Induction of Shiga toxins in *Escherichia coli* O157:H7 isolated from groundwater in the North West Province, South Africa using different antimicrobial agents

By

Daphney Pfarelo Shandukani (BSc. Hons -Microbiology)

(23409460)

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Department of Biological Sciences
Faculty of Agriculture, Science and Technology
North West University – Mafikeng Campus
Mafikeng – South Africa

Supervisor: Dr. C.N Ateba

Co-Supervisor: Dr. P.N Sithebe

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DECLARATION

I, the undersigned, declare that the thesis hereby submitted to the North-west University – Mafikeng Campus for the degree of Masters (Biology) and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university for a degree. All materials used have been acknowledged.

Signed DP SHANDUKANI this the 19th day of SEPTEMBER 2013 atebaRt

atebaRt
(SUPERVISOR)

19/09/2013

DEDICATION

This work is ddedicated to my daughter, Phathutshedzo Mvuko Rankhododo, my mother, Joyce Takalani Shandukani, who gave me guidance and love throughout the study and to my sisters, Mulalo, Hulisani and Patience.

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this thesis and follow the style recommended by the American Society for Microbiology for Journals.

ATCC	: American Type Culture Collection
Bp	: base pair
CT-SMAC	: cefixime, potassium tellurite - sorbitol-MacConkey agar
eaeA	: attachment and effacing gene
E. coli	: *Escherichia coli*
***E. coli* O157:H7**	: *Escherichia coli* O157:H7
EDTA	: Ethylene diamine tetra-acetic acid
HUS	: Haemolytic uraemic syndrome
HC	: Haemorrhagic colitis
HlyA	: Haemolysin A gene
LEE	: Locus of enterocyte effacement
MUG	: 4-methylumbelliferyl-β-D-glucuronide
NCTC	: National Collection of Type Culture
PCR	: Polymerase chain reaction
SMAC	: Sorbitol MacConkey Agar
LB	: Luria Bertani
STEC	: Shiga-toxin producing *Escherichia coli*
Stx	: Shiga toxins
TTP	: Thrombotic thrombocytopenic purpura
W/v	: weight per volume

DEFINITION OF CONCEPTS

Clone: Bacterial isolates that, although they may have been cultured independently from different sources in different locations and at different times, still have so many identical phenotypic and genotypic traits that the most likely explanation for this identity is a common origin within a relevant time span.

Cluster analysis: Comparative analysis of typing data collected for a variety of bacterial isolates in order to group the organisms according to their similarity in these data.

Fingerprint: Specific banding pattern displayed by an isolate on application of one or more typing methods.

Haemorrhagic colitis: A disease that is characterised by severe crampy abdominal pain, watery diarrhoea, followed by grossly bloody diarrhoea and little fever.

Haemolytic uraemic syndrome: A disease that is characterised by a triad of acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia that is usually preceeded by a bloody diarrhoeal illness.

Pathogenicity: Biological ability of a microbe to cause disease.

Pattern analysis: The process of comparing data patterns generated using one or more typing methods.

Polymerase chain reaction: PCR is a molecular method that is used to amplify specific regions of DNA many times over using primers.

Outbreak: Local, initially small-scale, cluster of disease that is generally caused by increased frequency of an infection in a distinct population.

Shiga-toxin producing *Escherichia coli*: *Escherichia coli* strain that produces shiga toxins.

Shiga toxins: Proteins produced by STEC *Escherichia coli* strains.

Sporadic: Infectious cases that are rare and they occur at different times in different localities.

Typing: A phenotypic and/or genetic analysis of bacterial isolates below the species level that is employed to generate strain specific fingerprints that could be used in investigating cross contaminations, transmission patterns and/or sources of infections in humans or consumers of a particular food product.

Virulence: The property of an infectious agent that determines the extent to which it can cause disease in an infected population.

CHAPTER 1

INTRODUCTION

1.1 RATIONALE FOR THE STUDY AND PROBLEM STATEMENT

Water is a very important resource for life. Access to safe drinking water is a fundamental human need and therefore, a basic right of every individual, since contaminated water jeopardises both the physical and social health of consumers (WHO, 2003). In many countries, including South Africa, shortage of water, human activities and industrialisation have been found to significantly affect the quality of drinking water (Martellini *et al.,* 2005). Consequently, about 1.1 billion individuals worldwide do not have access to potable drinking water supplies (WHO, 2005). In developing countries, 98% of infections recorded are as a result of consumption of contaminated water and food (WHO, 2004). Contamination with pathogenic micro-organisms may result from poor sanitation and improper hygiene practices (Garner and Simmons, 1983; WHO, 2004). In South Africa and the North West Province in particular, individuals residing in most rural communities depend on untreated surface and groundwater for drinking and household activities (Ateba and Maribeng, 2011). Water from these sources is usually of poor quality due to chemical and microbial contamination (Momba *et al.,* 2003, 2005, 2006; Obi *et al.,* 2006). This renders the water unsafe for drinking.

Escherichia coli is a Gram-negative rod-shaped bacterium that is usually harmless and occurs as normal flora in the intestines of humans and animals (Harakeh *et al.,* 2005). However, shiga-toxin producing *E. coli* (STEC) and particularly the serotype O157:H7, are known to be responsible for a number of waterborne infections in humans worldwide (Bouvet *et al.*, 2001; Dundas *et al.*, 2001; Botteldoorn *et al.,* 2003; Tutenel *et al.,* 2003). Moreover, some healthy domestic animals such as cattle, sheep and goats may harbour *E. coli* O157:H7 in their gastro-intestinal tract without presenting any clinical symptoms (Müller *et*

al., 2002; Cobbold *et al.,* 2007). This therefore increases the chances of contaminating water bodies if proper farm management techniques and hygiene practices are not implemented. On the contrary, in humans, *E. coli* O157:H7 can cause a variety of diseases that range from non-bloody to severe bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura (Graffin and Tauxe, 1991; Armstrong *et al.,* 1996; Verweyen *et al.*, 2000; Wier, 2000; Banatvala *et al.,* 2001; Sugiyama *et al.,* 2005; Momba *et al.,* 2008). Although these infections can be self-limiting in some patients, in immuno-compromised individuals, infants and the elderly, they may present life-threatening complications (Momba *et al.,* 2008). In South Africa, the prevalence of HIV and AIDS is relatively high, especially among individuals who reside in rural areas (Bessong *et al.,* 2008). Given the difficulties associated with managing *E. coli* O157:H7 infections especially in individuals suffering from chronic infections, it is therefore important to constantly monitor the occurrence of water and foodborne pathogens in drinking water sources (Bessong *et al.,* 2008). The data obtained would be more valuable if the investigations are conducted in water from rural communities.

The treatment of infections caused by pathogenic micro-organisms is usually achieved through the administration of antibiotics (Johnson *et al.,* 2006). However, there is some controversy involving the use of antibiotics in the treatment of *E. coli* O157:H7 infections in humans (Igarashi *et al.,* 1999; Wong *et al.,* 2000; Dundas *et al.,* 2001). Antibiotics have been reported to serve as inducers for bacteriophages in *E. coli* O157:H7 cells and the phages harbour the shiga-toxin genes (Zhang *et al.,* 2000; Dundas *et al.,* 2001). Therefore, in the presence of antibiotics, the shiga-toxin genes are expressed, thus increasing the chances of the disease to progress to the more severe clinical forms (Wong *et al.,* 2000; Zhang *et al.,* 2000; Dundas *et al.,* 2001). On the contrary, it has also been demonstrated that infections such as HUS could be prevented in infected individuals when antibiotics are administered during the early stages of *E. coli* O157:H7 infections (Slutsker *et al.,* 1997). However, a number of studies

conducted worldwide have indicated the presence of multiple antibiotic resistant *E. coli* O157H7 strains from food products, water and humans (Kim *et al.*, 1991; Carl *et al.,* 2002; Ateba and Bezuidenhout, 2008; Wose Kinge *et al.,* 2010; Phokela *et al.,* 2011). It is therefore suggested that antibiotics may not have a significant effect on these antibiotic resistant isolates. Faced with this confusion, it is suggested that the treatment of *E. coli* O157:H7 infections be based on supportive therapy, to replace fluids and electrolytes lost through diarrhoea (Charle, 2012).

The aim of this study is to investigate the occurrence of *E. coli* O157:H7 in water from boreholes and to determine antibiotic resistant profiles of the isolates. A further objective is to evaluate the ability of two antimicrobial agents, tetracycline and ampicillin,in inducing shiga-toxin proteins in broth cultures of some selected isolates. This was designed to assess the health risks associated with the uncontrolled administration of antibiotics in diarrhoeal patients without determining the causative agents.

1.2 AIM AND OBJECTIVES

1.2.1 Aim

The aim of the study is to isolate, identify, determine the antibiotic resistant profiles and evaluate the ability of two antimicrobial agents (tetracycline and ampicillin) in inducing shiga-toxin proteins in broth cultures of some selected *E. coli* O157:H7 isolates.

1.2.2. Objectives

The specific objectives of the study are to:

- isolate *E. coli* O157:H7 from groundwater samples
- identify the isolates using preliminary (Gram staining, oxidase test, TSI and citrate utilisation) and confirmatory identification (serotyping, specific PCR analysis)
- determine the presence of shiga toxins genes using specific PCR analysis

- determine the percentage and MAR profiles of confirmed *E. coli* O157:H7 isolates
- determine the presence and concentration of shiga toxins in supernatants of antibiotic induced cultures using an ELISA assay.

CHAPTER 2
LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

Escherichia coli is a Gram-negative rod-shaped bacterium that occurs as normal flora in the gastro-intestinal tract of humans and animals (Donnenberg and Whittam, 2001). However, some *E. coli* strains have been reported to cause diseases in these species (Bell, 2000; Leelaporn *et al.,* 2003; Nunes *et al.,* 2003). *E. coli* strains that belong to the serotype O157:H7 have attracted a lot of attention because of their ability to cause a number of outbreaks and/or sporadic cases of food-borne infections worldwide (Dundas *et al.,* 2001; Maruzumi *et al.,* 2005; Sugiyama *et al.,* 2005). *E. coli* O157:H7 usually ferments sorbitol and does not hydrolyze 4-methylumbelliferyl-β-D-glucuronide (Wells *et al.,* 1983; Doyle and Schoeni, 1984). This pathogen is known to cause infections through the production of toxins termed shiga-toxins or verotoxins (Riley *et al.,* 1983; Karch *et al.,* 2005; Duffy *et al.,* 2006; Razzaq, 2006; Walch *et al.,* 2006). Shiga toxins are known to be the principal virulence factors of *E. coli* O157:H7 and two main types that are designated stx_1 and stx_2, have been identified and characterised (Pierard *et al.,* 1997; Law, 2000). However, other accessory virulence factors such as *eaeA* and *hlyA* have been detected in pathogenic *E. coli* O157:H7 strains from diseased patients (Law, 2000; Yilmaz *et al.,* 2006; Ateba *et al.,* 2008).

2.2 Virulence properties of *E. coli*

According to the World Health Organisation (WHO), enterotoxigenic *E. coli* (ETEC) causes diarrhoea , this usually occurs without fever (WHO, 2010). ETEC strains are non-invasive, and do not leave the intestinal lumen. ETEC is the leading cause of bacterial diarrhoea in children in developing countries and the most common cause of traveler's diarrhoea (Northey *et al.,* 2007). Each year, ETEC causes more than 200 million cases of diarrhoea and 380,000 deaths (Wenneras and Erling, 2004). Enteropathogenic *E. coli* (EPEC) is a causative

agent of diarrhoea in humans, rabbits, dogs, cats and horses. Like ETEC, EPEC also causes diarrhoea, but the molecular mechanisms of colonisation and aetiology are different. EPEC lacks fimbriae, shiga-like-toxinsand heat liable toxins, but utilises an adhesin known as intimin that binds to host intestinal cells (Adu-Bobie *et al.,* 1998; Gansheroff *et al.,* 1999; Malish *et al.,* 2003). .Adherence to the intestinal mucosa results in rearrangement of actin in the host cell, causing significant deformation (Gouin *et al.,* 2005). EPEC cells are moderately invasive and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to attachment and effacement are likely the major cause of diarrhoea in those affected with EPEC (Arbeloa *et al.,* 2008).

