Evaluation of agricultural effluent and irrigation water as sources of antibiotic resistant *Escherichia coli*

by

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DECLARATION

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ABSTRACT

Food-borne disease outbreaks caused by *Escherichia coli* have been linked to the use of faecally-polluted irrigation waters. Thus the overall aim of this research was to evaluate irrigation water and agricultural effluents as sources of antibiotic resistant *E. coli* in the Western Cape. The aim of the first study was to enumerate and characterise *E. coli* present in irrigation water and in potential contamination sources. Maximum total coliform and *E. coli* counts for irrigation sites was log 7.862 and log 5.364 MPN.100 mL^{-1}, respectively. Five out of seven irrigation sites had *E. coli* counts exceeding national and international guidelines for 'safe' irrigation water (<1 000 counts.100 mL^{-1}), making it unsafe for the irrigation of fresh produce.

In this study, 46.6% of the *E. coli* strains were characterised in phylogenetic group B1. It has been shown that *E. coli* in group B1 have the ability to survive and persist in the external environment. Group B1 was also the most common group among isolates from irrigation sites (79.4%), while isolates from environmental sites grouped mainly in group A_0 (54.1%). It was concluded that the wide variation of *E. coli* types present in irrigation water is a concern that should be further investigated. This raises human health implications since the increased exposure to faecal organisms increases the risk of food-borne outbreaks.

The *E. coli* isolates (n = 120) and the marker (n = 37) and reference strains (n = 6), were evaluated for antibiotic resistance to seven medically-important antibiotics from different classes using the Kirby-Bauer disc diffusion method. Thirty-five strains (35/163 = 21.5%) exhibited resistance to one or more antibiotics. Piggery effluent was found to harbour the most antibiotic resistant *E. coli* isolates (9/35 = 25.7%). Among the resistant *E. coli* strains, the highest occurrence of antibiotic resistance was to trimethoprim (2.5 µg) (68.6%), tetracycline (30 µg) (57.1%), ampicillin (10 µg) (45.7%) and chloramphenicol (30 µg) (34.3%). Seventy-four percent (26/35) exhibited multiple antibiotic resistances to two or more antibiotics.

The antibiotic resistant *E. coli* strains were evaluated for the presence of pathotypes using Polymerase Chain Reaction analysis to detect Intestinal Pathogenic *E. coli* (InPEC) and Extra-intestinal Pathogenic *E. coli* (ExPEC). Five InPEC strains were characterised as four Entero-Pathogenic *E. coli* (EPEC) strains resistant to three or four antibiotics and one Entero-Aggregative *E. coli* (EAEC) strain resistant to trimethoprim. The antibiotic resistant EAEC strain also possessed the ExPEC-related gene *iutA*. Two *E. coli* isolated from the Mosselbank River were both resistant to chloramphenicol and trimethoprim and also possessed the ExPEC-related gene *iutA*. It was concluded that the diverse antibiotic resistances of *E. coli* pathotypes present in irrigation water is a concern that should be further investigated.

UITTREKSEL

Voedselverwante siekte uitbrake wat deur *Escherichia coli* veroorsaak word, is gekoppel aan die gebruik van fekale besoedelde besproeiingswater. Dus was die hoof doel van die navorsing om besproeiingswater en landbou-afvalwater te evalueer as bronne van antibiotika-weerstandbiedende *E. coli* in die Wes-kaap. Die doel van die eerste studie was om die getalle en eienskappe van *E. coli* te bepaal wat in besproeiingswater en in ander potensiële besmettingsbronne teenwoordig is. Maksimum totale koliforme en *E. coli*-tellings vir besproeiingspunte was onderskeidelik log 7.862 en log 5.364 MPN.100 mL^{-1}. Vyf uit sewe besproeiingspunte het *E. coli*-tellings gehad wat hoër is as die nasionale en internasionale riglyne vir 'veilige' besproeiingswater (<1 000 tellings.100 mL^{-1}). Dit maak dit onveilig vir die besproeiing van vars produkte.

In hierdie studie was 46.6% van die *E. coli*-stamme in filogenetiese groep B1 gegroepeer. Dit is reeds bewys dat *E. coli* in groep B1 oor die vermoë beskik om in die eksterne omgewing te oorleef en voort te bestaan. Groep B1 was ook die mees algemene groep onder die isolate van besproeiingspunte (79.4%), terwyl isolate van omgewingspunte meestal in groep A_0 (54.1%) gegroepeer is. Die breë variasie *E. coli* tipes in die besproeiingswater is bekommerniswaardig en sal gevolglik verder ondersoek moet word. Dit bring gesondsheidsimplikasies mee vir mense aangesien die verhoogde blootstelling aan fekale organismes die risiko van voedselverwante uitbrake verhoog.

Die *E. coli* isolate (n = 120) en die merker (n = 37) en verwysingsstamme (n = 6), is teen sewe medies belangrike antibiotikas uit verskillende klasse getoets vir antibiotika-weerstandbiedendheid. Die Kirby-Bauer skyfie diffusie metode is gebruik. Vyf-en-dertig stamme (35/163 = 21.5%) het weerstand teen een of meer antibiotika getoon. Dit is gevind dat vark-afvalwater die meeste antibiotika-weerstandbiedende *E. coli*-isolate (9/35 = 25.7%) bevat. Die weerstandbiedende *E. coli*-stamme het die hoogste antibiotika-weerstandheid getoon teen "trimethoprim" (2.5 µg) (68.6%), tetrasiklien (30 µg) (57.1%), ampisillien (10 µg) (45.7%) en chloramfenikol (30 µg) (34.3%). Vier-en-sewentig persent (26/35) het meervoudige weerstandbiedheid teen twee of meer antibiotikas getoon.

Die antibiotika-weerstandbiedende *E. Coli* stamme is getoets vir die teenwoordigheid van patogene deur van Polimerase Ketting Reaksie analise gebruik te maak om 'Intestinal Pathogenic' *E. coli* (InPEC) en 'Extra-intestinal Pathogenic' *E. coli* (ExPEC) op te spoor. Vyf InPEC-stamme is geklassifiseer as vier 'Entero-Pathogenic' *E. coli* (EPEC)-stamme wat weerstandbiedend teen drie of vier antibiotika getoon het en een 'Entero-Aggregative' *E. coli* (EAEC)-stam wat weerstandbiedendheid getoon het teen "trimethoprim". Die antibiotika-weerstandbiedende EAEC-stam het ook die ExPEC-verwante geen, *iutA*, besit. Twee *E. coli* isolate van die Mosselbankrivier het weerstand teen beide chloramfenikol en "trimethoprim"

getoon en het ook die ExPEC-verwante geen, *iutA*, besit. Daar is tot die gevolgtrekking gekom dat die diverse antibiotika-weerstandbiedenheid van *E. coli* patogene teenwoordig in besproeiingswaters bekommerniswaardig is en verder ondersoek behoort te word.

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CONTENTS

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion and conclusion. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1
INTRODUCTION

There is a growing concern with regards to the presence of antibiotics in the environment due to the increase in antibiotic-resistant microorganisms, especially in developing countries (Li *et al.*, 2009). The management of bacterial infections has become increasingly complicated due to the emergence of pathogen resistance to most first-line antibiotics such as penicillins, cephalosporins and fluoroquinolones (Akter *et al.*, 2012). This means that a first-line antibiotic might not be effective for the treatment of a particular infection so a second or third-choice antibiotic will be required that may be less effective, more toxic and more expensive (DeWaal *et al.*, 2012). It has been shown that resistance to penicillins, tetracyclines and sulphonamides are more prevalent in *Escherichia coli* than resistance to quinolones and aminoglycosides (Sáenz *et al.*, 2004).

It has been reported in literature that surface waters have become a major reservoir of multi-antibiotic resistant pathogenic bacteria due to contamination by agricultural waste, animal excreta, industrial effluent and sewage disposal (Lupo *et al.*, 2012). South Africa's water resources are utilised by different industrial, agricultural and domestic sectors which may contribute to contamination of the country's water resources (Paulse *et al.*, 2012). The link between use of antibiotics for food animals and subsequent emergence of antibiotic resistance in human pathogenic bacteria has been clearly demonstrated (Ahmed *et al.*, 2009). The presence of antibiotics in water sources has also been ascribed to the use of growth promoters (prophylactic antibiotics) to improve animal production yields (Baquero *et al.*, 2008). Studies on farms have shown an occurrence of multi-antibiotic resistant *E. coli* after the chronic exposure to antibiotics (Von Baum & Marre, 2005). A study by Tadesse *et al.* (2012) similarly described *E. coli* isolates from food animals with high levels of antibiotic-resistance to tetracycline, streptomycin and sulphonamide. Multi-antibiotic resistant *E. coli* isolated from a pig effluent lagoon on a farm were resistant to ten antibiotics from different medically-important classes (Sayah *et al.*, 2005).

Sanitation in communities with a lack of access to clean water is another factor that leads to the contamination of water sources (Olaniran *et al.*, 2009). In South Africa, poverty levels and overcrowded informal settlements in such areas are issues that further complicate the problem of antibiotic resistance (Krige, 2009). In more recent years, studies on the microbiological quality of water in many of South Africa's rivers revealed unacceptable and dangerous levels (Paulse *et al.*, 2012; Britz *et al.*, 2013). The presence of pathogenic *E. coli* in sewage-contaminated river water was confirmed by a South African study during 2001 (Müller *et al.*, 2001). Similarly, a study on rivers in Durban described *E. coli* isolates with virulence potential that were resistant to multiple antibiotics (Olaniran *et al.*, 2009). The

microbial quality of the Berg River used for irrigation of vegetables in South Africa has also been reported to fall far below European Union (EU) microbiological standards allowed for food production (Paulse *et al.*, 2012). If such waters are not disinfected before it is used for drinking or for irrigation purposes, it could result in waterborne diseases such as diarrhoea, dysentery, cholera and hepatitis (DeWaal *et al.*, 2012). Treatment of these diseases could be further complicated if pathogenic strains were also resistant to medically-important antibiotics.

Different mechanisms are used by pathogens to acquire resistance to certain antibiotics (Akter *et al.*, 2012). Horizontal gene transfer events (conjugation, transduction and transformation) are responsible for acquisition of antibiotic resistance mechanisms among different *Enterobacteriaceae* species (Yang *et al.*, 2009). Genes that encode for antibiotic resistance may be present on bacterial chromosomes, plasmids, transposons and integrons (Sayah *et al.*, 2005). Since antibiotic resistance genes are often found on mobile genetic elements, bacteria are able to freely exchange genetic material (Wright, 2011). This is of great concern because non-pathogenic bacteria that acquire an antibiotic resistance mechanism may later transfer that resistance mechanism to a pathogenic strain (Li *et al.*, 2009). Once the pathogenic strain has acquired the resistance mechanism, treatment of the infection caused by that pathogenic bacteria, will therefore no longer be successful (Tadesse *et al.*, 2012). Studies have demonstrated that *E. coli* may persist and multiply in the external environment outside the host and are also important vectors in the dissemination of antibiotic resistance (Power *et al.*, 2005).

Frequently a bacterial pathogen will become antibiotic resistant because it acquired a plasmid bearing one or more resistance genes (Chen *et al.*, 2011). For example, the New Delhi food-borne outbreak was caused by a pathogenic *E. coli* strain that gained an antibiotic resistance plasmid which conferred resistance to all β-lactam antibiotics (Pillai *et al.*, 2011). β-lactam antibiotics are important for the treatment of a diverse range of bacterial infections caused by both Gram-positive and Gram-negative bacteria including *Haemophilus*, *Salmonella* and *Shigella* (Lupo *et al.*, 2012). Plasmid-associated genes are frequently implicated not only in resistance to β-lactam antibiotics but also to aminoglycosides and chloramphenicol (Willey *et al.*, 2008). Aminoglycosides are most effective against Gram-negative pathogens which include infections caused by *Escherichia coli*, *Klebsiella* spp. and *Serratia* spp. (Wright, 2011). Similarly, chloramphenicol has a very broad spectrum of antibiotic activity but it is unfortunately quite toxic since the most common side effect is depression of bone marrow function (Von Baum & Marre, 2005). Transposons that contain antibiotic resistance genes are present in both Gram-positive and Gram-negative bacteria that encode for resistance to kanamycin, chloramphenicol and tetracycline (Yang *et al.*,

2009). Tetracycline has a broad spectrum of antibiotic activity that acts against Gram-negative and Gram-positive bacteria (DeWaal *et al.*, 2012).

Escherichia coli is a normal inhabitant of the gastro-intestinal tract of humans and warm-blooded animals but antibiotic resistant strains have been a serious problem in terms of human health (Von Baum & Marre, 2005). Certain pathotypes such as Shiga-Toxin producing *E. coli* (STEC), Enterohaemorhagic *E. coli* (EHEC) and Enterotoxigenic *E. coli* (ETEC) have been associated with waterborne-disease outbreaks and mortality in humans (Ram *et al.*, 2009). A study in one of China's most intensive poultry and livestock production areas described ETEC isolates from the Minjian River resistant to ampicillin, chloramphenicol and tetracycline (Chen *et al.*, 2011). ETEC is commonly responsible for infectious diarrhoea, vomiting, sunken eyes, massive dehydration and a collapse of the circulatory system due to poor sanitary conditions (Bhunia, 2008). A study in India described STEC isolates resistant to multiple antibiotics from a river that receives large quantities of untreated sewage and domestic waste (Ram *et al.*, 2009). STEC produce Shiga-like toxins (stx1 and stx2) after enteric infection that causes massive damage to kidney tubules, bloody urine and Haemorrhagic Uremic Syndrome (Laing *et al.*, 2011).

The gastro-intestinal tract serves as a reservoir for integron-bearing *E. coli* strains (Vinué *et al.*, 2008). Often multiple resistance genes are carried together as gene cassettes in an integron (Willey *et al.*, 2008). Class 1 integrons are frequently associated with resistance to multiple antibiotics such as penicillins, aminoglycosides and tetracyclines (Sáenz *et al.*, 2004). The relative ease with which *E. coli* exchange genetic material was demonstrated by the *E. coli* O104:H4 outbreak in Germany in 2011 (Laing *et al.*, 2011). This strain acquired genes that encoded for unique pathogenicity and resistance to multiple medically-important antibiotics (Safadi *et al.*, 2012). The intense media attention devoted to *E. coli* outbreaks for example, *E. coli* O157:H7 on spinach, lettuce and on sprouts demonstrates the importance of food safety in the mind of the public (Powell *et al.*, 2009).

Since information on the presence of pathogenic *E. coli* strains with antibiotic resistance is limited in South Africa, the overall objective of this research will be to evaluate agricultural effluents and irrigation waters in the Western Cape as sources of antibiotic resistant *E. coli*. The study will entail determining the coliform loads present in irrigation water and potential contamination sources using standard microbial methods (Colilert-18) for detection of indicator and potential *E. coli* pathogens. Agricultural effluent identified as potential contamination sources will include chicken, dairy and pig farms. Effluent from the Stellenbosch Sewage Works flows into a river that farmers further down-stream use for irrigation and will therefore be included in this study. Water from dams in Durbanville, Stellenbosch and Worcester will also be included in this study to evaluate the impact of wild birds on water quality. Isolates will be identified to species level using standard microbial

methods and the API-20E system. The identity of *E. coli* isolates will be confirmed and phylogenetic groups will be established. Antibiotic resistance profiles of the confirmed *E. coli* isolates will be determined and the distribution of antibiotic resistance within *E. coli* strains will be correlated to the source of contamination. *Escherichia coli* strains with antibiotic resistance will furthermore be tested for pathogenicity using molecular methods.

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CHAPTER 2
LITERATURE REVIEW

A. BACKGROUND

Escherichia coli is a facultative anaerobic species of the *Enterobacteriaceae* family which forms part of normal microflora of gastro-intestinal tracts of warm-blooded humans and animals (Olaniran *et al.*, 2009). Due to its high prevalence in the gut, *E. coli* is used as the preferred indicator to detect and measure faecal contamination of food and water (Bucci *et al.*, 2011). Bacterial contamination of surface water and particularly contamination with faecal-derived bacteria, has long been a water quality issue owing to the potential for disease transmission (Kinge *et al.*, 2010). The contamination path can be very complex and involves all aspects of human, animal and plant interfaces and their interactions with the ecosystem (Casarez *et al.*, 2007).

Generally harmless, some *E. coli* are pathogenic and can contaminate food, water and the environment (Adler *et al.*, 2011). Pathogenic *E. coli* are distinguished from other commensal *E. coli* by their ability to cause serious illness as a result of genetic elements present for toxin production, adhesion to and invasion of host cells, interference with cell metabolism and tissue destruction (Ram *et al.*, 2008). The epidemiology of each *E. coli* pathotype varies with the reservoir host, level of community hygiene, agriculture and food production systems (Kinge *et al.*, 2010).

Industrial effluent, sewage disposal, agricultural waste and animal husbandry effluent are aspects of urbanisation that have an adverse impact on surface water quality (Erb *et al.*, 2007). Faecal contamination of water sources are a major concern for many countries, especially in poorer developing countries (Olaniran *et al.*, 2009). Sanitation in communities with poor access to clean water is one of the main factors responsible for contamination of water sources (Yang *et al.*, 2009). Poverty and overcrowded informal settlements are also both critical contributing factors responsible for water contamination in South Africa (Mwabi *et al.*, 2012). Additional factors including minimal and under serviced sanitation works, inadequately maintained sewage systems, shortage of skilled workers and properly designed sanitation treatment plants compounds the problem (Krige, 2009).

Escherichia coli in faeces is consistently released into the environment, thereby contaminating water, soil and subsequently fruit and vegetables (Mwabi *et al.*, 2012). This is especially true if untreated manure is used as fertiliser for horticultural cultivation (Ibenyassine *et al.*, 2006). Organic waste plays an important role in agriculture since it supplies important nutrients to crops and also helps to maintain overall soil quality (Jiang & Shepherd, 2009). Inadequately composted manure poses a threat to human health and has

been implicated in multiple food-borne outbreaks (Ahmed *et al.*, 2010). Fresh fruit and vegetables risk contamination because they are generally grown in open fields with potential exposure to enteric pathogens from soil, irrigation water, manure, wildlife, workers and other sources (Mandrell, 2009). Additionally, fresh produce is often eaten raw, without cooking or other treatments that could eliminate pathogens that may be present (Ibenyassine *et al.*, 2006).

Many reasons have been suggested for the recent increase in illnesses caused by fresh-produce. These reasons include: an increase in global trade; a longer and more complex food supply chain; centralised processing plants with wide distribution networks; genetic changes increasing the pathogenicity of microorganisms and even an aging population that is more susceptible to food-borne illness (Anon., 2011). Leafy vegetables have been implicated in multiple food-borne outbreaks worldwide (Jiang & Shepherd, 2009). The intense media attention devoted to outbreaks for example, *E. coli* O157:H7 on spinach, lettuce and on sprouts demonstrates the importance of food safety in the mind of the public (Powell *et al.*, 2009). Multiple outbreaks of *E. coli* O157:H7 illness occurring worldwide have been associated usually with sprouts (e.g. alfalfa, mung bean and radish) grown from contaminated seeds (Mandrell, 2009). The seed may become contaminated in the field or during harvesting, storage or transportation (Anon., 2011). The seeds are harvested in different parts of the world under agricultural conditions that in many cases are not controlled sufficiently for microbial safety, considering the eventual ready-to-eat product to be produced (Powell *et al.*, 2009). The sprouting process involves ideal conditions for the growth of even a small concentration of pathogen that may contaminate even a small proportion of seeds (Anon., 2008).

It has been demonstrated that *E. coli* shed into the environment can survive for significant periods of time (Chandran & Mohamed-Hatha, 2005). Observed decay typically follows a biphasic pattern where the stationary growth phase consists of dying cells and cells better adapted to the low-nutrient environment (Bucci *et al.*, 2011). Mutations in genes allow for the utilisation of nutrients released from dying cells (Avery *et al.*, 2008). Long-term survivors in surface water cultures later out-compete cells without this ability and take over the subpopulation in surface water (Bucci *et al.*, 2011).

B. POSSIBLE IMPACT ON HUMAN HEALTH

Escherichia coli is the most common cause of Gram-negative, hospital- and community-acquired infections (Von Baum & Marre, 2005). Almost exclusively of faecal origin, it is transmitted through faecal contamination of foods and water, as well as cross-contamination, or by direct human contact during food preparation (Ahmed *et al.*, 2009). Meanwhile, the

primary exposure route appears to be through consumption of contaminated foods such as raw or undercooked ground meat products, raw milk and fresh produce (Anon., 2011). Frequent sources of food-borne infections include: unpasteurised dairy products and juices; insufficiently cooked and processed meat; raw fruits and vegetables and unsanitary handling and storage of prepared foods (DeWaal *et al.*, 2012). Prevention and control require a multidisciplinary approach in animal and plant production as well as risk-based approaches along the entire food-supply chain which include Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), Good Hygiene Practices (GHP) and Hazard Analysis Critical Control Point (HACCP) (Anon., 2011).

Hundreds of thousands of people become ill by pathogenic *E. coli* infections each year, with hundreds dying (Adler *et al.*, 2011). In recent years, there has been an increase in outbreaks with significant impact on health-care systems and agricultural production (Anon., 2008). Diarrhoeal diseases attributable to unsafe water stemming from lack of sanitation and hygiene are responsible for extensive morbidity and mortality particularly among children in developed and developing countries (Olaniran *et al.*, 2009). In many developing countries large populations depend on untreated water from rivers, lakes, wells and other surface water resources for drinking, bathing, laundry, recreation and other domestic purposes (Tadesse *et al.*, 2012). Regardless of the severity or absence of disease symptoms, infected individuals and animals can shed up to 10^6 to 10^9 *E. coli* colony forming units (cfu) per gram of faeces, shedding of pathogenic *E. coli* can also occur from asymptomatic carriers (Anon., 2011).

Escherichia coli can exchange genetic material via mobile genetic elements such as integrons and can adapt easily to new and stressful environments (Yang *et al.*, 2009). These factors are believed to contribute to the emergence of intestinal pathogenic types, some with enhanced survival and persistence in food systems or pathogenicity (Vinué *et al.*, 2008). The relative ease with which *E. coli* exchange genetic material was demonstrated in the case of the *E. coli* O104:H4 strain responsible for the outbreak in Germany in May/June 2011 (Safadi *et al.*, 2012). It was found to carry genetic material from both Enteroaggregative (EAEC) and Enterohaemorrhagic (EHEC) pathotypes. In addition, the strain was resistant to multiple antibiotics (Laing *et al.*, 2011).

In communities with poor sanitation and hygiene, Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC) and Enteropathogenic *E. coli* (EPEC) are prevalent (Krige, 2009). They are acquired by consumption of contaminated food and water and by cross-contamination through direct human contact (Kinge *et al.*, 2010). Food-borne pathogenic *E. coli* have emerged paradoxically in communities with better developed sanitation and hygiene (Begum *et al.*, 2007). However, the pathotypes differ and the transmission pathways often include raw or inadequately processed animal or horticulture products,

contact with animal manure, contaminated water and cross-contamination with raw food (Chandran & Mohamed-Hatha, 2005). Pathogenic *E. coli* are excreted in the faeces of either ill or healthy hosts (De Verdier *et al.*, 2012). Ruminants and wildlife appear to be major reservoirs of STEC/EHEC, while the human host may be more important for other pathotypes (Sayah *et al.*, 2005). Because of the wide dissemination of human and animal faecal material into the environment, bacteria have the potential to be present in areas used for food production (Salvadori *et al.*, 2004). For example, *E. coli* may be found in animal manure and sewage (until fully composted), farm and peri-urban environments contaminated by humans, livestock, wild animals and birds, manure-amended soils and contaminated water sources (Morgan-Linnell *et al.*, 2009).

C. ANTIBIOTICS

In order to understand how to treat and prevent disease, the control of microorganisms is essential (Van den Bogaard & Stobberingh, 2000). Microbial colonisation occurs when microorganisms grow on and within other organisms and can lead to disease, disability and even death (Li, 2005). The control or destruction of microorganisms that are present in bodies of humans and animals are therefore important.

Chemotherapeutic agents are chemical agents that are used to treat disease caused by microbial infections (Xia *et al.*, 2011). Chemotherapeutic agents destroy pathogenic microorganisms or inhibit their growth at concentrations low enough to avoid undesirable damage to the host (Coyne *et al.*, 2011). Many of these agents are antibiotics which are defined as microbial products or their derivatives that can kill or inhibit the growth of susceptible bacteria and other microorganisms (Lietzau *et al.*, 2006). Drugs such as sulphonamides, chloramphenicol and ciprofloxacin are sometimes called antibiotics although they are synthetic chemotherapeutic agents, not synthesised by a microorganism (DeWaal *et al.*, 2012). An increasing number of antibiotics are semi-synthetic e.g. ampicillin, carbenicillin and methicillin (De Verdier *et al.*, 2012). These are natural antibiotics that have been structurally modified by addition of chemical groups to make them less susceptible to inactivation by pathogens (Zhang *et al.*, 2009). In addition, many semi-synthetic antibiotics have an increased range of antibiotic action compared to the original molecule (Kümmerer, 2009). This is particularly true for semi-synthetic penicillins like ampicillin and amoxycillin when compared to naturally produced penicillin G and penicillin V (Li *et al.*, 2009). For the purpose of this discussion, the term ‘antibiotic’ will refer to chemotherapeutic agents used to treat bacterial infections, which are either microbial products or synthetic drugs.

A successful antibiotic must kill or inhibit the bacterial pathogen while damaging the host as little as possible (Baquero *et al.*, 2008). This is known as selective toxicity and may

be described in terms of therapeutic dose, therapeutic index and toxic dose (Willey *et al.*, 2008). The therapeutic dose is the concentration of antibiotic required for clinical treatment of a particular infection while the toxic dose is the concentration at which the antibiotic becomes too toxic for the host (Von Baum & Marre, 2005). Therapeutic index is the ratio of toxic dose to therapeutic dose and a high therapeutic index indicates an effective antibiotic (Adler *et al.*, 2011).

Antibiotics that disrupt bacterial function not found in eukaryotic animal cells often have a greater selective toxicity and higher therapeutic index (Li *et al.*, 2009). Antibiotics that kill bacteria are referred to as bactericidal and antibiotics that stop bacterial growth are bacteriostatic (Kümmerer, 2009). Antibiotics vary considerably in their range of effectiveness against treatment of infectious bacteria (De Verdier *et al.*, 2012). Many are narrow-spectrum antibiotics that are effective against a limited variety of pathogens (Costanzo *et al.*, 2005). Others are broad-spectrum antibiotics that attack many different kinds of pathogens (Sayah *et al.*, 2005). Antibiotics disrupt bacterial processes or structures that differ from those of the host (Ahmed *et al.*, 2010). They may damage pathogenic bacteria by hampering cell wall synthesis, inhibit protein or nucleic acid synthesis, disrupting membrane structure and function, or block metabolic pathways through inhibition of key enzymes (Baquero *et al.*, 2008).

The effectiveness of antibiotics depends on many factors: route of administration and location of the infection; presence of interfering substances; concentration of antibiotic in the body; nature of the pathogen; presence of antibtioc allergies and resistance of bacteria to the antibiotic (Wright, 2011). Some idea of effectiveness of a chemotherapeutic agent against a pathogen can be obtained from the Minimal Inhibitory Concentration (MIC) (Xia *et al.*, 2011). MIC is the lowest concentration of an antibiotic that prevents growth of a particular pathogen (DeWaal *et al.*, 2012). On the other hand, the Minimal Lethal Concentration (MLC) is the lowest antibiotic concentration that kills the pathogen (Lietzau *et al.*, 2006). A bactericidal antibiotic generally kills pathogens at levels only two to four times the MIC, whereas a bacteriostatic antibiotic kills at much higher concentrations (Willey *et al.*, 2008). The primary effect, mechanism of action, members of antibiotic groups, spectrum of antibiotic activity and possible side-effects of some common antibacterial antibiotics is illustrated in Table 1.

Table 1. Common antibiotics (Willey *et al.*, 2008; Wright, 2011; Lupo *et al.*, 2012)

Antibiotic Group	Primary Effect	Mechanism of Action	Members	Spectrum	Possible Side Effects
Cell Wall Synthesis Inhibition					
Penicillins	Bactericidal	Inhibit transpeptidation enzymes. Activate cell wall lytic enzymes.	penicillin G, penicillin V, methicillin, ampicillin, carbenicillin	Narrow (Gram +, some Gram -)	Allergic responses (diarrhoea, anaemia, hives, nausea, renal toxicity)
Cephalosporins	Bactericidal	Same as above	cephalothin, cefoxitin, cefaperazone, ceftriaxone	Broad (Gram +, some Gram -)	Allergic responses, thrombophlebitis, renal injury
vancomycin	Bactericidal	Prevents transpeptidation by binding to aminoacids.	vancomycin	Narrow (Gram +)	Ototoxic (tinnitus & deafness), nephrotoxic, allergic reactions
Protein Synthesis Inhibition					
Aminoglycosides	Bactericidal	Bind to 30S ribosomal subunit directly inhibits protein synthesis & misreading of mRNA.	neomycin, kanamycin, gentamicin, streptomycin	Broad (Gram -, mycobacteria)	Deafness, renal damage, loss of Balance, nausea, allergic responses
Tetracyclines	Bacteriostatic	Same as above	oxytetracycline, chlortetracycline	Broad (Gram + & Gram -, rickettsia, chlamydia)	Gastrointestinal upset, teeth discolouration, renal, hepatic injury
Macrolides	Bacteriostatic	Bind to 23S rRNA of 50S ribosomal subunit.	erythromycin, clindamycin	Broad (aerobic and anaerobic Gram +, some Gram -)	Gastrointestinal upset, hepatic injury, anaemia, allergic responses
chloramphenicol	Bacteriostatic	Same as above	chloramphenicol	Broad (Gram + & Gram -, rickettsia, chlamydia)	Depressed bone marrow function, allergic reactions
Nucleic Acid Synthesis Inhibition					
Quinolones & Fluoroquinolones	Bactericidal	Inhibit DNA gyrase & topoisomerase IV.	norfloxacin, ciprofloxacin, levofloxacin	Narrow (Gram – better than Gram +) Broad spectrum	Tendonitis, headache, faint, convulsions, allergic reactions
Anti-metabolites					
Sulphonamides	Bacteriostatic	Inhibits folic acid synthesis by competing with PABA*.	sulphamethoxazole, sulphasoxazole	Broad spectrum	Nausea, vomiting, diarrhoea
trimethoprim	Bacteriostatic	Inhibits folic acid synthesis by enzyme inhibition.	trimethoprim	Broad spectrum	Same as sulphonamides but less frequent

*PABA = ρ-aminobenzoic acid

Inhibitors of cell-walls

The most selective antibiotics are those that interfere with bacterial cell-wall synthesis (Rolain *et al.*, 2010). Penicillins, cephalosporins and vancomycin have a high therapeutic index because they target structures not found in eukaryotic cells (Salvadori *et al.*, 2004). These antibiotics also generally have a broad spectrum of activity, therefore making them effective first-line antibiotics in the treatment of certain infections (Erb *et al.*, 2007).

i. *Penicillins* - In 1928 Alexander Flemming noticed the killing effect of a mould accidentally blown onto an agar plate (Davies, 2007). After an attempt at isolation of the compound responsible, it was judged too unstable to be applied as an antibiotic until Florey and Chain in 1938 successfully isolated penicillin by lyophilisation (Willey *et al.*, 2008). In 1945 the structure of penicillin was shown by x-ray crystallography. Most penicillins (e.g. penicillin G or benzylpenicillin) are derivatives of 6-aminopenicillanic acid and differ with respect to the side-chain attached to the amino group (Li *et al.*, 2009). The most crucial feature of the molecule is the β-lactam ring, which is essential for bioactivity (Zou *et al.*, 2012). Many penicillin-resistant bacteria produce penicillinase (also called β-lactamase), an enzyme that inactivates the antibiotic by hydrolysing a bond in the β-lactam ring (Dolejska *et al.*, 2009).

