The Detection and Molecular Characterisation of Shiga Toxigenic *Escherichia coli* (STEC) O157 strains from humans, cattle and pigs in the North-West Province, South Africa

By

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Submitted

February 2006

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DECLARATION

I declare that, the dissertation for the Degree of Master of Science in Agriculture (Molecular Microbiology) at the North-West University – Mafikeng Campus hereby submitted, has not been submitted by me for a degree at this or other university, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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DEDICATION

This work is dedicated to Dr. P. T. Mmolawa (late), my uncle Mr. Ateba Benedict Belobo (late), my grandmothers; Mama Ewokolo Njie (late) and Mama Ateba Magaret Adjessa (late) and finally my aunt Miss Moki Agnes Mojoko (late).

ACKNOWLEDGEMENTS

This research work would not have been possible or even imaginable without the help and cooperation of many people and organisations.

First and foremost, my sincere gratitude goes to my supervisor Dr. C. C. Bezuidenhout. His constant supervision and contributions went a long way in formulating the style of this work.

I wish to thank the Management of the North-West University –Mafikeng Campus, for their cooperation throughout this work. I cannot forget the efforts put in by Dr. M. Molefe (H. O. D) -Department of Animal Health, in securing funds that was required to carry out this research. I would like to acknowledge my colleagues (M. S. Moneoang, L. L. N. Ramatlhape, K. M. Mokgejane and K. M. Kwenamore), the Microbiology postgraduate group, Mr. L. E. Motsei, and most especially, Prof. M. Mbewe who always assisted despite his busy schedule.

I am greatly indebted to my family for their constant encouragement and I would like to single out my loving parents Mr. and Mrs. Ateba G. N., as well as the following Ateba Olivia, Ateba Sarahtherese, Ateba Divine Mongou, Ateba Linus Ossendi, Ateba Benedict Belobo, Mokako Jude, Ngowo Magdaline, Papa Njie Mosoko, Mrs. Hannah Ndopin, Mr. and Mrs Junkwi James, Mr and Mrs. Eyong, Mr. Agwa Desmond Tah, Mr. Nkong Pascal and Dr. Charles Atangana Nama, all in Cameroon.

My foster family in South Africa, Mr. Kale and family, Ngomba Mbua, Mrs. Olivia Mutua, Mr. Henry Elango, Ikome Fritz Nganje, Dr and Mrs Blessed Okolle, Anjah Emmanuel, Celine Otang, Asonganyi Sylvanus, Mr and Mrs Ashu-Tako, Kenneth Jinkwa, Fuh Athanasius, Kamanyi Calvin, Bikim Paul, Fobiteh Rodex Bessong, Ambe Marcus, Mr. amd Mrs. Ambe, Mr Nfobin, Mr. Ocaya, Mr. and Mrs Ngwa and Dr. Eyono and Family. Thank you all for the encouragement and support.

The list would be incomplete without making mention of Miss T. P. Ngakane who may not have contributed a single idea to this work but her boundless love and support kept my head above the flood. Thank you for being there for me.

Also thanks to Dr. Ettienne Müller (Department of Medical Virology –University of Pretoria, South Africa) for providing the *E. coli* control strains that were used in this study.

ABSTRACT

The prevalence and antibiotic resistant profiles of shiga-toxin producing *Escherichia coli* O157 strains isolated from faeces samples of cattle, pigs and human stool samples were determined. The strains were further characterised by molecular methods for the presence of shiga-toxin virulence genes and antibiotic resistant genes. Seventy-six *Escherichia coli* O157 strains were isolated and the prevalence was higher among *E. coli* isolated from faeces from pigs (44.2% to 50%) than those from cattle faeces (5.4% to 20.0%) or human stool samples (7.5%). On testing *E. coli* O157 isolates for their resistance to 9 antimicrobial agents, multiple antibiotic resistance (MAR) was observed in all of the isolates arising from resistance to three or more antibiotics. Seventy (92.1%) of the *E. coli* O157 isolated from humans, cattle and pigs were resistant to tetracycline, 73 (96.1%) were resistant to sulphamethoxazole, 63 (82.9%) were resistant to erythromycin, 40 (52.6%) were resistant to streptomycin and 26 (34.2%) were resistant to ampicillin. The highest frequency of resistance was observed among the human isolates (n=3), where 3 (100%) of the isolates were resistant to tetracycline, sulphamethoxazole, erythromycin and ampicillin. Furthermore, among the pig isolates (n=60), 58 (96.7%) were resistant to tetracycline, 57 (95%) were resistant to sulphamethoxazole, 47 (78.3%) were resistant to erythromycin, 38 (63.3%) were resistant to streptomycin and 22 (36.7%) were resistant to ampicillin.

The MAR phenotypes S-Smx-T-E, Smx-T-Ap and Smx-T-E were the dorminant phenotypes among the *E. coli* O157 isolated from the faeces samples of communal pigs in 30.4%, 21.7% and 17.4% of these isolates, respectively. However, phenotypes

Smx-T-E and S-Smx-T-E-Ne were identified at 16.2% and 10.8%, respectively within the isolates obtained from commercial pig feaces. The phenotype Smx-T-E was the only MAR phenotype identified among the *E. coli* O157 isolated from the feacal samples of commercial cattle at Lichtenburg. Furthermore, MAR phenotypes Smx-T-E-C, K-S-Smx-T-E, S-Smx-T-E and Smx-T-E-Ap were obtained at 25%, respectively for the isolates obtained from communal cattle at Mogosane while Smx-T-E-Ap was the dorminant (66.7%) phenotype among the isolates of human origin. The phenotype Smx-T formed the basis of all the MAR phenotypes obtained and this was similar to the percentage antibiotic resistance data.

The distribution of the resistant determinants for tetracycline was determined by PCR analysis in resistant isolates. A *tetB* gene was detected in *E. coli* O157 of pig origin. Based on the characterisation of 30 isolates for the presence of STEC virulence genes by PCR, 18 (60%) possessed the *hlyA* gene, 7 (23.7%) possessed the *eae* gene and 5 (16.7%) harboured both genes.

The average MAR indices for pig, cattle and human *E. coli* O157 isolates were 0.4722, 0.3419 and 0.4814, respectively. Among the cattle isolates, the group MAR index was highest for the communal (Mogosane) population while the values for the commercial populations at Lichtenburg and Rustenburg were 0.33 and 0.22, respectively. *E. coli* O157 isolated from pigs revealed MAR index results that were 0.508 and 0.415 for the commercial and communal populations respectively and 0.1851 for the *E. coli* control strains.

Characterisation by cluster analysis to determine the commonness and resolve differences between the *E. coli* O157 isolated from the various sources revealed a close association between pig (Tlapeng and Mareetsane), cattle (Mogosane) and human isolates. Interestingly, *E. coli* O157 isolated from pigs occurred at the highest frequency in all the clusters, which suggested their role in the dissemination of resistant determinants.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1.1 INTRODUCTION AND PROBLEM STATEMENT

Shiga toxin producing *Escherichia coli* (STEC) have emerged as important pathogens of the gastrointestinal tract in individuals of all ages, but with an increased incidence and severity of illness in young children and the elderly (Griffin and Tauxe, 1991). STECs are pathogens responsible for outbreaks and sporadic cases of diarrhoea (Muhldorfer *et al.,* 1996; Head *et al.,* 1998). Approximately 100 STEC serotypes have been associated with human disease (Bryan *et al.,* 2004). However, the most well studied and frequently identified STEC serotype is *E. coli* O157:H7. Its clinical importance was recognised in 1982 when it was isolated from human stools during an outbreak of food-borne illness associated with the consumption of improperly cooked ground beef (Riley *et al.*, 1983).

STECs are distinguished from other types of diarrhoeagenic *E. coli* by the production of proteinaceous toxins called shiga-like toxins and other probable virulence factors (Riley *et al.*, 1983; Griffin, 1995). Two principal types of shiga-toxins (Stx) called shiga-like toxins 1 and 2 have been described and are designated SLT (VT)1 and SLT (VT)2 respectively. The toxins resemble the cytotoxin of *Shigella dysenteriae* type 1 biologically and structurally hence their name, but they are antigenically distinct (O'Brien and Holmes, 1987).

Cattle are known reservoirs of STEC (Chapman *et al.,* 1993; Armstrong *et al.,* 1996). However, epidemiological surveys have revealed that STEC strains are also prevalent in the gastrointestinal tracts of other domestic animals, including sheep, pigs, goats and cats. Thus the identification of STEC positive animals before slaughter may be

beneficial especially if proper farm management techniques and sanitary control measures at abattoirs are not adopted to minimise beef contamination with these pathogens (Mead and Griffin, 1998; Bonardi *et al.*, 2001; LeJeune and Kauffman, 2005).

The prevalence of *E. coli* O157 in food products should be as low as possible due to its pathogenic nature (Tutenel *et al.*, 2003). Moreover, its presence in human stool may result from the contamination of fresh beef with this pathogen (Pierard *et al.*, 1997a). Thus the capacity to rapidly determine whether a patient with diarrhoea is infected with STEC is extremely important from both the clinical and epidemiological viewpoints. However, development of a rapid, reproducible and easily comparable strain typing system for closely related enterohemorrhagic *Escherichia coli* such as O157:H7 has been a particular challenge (Indira *et al.*, 2002). Moreover, *E. coli* O157 strains that harbour the same virulence determinants may possess significant genomic diversities (Allison *et al.*, 2000; Preston *et al.*, 2000). Early knowledge of the infecting STEC serogroup is also valuable, because it may provide an indication that outbreaks of cases could occur and may also enable microbiological investigations of suspected foods to be targeted (Kim *et al.*, 1999; Pradel *et al.*, 2001).

Although a substantial volume of information on the causative STEC strain can be obtained by molecular analysis of mixed cultures, isolation of the STEC strain must be considered the definitive diagnostic procedure (Tutenel *et al.*, 2003). Isolation procedures are based on the identification of the biochemical characteristics of *E. coli* O157 strains that include the lack of beta-glucorunidase and non-sorbitol-fermenting

activities (Tutenel *et al.,* 2003). Apart from confirming the molecular data, isolation permits additional characterisation of STEC by a variety of methods that include, O:H serotyping (Bouvet *et al.,* 2002; Tutenel *et al.,* 2003), phage typing (Willshaw *et al.*, 1987; Watarai *et al.*, 1998; Preston *et al.*, 2000), restriction fragment length polymorphism (Bernhard and Field, 2000a), pulsed-field gel electrophoresis (Preston *et al.*, 2000; Simmons *et al.*, 2000; King and Standfield, 2002; Davis *et al.*, 2003; Tutenel *et al.,* 2003; Avery *et al.*, 2004), denaturing-gradient gel electrophoresis (Buchan *et al.*, 2001; Chee-Sanford *et al.*, 2001), Repetitive DNA sequences (Dombek *et al.*, 2000; Holloway, 2001), length heterogeneity PCR (Suzuki *et al.*, 1998; Bernhard and Field, 2000a), ribotyping (Carson *et al.*, 2001; Hartel *et al.*, 2002; Scott *et al.*, 2003) and 16S rRNA gene fragment sequence analysis (Gee *et al.*, 2004). While the latter characterisation may have limited clinical application, it is of great importance from an epidemiological point of view, particularly in an outbreak setting (Dundas *et al.* 2001). Unfortunately clinical amendable methodologies for the detection of STEC strains are not widely used, and the overall incidence of STEC – related disease is therefore unknown. The main purpose is to develop some methods that will provide early diagnosis of shiga-like toxigenic *E. coli* (STEC) O157 infections in domestic animals and humans. This will help in minimising the impact of STEC, thus limiting human sufferings due to food poisoning with a positive increase in their welfare and economic output.

Despite the controversy that surrounds the treating of STEC infections with antibiotics (Yoh and Honda, 1997; Igarashi *et al.,* 1999), several recent studies have documented antibiotic resistance among *E. coli* O157:H7 isolates (Kim *et al.,* 1994; Bettelheim *et*

al., 2003). There are significant disadvantages for STEC infections to be caused by multiple resistant strains as these strains may have a selective advantage and be more likely to cause an outbreak (Bettelheim *et al.*, 2003). Furthermore, the acquisition and dissemination of antibiotic resistant genes among *E. coli* O157 have a negative impact on therapy. Thus an investigation of the prevalence of antibiotic resistance within *E. coli* O157 would be more valuable from a clinical point of view.

1.2 AIMS OF THE STUDY

The aim of this study was to determine the prevalence of shiga-like toxin (verotoxin) producing *Escherichia coli* (STEC; *E. coli* O157) in some species of domestic animals and humans and to characterise these isolates using antibiotic susceptibility patterns and molecular methods.

1.3 OBJECTIVES OF THE STUDY

The objectives of the study were:

i) To isolate and characterise *E. coli* O157 from cattle, pigs and humans.

ii) To determine antibiotic resistant profiles of the *E. coli* O157 isolates.

iii) To evaluate a PCR technique for the detection of STEC virulence genes in isolates from the North-West Province of South Africa.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL INTRODUCTION AND PROPERTIES OF *ESCHERICHIA COLI*

Escherichia coli, a gram-negative non-spore-forming rod, is a commensal of warm-blooded animals and humans, and the most common facultative anaerobic bacterium in the gut (Donnenberg and Whittam, 2001). Pathogenic variants of *E. coli* cause diarrhoeal diseases as well as the urinary tract, or other extra-intestinal infections like meningitis and septicaemia (Nataro and Kaper, 1998). Diarrhoeagenic *E. coli* can be divided into five pathotypes on the basis of their pathogenicity mechanisms: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC), and enteroaggregative (EAEC) (Czeczulin *et al.*, 1999). The sixth pathotype is referred to as the diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998; Donnenberg and Whittam, 2001) and the seventh pathotype the cytolethal distending toxin (CDT) – producing *E. coli* (Nataro and Kaper, 1998; Clarke, 2001).

A typical two-layer cell wall of a gram-negative bacterium surrounds the cell. The outer membrane contains two types of lipids, lipopolysaccharides (LPS) and phospholipids, as well as a set of proteins. LPS comprises three regions: lipid A, an oligosaccharide core, and a polysaccharide O antigen. The O antigen is very variable and is used for serotyping. The cytoplasmic membrane borders the cytoplasm. Periplasm fills the space between the outer and cytoplasmic membrane, and contains a layer of murein or peptidoglycan. *E. coli* often have fimbria on the surface of the cell that the bacterium uses for adhering to intestinal epithelial cells (Levine *et al.*, 1984).

Shiga toxin producing *Escherichia coli* (STEC) strains have emerged as an important cause of serious human gastrointestinal disease, which may result in life threatening complications in both humans and domestic animals. These range from traveller's diarrhoea and infantile diarrhoea to haemolytic uraemic syndrome in humans especially in developing countries (Daniels *et al.*, 2000) to diarrhoea in calves, lambs, neonatal pigs and in weanling pigs (Mainil *et al.,* 1993).

Food-borne outbreaks of STEC disease appears to be increasing and such outbreaks have the potential to overwhelm acute-care resources, even in countries with advanced health care systems (Paton and Paton, 1998; Ostroff *et al.,* 1990; Bell *et al.*, 1994; Michino *et al.,* 1999; Paunio *et al.,* 1999; Cowden *et al.,* 2001; Dundas *et al.,* 2001; Crump *et al.,* 2002; NIH-Korea, 2001-2003). The morbidity and mortality associated with several recent large outbreaks of gastrointestinal disease caused by STEC have highlighted the threat these organisms pose to public health (Fukushima *et al.*, 1997).

Development of therapeutic and preventive strategies to combat STEC disease requires a thorough understanding of the mechanisms by which STEC organisms colonise the human intestinal tract and cause local and systemic pathological changes. Much attention has been focused upon this group of pathogens since their discovery, and there have been several excellent reviews covering either the field as a whole (Karmali, 1989), or specific aspects such as the toxin (Tesh and O' Brien, 1991), its structure and function (Jackson, 1990), its interaction with host cell receptors (Lingwood, 1996) and clinical aspects of the disease (Tarr, 1995).