Enteroinvasive *E coli* (EIEC) are another group found only in humans. EIEC infection causes a syndrome identical to shigellosis, with profuse diarrhoea and high fever (Rendón *et al.,* 2007).

Enterohemorrhagic *E. coli* (EHEC) is found in humans, domestic and wild animal species (FAO/WHO, 2008). The most famous member within this group of *E. coli* is the serotype O157:H7, which causes bloody diarrhoea not characterised by fever. EHEC can cause hemolytic uremic syndrome (HUS) known to be the major cause of kidney failure (Caprioli *et al.,* 1997; Verweyen *et al.,* 1999). It uses bacterial fimbriae for attachment (Rendón *et al.,* 2007; Roe *et al.,* 2001), is moderately invasive and possesses a phage-encoded Shiga toxin that can elicit an intense inflammatory response (Rendón *et al.,* 2007).

Enteroaggregative *E. coli* (EAEC) was first described in 1987 (Nataro *et al.,* 1987) in a child with acute diarrhoea and since then, they have been linked with persistent diarrhoea in children living in areas where EAEC is endemic (Wanke *et al.,* 1991). In several regions of the world, EAEC surpasses enterotoxigenic *E. coli* (ETEC) as the most common bacterial pathogen identified in diarrhoeal stool samples and in the United States, this emerging pathogen is becoming increasingly recognised as a leading cause of sporadic diarrhoea in healthy

adults and children (Cohen *et al.,* 2005; Nataro *et al.,* 2006). Contaminated food appears to be the main source of EAEC infection and has been implicated in several foodborne outbreaks of diarrhoea (Hedberg *et al.,* 1997; Itoh *et al.,* 1997). EAEC are non-invasive and produce a hemolysin and a shiga like enterotoxin similar to that of ETEC (Rendón, *et al.,* 2007).

2.3 Toxins produced by *E. coli* O157:H7

Shiga-toxin producing *E. coli* strains, particularly the serotype O157:H7, cause diseases in humans by producing toxins called shiga-toxins. Shiga toxins are a family of related toxins that resemble the toxins produced by *Shigella dysenteriae* in structure and biological activity (O'Brien *et al.,* 1983; Beutin, 2006). Two major groups that are designated stx_1 and stx_2 have been identified and characterised (Gannon *et al.,* 1990; Weinstein et al., 1998). Moreover, there are other variants of the stx_2 that are known to play a role in the pathogenesis of some strains (Schmidt *et al.,* 2000). The shiga toxin genes are considered to be part of the genome of lambdoid prophages (Friedman and Court, 2001). Shiga toxins inhibit protein synthesis within target cells by a mechanism similar to that of ricin toxin produced by *Ricinus communis* (Sandvig and van Deurs, 2000). After entering a cell, the protein functions as an N-glycosidase, cleaving a specific adenine nucleobase from the 28S RNA of the 60S sub-unit of the ribosome, thereby, halting the process protein synthesis (Sandvig *et al.,* 2010).

2.4 Antibiotic therapy and resistance

Bacterial infections are usually treated with antibiotics (Johnson *et al.,* 2006). However, the sensitivities of different strains of *E. coli* to particular antibiotics vary widely (Ateba and Bezuidenhout, 2008; Wose King *et al.,* 2010; Phokela *et al.,* 2011). Antibiotics which may be used to treat *E. coli* infection include amoxicillin, other semi-synthetic penicillins, cephalosporins, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and aminoglycosides (Johnson *et al.,* 2006). Currently, there is a lot of controversy involving the treatment of *E. coli* O157:H7 infections in humans (Igarashi et al., 1999; Wong *et*

al., 2000; Dundas *et al.,* 2001). Some studies have postulated that antibiotics may serve as inducers for bacteriophages that harbour the shiga toxin genes and this increases the chances of the disease to progress to more severe clinical forms (Craig *et al.*, 2000; Zhang *et al.,* 2000; Dundas *et al.,* 2001). Despite this, it has also been demonstrated that the administration of antibiotics during the early stages of *E. coli* O157:H7 infections may prevent the development of haemolytic uremic syndrome HUS (Slutsker *et al.,* 1997). Mitomycin C has been extensively studied to investigate its contribution in inducing shiga toxins in *E. coli* O157:H7 strains (Ptashne, 2004; Aertsen *et al.,* 2005; Wegrzyn and Wegrzyn, 2005). It is therefore important to evaluate the ability of other antimicrobial agents in inducing shiga-toxins from *E. coli* cells.

The overuse of antibiotics in humans and their use as growth promoters in animals (Johnson *et al.,* 2006) may account for the increased resistance reported worldwide (Ateba and Bezuidenhout, 2008; Wose King *et al.,* 2010; Phokela *et al.,* 2011). Moreover, the rate of adaptative mutations in *E. coli* is on the order of 10^{-5} per genome per generation, which is 1,000 times as high as previous estimates (Perfeito et al., 2007). Faced with these attributes, antibiotic-resistant *E. coli* may disseminate resistant genes to other bacteria species through a horizontal process. *E. coli* strains that carry multidrug resistant plasmids readily transfer the plasmids to other susceptible species when subjected to stress (Salyers *et al.,* 2004; Shakibaie *et al.,* 2009). The transfer is highly facilitated by the fact that *E. coli* is a frequent member in most biofilms, where they associate with many different bacteria species (Paul *et al.,* 1999).

2.5 Health effects of *E. coli* O157:H7

E. coli O157:H7 belongs to the group of strains that are highly pathogenic to humans (Tozzi *et al.,* 2003; Beutin *et al.,* 2004; Chang *et al.,* 2004; Lynn *et al.,* 2005). This strain has been identified as the cause of most food and water-borne infections reported in humans worldwide (Belongia *et al.,* 1993; Mead *et al.,* 1997; Müller *et al.,* 2001; Olsen *et al.,* 2002; Sugiyama *et al.,* 2005). Infections

caused by *E. coli* O157:H7 often range from non-bloody diarrhoea, abdominal cramps, bladder and kidney infections, pneumonia, neonatal meningitis and to the more complicated haemolytic uraemic syndrome, haemorrhagic colitis and thrombotic thrombocytopenic purpura (Mahon *et al.,* 1997; Nataro and Kaper, 1998; Igarashi *et al.,* 1999; Olsen *et al.,* 2002; Tozzi *et al.,* 2003; Chang *et al.,* 2004; Lynn *et al.,* 2005). Frequently, no fever is present. It should be noted that these symptoms are common to a variety of diseases, and may be caused by sources other than contaminated drinking water. In some individuals, particularly children under 5 years of age, elderly subjects and immuno-compromised individuals, the infections are usually more severe (Riley *et al.,* 1983; Dundas *et al.,* 2001; Duffy *et al.,* 2006; Razzaq, 2006; Walch *et al.,* 2006). Moreover, these infections are high in individuals who live in developing countries. Poor hygiene has been considered a valuable factor for transmission of the pathogen to healthy individuals (Daniels *et al.,* 2000).

2.6 Outbreaks of by *E. coli* O157:H7

The first recognised outbreaks of illness caused by *E. coli* O157:H7 happened in 1982. Undercooked hamburger meat was recognised as the carrier (Riley *et al.,* 1983). Outbreaks and/or sporadic cases of infections caused by *E. coli* O157:H7 appear to be increasing worldwide and these infections are reported even in countries such as the USA, France, Canada, Japan, Scotland, Wales and UK that have more advanced public health and health care facilities (Belongia *et al.,* 1991; Bell *et al.,* 1994; Willshaw *et al.,* 1997; Chapman *et al.,* 1997; Allison *et al.,* 2000; Vogt and Dippold, 2005; CDC 2011; 2012). The morbidity and mortality associated with several recent large outbreaks of gastro-intestinal diseases caused by *E. coli* O157:H7 strains have highlighted the threat these organisms pose to human health (Elliott and Nicholas, 2001; Feng, 2001; Heuvelink *et al.,* 2002; Tsuji *et al.,* 2002; Doorduyn *et al.,* 2006). A large propotion of outbreaks of *E. coli* O157:H7 infections have been reported to be caused by the consumption of contaminated food (Bouvet *et al.,* 2001; Botteldoorn *et al.,* 2003; Tutenel *et al.,* 2003; Anonymous, 2006). Food products such as chicken, lamb, pork, beef,

mince, vegetables, produce from manure-fertilized gardens and raw milk have been reported as major sources of infections (Richert *et al.,* 2000; Lejeune *et al.,* 2001; Guan and Levin, 2002; Davis and Kendall, 2005). Ground beef too remains an important cause of outbreaks (Davis and Kendall, 2005). A recent outbreak was reported in 2012, in which 58 individuals were infected with *E. coli* O157:H7 within 9 states in the USA. Collaborative laboratory investigations indicated that romaine lettuce was the source of the outbreak (CDC, 2012).

Despite the fact that most outbreaks of *E. coli* O157:H7 infections have resulted through the consumption of contaminated food products, contaminated water has also been identified as a potential source for the transmission of this pathogen to humans (Müller *et al.,* 2001; Momba *et al.,* 2008; Ateba *et al.,* 2011). Evidence for this can be drawn from the number of outbreaks reported worldwide (Paunio *et al.,* 1999; Olsen *et al.,* 2002; Said *et al.,* 2003; Schets *et al.,* 2005). Waterborne outbreaks are associated with the consumption of water contaminated with bacteria of faecal origin and this may include water from recreational sources (Licence *et al.,* 2001; Olsen *et al.,* 2002), (Cransberg *et al.,* 1996; Paunio *et al.,* 1999). In South Africa, outbreaks of *E. coli* O157:H7 infections have not been reported to date. However, *E. coli* O157:H7 has been isolated from animals and humans (Ateba *et al.,* 2008; Ateba *et al.,* 2011) and this increases the possibility of contaminating either surface or groundwater through improper deposition of faeces. Moreover, water supplied by drinking water companies is treated as opposed to water obtained from boreholes and catchment areas that is usually untreated. This phenomenon is common in rural communities where there is insufficient protection of these water bodies resulting in contamination through leaching of animal faeces (Olsen *et al.,* 2002). Given the fact that the specific monitoring of pathogens such as *E. coli* O157:H7 in water bodies may suggest possible health implications on consumers (Schets *et al.,* 2005) especially in developing communities, the importance of this study cannot be overemphasised.

CHAPTER 3
MATERIALS AND METHODS

3.1 AREA OF THE STUDY

The research was conducted at the North West University-Mafikeng Campus, North West Province. A total of 55 groundwater samples were collected from some rural communities in the North West Province, South Africa. Table 3.1 indicates the number of water samples collected from different sample sites.

3.2 SAMPLE COLLECTION

A total of 55 water samples were collected from borehole taps and storage tanks in some rural communities in the North West Province of South Africa. Before collecting the samples, taps were allowed to run for about 1 minute and water samples were collected in 500 ml Durham Schott bottles. The water samples were clearly labelled and immediately transported on ice to the laboratory for analysis. Upon arrival in the laboratory, all the samples were analysed for *E. coli* within 6 hours.

Table 3.1: Areas where water samples were collected during the study.

Sample source	Sampling area	Location	Number of samples
Ground water (Borehole)	Bekkersdal	Rural	2
	Khutsong	Rural	2
	Taung	Rural	2
	Zeerust	Rural	6
	Delaryville	Rural	2
	Deelpan	Rural	2
	Rustenburg	Rural	2
	Disaneng	Rural	8
	Tshidilamolomo	Rural	2
	Selosesha	Rural	2
	Makgobistad	Rural	2
	Logagane	Rural	2
	Mabule	Rural	2
	Dinareng	Rural	2
	Masamane	Rural	2
	Leporang	Rural	2
			2
Tap water (Storage tanks)	Stella	Rural	2
	Zeerust	Rural	3
	Rustenburg	Rural	2
	Deelpan	Rural	2
	Carltonville	Rural	2

3.3 ISOLATION OF *E. coli* O157:H7

3.3.1 Water Samples: Membrane filtration method

Membrane filtration method was used for the isolation of *E. coli* O157:H7 from water samples. Aliquots of 100 ml from each of the sample were filtered through 0.45µm Gridfilter-units (Type HA) using a Gelman Little Gaint pressure/vacuum pump machine (model 13156 – GelmanSciences, Michigan - USA). The filters were handled with sterile forceps and placed onto sorbitol-MacConkey agar (SMAC) for selective isolation of *E. coli* O157:H7. The plates were incubated at 37°C for 24 hours (Müller *et al.*, 2001).

