≥32

Source: (CLSI, 2011)

3.5 Evaluation of antibiotic resistance genes

Following susceptibility testing, the relevant antibiotic resistance determinants were evaluated using PCR. The target genes for the antibiotics that the organisms were resistant to are listed in Table 3.4.

Table 3.4: Details of target antibiotic resistance genes.

Antibacterial agent	Resistance gene	Sequence	Annealing Temp. (°C)	References
Streptomycin	aadA1	(F)TATCCAGCTAAGCGCGAACT (R)ATTTGCCGACTACCTTGGTC	58	Van *et al.*, 2008
Beta-lactams	bla	(F)TCGCCTGTGTATTATCTCCC (R)CGCAGATAAATCACCACAATG	52	Van *et al.*, 2008
Tetracycline	tetA	(F)CCTCAGCTTCTCAACGCGTG (R)GCACCTTGCTGATGACTCTT	56	Randall *et al.*, 2004
TMP-SMX	dfrA1	(F)GGAGTGCCAAAGGTGAACAGC (R)GAGGCGAAGTCTTGGGTAAAAAC	45	Toro *et al.*, 2005

CHAPTER FOUR : RESULTS

4.1 Confirmation of presumptive *E. coli* isolates by PCR

Seventy percent (278/400) of the presumptive *E. coli* isolates were confirmed via PCR as *E. coli* as they were positive for the *uid* gene (house-keeping gene for *E. coli*). Figure 4.1 below shows a gel picture of the amplicons of the expected size of 147 bp for some positive isolates.

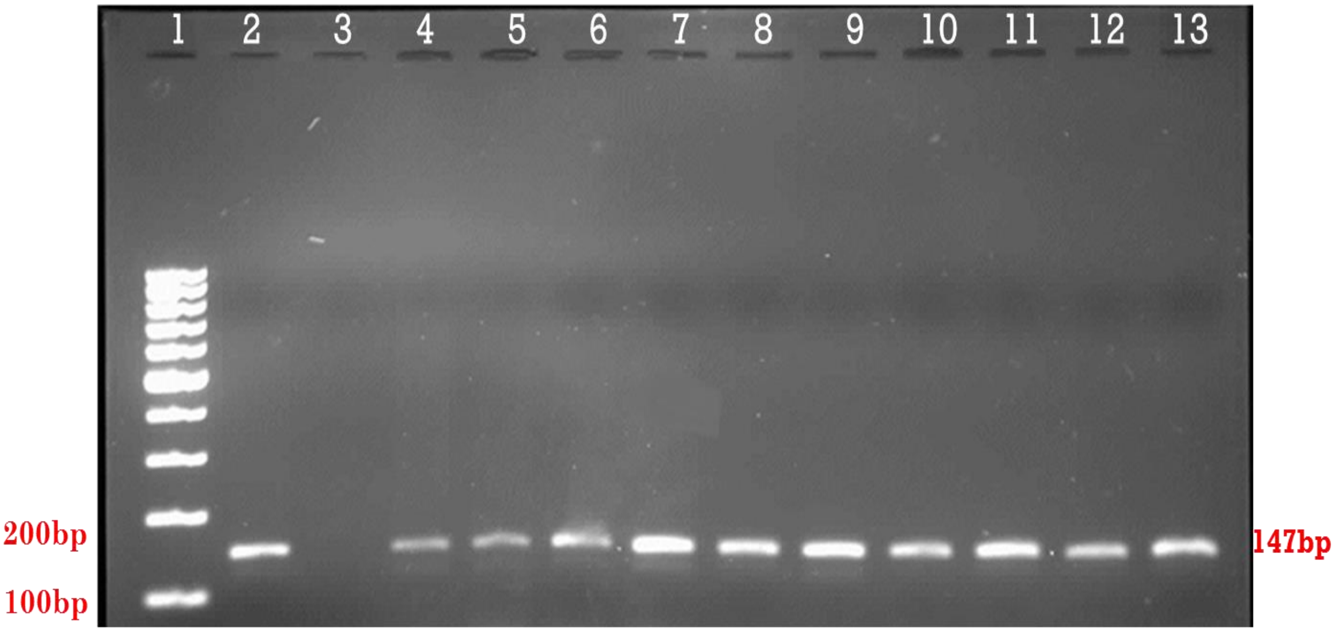

Figure 4.1: Gel electrophoresis of PCR products amplified with *uid*primers for the detection of *E. coli.* Lane 1: 100 bp Molecular Marker; Lane 2: Positive control, Lane 3: Negative control; Lanes 4-13: *E. coli* isolate