While our knowledge remains incomplete, studies (O'Brien and Holmes, 1987; Tesh *et al.*, 1993; Louise and Obrieg, 1995) have improved our understanding of these processes, particularly the complex interaction between shiga toxins and host cells, which is central to the pathogenesis of STEC disease. In addition, several putative accessory virulence factors have been identified and partly characterised. The feature that distinguishes STEC from other classes of pathogenic *E. coli,* is the production of a toxin with profound and irreversible cytopathic effect on Vero (African green monkey kidney) cells (Konowalchuk *et al.*, 1977). In the early 1980s, shiga toxin *E. coli* strains were linked to cases of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Moreover, shiga toxin *E. coli* strains associated with two outbreaks of HC belonged to a previously rare serotype O157:H7, as did two of eight verotoxigenic isolates from HUS patients (Kamali *et al.,* 1983).

O'Brien *et al.* (1983) purified and characterised the cytotoxin produced by one of Konowalchuk's isolates (strains H30; serotype O26:H11) and found that it had a strikingly similar structure and biological activity to shiga toxin produced by *Shigella dysenteriae* type 1. However, the two were antigenically distinct. Moreover, it could be neutralised by anti-shiga toxins (O'Brien *et al.,* 1982) resulting in the new nomenclature of shiga-like toxins (SLT). Shiga-like toxin and verotoxin (VT) nomenclature systems have been used interchangeably in this study. The situation was further complicated by the subsequent recognition that there are two major types of SLT / VT (SLT-I and SLT-II or VT1 and VT2), with additional sequence variants within these types. In an attempt to avoid further confusion, Calderwood *et al.* (1996) proposed a rationalisation of nomenclature, which recognises that all of these toxins

have a high degree of structural and functional homology and so belong to the shiga toxin family. The rationalised nomenclature system was used throughout this literature review.

2.2 VIRULENCE FACTORS OF STEC

2.2.1 Types, structure and mode of action of shiga – like toxins (Stx)

The toxin genes can be transferred or lost by strains (Karch *et al.,* 1992). STEC strains may express stx_1 or stx_2 or both or multiple forms of stx_2. Initial recognition of the presence of multiple shiga-like toxin types arose from the observation that anti- shiga-like toxins (anti-Stx) could not neutralise the cytotoxicity of some STEC strains (Scotland *et al.*, 1985). Stx1 differs from Stx of *S. dysenteriae* by only one amino acid in the A polypeptide, whereas Stx2 shows 55-60% homology (Paton and Paton, 1998). Stx1 is highly conserved while Stx2 has antigenic variation. The variability primarily comes from the sequence diversity in the B subunit, which may alter receptor-binding preference. The Stx2 variants also differ in toxicity for tissue cultures and animals. The different variants presently known are Stx2c, Stx2d, Stx2e and Stx2f (Schmidt *et al.,* 2000). Human pathogenic STEC O157 strains usually contain stx_2 or both stx_2 and stx_1 genes (Karmali, 1989; Paros *et al.*, 1993). In one study carried out in Australia, *E. coli* O157 strains, however, produced stx_1 only, which made them less virulent (Robins-Browne *et al.*, 1998). Stx_2c has been isolated from human strains but mostly from isolates that also contain stx_1, stx_2 or both genes (Furst *et al.*, 2000). Stx_2d has been isolated from asymptomatic carriers (Stephan and Hoezle, 2000). Stx_2e is mainly found with oedema disease in pigs (Marques *et al.*, 1987). Stx_2f has been isolated from pigeons (Dell'Omo *et al.,* 1998; Schmidt *et al.*, 2000). Stx2 is more cytotoxic than Stx1

and more often isolated from severe cases (Ostroff *et al.*, 1989a,b). The production of Stx was linked to HC and HUS in the mid 1980s (Karmali *et al.*, 1985a). Although shiga toxin genes are considered important in the pathogenesis of STEC, *E. coli* O157:H7 strains not possessing *stx* genes have been isolated from patients with diarrhoea and HUS (Schmidt *et al.*, 1999). Shiga toxins are important virulence factors in human disease but it is not clear whether they have a role in bovine infections (Rasmussen and Casey, 2001).

Members of the shiga toxin family are compound toxins (the holotoxin which is approximately 70KDa), comprising a single catalytic 32KDa A subunit and a multimeric B subunit (7.7 KDa monomers) that is involved in the binding of the toxin to specific glycolipid receptors on the surface of target cells (O'Brien and Holmes, 1987). The eukaryotic cell surface receptor for members of the shiga toxin family is globotriaosylceramide (Gb_3) (Lingwood *et al.*, 1987). The exception to this is the variant toxin Stx2e, which recognises globotetraosylceramide (Gb_4), preferentially over Gb_3 (Samuel *et al.*, 1990). The interaction between the Stx1 B subunit and its receptor has been studied extensively by molecular modelling; site directed mutagenesis, and crystallographic analysis and the amino acid Phe 30 was shown to play a critical role in this process. Despite the fact that certain regions of the *stx* genes are highly conserved, there is a lot of heterogeneity among them. Two possible Gb_3 binding sites have been identified on either side of this residue; one site is near the cleft between adjacent B monomers, while the other is a shallow indentation on the B subunit surface opposed to the plasma membrane (Nyholm *et al.*, 1996). A third putative Gb_3 binding site has also recently been identified in the vicinity of Trp 34 (Bast *et al.*, 1997). Verotoxins bind to

specific glycolipid receptor Gb3 found on endothelial cells of blood vessels, smooth muscle cells, renal endothelial cells, as well as red blood cells (Lingwood *et al.,* 1987). Once bound to a target cell membrane, toxin molecules are internalised by a process of receptor-mediated endocytosis (Sandvig and Deurs, 1996).

2.3 LOCUS FOR ENTEROCYTE EFFACEMENT (LEE)

The pathogenecity island LEE was originally discovered in EPEC strains. The LEE island of *E. coli* O157:H7 possesses all the genes found in EPEC LEE (Perna *et al.,* 1998). The LEE of *E. coli* O157 strain EDL933 consists of 54 genes. The type III secretion apparatus is fairly similar but the sequences coding for the secreted proteins of EPEC and STEC are more diverse. EPEC and EHEC have different modes of pathogenesis and colonise different sites in the intestine, due to differences in the *eae* and *tir* genes products that facilitate attachment of these bacteria to the mucosal cell walls of their hosts (Philips *et al.,* 2000). The initial colonisation and attachment site for *E. coli* O157 strains is the Payer's patches in the intestinal ileum (Philips *et al.,* 2000). In addition to *tir,* there is evidence for the existence of one or more host cell intimin receptors (Smith *et al.,* 2002). The STEC LEE has an *espF* gene that facilitates in the colonisation of the host mucosal cells by STEC strains resulting in death and loss of intestinal barrier function (Donnenberg and Whittam, 2001). Eleven intimin types have been identified, but *E. coli* O157:H7 and H-negative strains produce a γ type (Son *et al.,* 2002). Although shiga toxins are the primary virulence factor, the ability to produce eaeA and hlyA are also important (Gyles *et al.,* 1998; Lehmacher *et al.,* 1998; Boerlin *et al.,* 1999). While the *eae* gene is important in the colonisation and the pathogenesis of EHEC, STEC strains lacking *eae* have caused HUS cases (Paton *et al.,* 1999). This suggests that other colonisation factors must be present.

2.4 EPIDEMIOLOGY OF STEC DISEASE

2.4.1 Species and serotype distribution of shigatoxin producing *E. coli*

It has been recognised that STEC strains causing human disease belong to a very broad range of O and H serotypes (Karmali, 1989). In 1989, thirty-two O serotypes were known (Karmali, 1989). However, more serotypes were later discovered (Goldwater and Bettelheim, 1994). Although not represented in the initial group of STEC isolates described by Konowalchuk *et al.* (1977), serotype O157:H7 was the first STEC type to be linked to outbreaks of HC and HUS (Riley *et al.,* 1983). In many parts of the world, STEC strains belonging to this serotype appear to be the most common cause of human disease. However, the relative ease of isolation of this serotype on the basis of its inability to ferment sorbitol may be contributing to an overestimation of its prevalence with respect to other STEC serotypes. Other common STEC serogroups include O26, O91, O103 and O111. There have been several reports of multiple STEC serotypes being isolated from a single patient, and in such circumstances, the contribution of each type to the pathogenesis of disease could not be ascertained (Paton *et al.,* 1996). When one of the isolated types is O157, there is a (perhaps mistaken) tendency to ignore the potential aetiological significance of the other serotypes.

2.4.2 Host range of *E. coli* O157

Cattle have long been regarded as the principal reservoir of STEC strains, including those belonging to serotype O157:H7. However, epidemiological surveys have revealed that *E. coli* O157 strains are also prevalent in the gastrointestinal tracts of other domestic animals, including sheep, pigs, goats, dogs and horses (Beutin *et al.,* 1995; Trevena *et al.,* 1996). Estimation of the incidence of carriage of STEC is complicated

by the fact that faecal shedding may be transient and is almost certainly influenced by a range of factors including diet, stress, population density, geographical region and season (Clark *et al.,* 1994). Similarly the dynamics of these pathogens in animals and the environment is not well understood. Serological studies have suggested that the vast majority of cattle have been exposed to STEC at some point during their lives (Pirro *et al.,* 1995). STEC isolates from animal sources include the important human disease causing serotypes, as well as a number of O:H types that are yet to be associated with human infections (Beutin *et al.,* 1993).

While many domestic animals carrying STEC are asymptomatic, certain STEC strains are capable of causing diarrhoea in cattle, particularly calves (Gyles, 1992). STEC strains have also been detected in cats and dogs with diarrhoea (Abaas *et al.,* 1989). Natural and experimental infection of calves with an O111 STEC strain results in colitis with attachment and effacement (A/E) of the colonic mucosa (Gyles, 1992). Another study involving experimental infection with O157:H7 STEC showed that both adult cattle and calves could be transiently colonised, however, neonatal calves may develop significant intestinal lesions (Dean-Nystrom *et al.,* 1997).

STEC O157 can potentially enter the human food chain from a number of animal sources with the most common means being contamination of meat with faeces or intestinal contents after slaughter. Principally, transmission is through the consumption of these contaminated foods such as raw or undercooked ground meat products and raw milk (Clarke *et al.,* 1994). In a Canadian survey of local and imported ground beef, 4 to 16% of samples showed positive results for STEC depending on the source. However,

15 to 40% of the cultures were cytotoxic for Vero cells, and this may be a more accurate reflection of the proportion that was actually contaminated (Clarke *et al.*, 1994).

One of the more common sources of human STEC infection is hamburger patties made from ground beef and a number of outbreaks of O157:H7 infection have been linked to this source (Karmali, 1989). Ground beef may pose a particular risk for two reasons. Firstly, the prevalence of highly pathogenic STEC strains such as O157:H7 may be higher in cattle than in other animal species. Secondly, STEC contaminating the surface of meat becomes evenly distributed during the mincing process and unless hamburger patties are thoroughly cooked, STEC organisms in the centre may not be exposed to lethal temperatures. Massive outbreaks were shown when fast food restaurants used a common source of ground-beef and sub-optimal cooking procedures to make hamburgers. In one of these outbreaks, over seven hundred people became ill and over fifty of them developed HUS with four fatalities (Griffin *et al.*, 1994).

Other proven food sources of STEC infection include raw or inadequately pasteurised dairy products, fermented or dried meat products such as salami and jerky as well as fruit and vegetable products (Adak *et al.*, 1997). The largest outbreak of STEC disease yet reported occurred in Sakai, Japan, in 1996 and involved more than six thousand cases of HC and over one hundred cases of HUS (Fukushima *et al.*, 1997). The most-likely source appeared to have been radish spouts in mass- prepared school lunches. Transmission of STEC by the faecal–oral route and person-to-person is well documented and may account for a significant proportion of sporadic cases (Reida *et*

al., 1994). In a study of patients with O157 infection, the median duration of faecal shedding of STEC was 2 to 3 weeks. However, 13% of patients shed O157 for more than 1 month (the maximum was 18 weeks) and were clinically asymptomatic during the latter stages (Karch *et al.,* 1995). The sources of sporadic cases of STEC infections are often difficult to pinpoint, because of the lack of epidemiological correlation, but there is no reason to propose that the source of these infections differ from those listed above.

2.5 PATHOGENESIS OF STEC INFECTION

The production of a potent shiga-like toxin is essential for many of the pathological features as well as the life-threatening sequaelae of infection. Shiga toxin 2 (Stx_2) is considered to be the most important virulence factor associated with human disease (Ostroff *et al.,* 1989; Boerlin *et al.,* 1999). In addition, Stx_2 is about 400-fold more toxic to mice than Stx_1 and has also been shown to induce fetoplacental resorption, intra-uterine haematoma, fibrin deposition, and neutropile infiltration when injected intravenously into mice on the fifth day of pregnancy (Tesh *et al.*, 1993; Yoshimura *et al.,* 2000).

More importantly, differences in the degree of pathogenicity of STEC serotypes have been associated with variations in the stx2 subtype (Melton-Celsa and O'Brien, 1998). However, pathogenesis is a multistep process, involving a complex interaction between a range of bacterial and host factors. Orally ingested STEC (often in very low initial doses) must initially survive the harsh environment of the stomach and then compete with other gut microorganisms to establish intestinal colonisation. STEC organisms remain in the gut, and so shiga toxins produced in the lumen must be first absorbed by

the intestinal epithelium and then translocated to the bloodstream. This permits delivery to the specific toxin receptors on target cell surfaces inducing both local and systemic effects.

2.6 OTHER VIRULENCE FACTORS

Virtually all *E. coli* O157 isolates carry a large plasmid of approximately 90kb, which is called STEC plasmid (Karch *et al.*, 1998). The STEC plasmid carries genes for several putative virulence factors, including haemolysin (encoded by *hlyA*), catalase-peroxidase (encoded by *katP*), lymphocyte inhibition factor (*Lif*), EspP (encoded by *espP*), toxB (encoded by *toxb*) and a predicted type II secretion system (*etp*). Haemolysin is found in nearly all *E. coli* O157 strains and widely among non-O157 strains. However, the role of haemolysin in the pathogenesis is still unknown but serological response against haemolysin has been detected from HUS patients, which indicates an association with virulence (Schmidt *et al.*, 1995). The toxin lyses erythrocytes of human and bovine origin, and bovine but not human leukocytes. EspP cleaves pepsin and human coagulation factor V and may affect the normal coagulation cascade, increasing gastrointestinal haemorrhage (Brunder *et al.*, 1997) while *toxB* gene is needed in epithelial adherence (Tatsuno *et al.*, 2001).

2.7 ATTACHING AND EFFACING ADHERANCE (A/E)

Certain strains of STEC are capable of causing A/E lesions on enterocytes (Sherman. *et al.*, 1988). These lesions involve ultrastructural changes including loss of enterocyte microvilli and intimate attachment of the bacterium to the cell surface. An additional potential complication in the elucidation of the role of intimin in the pathogenesis of human disease is introduced by the significant sequence heterogeneity of the C-

terminal portion of the protein. Heterogeneity between STEC and EPEC intimin accounts for marked differences in tissue tropism but heterogeneity also occurs within STEC strains. Wieler *et al.* (1996) found that *eaeA* probe- positive STEC strains from only 8 of 170 serogroups tested were PCR positive with primers based on the 3' portion of O157:H7 *eaeA*. Such differences have been used as the basis for serotype-specific assays for STEC. It is however, not known whether variations affect the biological activity or receptor specificity of intimin.