Although penicillin's mechanism of action is still not completely known, the structure resembles the terminal D-alanine-D-alanine found on the peptide side-chain of the peptidoglycan subunit (Wright, 2011). It has been proposed that this structural similarity blocks the enzyme catalysing the transpeptidation reaction that forms the peptidoglycan cross-links (Kümmerer, 2009). Thus the formation of a completed cell-wall is blocked, leading to osmotic lysis (Zhang *et al.*, 2009). This mechanism is consistent with the observation that penicillins act only on actively growing bacteria that are synthesising new peptidoglycan (Lampang *et al.*, 2008).

Evidence has indicated that penicillin's mechanism of action is even more complex than previously imagined (DeWaal *et al.*, 2012). Penicillins bind to a number of periplasmic proteins namely Penicillin-Binding Proteins (PBPs) and it has been proposed that this may also activate autolytic enzymes (autolysins) that kill the bacteria (Nikaido, 1989). There is also some evidence that penicillin kills bacteria even in the absence of autolysins or murein hydrolases (Levy, 1992). Lysis could occur after bacterial viability has already been lost (Nikaido, 1989). Penicillin may stimulate special proteins called bacterial holins to form holes or lesions in the plasma membrane, leading directly to membrane leakage and death (Zou *et al.*, 2012). Murein hydrolases also could move through the holes, disrupt the peptidoglycan and lyse the cell (Zhang *et al.*, 2009).

The two naturally occurring penicillins, penicillin G and penicillin V, are narrow-spectrum antibtiocs (Kümmerer, 2009). Penicillin G is effective against gonococci, meningococci and several Gram-positive pathogens including streptococci and staphylococci (Von Baum & Marre, 2005). However, it must be administered intravenously because it is destroyed by stomach acid (Erb *et al.*, 2007). Penicillin V is similar to penicillin G in spectrum of activity but because of an increased resistance to acidic conditions, it can be orally administered (DeWaal *et al.*, 2012). Semi-synthetic penicillins, on the other hand, have a broader spectrum of activity (Baquero *et al.*, 2008). Ampicillin can be administered orally and is effective against Gram-negative bacteria such as *Haemophilus*, salmonellae and *Shigella* (Yang *et al.*, 2009). Carbenicillin and ticarcillin are potent against *Pseudomonas* and *Proteus* (Ibenyassine *et al.*, 2006).

An increasing number of bacteria have become resistant to natural penicillins and many of the semi-synthetic analogues (Zou *et al.*, 2012). Physicians frequently employ specific semi-synthetic penicillins that are not destroyed by β-lactamases to combat antibiotic-resistant pathogens such as methicillin and oxacillin (Lupo *et al.*, 2012). Methicillin is designed to be resistant to β-lactamases due to the steric shield on the side-chain to protect against β-lactam hydrolysis (Costanzo *et al.*, 2005). However, its use has been confounded by the emergence of methicillin-resistant bacteria such as *Staphylococcus aureus* (Dolejska *et al.*, 2009). It is ineffective against Gram-negative bacteria and has to be administered by injection since there is no electron withdrawing group on the side-chain (Lietzau *et al.*, 2006). Oxacillin is still resistant to β-lactamases and has more acid stability than methicillin (Lampang *et al.*, 2008). Ampicillin and amoxicillin have better Gram-negative activity due to the presence of a hydrophilic amino-substituent directly adjacent to the carbonyl of the side-chain (Ribeiro *et al.*, 2012). However, ampicillin and amoxicillin are still inactive against *Pseudomonas aeruginosa*, a particularly challenging pathogen (Davies, 2007). Carbenicillin has the best activity against Gram-negative organisms including *P. aeruginosa* (Li *et al.*, 2009). Penicillins are also used in combination where the second component prevents bacterial resistance to the first component e.g. amoxicillin/clavulanate and ticarcillin/clavulanate (Xia *et al.*, 2011).

ii. *Cephalosporins* - Cephalosporins are a family of antibiotics isolated in 1948 from the *Cephalosporium* fungus that was found in a sewer line on the island of Sardinia (Willey *et al.*, 2008). They contain a β-lactam structure that is very similar to that of the penicillins (Lietzau *et al.*, 2006). As might be expected from their structural similarities to penicillins, cephalosporins also inhibit the transpeptidation reaction during peptidoglycan synthesis (Von Baum & Marre, 2005). They are broad-spectrum antibiotics frequently given to patients with penicillin allergies (Lupo *et al.*, 2012).

There are many cephalosporins that are currently in use today. Cephalosporins are broadly categorised into four generations (groups of antibiotics that are sequentially developed) based on their spectrum of activity (Ibenyassine *et al.*, 2006). First-generation cephalosporins (e.g. cephalothin, cephaloridine and cephalexin) are more effective against Gram-positive than Gram-negative pathogens (DeWaal *et al.*, 2012). Cephalothin has less antibiotic activity than penicillin G against Gram-positive bacteria and more activity than penicillin G against Gram-negative bacteria (Lietzau *et al.*, 2006). The side-chain acetoxy group is a main point susceptible to hydrolysis leading to metabolic inactivation (Lampang *et al.*, 2008). Cephaloridine is a zwitterion, an overall neutral molecule with a positive and negative charge at different locations within the molecule (Costanzo *et al.*, 2005). A positively charged pyridinium group prevents the metabolic inactivation of the compound (Xia *et al.*, 2011).

Second-generation cephalosporins (cefoxitin and cefuroxime) have improved effects on Gram-negative bacteria with some anaerobe coverage (Costanzo *et al.*, 2005). Third-generation cephalosporins (cefotaxime, ceftriaxone and ceftazidime) are particularly effective against Gram-negative pathogens and some reach the central nervous system (Von Baum & Marre, 2005). This is of particular concern because many antibiotics do not cross the blood-brain barrier (DeWaal *et al.*, 2012). Ceftazidime combines activation of the antibiotic with steric shielding of the β-lactam ring to protect it from hydrolysis by β-lactamases (Dolejska *et al.*, 2009). Hydrophilic groups on the side-chain further improve activity against Gram-negative strains (Lietzau *et al.*, 2006). Finally, fourth-generation cephalosporins are broad-spectrum antibiotics with excellent Gram-positive and Gram-negative coverage and, like their third-generation predecessors, inhibits growth of the difficult opportunistic pathogen *P. aeruginosa* (Lupo *et al.*, 2012).

iii. *Vancomycin* - Vancomycin is a glycopeptide antibiotic produced by *Streptomyces orientalis* (Van den Bogaard & Stobberingh, 2000). It is a cup-shaped molecule composed of a peptide linked to a disaccharide (Lupo *et al.*, 2012). Vancomycin's peptide portion blocks the reaction that forms peptide cross-links during peptidoglycan synthesis known as transpeptidation (Lietzau *et al.*, 2006). This is achieved by binding particularly to the D-alanine-D-alanine terminal sequence of peptidoglycan (Nagar *et al.*, 2011). The antibiotic is bactericidal for staphylococci and some clostridia, bacilli, streptococci and enterococci (Ram *et al.*, 2008). It is given both orally and intravenously and has been particularly important in the treatment of antibiotic-resistant staphylococci and enterococci infections (Baquero *et al.*, 2008). However, vancomycin-resistant strains of enterococci have become widespread and cases of resistant *Staphylococcus aureus* have appeared (Lupo *et al.*, 2012). Vancomycin

has been considered “the drug of last resort” in cases of antibiotic-resistant *S. aureus* (Davies, 2007).

Protein Synthesis Inhibitors

Many antibiotics inhibit protein synthesis by binding with the prokaryotic ribosome (Zhang *et al.*, 2009). The therapeutic index is the ration between toxic dose and therapeutic dose of an antibiotic, used as a measure of the antibiotic’s relative safety (Willey *et al.*, 2008). Because these antibiotics discriminate between prokaryotic and eukaryotic ribosomes, their therapeutic index is fairly high but not as high as that of cell-wall inhibitors (Yang *et al.*, 2009). Some antibiotics bind to the 30S (small) ribosomal subunit, while others attach to the 50S (large) subunit (Gibson *et al.*, 2010). Several different steps in proteins synthesis can be affected: aminoacyl-tRNA binding, peptide bond formation, mRNA reading and translocation (Lupo *et al.*, 2012).

i. *Aminoglycosides* - Although there is considerable variation in structure among several important aminoglycoside antibiotics, all contain a cyclohexane ring and amino sugars (Kümmerer, 2009). Streptomycin, kanamycin, neomycin and tobramycin are synthesised by different species of the genus *Streptomyces,* whereas gentamicin comes from another actinomycete, *Micromonospora purpurea* (Lietzau *et al.*, 2006). Aminoglycosides bind to the 30S (small) ribosomal subunit and interfere with protein synthesis by directly inhibiting the synthesis process and also by causing misreading of the mRNA (Wright, 2011).

These antibiotics are bactericidal and tend to be most effective against Gram-negative pathogens (Ram *et al.*, 2008). Streptomycin’s usefulness has decreased greatly due to widespread antibiotic resistance but it is still effective in treating tuberculosis and plague (Yang *et al.*, 2009). Gentamicin is used to treat infections caused by *Proteus* spp., *Escherichia* spp., *Klebsiella* spp. and *Serratia* spp. (Willey *et al.*, 2008). However, aminoglycosides can be quite toxic and can cause deafness, renal damage, loss of balance, nausea and allergic responses (Lupo *et al.*, 2012).

ii. *Tetracyclines* - The tetracyclines are a family of antibiotics with a common four-ring structure to which a variety of side-chains are attached (Von Baum & Marre, 2005). Oxytetracycline and chlortetracycline are produced naturally by *Streptomyces* species while others are semi-synthetic antibiotics (Sáenz *et al.*, 2004). These antibiotics are similar to aminoglycosides and combine with the 30S (small) subunit of the ribosome (Van den Bogaard & Stobberingh, 2000). This inhibits binding of aminoacyl-tRNA molecules to the A-site of the ribosome (Kümmerer, 2009). Because their action is only bacteriostatic,

effectiveness of treatment depends on the host's active resistance to the pathogen (Sáenz *et al.*, 2004).

Tetracyclines are broad-spectrum antibiotics that are active against Gram-negative, as well as Gram-positive bacteria, *Rickettsia* spp., *Chlamydia* spp. and *Mycoplasma* spp. (Coyne *et al.*, 2011). High doses may result in nausea, diarrhoea, yellowing of teeth in children, damage to the liver and kidneys (Lupo *et al.*, 2012). Although their use has declined in recent years, they are still sometimes used to treat acne (DeWaal *et al.*, 2012).

iii. *Macrolides* - The macrolide antibiotics which include erythromycin, clindamycin and azithromycin, contain 12 to 22-carbon lactone rings linked to one or more sugars (Li *et al.*, 2009). Erythromycin is usually bacteriostatic and binds to 23S rRNA of the 50S (large) ribosomal subunit to inhibit peptide-chain elongation during protein synthesis (Lietzau *et al.*, 2006). Erythromycin is a relatively broad-spectrum antibiotic effective against Gram-positive bacteria, *Mycoplasma* spp. and a few Gram-negative bacteria (Von Baum & Marre, 2005). It is used with patients who are allergic to penicillins and in the treatment of whooping cough, diphtheria, diarrhoea caused by *Campylobacter* spp. and pneumonia from *Legionella* spp. or *Mycoplasma* spp. infections (Kümmerer, 2009). Clindamycin is effective against a variety of bacteria including staphylococci and anaerobes such as bacteriodes (Xia *et al.*, 2011). Azithromycin which has surpassed erythromycin in use, is particularly effective against *Chlamydia trachomatis* (Zhang *et al.*, 2009).

iv. *Chloramphenicol* - Chloramphenicol was first produced from cultures of *Streptomyces venezuelae* but it is now chemically synthesised (DeWaal *et al.*, 2012). Like erythromycin, this antibiotic inhibits the enzyme responsible for catalysis of amino-acid addition to the growing peptide chain (peptidyl transferase) and achieves this by binding to 23S rRNA on the 50S ribosomal subunit. (Li *et al.*, 2009). It has a very broad spectrum of activity but, unfortunately, can be toxic as there may be allergic responses or neurotoxic reactions (Wright, 2011). The most common side-effect is depression of bone marrow function, leading to aplastic anaemia and a decrease in white blood cells (Lupo *et al.*, 2012). Consequently, this antibiotic is used only in life-threatening situations when no other antibiotic is adequate (Costanzo *et al.*, 2005).

Metabolic Antagonists

Several valuable antibiotics act as anti-metabolites that antagonise, or block, the functioning of metabolic pathways by competitively inhibiting the use of metabolites by key enzymes (Erb *et al.*, 2007). These antibiotics can act as structural analogues, molecules that are

structurally similar to naturally occurring metabolic intermediates (Xia *et al.*, 2011). These analogues compete with intermediates in metabolic processes because of their similarity (Nagar *et al.*, 2011). However, they are just different enough to prevent normal cellular metabolism. Metabolic antagonists are bacteriostatic and broad-spectrum (Zhang *et al.*, 2009).

i. *Sulphonamides* - The first anti-metabolite to be used successfully as chemotherapeutic agents, were the sulphonamides, discovered by G. Domagk (Davies, 2007). Sulphonamides are structurally related to sulphanilamide, an analogue of ρ-aminobenzoic acid (PABA) (DeWaal *et al.*, 2012). PABA is used in the synthesis of the cofactor folic acid (Yang *et al.*, 2009). When sulphanilamide or another sulphonamide enters a bacterial cell, it competes with PABA for the active site of an enzyme involved in folic acid synthesis, causing a decline in folic acid concentration (Ibenyassine *et al.*, 2006). This decline is detrimental to the bacterium because folic acid is a precursor of purines and pyrimidines (Von Baum & Marre, 2005). Purines and pyrimidines are the bases used in the construction of DNA, RNA and other important cell constituents (Akter *et al.*, 2012). The resulting inhibition of purine and pyrimidine synthesis leads to termination of protein synthesis and DNA replication and subsequently the pathogen dies (Costanzo *et al.*, 2005). Sulphonamides are selectively toxic for many pathogens because these bacteria manufacture their own folic acid and cannot effectively take up this cofactor (DeWaal *et al.*, 2012). Humans do not synthesise folic acid as it is obtained from the diet therefore sulphonamides have a high therapeutic index (Yang *et al.*, 2009).

The increasing resistance of many bacteria to sulphonamides limits its effectiveness in treatment of disease (Ibenyassine *et al.*, 2006). Furthermore, as many as 5% of the patients receiving sulphonamides experience adverse side-effects, chiefly allergic responses such as fever, hives and rashes (Lupo *et al.*, 2012).

ii. *Trimethoprim* - Trimethoprim is a synthetic antibiotic that also interferes with the production of folic acid (DeWaal *et al.*, 2012). It does so by binding to dihydrofolate reductase (DHFR), the enzyme responsible for converting dihydrofolic acid to tetrahydrofolic acid, competing against the dihydrofolic acid substrate (Wright, 2011). It is a broad-spectrum antibiotic often used to treat respiratory and middle ear infections, urinary tract infection and traveller's diarrhoea (Yang *et al.*, 2009). It can be combined with sulphonamides to increase effectiveness of treatment by blocking two key steps in the folic acid pathway (Ibenyassine *et al.*, 2006). The inhibition of two successive steps in a single biochemical pathway means that less of each antibiotic is needed in combination than when

used alone (Esiobu *et al.*, 2002). This is termed a synergistic antibiotic interaction (Costanzo *et al.*, 2005).

The most common side effects associated with trimethoprim are abdominal pains, abnormal taste, diarrhoea, loss of appetite nausea, vomiting and swelling of the tongue (Lupo *et al.*, 2012). Some patients are allergic to trimethoprim, exhibiting rashes and itching, while others develop photosensitivity reactions (Zhang *et al.*, 2009).

Nucleic Acid Synthesis Inhibitors

Nucleic acid synthesis inhibitors are antibacterial antibiotics that inhibit nucleic acid synthesis function by inhibiting DNA polymerase, DNA helicase or RNA polymerase, thus blocking processes of replication or transcription, respectively (Baquero *et al.*, 2008). These antibiotics are not as selectively toxic as other antibiotics because prokaryotes and eukaryotes do not differ greatly with respect to nucleic acid synthesis (Fabrega *et al.*, 2008).

i. *Quinolones* - The quinolones are synthetic antibiotics that contain a 4-quinolone ring (Davies, 2007). Quinolones are important anti-bacterial agents that inhibit nucleic acid synthesis (Gibson *et al.*, 2010). They are increasingly used to treat a wide variety of infections (Kümmerer, 2009). The first quinolone, nalidixic acid was synthesised in 1962 and since generations of fluoroquinolones have been produced (Akter *et al.*, 2012). Ciprofloxacin, norfloxacin and ofloxacin are currently used in USA and more fluoroquinolones are being synthesised and tested (Fabrega *et al.*, 2008). Ciprofloxacin gained notoriety during the 2001 bio-terror attacks in the USA as one treatment for anthrax (Willey *et al.*, 2008).

Quinolones act by inhibiting the bacterial DNA gyrase and topoisomerase II (Davies, 2007). DNA gyrase introduces a negative twist in the DNA and thus helps separate its strands (Gibson *et al.*, 2010). Inhibition of DNA gyrase disrupts DNA replication and repair, bacterial chromosome separation during division and other cell processes involving DNA (DeWaal *et al.*, 2012). Fluoroquinolones also inhibit topoisomerase II, another enzyme that untangles DNA during replication (Costanzo *et al.*, 2005). It is not surprising that quinolones are bactericidal since the correct DNA function is crucial for survival (Von Baum & Marre, 2005).

Quinolones are broad-spectrum antibiotics that are highly effective against enteric bacteria such as *E. coli* and *Klebsiella pneumoniae* (Morgan-Linnell *et al.*, 2009). They can be used to treat *Haemophilus* spp., *Neisseria* spp., *P. aeruginosa* and other Gram-negative pathogens (Kümmerer, 2009). Quinolones are also active against Gram-positive bacteria such as *S. aureus*, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* (Davies, 2007). Currently, they are used in treating urinary tract infections, sexually transmitted

diseases caused by *Neisseria* spp. and *Chlamydia* spp., gastrointestinal infections, respiratory infections, skin infections and osteomyelitis (Fabrega *et al.*, 2008). Quinolones are effective when administered orally but can sometimes cause diverse side-effects, particularly gastrointestinal upset (Lupo *et al.*, 2012).

Over the past 70 years, a diverse variety of antibiotics for the treatment of a wide range of illnesses has been developed. Most classes of antibiotics including β-lactams, tetracyclines, aminoglycosides and macrolides were originally derived from natural sources and then further chemically modified to confer better properties (Wright, 2011). However, some important antibiotics including sulphonamides and quinolones are completely synthetic (Li, 2005). Evolution of bacterial antibiotic resistances, its spread and emergence represents one of the most threatening healt-care problems with worldwide proportions (Dolejska *et al.*, 2009). Resistance is an inevitable consequence of antibiotic use; the more antibiotics are used, the more bacteria will develop resistance (Ribeiro *et al.*, 2012).

D. ANTIBIOTIC RESISTANCE

Antibiotics are life-saving chemotherapeutic agents that have an enormous disadvantage when confronted by bacteria's specialised defence mechanisms (Li, 2005). The ability of bacteria to evolve resistance mechanisms to resist attack by antibiotics was recognised soon after the widespread deployment of the first antibiotics (Maynard *et al.*, 2004). In modern times resistance clinically emerges almost immediately after introduction of a new antibiotic (Baquero *et al.*, 2008). Since Fleming's discovery of penicillin in 1928, the main issue when treating patients with serious bacterial infections has been antibiotic resistance (Davies, 2007).

There has been great concern regarding environments containing antibiotics due to the possibility of antibiotic-resistant strains becoming dominant in bacterial ecosystems (Olaniran *et al.*, 2009). The problem of antibiotic resistance is not limited to pathogenic bacteria since it also involves commensal bacteria (Nagar *et al.*, 2011). Water also constitutes a route by which resistance genes are introduced in natural bacterial ecosystems (Zou *et al.*, 2012). This means that non-pathogenic *E. coli* could serve as a reservoir for resistance genes that could be later transferred to pathogenic *E. coli* strains (Rolain *et al.*, 2010). Despite the widespread use of antibiotics, very little attention is given to antibiotics as pollutants in the aquatic environment (Lupo *et al.*, 2012). Antibiotics enter the aquatic environment through sewage systems and via effluent from industry, farms, abattoirs and landfills (Kümmerer, 2009).

There is clear evidence of adverse human health effects due to resistant organisms resulting from the non-clinical application of antibiotics (Fabrega *et al.*, 2008). These

consequences include infections that would not otherwise occur, increased frequency of treatment failure and increased severity of infections as documented by fluoroquinolone-resistant human *Salmonella* infections (Gibson *et al.*, 2010). The largest use of antibiotics outside human medicine is in food animals for the treatment of illnesses and increase feed and growth efficiency (Shea, 2003). *Salmonella* species have been implicated in 82% of antibiotic-resistant food-borne outbreaks in USA from 1976 to 2011 (DeWaal *et al.*, 2012). *Salmonella typhimurium* was the most frequently identified serotype in dairy products and ground beef that showed the highest occurrence of resistance to tetracycline, streptomycin and ampicillin (DeWaal *et al.*, 2012). Of all the outbreaks included in the study, 45% occurred between 2000 and 2011 (DeWaal *et al.*, 2012).

Aeromonas caviae, A. veronni and *A. sobria* with virulence potential and antibiotic resistance was reported to be present on chicken, fish and ready-to-eat sprouts from various retail outlets in Mumbai, India (Nagar *et al.*, 2011). More than 60% of the isolates possessed virulent β-haemolytic activity and all food isolates were resistant to ampicillin and bacitracin (Nagar *et al.*, 2011). *Aeromonas* strains from aquaculture water systems are particularly resistant to antibiotics and frequently contain plasmids and integrons with multiple genes for antibiotic resistance (Zhang *et al.*, 2009). A Portuguese study on aquaculture water showed that the most frequently isolated gene cassette found in *Aeromonas* strains involved in antibiotic resistance, encoded resistance to aminoglycosides (Baquero *et al.*, 2008).

Resistance to quinolones/fluoroquinolones has emerged rapidly and can be achieved in several different ways (Li, 2005). Bacterial strains that express efflux-mediated quinolone resistance show cross-resistance to a number of structurally unrelated antibiotics such as tetracycline, chloramphenicol, β-lactams, trimethoprim and aminoglycosides (Gibson *et al.*, 2010). This is made possible by the broad substrate specificity of efflux systems, which are capable of accommodating a variety of clinically relevant antibiotics (Levy, 1992). Hydrophilic fluoroquinolones enter the bacterial cell through specific outer-membrane proteins called porins (Fabrega *et al.*, 2008). In *Enterobacteriaceae*, the main fluoroquinolone efflux system is encoded by *acrAB/tolC* genes, an efflux pump which is widely distributed amongst Gram-negative bacteria (Morgan-Linnell *et al.*, 2009). Protein targets for quinolones are toposisomerase II and DNA gyrase (Gibson *et al.*, 2010). The inhibition of these enzymes by interaction with quinolone molecules leads mainly to inhibition of replication, transcription and ultimately DNA synthesis (Li, 2005).

In *Salmonella* spp., as in other *Enterobacteriaceae*, a single point mutation in the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene can mediate resistance to nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin (Lupo *et al.*, 2012). The most frequent mutations of the *gyrA* gene in salmonellae result from amino acid substitutions of Ser83 to Phe and Aps87 to Gly, Tyr or Asn (Fabrega *et al.*, 2008).

According to Kümmerer *et al.* (2009), the most prominent medical examples of antibiotic resistance are vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* and multi-resistant pseudomonads. Barlow *et al.* (2004) describes multiple-resistance phenotypes by Class 2 integron-containing *P. stuartii* isolates from bovine faeces and antibiotic-resistant *A. caviae* isolates all with Class 1 integrons.

Bacteria isolated from dairy farm effluent and a lake near a hospital in Florida, USA displayed frequent antibiotic resistance (Esiobu *et al.*, 2002). *Pseudomonas* spp., enterococci and *Enterobacter* spp. were responsible for 77%, 75% and 70% resistance frequencies, respectively (Esiobu *et al.*, 2002). Tetracycline-resistance genes present on plasmids were most commonly accountable for resistance to the antibiotic (Esiobu *et al.*, 2002). Most environmental *tet* genes code for transport proteins, which pump the antibiotic out of the bacterial cell and keep the intercellular concentration low enough to maintain normal ribosomal function (Nikaido, 1989). Efflux genes *tet*A, B, C, D and E frequently appear in aquaculture, surface water and swine lagoons (Levy, 1992).

E. KNOWN *E. COLI* ANTIBIOTIC RESISTANCE PROFILES

Escherichia coli is well known for its ability to cause a variety of infections (Adler *et al.*, 2011). In addition to gastrointestinal infections usually in the form of diarrhoea, *E. coli* can also cause a variety of diseases outside the intestinal tracts of humans of animals (Gibson *et al.*, 2010). Urinary tract infections, meningitis, sepsis, abdominal infections and osteomyelitis are a few examples of Extraintestinal Pathogenic *E. coli* (ExPEC) infections (Bhunia, 2008). Treatment of any of these infections can be severely hampered if *E. coli* pathogens are antibiotic resistant (DeWaal *et al.*, 2012). The emergence of *E. coli* isolates with multiple antibiotic-resistances to four or more unrelated antibiotics are considered a serious health-concern (Sáenz *et al.*, 2004). Many examples of *E. coli* strains included in this literature review were reported to possess virulence characteristics; which further emphasises the potential risk that antibiotic-resistant bacteria have on food safety and human health (Begum *et al.*, 2007; Ahmed *et al.*, 2009; Ram *et al.*, 2008; Xia *et al.*, 2011). A summary of global reports of antibiotic-resistant *E. coli* isolated from different sources is presented in Table 2.

Table 2. Antibiotic resistant *E. coli* reports

Year	Location	Type	Source	Resistance	Reference
2004	Canada	ExPEC	human, animal	ampicillin, tetracycline & sulphonamide	Maynard *et al.*, 2004
2005	USA	*E. coli*	animal, human, surface water	cephalothin, tetracycline, streptomycin & sulphasoxazole	Sayah *et al.*, 2005
2008	India	STEC, ETEC	surface water	cephotaxime, gentamicin & norfloxacin	Ram *et al.*, 2008
2009	Japan	*E. coli*	retail chicken	ampicillin, streptomycin, tetracycline & trimethoprim	Ahmed *et al.*, 2009
2009	China	*E. coli*	human	ampicillin, chloramphenicol, trimethoprim & streptomycin	Zhang *et al.*, 2009
2010	Australia	ExPEC	dogs	fluoroquinolones	Gibson *et al.*, 2010
2010	England	*E. coli*	horse	ampicillin, chloramphenicol, tetracycline & trimethoprim	Ahmed *et al.*, 2010
2011	Germany	EAEC/STEC	human	all penicillins, cephalosporins & co-trimoxazole	Laing *et al.*, 2011
2011	USA	ExPEC	retail meats	tetracycline, sulphisoxazole & streptomycin	Xia *et al.*, 2011
2012	China	*E. coli*	river	β-Lactams	Zou *et al.*, 2012
2012	Sweden	ETEC	dairy calves	ampicillin, tetracycline, streptomycin & sulphonamide	De Verdier *et al.*, 2012
2012	France	*E. coli*	chalk aquifer	β-Lactams, chloramphenicol & tetracycline	Ribeiro *et al.*, 2012

Retail meats are often contaminated with *E. coli* strains and can serve as a vehicle for dissemination of antibiotic-resistant ExPEC (Sáenz *et al.*, 2004; Gibson *et al.*, 2010; Nagar *et al.*, 2011). A study in the USA found that most of the ExPEC isolates were obtained from ground turkey and chicken breasts (Xia *et al.*, 2011). Resistance to tetracycline was most prevalent and more than half of the isolates were resistant to three or more antibiotics (Xia *et al.*, 2011). Maynard *et al.* (2004) also described ExPEC isolates from humans and animals that were most resistant to ampicillin, tetracycline and sulphonamide. Relatively high resistance levels to tetracycline in the human isolates were observed, which was unexpected considering it is less frequently used in humans (Maynard *et al.*, 2004). A similar observation was made for kanamycin in animal isolates and although more widely used in human therapy than animal therapy, more animal isolates were resistant to this antibiotic (Maynard *et al.*, 2004). The study also demonstrated similar multi-antibiotic resistance patterns for human and animal isolates which can be explained by the

co-selection of resistance genes due to the use of different antibiotics (Maynard *et al.*, 2004). More than 50% of the isolates were resistant to three or more antibiotics and TEM *bla* (β-lactam) and *sul* (sulphonamide) resistance genes were the most common resistance genes (Maynard *et al.*, 2004).

A study done on retail chicken meat in Japan reported 41% of *E. coli* isolates resistant to multiple antibiotics (Ahmed *et al.*, 2009). Resistance phenotypes were mostly to tetracycline, ampicillin, streptomycin, sulphamethoxazole-trimethoprim and kanamycin (Ahmed *et al.*, 2009). Furthermore, Polymerase Chain Reaction (PCR) screening revealed the presence of Class 1 and Class 2 integrons with gene cassettes that encode for extended spectrum β-lactamases (ESBL) (Ahmed *et al.*, 2009). The emerging resistance to fluoroquinolones and ESBL by multi-antibiotic resistant *E. coli* strains has caused increasing concern over the last decade due to limited therapeutic options if infections with these strains occur (Erb *et al.*, 2007). In 2011, major media attention was focused on the German *E. coli* O104:H4 food-borne disease outbreak (Laing *et al.*, 2011). The outbreak strain exhibited characteristics typical of other EAEC but also produced Shiga-toxin 2 (Safadi *et al.*, 2012). The strain also acquired an antibiotic resistance plasmid, conferring resistance to all penicillins, cephalosporins and co-trimoxazole (Laing *et al.*, 2011).