3.3.2 Purification of presumptive *E. coli* isolates

Eight presumptive *E. coli* O157:H7 colonies from each sample were sub-cultured onto sorbitol-MacConkey agar. The plates were incubated at 37°C for 24 hours (Meichtri *et al.*, 2004). After incubation, colourless colonies were regarded as presumptive *E. coli* O157:H7 isolates. Pure cultures that were identified as sorbitol negative colonies and lactose positive were preserved for characterisation and further biochemical identification using standard tests for *E. coli*.

3.4 BACTERIAL IDENTIFICATION

Presumptive *E. coli* isolates were identified based on the following criteria:

3.4.1 Cellular Morphology

Isolates were Gram stained using standard techniques (Cruikshank *et al.*, 1975), which differentiates bacterial species into Gram positive and Gram negative based on the chemical and physical properties of their cell walls (Bergey *et al.*, 1994). In performing the test, colonies were revived on nutrient agar plates. The isolates were transferred onto clean microscope slides that contained a drop of distilled water. The water and the bacteria culture were mixed using a sterile wire loop to prepare a bacteria smear. The smear was heat-fixed and flooded with crystal violet for 2 minutes. Crystal violet solution was washed off using tap water

and the slide was flooded with iodine solution for 2 minutes. The iodine solution was washed off and 70% ethanol used to remove excess crystal violet stain from the slide. The slide was later flooded with safranin O for 2 minutes and washed with water. It was air dried and observed under a microscope.

3.4.2 Oxidase test

The oxidase test was performed on all *E. coli* isolates to screen for the presence of the cytochrome system. This test was performed using the TestOxidase™ reagent (PL.390) Mast Diagnostics (Neston, Wirral, U.K.) in accordance with the manufacturer's protocol. A large mass of pure bacteria was aseptically transferred using a sterile loop to a test strip. The strip was observed for up to three minutes. The area of inoculation that turned dark-blue to maroon or black were recorded as positive results. All the isolates that were oxidase positive were retained for further testing.

3.4.3 Triple Sugar Iron (TSI) agar test

The presumptive isolates were subjected to the Triple sugar iron (TSI) agar test (Biolab, Merck – South Africa). This test was used to assay the *E. coli* content against three sugars (glucose, sucrose and lactose) that are present at concentrations 0.1%, 1.0% and 1.0% respectively (Forbes and Weissfeld, 1998). In performing this test, a large mass of pure bacteria was stab inoculated into the butt of the TSI medium and later streaked on top of the slant using a sterile wire loop (USPC, 2001; Prescott, 2002). The culture was incubated at 37°C for 24 hours with the caps loosely closed. The results were interpreted based on colour change from red to yellow in the medium coupled with the production of hydrogen sulphide gas (H_2s).

3.4.4 Simmons Citrate agar test

In performing the test, pure colonies of the test organism were stab inoculated into the butt and streaked on the surface of the slant with a wire-loop. The tubes were then incubated at 37°C for 24 hours. A positive reaction was identified by

growth on the slant with an intense blue colour. All isolates that satisfied this preliminary identification criterion were subjected to confirmatory biochemical tests.

3.5 CONFIRMATORY BIOCHEMICAL IDENTIFICATION TESTS FOR ISOLATES

3.5.1 Analytical Profile Index (API 20E) test

In this study, API 20E system was used according to the manufacturer's specifications (BioMérieux, Marcy l'Etoile, France). Briefly, an incubation box (tray and lid) was prepared and 5 ml of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere. The strip was removed from its individual packaging and placed in the incubation box. A 24 hours bacterial suspension in 2 ml distilled water was prepared for each isolate. A sterile needle was used to fill the microtubes with the suspension as per manufacture instructions. The strip was placed in the incubation box and incubated at 37°C for 24 hours. The results were read with or without the addition of reagents. Indices were generated and the identities of the isolates were determined using the API web software.

3.5.2 Serotyping

Representative *E. coli* isolates from each sample were further analysed for characteristics of *E. coli* O157:H7 using the slide agglutination test with *E. coli* O157 and H7 specific antisera. The test was performed as per manufacture instructions (Mast Diagnostics, U.K). This was achieved by dividing a glass slide into two sections. A drop of saline was put in each section. A pure colony of the isolate was emulsified using a wireloop in each drop of saline to obtain a dense suspension. One drop of *E. coli* O157 or H7 monovalent specific antiserum was added to one of the suspensions and mixed. The other suspension served as a negative control. Agglutination was observed using indirect lighting over a dark

background. Agglutination that was strong and clearly visible within one minute was recorded as positive results.

3.6 MOLECULAR CHARACTERISATION OF ISOLATES

3.6.1 Extraction of Genomic DNA

Genomic DNA was extracted from all presumptive *E. coli* O157:H7 isolates using a modified cell boiling method (Tunung *et al.,* 2007). Fresh cultures were prepared by spread plating the isolates onto nutrient agar plates to revive the cells. Plates were incubated at 37°C for 24 hours. After incubation, 500 µl of sterile water was placed in 1.5 ml microfuge tube and pure cultures of the isolates were transferred into the tubes. The tubes were votexed vigorously to prepare a homogenous suspension. The cell suspension was incubated at 100°C in a heating block (Biorad, Digital dry bath) for 15 minutes and this was followed by centrifugation for 2 minutes at 13500 rpm. After centrifugation, the tube was placed on ice for 5 minutes and the supernatant was transferred to a new tube. An aliquot of 5µl of this supernatant was used for PCR analysis.

3.6.2 Multiplex PCR for the detection of two *E. coli* housekeeping genes

A multiplex PCR analysis was performed on all positively identified *E. coli* isolates to detect the presence of two housekeeping genes; the malate dehydrogenase (*mdh*) (Tarr *et al.,* 2002) and beta-galactosidase (*lacZ*) (Ram and Shanker, 2005). The primer combinations and cycling conditions used are shown in Table 3.2.

3.6.3 Identification of suspected *E. coli* O157:H7 isolates by PCR

The identities of the suspected *E. coli* O157:H7 isolates were confirmed through amplification of the $rfbO_{157}$ (Morin *et al.,* 2004) and the $fliC_{H7}$ gene fragments (Reischl *et al.,* 2002). PCR was performed using oligonucleotide primer combinations presented in Table 3.2. Amplifications were performed using a Peltier Thermal Cycler (model-PTC-220 DYAD™ DNA ENGINE). Standard 25 µl PCR reactions that constituted of 1 µg/µl of the template DNA, 50 pmol of each

oligonucleotide primer set, 1 X PCR master mix, 1U *Taq* DNA polymerase and RNase free distilled water were performed. All the PCR reagents used were Fermentas, USA products and supplied by Inqaba Biotec Ltd, South Africa. Amplification conditions for the rfb_{O157} and the $fliC_{H7}$ gene fragments are shown in Table 3.2. All PCR products were stored at 4°C until electrophoresis.

3.6.4 PCR for the detection of shiga-toxin genes in *E. coli* O157:H7 isolates

Specific PCR analysis was performed on all positively identified *E. coli* O157:H7 isolates to detect the presence of the shiga-toxin genes (Pass et al., 2000). The primer combinations and cycling conditions used are shown in Table 3.2.

3.6.5 Electrophoresis of PCR products

The PCR products were resolved by electrophoresis on a 2% (w/v) agarose gel. A horizontal Pharmacia biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia biotech, Sweden) was used to carry out electrophoresis. Gels were run for 5 hours at 60V using 1X TAE buffer (40mM Tris, 1mM EDTA and 20mM glacial acetic acid, PH 8.0). Each gel contained a 100bp DNA molecular weight marker (Fermentars, USA). The gels were stained in ethidium bromide (0.1µg/ml) for 15 minutes and amplicons were visualized under U.V light at 420 nm (Sambrook *et al.,* 1989). A Gene Genius Bio Imaging System (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 6.00.22) software. GeneTools (version 3.07.01) software (Syngene, Synoptics; UK) was used to analyse the images in order to determine the relative sizes of the amplicons.

Table 3.2: Oligonucleotide primers and the different cycling conditions used for molecular identification of *E. coli* O157:H7 isolated during the study.

Primer	Sequence (5' – 3')	Target Gene	Amplicon size (bp)	PCR Cycling Conditions	
RfbE F[d]	GCGCGAATTCGTGCTTTTGATATTTTTCCGAGTACATTGG	$rfbE_{O157}$	239	45x[f]	94 °C for 30s
RfbE R[d]	GCGCGAATTCTTTATATCACGAAAACGTGAAATTGCTGAT				60 °C for 90s
					72 °C for 90s
FliCH7 F[b]	GCTGCAACGGTAAGTGAT	$fliC_{H7}$	984	45x[f]	94 °C for 30s
FliCH7 R[b]	GGCAGCAAGCGGGTTGGT				60 °C for 90s
					72 °C for 90s
mdhF[c]	GGTATGGATCGTTCCGACCT	*mdh*	301	35x[g]	94 °C for 1min
mdh R[c]	GGCAGAATGGTAACACCAGAGT				59 °C for 1min
					72 °C for 1min
SLF[a]	CCCCTCTTTTAGTCAGTCAACTG	*stx*	159	35x[g]	94 °C for 1min
SLR[a]	GGCAGGATTACAACAAAGTTCACA				67 °C for 1min
					72 °C for 1min
lacZF[e]	CTGGCGTAATAGCGAAGAGG	*lacZ*	228	35x[g]	94 °C for 1min
lacZR[e]	GGATTGACCGTAATGGGATATG				59 °C for 1min
					72 °C for 1min

[f,g] Initial denaturing steps of (95°C for 10min) respectively and final strand extension steps of (72°C for 10min), [a]Pass *et al.*, 2000; [b]Reischl *et al.*, 2002; [c]Tarr *et al.*, 2002, [d]Morin *et al.*, 2004, [e]Ram and Shanker, 2005

3.7 ANTIBIOTIC SUSCEPTIBILITY TESTS

Antibiotic susceptibility test was performed on *E. coli* O157:H7 isolates to determine their antibiotic resistance profiles using the Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966). The susceptibilities of isolates against a panel of 7 different antibiotic discs obtained from Mast Diagnostic, United Kingdom were determined. Isolates were revived by culturing them on SMAC plates. The plates were incubated aerobically at 37°C for 24 hours. Bacterial suspensions were prepared using these pure isolates and aliquots of 100 µl from these suspensions were spread-plated on Mueller-Hinton agar (Biolab, Merck, South Africa). The antibiotic discs were gently pressed onto the inoculated Mueller-Hinton agar to ensure intimate contact with the surface and the plates were incubated aerobically at 37°C for 24 hours (CLSI, 1999). The antibiotic inhibition zone diameters were measured and the results obtained used to classify organisms as being resistant, intermediate and susceptible to a particular antibiotic based on standard reference values (NCLS, 1999). Table 3.3 indicates details of antibiotics that were used in the study. The antibiotics used were those to which isolates from animals and humans have been found to be resistant against in the area (Ateba and Bezuidenhout, 2008; Bezuidenhout and Moneoang, 2009).

Table 3.3: Details of antibiotics used in this study. The superscripts [a] and [b] indicate the generally accepted concentrations of the discs according to the manufacturer, Mast Diagnostics, Merseyside, United Kingdom.