Despite all this, a significant minority of human STEC isolates, including those from patients with HC and HUS, do not contain *eaeA,* indicating that intimin is not essential for human virulence (Barrett *et al.,* 1994). It was suggested that these strains produce additional, as yet uncharacterised virulence factors to compensate for the absence of *eaeA*. Wieler *et al.* (1996) found that only 65% of *eaeA* probe-positive bovine STEC isolates were positive by fluorescent actin staining of infected Hep-2 cells. Furthermore, only 19% of the isolates were positive for *espB* by PCR or low-stringency hybridisation. Thus the presence of *eaeA* does not necessarily imply that a given STEC strain is capable of production of functional intimin and generation of A/E lesions.

2.8 ROLE OF STX IN THE PATHOGENESIS OF DISEASE

2.8.1 Uptake and translocation of Stx by intestinal epithelial cells

Stx have direct enterotoxic properties, which result from selective targeting of Gb3-containing absorptive villus epithelial cells in the ileum based on studies carried out with rabbits. This susceptibility of rabbit intestinal cells is age related and correlates with up-regulation of net Gb_3 biosynthesis in the third week of life (Mobassaleh *et al.,*

1994). It is possible that the diarrhoea seen in human STEC infections is partly due to direct exposure of enterocytes to Stx in the gut lumen. However, the presence of Gb_3 in human enterocytes has not yet been demonstrated. There is the suggestion that many of the gastrointestinal pathological findings may be caused by a systemic toxin (Paton and Paton, 1998).

Intravenous injection of Stx1 into rabbits caused diarrhoea with edematous and haemorrhagic lesions in the mucosa and submucosa of the caecum (Richardson *et al.*, 1992). In the above study the microvascular endothelium appeared to be the principal cytotoxic target. STEC strains appear to be unable to invade gut epithelial cells to any significant extent. The generation of systemic sequelae must presumably involve translocation of shiga toxin produced by colonising bacteria from the gut lumen to underlying tissues and the bloodstream. One possible route might be through lesions in the mucosal barrier caused either by direct effects of shiga toxin or other factors such as intimin or perhaps through gaps between adjacent epithelial cells. Thus shiga toxin is capable of translocation across epithelial cells without apparent cellular disruption via a transcellular pathway (Acheson, 1998).

2.9 CLINICOPATHOLOGICAL FEATURES OF HUMAN STEC DISEASE

It is now recognised that there is a very broad spectrum of human diseases associated with shiga toxin-producing *E. coli* strains especially isolates of the serotype O157 (Griffin, 1995; O'Brien and Kaper, 1998; Kim *et al.*, 1998; NIH, Korea, 2001-2003). STEC-related disease may involve either sporadic cases or large outbreaks involving a common contaminated food source. Some individuals infected with STEC may be completely asymptomatic, in spite of the presence of large numbers of organisms as

well as free toxins in the faeces (Brian *et al.,* 1992). Very little is known of the true incidence of asymptomatic carriage.

Nevertheless, a significant number of survivors (approximately 30%) suffer a range of permanent disabilities including chronic renal insufficiency, hypertension, and neurological deficits (Tarr, 1995). STEC infections can also result in a variant form of HUS, sometimes referred to as thrombotic thrombocytopaenic purpurae (TTP). This "diarrhoea-associated TTP" is more common in adults than in children. The pathological features are essentially the same, but it differs from the typical form of HUS in that patients are more often febrile and have marked neurological involvement (Morrison *et al.,* 1985). However, there is another form of TTP without a diarrhoeal prodrome, which is not associated with STEC infection. Rare complications include pancreatitis, diabetes mellitus and pleural and pericardial effusions (Mead and Griffin, 1998).

Whether STEC – associated diarrhoeal disease progresses to life-threatening complications depends upon an interplay between bacterial and host factors. In an outbreak setting, the age of infected persons will have a significant influence on the proportion of infected persons who develop HUS, as well as the mortality rate. Characteristics of individual STEC strains will also have a major impact. Results obtained in a study indicated that females are at higher risk than males of developing HUS (Mead and Griffin, 1998). Despite this consideration when outbreaks of infections caused by O157 occur roughly 5% to 10% of individuals with diarrhoea progress to HUS (Griffin and Tauxe, 1991).

2.10 TREATMENT OF STEC INFECTIONS AND ANTIBIOTIC RESISTANCE OF *E. COLI* O157 STRAINS

The treatment of STEC infection with antibiotics is still regarded as controversial (Yoh and Honda, 1997; Igarashi *et al.,* 1999). Several studies suggested that, either there was no significant benefit associated with administration of antibiotics or that therapy actually increased the risk of developing HUS (Riley *et al.*, 1983; Carter *et al.,* 1987; Butler *et al.,* 1987; Cimolai *et al.,* 1990; Pavia *et al.*, 1990; Slutsker *et al.,* 1998; Wong *et al.,* 2000; Dundas *et al.,* 2001). Antibiotics act as inducers of bacteriophage liberation in *E. coli* O157. These phages carry the shiga-like toxin (*stx*) genes. This increases the free *stx* gene in the gut lumen by releasing cell-associated toxins and inducing gene expression that may result in the death of the individual (Zhang *et al.,* 2000). However, results obtained from other studies indicated that initiation of antibiotic therapy early in the stages of STEC infection was able to prevent the disease progression to HUS (Fukushima *et al.,* 1999; Ikeda *et al.,* 1999; Shiomi *et al.,* 1999). No specific therapy for STEC infections are available and most patients require prolonged clinical and outpatient treatment (Slutsker *et al.,* 1997). Initially, *E. coli* O157 strains were susceptible to many antibiotics (Ratnam *et al.,* 1988). However, several studies have documented antibiotic resistance among *E. coli* O157:H7 isolates (Kim *et al.,* 1994; Galland *et al.*, 2001; Zhao *et al* 2001; Khan *et al.,* 2002; Schroeder *et al.*, 2002; Bettelheim *et al.,* 2003; Wilkerson *et al.*, 2004).

STEC infections caused by multiple resistant strains may have a selective advantage to cause an outbreak (Bettelheim *et al.,* 2003). The resistance patterns observed for *E. coli* O157 isolates may vary depending on the types of antimicrobials tested. Furthermore,

the different classes of antimicrobials have different mechanisms of action and as such the bacterial isolates have different ways exhibiting resistance to antibiotics. Table 2.1 outlines the mechanisms of action of the different antibiotics and the mechanisms of resistance to the anitibiotics exhibited by bacterial isolates. The antibiotics tested in this study involve some of the drugs that are commonly utilised in both humans and animals (Table 2.1). These include the commonly used tetracycline, ampicillin and erythromycin (Iqbal *et al.,* 1996) as well as the less commonly used ones such as streptomycin, neomycin, kanamycin and chloramphenicol. Norfloxacin was included since it has been used in the induction of bacteriophages from STEC strains (Matsushiro *et al.*, 1999). Sulphamethoxazole is usually used for the treatment of diarrhoael diseases in cases where tetracycline is contraindicated and the drug is more effective when used in combination with trimethoprim (Iqbal *et al.,* 1996).

Table 2.1: Mechanisms of action of the various antibiotics and mechanisms of resistance to the antibiotics exhibited by bacteria isolates.

Antibiotic Group	Example	Action on Gram-Negative bacteria.	Mechanism of Resistance
Aminoglycosides	Kanamycin Neomycin Streptomycin	Bind to 30S subunit of ribosomes – inhibit protein synth	Aminoglycoside modifying enzymes[2] Flux mechanisms[2] RNA modifications[2]
Macrolides	Erythromycin	Bind to 50S subunit of ribosomes – inhibit protein synth	May be impermeable to G(-) ve Post-transcriptional modifications of 23S rRNA Efflux mechanisms – Cell membrane Hydrolysis enzymes
Tetracyclines	Tetracycline	Bind to 30S subunit of ribosomes – inhibit protein synth	Efflux mechanisms[2] 16S mutations[2]
Beta- Lactams	Ampicillin	Cell Wall synth. Inhibitor- act on penicillin binding proteins (PBP)	Penicillin-G impermeable to G(-) Mutations in PBP's Produce β-Lactamase (Ampicillin resistant to β-Lactamases)
Quinolones	Norfloxacin	Inhibits DNA gyrase synthesis	The presence of *qnr* gene that is plasmid encoded.[3]
Phenicols	Chloramphenicol	Inhibit bacterial protein synthesis by blocking the 50S subunit.	
Sulphonamide	Sulphamethoxazole	Inhibit bacterial protein synthesis	The presence of *sul3* gene or mutations[1]

[1](Perreten and Boerlin, 2003); [2](Fliut *et al.*, 2001); [3] (Xian-Zhi, 2005).

2.11 DIAGNOSTIC TYPING METHODS

Typing systems are used to define specific characteristics of the object under study (van Belkum *et al.*, 2001). An evaluation of a typing method involves a thorough consideration of some, if not all of the following factors; reproducibility, discriminating power, typeability, ease of interpretation and the ease of performance (Maslow *et al.*, 1993a). Using the criteria above in evaluating a typing method usually requires information that is unfortunately not available. Evaluation of a method involves analysis of adequate numbers of sporadic and epidemic isolates and directly comparing the results with those of previously studied approaches (Maslow *et al.*, 1993a). An ideal typing method should be inexpensive, rapid and technically simple.

Given that STEC strains, as with other diarrheagenic *E. coli* strains, are defined on the basis of virulence properties, there are two approaches or typing systems employed in the detection of STEC in the laboratory viz; phenotypic and genotypic (Nataro and Kaper, 1998). Phenotypic techniques are those that detect characteristics expressed by the microorganism while genotypic techniques are those involving DNA-based analysis of chromosomal or extra-chromosomal genetic elements (Maslow *et al.*, 1993a).

2.11.1 Phenotypic typing methods

2.11.1.1. Serotyping of E. coli

Serotyping of *E. coli* occupies a central place in the history of these pathogens (Lior, 1996). Prior to the identification of specific virulence factors in diarrheagenic *E. coli* strains, serotypic analysis was the predominant means by which pathogenic strains were differentiated. In 1944, Kauffman proposed a scheme for the serologic classification of *E. coli*, which is still used in modified form today. According to the schemes, *E. coli* are

serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Lior, 1996). A specific combination of O and H antigens defines the "serotype" of an isolate. *E. coli* of specific serogroups can be associated reproducibly with certain clinical syndromes, but the presence of serologic antigens does not generally confer virulence. However, the serotypes serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Whittam *et al.*, 1993).

2.11.1.2 Antibiotic sensitivity analysis

Antimicrobial susceptibility testing is a simple, robust and cost effective method, which might in some instances give additional information for the characterisation of bacterial isolates (Nataro and Kaper, 1998). Formally, *E coli* O157 isolates were almost universally susceptible to most of the antibiotics that are used in both human and animal medicine (Bopp *et al.,* 1987; Ratnam *et al.,* 1988) but antimicrobial resistance against these drugs has in recent years become more common and can sometimes be an epidemiological marker (Strockbine *et al.* 1998; Willis, 2000). Resistance data is usually reported as percentage resistant with respect to the number isolated.

2.11.1.3 Multiple Antibiotic Resistant (MAR) Phenotypes and indices

Multiple antibiotic resistant phenotype (MAR) of an isolate is a representation of its physical appearance and constitution with respect to resistance observed against a panel of antimicrobial agents (Meays *et al.,* 2004). The MAR phenotype is most often determined by the genetic constitution of the isolate and can be employed as a useful tool for the characterisation of isolates. Antibiotic resistance analysis (ARA) has proven

to be a useful tool in the characterisation of isolates from different sources (Harwood *et al.*, 2000; Hager, 2001a; Guan *et al.*, 2002).

The MAR index, which is a measure of the frequency of antibiotic resistance, has been used to differentiate *E. coli* of high-risk origin from those from other sources (Krumperman, 1983). Identifying *E. coli* of high-risk sources could facilitate the detection of sources of contamination (Kaspar *et al.*, 1990).

Studies have shown that isolates from different sources may react differently against a panel of antibiotics (Harwood *et al.*, 2000; Guan *et al.*, 2002). Furthermore, a related study indicated that the multiple antibiotic resistant (MAR) indexing was able to differentiate *E. coli* isolates from different sources based on the level of exposure to antibiotics (Krumperman, 1983). In a particular study, a greater proportion of isolates having identical MAR index and resistance profiles were from the same collection area (Kaspar *et al.*, 1990). It was suggested that MAR indices could be used as a tool in characterising subpopulations of *E. coli* isolates.

However, the antibiotic resistance patterns for sets of isolates from a particular source may change through the acquisition of resistant determinants. Classification of *E. coli* isolates from different sources has also been performed by cluster analysis using antibiotic susceptibility data (Guan *et al.*, 2002). The analysis was found to be more discriminating upon inclusion of the 16S rRNA sequence and amplified fragment length polymorphism (AFLP) data.

2.11.2 Genotypic Typing Methods

Genotypic (DNA-based) typing methods provide many advantages over phenotypic methods due to their usually greater discriminating power (Farber, 1996; van Belkum *et al.*, 2001). Commonly employed genotypic methods include, plasmid profile analysis, pulse-field gel electrophoresis (PFGE), ribotyping, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and genome sequencing.

2.11.2.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was introduced in 1985 and since then has been an important technique in molecular biology (Faloona *et al.*, 1985). Using PCR, a single piece of DNA can be used to generate an infinite supply of identical copies for a variety of research, clinical, forensic and commercial purposes. PCR has revolutionised how we detect infectious diseases such as immunodeficiency virus infection or tuberculosis, diagnose cancer, determine paternity and confirm criminal acts (Palmer and Stehens, 1993). PCR is fast, simple, inexpensive and consists of repetitive cycles of three precisely timed, consecutive reactions that require separate temperature conditions. It is performed in themocyclers that rapidly shift samples between several temperatures in a preset order. Each cycle involves strand separation, primer annealing and elongation of the strand (Paton and Paton, 1998; Tutenel *et al.,* 2003; Harakeh *et al.,* 2005; Yilmaz *et al.,* 2006).

PCR technology has been widely applied in various areas of molecular biology analysis and further developed into different applications. The most frequent application

involves the amplification of a certain portion of a specific gene with known flanking sequences. The amplified fragment can be further analysed to obtain sequence information or functional information. PCR has also been used in clinical diagnosis of infection, identification of a certain known disease molecular marker, or amplification of a specific gene and identification of mutations or polymorphisms (Abbas *et al.*, 2003; Shaila and Saumitra, 2005).

2.11.2.2 PCR for the detection of E. coli O157 strains.

PCR analysis is generally considered the most sensitive method for detecting the genes that code specific virulence determinants in *E. coli* O157 strains (Yu and Kaper, 1992; Paton and Paton, 1999). Several studies have employed the PCR technique for the detection of *E. coli* O157 from faeces and food samples (Meng *et al.,* 1998; Paton and Paton, 1998; Fratamico *et al.,* 2000; Holland *et al.*, 2000; Bovet *et al.,* 2001; Bovet *et al.,* 2002; Botteldoorn *et al.,* 2003; Tutenel *et al.,* 2003; Blanco *et al.,* 2004; Meichtri *et al.,* 2004; Harakeh *et al.*, 2005; Yilmaz *et al.,* 2006). In some of these studies, primer sets were used to amplify the different virulent molecular markers (stx_1, stx_2, *eaeA* and *hlyA*) that are usually carried on the *E. coli* O157 bacterial chromosome or plasmids (Paton and Paton, 1998; Osek and Gallein, 2002; Reischl *et al.,* 2002; Karch and Meyer, 1989; Holland *et al.*, 2000; Barkocy-Gallagher *et al.,* 2001, Chapman *et al.,* 2001; Bovet *et al.,* 2001; 2002; Botteldoorn *et al.,* 2003; Omisakin *et al.,* 2003; Tuntenel *et al.,* 2003; Meichtri *et al.,* 2004; Yilmaz *et al.,* 2006). However, identification of the *E. coli* O157 serotype can also be performed by PCR analysis using a set of primers that amplifies the O-antigen-encoding region (rfb region) of the *E. coli* O157 chromosome that codes for the rfb_{O157} gene (Paton and Paton, 1998). These PCR

assays have been specific for the identification of *E. coli* O157 and hence can be effectively employed in the genotypic detection of this pathogen.