Enterotoxigenic *E. coli* is a common cause of bacterial infection leading to acute watery diarrhoea in infants and young children (Bhunia, 2008). The prevalence of ETEC infections is especially high in developing countries such as India and Bangladesh (DeWaal *et al.*, 2012). Infection spreads through contaminated food and surface water e.g. ponds, rivers and lakes (Gibson *et al.*, 2010). A study by Begum *et al.* (2007) in Bangladesh demonstrated that almost all ETEC strains isolated from rural and urban surface waters expressed heat-stable diarrhoeal toxin (ST) either alone or in combination with heat-labile toxin (LT). Forty four % ETEC strains were found to express one or more colonisation factors (CF) thus enabling attachment to the intestinal mucosa (Begum *et al.*, 2007). Combined resistance to antibiotics generally used to treat enteric infections, such as erythromycin, ampicillin and tetracycline was observed (Begum *et al.*, 2007). The highest resistance levels were observed against erythromycin (70%) and streptomycin (50%) (Begum *et al.*, 2007).

The Ganga River and its tributaries is a major source of water supply in northern India (Ram *et al.*, 2008). The Gomti River, a tributary of the Ganga River supplies water to approximately 3.5 million people and receives 450 million litres of untreated domestic waste every day (Ram *et al.*, 2008). Fifty eight % of *E. coli* isolates from the Gomti River demonstrated multiple resistance patterns to three or more antibiotics (up to seven) from different antibiotic classes (Ram *et al.*, 2008). Thirty three % of the resistant isolates possessed EHEC virulence potential and 21% of the isolates were ETEC (Ram *et al.*, 2008).

Aquifers are porous and water seeps down through fissures and cracks to form springs that are utilised as sources of drinking water (Avery *et al.*, 2008). Aquifers like limestone systems are highly susceptible to bacterial contamination present in surface water (Bucci *et al.*, 2011). A study in France showed that high levels of antibiotic-resistant *E. coli* were present in springs used for drinking water (Ribeiro *et al.*, 2012). Ninety-seven % of the resistant isolates were resistant to chloramphenicol and tetracycline, while 86% of the resistant isolates were resistant to β-lactams (Ribeiro *et al.*, 2012). Polymerase Chain Reaction analysis and DNA sequencing showed isolates that contained Class 1 integrons with up to 10 different antibiotic-resistance gene cassettes (Ribeiro *et al.*, 2012).

Escherichia coli isolates from the Fu River in Southwest China were found to be resistant to trimethoprim-sulphamethoxazole (50%) and ampicillin (35%) (Zou *et al.*, 2012). The river is a large waterway that runs through the densely populated Sichuan Province with a population of approximately 11.4 million (Zou *et al.*, 2012). Water from the river is also used for drinking and irrigation purposes (Zou *et al.*, 2012). PCR analysis of the ampicillin-resistant isolates revealed the presence of ampicillin resistance genes bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$, which also causes resistance to many other β-lactam antibiotics (Zou *et al.*, 2012).

Diarrhoea in young calves is a major problem world-wide when calves are reared intensively and is responsible for substantial treatment costs (Jiang & Shepherd, 2009). In a study from Sweden, 61% of *E. coli* isolated from young calves was found to be resistant to at least one antibiotic and 28% was resistant to at least three antibiotics (De Verdier *et al.*, 2012). The highest resistance levels to streptomycin (55%), tetracycline (42%), sulphonamide (42%) and ampicillin (38%) were observed amongst the resistant isolates (De Verdier *et al.*, 2012). A study on *E. coli* isolates from horses in northwest England revealed high resistance levels to multiple antibiotics in horses that had been hospitalised (Ahmed *et al.*, 2010). The highest resistance levels were to trimethoprim (51%), tetracycline (35%) and ampicillin (34%) (Ahmed *et al.*, 2010). Polymerase Chain Reaction analysis confirmed the presence of the *dfr* gene in 93% of the trimethoprim-resistant isolates, *tet* gene in 87% tetracycline-resistant isolates and TEM β-lactamase in 91% ampicillin-resistant isolates (Ahmed *et al.*, 2010).

Resistance to at least one antibiotic was demonstrated in *E. coli* isolates from livestock, wildlife and surface water in a study from the USA (Sayah *et al.*, 2005). Similarities in resistance patterns were observed in faecal samples and farm environment samples by animals (Sayah *et al.*, 2005). In this study resistance phenotypes were most frequently demonstrated to tetracycline, cephalothin, streptomycin and sulphasoxazole (Sayah *et al.*, 2005). Resistance to multiple antibiotics were observed in isolates from a variety of sources, with the highest resistance levels in swine faecal samples (Sayah *et al.*, 2005). The surface water isolates were only resistant to cephalothin however, higher

resistance levels were observed in isolates from the farm environment compared to faecal isolates (Sayah *et al.*, 2005).

Infections due to β-lactam resistant *E. coli* strains that produce ESBL of the CTX-M family are emerging in European countries which include the United Kingdom (UK) and Spain (Rodriguez-Villalobos *et al.*, 2005). Community-acquired infections caused by these strains appear to be increasingly common in these countries (Pillai *et al.*, 2011). This represents a therapeutic problem, due to resistance to multiple antibiotics from several classes which include penicillins, cephalosporins, aminoglycosides and fluoroquinolones (Von Baum & Marre, 2005). A study in Belgium on hospital isolates revealed new emergence and rapid increase in the prevalence of CTX-M producing *E. coli* (Rodriguez-Villalobos *et al.*, 2005). Co-resistance to antibiotics other than β-lactams were also commonly seen in ESBL-producing *E. coli* isolates such as resistance to ciprofloxacin (64%), co-trimoxazole (54%) and gentamicin (44%) (Rodriguez-Villalobos *et al.*, 2005). PCR analysis revealed the majority of these isolates contained CTX-M enzymes whereas the remainder contained genes for TEM enzymes or SHV enzymes alone or in combination with TEM (Rodriguez-Villalobos *et al.*, 2005).

The proportion of *E. coli* isolated in Ireland with resistance to fluoroquinolones increased significantly by 12%, from 2002 to 2005 (Murchan & Cunney, 2006). Across Europe, increasing fluoroquinolone resistance was observed in 15 out of 26 European countries reporting to the European Antimicrobial Resistance Surveillance System (EARSS) between 2001 and 2004 (Murchan & Cunney, 2006). In 2004, four countries (Italy, Spain, Portugal and Malta) reported proportions over 25% (Murchan & Cunney, 2006).

Yang *et al.* (2009) observed the presence of integrons in 32% of clinical *E. coli* isolates in Taiwan using an integrase gene PCR assay. Among the 71 integron-positive isolates, 65 were Class 1 integrons and the remainder belonged to Class 2 (Yang *et al.,* 2009). DNA sequencing of Class 1 integrons showed gene cassettes that encode for resistance to streptomycin and trimethoprim (Yang *et al.,* 2009). Vinué *et al.* (2008) described integrons in 41% of *E. coli* isolates and 26 out of 29 integron-positive isolates contained Class 1 integrons (Vinué *et al.*, 2008). Integron-positive isolates displayed high resistance to streptomycin, ampicillin, tetracycline, trimethoprim and chloramphenicol (Vinué *et al.*, 2008).

Retail meats are often contaminated with *E. coli* strains and can serve as a vehicle for dissemination of antibiotic-resistant ExPEC (Sáenz *et al.*, 2004). Water-related illness caused by antibiotic-resistant ETEC is a major problem in many developing countries (Bhunia, 2008). The ETEC and STEC strains resistant to multiple antibiotics were common in rivers that flow through densely populated regions (Ram *et al.*, 2008; Zou *et al.*, 2012). The use of antibiotics in animal feed has been shown to be a major contributing factor to

increasing antibiotic resistance (Salvadori *et al.*, 2004). Antibiotic-resistant ETEC strains is a major problem world-wide when livestock are reared intensively and the highest resistance levels were observed in isolates from swine faecal samples (Sayah *et al.*, 2005; De Verdier *et al.*, 2012). A study by Sayah *et al.* (2005) described similarities in resistance patterns from faecal samples and farm environment samples to tetracycline, cephalothin, streptomycin and sulphasoxazole (Sayah *et al.*, 2005). Ribeiro *et al.*, (2012) described the presence of *E. coli* isolated from a spring used for drinking water in France that encoded resistance for up to ten different antibiotics (Ribeiro, *et al.*, 2012). Infections due to β-lactam and fluoroquinolone resistant *E. coli* strains increased significantly in Europe (Rodriguez-Villalobos *et al.*, 2005; Murchan & Cunney, 2006).

F. RESISTANCE MECHANISMS

Bacterial antibiotic resistance has over the past decade evolved into a calamity of international magnitude (Wright, 2011). Bacterial resistance to many and even all clinically approved antibiotics are increasingly common and can easily spread across continents (Shea, 2003). It is thus of great importance to better the knowledge of bacterial mechanisms of antibiotic resistance. A basic overview of the different bacterial mechanisms of antibiotic resistance is presented in Table 3.

Table 3. Resistance mechanisms (Erb *et al.*, 2007; Wright, 2011; Lupo *et al.*, 2012)

Type	Mechanism	Antibiotic
Prevent entrance	Gram – outer membrane, PBP mutations, Decrease in permeability,	penicillin, sulphonamide
Efflux systems	Plasma membrane translocases	tetracycline, multi-antibiotic resistance (nonspecific)
Inactivation by chemical modification	penicillinase, chloramphenicol acyltransferase, acetyltransferase	penicillin, chloramphenicol, aminoglycosides
Modification of target	Change in 23S rRNA, Change in dihydropteric acid synthetase	erythromycin, chloramphenicol, sulphonamide
Alternate metabolic pathways	Use preformed folic acid or increase folic acid production	sulphonamide, trimethoprim
Horizontal gene transfer	Class 1 integrons (Gram -)	Multi-antibiotic resistance (non-specific)

The first resistance strategy is when pathogens become resistant simply by preventing entrance of a specific antibiotic (Tadesse *et al.*, 2012). Many Gram-negative bacteria are unaffected by penicillin G because it cannot penetrate the cell envelope's outer membrane (Li *et al.*, 2009). Genetic mutations that lead to changes in penicillin binding proteins also render a cell resistant (Willey *et al.*, 2008). For example, a decrease in antibiotic permeability can lead to sulphonamide resistance (Levy, 1992). Mycobacteria can resist many antibiotics because of the high content of mycolic acids in a complex lipid layer outside their peptidoglycan, which is impermeable to most water-soluble antibiotics (Willey *et al.*, 2008).

A second resistance strategy is to pump the antibiotic out of the cell after it has entered (Kümmerer, 2009). Some pathogens have plasma membrane translocases, often called efflux pumps that expel antibiotics (Nikaido, 1989). These transport proteins are called multi-antibiotic resistance pumps because they are relatively nonspecific and can pump many different antibiotics (Levy, 1992). Many are antibiotic/proton anti-porters, where protons enter the cell as the antibiotic leaves. Such systems are present in *E. coli*, *P. aeruginosa* and *S. aureus* to name a few (Von Baum & Marre, 2005). The role of efflux pumps in conferring antibiotic resistance and multi-antibiotic resistances in bacteria has been extensively studied and reviewed (Lupo *et al.*, 2012).

Efflux systems in Gram-negative bacteria have structural genes that can be located on mobile genetic elements (Levy, 1992). This constitutes the main acquired mechanisms for antibiotic resistance efflux systems for tetracycline and chloramphenicol resistance (Baquero *et al.*, 2008). However, bacteria are intrinsically provided with chromosomally encoded efflux systems that are believed to participate in homeostasis of the cell, by extruding endo and/or exogenous toxic compounds, heavy metals, virulence factors and quorum sensing signals (Nikaido, 1989). In Gram-negative bacteria, resistance nodulation division (RND) systems exhibit a wide substrate spectrum, which usually includes antibiotics of different classes (Akter *et al.*, 2012). Lupo *et al.* (2012) described the role of these efflux pumps in a wide range of pathogenic and opportunistic bacterial species. A more complex regulation network, linking efflux to membrane permeability and other cellular functions, is likely to occur in these bacteria (Davies, 2007). This has been described for the multiple antibiotic resistant (*mar*) regulon in *E. coli* (Sáenz *et al.*, 2004).

Many bacterial pathogens resist attack by inactivating antibiotics through chemical modification (DeWaal *et al.*, 2012). The best-known example is the hydrolysis of the β-lactam ring of penicillins by the enzyme penicillinase (Zou *et al.*, 2012). Bacteria, especially Gram-negatives, can also resist β-lactams by the production of hydrolytic enzymes, β-lactamases (Kümmerer, 2009). Carbapenems have become progressively popular for the treatment of bacterial infections caused by ESBL in *Enterobacteriaceae*, mostly in *E. coli*

(Wright, 2011). New Delhi metallo-β-lactamase-1 (NDM-1) is a bacterial enzyme that renders all β-lactam antibiotics inactive and is potentially a global health problem (Rolain *et al.*, 2010). Bacteria that produce NDM-1 have also been associated with resistance to other antibiotic classes, severely limiting treatment options (Pillai *et al.*, 2011). Since 2010 spreading and dissemination has occurred, with several cases of EHEC producing NDM-1 reported internationally in the USA, Europe, Asia and Africa (Rolain *et al.*, 2010).

Detection of antibiotic resistant bacteria, in freshwater, harbouring β-lactamases has been reported from several studies and clinical occurrence of β-lactamase encoding genes has been extensively reviewed and recently much attention to their propagation in the environment has been given (Rodriguez-Villalobos *et al.*, 2005). Lupo *et al*. (2012) described the over-expression of *amp*C by mutations in the promoter region, in *E. coli* strains isolated from recreational beaches and drinking water. As for other β-lactamases, chromosomal cephalosporinases have been described to evolve by point mutation, hydrolysing a broader spectrum of β-lactams (Rodriguez-Villalobos *et al.*, 2005).

Antibiotics also are inactivated by the addition of chemical groups (Willey *et al.*, 2008). For example, chloramphenicol contains two hydroxyl groups that can be acetylated in a reaction catalysed by the enzyme chloramphenicol acyltransferase with acetyl CoA as the donor (Costanzo *et al.*, 2005). Aminoglycosides can be modified and inactivated in several ways; acetyltransferases catalyse the acetylation of the amino groups (Coyne *et al.*, 2011). Some aminoglycoside-modifying enzymes catalyse the addition to hydroxyl groups of either phosphates (phosphotransferases) or adenyl groups (adenyltransferases) (Sayah *et al.*, 2005).

Each antibiotic reacts on a specific target so resistance arises when the target enzyme or cellular structure is modified so that it is no longer susceptible to the antibiotic (Willey *et al*., 2008). For example, the affinity of ribosomes for erythromycin and chloramphenicol can be decreased by a change in 23S rRNA to which they bind (DeWaal *et al.*, 2012). Enterococci become resistant to vancomycin by changing the terminal D-alanine-D-alanine in their peptidoglycan to D-alanine-D-lactate, drastically reducing binding of the antibiotic (Murchan & Cunney, 2006).

Anti-metabolic action may be resisted through alteration of susceptible enzymes (Ibenyassine *et al.*, 2006). In sulphonamide-resistant bacteria the enzyme that uses PABA during folic acid synthesis often has a much lower affinity for sulphonamides (Xia *et al.*, 2011). Resistant bacteria use either an alternate pathway to bypass the sequence inhibited by the antibiotic or increase production of the target metabolite (Lupo *et al.*, 2012). For example, some bacteria are resistant to sulphonamides simply because they use preformed folic acid from their surroundings rather than synthesize it themselves (Akter *et al.*, 2012).

Other strains increase their rate of folic acid production and thus counteract sulphonamide inhibition (Von Baum & Marre, 2005).

Horizontal gene transfer by mobile integrons among bacteria plays an important role in the dissemination of antibiotic-resistance (Barlow *et al.*, 2004). Integrons are gene-capture and expression systems characterised by the presence of *intI* gene encoding an integrase, a recombination site (*attI*) and promoter (Cambray *et al.*, 2010). Integrons are able to capture gene cassettes from the environment and incorporate them by using site-specific recombination (Roe *et al.*, 2003). Often several resistance genes are carried together as gene cassettes in association with integrons, resulting in the development of multi-antibiotic resistance (Willey *et al.*, 2008). Many gene cassettes of integrons encoding antibiotic-resistance genes are found in Gram-negative bacteria and are commonly associated with members of the family *Enterobacteriaceae* (Vinué *et al.*, 2008).

Integrons are mostly characterised based on their homology to integrase enzymes that catalyse the excision and integration of specific gene cassettes (Cambray *et al.*, 2010). Among the integrons, Class 1 and Class 2 integrons are commonly observed in bacteria (Roe *et al.*, 2003). Several studies have highlighted the crucial role of integrons, particularly Class 1 integrons in the evolution of antibiotic resistances (Barlow *et al.*, 2004). Many gene cassettes of integrons encoding antibiotic resistance are found in Gram-negative bacteria, and more than 100 gene cassettes for antibiotic resistance are known (Cambray *et al.*, 2010). Class 1 integrons are most commonly found in clinical isolates of Gram-negative bacteria (Yang *et al.*, 2009). Previous studies have shown that integrons are widely present in human and animal isolates and play a significant contribution to antibiotic resistance (Roe *et al.*, 2003; Vinué *et al.*, 2008; Dolejska *et al.*, 2009; Yang *et al.*, 2009).

Bacteria often become resistant in several different ways and, unfortunately a particular resistance mechanism is not confined to a single class of antibiotic (Costanzo *et al.*, 2005). Two bacteria may use different resistance mechanisms to withstand the same antibiotic (Kümmerer, 2009). Furthermore, resistant mutants arise spontaneously and are then selected for in the presence of the antibiotic (Baquero *et al.*, 2008). Efflux systems in Gram-negative bacteria exhibit a wide substrate spectrum which usually includes antibiotics from different classes (Akter *et al.*, 2012). Bacteria that produce ESBL are resistant to multiple antibiotics which severely limit treatment options (Pillai *et al.*, 2011). Class 1 integrons play an important role in the dissemination of antibiotic-resistance among *Enterobacteriaceae* and is usually associated with multi-antibiotic resistance (Barlow *et al.*, 2004). These resistance mechanisms may be transferred from commensal bacteria to pathogenic bacteria resulting in serious health implications (Olaniran *et al.*, 2009). The concern is that these mechanisms may confer cross-resistance to other clinically important antibiotics (Li *et al.*, 2009).

G. GENERAL CONCLUSION

Microbial contamination of surface water, particularly contamination with faecal bacteria has long been a water quality issue owing to the potential for disease transmission. *Escherichia coli* in faeces is consistently released into the environment, thereby contaminating water, soil and subsequently fruit and vegetables. Generally harmless, some *E. coli* are pathogenic and may be distinguished from commensal strains by their ability to cause serious illness. In order to understand how to treat and prevent disease, it is essential to know what are the levels of contamination as well as the distribution of antibiotic resistance in surface water.

Previous reports on the microbiological quality of South African rivers showed that the water was unsafe for human consumption and for the use as irrigation water. In addition to the problem of the detection of pathogens in water samples with microbial levels far above the recommended international standards, are the wide antibiotic resistance profiles that have been reported in pathogens. In other cases as reported in the literature several pathogens including *E. coli* isolates have been shown to demonstrate significant increases in resistance to certain antibiotics over short periods. Reports have ascribed this new trend to either the impact of increased faecal pollution, selective pressure and antibiotic abuse or over-use in animal feeds. Industrial effluent, incorrect sewage disposal, agricultural and animal waste run-off are aspects of urbanisation that have an adverse impact on surface water quality. Additionally our country's water resources are under increasing pressure due to badly managed water resource systems and the subsequent chances of water-borne outbreaks increase.

Over the last decade the incidence of food-related outbreaks associated with the consumption of fresh produce has globally increased. Since antibiotics are used in animals to increase their growth and prevent infections and in crop production to control pathogens, there is concern about the development of antibiotic resistance in pathogens and subsequent transfer to humans through contaminated food. Drastically changing antibiotic resistance profiles have been reported for *E. coli* strains isolated from environmental sources and resistance to at least two types of antibiotics is generally now seen as normal. Even resistance to fluoroquinolones is not restricted to pathogenic strains but has also been reported to be present in environmental strains.

From the above literature study it is obvious that antibiotics are appearing in unexpected places. It is clear that antibiotic resistance must be seen as a serious food-safety problem. Previous research has shown that antibiotic-resistant bacteria may easily be carried over from polluted water resources to irrigated fresh produce and then ingested with vegetables and even meat. Antibiotic resistance among bacteria associated with food animals has been documented but research regarding resistance profiles of bacteria present

in water used specifically for irrigation purposes is limited. That is why it is important that the antibiotic resistance profiles be determined of water used for irrigation in South Africa.

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CHAPTER 3

ENUMERATION AND CHARACTERISATION OF *ESCHERICHIA COLI* FROM IRRIGATION WATER AND POTENTIAL CONTAMINATION SITES

SUMMARY

Nineteen sites were sampled in the Western Cape (irrigation, contamination and environmental sources) with the aim of characterising and determining the diversity of *Escherichia coli*. Total coliform and *E. coli* counts from contamination sites were recorded to be as high as log 8.837 and log 8.145 MPN.100 mL^{-1}, respectively. Maximum total coliform and *E. coli* counts for irrigation sites were log 7.862 and log 5.364 MPN.100 mL^{-1}, respectively. Maximum total coliform and *E. coli* counts from environmental sites were log 3.613 and log 2.491 MPN.100 mL^{-1}, respectively. Five out of seven irrigation sites had *E. coli* counts exceeding the guideline for 'safe' irrigation water (<1 000 counts.100 mL^{-1}) (WHO, 1989; DWAF, 1996).

The majority of the *E. coli* isolates represented irrigation (n = 34) and contamination water (n = 49), while 37 isolates were from 'environmental' sites. *Escherichia coli* marker (n = 37) and reference strains (n = 6) were included in the dataset as comparative controls. The Jaccard statistical method was used to create dendrogrammes which clustered similar *E. coli* strains on the basis of their biochemical profiles. It was observed that 36 clusters were formed in total, containing one to 117 strains in each. API 20E was used to identify isolates and Polymerase Chain Reaction (PCR) was used to confirm the identity of *E. coli* isolates.

Phylogenetic group B1 has been reported to contain strains with the ability to survive and persist in the external environment. In this study, 46.6% of the strains were assigned to group B1. Isolates from irrigation water showed similar phylogenetic distribution patterns and B1 was seen as the most common group (79.4%), while isolates from environmental sources were mainly assigned to group A_0 (54.1%). It was concluded that the variation of *E. coli* isolates present in irrigation water is a matter of concern that should be further investigated. This raises major human health implications since the increased exposure to faecal organisms increases the risk of disease transmission.

INTRODUCTION

Microbial food-borne diseases are a growing concern to food legislators, food manufacturers and consumers worldwide. Microbiological contamination can occur at any stage of the food-chain and therefore requires strict control and management. Water acts as a passive carrier of numerous organisms that can cause human illness (Derrien *et al.*, 2012). Irrigation

water is a key pre-harvest source of fresh produce contamination (Oliviera *et al.*, 2012). Poor irrigation water quality indicated by elevated faecal coliform counts has long been known to correlate with the incidence of human pathogens on leafy vegetables (Mandrell, 2009). *Escherichia coli* in particular has been responsible for numerous food-borne outbreaks linked to contaminated fresh produce (Lynch *et al.*, 2009). For example *E. coli* O157:H7 outbreaks on spinach, lettuce and sprouts received intense global media attention and demonstrates the importance of food safety in the mind of the public (Powell *et al.*, 2009). Survival studies demonstrated that *E. coli* O157:H7 can persist in contaminated manure and irrigation water for several months (Franz *et al.*, 2011).

Access to clean water is a major concern in many developing countries due to contamination of water sources (Krige, 2009). Factors responsible for the contamination of water sources include agricultural waste, animal effluent, industrial effluent and sewage disposal (Lupo *et al.*, 2012). Municipal wastewater and surface waters constitute important vehicles in the dissemination of *E. coli* in the urban environment (Figueira *et al.*, 2011). Sanitation in communities with a lack of access to clean water is another factor that leads to the contamination of water sources (Gemmell & Schmidt, 2012).

Studies over the past 10 years on the microbiological quality of water in many of South Africa's rivers revealed unacceptable and dangerous levels of faecal contamination (Lötter, 2010; Gemmell & Schmidt, 2012; Paulse *et al.*, 2012; Britz *et al.*, 2013). If such waters are not disinfected before being used for drinking or for irrigation purposes, it could result in serious health implications (DeWaal *et al.*, 2012). Agriculture in the Western Cape is one of the most important economic sectors in the country and produced close to 45% (R12.5 billion) of South Africa's agricultural exports in 2008 (Anon., 2012). However, faecal contamination of water used for irrigational purposes threatens the livelihood of this province. On most farms, the water does not undergo any treatment to improve water quality before it is administered to crops (Britz, T.J., 2012, Department of Food Science, Stellenbosch, South Africa, personal communication). The Berg River used for irrigation of vegetables in South Africa has also been reported to fall below the European Union's (EU) microbiological standard allowed for food production (Paulse *et al.*, 2012).

It is therefore essential to look at irrigation waters as well as possible contamination sources when estimating the risk of *E. coli* presence in water. Additionally, this could lead to a better understanding of the composition of the *E. coli* population found in natural water sources of South Africa. The objective of this study will therefore be to determine the level of microbial pollution of water sources used to irrigate crops in the Western Cape and to determine the prevalence of *E. coli* strains that are able to survive in natural water sources as well as contamination sources. This will entail quantification, characterisation and

identification of total coliforms and *E. coli* isolated from selected irrigation, contamination and 'environmental' sources.

MATERIALS AND METHODS

Site Selection

A total of 19 sites were selected (Tables 1, 2 and 3) and each sampled twice over a period of seven months. Of the 19 sites selected, seven sites were classified as irrigation sources, eight sites as contamination sources and four sites as environmental sources. The sites were mostly in the Stellenbosch and surrounding areas, with one site each in Durbanville, Kraaifontein, Paarl and Worcester.

Irrigation sites

The irrigation sites were selected from different types of water sources including four rivers, one dam and one grey water source (Table 1). Grey water is wastewater generated from domestic activities such as bathing, dishwashing and laundry. These sample sites were chosen because the water was used for irrigation of fresh produce either at the same point or further downstream from the point of extraction.

Table 1 Irrigation sites, their geographical locations and water application

Water source	Geographical location	Used to irrigate
Berg River	Paarl	Fresh produce and fruit
Grey water	Raithby	Fresh produce and fruit
Limberlost River	Annandale	Fresh produce and fruit
Middlevlei Dam (MV-Dam)	Stellenbosch	Fresh produce and fruit
Mosselbank River	Kraaifontein	Fresh produce and fruit
Plankenburg River (Plank-2)	Stellenbosch	Fresh produce and fruit
Veldwagters River	Stellenbosch	Fresh produce and fruit

Contamination source sites

This set of sampling sites (Table 2) was chosen to represent potential contamination sources from where *E. coli* can originate. These sites were therefore chosen with the expectation of high microbial loads, especially in terms of total coliform bacteria and *E. coli*. The contaminated water also had to reach another water source which could contribute to a river or dam which is used for irrigation.

Agricultural activity was represented by both a dairy and piggery; in both cases samples were taken from the water being used to wash the stalls. The wash-water (high in faecal contamination) from the stalls is then directed to primary and secondary fermentation dams. Water from primary and secondary fermentation dams of both the cow and pig stalls

were sampled (Muller, C.J.C., 2012, Institute for Animal Production, Stellenbosch, South Africa, personal communication). These dams could over-flow and reach a nearby marsh which signals the start of the Plankenburg River.

Water from a dam used to breed trout fish was sampled to include another possible source of contamination (Salie, K., 2012, Division of Aquaculture, Stellenbosch, South Africa, personal communication). Effluent water from a free-range chicken abattoir was also sampled since it was expected to have high loads of enteric bacteria (Steyn, C., 2012, Technical Manager, Elgin Free-Range Chickens, Grabouw, South Africa, personal communication). Equine faecal samples were collected from a stable that houses horses used for equestrian purposes. The horse faeces is sold to local farmers as manure, after it had been dried in large heaps (Kilian, G., 2012, Owner, Evergreen Stable, Paarl, South Africa, personal communication).

Water from the Plankenburg River after flowing passed Kayamandi, a large informal settlement in Stellenbosch, represented the human factor. Kayamandi has previously been implicated as a possible source of human faecal pollution (Barnes & Taylor, 2004; Van Blommestein, 2012). Water from a stream isolated after flowing passed a certain area ('Smartie Town') within Cloetesville, a low-income residential area, also represented the human factor. This stream is notorious for being highly polluted and cases of water-related illness have previously been reported in 'Smartie Town' (Esler, K., 2012, Deputy Chairperson, Department of Conservation Ecology and Entomology, Stellenbosch University, South Africa, personal communication).

Table 2 Possible contamination sites, their geographical locations and main source of contamination

Contamination Site	Geographical location	Contamination source
Aquaculture Dam	Stellenbosch	Fish
Abattoir effluent	Grabouw	Chicken
Dairy effluent	Elsenburg	Cow
Large Dam (L-Dam)	Elsenburg	Cow, storm water
Horse stables	Paarl	Horse
Piggery effluent	Elsenburg	Pig
Plankenburg River (Plank-1)	Stellenbosch	Human
Smartie Town	Stellenbosch	Human

Environmental sites

The environmental sites were selected to represent a control where no direct source of faecal contamination was expected (Table 3). The Plankenburg River (Plank-0) was sampled at a site that is south of Stellenbosch, before it passes the large informal settlement, Kayamandi. It was the furthest point upstream in the Plankenburg River that was sampled where no direct sources of faecal contamination were apparent. Water from dams

in Durbanville, Stellenbosch and Worcester were also included in this study to evaluate the impact of wild birds and fish on water quality.

Table 3 Environmental sites, their geographical locations and source of contamination

Environmental Site	Geographical location	Contamination source
Eversdal Dam (E-Dam)	Durbanville	Geese, environmental
House-Horizon Dam (HH-Dam)	Stellenbosch	Environmental
Plankenburg River (Plank-0)	Stellenbosch	Environmental
Worcester Dam (W-Dam)	Worcester	Fish, environmental

Sampling Frequency

Samples were collected over a period of seven months, from March to September 2012. During this time all sites were sampled in duplicate. It was also decided that if the results of the duplicate samples drastically contradicted one another, that a third sample would be taken. Samples were taken in such a way that follow-up samples from a single site were approximately two months apart. This was to ensure that sufficient time could pass between sampling opportunities so that it was confirmed that the contamination in the water system was constant and not just a once-off contamination.

Sample Collection

Surface water – The sampling of surface water was conducted according to the SANS 5667-6 method (SANS, 2006). All necessary safety measures were taken into account. Care was taken not to disturb the sediment, and a sample was taken as near to the middle of the river as possible. A sterile bottle was submerged to 30 cm under the surface pointing toward the direction of flow, before the cap was removed and the bottle filled. The bottles were transported on ice and analysed as soon as possible.