Group	Antibiotic	Disc conc.	R	I	S
Aminoglycosides	Ne	30µg[b]	≤12	13-16	≥17
	K	30µg[b]	≤13	14-17	≥18
Beta-Lactams	Ap	10µg[a]	≤11	12-14	≥15
	A	10µg[a]	≤13	14-16	≥17
	PG	10µg[a]	≤20	21-28	≥29
Tetracycline	OT	30µg[b]	≤14	15-18	≥19
Phenols	C	30µg[b]	≤12	13-17	≥18

Ap (ampicillin), K (kanamycin), C (chloramphenicol), PG (penicillin G), A (amoxicillin), OT (oxytetracycline) and Ne (neomyxin).

3.8. MULTIPLE ANTIBIOTIC RESISTANT (MAR) PHENOTYPES

MAR phenotypes were generated for isolates that were resistant to 3 and more antibiotics (Rota *et al.,* 1996). Phenotypes were generated using the abbreviations that appeared on the antibiotic discs.

3.9 CYTOTOXICITY ASSAY

E. coli O157:H7 isolates that were positively identified by PCR analysis to possess the shiga-toxin genes were incubated for 24, 48 and 72 hours respectively in Luria Bertani broth while shaking at 170 rpm. Prior to shaking, either ampicillin or tetracycline was added to the broth at concentrations of 10 µg/ml or 30 µg/ml respectively. After incubation, the entire broth sample was centrifuged for 15 minutes at 3000 rpm. Aliquots of 100 µl from the clear supernatants were used in performing the ELISA test. The ELISA test was performed using a RIDASCREEN® Verotoxin (C2201). The test is an *in vitro* diagnostic assay for the quantitative determination of shiga-like toxins 1 and 2 of *Escherichia coli* strains in stool and enrichment cultures.

In performing the test 100µl of the positive control, negative control and the test samples were pipetted into the wells of a 96 well micro-titre plate. The samples were incubated at room temperature (20 – 25°C) for 60 minutes. The samples were emptied and the plate inverted on absorbent paper in order to remove the residual moisture. The plate was washed five times using 300 µl of the wash buffer and then 100 µl of the enzyme conjugate was added to the wells and incubated at room temperature for 30 minutes. The plate was washed five times using 300µl wash buffer and 100µl of the substrate added to each well. The plate was then incubated at room temperature for 15 minutes in the dark. After incubation, the reactions were stopped by adding 50 µl of the stop reagent to each well. The extinction was measured spectrophotometrically using a micro-plate reader (LT-4000MS – Labtech International Ltd, Sussex, UK) at a wavelength of 450 nm. In validating the results obtained, the test was considered correctly carried out if the extinction value of the positive control was greater than 0.8 at 450 nm and that of the negative control less than 0.2 nm at 450 nm. In calculating the cut-off, the following formula was used:

Culture supernatant: Cut-off = Extinction for the negative control + 0.1

Therefore, 0.2 was considered the threshold above which all tests were seen to be positive for the shiga-toxin genes and results were recorded as positive when their extinction was greater than the calculated cut-off value.

CHAPTER 4

RESULTS AND INTERPRETATION

4.1 Detection of *E. coli* isolates in groundwater samples using preliminary and confirmatory biochemical tests

Fifty five water samples were analysed for the presence of *E. coli* using modified selective media. Only isolates that satisfied both the preliminary and confirmatory biochemical identification tests were considered. A total of 400 isolates were screened for characteristics of *E. coli*. Of these isolates 41.5% and 16.8% were positively identified using API 20E and serotyping respectively. Large proportions of the isolates (47% and 67.5%) were oxidase positive, could not utilise citrate, able to ferment the three sugars in the TSI medium but with only 42% able to produce gas and hydrogen sulphide. The results showed that *E. coli* was frequently isolated from samples obtained in villages around Zeerust and Rustenburg. All the samples that were positive for *E. coli* from these areas were collected from borehole tanks placed next to the animal manure storage. Despite the fact that *E. coli* was detected in water obtained from Deelpan and Tshidilamolomo, none of these isolates were positively identified with API 20E and serotyping tests. The results in Table 4.1 indicate the number of isolates screened from the different sampling areas and those that were positive for the different tests. All the isolates that were positively identified as *E. coli* by API 20E and serotyping, were subjected to confirmatory identification using molecular methods.

Table 4.1: Results for preliminary and confirmatory biochemical test

Sample source	Gram staining (-ve rod)	Oxidase (+ve)	Lactose (+ve)	Glucose (+ve)	H_2s (-ve)	Gas (+ve)	SCT (-ve)	Serotyping	No. of *E. coli* isolates using API 20E test
Carltonville	8	6	5	5	6	6	8	8	4
Khutsong	16	10	11	10	15	10	11	0	10
Bekkersdal	16	9	10	11	10	9	10	2	14
Taung	20	8	7	8	4	5	5	10	4
Vryburg	16	7	11	11	11	11	7	0	5
Delaryville	16	15	10	11	10	10	7	5	12
Zeerust	100	80	55	55	65	66	40	25	7
Deelpan	24	18	15	15	14	3	0	0	0
Tshidilamolomo	8	7	5	5	6	5	2	0	0
Rustenburg	100	12	8	10	75	70	33	5	32
Dinotateng	18	15	6	7	4	3	5	0	1
Disaneng	8	7	7	6	4	5	5	0	2
Logagane	8	4	6	6	6	7	6	3	1
Leporang	8	4	4	6	3	6	6	2	1
Makgobistad	8	6	4	4	5	4	7	0	1
Mabule	8	7	4	4	4	5	4	2	3
Masamane	8	5	5	4	4	4	4	1	0
Selosesha	8	4	6	5	7	4	3	1	0
Stella	12	4	4	5	6	6	5	3	1
Total No.	400	218	188	259	239	168	270	67	166

4.2 Molecular characterisation of *E. coli* isolates from groundwater

4.2.1 PCR for the detection of two *E. coli* housekeeping genes

A total of 67 *E. coli* isolates that were positively identified by serotyping were subjected to specific and multiplex PCR analysis for the detection of two *E. coli* housekeeping genes, the *mdh* and *lacZ* genes. The *mdh* gene codes for malate dehydrogenase and the *lacZ* is a structural gene within the lac operon that codes for the enzyme β-galactosidase. This operon is required for transporting and metabolising lactose in *E. coli* cells. Figure 4.1 shows a 2% agarose (w/v) gel depicting the *mdh* and *lacZ* gene fragments that were amplified by PCR using genomic DNA extracted from *E. coli* isolates. The *mdh* and *lacZ* fragments with the expected amplicon sizes of 301bp and 228bp respectively were obtained. Table 4.2 indicates the number of isolates that were screened from the different sampling points and those that were positive for the targeted genes. As shown in Table 4.2, a total of 23 (34%) of the potential *E. coli* O157:H7 isolates were positively identified by specific PCR analysis. Despite this, none of the isolates were positive for the shiga-toxin genes. However, a large proportion 57 (85%) of these isolates that were screened, were positive for the *mdh* gene fragments.

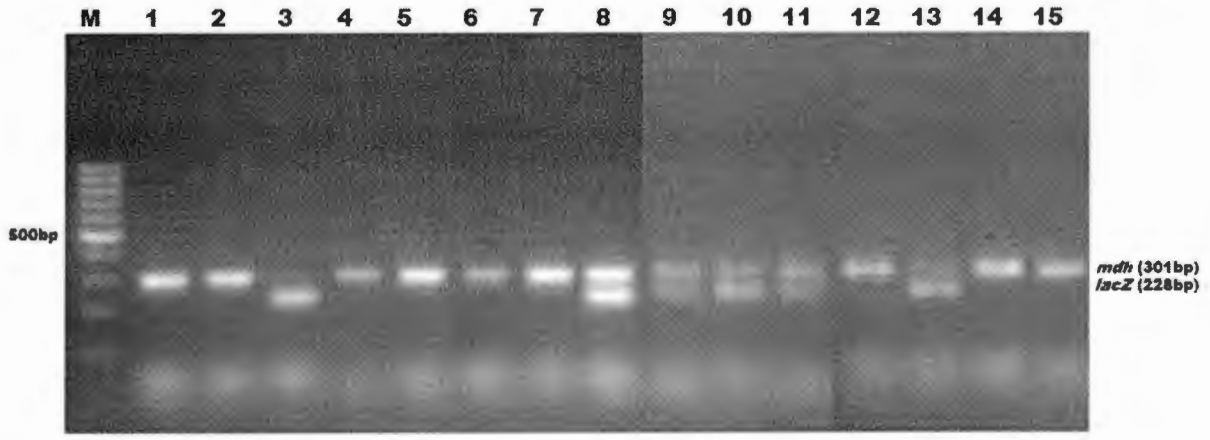

Figure 4.1: PCR for the detection of *E. coli* housekeeping genes *mdh* and *lacZ*. Lane M = 100 bp DNA marker; Lanes 1-2=*mdh* genes from Zeerust isolates; lane 3=*lacZ* from Zeerust isolates; lanes 4-7=*mdh* genes from Taung isolates; Lane 8-11=*mdh* and *lacZ* genes from Zeerust isolates, Rustenburg and Taung isolates; Lane 12=*mdh* from Rustenburg isolates; Lane 13= *lacZ* gene from Rustenburg isolates and Lane 14-15=*mdh* from Rustenburg isolates.

4.2.2 PCR for the identification of *E. coli* O157:H7 isolates using rfb_{O157} and $Flic_{H7}$ primers

A total of 57 and 31 *E. coli* isolates that possessed the *mdh* and *lacZ* genes were subjected to specific PCR analysis for identification as *E. coli* O157:H7. This was achieved through amplification of the $rfbO_{157}$ and $fliC_{H7}$ gene fragments specific for *E. coli* O157:H7 isolates. Only 15 (40.1%) of the isolates were positively identified as *E. coli* O157:H7 and results are shown in Table 4.2. Figure 4.2 indicates a 2% (w/v) agarose gel showing PCR rfb_{O157} gene fragments that were amplified. As shown in Table 4.2, the number of *E. coli* O157:H7 isolates obtained were higher in samples obtained from Zeerust (47.8%) and Taung (30.4%).

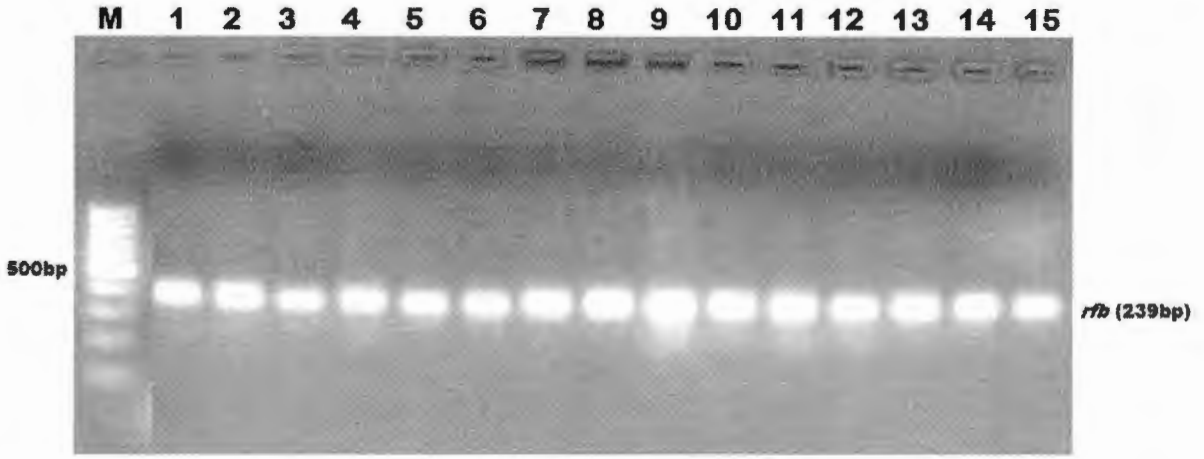

Figure 4.2: PCR for the detection of *E. coli* gene rfb_{O157}. Lane M=100bp DNA marker; Lanes 1-5= *rfb* gene from *E. coli* O157:H7 isolated from water samples in Zeerust; lanes 6-10 = *rfb* gene from *E. coli* O157:H7 isolated from water samples in Rustenburg and Lanes 11-15= *rfb* gene from *E. coli* O157:H7 isolated from water samples in Taung.