Multiplex PCR assays have also been developed to facilitate the simultaneous detection of these virulence genes within *E. coli* O157 isolates (Paton and Paton, 1998; Fagan *et al.,* 1999; Fratamico *et al.,* 2000; Radu *et al.,* 2001; Goiffré *et al.,* 2002; Botteldoorn *et al.,* 2003; Blanco *et al.,* 2004; Meichtri *et al.,* 2004; Yilmaz *et al.,* 2006).

Although PCR analysis is very sensitive for the detection of this pathogen in samples, more reliable results are obtained when using DNA extracted from broth cultures as compared to direct extracts from faeces and food samples (Gannon *et al.*, 1992; Paton *et al.*, 1993; Begum and Jackson, 1995).

2.12 SUMMARY

The preceding literature review considered a number of aspects regarding the biology and epidemiology of *E. coli* O157. The literature also clearly demonstrate that in developing countries proper hygienic food standards are not always strickly implemented and food is an important vehicle for the transmission of many diseases (Harakeh *et al.,* 2005). Outbreaks of infections may result from the consumption of food contaminated with animal faecal matter (Borczyk *et al.,* 1987; Belongia *et al.,* 1991). *E. coli* O157 strains were formally not resistant most antibiotics used in both animal and human medicine. However, the emergence of antibiotic resistant among *E. coli* O157 have made infections caused by these resistant pathogenic bacterial strains to result in an increase in the fatality rate in humans. This phenomenon is further compounded by the borden of HIV/AIDs and other immuno-compromised conditions (Holmberg *et al.*, 1984).

The identification of pathogenic strains of *E. coli* is highly important to aid in the surveillance, prevention and control of its associated diseases. However, the epidemiological investigation of *E. coli* O157 strains is usually hampered by its ubiquitous nature and the lack of heterogeneity among clonal strains (Asis *et al.*, 2002). The discriminatory capacity of genotypic and phenotypic methods that are used to characterise *E. coli* O157 strains is enhanced by the ability to minimise the little variation between strains (Galland *et al.*, 2001; Radu *et al.*, 2001). These typing methods are important for the identification of the sources of *E. coli* O157 contamination, routes of transmission between species and make suggestions for the implementation of intervention strategies.

CHAPTER 3

MATERIALS AND METHODS

3.1 AREA OF THE STUDY

This research was carried out at the North-West University – Mafikeng Campus, - North-West Province. Cattle samples were collected from commercial farms at Litchtenburg and Rustenburg and from a communal farm at Mogosane. Pig samples were collected from communal and commercial farms at Tlapeng and Mareetsane respectively, while human samples were collected from diarrhoeagenic patients that visited Mafikeng Provincial Hospital. The samples were used for the isolation of *E. coli* O157 only and were provided without any indication of patient identity. The human stool samples were handled with care during bacterial isolation and incinerated immediately after analysis. Laboratory analysis was performed at the veterinary microbiology diagnostic laboratory of the Animal Health Department, (Mafikeng Campus) of the North-West University, situated at Molelwane village. Table 3.1 indicates the distance between the different sampling stations with reference to the animal health deparment (Mafikeng Campus).

Table 3.1: The distance (in Kilometers-km) between sampling stations using the Animal Health Department of the Mafikeng campus as the reference point.

Sample source	Sampling station	Distance (Km)
Pigs	Tlapeng	50
	Mareetsane	90
Cattle	Rustenburg	215
	Lichtenburg	75
	Mogosane	25
Humans	Mafikeng Provincial Hospital	25

3.2 COLLECTION OF SAMPLES AND CONTROL BACTERIAL STRAINS

3.2.1 Collection of samples

Samples were collected directly from the rectum of animals using sterile arm-length gloves and were placed in sterile sample collection bottles. The samples were immediately placed on ice and transferred to the laboratory for analysis.

3.2.2 *E. coli* control strains

E. coli O157:H7 (ATCC 43889) and *E. coli* O145 were used as positive controls for the shiga toxin genes. They both possess *eae* and *hlyA* genes. *E. coli* O157:H7 (ATCC 43888) that does not contain any one of these genes was used as a negative control.

3.3 MICROBIOLOGICAL ANALYSIS

3.3.1 Enrichment for *E. coli*

Sample enrichment was achieved by inoculating 1g of faecal sample into 1 ml of MacConkey broth (Mi-Yeong *et al.,* 2004) and incubated aerobically at 37°C for 18 to 24 hours.

3.3.2 Culturing

After incubation, a ten-fold serial dilution of the bacteria culture was performed using MacConkey broth as the diluent. Aliquots (100µl) of each dilution were transferred and spreaded onto Sorbitol MacConkey agar. Plates were incubated aerobically at 37°C for 18 to 24 hours. After incubation, single colonies that were either Sorbitol negative (colourless) or Sorbitol positive (pink) were picked and purified by streaking on Sorbitol MacConkey agar. Plates were incubated aerobically at 37°C for 18 to 24 hours.

3.3.3 Purification of presumptive *E. coli* isolates

Purification was achieved by streaking presumptive *E. coli* isolates from plates with mixed cultures on new Sorbitol MacConkey agar and plates were incubated aerobically at 37°C for 18 to 24 hours. Pure cultures that were identified as Sorbitol negative colonies and lactose positive were preserved for characterisation and further biochemical identification. Presumptive *E. coli* isolates were subjected to Gram staining.

3.4 PRELIMINARY IDENTIFICATION TESTS

Escherichia coli is a gram-negative rod and to achive more reliable results during its identification Gram staining was required for the confirmation of isolates.

3.4.1 Gram Staining

Gram staining was performed on the isolated single pure colonies according to the standard methods (Cruikshank *et al.*, 1975). Gram-negative rods were subjected to biochemical tests (oxidase, and the triple sugar iron agar; TSI), which are distinctive for the genus. Results are indicated in Appendix C.

3.4.2 Oxidase Test

This test was used as a screening test to identify *E. coli* isolates for the presence of the cytochrome system. Cytochromes are haem-containing proteins and are oxidative enzymes in the respiratory chain of bacteria, which utilise free oxygen as a terminal electron acceptor (Cowan and Steel, 1966). The test was performed as instructed by the manufacturer (Pro-Lab Diagnostics – United Kingdom). One or two drops of oxidase reagent was placed on a filter paper and left for about two minutes to ensure proper

redistribution of the reagent. A pure colony of isolate was placed on a filter paper and rubbed onto the reagent-saturated area. The filter paper was observed for colour change within thirty seconds. The production of a distinct blue or purple colour was regarded as a positive reaction and vise versa (Appendix C). All isolates that were oxidase negative were retained for further testing.

3.4.3 Triple sugar iron agar test (TSI)

Oxidase negative isolates were subjected to the TSI test, which evaluates the ability of the organisms to utilise the three sugars; glucose, lactose and sucrose present at different concentrations in the medium, producing acids and gas (MacFaddin, 1985; Forbes and Weissfeld 1998; Prescott 2002). This test was performed by stab inoculating a pure colony into the butt of a TSI culture medium and later performing an "S" on the surface of the slant (USPC, 2001; Prescott 2002). The bottle was closed loosely and incubated aerobically at 37°C for 18 to 24 hours. After incubation a colour change of the media from pink to yellow at the butt and slant coupled with the production of gas that was identified by a splint in the media, was regarded as a positive reaction for all three sugars. This is explained from the fact that glucose, which has the lowest concentration, is first utilised and later lactose and sucrose that are at higher concentrations. The colour change is due to the production of acids. Results were as indicated in Appendix C.

3.5 SECONDARY BIOCHEMICAL TEST

3.5.1 Analytical profile index (API 20E) test

The API 20E is a standardised identification system for *Enterobacteriaceae* and other non – fastidious rods. The test was employed according to the manufacturer's

instructions (Biomeriux, France). The API 20E strip consists of 20 micro tubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism of bacteria produces colour changes that are either spontaneous or revealed by the addition of reagents. Isolates were identified by referring numbers obtained on the API 20E computerised identification system and results are indicated in Appendix C.

3.6 SEROTYPING OF *E. COLI* O157 ISOLATES

The isolates were examined by slide agglutination with *E. coli* O157 specific antisera. The test was performed as instructed by the manufacturer (Mast diagnostics, Merseyside, United Kingdom). This was achieved by transferring a drop of saline each at reasonable distance on a microscope slide. One of the drops served as the test, while the other was the control. A single pure colony of isolate was added to both the test and control. The colonies were mixed to obtain a dense suspension. To the test sample a drop of *E. coli* O157 monovalent specific antisera was added while a drop of saline was added to the control. The slide was placed on the roter model VRN-200, to ensure that contents are well mixed. The formation of clots on the test sample was regarded as a positive reaction and results are as reflected in Appendix C.

3.7 ANTIBIOTIC SENSITIVITY TEST

Antibiotic sensitivity tests were performed on all isolates using the paper disk diffusion method (Kirby-Bauer *et al.*, 1966) to determine the antibiotic resistant profiles of *E. coli* O157 isolated from the various species. In performing the test, a bacterial suspension was prepared by transferring a single pure colony into an eppendorf tube containing 1ml of sterile distilled water. A ten-fold serial dilution of the suspension was

performed and an aliquot (100µl) of the dilution (10^{-5}) was transferred onto Mueller Hinton agar plates and cultured by spreading. Selected antibiotic disks that contained therapeutic concentrations of antibiotics (Table 3.2) were placed on the media using sterile forceps and plates were incubated at 37°C overnight. Antibiotics impregnated on the disks diffuse out into the surrounding environment inhibiting growth. After incubation antibiotic inhibition zone diameters were determined and the antibiotic profiles of isolates were further deduced (Table 3.2). Isolates identified to be resistant against particular antibiotics were subjected to a further screening test to determine if such phenotypic characters were as a result of presence of antibiotic resistant markers or plasmids. Table 3.2 indicates the details of antibiotics that were used in this study.

Table 3.2: Details of antibiotics that were used in this study. The concentration used as well as the inhibition zone measurements (in mm) that were considered resistant (R), intermediate resistant (I) and susceptible (S) are shown and was according to NCCLS (1999). The abbreviations (Abbrev.) were as they appeared on the antibiotic disks.

Group	Antibiotic	Abbrev.	Disk conc.	R	I	S
Aminoglycosides	Kanamycin	K	30µg[c]	≤13	14-17	≥18
	Neomycin	Ne	30µg[c]	≤12	13-16	≥17
	Streptomycin	S	10µg[a]	≤11	12-14	≥15
Macrolides	Erythromycin	E	15µg[b]	≤13	14-22	≥23
Tetracyclines	Tetracycline	T	30µg[c]	≤14	15-18	≥19
Beta- Lactams	Ampicillin	Ap	10µg[a]	≤11	12-14	≥15
Quinolones	Norfloxacin	Nor	10µg[a]	≤12	13-16	≥17
Phenicols	Chloramphenicol	C	30µg[c]	≤12	13-17	≥18
Sulphonamide	Sulphamethoxazole	Smx	10µg[a]	≤10	11-15	≥16

The superscripts [a] to [c] indicate the generally accepted concentrations of the disks according to the standard method stipulated by the manufacturer, Mast diagnostic, Merseyside, United Kingdom.

3.8 MULTIPLE ANTIBIOTIC RESISTANT (MAR) INDEX

The MAR indices were determined using selected antibiotics (Section 3.6, Table 3.2). The antibiotics used were those commonly added to animal feeds and/or used in clinical applications (Krumperman, 1983). The MAR index for each group of *E. coli* O157 isolates were determined as previously described (Kaspar *et al.,* 1990) and was calculated as follows:

$$\text{MAR} = \frac{\text{Number of isolates resistant to all antibiotics in a specific sample population}}{\text{(number of antibiotics tested) x (total number of organisms in sample)}}$$

This index was a measure of the extent of drug resistance for isolates in the group. The MAR index of an isolate was calculated by dividing the number of antibiotics to which the isolate was resistant by the number of antibiotics used. The antibiotic resistance pattern comparisons were performed using cluster analysis for antibiotic susceptibility data.

3.9 HAEMOLYSIS ON BLOOD AGAR

Haemolysis was determined by culturing on blood agar supplemented with 5% sheep blood (Beutin *et al.*, 1998) and results were as indicated in Appendix C. The genotype was confirmed by using specific primers as previously described (Paton and Paton, 1998) with the conditions obtained through optimisation.

3.10 INDUCTION OF BACTERIOPHAGES

Induction of phages was performed as previously described (Matsushiro *et al.,* 1999), Appendix A, (Section A.7). However, all attempts to induce bacteriophages from the *E. coli* isolates and control strains failed.

3.11 MOLECULAR CHARACTERISATION OF *E. COLI* O157 ISOLATES

3.11.1 Extraction of genomic DNA

Genomic DNA was extracted from bacteria using a modification of the hot (65°C) CTAB – PVP DNA extraction procedure of Dolye and Dolye, (1990). Overnight cultures were prepared by inoculating the isolates into 5ml Luria-Bertani broth. After incubation, 1.0 ml of the overnight culture was transferred into a 1.5 ml microfuge tube. The contents of the tube were centrifuged at 13500 rpm in a Heraeus microfuge (Kendro, Germany) for 5 minutes and the resulting supernatant poured off. The sediment was resuspended in 100µl of TE buffer and 0.1mg Lyzozyme suspension. The contents of the tube were incubated at 37°C for 30 minutes. After incubation 2X hot (65°C) CTAB isolation buffer (100mM Tris-Cl buffer, 20mM EDTA, 1.4M sodium chloride, 2% CTAB and 0.2% 2-mercaptoethanol), 0.25% PVP and 0.2mg proteinase K were added to the final concerntration of the sample. The tubes were incubated in a water bath held at a temperature of 65°C for 30 minutes while inverting the tubes 3 to 4 times every 3 minutes. An equal volume of TE buffered Phenol:Chloroform:Isoamyl: (25:24:1) was added and DNA was extracted for 10 minutes at room temperature while gently inverting the tube 3 to 4 times every 3 minutes. The contents of the tube were centrifuged at 13500 rpm for 5 minutes. The amount of aqueous phase obtained was measured and transferred into a new sterile microfuge tube. DNA was re-extracted with an equal volume of TE buffered chloroform:Isoamyl: (24:1) for 10 minutes at room

temperature while gently inverting the tube 3 to 4 times every 3 minutes. The contents of the tube were centrifuged at 13400 rpm for 5 minutes and the resulting aqueous phase transferred to a new sterile microfuge tube. Furthermore, 1.2M NaCl and 1ml of ice cold 95% ethanol were added and DNA was precipitated at –80°C for 1 hour. Following precipitation the tube was centrifuged at 13500 rpm for 5 minutes at 4°C and the supernatant was gently poured off. The pellets were washed in 1ml ice-cold 70% ethanol to remove NaCl. The contents of the tube were later centrifuged at 13500 rpm for 5 minutes at 4°C using the Sorvall (model RC 5B+) centrifuge. The supernatant was gently poured off and the pellets were dried under vacuum using the Tomy Micro Vac_{TM} mv-100 (Tomy Medico, Japan) vaccum dryer. The DNA was resuspended in 50µl of TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) and incubated at 65°C for 1 hour to reconstitute the DNA.

3.11.2 Agarose gel electrophoresis

Extracted DNA (10µl) was mixed with 5µl tracking dye (0.25% bromophenolblue, 0.25% xylene cyanol, 30% glycerol). The final reaction mixture was transferred into the wells of a 1% (w/v) agarose gel that contained ethidium bromide (0.001µg/ml) Electrophoresis was conducted in a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia biotech, Sweden) for 1.0 hour at 80 V using 1 x TAE (40mM Tris, 1mM EDTA and 20mM glacial acetic acid, pH 8.0). Each gel contained a DNA molecular weight (Lambda DNA -Hind III digest, Roche Biochemicals, Germany). DNA was visualised under UV light (420nm) as previously reported (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. Images were analysed using GeneTools (version 3.00.22) software (Syngene, Synoptics; UK) to determine the relative sizes.