Equine Faecal samples – Faecal matter was sampled according to the method described by Graves *et al.* (2011) as a guideline. All necessary safety measures were always taken into account and care was taken to sample fresh faecal samples. Sterile autoclavable bags were used to sample faeces in triplicate. The sterile bags were transported on ice and analysed after sampling as soon as possible. Faecal matter (10 g) was weighed aseptically before it was mixed thoroughly with 90 mL Sterile Saline Solution (SSS) (0.85% m/v NaCl) in a sterile bag for two minutes using a BagMixer (Interscience, France). The solid matter was allowed to settle before the mixture was used for further analysis.

Sample Analysis

Total coliforms and Escherichia coli counts

Water analysis was done according to the standard method described by SANS 9308 (SANS, 2012). The QuantiTray system was used to enumerate total coliforms and *E. coli* using the Colilert 18 kit (IDEXX, South Africa). The dilutions of the samples used with the QuantiTray system varied according to source types and potential contamination load. QuantiTrays were incubated at 37°C for 18 h and subsequently examined for the presence of total coliforms (yellow wells) and *E. coli* (fluorescent wells). The number of total coliforms and *E. coli* were then calculated by means of a conversion table.

Isolation of E. coli

Wells showing fluorescence on the QuantiTrays at 365 nm were marked, and the total area of the large wells of each tray was divided into quarters. A maximum of two fluorescent wells were chosen at random from each quarter, 1 mL of the contents of each chosen well were aseptically removed and placed into a sterile McCartney bottle. A maximum sample size of 8 mL was therefore generated for further analysis. A loop-full of this collected sample was placed in a McCartney bottle with 9 mL sterile saline solution (SSS) (0.85% m/v NaCl), and vortexed (Vacutec, South Africa). This was used as the 'concentrated' solution and from this a 10^{-3} dilution series was prepared using SSS. Eosin Methylene-Blue Lactose Sucrose Agar (L-EMB) (Oxoid, South Africa) plates were inoculated with 100 µL aliquots of the 10^{-2} and 10^{-3} bacterial suspensions by means of the spread-plate method and subsequently incubated for 24 h at 37°C.

After incubation, colonies showing a metallic green sheen that denotes typical *E. coli* growth (Merck, 2007), were regarded as presumptive *E. coli* colonies. A minimum of five colonies showing typical *E. coli* growth on L-EMB agar were selected using the Harrison Disk method (Harrigan & McClance, 1976). These colonies were then streaked onto Brilliance™ *E. coli* coliform selective agar (Oxoid, South Africa) and incubated for 24 h at 37°C. The streaking out of each isolate onto Brilliance™ *E. coli* coliform selective agar (Oxoid, South Africa), was repeated if necessary until pure cultures were obtained on this agar. Typical *E. coli* forms round, smooth, convex colonies which are deep purple in colour (Merck, 2007). Any atypical colonies resulting from the purification process were also isolated for further analysis.

Characterisation and confirmation of E. coli identification

Each isolate was then streaked out on Nutrient Agar (Biolab, South Africa) and incubated for 24 h at 37°C. The API 20E system (BioMérieux, South Africa) was used in conjunction with Gram staining and catalase testing (Gerhardt *et al.*, 1981) to create a unique 'profile number' for each isolate. This profile number was then entered into the APIweb™ (BioMérieux, South Africa) database and the isolates were identified. Isolates were then stored in the presence of 40% (v/v) glycerol (Fluka Analytical, Germany) in cryotubes at -80°C.

Reference and Marker Strains

Six American Type Culture Collection (ATCC) *E. coli* reference strains were included as comparative controls during all API and PCR analyses (Addendum A). An additional set of 37 *E. coli* marker strains from the Food Science collection were also included in the dataset of isolates (Addendum A). Twenty-seven marker strains were isolated in previous studies from natural water sources which include rivers and ground water used for irrigation in Stellenbosch and surrounding areas. Ten marker strains isolated from green beans that were irrigated with contaminated water from the Plankenburg River were also included in this study.

API 20E Data Analysis

The API 20E (BioMérieux, South Africa) system uses 27 biochemical tests for the phenotypic characterisation of a microorganism. The APIweb™ (BioMérieux, South Africa) program is used to compare results from the API 20E analysis and give an identification based on the biochemical profiles of the isolates. Isolate profiles were converted into a series of ones and zeros denoting positive and negative attributes, respectively. Agglomerative Hierarchical Clustering (AHC) analysis (XLSTAT, 2012.4.03) was used to create unsorted dissimilarity matrices. The unsorted matrices were then sorted by means of the Jaccard (S_J) coefficient and constructed dendrogrammes. Dendrogrammes from the S_J analysis were used to determine the degree of similarity/dissimilarity of the isolates that were characterised. The calculation of dendogramme distances were based on the biochemical test results of individual isolates (Lockhart & Liston, 1970).

PCR Methods

*DNA template preparation (*Altahi *et al.*, 2009)

Isolates were cultivated on Tryptone Soya Agar (TSA) (Oxoid, England) for 24 h at 37°C. Following this, a colony of each culture was boiled in a 1.5 mL micro-centrifuge tube with

100 µL nuclease-free water for 13 min to lyse the cells and release its content. The tubes were cooled on ice and centrifuged (Vacutec, South Africa) for 15 min at 14 000 x g to pellet the cell debris. The supernatant was subsequently transferred to a sterile tube and stored at -18°C until it was required for analysis.

E. coli uidA-PCR Analysis

After the basic identification and phenotypic characterisation of isolates, the presence of the *E. coli uidA* gene was determined according to Heijnen & Medema (2006). The *uidA* gene encodes for the enzyme β-glucuronidase which *E. coli* use to break-down liver conjugates such as steroid glucuronides in the human gut (McIntosh *et al.*, 2012). Each PCR reaction of 25 µL contained 0.4 µM of each primer (Table 4), 2.5 mM $MgCl_2$, 1 X KapaTaq Hotstart buffer, 0.2 mM of each dNTP, 0.625 U KapaTaq Hotstart DNA Polymerase and 0.5 µL template DNA.

Table 4 Primer sequences and amplicon sizes used for *uidA*-PCR (Heijnen & Medema, 2006)

Target gene	Primer	Primer sequence (5' - 3')	Size (bp)
uidA	UAL1939b (F) UAL2105b (R)	ATGGAATTTCGCCGATTTTGC ATTGTTTGCCTCCCTGCTGC	187

(F) - Forward primer; (R) - Reverse primer

A positive control (*E. coli* ATCC 25922) as well as a negative control (nuclease-free water) were included in all PCR analyses. All PCR tubes were briefly centrifuged and transferred to the G-Storm thermal cycler (Vacutec, South Africa). The cycling protocol is specified in Table 5.

Table 5 Summary of *uidA*-PCR reaction conditions

Action	Temperature (°C)	Time (mm:ss)
Initial Denaturation	95	03:00
30 cycles of:		
Denaturation	95	00:30
Primer annealing	59.7	00:30
Extension	72	00:30
Final Extension	72	05:00

The PCR products were analysed using gel electrophoresis in a 1% agarose (SeeKem, Switzerland) gel containing 1 µg.mL^{-1} ethidium bromide (Sigma, Germany). Gel

electrophoresis was performed at 210 V for 20 min and the PCR bands were visualised on a UV-transilluminator (Vacutec, South Africa).

Determination of Phylogenetic Groups with PCR Analysis

Triplex PCR (t-PCR) was conducted on all the *uidA*-PCR confirmed *E. coli* isolates to determine the phylogenetic groups based on the method described by Clermont *et al.* (2000). Each 12.5 µL reaction volume consisted of 1 X KAPA 2G Fast Multiplex PCR master mix (KAPA Biosystems, South Africa), 0.2 µM of each primer (Table 6) and 0.25 µL template DNA.

Table 6 List of primer sequences and amplicon sizes used for t-PCR (Clermont *et al.*, 2000)

Target gene	Primer	Primer sequence (5' - 3')	Size (bp)
chuA	chuA (F) chuA (R)	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279
yjaA	yjaA (F) yjaA (R)	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211
TspE4.C2	TspE4.C2 (F) TspE4C2 (R)	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152

(F) - Forward primer; (R) - Reverse primer

A positive control (*E. coli* ATCC 25922) which contained genetic markers *chuA*, *yjaA*, as well as the DNA fragment TspE4.C2 was included in each PCR assay. Similarly, a negative control (nuclease-free water) was also included. All tubes were then placed in a G-Storm thermal cycler (Vacutec, South Africa) and reaction conditions in Table 7 were applied.

Table 7 Summary of t-PCR reaction conditions

Step	Temperature (°C)	Time (mm:ss)
Initial Denaturation	95	3:00
30 cycles of:		
Denaturation	95	00:30
Primer annealing	60	00:30
Extension	72	0:30
Final Extension	72	05:00

A 2% (m/v) agarose (SeeKem, Switzerland) containing 1 µg.mL^{-1} ethidium bromide (Sigma, Germany) was used for the analysis of PCR products. Gel electrophoresis was performed at 210 V for 20 min. Thereafter band patterns were observed on a UV-

transilluminator (Vacutec, South Africa) to determine which combination of the three DNA fragments were amplified in each strain. This allowed each strain to be assigned to one of four main phylogenetic groups (A, B1, B2 or D) which could further be divided into seven phylogenetic subgroups (A_0, A_1, B1, $B2_2$, $B2_3$, D_1 or D_2) (Carlos *et al.*, 2010) as shown in Table 8.

Table 8 Distribution of genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2 denoting each of the seven phylogenetic subgroups (Carlos *et al.*, 2010)

Phylogenetic group	*chuA*	*yjaA*	TspE4.C2
A_0	-	-	-
A_1	-	+	-
B1	-	-	+
$B2_2$	+	+	-
$B2_3$	+	+	+
D_1	+	-	-
D_2	+	-	+

RESULTS AND DISCUSSIONS

Prevalence of Total Coliforms and *E. coli*

The coliform counts exceeded the *E. coli* counts in all the collected samples (Figs. 1, 2 and 3). This was expected since *E. coli* usually only represents a fraction of the total coliform population (Gemmell & Schmidt, 2012). It was also observed that high total coliform counts were not always indicative to the presence of high *E. coli* counts in this study. This was seen in certain samples (Aquaculture, L-Dam and Smartie Town) (Fig. 2) where high total coliform counts were observed (as high as log 5.062 MPN.100 mL^{-1}) only low *E. coli* counts (log 2.301 MPN.100 mL^{-1}) were detected.

The Colilert 18 method uses 4-methylumbelliferyl-β-D-glucuronide (MUG) to detect the presence of *E. coli* in water samples, which fluoresces in the presence of β-glucuronidase (IDEXX, South Africa). *Escherichia coli* in the human gut use β-glucuronidase to break-down liver conjugates such as steroid glucuronides (McIntosh *et al.*, 2012). It should be noted that there may be some under-estimation of *E. coli* numbers when using the Colilert 18 method, as some *E. coli* strains lack β-glucuronidase activity and consequently do not fluoresce in the presence of MUG (Maheux *et al.*, 2011). *Escherichia coli* O157:H7 strains that lack β-glucuronidase activity have been reported to be frequently responsible for large outbreaks of severe enteric infections (Rump *et al.*, 2012).

It is important to note the large variation in total coliform and *E. coli* counts from site-to-site, as well as the number of sites (10/19 = 52.6%) showing counts above the recommended *E. coli* guidelines of 1 000 counts.100 mL^{-1} (WHO, 1989; DWAF, 1996) for 'safe' irrigation water. The Department of Water Affairs and Forestry (DWAF) guidelines

(DWAF, 1996) also associate risk with various levels of *E. coli* present in irrigation water utilised for fresh produce. Water containing undetectable loads of *E. coli* is classified as having 'no risk' associated when using the water for irrigation of fresh produce. Water with *E. coli* counts ranging between one and 999 *E. coli* counts.100 mL^{-1} is classified as 'low risk' and water with an *E. coli* level ranging between 1 000 and 3 999 *E. coli* counts.100 mL^{-1} is a 'high risk' (DWAF, 1996). Although the risk of disease transmission rises when unacceptable *E. coli* levels are detected in water sources, it must be stated that even the presence of *E. coli* in low numbers in water can serve as a possible risk of disease.

Irrigation sites

The total coliform and *E. coli* counts in all sampled irrigation sites (Fig. 1) with the exception of grey water and Veldwagters River were considerably lower than those of the contamination source sites (Fig. 2). Total coliforms and *E. coli* counts across all the irrigation sites ranged from log 3.477 to log 7.862 MPN.100 mL^{-1} and log 2.301 to log 5.364 MPN.100 mL^{-1}, respectively. The high total coliforms and *E. coli* loads that were detected in this study were similar to previous studies on the microbiological quality of surface waters used for irrigation purposes in the Western Cape (Lötter, 2010; Britz *et al.*, 2013).

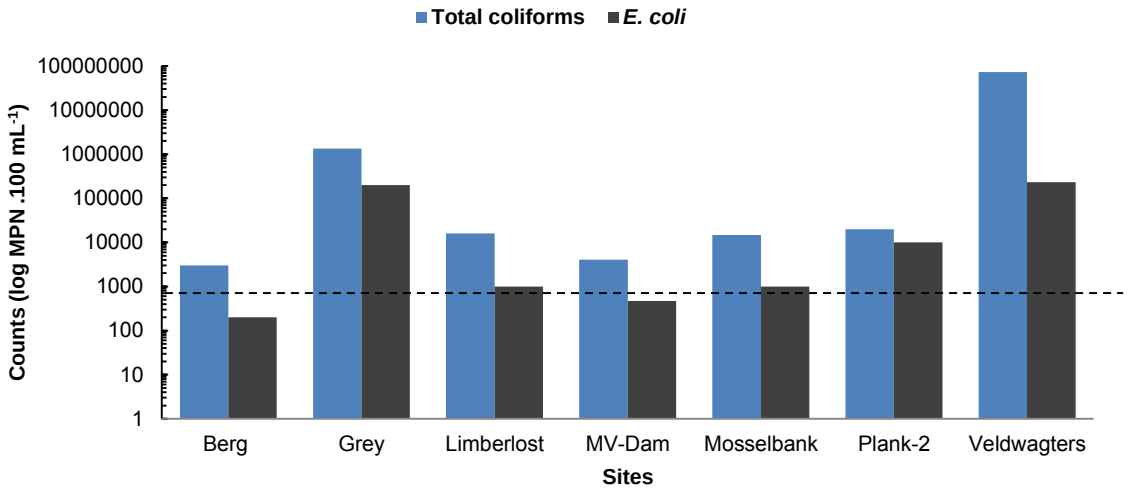

Figure 1 Maximum total coliforms and *E. coli* levels present in the water from the sampled irrigation sources (dotted line is upper limit for 'low risk' by DWAF)

When comparing the low and high risk guideline of DWAF (1996) to the *E. coli* counts present in irrigation water monitored in this study, it can be seen that the Berg River and MV-Dam samples were the only sites that had *E. coli* counts lower than the log 3 MPN.100 mL^{-1} guideline. This means that this water can be considered a 'low risk' when used to irrigate fresh produce as stated in the published guideline (DWAF, 1996). Both the Limberlost and

Mosselbank River sites had *E. coli* counts between log 3 and log 3.602, suggesting that these waters might have a slightly higher risk (DWAF, 1996) associated when used to irrigate fresh produce.

On the top end of the scale and exceeding the upper limit for 'high risk' water (log 3.602 and higher) as stated in the published guidelines (DWAF, 1996) are the grey water, Plank-2 and Veldwagters River waters. Grey water is wastewater generated from domestic activities (such as laundry, dishwashing and bathing) and is often used to irrigate plant crops (Li *et al.*, 2009). Although grey water excludes domestic sewage discharge, high *E. coli* counts have been reported in previous studies (Maimon *et al.*, 2010). Previous studies on the microbial quality of the Plankenburg River have also reported high *E. coli* counts at the Plank-2 site (Lötter, 2010; Huisamen, 2012; Van Blommestein, 2012). The source of contamination at the Plank-2 site is expected to be similar to that of the Plank-1 site, as it is situated downstream from the Kayamandi informal settlement. The high *E. coli* counts observed in the Veldwagters River samples could be as result of the treated effluent from the Stellenbosch Sewage Works that enters the river not far from this sampling point. This is in agreement with a study by Okeke *et al.* (2011) who observed high *E. coli* counts in a river polluted with treated effluent from a sewage works.

Contamination sites

In this study contamination sources were considered potential sources of direct faecal contamination and reservoirs for high concentrations of *E. coli*. As such, it was expected that *E. coli* counts recorded in water from these sites would exceed the recommended guideline of log 3.000 MPN.100 mL^{-1} for 'safe' irrigation water (DWAF, 1996).

Total coliform and *E. coli* counts in the studied contamination source sites ranged from log 3.037 to log 8.837 MPN.100 mL^{-1} and from log 1.000 to log 8.145 MPN.100 mL^{-1}, respectively (Fig. 2). It was observed that all contamination source sites with the exception of the Aquaculture, L-Dam and Smartie Town site had *E. coli* counts that exceeded the log 3 MPN.100 mL^{-1} guideline for the irrigation of fresh produce (DWAF, 1996). This therefore confirms that farm (dairy, piggery and horse) and chicken abattoir effluent could carry high loads of coliform bacteria as well as *E. coli*.

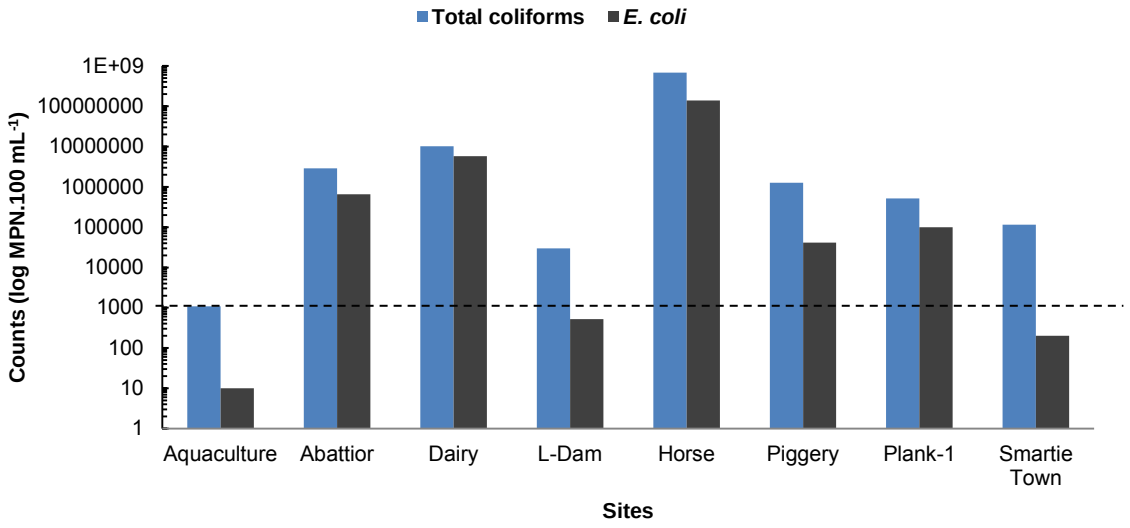

Figure 2 Maximum total coliforms and *E. coli* levels present in the water from the sampled contamination sites (dotted line is upper limit for 'low risk' by DWAF)

Although water from the contamination sources sampled in this study (Fig. 2) are not directly used for irrigation, they do all eventually merge with or cross paths with a river/dam which is used for irrigation. It can also be deduced that the higher the microbial counts present in the water at the source, the higher the chances of it still having microbial contamination levels when it reaches groundwater and surface water catchment areas. Along with this, sampling of contamination sources and characterisation of *E. coli* present will possibly allow for tracing the microbial source of contamination (Derrien *et al.*, 2012).

It can also be concluded that human pollution plays a significant role in introducing microbial contaminants into the water sources, by looking at the Plankenburg River before (Plank-0) and after (Plank-1) an informal settlement. The Plankenburg River was sampled both before and after the Kayamandi informal settlement and it was found that there was a substantial increase in the levels of both total coliforms (log 3.130 to log 5.716 MPN.100 mL^{-1}) and *E. coli* (log 2.491 to log 5.000 MPN.100 mL^{-1}). Where water was collected five km before the informal settlement (Plank-0), it had not passed any informal settlement. While the Plank-1 site which is situated immediately after the informal settlement, showed an increase in both total coliforms and *E. coli* counts to log 5.716 MPN.100 mL^{-1} and log 5.000 MPN.100 mL^{-1}, respectively. This gives an indication of the effect of an informal settlement on water quality.

Environmental sites

These sampling sites (Table 3) were selected to represent environmental sites with no direct source of faecal contamination and subsequently low total coliforms and *E. coli* levels were

therefore expected (Plank-0). Water from these dams was also included in this study to evaluate the impact that wild birds and fish can have on water quality (E-Dam, HH-Dam and W-Dam). In all environmental sites sampled both total coliform and *E. coli* counts were substantially lower than those of contamination source and irrigation sites (Fig. 3).

Total coliforms and *E. coli* levels from environmental sites ranged from log 3.130 to log 3.613 MPN.100 mL^{-1} and log 2 to log 2.491 MPN.100 mL^{-1}, respectively. None of the environmental sites therefore had *E. coli* levels that exceeded the guidelines for irrigation water and could be considered as a low risk for bacterial transfer (DWAF, 1996). Although low *E. coli* counts were detected in water samples from environmental sites, the possible presence of *E. coli* pathotypes could still be a considerable risk to human health. This is confirmed by Masters *et al.* (2011), who reported that *E. coli* numbers in surface waters do not correlate with the presence of *E. coli* virulence genes.

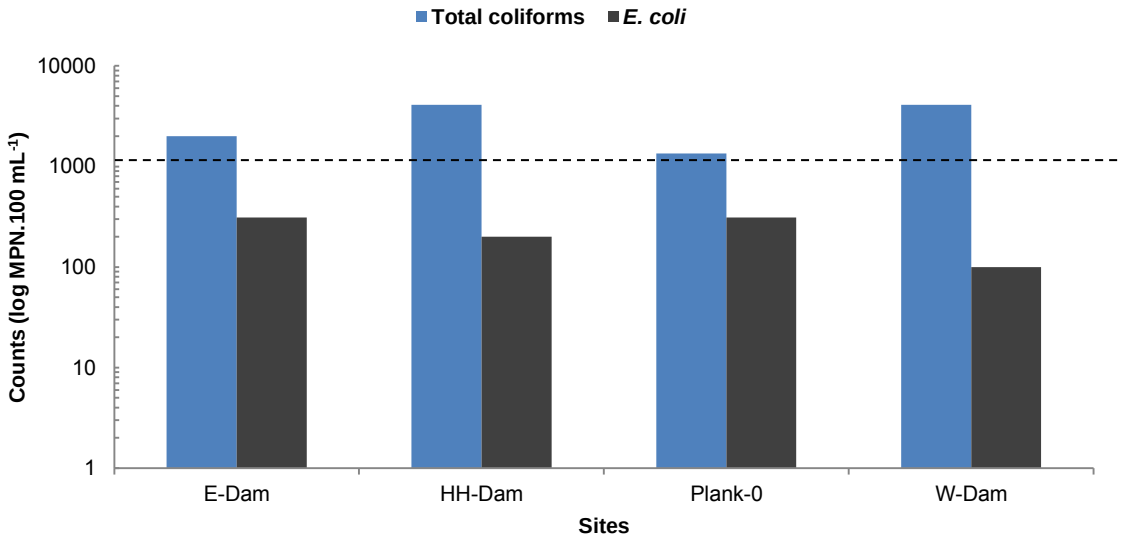

Figure 3 Maximum total coliforms and *E. coli* levels present in the water from the sampled environmental sites (dotted line is upper limit for 'low risk' by DWAF)

Identification of Isolates

The investigation of water samples from the irrigation, contamination source and environmental sites led to the isolation of 140 coliform isolates in total, of which 120 isolates were identified as *E. coli* (Addendum A). The API 20E system (BioMérieux, South Africa) was used to identify isolates and the *uidA*-PCR analysis was used to confirm the identity of presumptive *E. coli* isolates. All 120 *E. coli* isolates that were identified as *E. coli* by the API 20E system yielded positive results in the *uidA*-PCR which confirmed the identity of *E. coli* isolates.

It was found that *Citrobacter*, *Enterobacter*, *Kluyvera*, *Leclercia* and *Serratia* species, in addition to *E. coli,* were identified amongst the isolates. These coliform isolates (n =20) tested negative with the *uidA*-PCR, which was expected, since these coliform isolates mostly lack β-glucuronidase activity (Maheux *et al.*, 2011). *Escherichia vulneris* from the same genus as *E. coli* (Merck, 2007), was also identified amongst the isolates and also tested positive for the *uidA* gene. A study by Rice *et al.* (1995) detected at least a small portion of *E. vulneris* isolates as false positives in the PCR detection of *E. coli* using the *uidA* gene.

Other studies on irrigation water from natural sources in the Stellenbosch and surrounding areas have found high levels of *Citrobacter* spp. in the water (Lötter, 2010; Van Blommestein, 2012). This could mean that *Citrobacter* spp. has been isolated due to their prevalence or because they possibly out-competed some of the *E. coli* isolates. The presence of *Enterobacter* spp. can be explained by the use of L-EMB agar as a growth medium to isolate *E. coli*. Although typical *E. coli* growth on L-EMB agar is a colony with a green metallic sheen, it is known that *Enterobacter cloacae* may also show this attribute (Merck, 2007). The *Kluyvera* spp. isolates from the trout aquaculture dam can be justified by the study of Navarrete *et al.* (2012) that observed *Kluyvera* spp. to be one of the dominant microbial species in rainbow trout. Ewing (1986) described *Leclercia adecarboxylata* isolates with phenotypic characteristics similar to *E. coli* because it had the distinctive ability to utilise lactose, not utilise citrate and also produce indole. This could possibly explain why *Leclercia* spp. was isolated during this study.

API 20E and AHC Analysis

The dissimilarity dendrogramme (Fig. 4) was created by using the Jaccard statistical method (S_J) which included all the sampled isolates (n = 140), marker (n = 37) and ATCC reference strains (n = 6) in Addendum A. The variation within the dataset of strains (n = 183) is clearly expressed in terms of their biochemical profiles generated by the API 20E system. According to the dissimilarity matrix illustrated in Fig. 4, a large characteristic variation is depicted among the *E. coli* strains. It can be seen that there are 36 smaller clusters containing anything from one single strain to a group of 117 strains. In this case, the strains contained in each of these groups all have an identical biochemical profile that results in 0% variance within a cluster.

The 36 smaller clusters are reduced to just five by AHC analysis which statistically cuts off the groups at the level illustrated by the dotted line in Fig. 4 (XLSTAT, 2012.4.03). Each of these clusters had a maximum variation of 61.2%, while the variation between clusters was at least 38.8% (Ntushelo, N., 2013, Biometrical Services, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication). This 61.2% can be seen

as intrinsic error that illustrates the degree of genetic diversity within the major clusters and the same species.

By statistically dividing the clusters into five major groups, namely A to E (Fig. 4), the clusters are easier to work with and grouping of strains can be seen more conclusively. In Table 9, the distribution of strains among the five major dendrogramme clusters is presented. The first observation to be made is that clusters D and E only contain one strain. Cluster D has the only isolate that was identified by API 20E as *Serratia marascens* (M112) and cluster E has the only isolate identified as *Citrobacter youngae* (M138). *Enterobacter* spp. and *Leclercia* spp. isolates were grouped into cluster C (n = 7) and all the *E. coli* isolates were grouped into cluster A (n = 57) and B (n = 117). It should be noted that the four *E. vulneris* isolates were also grouped into cluster A among the *E. coli* isolates. The similar biochemical profiles could be explained by *E. vulneris* that belongs to the same genus as *E. coli* (Merck, 2007). Eight *Kluyvera* spp. isolates also grouped among the *E. coli* isolates in cluster B since they had very similar biochemical profiles. Only the tests for the fermentation/oxidation of D-saccharose (SAC) and amygdalin (AMY) in the *Kluyvera* spp. isolates differed to that of the *E. coli* isolates.

According to the data in Table 9, filled cells contain isolates from contamination sources, unfilled cells contain isolates from irrigation sites, bordered cells contain isolates from environmental sites, horizontal striped cells contain marker strains and vertical striped cells contain ATCC reference strains. The distribution of strains among the 5 major groups (A to E) can be seen in Table 9. Of the 57 strains in cluster A, 26 were isolated from contamination sources (26/57 = 45.6%), 18 from environmental sites (18/57 = 31.6%), 11 were marker strains (11/57 = 19.3%) and 2 from irrigation sites (2/57 = 3.5%) (Table 9). Of the 117 strains in cluster B, 35 were isolated from contamination sources (35/117 =29.9%), 32 from irrigation sites (32/117 = 27.4%), 24 were marker strains (24/117 = 20.5%), 20 from environmental sites (20/117 = 17.1%) and 6 were reference strains (6/117 = 5.1%) (Table 9). Of the 7 strains in cluster C, 2 were isolated from contamination sources, 2 from environmental sites, 2 were marker strains and 1 from an irrigation site (Table 9). The single isolate in clusters D and E were from an irrigation and environmental site, respectively (Table 9).

It can be concluded that most of the strains that were observed to be grouped into clusters A and B were from contamination source sites. The distribution of *E. coli* isolated from environmental sites between clusters A and B were very similar, while the distribution of *E. coli* from irrigation sites and marker strains were more erratic. Only 2 *E. coli* isolated from irrigation sites were grouped into cluster A, while 32 *E. coli* from irrigation sites were grouped into cluster B. Only 11 marker strains were grouped in cluster A, while 24 marker strains were grouped into cluster B. Cluster A had the highest degree of biochemical variation

indicative to diverse metabolic capacities of strains in this cluster, as 16 minor clusters were apparent within this major cluster.

The main biochemical differences between the *E. coli* strains were the tests for L-ornithine decarboxylase (ODC) activity, followed by the fermentation/oxidation of SAC and D-sorbitol (SOR) tests. This correlates with the study by Janezic *et al.* (2013), where biochemical differences were also observed for the ODC and SAC tests for *E. coli* isolated from untreated surface waters. Similar biochemical profiles were also observed for *E. coli* isolated from soil in the study by Brennan *et al.* (2010).

Escherichia coli are able to induce a number of amino-acid decarboxylases such as ODC in response to reduced pH conditions (Kanjee *et al.*, 2011). Of the 117 *E. coli* strains in cluster B, 98.3% were observed to possess ODC activity, while 91.2% of the 57 *E. coli* strains in cluster A lacked ODC activity. The *E. coli* strains in cluster B could have been exposed to certain environmental stresses that enable ODC activity. Since the *E. coli* strains in cluster A did not possess ODC activity, they possibly were not exposed to the same environmental stresses as experienced by the *E. coli* strains in cluster B.