4.2 Pathotype characterization

The confirmed isolates were further analysed for the presence of 8 pathogenic genes (Table 3.2). The most prevalent pathotype detected was the Enterotoxigenic *E. coli* as 47% of the isolates were positive for the *lt* gene. This was followed by Enteropathogenic *E. coli* (6%); Neonatal meningitis *E. coli* (5%);and Uropathogenic *E. coli* (2%)**.** The *flicH7* gene for Enterohaemorhaegic *E. coli* was detected in one isolate. So also for the diffusely adherent *E. coli* pathotype. The *aafII* (Enteroaggregative *E. coli*) and *ipa* (Entero-invasive *E. coli*) target genes were not detected in all the isolates. Shown on Figures (4.2 – 4.7) below are the gel pictures for the PCR products of the respective pathotype delineation..

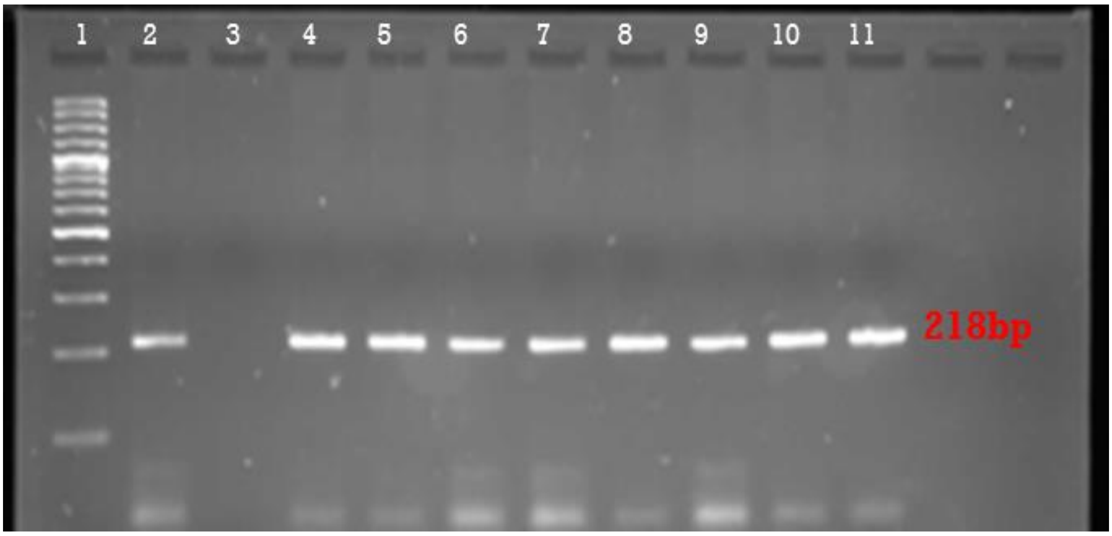

Figure 4.2: Gel electrophoresis of PCR products amplified with the *lt (*heat-labile*)* primers for the detection of Enterotoxigenic *E. coli.* Lane 1: Molecular Marker; Lane 2: Positive control, Lane 3: Negative control; Lanes 4-11: Enterotoxigenic *E. coli.*