3.11.3 The polymerase chain reaction (PCR)

PCRs were performed to amplify bacterial 16S rRNA gene fragments (Muyzer *et al.*, 1995) as well as for detection of STEC virulence genes (Paton and Paton 1998) and tetracycline resistant genes (Miranda *et al.*, 2003). A Peltier Thermal Cycler (model-PTC-220 DYAD^{TM} DNA ENGINE; MJ Research Inc. USA) was used for PCR amplifications using genomic DNA isolated from *E. coli* O157 (Section 3.11.1). The oligonucleotide primer combinations used to amplify various genes were as indicated in the table that follows, (Section 3.11.3, Table 3.3). Amplification of bacterial genomic DNA was performed in 25µl volumes containing 1µl of the prepared template DNA, 50pmol each of the oligonucleotide primers, 12.5µl of 2X PCR master mix, 8.3µl Dnase-Rnase free distilled water, 50ng bovine serum albumen and 1U Taq DNA polymerase. The amplifications were performed under the following conditions: an initial denaturation step at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds; primer annealing at 62°C; primer elongation at 72°C for 60 seconds and a final elongation step at 72°C for 5 minutes. PCR products were cooled at 4°C and visualised by gel electrophoresis as indicated in Section 3.11.2.

Table 3.3: Summary of oligonucleotide primers used in the study.

Primer	Sequence (5'–3')	Specificity	Amplicon Size (bp)
GM5F[c]	TACGGGAGGCAGCAG	16S bacterial ribosomal genes	550
907R[c]	CCGTCAATTCCTTTGAGTTT		
stx 1F[a]	ATAAATCGCCATTCGTTGACTAC	A subunit coding region of *stx*$_1$	180
stx 1R[a]	AGAACGCCCACTGAGATCATC		
stx 2F[a]	GGCACTGTCTGAAACTGCTCC	A subunit coding region of *stx*$_2$	255
stx 2R[a]	TCGCCAGTTATCTGACATTCT		
eae F[a]	GACCCGGCACAAGCATAAGC	*Eae* virulence gene (*eae*)	384
eae R[a]	CCACCTGCAGCAACAAGAGG		
hlyAF[a]	GCATCATCAAGCGTACGTTCC	Haemolysin A virulence gene (*hlyA*)	534
hlyAR[a]	AATGAGCCAAGCTGGTTAAGCT		
TetA1[b]	CGA GCC ATT CGC GAG AGC	*TetA*	500
TetA3[b]	GCC TCC TGC GCG ATC TGG		
TetB BF[b]	CAG TGC TGT TGT TGT CAT TAA	*TetB*	500
TetB BR[b]	GCT TGG ATT ACT GAG TGT AA		
TetC CI[b]	CTT GAG AGC CTT CAA CCC AG	*TetC*	500
TetC CR[b]	TGG TCG TCA TCT ACC TGC C		
TetD DF[b]	GGA TAT CTC ACC GCA TCT GC	*TetD*	500
TetD DR[b]	CAT CCA TCC GGA AGT GAT AGC		

[a]Paton and Paton 1998; [b]Miranda *et al.*, 2003; [c]Muyzer *et al.*, 1995

3.11.4 Sequence analysis

Amplified 16S rRNA gene fragments were sequenced by Inqaba Biotec, South Africa. Blast searches (http://www.ncbi.nlm.nih.gov/BLAST) were used to confirm the identity of the amplified sequences.

3.11.5 Extraction of plasmid DNA

Plasmid DNA was extracted by one of two methods; the rapid plasmid extraction protocol which was an adaptation from the method of Birnboim and Doly, (1979) and

the small-scale alkali lysis method using a modification of the procedure of Birnboim and Doly (1979), Appendix A, (Sections A1.8.1 and A1.8.2).

3.11.6 Statistical analysis

Statistical analysis was done using Minitab Release Version 13.31 to determine the percentage antibiotic resistance of *E. coli* O157 isolated from various species at the different farms. Pearson's correlation product of moment was used to determine whether *E. coli* O157 isolated from the various species and/or sampling stations exhibited similar reactions against the antibiotics tested. The correlation value obtained was considered significant at $P<0.05$. Furthermore, cluster analysis based on the antibiotic inhibition zone diameter data of *E. coli* O157 isolated from the different stations was determined using Wards algorithm and Euclidean distances of Statistica, version 7.

CHAPTER 4

RESULTS AND INTERPRETATION

4.1 PREVALENCE OF *E. COLI* O157 IN CATTLE, PIG AND HUMAN FAECES IN NWP, S. A.

Eight hundred samples were analysed but only those isolates from the various sample stations that satisfied all primary and secondary identification criteria (Sections 3.3 and 3.4) for *E. coli* were used. A total of 294 *E coli* isolates were further analysed for characters of *E. coli* O157 and 76 were positively identified. Data sheets for all the *E. coli* O157, showing phenotypic (biochemical and serotype) characteristics as well as antibiotic inhibition zone data, are provided in Appendix C. The results in Table 4.1 shows the number of *E. coli* samples from the various species, sample stations and the numbers and percentages of *E. coli* O157 isolated.

Table 4.1: The percentages of *E. coli* versus *E. coli* O157 isolates at the different sampling stations. Percentages were expressed as number of *E. coli* O157 per number of *E. coli* per sample station.

Species	Area Sampled	No. of *E. coli*	No. of *E. coli* O157	Percentage of *E. coli* O157 isolated
Cattle	Lichtenburg	25	5	20.0
	Rustenburg	29	4	13.8
	Mogosane	74	4	5.4
Pigs	Mareetsane	74	37	50.7
	Tlapeng	52	23	44.2
Humans	Mafikeng Provincial Hospital	40	3	7.5

The results in Table 4.1 indicated that the observed prevalence of *E. coli* O157 was higher among *E. coli* isolated from faeces of pigs (44.2% to 50%) than those from cattle faeces (5.4% to 20.0%), as well as those from human stool samples (7.5%). Samples from the Mogosane communal farm were all obtained from diarrhoeal cattle. Despite this, the prevalence of *E. coli* O157 was lower compared to those from the non-

diarrhoeal commercial cattle (Table 4.1). Additionally, the human stool samples from which the *E. coli* O157 were isolated were also all from diarrhoea patients. It thus appears that in some of the cases *E. coli* O157 could have been the cause of the diarrhea. However, it was not confirmed with the hospital whether *E. coli* O157 was the cause of diarrhoea. This is mainly due to the fact that other pathogens have also been linked to outbreaks of diarrhoea. *E. coli* O157 prevalence trends in cattle have been reported to range from as low as 1.8% (Hancock *et al.*, 1997) to as high as 27% (Chapman *et al.*, 1997; Elder *et al.*, 2000). The results indicated in Table 4.1 are similar to those from previously published data and will be discussed in chapter 5.

4.2 ANTIBIOTIC RESISTANT DATA OF *E. COLI* O157 ISOLATED FROM COMMUNAL (TLAPENG) AND COMMERCIAL (MAREETSANE) PIG FAECES.

Antibiotic phenotypic characterisation of *E. coli* O157 isolated from all the farms was achieved by performing the disc diffusion method (Kirby-Bauer *et al.*, 1966) on Mueller-Hinton agar, using selected antibiotics. A standard table (Section 3.6, Table 3.2) was used to interpret the results obtained in order to classify isolates as being resistant, intermediate resistant or susceptible to a particular antibiotic. Thirty-seven and 23 *E. coli* O157 isolates from commercial (Mareetsane) and communal (Tlapeng) farms respectively, were tested to evaluate their susceptibilities to different antibiotics. Results obtained are depicted in Figure 4.1. The latter figure was generated using data that appear in Appendix E, Table 1E.

A summary of the percentage antibiotic resistance exhibited by *E. coli* O157 isolated from communal and commercial pigs at Tlapeng and Mareetsane respectively, is provided in Figure 4.1.

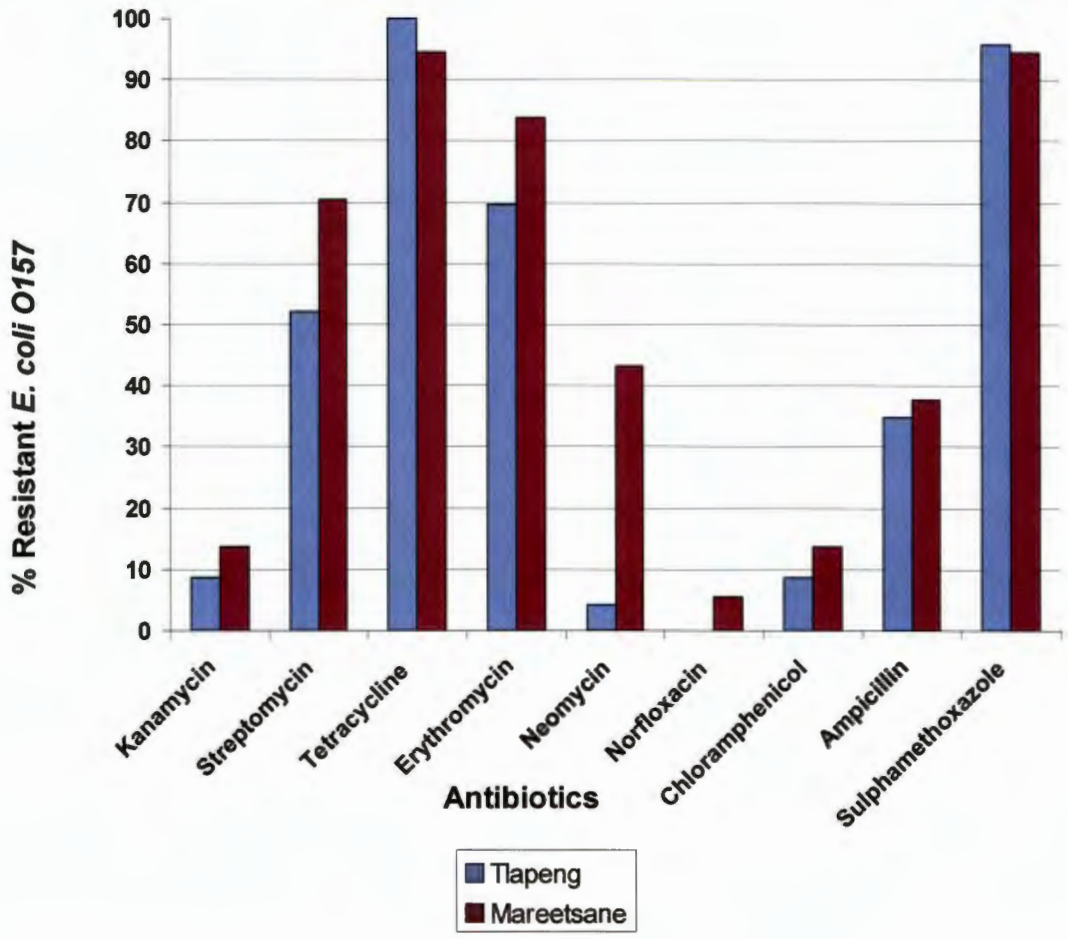


Figure 4.1: Antibiotic resistant pattern of *E. coli* O157 isolated from communal and commercial pigs at Mareetsane and Tlapeng, respectively.

As indicated in Figure 4.1, a high percentage of *E. coli* O157 isolated from pig faeces from Tlapeng communal and Mareetsane commercial farms were resistant to tetracycline (98% to 100%). A large proportion of *E. coli* O157 (83.8% - 95.7%) of the commercial and communal pig isolates were resistant to erythromycin and sulphamethoxazole. A high percentage of *E. coli* O157 (52.2% and 70.3%) from

commercial and communal pigs, respectively were resistant to streptomycin. These antibiotics belong to different groups but have a similar mode of action, which is mainly inhibiting protein synthesis.

A low percentage of *E. coli* O157 (4.3%) isolated from pig faeces from the communal farm were resistant to the aminoglycoside, neomycin. Moreover, a larger proportion (43.2%) of the *E. coli* O157 from pig faeces from the commercial farm showed resistance to neomycin. However, the *E. coli* O157 isolates from the faeces of commercial pigs showed a similar low resistance to norfloxacin (5.4%). Furthermore, a small proportion of *E. coli* O157 isolated from pig faeces from both farms (8.7%, Tlapeng) and (13.5%, Mareetsane) were resistant to chloramphenicol and kanamycin. These antibiotics are from different groups (phenicols and aminoglycosides) and have different modes of resistance mechanisms.

4.3 MAR PHENOTYPES OF *ESCHERICHIA COLI* O157 FROM PIGS.

MAR phenotypes were generated from isolates showing resistance to 3 and more antibiotics (Rota *et al.*, 1996). The antibiotic resistant phenotypes for *E. coli* O157 isolated from both farms were determined and results are shown in Appendix B, (Tables 1B and 2B). Table 4.2 that follows, shows the predominant antibiotic resistant phenotypes that were obtained between the commercial and communal isolates respectively. Multiple antibiotic resistant phenotypes (MAR) were expressed using the abbreviation letters as they appear on the antibiotic paper discs with a modification of small letters.

Table 4.2: Predominant multiple antibiotic resistant (MAR) phenotypes for *E. coli* O157 isolated from communal and commercial pigs at Tlapeng and Mareetsane respectively. Percentages were obtained by dividing the number of a particular phenotype obtained with the total number of isolates in the given area.

Communal pigs (Tlapeng)			Commercial pigs (Mareetsane)		
Phenotype	Number observed	%	Phenotype	Number observed	%
Smx-T-E-Ap	2	8.7	Smx-T-E	6	16.2
Smx-T-Ap	5	21.7	S-Smx-T-E-Ne	4	10.8
Smx-T-E	4	17.4	S-Smx-T-E	3	8.1
S-Smx-T-E	7	30.4	K-S-Smx-T-E-Ap-Ne	2	5.4
			S-Smx-T-E-Ap-Ne	2	5.4
			S-Smx-T	2	5.4
			S-Smx-T-E-Ap	2	5.4
			S-Smx-T-Ap	2	5.4

Ap (ampicillin), T (tetracycline), S (streptomycin), E (erythromycin), Ne (neomycin), K (kanamycin), Smx (sulphamethoxazole).

From Table 4.2, it is evident that several different and similar multiple antibiotic resistant (MAR) phenotypes were observed in the communal and commercial *E. coli* O157 populations. The predominant MAR phenotypes were S-Smx-T-E and Smx-T-E in 30.4% and 16.2% of the communal and commercial isolates respectively. Phenotype Smx-T-Ap, Smx-T-E and Smx-T-E-Ap were the other dorminating phenotypes within the *E. coli* O157 isolates from communal pigs and occurring in the following proportions 21.7%, 17.4% and 8.7%, respectively. Furthermore, phenotypes S-Smx-T-E-Ne, S-Smx-T-E and K-S-Smx-T-E-Ap-Ne occurred at higher frequencies (10.8%, 8.1% and 5.4% respectively) within the *E. coli* O157 isolated from commercial pigs than in communal pigs. It was also evident that Smx-T-E formed the basis of the other phenotypes for both farms as depicted in Table 4.2.

Figure 4.2 indicates the proportion of *E. coli* O157 isolated from commercial (Mareetsane) and communal (Tlapeng) pigs resistant to the various antibiotics. This

figure also indicates a summary of the number of antibiotics to which isolates were resistant.