According to the API 20E system, *E. coli* strains are classified as either *E. coli* type 1 or type 2 (BioMérieux, South Africa). Based on the results in this study, *E. coli* type 1 was the most common type to be isolated from water sources since all 163 *E. coli* strains were characterised as *E. coli* type 1. The lack of ODC activity as well as the lack of fermentation of SAC is associated with *E. coli* type 2 strains (BioMérieux, South Africa). The results in this study emphasise the ability of *E. coli* to adapt to different environmental niches in order to survive (Franz *et al.*, 2011; Lupo *et al.*; 2012).

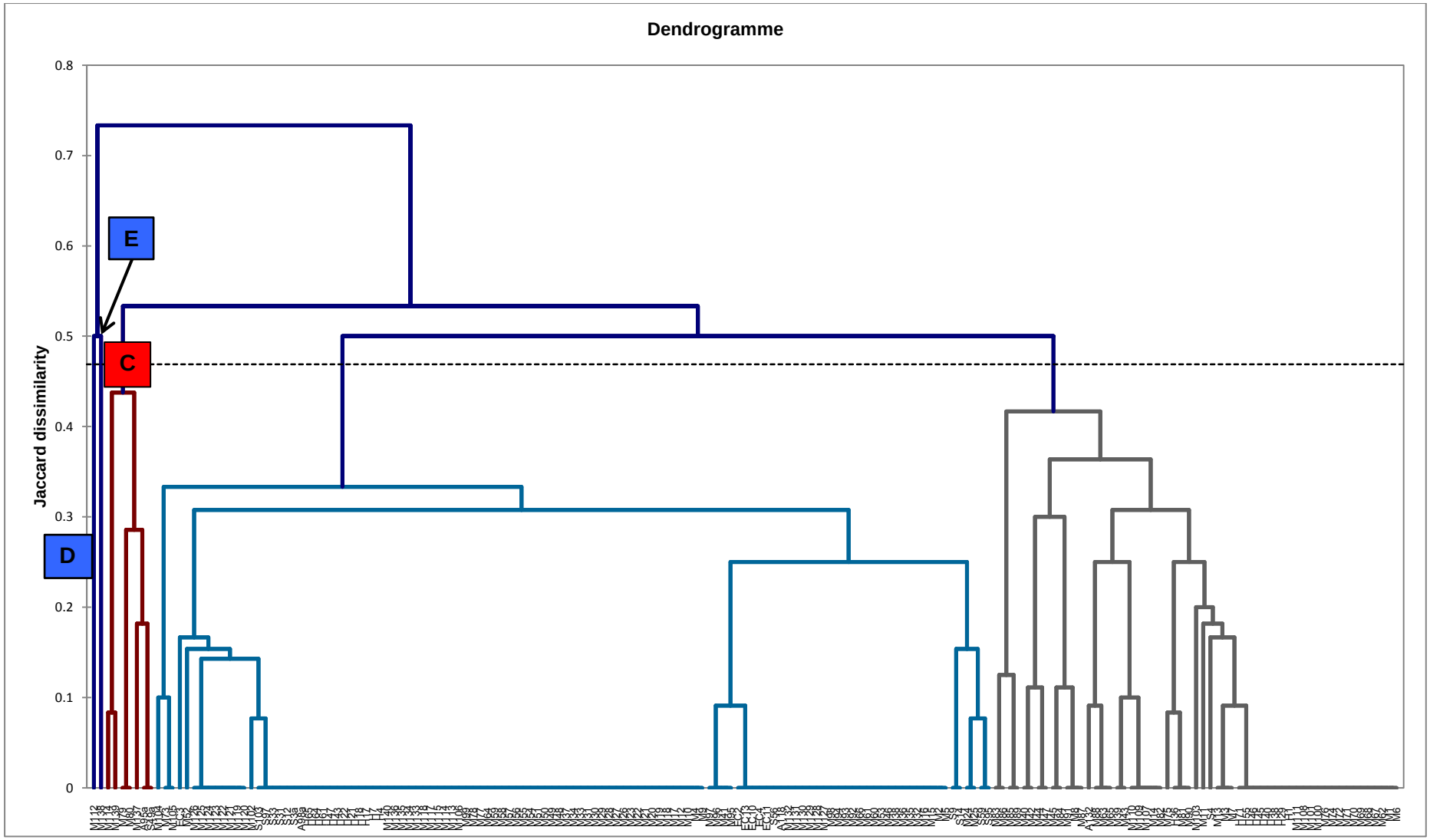

Figure 4 Dissimilarity dendrogramme, based on the S_J dissimilarity coefficient of all isolates including the non *E. coli* using API 20E data as clustering basis

Table 9 Distribution of isolates amongst the five major clusters as determined by the S_J dendrogramme from data generated by the API 20E system (* = non-*E. coli* isolates)

Cluster A	Cluster A (cont.)	Cluster B	Cluster B (cont.)	Cluster B (cont.)	Cluster C	Cluster D	Cluster E
M1	M103	M2	M56	M131	M79*	M112*	M138*
M3	M107	M4	M57	M132	M80*		
M6	M108	M5	M58	M133	M114*		
M7	M109	M9	M59	M134	M137*		
M8	M110	M10	M60	M135	M139*		
M11	M111	M12	M61	M136	A95a		
M13	H1	M15	M64	M140	S49a		
M14	H29	M16	M66	H4			
M39	H36	M17	M73	H7			
M40	H38	M18	M77	H17			
M42	H40	M19	M78	H18			
M43	H45	M20	M92	H21			
M44	H46	M21	M93	H22			
M45	H55	M22	M94	H43			
M47	H71	M23	M95	H47			
M62	A132	M24	M96	H61			
M63	S4	M25	M97	H64			
M65		M26	M98	H65			
M67		M27	M99	A98a			
M68		M28	M102	A118			
M69		M29	M104	S3a			
M70		M30	M105	S9			
M71		M31	M106	S12			
M72		M32	M113	S14			
M74		M33	M114	S31			
M75		M34	M115	S33			
M76		M35	M117	S56			
M81		M36	M118	S59			
M82		M37	M119*	S95			
M83*		M38	M120*	S97			
M84		M41	M121*	S103			
M85*		M46	M122*	EC11			
M86*		M48	M123*	EC4			
M87		M49	M124*	EC10			
M88		M50	M125*	EC13			
M89*		M51	M126*	EC2			
M90		M52	M127	EC3			
M91		M53	M128				
M100		M54	M129				
M101		M55	M130				

Filled cells = contamination sites, unfilled cells = irrigation sites, bordered cells = environmental sites, horizontal striped cells = marker strains and vertical striped cells = ATCC reference strains

E. coli Phylogenetic Group Analysis

Phylogenetic group analysis was used to further characterise the *E. coli* isolates and interpret data generated by the API 20E system and AHC analysis. The PCR analysis of *E. coli* using the method of Clermont *et al.* (2000) enabled the grouping of isolates into one of the four main phylogenetic groups, namely A, B1, B2 and D (Silva *et al.*, 2011). These groups can be further divided into subgroups, namely A_0, A_1, B1, $B2_2$, $B2_3$, D_1 and D_2 (Salehi, 2012). Many deductions can be made with regards to the possible source and potential virulence by determining the phylogeny of an *E. coli* isolate (Obeng *et al.*, 2012).

Two genetic markers (*chuA* and *yjaA*) as well as a DNA fragment (Tsp.E4.C2) were used to determine the phylogenetic groups (Clermont *et al.*, 2000). An example of the PCR amplified genetic markers and DNA fragment after separation on a 2% agarose gel can be seen in Fig. 5. The banding patterns present in lanes 2-6 each represent a different phylogenetic subgroup (A_1, B1, $B2_3$ and D_2) (Fig. 5). DNA fragments that had been amplified in each isolate could be determined by using the *E. coli* reference strain (ATCC 25922) as a positive control (lane 7). The combination of the amplified fragments led to the allocation of each isolate to a specific phylogenetic group as shown in Fig. 5.

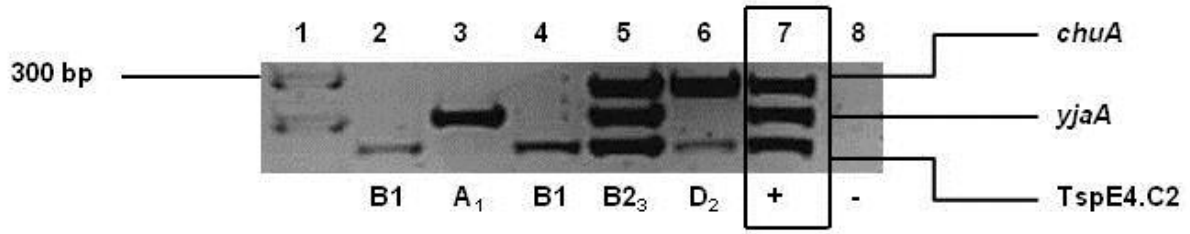

Figure 5 Agarose gel (2% agarose and 1 µg.mL^{-1} ethidium bromide) with triplex PCR amplicons. Lane 1 = 100 bp marker; lane 2–6 = *E. coli* phylogroups B1, A_1, B1, $B2_3$, and D_2.; lane 7 = positive control (ATCC 25922); lane 8 = negative control

In total, 163 *E. coli* strains were analysed that were isolated from irrigation (n = 34), contamination source (n = 49) and environmental sites (n = 37) in Stellenbosch and surrounding areas. *Escherichia coli* marker strains (n = 37) from the Food Science collection and ATCC *E. coli* reference strains (n = 6) were also included in this set of data. The phylogenetic distribution of the *E. coli* strains that were included in this study is presented in Table 10 and Fig. 6.

Pig

The 10 *E. coli* isolates from the piggery clustered mainly in subgroups A_0 (5/10) and A_1 (3/10), which means that 80% of isolates from the piggery were classified in group A (Table 10). In previous studies, group A had been reported to contain mainly commensal and intestinal pathogenic *E. coli* isolates from a variety of hosts (Van Elsas *et al.*, 2011). A study by Carlos *et al.* (2010) showed a similarity between the *E. coli* population structure of humans and pigs that are both omnivorous mammals.

The other 20% (2/10) of the isolates from the piggery were assigned to groups B1 and D_1. Isolates in group B1 have also been found to persist and survive in the external environment (Walk *et al.*, 2007). As viable *E. coli* were isolated from a number of natural water sources in this study, it was expected that a large proportion of the isolates would be assigned to group B1. This was the case as seen in Fig. 6 which therefore concurs with the findings made by Walk *et al.* (2007). As none of the 11 *E. coli* isolates from the piggery were assigned to groups B2 or D_2, it can possibly indicate that only a small proportion of the *E. coli* population in pigs belong to these groups. This concurs with the findings made by Carlos *et al.* (2010), as only 5.1% (2/39) of their pig isolates were found to belong to group B2 or D_2.

Table 10 Distribution of *E. coli* phylogenetic groups among the strains from the irrigation, contamination source, and environmental sites (with marker and ATCC reference strains)

Phylogenetic group	Contamination sources						Irrigation sites	Environmental sites	Marker strains	ATCC strains
	Pig	Cow	Horse	Human	Fish	Chicken				
A_0	5	4	7	1	-	1	5	20	1	-
A_1	4	3	3	-	-	1	-	1	12	-
B1	-	1	6	5	6	-	27	12	16	3
$B2_2$	-	-	-	-	-	-	-	-	-	-
$B2_3$	-	-	-	1	-	-	1	1	4	3
D_1	1	-	-	-	-	-	1	3	3	-
D_2	-	-	-	-	-	-	-	-	1	-
Total	10	8	16	7	6	2	34	37	37	6

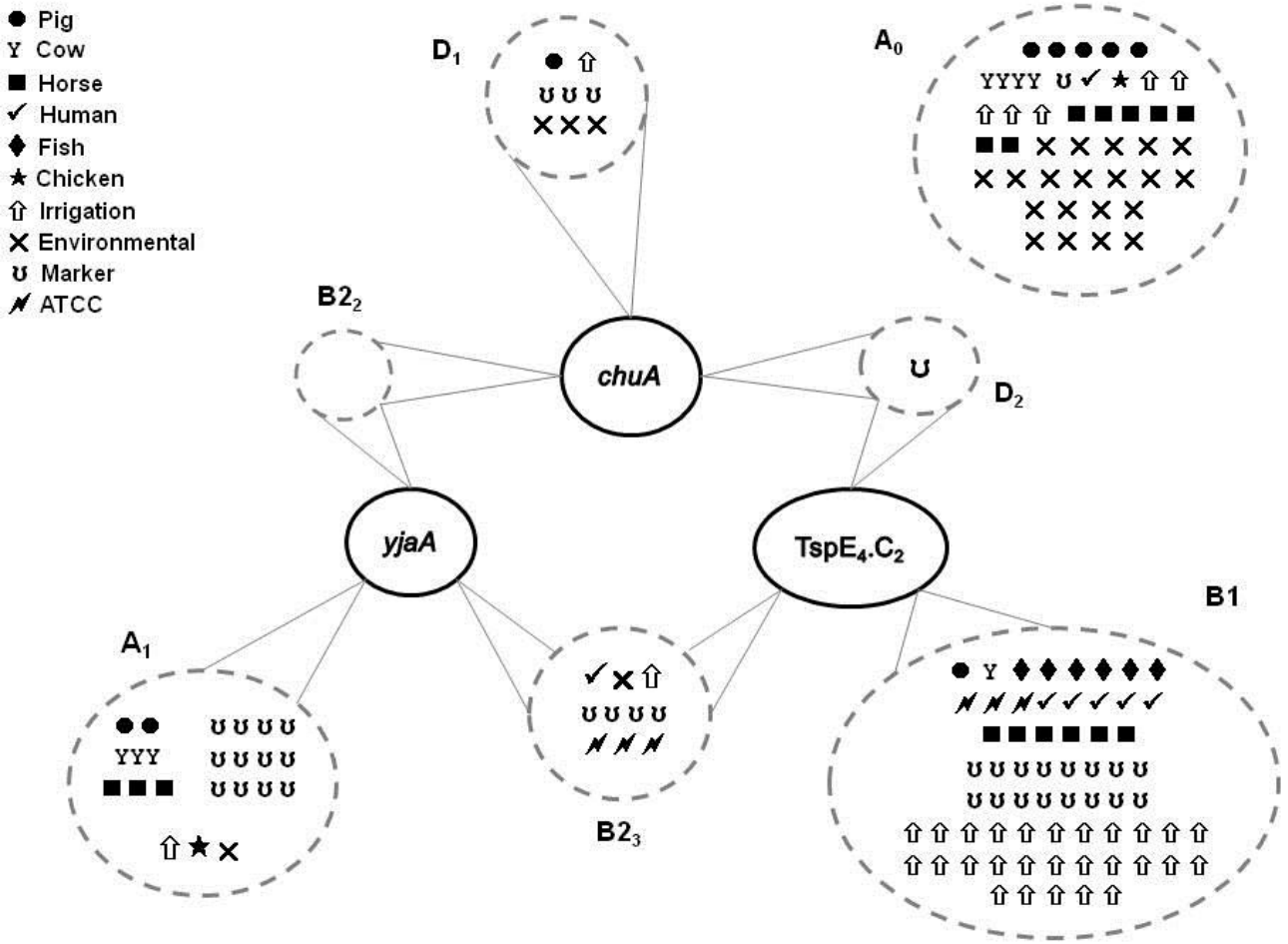

Figure 6 Graphic representation of the occurrence of genetic markers in the *E. coli* strains. Circles with a solid outline represent each genetic marker (*chuA* and *yjaA*) and the DNA fragment (TspE4.C2). Isolates from different sources are represented by different shapes. Lines leading from the genetic markers to subgroups (outlined in dotted lines) show that the genetic marker was present in strains from that subgroup. Based on the representation scheme developed by Carlos *et al.* (2010).

Cow

Escherichia coli isolates from the dairy (8) were mainly (7/8 = 87.5%) assigned to group A (Table 10). Only one dairy isolate was grouped in B1 but none in B2 or D. According to Escobar-Páramo *et al.* (2006) *E. coli* from domestic animals are mostly associated with groups A and B1. As none of the *E. coli* isolates from the dairy were assigned to groups B2 or D, it is possible that only a small proportion of the *E. coli* population in cows belong to these groups. This concurs with the studies by Carlos *et al.* (2010) and Tenaillion *et al.* (2011) that a low proportion of strains in groups B2 and D occur in domesticated animals. According to White *et al.* (2011), virulent *E. coli* pathotypes belong mostly to groups B2 and D.

Horse

The *E. coli* isolates from the horse stables clustered mostly in groups A (10/16 = 62.5%) and B1 (6/16 = 37.5%) (Table 10). This concurs with previous studies that *E. coli* strains in groups A and B1 appear to be best adapted to animals (Silva *et al.*, 2011). As none of the 16 *E. coli* isolates from horses were assigned to groups B2 or D, it might be possible to indicate that only a small proportion of the *E. coli* population in horses belong to these groups. This concurs with the observation that a decreased proportion of strains in groups B2 and D occur in domesticated animals (Carlos *et al.*, 2010; Tenaillon *et al.*, 2010).

Human impact

The impact of informal housing was represented by the seven *E. coli* isolates from a stream that passed a certain area (‘Smartie Town’) within Cloetesville, a low-income residential area in Stellenbosch. The main group (5/7 = 71.4%) was reported to be B1 (Table 10). One isolate was assigned to group B2 (specifically $B2_3$) and one isolate was assigned to group D. This does not concur with previous studies that reported isolates from humans to be predominantly assigned to groups A and B2 (Gordon, 2010; Figueira *et al.*, 2011). These isolates were not directly isolated from human faecal matter but rather from water that was assumed to be contaminated with human faecal matter. Based on the phylogenetic group results, it was concluded that the source of the *E. coli* isolates was probably not only of human origin.

Fish

Escherichia coli is considered a commensal inhabitant of the lower intestinal tract of mammals (Power *et al.*, 2005). Although *E. coli* is not a normal inhabitant of fish, previous

studies have shown its presence in the stomach and intestines of fresh water fish (Guzmán *et al.*, 2004; Stachowiak *et al.*, 2010). All *E. coli* isolates (6/6 = 100.0%) from the trout-breeding dam were assigned to group B1. This is in agreement with Walk *et al.* (2007) that *E. coli* from group B1 are able to persist in the aquatic environment. This also concurs with the findings made by Koo *et al.* (2012), as 17/34 = 50.0% of their fish isolates were assigned to group B1. Phylogenetic group B1 is seen by Tenaillon *et al.* (2010) as a 'generalist' group that contains *E. coli* isolates from fish, reptiles and birds.

Chicken

The two *E. coli* isolates from the free-range chicken abattoir effluent were assigned to groups A_0 and A_1. This is in agreement with the findings made by Obeng *et al.* (2012) since their free-range chicken isolates mainly clustered in group A (65/193 = 33.7%). Salehi *et al.* (2012) described *E. coli* isolates from broiler chickens to also be predominantly characterised into group A (176/241 = 73.0%). According to the study by Koo *et al.* (2012), most food-borne isolates from poultry in Korea were also assigned to group A (38/61 = 62.3%).

Irrigation sites

The phylogenetic distribution of the 34 *E. coli* isolates from irrigation water (Berg River, Mosselbank River, MV-Dam and Limberlost River), showed a majority (27/34 = 79.4%) assigned to group B1. When considering that Walk *et al.* (2007) found that isolates from group B1 survive in the environment with ease, it is possible to assume that the majority of *E. coli* isolates found in the environment will fall within this group. Some of the irrigation water isolates were also distributed in groups A_0 (5/34 = 14.7%), D (1/34 = 2.9%) and B2 (1/34 = 2.9%) (Table 10). A study by Koczura *et al.* (2013) observed that the majority of *E. coli* isolated from river water belonged to group A. The smaller percentages of isolates that were assigned to these groups can be explained by outside factors that constantly introduce new contaminants from a variety of sources. Surface waters may also cross-contaminate other rivers and dams, particularly in the rainy season when rivers and dams overflow (Oliviera *et al.*, 2012).

Environmental sites

In this study, isolates from 'environmental' sites were defined as isolates that were found in rivers before potential contamination source sites were able to affect the water quality. These isolates therefore are those which are considered to be naturally present in water systems, hence they do not come from a particular contamination source. Water from dams

was also included in this study to evaluate the phylogenetic distribution of *E. coli* from wild birds and fish. Isolates that were classed as 'environmental' were isolated from the Plankenburg River at site Plank-0 and dams (E-Dam, HH-Dam and W-Dam).

When investigating the population structure of the 37 *E. coli* isolates from environmental sites, it was found that most of the isolates were assigned to group A (21/37 = 56.8%) and B1 (12/37 = 32.4%), while the remainder were characterised as D (3/37 = 8.1%), A_1 (1/37 = 2.7%) and B2 (1/37 = 2.7%) (Table 10). Escobar-Páramo *et al.* (2006) described the occurrence of groups B1 and D to be predominantly present in birds. When taking the work of White *et al.* (2011) into consideration, it was thought that isolates from water sources would be mostly assigned to group B1. This was not the case in this study.

It was concluded that it is not possible to isolate a group of *E. coli* from the environment with the expectation that they are purely environmental isolates that have originated in the environment. This is because there is so much cross-contamination as well as historical practices which could have led to the introduction of *E. coli* from a variety of sources into the surface water.

Marker and reference strains

Escherichia coli marker strains (n = 37) included in this data set were mainly grouped into B1 (16/37 = 43.2%) and A (13/37 = 35.1%) groups. The remaining *E. coli* marker strains were grouped in B2 (4/37 = 10.8%) and D (4/37 = 10.8%), respectively. The high amount of strains that were clustered in group B1 correlates with the previous work done by Walk *et al.* (2007). A similar phylogenetic distribution was expected for the markers strains and isolates from irrigation sites included in this study, since the marker strains were isolated from similar irrigation sources. The ATCC *E. coli* reference strains included in this study (n = 6) were grouped into B1 (3/6 = 50.0%) and B2 (3/6 = 50.0%).

Escherichia coli population structures

The data in Table 10 shows the distribution of the *Escherichia coli* isolates across the seven phylogenetic subgroups from sampled sites (irrigation, contamination and environmental sources) included in this study. *Escherichia coli* marker strains and ATCC reference strains were also included to compare and identify similar distribution patterns across the phylogenetic groups. The natural habitat of *E. coli* is the gastro-intestinal tract of warm-blooded animals (Van Elsas *et al.*, 2011). In a study conducted by Tenaillion *et al.* (2011), it was established that dietary requirements and type of digestive system of the primary host do have a role in determining the *E. coli* population structure. The findings in this study are similar to the results in the study by Tenaillion *et al.* (2011) as different phylogenetic

distributions were apparent among the *E. coli* isolates from irrigation, contamination source and environmental sites.

Most of the *E. coli* strains belonged to group B1 (77/163 = 47.2%) followed by group A (67/163 = 41.1%). Six percent (9/163) of strains were assigned to group D and four percent (7/163) were assigned to group B2. Isolates from pigs, cows, horses and chickens had a similar distribution of isolates among the phylogenetic groups. Contamination source sites did not exhibit such a large amount of *E. coli* isolates in group B1. However, it was found that water sampled from irrigation sites mostly contained isolates assigned to group B1 (27/34 = 79.4%). Water sampled from environmental sites had isolates that mostly grouped into A_0 (20/37 = 54.1%) followed by B1 (12/37 = 32.4%). Marker strains grouped mainly into B1 (16/37 = 43.2%) followed by A_1 (12/37 = 32.4%). This may be useful when undertaking source tracking of *E. coli* strains, as this information allows for linking a population attribute from irrigation to contamination water.

CONCLUSIONS AND RECOMMENDATIONS

The study on the occurrence of *E. coli* from the sampled sites revealed that more than half (10/19 = 52.6%) of the sample sites had *E. coli* counts that exceeded the WHO and DWAF guidelines for irrigation water of fresh produce to be consumed raw or minimally processed (WHO, 1989; DWAF, 1996). Bacterial species from the *Enterobacteriaceae* family were isolated from all sampling sites, which indicated that these sites were subjected to faecal pollution. The investigation of irrigation sites determined that 5/7 = 71.4% of the surface waters sampled were deemed as 'unsafe' for irrigation purposes. It can be concluded from this study that the presence of total coliform bacteria and *E. coli* in water used for irrigation poses a definite threat to farmers who use these natural water sources to irrigate fresh produce. Even though *E. coli* was only detected at low concentrations in the environmental samples, some pathogenic *E. coli* have very low infectious doses and could therefore still cause disease, even when only a few bacterial cells are present.

The ease with which *E. coli* is able to incorporate and transfer genetic material enables this bacterium to easily adapt to different environments. Adaptations can include the acquisition of genes that improves the survival of a bacterium in a particular environment such as genes for pathogenic potential, toxin production and resistance to antibiotics. The large degree of phylogenetic and biochemical variation (especially within the *E. coli* species), emphasises the ability of this bacterium to adapt to different environments, making them particularly dangerous if they are pathogenic. The large degree of variation present in irrigation water therefore could have human health implications and ought to be further investigated.

The wide antibiotic resistance profiles that have been reported in pathogens further complicate the problem of pathogens associated with poor microbiological quality of irrigation water. There is growing concern about the development of antibiotic resistance in pathogens and subsequent transfer to humans through contaminated food. Antibiotic resistant bacteria associated with food animals have been extensively documented but research regarding resistant bacteria in irrigation water is limited. There is therefore a need for a further study to determine the presence of antibiotic resistant *E. coli* in irrigation and other surface waters.

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Addendum A Isolate identification according to API 20E (including percentage certainty and identification rating) and PCR (*uidA* and triplex)

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
Elsenburg L-Dam	M1	*E. coli*	99.9%	Excellent	+	A_1
Elsenburg Pig	M2	*E. coli*	99.9%	Excellent	+	A_0
Elsenburg Pig	M3	*E. coli*	97.5%	Good	+	A_1
Elsenburg Cow	M4	*E. coli*	99.5%	Very Good	+	A_0
Elsenburg Pig	M5	*E. coli*	99.9%	Excellent	+	A_0
Elsenburg L-Dam	M6	*E. coli*	99.9%	Excellent	+	A_1
Elsenburg Pig	M7	*E. coli*	97.5%	Good	+	A_1
Elsenburg Pig	M8	*E. coli*	99.9%	Excellent	+	A_0
Elsenburg Cow	M9	*E. coli*	99.5%	Very Good	+	A_0
Elsenburg Cow	M10	*E. coli*	99.5%	Very Good	+	A_0
Elsenburg L-Dam	M11	*E. coli*	99.6%	Very Good	+	A_1
Elsenburg Cow	M12	*E. coli*	99.5%	Very Good	+	B1
Elsenburg Pig	M13	*E. coli*	97.5%	Good	+	A_1
Elsenburg Pig	M14	*E. coli*	98.0%	Good	+	D_1
Elsenburg Pig	M15	*E. coli*	99.9%	Excellent	+	A_0
Elsenburg Pig	M16	*E. coli*	99.9%	Excellent	+	A_0
Elsenburg Cow	M17	*E. coli*	99.5%	Very Good	+	A_0
Elsenburg Pig	M18	*E. coli*	99.5%	Very Good	+	B1
Berg River	M19	*E. coli*	99.5%	Very Good	+	B1
Berg River	M20	*E. coli*	99.5%	Very Good	+	B1
Berg River	M21	*E. coli*	99.5%	Very Good	+	B1
Berg River	M22	*E. coli*	99.5%	Very Good	+	B1
Berg River	M23	*E. coli*	99.5%	Very Good	+	B1
Berg River	M24	*E. coli*	99.2%	Doubtful Profile	+	A_0
Berg River	M25	*E. coli*	99.2%	Doubtful Profile	+	B1

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
Berg River	M26	*E. coli*	99.5%	Very Good	+	A_0
Mosselbank	M27	*E. coli*	99.5%	Very Good	+	B1
Mosselbank	M28	*E. coli*	99.5%	Very Good	+	B1
Mosselbank	M29	*E. coli*	99.5%	Very Good	+	B1
Mosselbank	M30	*E. coli*	99.5%	Very Good	+	B1
Mosselbank	M31	*E. coli*	99.5%	Very Good	+	B1
Smartie Town	M32	*E. coli*	99.9%	Excellent	+	B1
Smartie Town	M33	*E. coli*	99.5%	Very Good	+	B1
Smartie Town	M34	*E. coli*	99.5%	Very Good	+	A_0
Smartie Town	M35	*E. coli*	99.9%	Excellent	+	B1
Smartie Town	M36	*E. coli*	99.9%	Excellent	+	B1
Smartie Town	M37	*E. coli*	99.5%	Very Good	+	$B2_3$
Smartie Town	M38	*E. coli*	99.9%	Excellent	+	B1
Horse	M39	*E. coli*	99.4%	Very Good	+	B1
Horse	M40	*E. coli*	98.3%	Good	+	B1
Horse	M41	*E. coli*	99.9%	Excellent	+	A_0
Horse	M42	*E. coli*	98.3%	Good	+	A_0
Horse	M43	*E. coli*	99.4%	Very Good	+	B1
Horse	M44	*E. coli*	99.9%	Excellent	+	B1
Horse	M45	*E. coli*	99.4%	Very Good	+	A_0
Horse	M46	*E. coli*	99.9%	Excellent	+	A_0
Horse	M47	*E. coli*	96.8%	Good	+	A_0
Limberlost	M48	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M49	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M50	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M51	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M52	*E. coli*	98.5%	Good	+	B1

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
Limberlost	M53	*E. coli*	99.9%	Excellent	+	B1
Limberlost	M54	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M55	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M56	*E. coli*	99.5%	Very Good	+	$B2_3$
Limberlost	M57	*E. coli*	99.5%	Very Good	+	B1
Limberlost	M58	*E. coli*	99.5%	Very Good	+	B1
MV-Dam	M59	*E. coli*	99.5%	Very Good	+	A_0
MV-Dam	M60	*E. coli*	99.9%	Excellent	+	B1
MV-Dam	M61	*E. coli*	99.9%	Excellent	+	B1
MV-Dam	M62	*E. coli*	99.9%	Excellent	+	A_0
MV-Dam	M63	*E. coli*	99.9%	Excellent	+	D_1
HH-Dam	M64	*E. coli*	99.5%	Very Good	+	B1
HH-Dam	M65	*E. coli*	99.9%	Excellent	+	$B2_3$
HH-Dam	M66	*E. coli*	99.9%	Excellent	+	B1
Eversdal	M67	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M68	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M69	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M70	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M71	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M72	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M73	*E. coli*	99.2%	Very Good	+	A_0
Eversdal	M74	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M75	*Serratia odorifera 2*	95.1%	Good	-	A_0
Eversdal	M76	*E. coli*	99.9%	Excellent	+	A_0
Chicken	M77	*E. coli*	99.5%	Very Good	+	A_0
Chicken	M78	*E. coli*	99.5%	Very Good	+	A_1
Chicken	M79	*Enterobacter cloacae*	59.2%	Excellent ID To Genus	-	N.A