4.2.3 PCR for the detection of *stx* genes of *E. coli* O157:H7

A total of 67 potential *E. coli* O157:H7 isolates were screened by specific PCR for the presence of the *stx* genes (Table 4.2). None of the isolates from the different sample stations were positive for shiga-toxin genes. Despite this, the isolates may still have the potential to cause water and foodborne infections in humans since there are other *E. coli* O157:H7 accessory virulence gene determinants that are currently associated with pathogenesis of these bacteria strains.

Table 4.2: Number of *E. coli* isolates that were positive for the targeted genes

Sample source	No of isolates tested	No of isolates positive for the targeted genes				
		mdh	***lacZ***	***rfb*$_{O157}$**	***fliC*$_{H7}$**	***stx***
Carltonville	8	8	0	3	3	0
Bekkersdal Dam	2	2	0	0	0	0
Taung	10	10	10	7	7	0
Delaryville	5	0	0	0	0	0
Zeerust	25	24	11	11	11	0
Rustenburg	5	3	0	0	0	0
Logagane	3	0	0	0	0	0
Leporang	2	1	1	2	2	0
Mabule	2	1	1	0	0	0
Masamane	1	0	0	0	0	0
Selosesha	1	3	0	0	0	0
Stella	3	3	3	0	0	0
Total No.	**67**	**53**	**31**	**23**	**23**	**0**

4.3 Percentage antibiotic resistant data of *E. coli* isolated from the ground water

A total of 67 *E. coli* isolates from different sampling sites in the North West Province were tested to evaluate their susceptibilities to 7 different antibiotics. Results obtained are shown in Table 4.5. All the *E. coli* O157H7 isolates from water samples obtained from Bekkersdal, Carltonville, and Taung were resistant to amocyxillin, ampicillin, chloramphenicol and penicillin G. Moreover, all the isolates from Delaryville, Logagane, Leporang, Mabule, Selosesha and Stella were resistant of amocyxillin, ampicillin, oxytetracycline and penicillin G. On the contrary, none of the isolates from Khutsong, Vryburg, Deelpan, Tshidilamolomo, Dingateng, Disaneng and Makgobistad were resistant to any of the antimicrobial agents tested. A large proportion (66.7% to 100%) of the isolates from Mabule, Selosesha, Stella and Rustenburg were resistant to kanamycin. However, none of the isolates from Carltonville, Khutsong, Vryburg, Deelpan, Tshidilamolomo, Dingateng, Disaneng, Logagane, Leporang Makgobistad and Masemane were resistant to kanamycin and neomycin. Despite this, 8.3% to 17% of the isolates from the aforementioned villages including Stella and Taung were resistant to neomycin. In general, ampicillin, amoxicillin, tetracycline and penicillin G are the drugs to which most of the isolates from the different sources were resistant to (Table 4.1). However, these isolates were highly susceptible to neomycin.

Table 4.3: Percentage antibiotic resistance data for *E. coli* O157:H7 isolates

Sampling site	No. of isolates	A (10)	Ap (10)	K (30)	Ne (30)	OT (30)	C (30)	PG (10)
Carltonville	8	100	100	0	0	12.5	100	100
Bekkersdal	2	100	100	50	0	50	100	100
Taung	10	100	100	30	0	100	60	100
Delaryville	5	100	100	20	0	100	0	100
Zeerust	25	100	100	40	8	80	36	100
Rustenburg	5	100	100	80	60	100	100	100
Logagane	3	100	100	0	0	100	66.7	100
Leporang	2	100	100	0	0	100	100	100
Mabule	2	100	100	100	100	100	100	100
Masamane	1	0	100	0	0	100	100	100
Selosesha	1	100	100	100	100	100	100	100
Stella	3	100	100	66.7	0	100	66.7	100

A= amoxycillin; Ap = ampicillin; K = kanamycin; Ne = neomycin; OT = oxytetracycline; C = chloramphenicol; PG = penicillin G

4.4 Multiple Antibiotic Resistant phenotypes of *E. coli* isolates from ground water

MAR phenotypes were generated from isolates showing resistance to three or more antibiotics according to Rota *et al.,* (1996). All the isolates from Mabule and Selososha were resistant to all the antibiotics tested. Thus, the multiple antibiotic resistant phenotypes (MAR) for these isolates were A-AP-K-NE-OT-C-PG. Moreover, a large proportion (60%) of the isolates from Rustenburg possessed this phenotype. The predominant MAR phenotypes for isolates from Carltonville and Delaryville were A-AP-C-PG and A-AP-OT-PG and were obtained at 87.5% and 80% respectively. Moreover, the MAR phenotypes A-AP-PG, A-AP-K-OT-C-PG and A-AP-OT-PG were obtained at percentages of 20%, 28% and 40% respectively among *E. coli* O157:H7 isolated from Zeerust. Similarly, the MAR phenotypes for isolates from Taung, included A-AP-K-OT-C-PG and A-AP-OT-C-PG which occurred at 30% each and A-AP-OT-PG was identified among 40% of the isolates. These results indicate that in the present study, MAR *E. coli* O157:H7 were isolated from groundwater. It is therefore suggested that these MAR isolates may have severe health implications in individuals who consume water from these sources.

Table 4.4: Predominant multiple antibiotic resistant (MAR) phenotypes for *E. coli* O157:H7 isolates from groundwater

Sampling site	Phenotypes	No. observed	Percentage (%)
Carltonville	A-Ap-OT-C-PG	1	12.5
	A-Ap-C-PG	7	87.5
Bekkersdal	A-Ap-OT-C-PG	1	50
	A-Ap-K-C-PG	1	50
Taung	A-Ap-K-OT-C-PG	3	30
	A-Ap-OT-PG	4	40
	A-Ap-OT-C-PG	3	30
Delaryville	A-Ap-OT-PG	4	80
	A-Ap-K-OT-PG	1	2
Zeerust	A-Ap-K-NE-OT-C-PG	2	8
	A-Ap-K-OT-C-PG	7	28
	A-Ap-K-OT-PG	1	4
	A-Ap-OT-PG	10	40
	A-Ap-PG	5	20
Rustenburg	A-Ap-K-NE-OT-C-PG	3	60
	A-Ap-K-OT-C-PG	1	20
	A-Ap-OT-C-PG	1	20
Logagane	A-Ap-OT-C-PG	2	66.7
	A-Ap-OT-PG	1	33
Leporang	A-Ap-OT-C-PG	2	100
Mabule	A-Ap-K-NE-OT-C-PG	2	100
Selososha	A-Ap-K-NE-OT-C-PG	1	100
Masamane	Ap-OT-C-PG	1	100
Stella	A-Ap-K-OT-C-PG	2	66.7
	Ap-OT-PG	1	33

A= amoxycillin; Ap = ampicillin; K = kanamycin; Ne = neomycin; OT = oxytetracycline; C = chloramphenicol; PG = penicillin G

4.5 ELISA assay for the detection of shiga toxins

A total of 51 *E. coli* O157:H7 isolates were selected and divided into two groups randomly. These isolates were subjected to an ELISA test to determine their potential to produce shiga toxins in the presence of two antimicrobial agents (ampicillin and tetracycline) in Luria Bertani broth cultures. Figure 4.3 shows the results of the ELISA test for 22 isolates after they had been grown in the LB broth for 24 hours in the presence of ampicillin or tetracycline. Details of the extinction values for the individual isolates are shown in Appendix Table 2A. The data from Appendix Table 2A was translated in a graph (Figure 4.3). As shown in Figure 4.3, none of the isolates were positive for the shiga-toxins after 24 hours induction with both ampicillin and tetracycline. This is mainly due to the fact that the extinction (O.D) for the samples and the negative control was less than 0.2 at 450nm. However, it is evident that the extinction values for the positive controls were 2.054 for tetracycline and ampicillin respectively. This observation therefore indicates that the low extinction values obtained for the test isolates was mainly due to the fact that shiga toxins were not released in the broth cultures.

The remaining 29 *E. coli* O157:H7 isolates were later subjected to the same antibiotics (ampicillin and tetracycline) for induction of shiga-toxins. Samples were taken out at 72 hours of incubation and the extinction values are shown in Appendix Table 2B. Results from Appendix Table 2B were used to produce graphs (Figures 4.4 and 4.5). As shown in Figure 4.4, when tetracycline was used, all except one [Z10-2(4)] of the isolates were negative for the shiga-toxins when compared to the positive control. On the contrary, when these isolates were induced with ampicillin, all except for 3 (KV1, KV2 and KV6) of the isolates and the negative control were positive for the shiga-toxins (Figure 4.5). Interestingly, all these 3 isolates were obtained from the same sampling site (Carltonville). From these results, it was identified that the duration of exposure of *E. coli* O157:H7 isolated to the antibiotics may significantly affect the release of shiga-toxins in broth cultures. Moreover, tetracycline could serve as a better inducer than ampicillin for shiga-toxins in these pathogens.

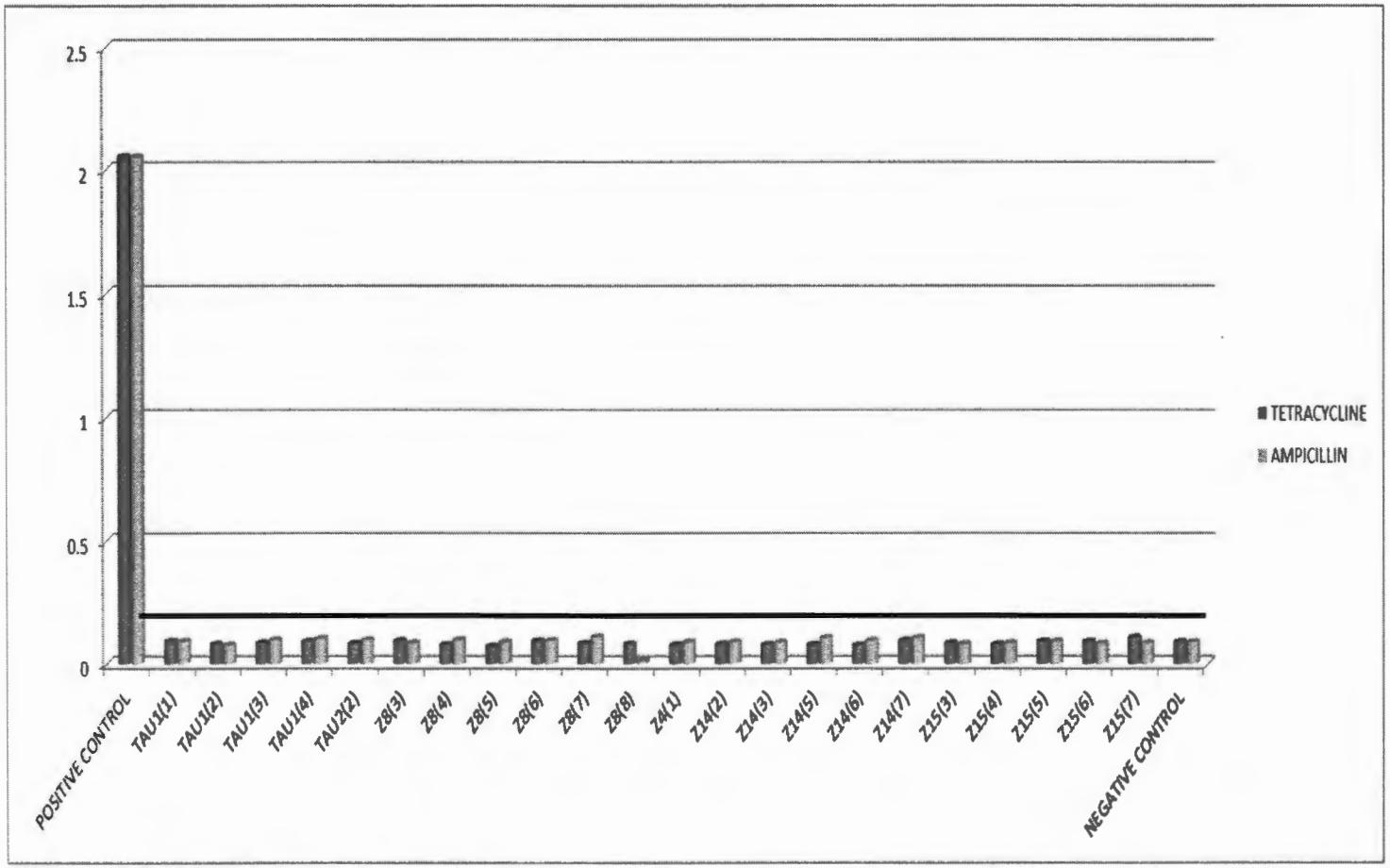

Figure 4.3: ELISA results for shiga-toxins of *E. coli* O157:H7 isolates induced with ampicillin and tetracycline after 1 day of incubation. Z = Zeerust; TAU =Taung