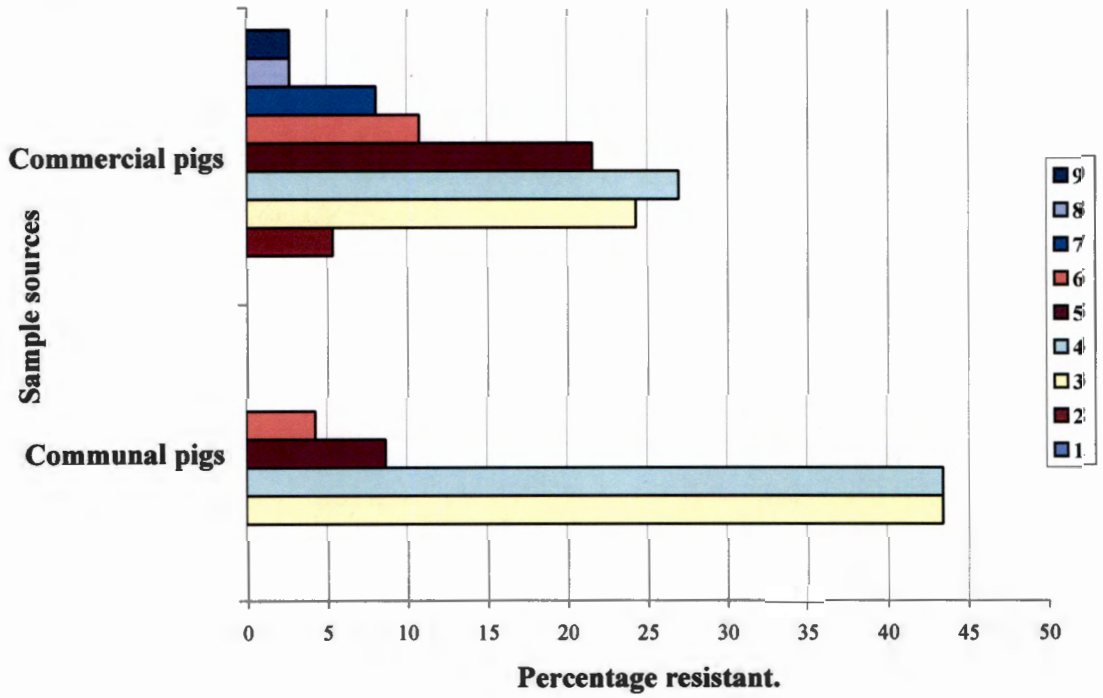


Figure 4.2: The proportion of *E. coli* O157 isolated from pigs resistant to antibiotics for Mareetsane and Tlapeng and number of antibiotics.

As shown in Figure 4.2, multiple antibiotic resistances (MAR) were observed amongs the communal and commercial pig isolates. Comparing the communal and commercial isolates for MAR, 10 (43.5%) of the communal pig isolates were resistant to 3 and 4 of the 9 antibiotics. Similarly, 10 (27%) of the commercial isolates were resistant to 4 of the 9 antibiotics used. However, 9 (24.3%) and 8 (21.6%) of *E. coli* O157 isolated from commercial pigs were resistant to 3 and 5 respectively of the 9 antibiotics. Interestingly, only the commercial pig isolates were resistant to more than 6 of the 9 antibiotics used. In this population 3 (8.1%) were resistant to 7 of the 9 antibiotics of

which 1 (2.7%) and 1 (2.7%) were resistant to 8 and 9 of the 9 antibiotics used respectively.

Upon comparing the percentage antibiotic resistant data positive correlations were observed between the faeces samples from communal and commercial pigs ($r=0.50$, $p<0.05$). Furthermore, a comparison of the mean antibiotic inhibition zone diameter for the antibiotics to which isolates were resistant was made. There was a significant difference for the drug erythromycin between the commercial and communal pig isolates ($\bar{x}$ 10.9 ± 1.31, $\bar{x}$ 11.8 ± 0.93; $p<0.05$).

One hundred and twenty eight *E. coli* isolated from cattle faeces from both commercial and communal farms were analysed for characters of *E. coli* O157 (Sections 3.4 – 3.6). Nineteen *E. coli* O157 isolates were obtained that comprised 5 from Lichtenburg cattle, 4 from Rustenburg cattle and 10 from Mogosane cattle. The *E. coli* O157 isolates were subjected to antibiotic phenotypic characterisation (Section 3.7) by the disc diffusion method (Kirby-Bauer *et al.*, 1966). The percentage resistance for a particular antibiotic was determined by dividing the number of resistant *E. coli* O157 isolates by the number of *E. coli* O157 isolates tested.

4.4 ANTIBIOTIC RESISTANT DATA OF *E. COLI* O157 ISOLATED FROM COMMUNAL (MOGOSANE) AND COMMERCIAL (RUSTENBURG AND LICHENBURG) CATTLE FAECES.

Figure 4.3 indicates the percentage antibiotic resistance exhibited by *E. coli* O157 isolated from commercial cattle from both farms as well as the communal cattle.

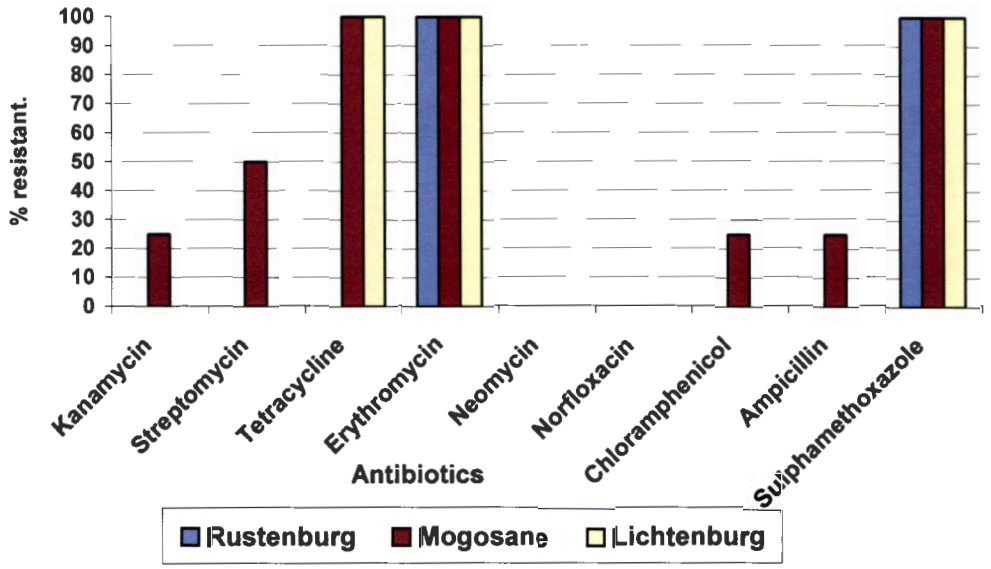


Figure 4.3: Antibiotic resistance pattern of *E. coli* O157 isolated from Lichtenburg and Rustenburg commercial cattle and Mogosane communal cattle.

Figure 4.3 indicates that all the *E. coli* O157 isolates from the commercial and communal farms from Lichtenburg, Rustenburg and Mogosane were resistant to erythromycin and sulphamethoxazole. Furthermore, all the isolates from both the commercial (Lichtenburg) and communal (Mogosane) farms were resistant to tetracycline. A lower percentage (25% to 50%) of *E. coli* O157 isolated from communal cattle from Mogosane were resistant to chloramphenicol, ampicillin kanamycin and streptomycin. A greater proportion of the isolates from the Lichtenburg and Mogosane farms were resistant to the tetracyclines, macrolides, and sulphonamide. These antibiotics have different modes of action that range from binding to ribosomes (tetracyclines and macrolides) and other mechanisms to inhibit of protein synthesis (sulphonamides).

4.5 MAR PHENOTYPES OF *ESCHERICHIA COLI* O157 FROM CATTLE.

The MAR phenotypes for *E. coli* O157 isolated from cattle were determined as previously outlined (Section 3.8) and the resulting antibiotic resistant phenotypes for *E. coli* O157 isolates are depicted in Table 4.3.

Table 4.3: Antibiotic resistant phenotypes for *E. coli* O157 from cattle at commercial (Lichtenburg) and communal (Mogosane) farms.

Commercial cattle (Lichtenburg)			Communal cattle (Mogosane)		
Resistance Phenotype	Number observed	%	Resistance Phenotype	Number observed	%
Smx-T-E	5	100	Smx-T-E-C	1	25.0
			K-S-Smx-T-E	1	25.0
			S-Smx-T-E	1	25.0
			Smx-T-E-Ap	1	25.0

Ap (ampicillin), C (chloramphenicol), T (tetracycline), Nor (norfloxacin), S (streptomycin), E (erythromycin), K (kanamycin), Smx (sulphamethoxazole).

As shown in Table 4.3, the dominating multiple antibiotic resistance (MAR) phenotype for *E. coli* O157 isolated from the commercial farm from Lichtenburg was Smx-T-E in 100% of the isolates. However, the MAR phenotypes Smx-T-E-C, K-S-Smx-T-E, S-Smx-T-E and Smx-T-E-Ap each constituted 25% among the communal isolates. Smx-T-E formed the basis of antibiotic resistant phenotypes for *E. coli* O157 cattle isolates as it appeared in all the other phenotypes with the addition of one or two antibiotics. Antibiotic resistant phenotypes for *E. coli* O157 isolated from commercial cattle in Rustenburg were not included, as resistance was shown to only two antibiotics (Table 4.3).

Despite the fact that isolates were resistant to three or more antibiotics, interestingly, none of them were resistant to more than 6 antibiotics used in the study. All the isolates

from the commercial farms (Lichtenburg and Rustenburg) were resistant to 3 antibiotics. Furthermore, most of the isolates (75% and 25%) from the communal farm at Mogosane were resistant to 7 antibiotics (Appendix E; Table 2E). The results above indicate that multiple antibiotic resistance (MAR) is common among *E. coli* O157 isolates from the various cattle sampling stations.

A comparison of the percentage antibiotic resistant data for all *E. coli* O157 isolated from cattle at the various stations (Appendix D; Table 1D) revealed strong positive correlations, between the faeces of cattle from commercial (Lichtenburg) and communal (Mogosane) farms ($r=0.94$, $p<0.05$) and the communal (Mogosane) and commercial (Rustenburg) farms ($r=0.71$, $p<0.05$), respectively.

The following section gives an overview of the proportion of *E. coli* O157 isolated from cattle resistant to antibiotics for Lichtenburg (commercial), Rustenburg (commercial) and Mogosane (communal) and the number of antibiotics. As indicated in (Appendix E; Table 2E), all the *E. coli* O157 isolated from cattle from the commercial (Lichtenburg) farm were resistant to 3 of the 9 antibiotics. However, a large proportion (66.7%) of *E. coli* O157 isolated from cattle at the communal (Mogosane) were resistant to 4 of the 9 antibiotics and a smaller proportion (33.3%) were resistant to 5 of the 9 tested antibiotics, respectively. Moreover, none of the *E. coli* O157 isolates from both farms (Lichtenburg and Mogosane) were resistant to more than 6 of the 9 antibiotics. Furthemore, MAR was common among the *E. coli* O157 isolates except for the isolates from cattle from the commercial (Rustenburg) farm that were resistant to less than 3 of the 9 antibiotics.

4.6 ANTIBIOTIC RESISTANT DATA OF *E. COLI* O157 ISOLATED FROM HUMAN STOOL SAMPLES AND *E. COLI* CONTROL STRAINS.

Fourty *E. coli* O157 (Table 4.1) isolated from human stool samples obtained from patients at the Mafikeng Provincial hospital with cases of diarrhoea were analysed for characters of *E. coli* O157. Three isolates were positively identified as *E. coli* O157 and were later subjected to antibiotic phenotypic characterisation using the antibiotics (Section 3.6). The *E. coli* O157 control strains (ATCC 43889 and ATCC 43888) and *E. coli* O145 were also phenotypically characterised using the same antibiotics. These strains were used as controls in the molecular characterisations by PCR of *E. coli* O157 isolated from the different species.

Table 4.4: Illustrating the antibiotic resistant data obtained for *E. coli* O157 isolated from human stool samples and *E. coli* control strains. The number and percentages of isolates that were resistant to each of the antibiotic tested are given. Percentages were obtained from a fraction of the number of isolates resistant to particular antibiotics and total number of isolates from the specie and / or sample source.

		K	S	T	E	Ne	Nor	C	Ap	Smx
Human	No. Resistant	0	0	3	3	0	0	1	3	3
	% resistant	0	0	100	100	0	0	33.3	100	100
Control Strains	No. Resistant	0	0	1	3	0	0	0	0	2
	% resistant	0	0	33.3	100	0	0	0	0	66.7

Ap (ampicillin), C (chloramphenicol), T (tetracycline), Nor (norfloxacin), S (streptomycin), E (erythromycin), Ne (neomycin), K (kanamycin), Smx (sulphamethoxazole

As indicated in Table 4.4 all three of the *E. coli* O157 isolated from human stool samples were resistant to erythromycin, tetracycline, ampicillin and sulphamethoxazole. Similarly, all the three *E. coli* control strains were resistant to erythromycin. Two of the three control strains were resistant to sulphamethoxazole and

only one was resistant to tetracycline. Neither the control strains nor the *E. coli* O157 isolated from humans were resistant to the quinolone (norfloxacin), kanamycin or streptomycin.

4.7 MAR PHENOTYPES OF *ESCHERICHIA COLI* O157 FROM HUMANS AND *E. COLI* CONTROL STRAINS.

The MAR phenotypes for *E. coli* O157 isolated from humans were determined as previously outlined (Section 3.8) and antibiotic resistant phenotypes for the *E. coli* O157 isolates were as shown in Table 4.5.

Table 4.5: Multiple antibiotic resistant (MAR) phenotypes of *E. coli* O157 isolated from human stool samples of patients who visited the Mafikeng Provincial Hospital.

	Human isolates	
Resistant Phenotype	Number observed	%
Smx-T-E-C-Ap	1	33.3
Smx-T-E-Ap	2	66.7

Ap (ampicillin), C (chloramphenicol), T (tetracycline), E (erythromycin), Smx (sulphamethoxazole).

The antibiotic resistant phenotypes obtained for *E. coli* O157 isolated from human stool samples were Smx-T-E-Ap in two of three and Smx-T-E-Ap in one of three. It is evident that Smx-T-E-Ap formed the basis of the resistant phenotypes. MAR phenotypes for control strains were not included.

In conclusion, when the percentage antibiotic resistant data for *E. coli* O157 isolated from the different species and sources (Appendix E; Table 1E) were compared, strong positive correlations were obtained between the faeces of pigs and stool samples of humans ($r=0.73$, $p<0.05$), pig and cattle faeces respectively ($r=0.87$, $p<0.05$) and cattle faeces and human stool samples ($r=0.76$, $p<0.05$).

4.8 MULTIPLE ANTIBIOTIC RESISTANCE INDEX OF *E. COLI* O157 ISOLATES

A total of 76 *E.coli* O157 isolated from faecal samples of pigs, cattle and humans were subjected to the MAR index test as outlined in Materials and Methods (Section 3.8). The aim was to determine the exposure of the *E. coli* O157 isolated from the different species and/or sampling stations to the antibiotics tested in the present study using the *E. coli* control strains as reference. The multiple antibiotic resistant (MAR) indices were determined for the MAR resistant *E. coli* O157 isolates obtained; and of the *E. coli* O157 isolates examined and *E. coli* control strains, twenty-seven distinctive antibiotic resistant patterns were observed. The average MAR indices for human, cattle and pig isolates were 0.4814, 0.3419 and 0.4722, respectively.

Among the cattle isolates, the group MAR index was highest for the communal population (0.4722) while the values for *E. coli* O157 from cattle from the commercial farms in Lichtenburg and Rustenburg were 0.33 and 0.22, respectively. *E. coli* O157 isolates from pigs revealed MAR index results that were 0.508 and 0.415 for the commercial and communal farms, respectively and 0.1851 for the *E. coli* control strains.

The multiple antibiotic resistant (MAR) indices reveal the spread of bacteria resistance in a given population (Krumpermann, 1983). Interestingly, some of the *E. coli* O157 isolates from cattle exhibited lower MAR indices than from pigs and humans, (Appendix D; Table 1D). These results are consistent with other reports from previous studies (Guan *et al.,* 2002) except for the observation that the MAR index result for humans was higher than that of pigs. The number of *E. coli* O157 with identical isolate

MAR indices and resistance profiles from the various sample areas / species are shown in (Appendix D; Table 1D). The high MAR indices obtained for *E. coli* O157 isolated from human stool samples clearly illustrate the transmission of these bacteria from animals to humans.