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
Chicken	M80	*Enterobacter cloacae*	59.2%	Excellent ID To Genus	-	N.A
Horse	M81	*E. coli*	95.9%	Good	+	A_1
Horse	M82	*E. coli*	98.0%	Good	+	A_0
Horse	M83	*E. vulneris*	83.4%	Acceptable	+	B1
Horse	M84	*E. coli*	99.4%	Very Good	+	A_0
Horse	M85	*E. vulneris*	95.5%	Very Good ID To Genus	+	B1
Horse	M86	*E. vulneris*	83.4%	Acceptable	+	B1
Horse	M87	*E. coli*	99.9%	Excellent	+	A_1
Horse	M88	*E. coli*	99.9%	Excellent	+	B1
Horse	M89	*E. vulneris*	95.5%	Very Good ID To Genus	+	B1
Horse	M90	*E. coli*	95.9%	Good	+	A_1
Horse	M91	*E. coli*	99.9%	Excellent	+	B1
Eversdal	M92	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M93	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M94	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M95	*E. coli*	99.9%	Excellent	+	D_1
Eversdal	M96	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M97	*E. coli*	99.9%	Excellent	+	D_1
Eversdal	M98	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M99	*E. coli*	99.5%	Very Good	+	B1
Eversdal	M100	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M101	*E. coli*	99.9%	Excellent	+	A_0
Eversdal	M102	*E. coli*	99.8%	Very Good	+	B1
Eversdal	M103	*E. coli*	99.1%	Very Good	+	A_1
Eversdal	M104	*E. coli*	97.9%	Good	+	A_0
Eversdal	M105	*E. coli*	99.2%	Very Good	+	A_0
HH-Dam	M106	*E. coli*	99.5%	Very Good	+	B1

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
HH-Dam	M107	*E. coli*	98.0%	Good	+	B1
HH-Dam	M108	*E. coli*	99.9%	Excellent	+	A_0
HH-Dam	M109	*E. coli*	98.0%	Good	+	B1
HH-Dam	M110	*E. coli*	98.0%	Good	+	A_0
HH-Dam	M111	*E. coli*	99.9%	Excellent	+	D_1
MV-Dam	M112	*Serratia marcescens*	98.1%	Good	-	N.A
MV-Dam	M113	*E. coli*	99.5%	Very Good	+	A_0
MV-Dam	M114	*E. coli*	99.5%	Very Good	+	B1
MV-Dam	M115	*E. coli*	99.5%	Very Good	+	B1
MV-Dam	M116	*Enterobacter amnigenus*	80.2%	Low Discrimination	-	N.A
MV-Dam	M117	*E. coli*	99.5%	Very Good	+	B1
MV-Dam	M118	*E. coli*	99.5%	Very Good	+	D_1
Aquaculture	M119	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M120	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M121	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M122	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M123	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M124	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M125	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M126	*Kluyvera spp*	83.1%	Low Discrimination	-	N.A
Aquaculture	M127	*E. coli*	99.9%	Excellent	+	B1
Aquaculture	M128	*E. coli*	99.9%	Excellent	+	B1
Aquaculture	M129	*E. coli*	99.9%	Excellent	+	B1
Aquaculture	M130	*E. coli*	99.9%	Excellent	+	B1
Aquaculture	M131	*E. coli*	99.9%	Excellent	+	B1
Aquaculture	M132	*E. coli*	99.9%	Excellent	+	B1
W-Dam	M133	*E. coli*	99.5%	Very Good	+	B1

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
W-Dam	M134	*E. coli*	99.5%	Very Good	+	B1
W-Dam	M135	*E. coli*	99.5%	Very Good	+	B1
W-Dam	M136	*E. coli*	99.5%	Very Good	+	B1
W-Dam	M137	*Leclercia adecarboxylata*	85.9%	Low Discrimination	-	N.A
W-Dam	M138	*Citrobacter youngae*	72.7%	Doubtful Profile	-	N.A
W-Dam	M139	*Enterobacter amnigenus*	84.0%	Low Discrimination	-	N.A
W-Dam	M140	*E. coli*	99.5%	Very Good	+	B1
Marker (Plank 1)	H1	*E. coli*	99.9%	Excellent	+	A_0
Marker (Plank 1)	H4	*E. coli*	99.5%	Very Good	+	B1
Marker (Bean)	H7	*E. coli*	99.5%	Very Good	+	B1
Marker (Plank 1)	H17	*E. coli*	99.9%	Excellent	+	D_1
Marker (Plank 1)	H18	*E. coli*	99.9%	Excellent	+	B1
Marker (Bean)	H21	*E. coli*	99.5%	Very Good	+	B1
Marker (Bean)	H22	*E. coli*	99.9%	Excellent	+	D_1
Marker (Bean)	H29	*E. coli*	99.4%	Very Good	+	A_1
Marker (Plank 1)	H36	*E. coli*	98.3%	Good	+	A_1
Marker (Bean)	H38	*E. coli*	99.9%	Excellent	+	A_1
Marker (Bean)	H40	*E. coli*	98.3%	Good	+	A_1
Marker (Plank 1)	H43	*E. coli*	99.4%	Very Good	+	B1
Marker (Plank 1)	H45	*E. coli*	99.9%	Excellent	+	D_1
Marker (Plank 1)	H46	*E. coli*	99.4%	Very Good	+	A_1
Marker (Bean)	H47	*E. coli*	99.9%	Excellent	+	B1
Marker (Plank 1)	H55	*E. coli*	96.8%	Good	+	A_1
Marker (Plank 1)	H61	*E. coli*	99.5%	Very Good	+	A_1
Marker (Bean)	H64	*E. coli*	99.5%	Very Good	+	B1
Marker (Bean)	H65	*E. coli*	99.5%	Very Good	+	B1
Marker (Bean)	H71	*E. coli*	99.5%	Very Good	+	A_1

Source	Isolate	API ID	API % certainty	API ID Rating	*uidA*-PCR (+/-)	t-PCR Phylogroup
Marker (Plank 2)	A95a	*E. coli*	88.2%	Acceptable	+	$B2_3$
Marker (Plank 2)	A98a	*E. coli*	99.5%	Very Good	+	B1
Marker (Plank 2)	A118	*E. coli*	99.9%	Excellent	+	B1
Marker (Plank 2)	A132	*E. coli*	99.5%	Very Good	+	$B2_3$
Marker (Borehole)	S3a	*E. coli*	99.5%	Very Good	+	A_1
Marker (Borehole)	S4	*E. coli*	99.9%	Doubtful Profile	+	A_1
Marker (Borehole)	S9	*E. coli*	99.6%	Doubtful Profile	+	A_1
Marker (Borehole)	S12	*E. coli*	99.5%	Very Good	+	B1
Marker (Borehole)	S14	*E. coli*	99.6%	Doubtful Profile	+	A_1
Marker (Borehole)	S31	*E. coli*	99.2%	Doubtful Profile	+	D_2
Marker (Borehole)	S33	*E. coli*	99.5%	Very Good	+	B1
Marker (Plank 2)	S49a	*E. coli*	88.2%	Acceptable	+	$B2_3$
Marker (Olifants)	S56	*E. coli*	99.9%	Excellent	+	B1
Marker (Olifants)	S59	*E. coli*	99.9%	Doubtful Profile	+	B1
Marker (Spring)	S95	*E. coli*	99.9%	Doubtful Profile	+	B1
Marker (Spring)	S97	*E. coli*	99.5%	Very Good	+	B1
Marker (Dam)	S103	*E. coli*	99.5%	Very Good	+	$B2_3$
ATCC (11775)	EC11	*E. coli*	99.9%	Excellent	+	$B2_3$
ATCC (4350)	EC4	*E. coli*	99.9%	Excellent	+	B1
ATCC (10799)	EC10	*E. coli*	99.9%	Excellent	+	B1
ATCC (13135)	EC13	*E. coli*	99.9%	Excellent	+	B1
ATCC (25922)	EC2	*E. coli*	99.2%	Very Good	+	$B2_3$
ATCC (35218)	EC3	*E. coli*	99.9%	Excellent	+	$B2_3$

CHAPTER 4

DETERMINATION OF ANTIBIOTIC RESISTANCE AND PATHOTYPES OF *ESCHERICHIA COLI* FROM IRRIGATION WATER AND POTENTIAL CONTAMINATION SITES

SUMMARY

Escherichia coli isolates from irrigation, contamination and environmental sources were evaluated for antibiotic resistance by means of the Kirby-Bauer disc diffusion assay. In this study, 163 *E. coli* strains were screened for antibiotic resistance to seven medically-important antibiotics from different classes. Thirty-five out of 163 (21.5%) *E. coli* strains exhibited resistance to at least one antibiotic. Most antibiotic resistant *E. coli* strains were assigned to phylogenetic groups A_1 (37.1%), B1 (28.6%) and A_0 (22.9%), while 11.4% were assigned to group B2. It should be noted that no antibiotic resistant *E. coli* strains were assigned to group D.

Piggery effluent was the source with the highest percentage of antibiotic resistant *E. coli* isolates (9/10 = 90.0%) resistant to chloramphenicol (30 µg), tetracycline (30 µg) and trimethoprim (2.5 µg) singly or in different combinations. Among the resistant *E. coli* strains, the highest percentage of antibiotic resistance was against trimethoprim (68.6%), tetracycline (57.1%), ampicillin (10 µg) (45.7%) and chloramphenicol (34.3%). Forty-nine percent (17/35) of the resistant *E. coli* strains displayed multi-antibiotic resistance to three (16/35) or four antibiotics (1/35), while the remaining 51.4% (18/35) displayed resistance to one (9/35) or two (9/35) antibiotics.

The antibiotic resistant *E. coli* strains were evaluated for potential pathogenicity using Polymerase Chain Reaction to detect Intestinal Pathogenic *E. coli* (InPEC) and Extra-intestinal Pathogenic *E. coli* (ExPEC). In this study, five InPEC strains were characterised, four Entero-Pathogenic *E. coli* (EPEC) strains resistant to three or four antibiotics and one Entero-Aggregative *E. coli* (EAEC) strain resistant to trimethoprim. The antibiotic resistant EAEC strain also had possessed the ExPEC-related aerobactin receptor gene *iutA*. Two *E. coli* isolates from the Mosselbank River both resistant to chloramphenicol and trimethoprim were also carriers of the ExPEC-related gene *iutA*.

INTRODUCTION

The occurrence of microbial antibiotic resistance has increased rapidly over the last decade and is a major public health threat throughout the world, particularly in developing countries (Li *et al.*, 2009). When pathogens become resistant to antibiotics they can pose a greater

human health risk as a result of potential treatment failure, decrease in treatment options and subsequent increased severity of disease (DeWaal *et al.*, 2012). The management of microbial infections has become increasingly complicated due to the emergence of pathogen resistance to most first-line antibiotics (Akter *et al.*, 2012). This means that a first-line antibiotic may not be effective for treatment of a certain infection so a second or third-choice antibiotic will be required that may be less effective, more toxic and more expensive (Wright, 2011).

Of particular concern, are the implications of resistance to antibiotics that are used in human medicine and food animals (Powell *et al.*, 2009; Laing *et al.*, 2011). Studies on farms have shown an association of multi-antibiotic resistant *E. coli* with the intensive exposure to antibiotics (Lampang *et al.*, 2008; Obeng *et al.*, 2012). A study by Tadesse *et al.* (2012) similarly described *E. coli* isolates from food animals with high percentages of antibiotic resistance.

Sanitation in communities with a lack of access to clean water is another factor that leads to the contamination of water sources (Paulse *et al.*, 2012). In South Africa, poverty levels and overcrowded informal settlements in such areas are issues that further complicate the problem of antibiotic resistance (Kinge *et al.*, 2010). In more recent years, studies on the microbiological quality of water in many of South Africa's rivers revealed unacceptable and dangerous *E. coli* levels (Gemmell & Schmidt, 2012; Britz *et al.*, 2013). The presence of pathogenic *E. coli* in contaminated rivers of South Africa was confirmed by previous studies (Huisamen, 2012; Van Blommestein, 2012). Similarly, a study on rivers in Durban described *E. coli* isolates with virulence potential that were resistant to multiple antibiotics (Olaniran *et al.*, 2009). If faecally contaminated waters are not disinfected before they are used for drinking or for irrigation purposes, it could result in waterborne diseases such as diarrhoea, dysentery, cholera and hepatitis (Nagar *et al.*, 2011). Treatment of these diseases could be further complicated if pathogenic isolates were also resistant to medically important antibiotics.

Since antibiotic resistance genes are often found on mobile genetic elements, bacteria are able to freely exchange genetic material (Cambray *et al.*, 2010). This is of great concern because commensal bacteria that acquire an antibiotic resistance mechanism may later transfer that resistance mechanism to a pathogenic strain (Li *et al.*, 2009). Studies have demonstrated that *E. coli* may persist and multiply in the external environment outside the host and are important vectors in the dissemination of antibiotic resistance (Bucci *et al.*, 2011; Van Elsas *et al.*, 2011).

Escherichia coli is a normal inhabitant of the gastro-intestinal tract of humans and warm-blooded animals (Ahmed *et al.*, 2010). However, *E. coli* strains that are able to resist antibiotics have become a serious problem in terms of human health (Chen *et al.*, 2011).

Certain pathotypes such as Shiga-Toxin producing *E. coli* (STEC), Enterohaemorhagic *E. coli* (EHEC) and Enterotoxigenic *E. coli* (ETEC) have been associated with waterborne-disease outbreaks and mortality in humans (Ram *et al.*, 2008). The transfer of resistant pathogens from the farm to food production environment creates the possibility of life-threatening and untreatable infections in patients that acquire food-borne illness (Tadesse *et al.*, 2012).

The objective of this study will be to determine the prevalence of antibiotic resistant *E. coli* in water sources used for irrigation purposes as well as potential contamination sources in the Western Cape. Antibiotic resistant *E. coli* will also be screened for pathogenic strains using Polymerase Chain Reaction (PCR) analysis.

MATERIALS AND METHODS

Isolates

The investigation of water samples from the irrigation, contamination sources and environmental sites (Table 1) led to the isolation of 120 *E. coli* isolates (see Chapter 3 of this thesis) that were positively identified as *E. coli* using the API 20E system (BioMérieux, South Africa) and the *uidA*-PCR. Six American Type Culture Collection (ATCC) *E. coli* reference strains and a set of 37 *E. coli* marker strains from the Food Science collection were also included in the dataset of strains (Table 1). Overall, 163 *E. coli* strains were evaluated for antibiotic resistance (Table 1).

Table 1 Number of *E. coli* isolated from the sources included in this study

Source	No. of isolates/strains	Description
Irrigation	34	Surface water used for irrigation of fresh produce
Contamination	49	Sources of *E. coli* with the potential to contaminate irrigation water
Environmental	37	No direct source of faecal contamination
Marker	37	*E. coli* isolated from irrigation sources during previous studies
ATCC	6	*E. coli* reference strains as comparative controls

Determination of Antibiotic Resistance of Escherichia coli

Antibiotic resistance tests were performed using the Kirby-Bauer disc diffusion assay (Andrews, 2009; CLSI, 2009) on all 163 *E. coli* strains that were identified using the API 20E system (BioMérieux, South Africa) and the *uidA*-PCR. Before antibiotic resistance testing, *E. coli* strains were revived that were stored in the presence of 40% (v/v) glycerol (Fluka Analytical, Germany) in cryotubes at -80°C. This was done by inoculating 5 mL sterile Nutrient Broth (NB) (Merck, South Africa) with 100 µL aliquots of the bacterial suspension from the cryotubes and subsequently incubating at 37°C for 24 h. After incubation, a 10^{-3} dilution series was prepared from each nutrient broth culture using Sterile Saline Solution (SSS) (0.85% m/v NaCl). Purity of the *E. coli* isolates was confirmed by inoculating Eosine Methylene-Blue Lactose Sucrose Agar (L-EMB) (Oxoid, South Africa) plates with 100 µL aliquots of the 10^{-2} and 10^{-3} bacterial suspensions by means of the spread-plate method and subsequently incubating at 37°C for 24 h. Colonies that showed a metallic green sheen after incubation denoted typical *E. coli* growth (Merck, 2007) and were regarded as *E. coli* colonies. Bacterial suspensions of pure *E. coli* strains were prepared by inoculating sterile NB with typical *E. coli* colonies and incubating at 37°C for 24 h. The turbidity of the bacterial suspension after incubation was visually adjusted to 0.5 McFarland standard (BioMérieux, South Africa) using SSS.

Mueller-Hinton Agar (MH) (Oxoid, South Africa) plates were inoculated in duplicate with 100 µL aliquots of the adjusted bacterial suspension by means of the spread-plate method. MH plates were dried for 3 to 5 min in a laminar flow cabinet before applying the antibiotics discs. Seven antibiotic discs were applied to the surface of the MH plates with a disc dispenser (MAST, South Africa) and subsequently incubated at 37°C for 24 h. The antibiotic discs included ampicillin 10 µg (MAST, South Africa), cephalothin 30 µg (MAST, South Africa), chloramphenicol 30 µg (MAST, South Africa), ciprofloxacin 5 µg (MAST, South Africa), streptomycin 10 µg (Liofilchem, Italy), tetracycline 30 µg (MAST, South Africa) and trimethoprim 2.5 µg (MAST, South Africa).

The above antibiotics were selected based on the significance in the treatment of different *Enterobacteriaceae* infections as given by Doyle *et al.* (2013) and the WHO's (World Health Organisation) list of critically important antibiotics for human health (WHO, 2012). The antibiotics listed as part of the standardised method for *Enterobacteriaceae* antibiotic resistance tests (Andrews, 2009) and previous reports of resistant *E. coli* isolated from similar sources (De Verdier *et al.*, 2012; Zou *et al.*, 2012) were also used as the selection criteria for antibiotics.

The interpretation of inhibition zones was based on internationally accepted break-points as summarised by the manufacturer (Liofilchem, Antibiotic disc interpretative criteria

and quality control F1403 Rev.5, 2011) and the standardised method of Andrews (2009) (Table 2). The Minimal Inhibitory Concentration (MIC) is the lowest concentration of an antibiotic that prevents growth of a particular pathogen (DeWaal *et al.*, 2012) and is seen as an inhibition zone on the MH plate. The MIC data is converted to break-points (indicated by S, I and R in Table 2) that are used to interpret the susceptibility/resistance of microorganisms. An *E. coli* strain was considered to be susceptible to a certain antibiotic if the diameter (mm) of the inhibition zone was the same or larger (S ≥) than the corresponding break-point (Table 2). An *E. coli* strain was intermediately resistant (I) to a certain antibiotic if the diameter (mm) of the inhibition zone was within the range of the corresponding break-point (Table 2). Resistance to a certain antibiotic was observed if the diameter (mm) of the inhibition zone was the same or smaller (R ≤) than the corresponding break-point (Table 2).

Table 2 Inhibition zone criteria for interpreting antibiotic resistance of *E. coli* strains (Andrews, 2009; Liofilchem, Antibiotic disc interpretative criteria and quality control F1403 Rev.5, 2011)

Antibiotic	**Diameter of Inhibition Zone**		
	S ≥ (mm)	**I (mm)**	**R ≤ (mm)**
ampicillin 10 µg	17	14-16	13
cephalothin 30 µg	18	15-17	14
chloramphenicol 30 µg	18	13-17	12
ciprofloxacin 5 µg	21	16-20	15
streptomycin 10 µg	15	12-14	11
tetracycline 30 µg	15	12-14	11
trimethoprim 2.5 µg	17	14-16	13

S = susceptible, I = intermediate and R = resistant

The *E. coli* ATCC 25922 reference strain was included in all antibiotic resistance analyses as a negative control as it is susceptible to all the antibiotics included in this study (ATCC, 2013). The *E. coli* ATCC 35218 reference strain was included as a positive control since it produces the non-extended β-lactamase TEM-1 and is subsequently resistant to ampicillin (ATCC, 2013). A quality-control plate that was inoculated with only the antibiotics and contained no organism was also left open in the laminar flow cabinet to check for contamination during the drying process of every antibiotic resistance assay. The interpretation of resistance and susceptibility of an *E. coli* strain to the antibiotics included in this study is illustrated in Fig. 1. It can be seen in Fig. 1 that the *E. coli* strain was resistant to two antibiotics since no inhibition zones were apparent (indicated by R) and was susceptible to five antibiotics (indicated by S).

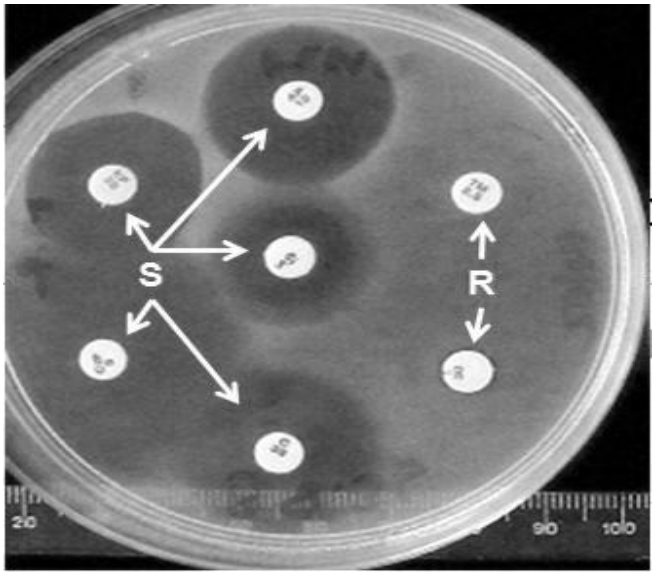

Figure 1 Muellller-Hinton Agar plate inoculated with an *E. coli* strain and seven antibiotic discs (S indicates susceptible and R indicates resistant)

PCR Methods

DNA template preparation

The DNA template of the strains was prepared according to the method of Altahi *et al.* (2009). All the strains were cultivated on Tryptone Soya Agar (TSA) (Oxoid, South Africa) for 24 h at 37°C. Following this, a colony of each was boiled in a 1.5 mL micro-centrifuge tube with 100 µL nuclease-free water for 13 min to lyse the cells and release its content. The tubes were cooled on ice and centrifuged (Vacutec, South Africa) for 15 min at 14 000 x g to pellet the cell debris. The supernatant that contained the DNA template was subsequently transferred to a sterile tube and stored at -18°C.

PCR detection of Intestinal Pathogenic E. coli (InPEC)

The 35 *E. coli* strains that exhibited resistance to at least one of the antibiotics included in this study were further screened for the presence of InPEC using a modified method of Omar & Barnard (2010). This was done to possibly correlate antibiotic resistance with *E. coli* pathotypes since resistance is often associated with mobile genetic elements that may also carry *E. coli* pathotype genes (Da Silva & Mendonça, 2012). Each 12.5 µL PCR reaction volume consisted of 1 x KAPA 2G fast multiplex PCR master mix (KAPA Biosystems, South Africa), 0.2 µM of each primer (Table 3) and 0.25 µL template DNA.

Table 3 Multiplex PCR primers for the detection of InPEC pathotypes

Pathotype (gene)	Primer	Primer sequence (5' – 3')	Size (bp)	Reference
Control (*mdh*)	Mdh01 Mdh02	GTATGGATCGTTCCGACCT GGCAGAATGGTAACACCAGAGT	300	Tarr *et al.* (2002)
EIEC (*ial*)	L-ial (F) Ial (R)	GGTATGATGATGATGAGTCCA GGAGGCCAACAATTATTTCC'	650	Lopez-Suacedo *et al.* (2003)
EPEC/EHEC (*eaeA*)	L-eaeA (F) L-eaeA (R)	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGC	384	Lopez-Suacedo *et al.* (2003)
EHEC (*stx1*, *stx2*)	Stx1 (F) Stx1 (R) Stx2 (F) Stx2 (R)	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCACTTTG	614 779 	Moses *et al.* (2006) Moses *et al.* (2006)
ETEC (*LT*, *ST*)	LT (F) LT (R) ST (F) ST (R)	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT TTTCCCCTCTTTTAGTCAGTCAACTG GGCAGGATTACAACAAAGTTCACA	450 160 	Lopez-Suacedo *et al.* (2003) Omar & Barnard (2010)
EAEC (*eagg*)	Eagg (F) Eagg (R)	AGACTCTGGCGAAAGACTGTATC ATGGCTGTCTGTAATAGATGAGAAC	194	Pass *et al.* (2000)

(F) - Forward primer; (R) - Reverse primer

As positive control, a standard culture mix (SCM) was prepared which contained genetic markers (*ial*, *eaeA*, *stx1*, *stx2*, *LT*, *ST* and *eagg*) representing the five InPEC classes (EAEC, EHEC, EIEC, EPEC and ETEC) (Table 3). The positive control reaction tube was prepared using 1.25 µL SCM as DNA template. A negative control (nuclease-free water) was included with each PCR assay. All PCR reaction tubes were placed in a G-Storm thermal cycler (Vacutec, South Africa) and reaction conditions outlined in Table 4 were applied.

Table 4 PCR Cycling Protocol for the detection of InPEC pathotypes

Step	Temperature (°C)	Time (mm:ss)
Initial Denaturation	95	03:00
30 cycles of:		
Denaturation	95	00:15
Primer annealing	55	00:30
Extension	68	00:30
Final Extension	72	05:00

PCR products were analysed with gel electrophoresis in a 1.25 % agarose (SeeKem, Switzerland) gel containing 1 µg.mL^{-1} ethidium bromide (Sigma, Germany). Gel electrophoresis was performed at 120 V for 90 min and the PCR bands were visualised on a UV-transilluminator (Vacutec, South Africa).

PCR detection of Extra-intestinal Pathogenic E. coli (ExPEC)

All 35 *E. coli* strains that exhibited resistance to at least one antibiotic were screened for the six most abundant ExPEC gene sequences using the modified method of Xia *et al.* (2011). Each 12.5 µL PCR reaction volume consisted of 1 x KAPA 2G fast multiplex PCR master mix (KAPA Biosystems, South Africa), 0.2 µM of each primer (except *afa/dra*) in Table 5 and 0.25 µL template DNA. All strains were screened separately for the presence of the *afa/dra* gene sequence in a simplex PCR under the same reaction conditions.

Table 5 Multiplex PCR primers for the detection of ExPEC pathotypes (Xia *et al.*, 2011)

Target gene	Primer	Primer sequence (5' – 3')	Size (bp)
papA	papA (F) papA (R)	ATGGCAGTGGTGTCTTTTGGTG CGTCCCACCATACGTGCTCTTC	717
papC	papC (F) papC (R)	GTGGCAGTATGAGTAATGACCGTTA ATATCCTTTCTCAGGGATGCAATA	205
sfa/foc	sfa/foc (F) sfa/foc (R)	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410
afa/dra	afa/dra (F) afa/dra (R)	GGCAGAGGGCCGGCAACAGGC CCCGTAACGCGCCAGCATCTC	592
iutA	uitA (F) uitA (R)	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	302
kpsMT 2	kpsMT 2 (F) kpsMT 2 (R)	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272

(F) = Forward primer; (R) = Reverse primer

As positive control, a SCM was prepared by combining cell extracts of ATCC 25922 and a clinical strain JUL 1211, which contained ExPEC genetic markers (*papA*, *papC*, *sfa/foc*, *iutA* and *kpsMT* 2) (Table 5). These were previously identified as the most prominent genetic markers indicative of ExPEC and the presence of two or more of these genetic markers determined ExPEC status (Xia *et al.*, 2011). The positive control reaction tube was made up using 1.25 µL SCM which replaced the DNA template. A negative control where nuclease-free water replaced template DNA was included with each PCR assay. All tubes were placed in a G-Storm thermal cycler (Vacutec, South Africa) and reaction conditions outlined in Table 6 were applied.

Table 6 PCR Cycling Protocol for the detection of ExPEC pathotypes (Xia *et al.*, 2011)

Step	Temperature (°C)	Time (mm:ss)
Initial Denaturation	95	03:00
30 cycles of:		
Denaturation	95	00:15
Primer annealing	61	00:30
Extension	68	00:30
Final Extension	72	05:00

PCR products were analysed with gel electrophoresis in a 1.25 % agarose (SeeKem, Switzerland) gel containing 1 $\mu g.mL^{-1}$ ethidium bromide (Sigma, Germany). Gel electrophoresis was performed at 120 V for 90 min and the PCR bands were visualised on a UV-transilluminator (Vacutec, South Africa).

RESULTS AND DISCUSSIONS

Antibiotic Resistant *E. coli*

Overall, 120 *E. coli* isolated from irrigation (n = 34), contamination sources (n = 49) and environmental sites (n = 37) were included in this study. *Escherichia coli* marker strains (n = 37) and ATCC reference strains (n = 6) were also included in the dataset. The susceptibility of the *E. coli* strains studied to the seven antibiotics included is shown in Table 7. Of the 163 *E. coli* strains in total that were included, 35 strains (35/163 = 21.5%) exhibited resistance to at least one antibiotic (Table 7).

The highest percentage of antibiotic resistance amongst the *E. coli* strains was for trimethoprim (24/35 = 68.6%), followed by tetracycline (20/35 = 57.1%), ampicillin (16/35 = 45.7%), chloramphenicol (12/35 = 34.3%) and streptomycin (7/35 = 20.0%) (Table 7). Of the 163 *E. coli* strains that were included in this study, 14.7% (24/163) of the strains were resistant to trimethoprim (Table 7).

Table 7 Percentage of antibiotic resistant *E. coli* strains from the different sources included in this study (n = 35)

Antibiotic	Irrigation % (n)	Contamination % (n)	Environmental % (n)	Marker % (n)	ATCC % (n)	Total % (n)
AMP	20.0 (7)	0.0 (0)	0.0 (0)	22.9 (8)	2.9 (1)	45.8 (16)
KF	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
C	5.8 (2)	20.0 (7)	2.9 (1)	2.9 (1)	2.9 (1)	34.5 (12)
CIP	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
T	2.9 (1)	25.7 (9)	2.9 (1)	25.7 (9)	0.0 (0)	57.2 (20)
TM	20.0 (7)	14.3 (5)	2.9 (1)	31.4 (11)	0.0 (0)	68.6 (24)
STR	11.4 (4)	0.0 (0)	0.0 (0)	5.8 (2)	2.9 (1)	20.0 (7)

AMP = ampicillin, KF = cephalothin, C = chloramphenicol, CIP = ciprofloxacin, T = tetracycline, TM = trimethoprim and STR = streptomycin

When the results (Table 7) from this study are compared to results reported in the literature, similar *E. coli* antibiotic resistance profiles can be seen. In the literature Su *et al.* (2011) reported a high occurrence of trimethoprim resistance (1403/3456 = 40.6%) in *E. coli* isolated from the Dongjiang River, a major source of drinking water and reservoir of various wastewaters in south China. A high occurrence of ampicillin, chloramphenicol and tetracycline resistance was found in *E. coli* isolated from different water sources in the North-West Province of South Africa (Kinge *et al.*, 2010). No *E. coli* strains in this study were resistant to cephalothin or ciprofloxacin (Table 7). Similar results were reported in another study, where low numbers of *E. coli* isolated from a river were found to be resistant to cephalothin and ciprofloxacin (Li *et al.*, 2009).