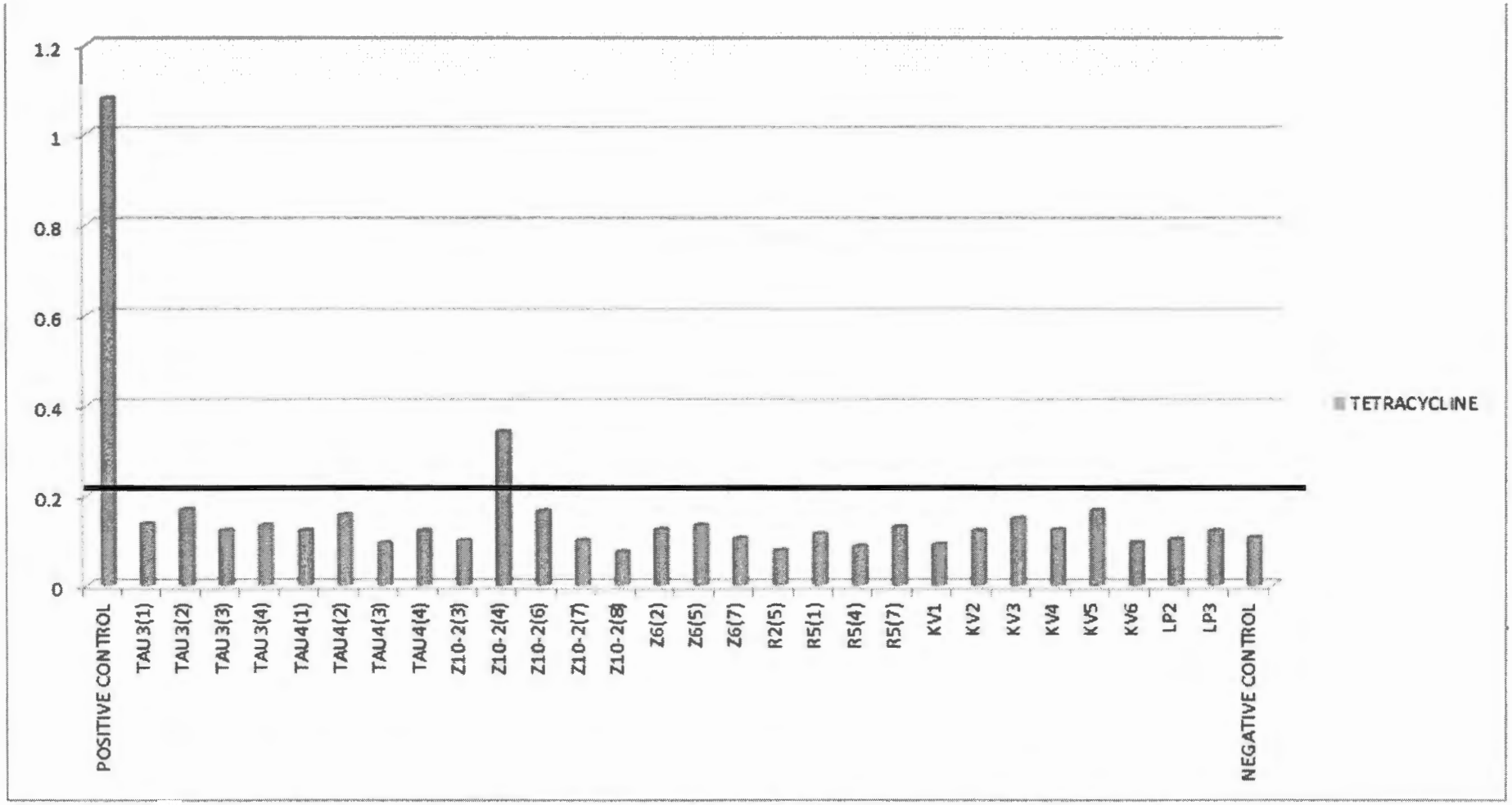

Figure 4.4: ELISA results for shiga-toxins of *E. coli* O157:H7 isolates induced with tetracycline after 2 days of incubation. TAU=Taung; Z=Zeerust; LP=Leporang; R=Rustenburg; KV=Carltonville

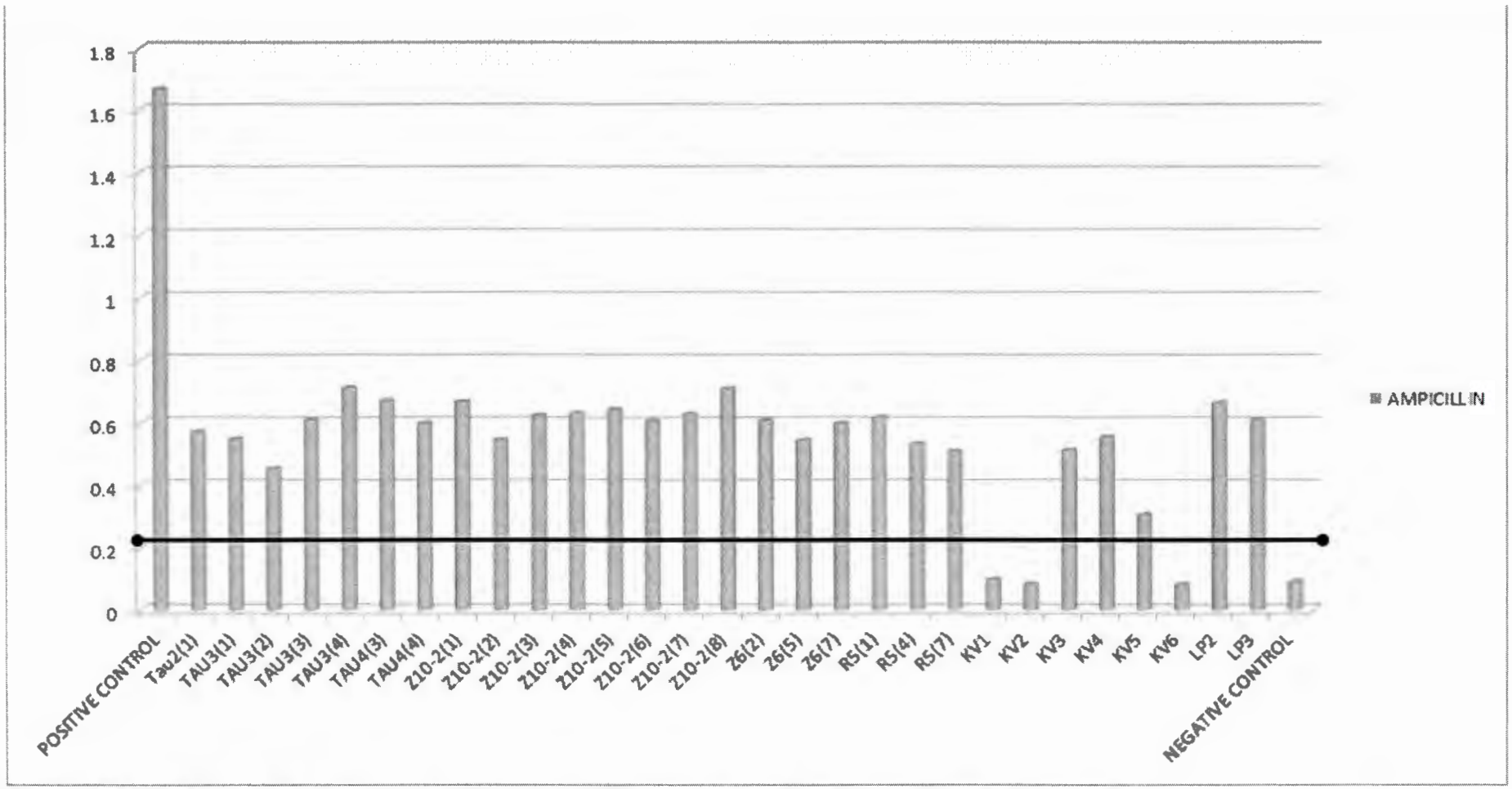

Figure 4.5: ELISA results for shiga-toxins of *E. coli* O157:H7 isolates induced with Ampicillin after 3 days of incubation. TAU=Taung; Z=Zeerust; R=Rustenburg; KV=Carltonvill; LP= Leporang

CHAPTER 5

DISCUSSION

5.1 GENERAL DISCUSSION

The primary objective of the study was to isolate and identify *E. coli* O157:H7 from groundwater obtained from some rural communities around the North West Province, South Africa. A motivation for this is that waterborne transmission of pathogenic microorganisms including *E. coli* O157:H7 has been reported from both untreated drinking water and water from recreational sources (Cransberg *et al.,* 1996; Paunio *et al.,* 1999; Licence *et al.,* 2001; Olsen *et al.,* 2002; Said *et al.,* 2003; Schets *et al.,* 2005). Despite the fact that water and foodborne outbreaks of *E. coli* O157:H7 infections have not been reported to date in South Africa, the pathogen has been isolated from animals and humans in the area (Ateba *et al.,* 2008; Ateba *et al.,* 2011). This therefore indicates that there is a possibility of these isolates contaminating either surface or groundwater through improper deposition of faeces from both humans and animals. Contamination of these water bodies could also result through leaching from rainfall runoffs.

Reasonable attention has been given to pathogenic *E. coli* strains especially those belonging to the serotype O157:H7 in developed countries (Richert *et al.*, 2000; Bouvet *et al.,* 2001; Elliott and Nicholas, 2001; Feng, 2001; LeJeune *et al.*, 2001; Guan and Levin, 2002; Heuvelink *et al.*, 2002; Tsuji *et al.,* 2002; Botteldoorn *et al.,* 2003; Dontorou *et al.,* 2003; Tutenel *et al.,* 2003; Havelaar *et al.*, 2004; Jay *et al.,* 2005; Maruzumi *et al.,* 2005; Sugiyama *et al.,* 2005; Cransberg, 2006; Doorduyn *et al.*, 2006). However, there is currently very little information on the prevalence of this pathogen and its associated virulence determinants in South Africa (Armstrong *et al.,* 1996; WHO, 1997; Browning *et al.,* 1990; Müller *et al.,* 2001; Ateba *et al.*, 2008; Momba *et al.,* 2008; Ateba and Mbewe, 2011). While our knowledge on the occurrence of *E. coli* O157:H7 in South Africa among animals and the environment including water remains incomplete, it is important to evaluate food products and water samples for the presence of these pathogens.

In the present study, *E. coli* O157:H7 was successfully isolated and identified in some of the water samples. The number of isolates were higher in water samples obtained from villages in Zeerust and Taung compared to the others. The exact reason for the distribution pattern observed for this pathogen cannot be explained at the moment. However, pathogens such as *E. coli* and other faecal coliform bacteria that are capable of causing diarrhoea-related illnesses are commonly derived from human faecal material (Pritchard et al., 2008). These pathogens can therefore readily be washed into water bodies such as shallow wells. During the collection of samples, it was identified that the boreholes in these villages were constructed very close to pit toilets. This may favour the transmission and cross contamination with pathogenic micro-organisms (Pritchard et al., 2008).