4.9 CLUSTER ANALYSIS OF *E. COLI* O157 FOR MULTIPLE ANTIBIOTIC RESISTANCE (MAR) RELATIONSHIP ON A DENDOGRAM

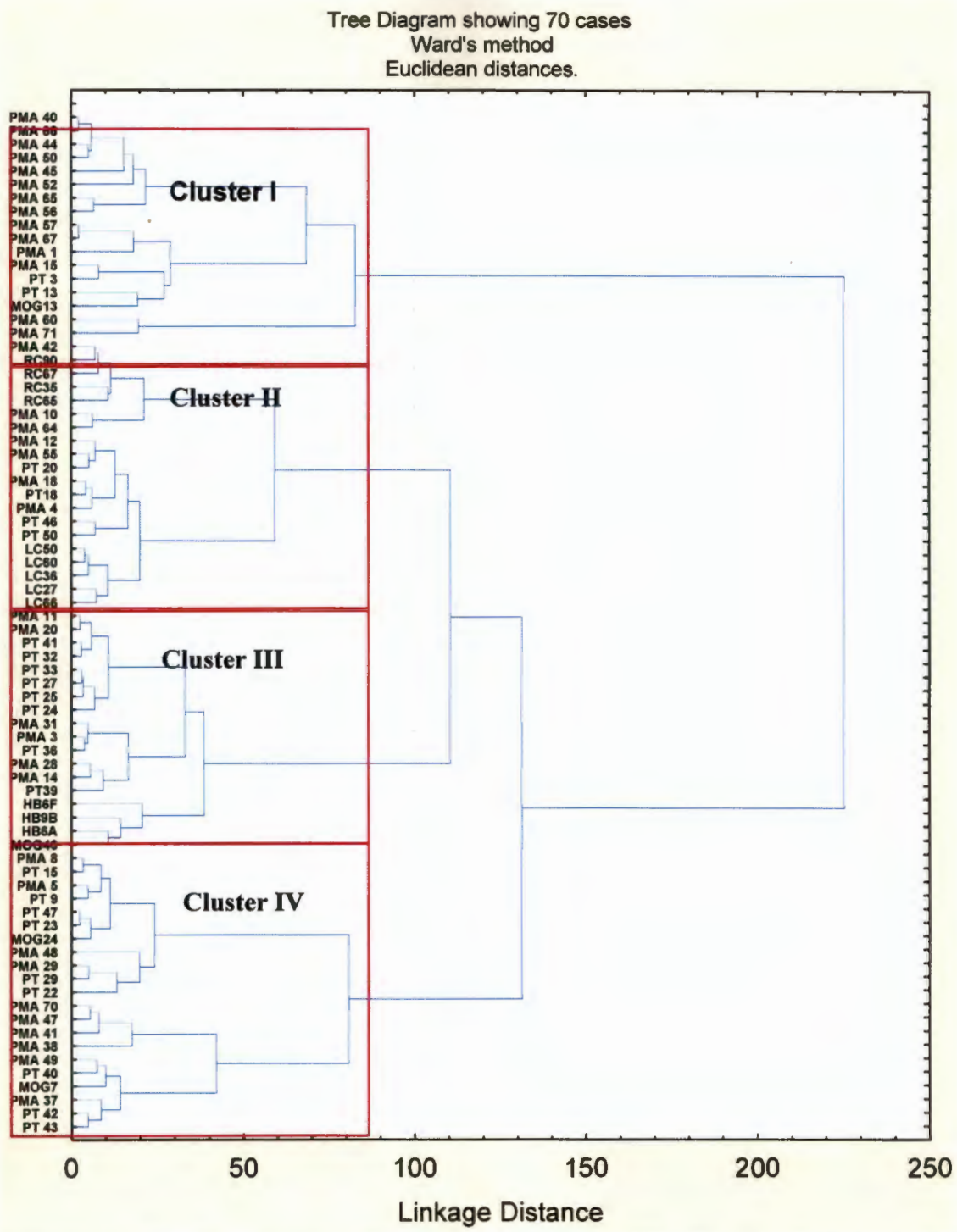
A total of 76 *E. coli* O157 isolated from pigs, cattle and humans were subjected to antibiotic sensitivity testing as outlined in Materials and Methods (Section 3.7) and their MAR patterns were determined as previously described (Rota *et al.,* 1996). These isolates were subjected to phenotypic characterisation by clustering analysis as outlined in Materials and Methods (Section 3.11.5). A dendogram was generated using the antibiotic inhibition zone diameter data (Appendix C) obtained for all *E. coli* O157 isolates. The analysis obtained was used as a tool in determining the commonness and resolve differences between the MAR phenotypes of *E. coli* O157 isolates. There exists evidence of efficient exchange of genetic material between *E. coli* species and other species, even pathogens (Levy, 1997). The level of antibiotic resistance of *E. coli* also serves as an indicator of antibiotic resistance in the intestinal community (Levy *et al.,* 1988; Levin *et al.*, 1997).

Figure 4.4 indicates the clustering patterns obtained for the *E. coli* O157 isolated from the various species. The clusters were further analysed for patterns of associations of isolates from the different species (Table 4.6). Samples were divided based on the specie type and the sampling station; hence pigs communal and commercial; cattle communal and commercial, and humans.

Table 4.6 shows the numbers and/ or percentages of *E. coli* O157 isolated from the different species within the different clusters.

Table 4.6: The percentage representation of *E. coli* O157 isolated from different species within the various clusters.

Specie	Sample type / Site name	Cluster I N=18	Cluster II N=19	Cluster III N=18	Cluster IV N=21
Pigs	Communal (Tlapeng)	2 (11.1%)	4 (21.1%)	8 (44.4%)	9 (42.9%)
	Commercial (Mareetsane)	15 (83.3%)	6 (31.6%)	6 (33.3%)	10 (47.6%)
Cattle	Communal (Mogosane)	1 (5.6%)	0 (0%)	1 (5.6%)	2 (9.5%)
	Commercial (Lichtenburg)	0 (0%)	5 (25.0%)	0 (0%)	0 (0%)
	Commercial (Rustenburg)	0 (0%)	4 (20%)	0 (0%)	0 (0%)
Human	Mafikeng Provincial Hospital	0 (0%)	0 (0%)	3 (16.7%)	0 (0%)



Designation: PT (Pig Tlapeng), PMA (Pig Mareetsane), HB (Human –Bophelong), RC (CattleRustenburg), LC (Cattle Lichtenburg) and MOG (Cattle Mogosane).

Figure 4.4: Dendogram showing the relationship between *E. coli* O157 isolated from faeces samples of pigs and cattle and human stool samples based on inhibition zone diameter (IZD) data. Bacterial designation prefixes are based on host and sample station origin. The tree was constructed using Ward's method and Euclidean distances in Statistica, version 7.

The dendogram in Figure 4.4 and Table 4.6 indicate four major clusters (I – IV). Of the *E. coli* O157 isolated from pigs, 19 (90.5%) were present in the largest cluster (cluster IV) along with 2 (9.5%) of the *E. coli* O157 isolated from communal cattle at Mogosane. The second largest cluster (cluster II) contained 10 (52.7%) of the *E. coli* O157 isolated from pigs and favoured the *E. coli* O157 isolates from faeces of pigs and cattle on all commercial farms. However, the isolates from cattle at both commercial farms were seen to cluster at both ends indicating the similarity of their inhibition zone diameter data. Cluster III was identified as a mixed cluster containing *E. coli* isolated from all three species. *E. coli* O157 isolated from human stool samples were all present in this cluster including a large proportion 14 (77.8%) isolates from pig faeces. The smaller clusters (clusters I and II) were represented by mostly *E. coli* O157 isolated from pigs at 94.4% and 77.7% respectively.

E. coli O157 isolated from faeces of pigs at Tlapeng and Mareetsane and cattle at Mogosane clustered together (Cluster III). These villages are in close proximity to the Mafikeng Provincial hospital that serves the area and could possibly indicate that the individuals sampled might have come from these villages. However, information was not available on the residence of the individuals from which the samples were obtained.

The results obtained in the present study indicated that antibiotic resistance of *E. coli* O157 isolates could possibly be associated with the diet of their hosts. Rainfall runoff may have contributed to the distribution of antibiotic resistant bacteria or genes within populations. Antibiotics are more widely used on cattle farms than on pig farms; thus the higher prevalence of antibiotic resistance in pigs rather than cattle was of concern.

However, pigs, especially in the communal setting, feed on human excreta while humans feed on both animals that may explain the distribution of antibiotic resistance patterns observed.

The results in Figure 4.4 indicate that cluster analysis based on various multiple antibiotic resistance profiles identified at the different species and/or sampling points might be suitable for predicting the source of antibiotic resistance contamination and its transfer from one specie to another. Similar results were obtained when the multiple antibiotic resistance of *E. coli* isolates from three sources (human, livestock and wildlife) were compared (Guan *et al.,* 2002).

4.11 MOLECULAR CHARACTERISATION OF *E. COLI* O157

4.11.1 Bacterial Strains

Thirty *E. coli* O157 isolates (20 from pigs, 7 from cattle and 3 from human stool samples) were selected and characterised by PCR analysis for the presence of shiga toxin virulence and tetracycline resistant genes. *E. coli* control strains used are as indicated in Section 3.2.2.

Genomic DNA extraction was performed using the hot CTAB-PVP protocol as outlined in Materials and Methods, (Section 3.11.1). The quality of the DNA was determined by electrophoresis (Section 3.11.2). Figure 4.5 indicates an image of an agarose (1% w/v) gel depicting genomic DNA extracted from the *E. coli* O157 isolates and *E. coli* control strains. The DNA extracted was of good quality, with little RNA and no fragmentation.

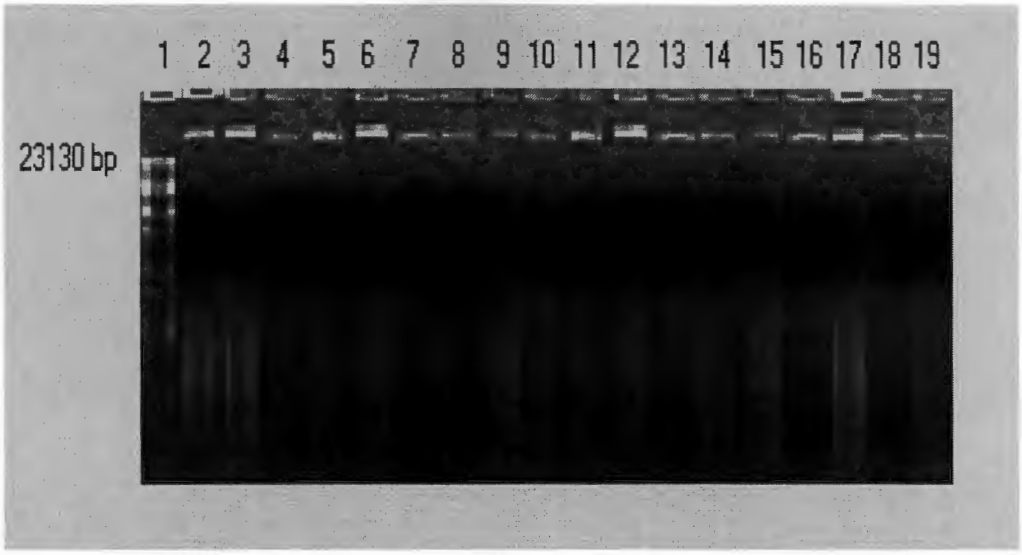


Figure 4.5: Image of an agarose (1% w/v) gel depicting genomic DNA extracted from the *E. coli* O157 isolates and *E. coli* control strains. Lane 1 (Lambda DNA-Hind III digest); Lanes 2-9 (DNA extracted from *E. coli* O157 isolated from pigs –Tlapeng and Mareetsane respectively); Lanes 10-13 (DNA extracted from *E. coli* O157 isolated from cattle –Rustenburg, Lichtenburg and Mogosane respectively); Lanes 14-16 (DNA extracted from *E. coli* isolated from human stool samples – Mafikeng Provincial Hospital); Lanes 17-19 (DNA extracted from *E. coli* O157:H7 (ATCC 43889), *E. coli* O157:H7 (ATCC 43888) and *E. coli* O145 respectively.

Figure 4.6 shows an image of an agarose (1% w/v) gel indicating 16S rRNA gene fragments generated by PCR using genomic DNA extracted from *E. coli* O157 isolated from the various species and *E. coli* control strains.

PCR analysis of the 16S rRNA gene fragment was performed as outlined (Section 3.11.3) using the primer set as indicated (Table 3.3). Gel electrophoresis of PCR products revealed the desired 550 bp fragment.

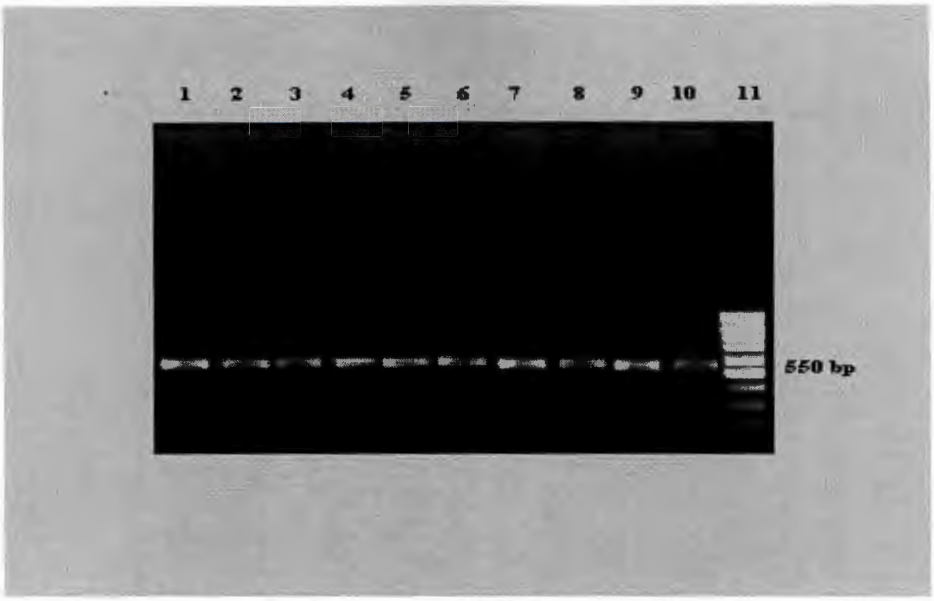


Figure 4.6: Image of an agarose (1% w/v) gel indicating 16S rRNA gene fragments generated by PCR analysis using genomic DNA extracted from *E. coli* O157 isolated from the various species and *E. coli* control strains. Lanes 1-3 (16S rRNA gene fragment from DNA extracted from *E. coli* O157 isolated from cattle –Rustenburg, Lichtenburg and Mogosane respectively); Lanes 4-5 (16S rRNA gene from DNA extracted from *E. coli* O157 isolated from pigs – Mareetsane); Lanes 6-7 (16S rRNA gene from DNA extracted from *E. coli* O157 isolated from pigs – Tlapeng); Lane 8 (16S rRNA gene fragment from DNA extracted from *E. coli* isolated from human stool samples – Mafikeng Provincial Hospital); Lanes 9-10 (16S rRNA gene fragment from DNA extracted from *E. coli* O157:H7 (ATCC 43889) and *E. coli* O157:H7 (ATCC 43888) control strains respectively. Lane 11 (100 bp –DNA Ladder).