Trimethporim is used for the treatment of urinary tract infections caused by *E. coli* pathotypes (McMurdo *et al.*, 2009). In contrast, tetracycline is extensively used as growth promoters in animal feed and for the treatment of human and animal infections (Thaker *et al.*, 2010). Ampicillin is a broad-spectrum antibiotic used for the treatment of meningitis and neonatal sepsis (Puopolo *et al.*, 2010), while chloramphenicol has a broad spectrum of activity and is the approved antibiotic for the control of respiratory tract infections (Lang *et al.*, 2010). Aminoglycosides such as streptomycin is used for the treatment of serious Gram-negative infections of the urinary tract, respiratory tract and central nervous system (Avent *et al.*, 2011). The treatment of illnesses caused by *E. coli* infections resistant to the above mentioned antibiotics will further be complicated since an alternative therapy may be required. According to the study by DeWaal *et al.* (2012) alternative therapy may be less

effective for a particular infection, more expensive and more detrimental to the patient's health.

The occurrence of antibiotic resistant *E. coli* isolated from the different sources included in this study is shown in Fig. 2. Marker strains (12/35 = 34.3%) and *E. coli* isolated from contamination source sites (12/35 = 34.3%) had the highest occurrence of antibiotic resistant *E. coli*, followed by irrigation sites (9/35 = 25.7%) (Fig. 2). One *E. coli* ATCC reference strain (1/35 = 2.9%) and one environmental *E. coli* isolate (1/35 = 2.9%) displayed resistance to at least one antibiotic (Fig. 2).

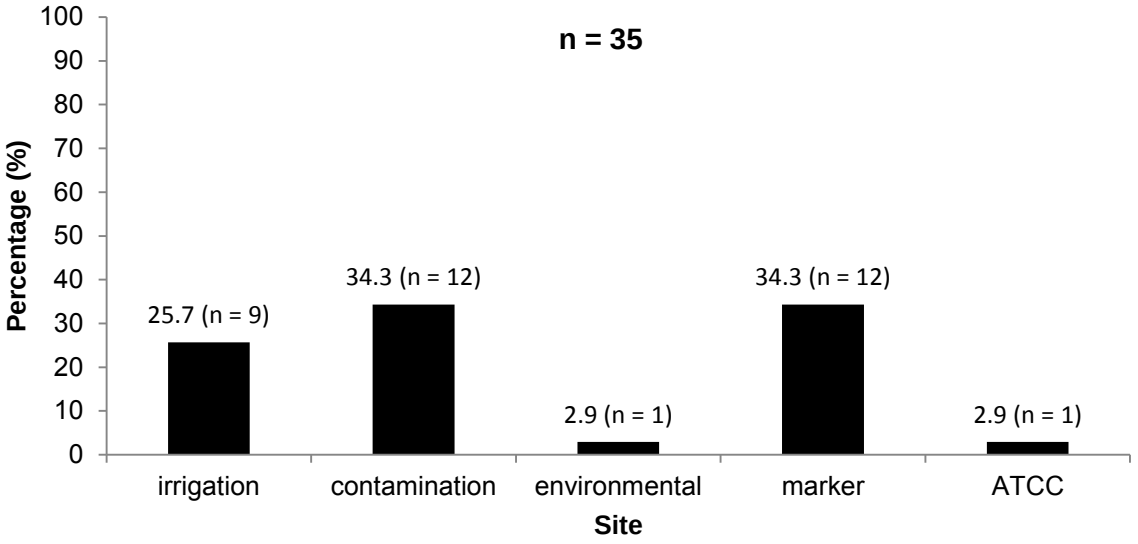

Figure 2 Percentage of antibiotic resistant *E. coli* isolated from the irrigation, contamination source and environmental sites. The marker and reference strains have been included.

Of the *E. coli* isolated from contamination source sites, only pig and cow isolates displayed resistance to one or more antibiotics (Addendum B). Pig isolates had the highest percentage of antibiotic resistant *E. coli* (9/35 = 25.7%), followed by the cow isolates (3/35 = 8.6%) (Addendum B). A high occurrence of antibiotic resistance in *E. coli* isolated from pigs concurs with the study by Córtes *et al.* (2010). No antibiotic resistance was observed for *E. coli* isolated from horses, fish and chickens that were included in this study (Addendum A). This does not correlate with results reported in the literature since high percentages of antibiotic resistant *E. coli* isolated from horses, fish and chickens have been reported in numerous studies (Ahmed *et al.*, 2010; Jiang *et al.*, 2012; Obeng *et al.*, 2012). It should be noted that the *E. coli* isolated in this study from chickens were from free-range and not broiler chickens. The study by Obeng *et al.* (2012) reported a high occurrence of antibiotic resistance amongst *E. coli* isolated from free-range chickens resistant to tetracycline (63/193 = 32.6%) and ampicillin (52/193 = 26.9%).

Escherichia coli marker strains (n = 37) from the Food Science collection were included in this study because they had been isolated in previous studies from similar sources. Of the 37 marker strains, 12 strains (12/37 = 32.4%) displayed resistance to at least one antibiotic (Addendum B). Seven of these 12 antibiotic resistant marker strains were isolated from the Plankenburg River (Plank 1 and Plank 2) (Addendum B). The other remaining five antibiotic resistant marker strains were originally isolated from the surface of green beans that had been irrigated with water from the Plankenburg River (Plank 1) (Addendum B). Similar antibiotic resistance profiles were observed for the marker strains from green beans and the marker strains from the irrigation water (Addendum B). The similar antibiotic resistance profiles among the *E. coli* marker strains may emphasise the potential transfer of *E. coli* from the contaminated irrigation water to fresh produce.

API 20E Cluster Analysis

The API 20E system (BioMérieux, South Africa) was used to identify and characterise the 163 *E. coli* strains included in this study based on their biochemical profiles. Agglomerative Hierarchical Clustering (AHC) analysis (XLSTAT, 2012.4.03) was applied to the API 20E data to generate the dissimilarity dendrogramme (see Fig. 4 in Chapter 3 of this thesis). The clusters were statistically divided into five main groups namely A to E (Table 8) with the Jaccard method (S_J).

The distribution of the 35 antibiotic resistant *E. coli* strains among non-resistant strains in the three major dendrogramme clusters is presented in Table 8. According to the groupings in Table 8, shaded cells contain *E. coli* isolates from contamination sources, unshaded cells contain isolates from irrigation sites, bordered cells contain isolates from environmental sites, horizontal striped cells contain marker strains and vertical striped cells contain the ATCC reference strains. Strain numbers in bold text were *E. coli* that displayed resistance to one or more of the antibiotics included in this study (Table 8).

The antibiotic resistant *E. coli* strains were mostly grouped in cluster B (19/35 = 54.3%) and cluster A (14/35 = 40.0%), while the remainder grouped in cluster C (2/35 = 5.7%) (Table 8). Of the 19 antibiotic resistant *E. coli* strains in cluster B, nine (9/19 = 47.4%) were from irrigation sites, five (5/19 = 26.3%) were marker strains, four (4/19 = 21.1%) from contamination source sites and one (1/19 = 5.3%) was an ATCC reference strain (EC3) (Table 8). Of the 14 antibiotic resistant *E. coli* strains in cluster A, eight (8/14 = 57.1%) were from contamination source sites, five (5/14 = 35.7%) were marker strains and one (1/14 = 7.1%) was from an environmental site (Table 8). Two antibiotic resistant *E. coli* marker strains were grouped in cluster C (Table 8).

It can be seen that an uneven distribution among the three major clusters was apparent with regards to antibiotic resistant *E. coli* isolated from the different sources (except for marker strains). No antibiotic resistant *E. coli* isolated from irrigation sites were grouped in cluster A, while nine isolates were in cluster B. Eight antibiotic resistant *E. coli* isolated from contamination source sites were grouped in cluster A, while only four isolates were in cluster B. Cluster A had the only antibiotic resistant environmental isolate, while the only antibiotic resistant ATCC reference strain was grouped in cluster B. No antibiotic resistance was present in clusters D and E (not shown in Table 8).

It should be noted that of the three major clusters containing antibiotic resistant *E. coli* strains, cluster A had the highest degree of biochemical variation. This is shown by the high degree of variation within this cluster of 80.3% (Ntushelo, N., 2013, Biometrical Services, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication) and is illustrated by the seven minor clusters (see Fig. 4 in Chapter 3 of this thesis) containing antibiotic resistant *E. coli* strains. The high degree of biochemical variation is indicative to diverse metabolic capabilities of strains in cluster A.

Biochemical differences were seen for the L-ornithine decarboxylase test (ODC) when comparing the biochemical profiles of the *E. coli* strains in cluster A to the ATCC reference (EC3) in cluster B. Since the reference strains are well characterised lab strains and not adapted to survival in the environment, some differences in biochemical profiles were expected when compared to the *E. coli* strains in this study. The *E. coli* strains in cluster A lacked ODC activity while the *E. coli* reference strain was positive for this test. It may have been that strains in cluster A were exposed to different environmental stresses since *E. coli* are able to induce amino-acid decarboxylases (such as ODC) in response to reduced pH conditions (Kanjee *et al.*, 2011). ODC negative *E. coli* strains have been isolated from soil and water before (Brennan *et al.*, 2010; Janezic *et al.*, 2010).

Table 8 Distribution of antibiotic resistant *E. coli* strains amongst the three major clusters as determined by the S_J dendrogramme from phenotypic data generated using the API 20E system (bold text = antibiotic resistant *E. coli* strains)

Cluster A	Cluster A (cont.)	Cluster B	Cluster B (cont.)	Cluster B (cont.)	Cluster C
M1	M110	**M2**	M56	H7	**A95a**
M3	M111	M4	M57	H17	**S49a**
M6	H1	**M5**	M58	H18	
M7	**H29**	M9	M59	H21	
M8	H36	M10	M60	H22	
M11	**H38**	M12	M61	H43	
M13	H40	**M15**	M64	**H47**	
M14	**H45**	**M16**	M66	H61	
M39	H46	M17	M73	**H64**	
M40	**H55**	M18	M77	**H65**	
M42	H71	M19	M78	**A98a**	
M43	**A132**	M20	M92	**A118**	
M44	S4	M21	M93	S3a	
M45		M22	M94	S9	
M47		M23	M95	S12	
M62		**M24**	M96	S14	
M63		M25	M97	S31	
M65		**M26**	M98	S33	
M67		M27	M99	S56	
M68		M28	M102	S59	
M69		**M29**	M104	S95	
M70		**M30**	M105	S97	
M71		M31	M106	S103	
M72		M32	**M113**	EC11	
M74		M33	**M114**	EC4	
M75		M34	**M115**	EC10	
M76		M35	**M117**	EC13	
M81		M36	M118	EC2	
M82		M37	M127	**EC3**	
M84		M38	M128		
M87		M41	M129		
M88		M46	M130		
M90		M48	M131		
M91		M49	M132		
M100		M50	M133		
M101		M51	M134		
M103		M52	M135		
M107		**M53**	M136		
M108		M54	M140		
M109		M55	H4		

Shaded cells = contamination sites, unshaded cells = irrigation sites, bordered cells = environmental sites, horizontal striped cells = marker strains and vertical striped cells = ATCC reference strains

Multiple Antibiotic Resistances

Numerous studies have attributed *E. coli* strains with resistance to multiple antibiotics (Graves *et al.*, 2011; Su *et al.*, 2012; Zou *et al.*, 2012). Multiple antibiotic resistance (MAR) refers to the resistance to two or more classes of antibiotics (Kinge *et al.*, 2010). The percentage of MAR *E. coli* isolated from the different sources included in this study is shown in Table 9. Of the 35 antibiotic resistant *E. coli* strains, 26 (26/35 = 74.3%) strains were characterised as MAR (Table 9). These MAR *E. coli* strains were resistant to two or more antibiotics (Table 9). Of the 163 *E. coli* strains, 16.0% (26/163) were identified as MAR (Table 9). The highest percentage of MAR was to three antibiotics (16/35 = 45.7%), followed by two (9/35 = 25.7%) and one (9/35 = 25.7%) antibiotic (Table 9).

It should be noted that the *E. coli* ATCC 35218 (EC3) was resistant to three antibiotics (ampicillin, chloramphenicol and streptomycin) (Table 9). This was not expected since according to the ATCC (ATCC, 2013), this *E. coli* reference strain was recorded to be resistant to only ampicillin. No reports of this reference strain resistant to chloramphenicol and streptomycin were in the literature. One marker strain was resistant to four different antibiotics (1/35 = 2.9%) (Table 9). Of the 12 antibiotic resistant *E. coli* isolated from contamination source sites, seven (7/12 = 58.3%) were resistant to two or more antibiotics (Table 9).

Table 9 Percentage of MAR *E. coli* isolated from the different sites. Marker and reference strains have been included (n = 35).

No. of antibiotics	Irrigation % (n)	Contamination % (n)	Environmental % (n)	Marker % (n)	ATCC % (n)	Total % (n)
1	5.7 (2)	14.3 (5)	0.0 (0)	5.7 (2)	0.0 (0)	25.7 (9)
2	5.7 (2)	14.3 (5)	0.0 (0)	5.7 (2)	0.0 (0)	25.7 (9)
3	14.3 (5)	5.7 (2)	2.9 (1)	20.0 (7)	2.9 (1)	45.7 (16)
4	0.0 (0)	0.0 (0)	0.0 (0)	2.9 (1)	0.0 (0)	2.9 (1)
Total % (n)	25.7 (9)	34.3 (12)	2.9 (1)	34.3 (12)	2.9 (1)	100.0 (35)

Further analysis of the antibiotic resistant *E. coli* isolated from contamination sites, revealed that seven of the nine pig isolates (7/9 = 77.8%) were resistant to two or more antibiotics (Addendum B). The observation of multiple antibiotic resistant *E. coli* isolated from pigs concurs with the study by Unno *et al.* (2010), where *E. coli* isolated from pigs were resistant to the greatest number of antibiotics. This is further emphasised by another study, where 90% of the *E. coli* isolated from pigs (manure and lagoon effluent) were reported to be resistant to three or more antibiotics (Graves *et al.*, 2011). The distribution of MAR *E. coli*

types isolated from the different sources, including the reference and marker strains are shown in Table 10.

Table 10 Distribution of MAR phenotypes for *E. coli* strains. Marker and reference strains have been included (n = 26).

MAR phenotype	Percentage (%)	Number (n)
AMP-T-TM	23.1	6
C-T	19.2	5
AMP-TM-STR	15.4	4
C-T-TM	11.5	3
C-TM	7.7	2
T-TM	7.7	2
AMP-T-TM-STR	3.8	1
AMP-C-STR	3.8	1
AMP-C-TM	3.8	1
AMP-T-STR	3.8	1
Total	100.0	26

AMP = ampicillin, C = chloramphenicol, STR = streptomycin, T = tetracycline and TM = trimethoprim

According to Table 10, the MAR phenotype with the highest occurrence was the combination of AMP-T-TM (6/26 = 23.1%), followed by C-T (5/26 = 19.2%) and AMP-TM-STR (4/26 = 15.4%). When comparing the results in this study to results in the literature, similar MAR phenotypes for *E. coli* strains can be seen. In the literature Olaniran *et al.* (2009) reports a high occurence of MAR *E. coli* isolated from rivers in Durban, South Africa that were resistant to similar antibiotics that were included in this study. Similarly, a high prevalence of MAR *E. coli* resistant to different combinations of AMP, C and T were isolated from different water sources in the North-West Province of South Africa (Kinge *et al.*, 2010).

The high amount of MAR *E. coli* strains characterised in this study poses a definite threat to the farmers who use this contaminated water for irrigational purposes. Major public health implications are involved since antibiotic resistance can be transferred from commensal to pathogenic strains (Ahmed *et al.*, 2010). This suggests a negative impact on therapy with these antibiotics as alternative antibiotics for a particular infection may not be available. According to WHO's list of critically important antibiotics for human medicine, ampicillin and streptomycin are listed as critically important while chloramphenicol, tetracycline and trimethoprim are highly important (WHO, 2012). Therefore, the results shown in this study indicates that the use of these antibiotics should be restricted in order to minimise the spread of antibiotic resistance.

E. coli Phylogenetic Group Analysis

Phylogenetic group analysis was used to further characterise the antibiotic resistant *E. coli* isolates. The method of Clermont *et al.* (2000) was applied to try and correlate antibiotic

resistance to *E. coli* phylogenetic groups. The PCR analysis of *E. coli* enabled the grouping of isolates into one of the four main phylogenetic groups, namely A, B1, B2 and D (Silva *et al.*, 2011). These groups were further divided into subgroups, A_0, A_1, B1, B2, D_1 and D_2 (Salehi, 2012).

Two genetic markers (*chuA* and *yjaA*) as well as a DNA fragment (Tsp.E4.C2) were used to determine the phylogenetic groups (Clermont *et al.*, 2000). An example of the PCR amplified genetic markers and DNA fragment after separation on a 2% agarose gel can be seen in Fig. 3. The banding patterns present in lanes 2-6 each represent a different phylogenetic subgroup (A_1, B1, $B2_3$ and D_2) (Fig. 3). DNA fragments that had been amplified in each isolate could be determined by using the *E. coli* reference strain (ATCC 25922) as a positive control (lane 7) (Fig. 3). The combination of the amplified fragments led to the allocation of each isolate to a specific phylogenetic group (A_0, A_1, B1, $B2_3$, D_1 or D_2) as shown in Fig. 3.

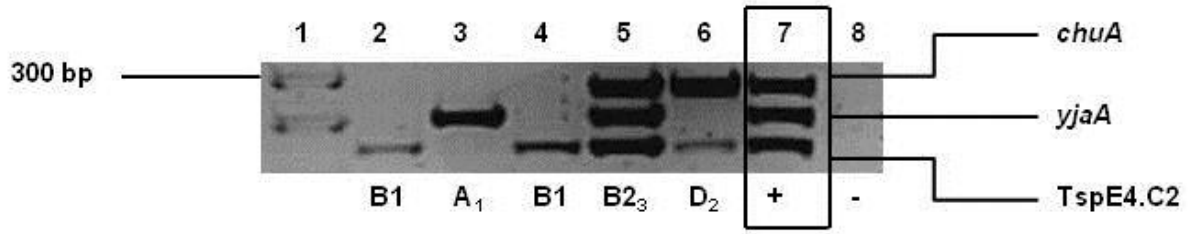

Figure 3 Agarose gel (2% agarose and 1 µg.mL^{-1} ethidium bromide) with triplex-PCR amplicons. Lane 1 = 100 bp marker; lane 2–6 = *E. coli* phylogroups B1, A_1, B1, $B2_3$, and D_2.; lane 7 = positive control (ATCC 25922); lane 8 = negative control

Overall, 163 *E. coli* strains were analysed from irrigation (n = 34), contamination source (n = 49) and environmental sites (n = 37) in Stellenbosch and the surrounding areas. *Escherichia coli* marker strains (n = 37) from the Food Science collection and ATCC *E. coli* reference strains (n = 6) were also included in this set of data. The phylogenetic distribution of antibiotic resistant *E. coli* strains is shown in Table 11, Fig. 4 and Fig. 5 (marker and reference strains have been included).

Table 11 Number of antibiotic resistant *E. coli* strains in the different phylogenetic groups (A_0, A_1, B1, $B2_3$ and D_2) (n = 35)

Phylogenetic group	Contamination source						Irrigation sites	Environmental sites	Marker strains	ATCC strains
	Pig	Cow	Horse	Human	Fish	Chicken				
A_0	5	-	-	-	-	-	3	-	-	-
A_1	4	3	-	-	-	-	-	1	5	-
B1	-	-	-	-	-	-	6	-	4	-
$B2_2$	-	-	-	-	-	-	-	-	-	1
$B2_3$	-	-	-	-	-	-	-	-	3	-
D_1	-	-	-	-	-	-	-	-	-	-
D_2	-	-	-	-	-	-	-	-	-	-
Total	9	3	0	0	0	0	9	1	12	1

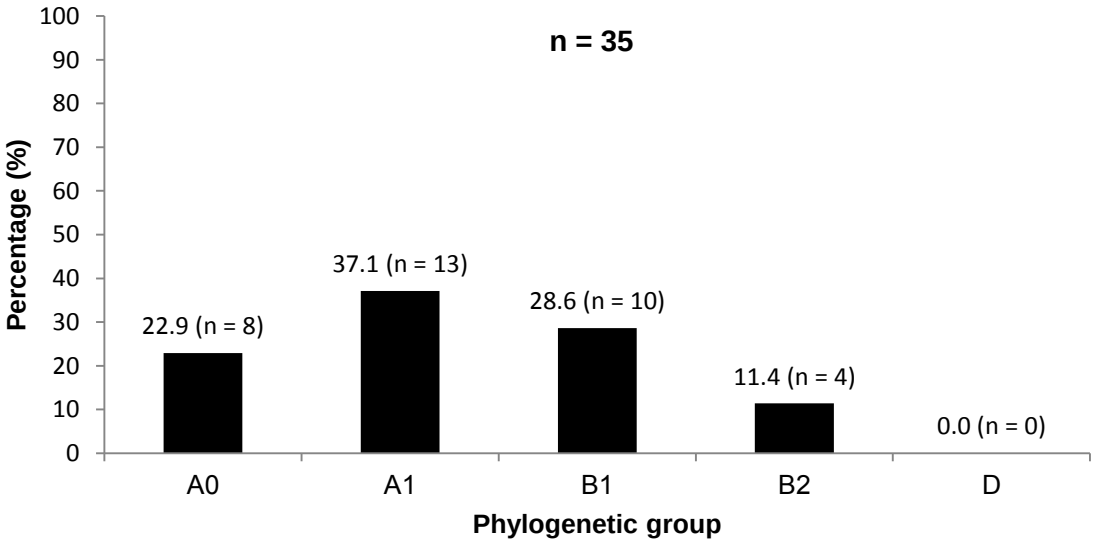

Figure 4 Percentage of antibiotic resistant *E. coli* isolates in each phylogenetic group

According to Table 11, all nine antibiotic resistant *E. coli* isolated from pigs were characterised into the main group A, while five (5/9 = 55.6%) belonged to sub-group A_0 and four (4/9 = 44.4%) to sub-group A_1. All three antibiotic resistant cow isolates were characterised as group B1, while none of the horse, human, fish and chicken isolates

exhibited antibiotic resistance (Table 11). Of the nine antibiotic resistant *E. coli* isolated from irrigation sites, six (6/9 = 66.7%) were characterised as group B1 and three (3/9 = 33.3%) as A_0 (Table 11). Of the 12 antibiotic resistant marker strains, 5 (5/12 = 41.7%) were characterised as group A_1, 4 (4/12 = 33.3%) as B1 and 3 (3/12 = 25.0%) as $B2_3$ (Table 11). The one antibiotic resistant *E. coli* isolated from an environmental site (M103) was characterised into group B1 and the antibiotic resistant *E. coli* ATCC 35218 (EC3) belonged to group $B2_3$ (Table 11).

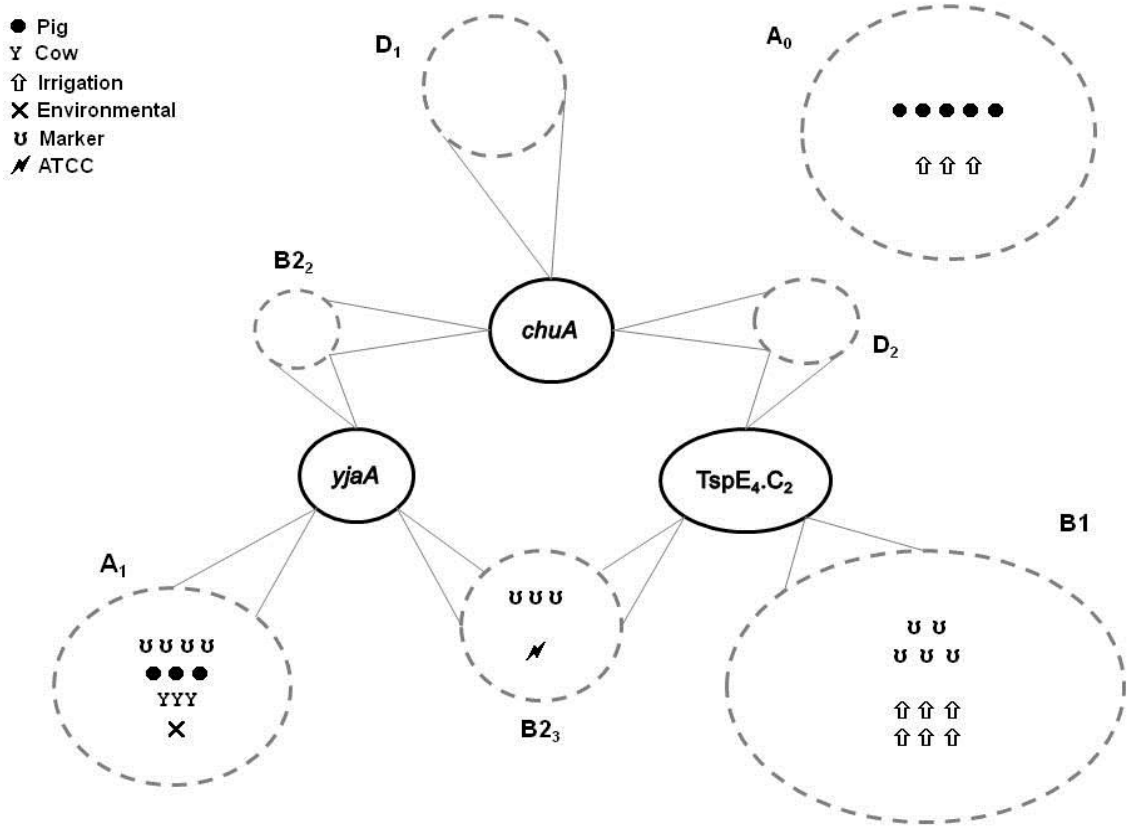

Figure 5 Graphic representation of the occurrence of genetic markers in the antibiotic resistant *E. coli* strains based on the scheme developed by Carlos *et al.* (2010). Circles with a solid outline represent each genetic marker (*chuA* and *yjaA*) and the DNA fragment (TspE4.C2). Isolates from different sources are represented by different shapes. Lines leading from the genetic markers to subgroups (outlined in dotted lines) show that the genetic marker was present in isolates from that subgroup.

The percentage of antibiotic resistant *E. coli* strains in each phylogenetic group is presented in Fig. 4. It was observed that the antibiotic resistant *E. coli* strains were mostly assigned to groups A_1 (13/35 = 37.1%) and B1 (10/35 = 28.6%), followed by A_0 (8/35 = 22.9%) and B2 (4/35 = 11.4%) (Fig. 4). In a previous study, high levels of antibiotic resistant *E. coli* isolated from waste and surface waters were also reported to be from groups A and

B1 (Figueira *et al.*, 2011). In this study no resistant isolates were characterised in group D (Fig. 4). The occurrence of lower amounts of antibiotic resistant *E. coli* strain in groups B2 and D was also reported in the study Garcia-Aljarpo *et al.* (2009).

E. coli Pathotype Analysis

The 35 *E. coli* isolates that exhibited resistance to at least one antibiotic were assessed for the presence of InPEC and ExPEC using the multiplex-PCR. The antibiotic resistance profiles of the *E. coli* pathotypes that were characterised in this study and corresponding genogroups are shown in Table 12. According to the study by Xia *et al.* (2011), an *E. coli* strain was characterised as an ExPEC pathotype if it possessed two or more ExPEC genes (Xia *et al.*, 2011)

Table 12 Antibiotic resistance profiles and phylogenetic groups of *E. coli* pathotypes

Source	Isolate	InPEC/ExPEC	Pathotype	Antibiotic Resistance Profile	Phylogenetic group
Marker (P1)	H45	InPEC, 'potential' ExPEC	EAEC, *iutA*	TM	A_1
Marker (P2)	A95a	InPEC	EPEC	AMP, T, TM	$B2_3$
Marker (P2)	A132	InPEC	EPEC	AMP, T, STR	$B2_3$
Marker (P2)	A118	InPEC	EPEC	AMP, T, TM, STR	B1
Marker (P2)	S49a	InPEC	EPEC	AMP, C, TM	$B2_3$
ATCC 35218	EC3	ExPEC	*papA, sfa/foc, kpsMT* 2, *papC*	AMP, C, STR	$B2_3$
Mosselbank	M29	'potential' ExPEC	*iutA*	C, TM	B1
Mosselbank	M30	'potential' ExPEC	*iutA*	C, TM	B1

P1 indicates site Plank-1, P2 indicates site Plank-2 and P3 indicates site Plank-3.

An example of the PCR amplified genetic markers of InPEC isolates after separation on a 1.25% agarose gel can be seen in Fig. 6. The banding patterns present in lane 2 represents an EAEC isolate and in lanes 3-7 represent EPEC isolates (Fig. 6). The genetic markers (*mdh*, *ial*, *eaeA*, *stx1, stx2*, *LT*, *ST* and *eagg*) that had been amplified in each isolate could be determined by using the SCM as positive control (lane 8) (Fig. 6). The combination of the amplified fragments led to the identification of InPEC isolates as shown in Fig. 6.

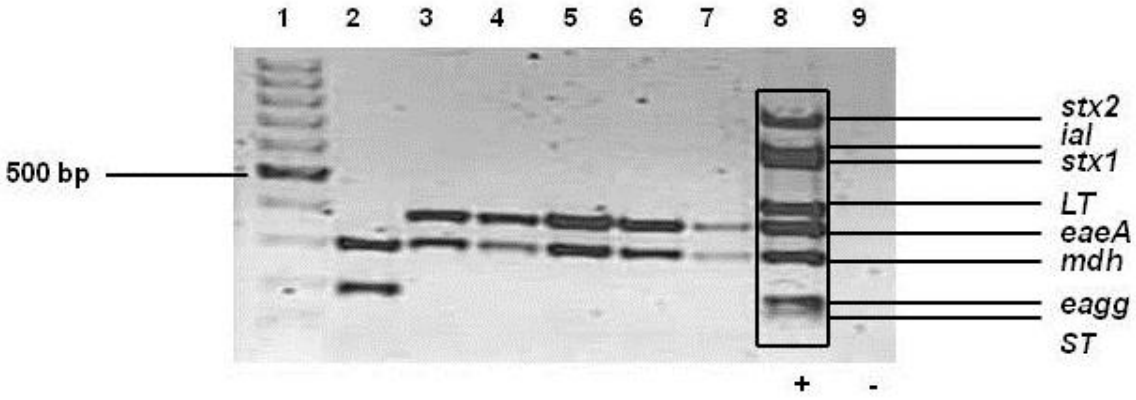

Figure 6 Agarose gel (1.25% agarose and 1 µg.mL^{-1} ethidium bromide) with InPEC PCR amplicons. Lane 1 = 100 bp marker; lane 2 = EAEC; lane 3-7 = EPEC; lane 8 = positive control (SCM); lane 9 = negative control

An example of the PCR amplified genetic markers of ExPEC isolates after separation on a 1.25% agarose gel can be seen in Fig. 7. The banding patterns present in lanes 2-4 represent isolates that carry one ExPEC-related gene sequence but does not have ExPEC status (Fig. 7). The banding patterns present in lane 6 represent an *E. coli* ATCC reference strain (ATCC 35218) with ExPEC status (Fig. 7). The genetic markers (*papA*, *papC*, *sfa/foc, iutA and kpsMT 2)* that had been amplified in each isolate could be determined by using the SCM as positive control (lane 6) (Fig. 7). The combination of the amplified fragments led to the identification of ExPEC isolates as shown in Fig. 7.