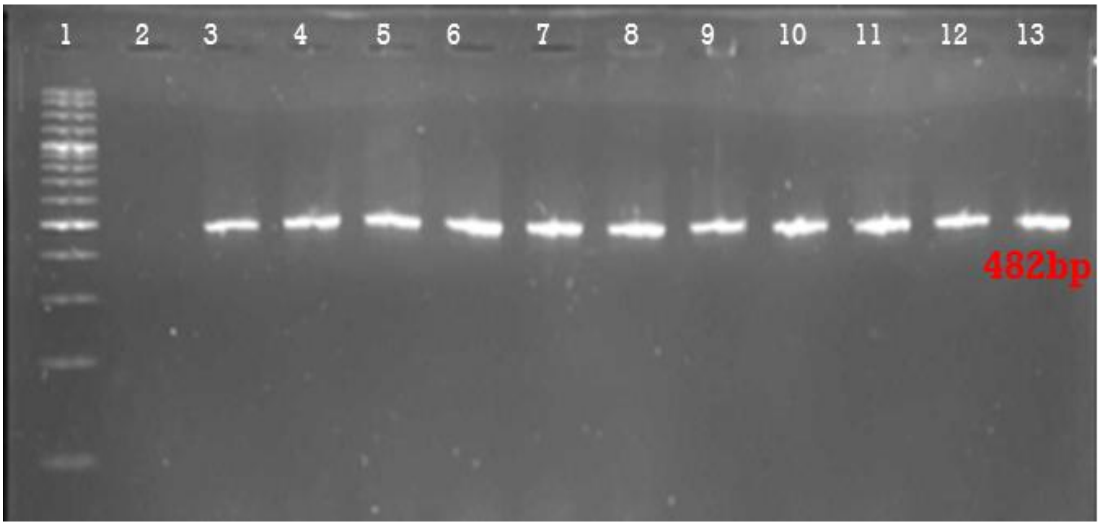

Figure 4.3: Gel electrophoresis of PCR products amplified with *eae (*intimin*)* primers for the detection of Enteropathogenic *E. coli.* Lane 1: Molecular Marker; Lane 2: Negative control, Lane 3: Positive control; Lanes 4-13: Enteropathogenic *E. coli*.

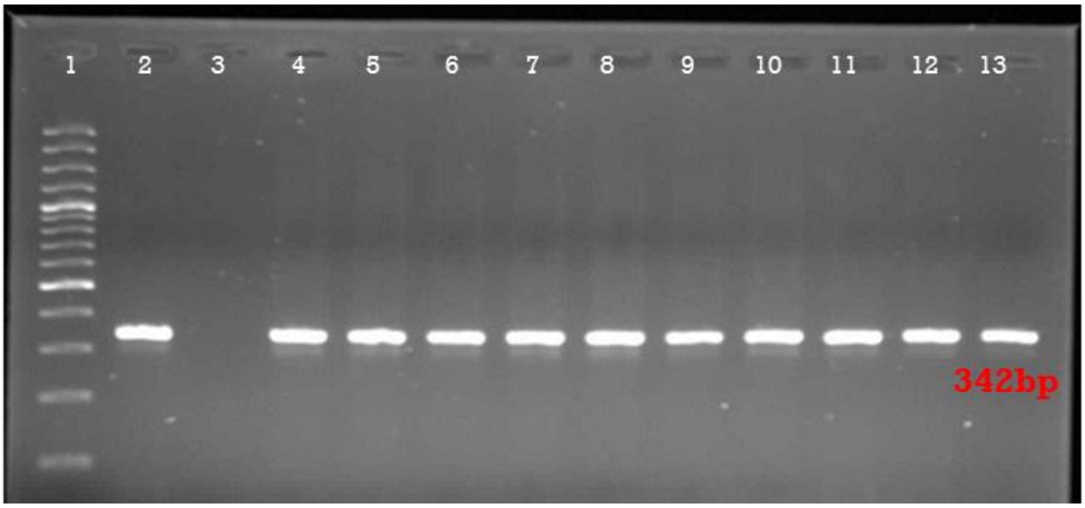

Figure 4.4: Gel electrophoresis of PCR products amplified with *IbeA (*Invasion of the brain endothelial*)* primers for the detection of Neonatal-meningitis *E. coli.* Lane 1: Molecular

Marker; Lane 2: Positive control, Lane 3: Negative control; Lanes 4-13: Neonatal-meningitis *E. coli*.