E. coli O157:H7 has a very low infectious dose and therefore, the results obtained in the present study may have a significant public health implication. Moreover, water from boreholes and surface water bodies tested are not treated and individuals in these rural communities who do not have access to treated water supplies, use the water in the state in which it is obtained (Olsen *et al.,* 2002). This therefore exposes them to a number of *E. coli* O157:H7 infections (Petridis *et al.*, 2005; Schets *et al.,* 2005). This is even aggravated by the fact that most residents in these communities do not have any knowledge about the health implications of these pathogens. While the government can be commended for its effort in providing individuals who live in rural areas with potable water, it is suggested awareness on the implications of pathogenic micro-organisms particularly *E. coli* O157:H7, may be of great importance. This may be motivated by the fact that specific identification and/or monitoring of pathogens such as *E. coli* O157:H7 in water bodies may suggest possible health implications on consumers (Schets *et al.,* 2005) especially in developing communities. Considering the burden of diseases caused by *E. coli* O157:H7 in humans in countries with more advanced public and health care systems (Belongia *et al.,* 1991; Bell *et al.,* 1994; Willshaw *et al.,* 1997; Chapman *et al.,* 1997; Allison *et al., 2*000; Vogt and Dippold, 2005; CDC 2011; 2012), there is need to reduce contact with these pathogens especially in rural communities within South Africa, where proper hygiene is not practiced.

Another objective of the study was to determine the presence of shiga-toxins genes using specific PCR analysis. The *stx* genes are known to be the primary virulence factors of *E. coli* O157:H7 isolates and are therefore, responsible for most of the pathogenicity observed (Paton and Paton, 1998; Johnsen *et al.*, 2001; Villani *et al.*, 2005). In the present study, none of the isolates were positive for the *stx* genes. Similar observations have been reported (Frydendahl, 2002; Hornitzky *et al.*, 2002; Ateba and Bezuidenhout, 2008). However, the isolates from these studies were obtained from animal species. Despite the fact that the *stx* genes have been reported to be highly associated with other *E. coli* O157:H7 virulence genes (Pradel *et al.*, 2000; Johnsen *et al.*, 2001) there are some studies in which isolates from diseased humans did not possess any of the shiga-toxin genes. Unfortunately, the *E. coli* O157:H7 obtained in the present study were not screened for the presence of other accessory virulence genes. It is therefore suggested that these isolates may have the potential to cause diseases in humans if consumed in contaminated water.

A further objective of the study was to determine the antibiotic resistance profiles of *E. coli* O157:H7 isolates obtained from water samples. Most *E. coli* O157:H7 strains considered to be susceptible to antibiotics and antimicrobial agents may serve as inducers for bacteriophages that result in cell lysis (Craig *et al.*, 2000; Zhang *et al.*, 2000; Dundas *et al.*, 2001). However, recent studies have revealed a trend towards increased antibiotic resistance among *E. coli* O157:H7 isolates (Amornrut *et al.*, 2000; Magwira *et al.*, 2005; Ateba and Bezuidenhout, 2008). Moreover, the antibiotic resistance profiles of isolates from the same region may vary considerably depending on certain factors. Antibiotic resistance may occur either spontaneously by selective pressure or because of antibiotic mis-use by humans or over-use by farmers on their beef cattle (Schroeder *et al.*, 2002). Therefore, it is important to determine the antibiotic resistance profiles of *E. coli* O157:H7 isolates as this would provide an indication of therapeutic options that could be used in the management of life threatening infections in humans.

In the present study, multiple antibiotic resistance (MAR), defined as resistance to three or more different classes of antibiotics, was observed in all (100%) of the *E. coli* O157:H7 isolated. A large proportion of the isolates were resistant to amocyxillin,

ampicillin, chloramphenicol and penicillin G. Consequently, the MAR phenotype A-AP-C-PG was present in most phenotypes. In a previous study, only a small proportion (0% to 37.8) of *E. coli* O157:H7 isolates were resistant to chloramphenicol and ampicillin (Ateba and Bezuidenhout, 2008; Wose King *et al.,* 2010; Phokela *et al.,* 2011). However, these isolates were obtained from animal and human stool samples. On the contrary, only a small proportion (23.8% to 25.4%) of *E. coli* O157:H7 isolates were resistant to tetracycline and ampicillin respectively (Khan *et al.,* 2002). More than one third of the isolates screened in this study were resistant to tetracycline. Despite the fact that some isolates obtained in the present study were highly resistant to kanamycin, a large proportion of these isolates were susceptible to this drug. These findings are similar to those obtained in previous studies (Khan *et al.,* 2002; Ateba and Bezuidenhout, 2008).

Results obtained from a study conducted in Gaborone, Botswana, showed that *E. coli* O157:H7 strains isolated from meat and meat products were highly resistant to cephalothin, sulfatriad, colistin sulfate and tetracycline (Magwira *et al.,* 2005). It is therefore suggested that the antibiotic resistant profiles of isolates may depend of level of exposure and varies between regions. These resistant determinants may be transferred easily to other microbes with which they share common habitats (Lin and Biyela, 2005). Moreover, these isolates may find their way into the environment, water bodies and grazing animals (Kidd *et al.,* 2002; Dancer, 2004) if proper hygiene is not practiced. Resistant bacteria have been isolated from a variety of sources, including domestic sewage, drinking water, rivers, and lakes in the area (Mulamattathil *et al.,* 2000). Apart from being used in veterinary and human medicine, antimicrobial agents are routinely used for disease prevention and growth promotion in animal production (Schoederb *et al.,* 2002). They also have a widespread use in animal husbandry, aquaculture, agriculture and food technology (Barbosa and Levy, 2000). It is therefore suggested that the widespread and inappropriate use of antibiotics is a significant contributing factor to the development and spread of bacterial resistance to antimicrobial agents (Mincey and Parkulo, 2001). The data obtained from this study could inform the usage of antibiotics in the area and therefore reduce the development antibiotic resistance within populations.

Another objective of the study was to determine the potential of two antimicrobial agents (ampicillin and tetracycline) in inducing the release of shiga-toxins in broth cultures. Despite the fact that antimicrobial agents may serve as inducers for bacteriophages and consequently the shiga-toxins in STEC *E. coli* (Craig *et al.*, 2000; Zhang *et al.*, 2000; Dundas *et al.*, 2001), there is controversy regarding the use of these agents in the treatment of *E. coli* O157:H7 infections. Tetracycline and ampicillin are easily accessible over the counter and could pose severe health implications on humans infected with *E. coli* O157:H7 isolates. Moreover, there is no data available about the effect of antibiotics on the release of shiga-toxins in *E. coli* O157:H7 isolates in South Africa. Considering the fact that shiga toxin genes have been detected in some studies within the area, it was necessary to carry out such an investigation. Using an ELISA assay, it was observed that the antimicrobial agent (ampicillin) was more effective in the release of shiga-toxins in broth cultures compared to tetracycline. Moreover, the ability of tetracycline to induce these shiga-toxin proteins in broth cultures depended largely on the duration of exposure of *E. coli* O157:H7 cells. These findings are important for public health and preventive medicine. Therefore, emergency cautions are necessary to decrease the incidence of *E. coli* O157:H7 infections in people and also provide an informed platform to understand the hazards associated with the uncontrolled administration of antimicrobial agents without consultation.

CHAPTER 6

CONCLUSIONS AND RECOMENDATIONS

6.1 CONCLUSIONS

In the present study, *E. coli* O157:H7 was successfully isolated and identified. The identification of pathogenic *E. coli* strains is highly important to assist in the surveillance, prevention and control of its associated diseases. The *E. coli* O157:H7 isolates were detected from water samples using PCR technique that is reliable and robust. The presence of this pathogen in water consumed without any treatment is a cause for concern. This is drawn from the fact that *E. coli* O157:H7 has been reported to cause outbreaks of infections even in countries with advanced public and health care facilities (Belongia *et al.*, 1991; Bell *et al.*, 1994; Willshaw *et al.*, 1997; Chapman *et al.*, 1997; Allison *et al.*, 2000; Strachan *et al.,* 2001; Olsen *et al.,* 2002; Teunis *et al.*, 2004; Anon, 2005; Maruzumi *et al.,* 2005; Strachan *et al.,* 2005; Vogt and Dippold, 2005). In one of these studies, a waterborne outbreak of *E. coli* O157:H7 infection resulted from the consumption of water from small, unprotected and unchlorinated water systems (Olsen *et al.,* 2002). The implication is that there is need to minimise the exposure of this pathogen to consumers. This can be achieved by proper monitoring of its occurrence in animal species, humans and water bodies. Moreover, the implementation of proper hygiene and provision of treated water to individuals who live in rural communities cannot be over-emphasised.

A large proportion of the *E. coli* O157:H7 isolates obtained in the present study were resistant to 3 or more antibiotics. MAR isolates obtained may serve as reservoirs for the transmission of antibiotic resistant genes to other bacterial species and this has a severe consequence on therapy. Despite the fact that the MAR isolates obtained in the present study did not possess the shiga-toxin genes as revealed by PCR analysis, they were able to release these toxins when induced with the selected antibiotics in broth cultures. A large proportion of the isolates produced the shiga toxins that were detected by an ELISA assay when subjected to treatment with tetracycline when compared to ampicillin. Despite the fact that the mitomycin and norfloxacin have been extensively studied and are generally considered as inducers of shiga-toxins in *E. coli* O157:H7 cells (Ptashne, 2004; Aertsen *et al.,* 2005;

Wegrzyn and Wegrzyn, 2005), this is the first report in the study area in which antibiotics that are commonly used in both human and veterinary medicine have been assessed to determine their potential in releasing these toxins from the pathogen. The findings from this study therefore indicate that antibiotics may result in the release of shiga-toxin genes in *E. coli* O157:H7 cells and thus, increase the chances of the disease to progress to the more severe clinical forms (Wong *et al.*, 2000; Zhang *et al.*, 2000; Dundas et al., 2001). Tetracycline and ampicillin are readily available over the counter and are most often used in animal medicine. It is therefore suggested that these antimicrobial agents may positively contribute in the release of shiga-toxins in *E. coli* O157:H7 cells and hence amplify the need to perform appropriate laboratory diagnostic tests especially in diarrhoeal patients before administering antibiotics.

6.2 RECOMMENDATIONS

The present study examined the occurrence of *E. coli* O157:H7 in untreated ground and surface water systems consumed directly or used for daily and recreational purposes. *E coli* O157:H7 was positively identified and the following recommendations are suggested:

- Stronger enforcement and broadening of current groundwater regulations to involve chlorination of water from these unprotected systems. This might significantly reduce and even prevent waterborne infections in individuals who live in these rural communities
- The detection of *E coli* O157:H7 in these untreated groundwater sources used by individuals in rural communities who do not have access to potable water supplies confirms the usefulness of routine water quality testing
- Routine monitoring of coliform bacteria and *E. coli* as required by the South African drinking water legislation may not indicate the presence of *E coli* O157:H7. Therefore, specific monitoring of this pathogen is recommended as this may suggest possible health consequences on consumers
- Antibiotics such as ampicillin and tetracycline that are easily accessible over the counter have been found to induce shiga toxins in *E coli* O157:H7 strains

that were negative for the *stx* genes by PCR analysis. It is therefore recommended that proper testing be performed to identify the causative agents before administering antimicrobial agents.

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APPENDIX

1. Liquid Media

1.1 Luria Bertani broth

Tryptone	12.0 g
Sodium Chloride	12.0 g
Yeast Extract	6.0 g

Twenty four grams of the medium was dissolved in 800 ml of distilled water and heated gently to ensure that it dissolves completely. Aliquots of 5 ml of the medium was distributed into McCartney bottles and sterilized by autoclaving at 121°C for 15 min. This growth medium was used for the cultivation and maintenance of *E. coli* O157:H7 isolates for molecular studies.