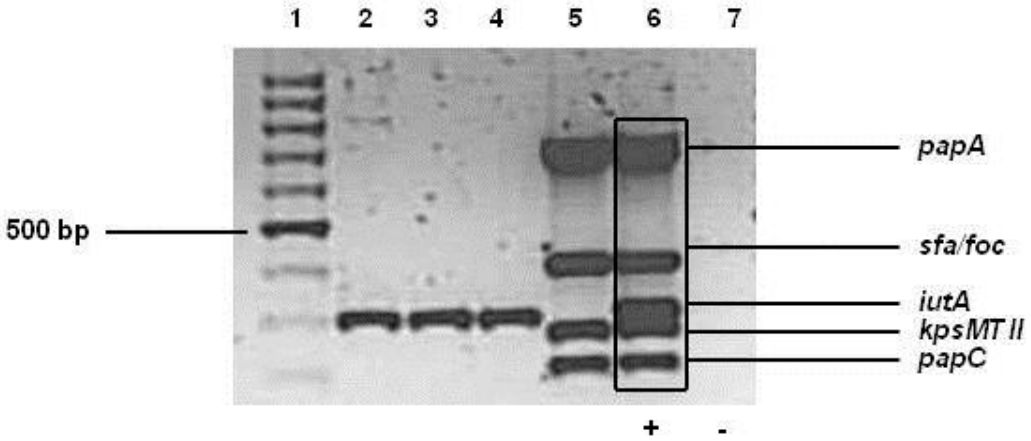

Figure 7 Agarose gel (1.25% agarose and 1 µg.mL^{-1} ethidium bromide) with ExPEC PCR amplicons. Lane 1 = 100 bp marker; lane 2-4 = 'potential' ExPEC; lane 5 = ATCC 35218; lane 6 = positive control (SCM); lane 7 = negative control

Of the 35 antibiotic resistant *E. coli* strains, five InPEC and one ExPEC strain was characterised (Table 12). The five InPEC marker strains were characterised as four EPEC and one EAEC pathotype (Table 12). The *E. coli* ATCC reference strain (EC3) that was

characterised as an ExPEC strain possessed all the ExPEC genes except the aerobactin receptor gene *iutA* (Table 12). Two *E. coli* isolated from the Mosselbank River (M29 and M30) as well as the one EAEC pathotype (H45) also possessed the *iutA* gene (Table 12). This gene encodes for an outer membrane receptor for ferric iron (Fe^{2+}) uptake into the cell and is a specialised adaptation mechanism associated with ExPEC pathotypes (Garcia *et al.*, 2011).

Three EPEC strains (A95a, A132, and S49a) were observed to be resistant to three antibiotics, while one EPEC was resistant to the combination AMP-T-TM-STR (Table 12). This correlates with the study by Ram *et al.* (2008), where MAR InPEC strains were isolated from the Saryu River in India. The *E. coli* ATCC reference strain that was identified as an ExPEC pathotype was resistant to ampicillin, chloramphenicol and streptomycin (Table 12). The two *iutA*-positive isolates from the Mosselbank River were both resistant to chloramphenicol and trimethoprim (Table 12). The EAEC isolate was resistant to trimethoprim and was the only pathotype that was not resistant to at least three antibiotics (Table 12).

Of the eight strains characterised as *E. coli* pathotypes, five (5/8 = 62.5%) (Table 12) were marker strains isolated in previous studies from the Plankenburg River. The five marker strains characterised as *E. coli* pathotypes were isolated from water samples that were drawn from the Plankenburg River after it had passed the Kayamandi informal settlement (Table 12). The one EAEC marker strain was isolated from the Plankenburg River directly after it had passed Kayamandi (Plank 1), while the four EPEC marker strains were isolated from the same point further on that farmers extract water for irrigation purposes (Plank 2) (Table 12). These results indicate that the Kayamandi informal settlement could be a source of MAR *E. coli* pathotypes that pollute the Plankenburg River. The MAR profiles reported in this study for *E. coli* pathotypes isolated from this river could have major public health implications associated with the use of this contaminated water for irrigation purposes.

CONCLUSIONS AND RECOMMENDATIONS

The study on the occurrence of antibiotic resistant *E. coli* in irrigation waters showed the presence of diverse antibiotic resistances. Of the 19 sites sampled in this study, antibiotic resistant *E. coli* was isolated from 36.8% (7/19) of the sampled sites. The majority of the *E. coli* isolates were resistant to trimethoprim, an antibiotic commonly used for the treatment of urinary tract infections caused by Gram-negative bacteria (McMurdo *et al.*, 2009). Most of the antibiotic resistant strains were resistant to two or more antibiotics and were characterised as MAR *E. coli*.

Major implications are associated with the high occurrence of MAR *E. coli* strains since antibiotic resistance determinants can be transferred on mobile genetic elements from commensal to *E. coli* pathotypes (Cambray *et al.*, 2010). The health risk associated with MAR *E. coli* greatly increases when *E. coli* pathotypes become resistant to medically important antibiotics since alternative treatment of a particular infection may not be possible. The consumption of raw or minimally processed fresh produce contaminated with antibiotic resistant *E. coli* from polluted irrigation water could therefore cause serious food infections.

Natural water sources may be a major reservoir of antibiotic resistant bacteria and play an important role in the dissemination of antibiotic resistance. The investigation of irrigation sites determined that antibiotic resistant *E. coli* was present in 57.1% (4/7) of the sampled surface waters. The problem of pathogens associated with poor irrigation water quality is further complicated by the occurrence of antibiotic resistant *E. coli* pathotypes. The marker strains characterised as MAR *E. coli* pathotypes isolated from the Plankenburg River is indicative to this problem.

The results in this study emphasise the potential role that natural water sources have in the dissemination of antibiotic resistant bacteria. It can be concluded that the presence of antibiotic resistant *E. coli* in water used for irrigation purposes poses a definite threat to farmers who utilise these natural water sources to irrigate fresh produce.

Various lists of critically important antibiotics, such as those published by the WHO prioritise the importance of strict regulatory use of antibiotics. Therefore, it is important to monitor the occurrence of antibiotic resistant bacteria in waters used for irrigation purposes since these bacteria are able to spread through food to humans. Subsequent action should be taken to enforce the restricted use of certain antibiotics that are critically important for the treatment of human illnesses.

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Addendum A Inhibition and susceptibility data of antibiotics used in this study for the 163 *E. coli* strains according to the Kirby-Bauer method (AMP = ampicillin, KF = cephalothin, C = chloramphenicol, CIP = ciprofloxacin, T = tetracycline, TM = trimethoprim, STR = streptomycin, S = susceptible, I = intermediate and R = resistance)

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Cow	M1	22	S	23	S	22	S	27	S	20	S	0	R	16	S
Pig	M2	22	S	21	S	0	R	24	S	0	R	0	R	16	S
Pig	M3	22	S	22	S	11	R	25	S	0	R	18	S	17	S
Cow	M4	19	S	21	S	20	S	29	S	19	S	22	S	17	S
Pig	M5	21	S	22	S	14	R	26	S	0	R	18	S	17	S
Cow	M6	21	S	23	S	22	S	28	S	18	S	0	R	16	S
Pig	M7	23	S	23	S	11	R	24	S	0	R	18	S	16	S
Pig	M8	21	S	20	S	12	R	24	S	0	R	17	S	17	S
Cow	M9	21	S	21	S	24	S	29	S	19	S	22	S	17	S
Cow	M10	19	S	22	S	22	S	29	S	20	S	24	S	16	S
Cow	M11	22	S	23	S	22	S	27	S	21	S	0	R	16	S
Cow	M12	21	S	20	S	21	S	28	S	19	S	23	S	16	S
Pig	M13	23	S	23	S	6	R	25	S	0	R	15	I	16	S
Pig	M14	22	S	22	S	0	R	25	S	0	R	0	R	16	S
Pig	M15	23	S	23	S	20	S	26	S	0	R	17	S	19	S
Pig	M16	22	S	22	S	19	S	25	S	0	R	17	S	18	S
Cow	M17	21	S	21	S	23	S	28	S	20	S	22	S	18	S
Pig	M18	22	S	21	S	21	S	27	S	19	S	22	S	17	S
Berg	M19	20	S	18	S	19	S	27	S	20	S	24	S	16	S

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Berg	M20	19	S	18	S	21	S	29	S	19	S	24	S	16	S
Berg	M21	21	S	21	S	21	S	29	S	20	S	24	S	18	S
Berg	M22	21	S	21	S	24	S	28	S	19	S	24	S	16	S
Berg	M23	19	S	19	S	18	S	28	S	18	S	24	S	16	S
Berg	M24	0	R	18	S	19	S	23	S	18	S	24	S	16	S
Berg	M25	21	S	20	S	20	S	28	S	19	S	24	S	16	S
Berg	M26	0	R	20	S	21	S	25	S	18	S	24	S	16	S
Mossel	M27	21	S	19	S	23	S	28	S	18	S	24	S	17	S
Mossel	M28	23	S	23	S	23	S	28	S	19	S	24	S	17	S
Mossel	M29	21	S	21	S	11	R	27	S	18	S	0	R	15	S
Mossel	M30	21	S	21	S	12	R	25	S	19	S	0	R	17	S
Mossel	M31	23	S	22	S	22	S	28	S	18	S	24	S	16	S
Smartie	M32	22	S	22	S	22	S	23	S	18	S	23	S	16	S
Smartie	M33	21	S	22	S	23	S	23	S	19	S	23	S	16	S
Smartie	M34	21	S	22	S	22	S	23	S	18	S	23	S	16	S
Smartie	M35	21	S	21	S	22	S	25	S	18	S	24	S	16	S
Smartie	M36	20	S	21	S	21	S	23	S	18	S	24	S	16	S
Smartie	M37	22	S	22	S	22	S	28	S	20	S	22	S	16	S
Smartie	M38	22	S	21	S	23	S	23	S	18	S	23	S	16	S
Horse	M39	20	S	19	S	22	S	24	S	19	S	23	S	17	S
Horse	M40	20	S	21	S	22	S	24	S	19	S	24	S	17	S

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Horse	M41	21	S	21	S	23	S	27	S	20	S	23	S	17	S
Horse	M42	20	S	19	S	22	S	26	S	20	S	21	S	15	S
Horse	M43	21	S	18	S	22	S	26	S	18	S	23	S	17	S
Horse	M44	21	S	21	S	21	S	25	S	20	S	23	S	17	S
Horse	M45	22	S	20	S	21	S	28	S	20	S	22	S	18	S
Horse	M46	23	S	23	S	23	S	27	S	19	S	22	S	17	S
Horse	M47	22	S	21	S	25	S	27	S	22	S	23	S	18	S
Limber	M48	21	S	21	S	23	S	27	S	20	S	23	S	17	S
Limber	M49	21	S	21	S	21	S	26	S	19	S	24	S	18	S
Limber	M50	21	S	21	S	22	S	26	S	19	S	24	S	17	S
Limber	M51	21	S	21	S	24	S	26	S	20	S	24	S	18	S
Limber	M52	21	S	21	S	22	S	26	S	19	S	24	S	17	S
Limber	M53	0	R	21	S	22	S	27	S	0	R	0	R	12	I
Limber	M54	20	S	21	S	21	S	27	S	19	S	24	S	17	S
Limber	M55	19	S	19	S	21	S	26	S	18	S	24	S	17	S
Limber	M56	22	S	21	S	22	S	26	S	19	S	24	S	17	S
Limber	M57	21	S	21	S	21	S	26	S	18	S	24	S	17	S
Limber	M58	21	S	21	S	22	S	21	S	18	S	24	S	17	S
Middle	M59	21	S	21	S	23	S	27	S	20	S	23	S	17	S
Middle	M60	18	S	20	S	20	S	25	S	19	S	22	S	16	S
Middle	M61	18	S	20	S	20	S	27	S	18	S	22	S	16	S

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Middle	M62	23	S	23	S	21	S	26	S	20	S	24	S	15	S
Middle	M63	20	S	18	S	20	S	27	S	20	S	23	S	15	S
House	M64	19	S	19	S	22	S	27	S	19	S	21	S	15	S
House	M65	21	S	20	S	22	S	27	S	19	S	22	S	15	S
House	M66	17	S	19	S	19	S	25	S	18	S	23	S	16	S
Eversdal	M67	22	S	22	S	21	S	25	S	18	S	21	S	17	S
Eversdal	M68	21	S	21	S	22	S	25	S	19	S	22	S	16	S
Eversdal	M69	20	S	21	S	22	S	27	S	22	S	22	S	17	S
Eversdal	M70	21	S	21	S	21	S	26	S	19	S	22	S	16	S
Eversdal	M71	21	S	21	S	21	S	26	S	18	S	24	S	16	S
Eversdal	M72	21	S	22	S	20	S	27	S	19	S	26	S	16	S
Eversdal	M73	19	S	18	S	21	S	25	S	19	S	21	S	15	S
Eversdal	M74	20	S	21	S	20	S	24	S	20	S	26	S	17	S
Eversdal	M76	21	S	24	S	20	S	27	S	19	S	27	S	17	S
Chicken	M77	21	S	21	S	20	S	25	S	20	S	26	S	16	S
Chicken	M78	21	S	20	S	21	S	25	S	20	S	25	S	16	S
Horse	M81	21	S	21	S	21	S	26	S	20	S	23	S	16	S
Horse	M82	21	S	21	S	20	S	21	S	18	S	24	S	16	S
Horse	M83	20	S	20	S	18	S	23	S	18	S	22	S	17	S
Horse	M84	21	S	20	S	18	S	23	S	18	S	21	S	17	S
Horse	M85	21	S	19	S	20	S	25	S	20	S	21	S	17	S

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Horse	M86	17	S	18	S	18	S	23	S	19	S	20	S	16	S
Horse	M87	20	S	21	S	20	S	24	S	18	S	22	S	11	R
Horse	M88	20	S	19	S	18	S	26	S	18	S	24	S	16	S
Horse	M89	18	S	18	S	19	S	25	S	18	S	22	S	16	S
Horse	M90	22	S	22	S	18	S	23	S	19	S	22	S	17	S
Horse	M91	19	S	20	S	18	S	23	S	19	S	21	S	16	S
Eversdal	M92	19	S	20	S	20	S	26	S	19	S	24	S	17	S
Eversdal	M93	19	S	19	S	19	S	25	S	19	S	25	S	16	S
Eversdal	M94	19	S	20	S	20	S	25	S	18	S	25	S	16	S
Eversdal	M95	21	S	23	S	19	S	25	S	18	S	24	S	16	S
Eversdal	M96	19	S	19	S	19	S	21	S	16	S	24	S	16	S
Eversdal	M97	21	S	23	S	19	S	22	S	18	S	23	S	16	S
Eversdal	M98	19	S	20	S	20	S	21	S	17	S	24	S	16	S
Eversdal	M99	19	S	20	S	20	S	21	S	18	S	23	S	16	S
Eversdal	M100	20	S	20	S	19	S	23	S	18	S	24	S	17	S
Eversdal	M101	21	S	21	S	19	S	23	S	17	S	24	S	17	S
Eversdal	M102	23	S	24	S	18	S	25	S	20	S	24	S	16	S
Eversdal	M103	17	S	19	S	11	R	23	S	0	R	13	R	15	S
Eversdal	M104	18	S	16	I	20	S	25	S	19	S	22	S	15	S
Eversdal	M105	19	S	17	I	20	S	25	S	19	S	22	S	15	S
House	M106	19	S	18	S	20	S	25	S	19	S	21	S	15	S

Source	Isolate	AMP 10 µg Inhibition Zone (mm)	S, I or R	KF 30 µg Inhibition Zone (mm)	S, I or R	C 30 µg Inhibition Zone (mm)	S, I or R	CIP 5 µg Inhibition Zone (mm)	S, I or R	T 30 µg Inhibition Zone (mm)	S, I or R	TM 2.5 µg Inhibition Zone (mm)	S, I or R	STR 10 µg Inhibition Zone (mm)	S, I or R
House	M107	19	S	19	S	19	S	23	S	18	S	20	S	15	S
House	M108	19	S	21	S	20	S	24	S	17	S	21	S	16	S
House	M109	19	S	20	S	20	S	24	S	18	S	20	S	16	S
House	M110	21	S	21	S	20	S	23	S	17	S	20	S	15	S
House	M111	21	S	20	S	18	S	23	S	17	S	21	S	15	S
Middle	M113	0	R	17	I	19	S	24	S	18	S	0	R	11	R
Middle	M114	0	R	17	I	20	S	25	S	17	S	0	R	10	R
Middle	M115	0	R	17	I	19	S	25	S	19	S	0	R	10	R
Middle	M117	0	R	17	I	19	S	23	S	18	S	0	R	10	R
Middle	M118	19	S	18	S	20	S	21	S	17	S	24	S	16	S
Jonkers	M127	20	S	19	S	19	S	25	S	18	S	24	S	15	S
Jonkers	M128	20	S	19	S	20	S	25	S	18	S	24	S	16	S
Jonkers	M129	20	S	19	S	19	S	24	S	18	S	23	S	15	S
Jonkers	M130	19	S	19	S	20	S	25	S	18	S	24	S	16	S
Jonkers	M131	20	S	19	S	20	S	23	S	16	S	22	S	15	S
Jonkers	M132	20	S	19	S	20	S	25	S	17	S	24	S	16	S
W-Dam	M133	20	S	19	S	19	S	24	S	18	S	24	S	16	S
W-Dam	M134	20	S	18	S	18	S	24	S	17	S	25	S	16	S
W-Dam	M135	19	S	18	S	18	S	24	S	17	S	23	S	16	S
W-Dam	M136	20	S	18	S	18	S	24	S	17	S	22	S	16	S
W-Dam	M140	19	S	19	S	19	S	25	S	17	S	23	S	16	S

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Marker	H1	19	S	19	S	19	S	26	S	18	S	22	S	15	S
Marker	H4	20	S	19	S	20	S	26	S	19	S	21	S	15	S
Marker	H7	19	S	19	S	19	S	26	S	18	S	22	S	15	S
Marker	H17	21	S	17	S	20	S	26	S	19	S	23	S	15	S
Marker	H18	21	S	19	S	23	S	27	S	20	S	23	S	16	S
Marker	H21	20	S	21	S	22	S	26	S	21	S	21	S	16	S
Marker	H22	22	S	22	S	21	S	26	S	20	S	23	S	15	S
Marker	H29	21	S	21	S	21	S	26	S	0	R	0	R	16	S
Marker	H36	20	S	21	S	20	S	27	S	20	S	22	S	15	S
Marker	H38	21	S	21	S	20	S	26	S	0	R	0	R	15	S
Marker	H40	19	S	20	S	21	S	27	S	19	S	25	S	17	S
Marker	H43	20	S	21	S	22	S	26	S	20	S	25	S	18	S
Marker	H45	0	R	19	S	21	S	25	S	19	S	0	R	16	S
Marker	H46	21	S	20	S	21	S	26	S	18	S	25	S	16	S
Marker	H47	0	R	19	S	21	S	25	S	0	R	0	R	15	S
Marker	H55	0	R	19	S	21	S	27	S	0	R	0	R	10	R
Marker	H61	20	S	21	S	21	S	25	S	19	S	25	S	16	S
Marker	H64	0	R	18	S	22	S	26	S	0	R	0	R	15	S
Marker	H65	0	R	20	S	23	S	26	S	0	R	0	R	15	S
Marker	H71	21	S	21	S	21	S	26	S	20	S	25	S	17	S
Marker	A95a	0	R	16	I	19	S	24	S	0	R	0	R	15	S

Source	Isolate	AMP 10 µg	S, I or R	KF 30 µg	S, I or R	C 30 µg	S, I or R	CIP 5 µg	S, I or R	T 30 µg	S, I or R	TM 2.5 µg	S, I or R	STR 10 µg	S, I or R
		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)		Inhibition Zone (mm)	
Marker	A98a	18	S	18	S	18	S	26	S	16	S	0	R	16	S
Marker	A118	0	R	19	S	23	S	25	S	0	R	0	R	0	R
Marker	A132	0	R	22	S	20	S	26	S	20	S	0	R	10	R
Marker	S3a	18	S	19	S	19	S	25	S	18	S	21	S	15	S
Marker	S4	22	S	21	S	20	S	25	S	18	S	23	S	16	S
Marker	S9	22	S	21	S	20	S	25	S	18	S	22	S	16	S
Marker	S12	19	S	20	S	19	S	24	S	19	S	24	S	16	S
Marker	S14	21	S	21	S	19	S	25	S	19	S	21	S	16	S
Marker	S31	19	S	20	S	19	S	24	S	18	S	24	S	16	S
Marker	S33	19	S	21	S	19	S	25	S	18	S	25	S	16	S
Marker	S49a	0	R	15	S	0	R	25	S	19	S	0	R	15	S
Marker	S56	19	S	18	S	20	S	26	S	18	S	24	S	16	S
Marker	S59	19	S	18	S	20	S	26	S	18	S	23	S	16	S
Marker	S95	19	S	21	S	19	S	25	S	18	S	25	S	16	S
Marker	S97	19	S	19	S	20	S	26	S	18	S	24	S	16	S
Marker	S103	19	S	18	S	20	S	26	S	18	S	23	S	16	S
ATCC 25922	EC2	21	S	21	S	21	S	25	S	17	S	23	S	17	S
ATCC 35218	EC3	0	R	20	S	8	R	27	S	21	S	24	S	11	R

Addendum B Antibiotic resistance profiles, phylogroups and pathotypes of the 35 resistant *E. coli* strains (AMP = ampicillin, KF = cephalothin, C = chloramphenicol, CIP = ciprofloxacin, T = tetracycline, TM = trimethoprim and STR = streptomycin)

Source	Isolate	Antibiotic	Phylogroup	Pathotype	
				InPEC	ExPEC
Cow	M1	TM	A_1	-	-
Pig	M2	C, T, TM	A_0	-	-
Pig	M3	C, T	A_1	-	-
Pig	M5	C, T	A_0	-	-
Cow	M6	TM	A_1	-	-
Pig	M7	C, T	A_1	-	-
Pig	M8	C, T	A_0	-	-
Cow	M11	TM	A_1	-	-
Pig	M13	C, T	A_1	-	-
Pig	M14	C, T, TM	A_1	-	-
Pig	M15	T	A_0	-	-
Pig	M16	T	A_0	-	-
Berg	M24	AMP	A_0	-	-
Berg	M26	AMP	A_0	-	-
Mosselbank	M29	C, TM	B1	-	*iutA*
Mosselbank	M30	C, TM	B1	-	*iutA*
Limberlost	M53	AMP, T, TM	B1	-	-
Eversdal	M103	C, T, TM	A_1	-	-
Middelvlei	M113	AMP, TM, STR	A_0	-	-
Middelvlei	M114	AMP, TM, STR	B1	-	-
Middelvlei	M115	AMP, TM, STR	B1	-	-
Middelvlei	M117	AMP, TM, STR	B1	-	-
Marker (Bean)	H38	T, TM	A_1	-	-
Marker (Bean)	H29	T, TM	A_1	-	-
Marker (Bean)	H47	AMP, T, TM	B1	-	-

Source	Isolate	Antibiotic	Phylogroup	Pathotype	
				InPEC	ExPEC
Marker (Plank 1)	H55	AMP, T, TM	A_1	-	-
Marker (Bean)	H64	AMP, T, TM	B1	-	-
Marker (Bean)	H65	AMP, T, TM	B1	-	-
Marker (Plank 1)	H45	AMP, TM	A_1	EAEC	*iutA*
Marker (Plank 2)	A95a	AMP, T, TM	B2	EPEC	-
Marker (Plank 2)	A132	AMP, T, STR	B2	EPEC	-
Marker (Plank 2)	A98a	TM	B1	-	-
Marker (Plank 2)	A118	AMP, T, TM, STR	B1	EPEC	-
Marker (Plank 2)	S49a	AMP, C, TM	B2	EPEC	-
ATCC (35218)	EC3	AMP, C, STR	B2	-	*papA, sfa/foc, iutA, kpsMT* 2, *papC*

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

Irrigation water has been shown to be a source of faecal contamination and food-borne outbreaks have been linked to its use. Research has shown (Paulse *et al.*, 2012; Britz *et al.*, 2013) that many South African irrigation water sources have *Escherichia coli* levels above the WHO and DWAF recommended guideline of 1 000 *E. coli* counts.100 mL^{-1} (WHO, 1989; DWAF, 1996). As no regulations have been set for irrigation water in this country and only guidelines exist, the microbial quality of such waters are not well known. The overall objective of the study was to evaluate the microbial quality of surface waters used for irrigation purposes in the Western Cape. Furthermore, irrigation waters and potential contamination sources were evaluated as reservoirs of antibiotic resistant *E. coli* strains.

The aim of the first study was to determine total coliforms and *E. coli* loads in water samples from irrigation, contamination and environmental sites. Environmental sites were selected to represent controls where no direct source of faecal contamination was expected. It was concluded from the findings that with the abnormally high *E.* coli loads at various sites, there is an increased risk for disease transmission when fresh produce is irrigated with this water and consumed raw or after it has been minimally processed. The loads, source of contamination and the characteristics of the *E. coli* are important when determining the risk of using contaminated water for irrigation purposes.

The *E. coli* strains were subjected to phylogenetic analysis using triplex-PCR to characterise their phylogenetic groups. The *E. coli* isolates, marker and reference strains were grouped into all four of the main phylogenetic groups (A, B1, B2 and D) and six of the seven standard subgroups (A_0, A_1, B1, $B2_3$, D1 and D_2). This indicates that a wide variety of *E. coli* types were isolated from the different sources that were studied. Phylogenetic group B1 has been reported to contain strains with the ability to survive and persist in the external environment (Carlos *et al.*, 2010) and concurs with the results in this study. Similar phylogenetic distribution patterns were seen amongst *E coli* isolates from irrigation water. There is an increased risk with the consumption of fresh produce irrigated with contaminated water containing pathogenic *E. coli* strains in phylogenetic group B1, as these strains may persist and survive on the surface of fresh produce. It was concluded, that the high occurrence of *E. coli* isolates in phylogenetic group B1 from irrigation water is a concern that should be further investigated.

There is a global concern that the widespread use of antibiotics in agriculture, food processing and human medicine may lead to the emergence of food-borne pathogens that are resistant to antibiotics. Surface waters have been identified as a major reservoir of multi-antibiotic resistant pathogenic bacteria as a result of continuous contamination with

agricultural waste, animal excreta, industrial effluent and sewage disposal. Intensively reared food animals such as pigs and cows harbour large numbers of antibiotic resistant bacteria (Graves *et al.*, 2011). This was also found in this study.

The aim of the second part of this study was to evaluate the presence of antibiotic resistance in *E. coli* from water sources used for irrigation purposes in the Western Cape. The occurrence of antibiotic resistant *E. coli* (9/35 = 25.7%) present in irrigation water represents an increased risk for food-borne outbreaks when fresh produce is irrigated with this water and consumed raw. Major clinical implications are associated with the high occurrence of resistance to the antibiotics included in this study. Ampicillin and streptomycin are included in the WHO's list of critically important antibiotics for human medicine; while chloramphenicol, tetracycline and trimethoprim are listed as highly important (WHO, 2012). The antibiotics tested were also all from different classes of antibiotics. It can be argued that if an *E. coli* infection were to occur after the consumption of fresh produce contaminated with a pathogenic *E. coli* strain resistant to any of the seven antibiotics studied, it might also be resistant to other antibiotics from the same classes. Alternative therapy subsequently may not be available to control the particular infection.

The 35 antibiotic resistant *E. coli* strains found were subjected to triplex-PCR to place them into the different genogroups. Three of the four main genogroups (A, B1 and B2) and four of the seven subgroups (A_0, A_1, B1 and $B2_3$) were present among the antibiotic resistant *E. coli* strains. This indicates a wide variation in antibiotic resistance and furthermore even from the various sources studied. Most antibiotic resistant *E. coli* strains were grouped in genogroups A_1 and B1. Similar phylogenetic distribution patterns were seen amongst the antibiotic resistant *E. coli* isolates from irrigation water. The implications are that most antibiotic resistant *E. coli* strains are able to survive in the external environment and may even result in the dissemination of antibiotic resistance among other environmental *Enterobacteriaceae*.

The occurrence of Multiple Antibiotic Resistant (MAR) *E. coli* strains as found in this study can have major medical implications since it is well known that antibiotic resistance can be transferred from commensal *E. coli* to *E. coli* pathotypes. The *E. coli* strains that exhibited resistance to at least one antibiotic were subsequently screened for the occurrence of *E. coli* pathotypes using multiplex-PCR. Class 1 integrons have been reported to mostly harbour MAR determinants and are widely distributed amongst members of the *Enterobacteriaceae* family (Cambray *et al.*, 2010). It may be possible that Class 1 integrons were responsible for the MAR profiles of *E. coli* strains in this study. Further research for the detection of Class 1 integrons in the MAR *E. coli* strains will need to be done to confirm this possibility. The health risk implications of the presence of MAR *E. coli* greatly increases when *E. coli* pathotypes become resistant to medically-important antibiotics since alternative

treatment of a particular infection may not be possible. The consumption of raw or minimally processed produce contaminated with antibiotic resistant pathogenic *E. coli* from polluted irrigation water may therefore lead to serious food-borne infections.

In this study, risk was defined as the presence of antibiotic resistance and *E. coli* pathotypes in the irrigation water samples. The presence of MAR *E. coli* pathotypes in this study increases the health risk to consumers. What is more important is that these strains were present at irrigation sites along the Plankenburg River. The risk of produce being contaminated with antibiotic resistant *E. coli* strains whether pathogenic or commensal, should be of concern to both produce farmers and the food industry.

It is recommended that in future studies the occurrence and persistence of faecal contamination and antibiotic resistance at irrigation sites included in this study be continuously monitored to determine increases in risk. Pre-treatment of river water prior to irrigation of fresh produce is also recommended. Since the extent of antibiotic resistance is unknown for *E. coli* strains in this study, they may be resistant to antibiotics other than those included in this study. Thus, the inclusion of additional antibiotics may determine the extent of resistance within the same antibiotic classes. The means of acquisition and dissemination of antibiotic resistance is unknown for *E. coli* strains in this study. Genetic analysis of antibiotic resistance mechanisms on mobile genetic elements (such as integrons, transposons and plasmids) should therefore be done to understand the different adaptive mechanisms of *E. coli*. It can be concluded that faecal contamination of irrigation waters with antibiotic resistant *E. coli* is a major threat to human health.

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