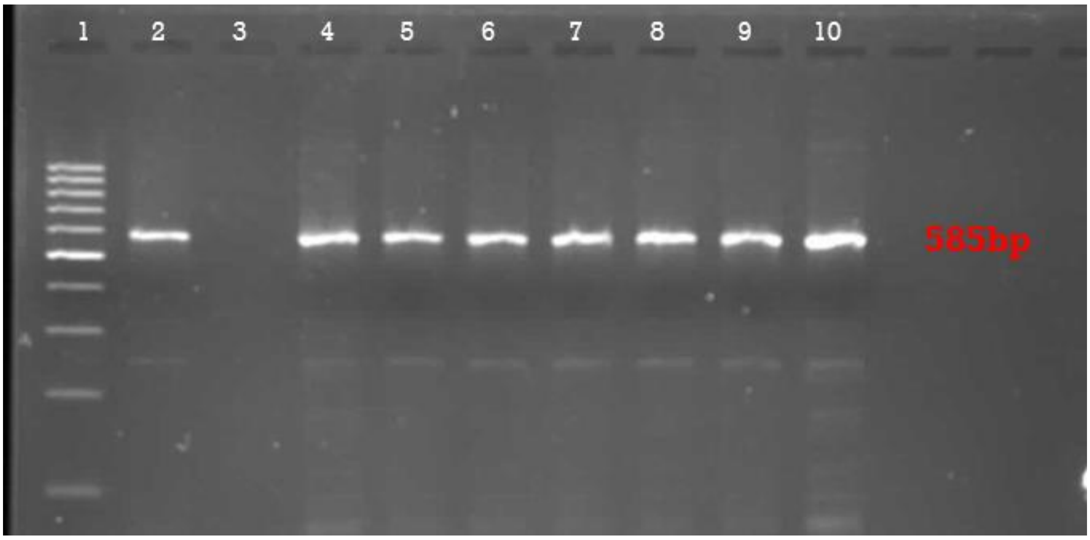

Figure 4.5: Gel electrophoresis of PCR products amplified with *pap (*pyelonephritis-associated pili*)* primers for the detection of Uropathogenic *E. coli.* Lane 1: Molecular Marker; Lane 2: Positive control, Lane 3: Negative control; Lanes 4-10: Uropathogenic *E. coli*

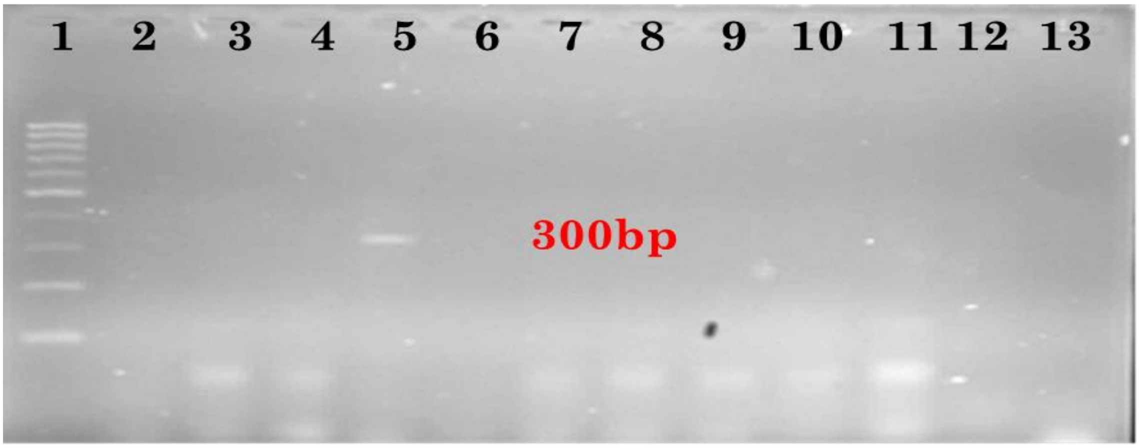

Figure 4.6: Gel electrophoresis of PCR products amplified with *daaE (F1845 fimbrae)* for the detection of diffusely adherent *E. coli.* Lane 1: Molecular Marker; Lane 5: Diffusely adherent *E. coli*