2. Solid Media

2.1 Sorbitol-MacConkey agar

Peptone	20.0 g
Lactose	10.0 g
Bile Salts	1.5 g
Sodium Chloride	5.0 g
Neutral Red	0.03 g
Crystal Violet	0.001 g
Sorbitol	8.0 g
Agar	13.8 g

Forty grams of MacConkey agar (Merck, S. A.) and 10 g of sorbitol (Merck, S. A.) were dissolved in 1000 ml of distilled water and autoclaved at 121°C for 15 min. The medium was cooled to 50 °C. The selectivity of the medium was enhanced by the incorporation of cefixime (Merck, S. A.) and potassium tellurite (Merck, S. A) at final concentrations of (50 ng/ml) and (25 mg/ml), respectively (Meichtri *et al.,* 2004). The media was then poured into Petri dishes, allowed to set and used for the cultivation and selective isolation of *E. coli* O157:H7 from samples.

2.2 Mueller – Hinton Agar

Meat Infusion	5.0
Casein Hydrolysate	17.5

Soluble Starch	1.5
Agar	14.0

Thirty eight grams of Mueller-Hinton agar (Merck, S. A.) was dissolved in 1000 ml of distilled water. It was boiled whilst starring until it was completely dissolved and autoclaved at 121°C for 15 min. The medium was cooled to 50 °C and poured into Petri dishes.

2.3 Nutrient Agar

Meat Extract	10.0 g
Peptone	5.0 g
Yeast Extract	2.0 g
Sodium Chloride	8.0 g
Agar	15.0 g

This growth medium was used for long term storage of *E. coli* O157:H7 isolates. It was prepared by dissolving 24.8 g of the medium in 800 ml of distilled water and autoclaved at 121°C for 15 min. The medium was cooled to 50 °C and poured into Petri dishes.

3. STOCK SOLUTIONS

3.1 50X TAE buffer

Tris (hydroxylmethyl) aminomethane	2 M
0.5 M EDTA (pH 8.0)	50 mM
Glacial acetic acid	57.1 ml

About 600 ml of distilled water was placed in a 1000 ml beaker. While stirring, 2 M of Tris (hydroxylmethyl) aminomethane was added. Thereafter, 50 mM of 0.5 M EDTA (pH 8.0) was added and the volume adjusted to 900 ml using distilled water. The solution was stirred until Tris dissolved completely and the volume was adjusted to 1000 ml. The solution was then transferred into a 1000 ml bottle and autoclaved at 121°C for 15 min. A 1X TAE working solution was prepared and used as an electrophoresis running buffer.

3.2 6X Loading dye

Xylene Cyanol	0.25%
Bromophenol blue	0.25%
Glycerol	50%

Fifty percent glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol were mixed to the desired volume. This solution was used as a loading buffer during electrophoresis of either extracted DNA or amplified PCR products.

3.3 Ethidium bromide

A stock solution of 10 mg/ml was prepared by dissolving the powder in distilled water. A final concentration 0.1µl/ml was used for staining extracted DNA and PCR products.

4. ENZYMES

4.1 Taq DNA Polymerase

A 5U *Taq* DNA polymerase (Fermentas, USA) obtained from Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria – South Africa and used during certain PCR amplifications

4.2 Oligonucleotide Primers

Different primer sets were used in the study to amplify the various genes that were targeted and for determining the genetic diversity of *E. coli* O157:H7 isolated. The sequences of these primers are indicated in the relevant sections. The primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria – South Africa. One hundred micro-molar (100 µM) stock solutions of the primers were prepared by adding the appropriate amount of sterile distilled water as indicated in the synthesis report. Primer stocks were stored at -4°C.

4.3 2X PCR Master Mix

The 2X PCR Master mixes (0.05U/µl *Taq* DNA polymerase in reaction buffer, 4mM $MgCl_2$, 0.4mM dATP, 0.4mM dCTP, 0.4mM dGTP and 0.4mM dTTP) used for amplification were Fermentas, USA products and were supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa.

LIST OF APPENDIX TABLES

Table 1A: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenoytypes
Zeerust 4	1	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
Zeerust 6	2	1	1	2	2	1	2	1	A-AP-OT-PG
	5	1	1	1	2	1	2	1	A-AP-K-OT-PG
	7	1	1	2	2	1	2	1	A-AP-OT-PG
Zeerust 8	3	1	1	2	2	1	2	1	A-AP-OT-PG
	4	1	1	2	2	1	2	1	A-AP-OT-PG
	5	1	1	2	2	1	2	1	A-AP-OT-PG
	6	1	1	2	2	1	2	1	A-AP-OT-PG
	7	1	1	2	2	1	2	1	A-AP-OT-PG
	8	1	1	2	2	1	2	1	A-AP-OT-PG
Zeerust 10(2)	1	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	2	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	3	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	4	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	5	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	6	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
	7	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	8	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
Zeerust 14	2	1	1	2	0	0	0	1	A-AP-PG
	3	1	1	2	2	0	0	1	A-AP-PG
	5	1	1	2	0	0	0	1	A-AP-PG
	6	1	1	2	2	0	0	1	A-AP-PG
	7	1	1	2	2	0	0	1	A-AP-PG
Zeerust 15	3	1	1	2	2	1	2	1	A-AP-OT-PG
	4	1	1	2	2	1	2	1	A-AP-OT-PG

Table 1B: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Rustenburg 2	5	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
	6	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
Rustenburg 5	1	1	1	2	2	1	1	1	A-AP-OT-C-PG
	4	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	7	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG

Table 1C: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Bekersdal 1	1	1	1	2	2	1	1	1	A-AP-OT-C-PG
	2	1	1	1	2	2	1	1	A-AP-K-C-PG

Table 1D: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Carltonville	1	1	1	2	2	1	1	1	A-AP-OT-C-PG
	2	1	1	0	0	2	1	1	A-AP-C-PG
	3	1	1	0	0	2	1	1	A-AP-C-PG
	4	1	1	0	0	2	1	1	A-AP-C-PG
	5	1	1	0	0	2	1	1	A-AP-C-PG
	6	1	1	0	0	2	1	1	A-AP-C-PG
	7	1	1	0	0	2	1	1	A-AP-C-PG
	8	1	1	0	0	2	1	1	A-AP-C-PG

Table 1E: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Leporang	1	1	1	0	0	1	1	1	A-AP-OT-C-PG
	2	1	1	0	2	1	1	1	A-AP-OT-C-PG
Area	**Isolate No.**	**A**	**AP**	**K**	**NE**	**OT**	**C**	**PG**	**Phenotypes**
Logagane	1	1	1	0	0	1	1	1	A-AP-OT-C-PG
	2	1	1	0	2	1	2	1	A-AP-OT-PG
	3	1	1	0	0	1	1	1	A-AP-OT-C-PG

Table 1F: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Mabule	1	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
	2	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
Area	**Isolate No.**	**A**	**AP**	**K**	**NE**	**OT**	**C**	**PG**	**Phenotypes**
Delareyville 1	1	1	1	2	0	1	0	1	A-AP-OT-PG
	2	1	1	2	0	1	2	1	A-AP-OT-PG
	3	1	1	1	0	1	0	1	A-AP-K-OT-PG
	4	1	1	2	0	1	0	1	A-AP-OT-PG
	5	1	1	2	0	1	0	1	A-AP-OT-PG
Area	**Isolate No.**	**A**	**AP**	**K**	**NE**	**OT**	**C**	**PG**	**Phenotypes**
Stella	1	1	1	0	0	1	0	1	A-AP-OT-PG
	2	1	1	1	2	1	1	1	A-AP-K-OT-C-PG
	3	1	1	1	0	1	1	1	A-AP-K-OT-C-PG

Table 1G: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Taung 1	1	1	1	1	0	1	1	1	A-AP-K-OT-C-PG
	2	1	1	2	0	1	0	1	A-AP-OT-PG
	3	1	1	2	0	1	1	1	A-AP-OT-C-PG
	4	1	1	2	2	1	2	1	A-AP-OT-PG
Taung 2	1	1	1	2	0	1	1	1	A-AP-OT-C-PG
	2	1	1	1	0	1	1	1	A-AP-K-OT-C-PG
Taung 3	1	1	1	0	0	1	0	1	A-AP-OT-PG
	2	1	1	0	0	1	0	1	A-AP-OT-PG
Taung 4	1	1	1	2	2	1	1	1	A-AP-OT-C-PG
	2	1	1	1	2	1	1	1	A-AP-K-OT-C-PG

Table 1H: Antibiotic Susceptibility Profiles Data for *E. coli* isolates (R=1; I=2; S=0)

Area	Isolate No.	A	AP	K	NE	OT	C	PG	Phenotypes
Selosesha	1	1	1	1	1	1	1	1	A-AP-K-NE-OT-C-PG
Area	**Isolate No.**	**A**	**AP**	**K**	**NE**	**OT**	**C**	**PG**	**Phenotypes**
Masamane	1	0	1	2	0	1	1	1	AP-OT-C-PG

Table 2A: Details of ELISA test for shiga-toxins of *E. coli* O157:H7 isolates when induced with tetracycline and ampicillin after 24 hours of incubation.

Sample ID	OD Tetracycline	OD Ampicillin	Sample cut-off for ampicillin and tetracycline
Positive control	2.054	2.054	
TAU1(1)	0.093	0.093	0.19
TAU1(2)	0.08	0.077	0.19
TAU1(3)	0.086	0.098	0.19
TAU1(4)	0.092	0.103	0.19
TAU2(2)	0.083	0.098	0.19
Z8(3)	0.094	0.085	0.19
Z8(4)	0.078	0.098	0.19
Z8(5)	0.072	0.09	0.19
Z8(6)	0.094	0.095	0.19
Z8(7)	0.084	0.11	0.19
Z8(8)	0.081	0.0127	0.19
Z4(1)	0.078	0.089	0.19
Z14(2)	0.081	0.089	0.19
Z14(3)	0.079	0.087	0.19
Z14(5)	0.079	0.106	0.19
Z14(6)	0.076	0.095	0.19
Z14(7)	0.096	0.107	0.19
Z15(3)	0.085	0.081	0.19
Z15(4)	0.08	0.085	0.19
Z15(5)	0.093	0.093	0.19
Z15(6)	0.091	0.084	0.19
Z15(7)	0.109	0.085	0.19
Negative control	0.090	0.090	

Table 2B: Details of ELISA test for shiga-toxins of *E. coli* O157:H7 isolates when induced with tetracycline for 2 days and ampicillin for 72 hours of incubation.

Sample ID	OD Tetracycline	Sample cut-off	OD Ampicillin	Sample cut-off
Positive control	1.079		1.668	
TAU3(1)	0.137	0.207	0.55	0.192
TAU3(2)	0.169	0.207	0.455	0.192
TAU3(3)	0.122	0.207	0.609	0.192
TAU3(4)	0.134	0.207	0.714	0.192
TAU4(1)	0.122	0.207	NT	0.192
TAU4(2)	0.157	0.207	0.603	0.192
TAU4(3)	0.094	0.207	0.672	0.192
TAU4(4)	0.122	0.207	0.601	0.192
Z10-2(1)	NT	0.207	0.66	0.192
Z10-2(2)	NT	0.207	0.547	0.192
Z10-2(3)	0.099	0.207	0.625	0.192
Z10-2(4)	0.841	0.207	0.631	0.192
Z10-2(6)	0.165	0.207	0.606	0.192
Z10-2(7)	0.10	0.207	0.629	0.192
Z10-2(8)	0.075	0.207	0.711	0.192
Z6(2)	0.125	0.207	0.608	0.192
Z6(5)	0.134	0.207	0.545	0.192
Z6(6)	0.105	0.207	0.600	0.192
R2(5)	0.078	0.207	NT	0.192
R5(3)	0.115	0.207	0.619	0.192
R5(4)	0.087	0.207	0.534	0.192
R5(6)	0.13	0.207	0.510	0.192
KV1	0.091	0.207	0.100	0.192
KV2	0.122	0.207	0.085	0.192
KV3	0.149	0.207	0.514	0.192
KV4	0.124	0.207	0.556	0.192
KV5	0.167	0.207	0.307	0.192
KV6	0.096	0.207	0.082	0.192
LP2	0.103	0.207	0.666	0.192
LP3	0.122	0.207	0.611	0.192
Negative control	0.107		0.092	

NT=Not tested