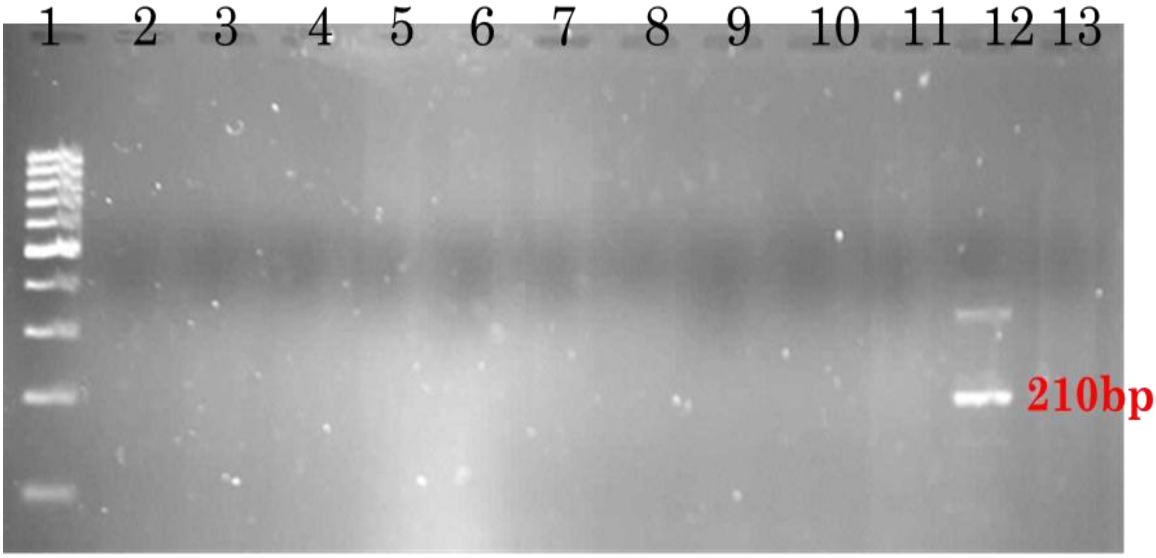

Figure 4.7: Gel electrophoresis of PCR product amplified with *flicH7* primers for the detection of the Enterohaemorhaegic *E. coli.* Lane 1: MW Marker; Lane 12: Enterohaemorrhagic *E. coli.*

4.3 Antibiotic Susceptibility Testing

The antibiotic susceptibility profile of the confirmed isolates is presented on Table 4.1 and Figure 4.8 shows the sensitivity patterns of the isolates towards the tested antibiotics. The highest susceptibility for the isolates was observed for norofloxacin (98%), amikacin (97%), and ciprofloxacin (93%) followed by streptomycin (77%), tetracycline (75%) and chloramphenicol (73%). All the isolates were resistance to penicillin G while 98% of the isolates were resistant to ampicillin.

Table 4.1: Antibiogram of *E. coli* isolated from Kat River and Fort Beaufort abstraction water.

Antibacterial agent	Concentration (µg)	Number of isolates (n = 278)		
		Resistant	Intermediate	Susceptible
Ampicillin (AP)	10	272(98%)	0 (0%)	6 (2%)
Penicillin G (PG)	10	278(100%)	0 (0%)	0 (0%)
Tetracycline (T)	30	37(13%)	33(12%)	208(75%)
Gentamycin (G)	10	0 (0%)	20(7%)	258(93%)
Chloramphenicol (C)	30	0 (0%)	75(27%)	203(73%)
Trimethoprim-Sulphamethoxazole (TS)	1.25/23.75	105(38%)	23(8%)	150(54%)
Streptomycin (S)	10	22(8%)	44(16%)	212(77%)
Ciprofloxacin (CIP)	5	0 (0%)	19(7%)	259(93%)
Norofloxacin (NOR)	10	0 (0%)	8 (2%)	272(98%)
Amikacin (AM)	30	0 (0%)	9 (3%)	269(97%)

Table 4.2: The predominant multiple antibiotic resistant (MAR) phenotypes among all isolates.

Surface water isolates (n=278)	
MAR phenotypes	**Number observed (%)**
PG-Amp	**272 (98%)**
Amp-PG-TS	**121 (44%)**
S-Amp-TS-PG	**22 (8%)**

Key: **PG**-Penicillin G; **Amp**-Ampicillin; **TS**-Trimethoprim-Sulphamethoxazole; **S**-Streptomycin

Table 4.2 above shows the predominant multiple antibiotic resistant (MAR) phenotypes among all the tested isolates. Ninety eight percent of the isolates were resistant to two antibiotics; penicillin G and ampicillin, 44% of the isolates were resistant to 3 antibiotics (ampicillin, penicillin and trimethoprim-sulphamethoxazole), while 8% of the isolates were resistant to 4 (streptomycin, ampicillin, penicillin G and trimethoprim-sulphamethoxazole).

4.4 Antibiotic Resistance Determinants

PCR was used to screen for antibiotic resistance genes mediating resistances to streptomycin, tetracycline, trimethoprim-sulphamethoxazole, ampicillin and penicillin G. The results showed that 8% of the isolates were positive for *aadA* and this correlated with the phenotypic resistance profile observed against streptomycin (Figure 4.9). However, none of the bacterial isolates tested positive for *tetA* although some isolates were found to be resistance to tetracycline. Among the

272 ampicilin and penicillin resistant strains, the *bla* gene was detected in 148 isolates and Figure 4.10 shows the PCR products of some of these isolates in this regards.

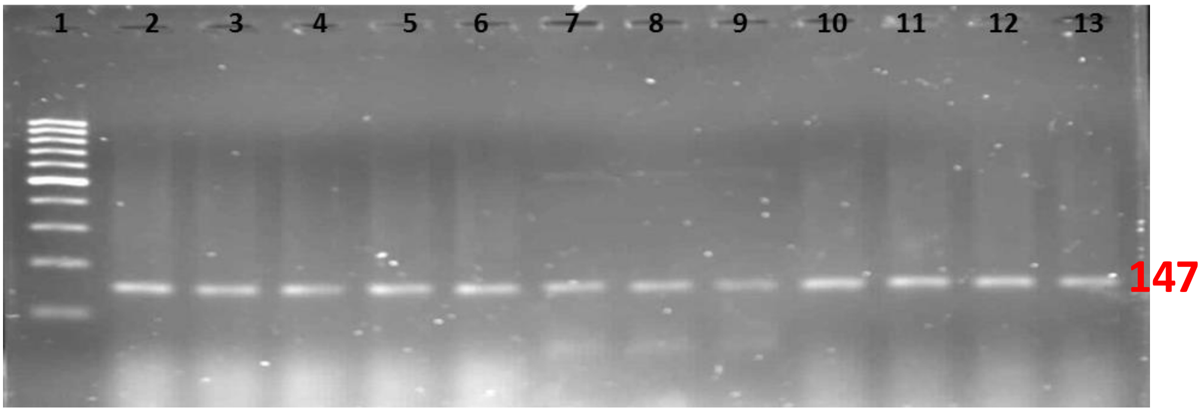

Figure 4.8: PCR products amplified with streptomycin (*aadA*) primers for the detection streptomycin-resistance determinants. Lane 1: Molecular marker; Lane 2-13: Streptomycin *aadA* sample.

CHAPTER FIVE : Discussion and Conclusion

This study investigated the distribution of defined pathotypes and antimicrobial resistant *E. coli* over a three month period, in water samples from Kat River and Fort Beaufort abstraction water. These surface waters are an important shared resource for people in the surrounding rural settlements from Seymour through to the Fort Beaufort community. Water quality is a significant concern for many communities that rely on source waters for drinking and recreation and concern is being raised about pathogens pollution and the spread of antibiotic resistant bacteria in surface waters (Anon, 2004)

The Kat River and Fort Beaufort abstraction water have previously been found to contain a high number of *E. coli* counts which exceeded the set guidelines for drinking water (DWAF, 2006). Considering the high counts of *E. coli* observed in Kat River and Fort Beaufort abstraction water sources, if this water are consumed untreated, or inadequately treated, chances are high that it could result in illnesses. Diseases acquired from contact with contaminated water include gastrointestinal illness, skin, ear, respiratory, eye, neurologic, and wound infections (CDC, 2013) and the most commonly reported symptoms are stomach cramps, diarrhea, nausea, vomiting, and low-grade fever.

The results obtained in this study showed that *E. coli* were found to occur in both the Kat River and Fort Beaufort abstraction water, with about 70% of the recovered isolates confirmed to be *E. coli* bacteria..

While this study did not determine the specific source of the *E. coli* isolates found in the understudied water resources, it is highly likely that the isolates were from human and animal excreta. This assumption is supported by the fact that during our sampling regimes human and animal excreta were observed at the banks of the river and also that livestock were observed drinking at these water sources. This further implicates both humans and animals as potential sources for *E. coli* pathotypes. Another interesting finding was the high prevalence of *E. coli* isolates (Table 4.1) carrying antimicrobial resistance genes at both sampling sites. This may reflect the influence of human antibiotic use, again suggesting the importance of humans and animals as potential sources of antimicrobial resistant *E. coli* pathotypes.

A number of isolates exhibited resistance to streptomycin, tetracycline, trimethoprime-sulphametoxazole and the β-lactam class of antimicrobials. The resistance to these specific antimicrobials is sometimes encoded by plasmids, which may distribute resistance in susceptible bacteria through horizontal gene transfer (Hall and Barlo, 2004; Sayah *et al.,* 2005). Our findings indicate that the *E. coli* recovered in this study express high levels of resistance to antimicrobials that are commonly used in clinical medicine. This could contribute to the spread and persistence of antimicrobial resistant bacteria and resistance determinants in humans and the environment. One of the most important factors contributing to the spread of antimicrobial resistance in bacteria could be the fact that in most developing countries, diarrheal diseases are treated with an inadequate regimen of antimicrobials and often without first identifying the pathogen (Yoh and Honda, 2000). Use of antibiotics in animal husbandry as growth promoters could be another factor contributing to the recovery of resistant bacteria in these water sources as the gut microbial flora of these animals end up developing resistance to these antimicrobials, and passing the same to autochthonous bacteria in surface systems.

The dissemination of antimicrobial resistance in these pathogens may have potential negative clinical implications for therapeutic advancement. *E. coli* with multiple antimicrobial resistances in surface water and other environmental media have been reported (Ram *et al*., 2007; Sayah *et al*., 2005). Among drinking-water isolates, the emergence of resistance and decreasing levels of susceptibility (intermediates) of *E. coli* to a wide spectrum of antimicrobials is a matter of concern as it may limit the availability of antimicrobials for clinical management of waterborne outbreaks in the future.

Multiple antibiotic resistances in this study was observed for 44% of the *E. coli* isolates. Results showing the *bla, tet*(A) and *dfr*A genes as the most abundant antimicrobial resistant genes are consistent with those of some reports demonstrating similar antimicrobial gene occurrences in the environment (Hameli *et al.*, 2006; Sayah *et al*., 2005). The increasing incidence of multidrug resistance is a significant health problem that has greatly impacted the treatment of infectious diseases and cancer (CDC, 2012). It also reduces the effectiveness of treatment, thus infected people remain infectious for a longer time, increasing the risk of spreading resistant microorganisms to others. The World Health Organization has reported that multidrug-resistant bacteria can account for up to 60% of all infections globally (WHO, 2002).

CONCLUSION

The presence of *E. coli* in surface water collected from Kat River and Fort Beaufort abstraction water poses a health risk to people around the Nkonkobe district using the domestic water supply for drinking and other domestic purposes. The results of this study emphasize the human health risk associated with exposure to contaminated water due to the presence of antimicrobial-resistant *E. coli* exhibiting resistant genes specific for pathogenic *E. coli.* Therefore, the presence of these antimicrobial resistant and pathogenic strains of *E. coli* in these surface waters requires increased surveillance for risk assessment and prevention strategies to protect public health. Because antimicrobial resistance is a complex problem driven by many interconnected factors, single and isolated interventions have little impact and coordinated actions are required.

RECOMMENDATIONS

The high percentage of *E. coli* isolates in the Kat River and Fort Beaufort abstraction dam suggest that these water sources pose a potential health threat to humans and animals in the area. The indiscriminate use of antibiotics in humans and animals cause for great concern as antibiotic resistance has a negative impact on therapy.

- Strict quality control measures should be put in place to ensure proper treatment of these water sources as the surrounding communities use them for drinking and domestic purposes when the municipal supplies fail to deliver potable water on time.
- The Kat River is also used for irrigation of the large citrus orchards in the Kat River Valley and if this contaminated water is continually used it will further exacerbate the

problem of the spread of resistant *E. coli* as these fruits are consumed raw. Different measures can be taken to reduce the spread of pathogenic *E. coli* and the impact of resistance gene pollution.

- Water pathways should be protected so as to prevent runoff pollution as well as contamination from human and animal waste.
- Waste treatment methods that favour the degradation of toxic compounds should be implemented. Because, while judicious use of antibiotics in non-therapeutic purposes is needed, it is unclear that the total amount of antibiotics used for all purposes and released into the environment will decrease in the near future.
- Physicians should also provide patients with sufficient information and guidelines for treatment and infection control when prescribing antibiotics to eliminate misuse of antibiotics.
- Official government policies should also be made on the rational use of antibiotics in both public and private hospitals as well as sufficient scientific information for physicians on antibiotic use to guide treatment and control infections

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