The identity of the isolates was confirmed by sequencing of the 16S rRNA gene fragments (Section 3.11.4). In Appendix F, the 16S rRNA gene fragment sequence of one of the randomly selected representatives of the *E. coli* O157 isolated from the North-West Privince is presented. This sequence is similar (98% identy and 2 gaps) to *E. coli* O157:H7 deposited in GenBank (AY513502).

Specific PCR analysis for the detection of STEC virulence genes (*eae* and *hlyA*) were performed using specific primer sets for the targeted genes and conditions as indicated

in Materials and Methods, (Section 3.11.3; Table 3.3). PCR products were detected by electrophoresis (Section 3.11.2).

Figure 4.7 indicates an image of an agarose (1% w/v) gel depicting the PCR fragments generated with the *hlyA* specific primers. The amplicons were of the expected size (534 bp).

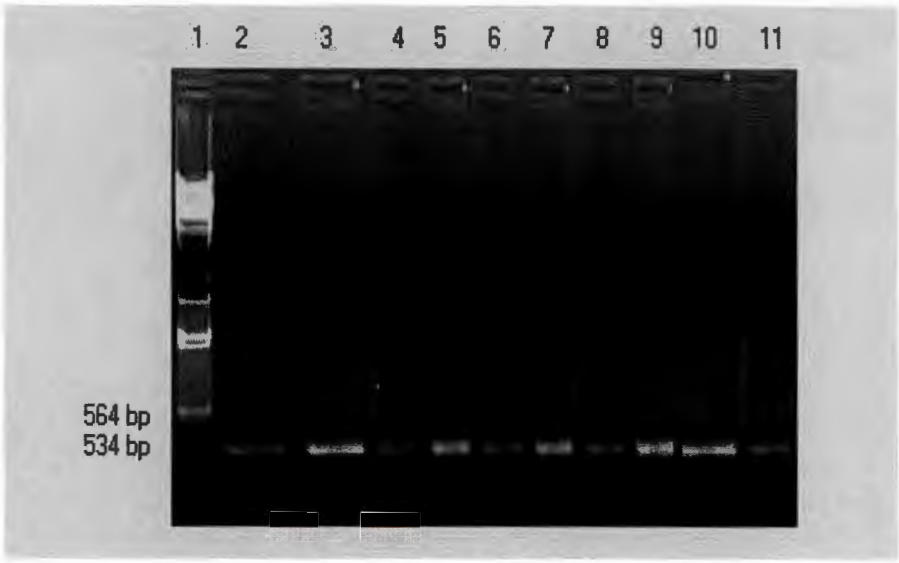


Figure 4.7: Image of an agarose (1% w/v) gel depicting the PCR fragments generated with the *hlyA* specific primers. Lane 1 (Lambda DNA-Hind III digest). Lanes 2-3 (*hlyA* gene from DNA extracted from *E. coli* O157 isolated from pigs –Tlapeng and Mareetsane respectively); Lanes 4-7 (*hlyA* gene fragment from DNA extracted from *E. coli* O157 isolated from cattle –Rustenburg, Lichtenburg and Mogosane respectively); Lane 8-9 (*hlyA* gene fragment from DNA extracted from *E. coli* isolated from human stool samples – Mafikeng Provincial Hospital); Lanes 10-11 (*hlyA* gene fragment from DNA extracted from *E. coli* O157:H7 (ATCC 43889) and *E. coli* O145 control strains respectively.

Figure 4.8 indicates an image of an agarose (1% w/v) gel depicting the PCR fragments generated with the *eae* specific primers. The amplicons were of the expected size (384 bp).

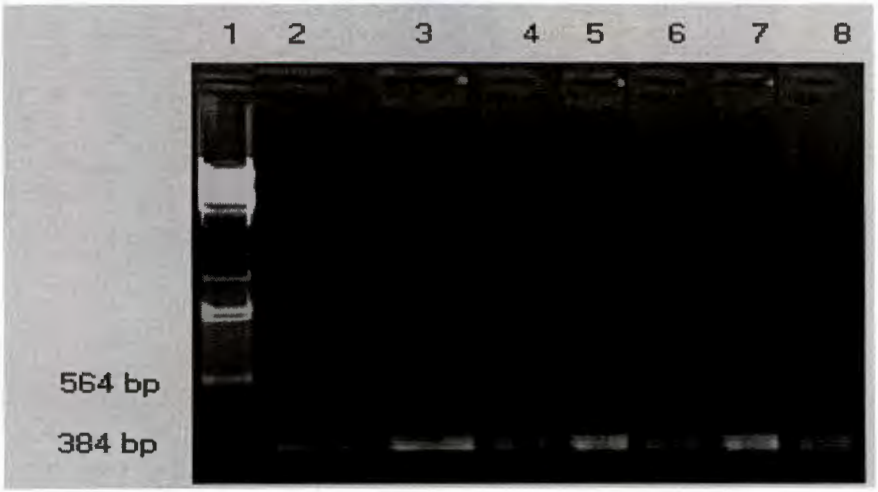


Figure 4.8: Image of an agarose (1% w/v) gel depicting the PCR fragments generated with the *eae* specific primers. Lane 1 (Lambda DNA-Hind III digest). Lanes 2-3 (*eae* gene from DNA extracted from *E. coli* O157:H7 (ATCC 43889) and *E. coli* O145 control strains respectively); Lanes 4-5 (*eae* gene fragment from DNA extracted from *E. coli* O157 isolated from cattle- Mogosane and human stool samples respectively) and Lane 6-8 (*eae* gene fragment from DNA extracted from *E. coli* O157 isolated from faeces samples of pigs –Tlapeng and Mareetsane respectively).

Table 4.7 depicts a summary of the *eae* and *hlyA* gene distribution among *E. coli* O157 isolated from selected pig and cattle populations as well as humans from the North-West Province. The results for the control strains are also indicated.

Table 4.7: Prevalence of the STEC virulence genes within the different species.

Virulence gene(s)	*E. coli* O157 (ATCC) 43889	*E. coli* O157 (ATCC) 43888	*E. coli* O145	No. of strains with indicated virulence gene(s).			
				Humans	Cattle	Pigs	Total (%)
stx$_1$	-	-	-	0	0	0	0
stx$_2$	-	-	-	0	0	0	0
eae	+	-	+	1	1	5	7 (23.7%)
hlyA	+	-	+	2	5	11	18 (60.0%)
eae and *hlyA*	+	-	+	0	1	4	5 (16.7%)

As indicated in Table 4.7, a large proportion (60%) of the *E. coli* O157 isolates possessed the enterohaemolysin A (*hlyA*) gene while only a small proportion (23.7%) possessed the *eae* gene. Furthermore, only 5 (16.7%) of the isolates possessed both genes. There was a higher prevalence of the *hlyA* gene in *E. coli* isolated from pigs than in cattle and than in humans. Moreover, none of the *E. coli* O157 isolated from human stool samples possessed both genes.

Specific PCR analysis was performed to detect the presence of tetracycline resistant genes using the protocols as outlined (Section 3.11.3). Figure 4.9 depicts an image of an agarose (1% w/v) gel depicting *tetB* fragments (500 bp) amplified from *E. coli* O157 isolated from pigs from Mareetsane.

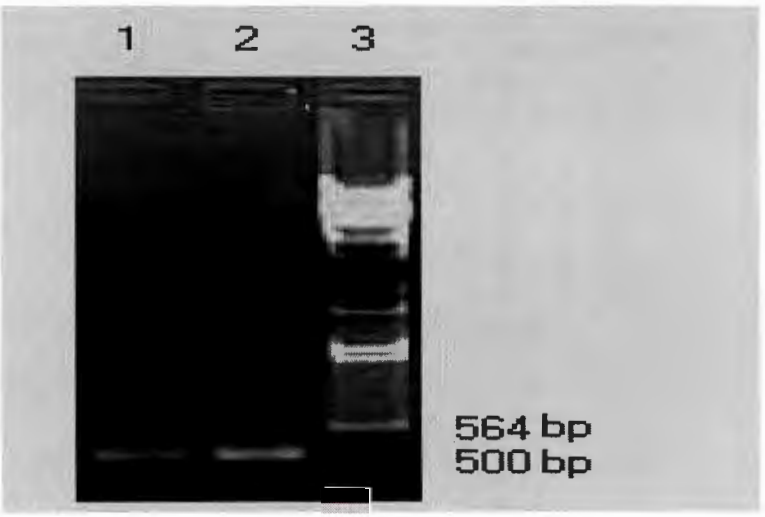


Figure 4.9: Image of agarose gel showing an amplified *tetB* gene fragment. Lane 1 and 2 (*tetB* gene fragment from *E. coli* O157 isolated from pigs at Mareetsane). Lane 3, molecular weight marker (Lambda DNA- Hind III digest).

The present study demonstrated that an *E. coli* O157 strain isolated from pig faeces possessed the *tetB* gene fragment that was responsible for resistance to tetracycline. Although the *tetB* gene fragment was detected in a small proportion (3.3%) of the

isolates, none of the other three tetracycline resistant genes were detected. Studies carried out to determine the cause of resistance to tetracycline have identified various tetracycline genes but in different proportions.

The results presented herein indicated that *E. coli* were successfully isolated and *E. coli* O157 identified amongst them. The identities of the isolates were later confirmed by analysis of the 16S rRNA gene fragments. The *E. coli* O157 isolates were characterised by the antibiotic susceptibility test and a large number of the isolates were resistant to the antibiotics tested. Clustering analysis of antibiotic inhibition zone diameter data revealed similarities among the isolates from the different species. The isolates were further characterised for the presence of STEC virulent genes. *E. coli* O157 isolates from the different species harboured the targeted genes and amplicons were of expected sizes.

CHAPTER 5
DISCUSSION

5.1 GENERAL DISCUSSION

The primary goal of this research was to isolate and determine the prevalence of shiga-like toxin (verotoxin) producing *Escherichia coli* (STEC; *E. coli* O157) in pigs, cattle and humans. This study was intended to isolate these bacteria from animals and humans and suggests some routes of transmission. Information obtained from the present study could help in managing outbreaks and most-specifically create awareness about the prevalence of this pathogen within the area of this study.

A motivation for this study was that domestic ruminants, mainly cattle, sheep and goats, have been implicated as the principal reservoir of *E. coli* O157 (Blanco *et al.*, 2001; 2003). *E. coli* O157 was successfully isolated from pigs, cattle and humans and the prevalence of this pathogen in these species was determined. The results indicated that the prevalence of *E. coli* O157 was higher among *E. coli* isolated from the faeces of pigs (44.2% to 50%) than those from cattle faeces (5.4% to 20.0%) as well as those from human stool samples (7.5%).

This strain of STEC represents a significant cause of sporadic cases of human diarrhoea infections. *E coli* O157 is a faeces–transmitted microorganism and poor hygiene, contamination of water bodies with human stool and animal faeces have been identified as routes that could lead to human infections (Petridis *et al.*, 2005). Residents of rural communities have greater exposure to *E coli* O157 due to contact with animal manure. In such communities there are sometimes poor hygienic practices during slaughtering rituals that may result in the meat coming into contact with faeces (Griffin, 1995). Elder

et al., (2000) showed that the prevalence of *E. coli* O157 on bovine carcass surfaces is related to its prevalence in the faeces of live cattle before slaughter. Consumers of these meat products are thus exposed to the risks of acquiring these bacteria (Petridis *et al.*, 2005). Ground beef, contaminated during the slaughtering process, may also be the route of transmission of *E. coli* O157 to humans as reported by Paton and Paton (1998).

The incidence of human disease associated with *E. coli* O157 is very high during summer. This could be attributed to an increase in faecal shedding during this period (Griffin 1995; Van Donkersgoed *et al.*, 1999). In the Southern hemisphere, the Christmas period falls within the summer months of the year. This is a time during which large numbers of cattle are slaughtered in order to meet the demands of the market. The large volumes of carcasses could lead to a decrease in hygienic practices in the abattoirs, hence facilitating this route of transmission of *E. coli* O157 to humans. This is more common within the rural communities where the animals are, at times, slaughtered within the living areas of the community, increasing the risk of contaminating the meat with faecal matter. Relatively high ambient temperatures (15°C at night and 30°C during the day) could also increase the risk of *E. coli* O157 infections.

Studies have shown that a reduction of both the magnitude of faecal excretion and the faecal prevalence of *E. coli* O157 in cattle prior to slaughter could significantly decrease the incidence of human disease associated with this organism (Pierard *et al.*, 1997a; Marks *et al.*, 1998; Jordan *et al.*, 1999; LeJeune and Kauffman, 2005; LeJeune *et al.*, 2006). Unfortunately, there are no methods currently available to reduce the

faecal prevalence of *E. coli* O157 in cattle. However, an understanding of the faecal shedding of *E. coli* O157 by cattle, its epidemiology and ecology and especially the source of exposure of cattle, may provide useful information that could be used in the design of slaughter control measures for *E. coli* O157 (LeJeune *et al.*, 2004).

The results obtained on the prevalence of *E. coli* O157 in cattle (5.4% to 20.0%) coincide with published data (Wells *et al.*, 1991; Chapman *et al.*, 1997; Van Donkersgoed *et al*, 1999; Müller *et al.*, 2002). In those studies the prevalence of *E. coli* O157 in cattle were found to be 7.5 % (Van Donkersgoed *et al*, 1999), 8% (Wells *et al.*, 1991) 15.7% (Chapman *et al.*, 1997) and 16.67% in South Africa (Müller *et al.*, 2002). However, the prevalence rates of *E. coli* O157 may be variable within herds (Butain, 1996). Studies have found that *E. coli* O157 faecal shedding rates in cattle vary from 0% to as high as 63.5% on some farms (Asis *et al.*, 2002). No factors have been identified, other than seasonality that consistently affects the *E. coli* O157 shedding rates of cattle (Van Donkersgoed *et al.*, 1999; Elder *et al.*, 2000). Unfortunately, in the study presented here collections were not conducted seasonally. The effects of seasonality on the prevalence of *E. coli* O157 isolated is thus still unknown. Previous studies on the bovine hide and beef carcasses have shown that *E. coli* O157 strains can be transferred to the carcass pre-evisceration, during hide removal operations (Elder *et al.*, 2000; McEvoy *et al.*, 2003). Once on the carcass surface, the pathogen can be spread by the handling and trimming operations to the beef trimmings (Gill and McGinnis, 2000). The results obtained revealed a potentially high level of exposure of animals to *E. coli* O157. These pathogens could be transmitted to humans if proper slaughtering practices are not properly implemented.

E. coli O157 does not appear to cause clinical diseases in pigs (Cornick *et al.*, 2000b). Therefore, its presence in a pig herd is of little economic significance to the farmer. Despite the aforementioned fact, pigs infected with *E. coli* O157 do present a risk to human health. The results obtained indicated that the prevalence of *E. coli* O157 among the *E. coli* isolated from pigs was generally higher than that of cattle and humans.

Several reports on the prevalence of *E. coli* O157 in pigs have been recorded and are discussed below. *E. coli* O157 have rarely been isolated from pigs in the United Kingdom, Europe or North America. When it was detected, isolation was from slaughter houses. A very low prevalence rate (0.7%) had been reported in a study in the Netherlands (Heuvelink *et al.*, 1999). Similar reports were obtained in studies carried out in Canada (Gill and Jones, 1998) in which 5% were detected. In Norway, the prevalence was 0.1% in slaughtered pigs (Johnsen *et al.*, 2001). The results of these studies suggested that pigs were not usually a source of *E. coli* O157 in these areas (Frydendahl, 1999). Such low prevalence of (1.4%) *E. coli* O157 was also observed in pigs from three different farms in Japan (Nakazawa *et al.*, 1999). This was similar to the prevalence rate of *E. coli* O157 in cattle in Japan. In another study carried out in Japan, *E. coli* O157 was isolated from faecal samples from pigs and human stool samples of patients suffering from HUS, respectively (Rios *et al.*, 1999). Furthermore, the two sets of *E. coli* O157 isolates were characterised genotypically. Results showed that there was a clonal relationship between the isolates from humans and pigs. This suggested that pigs in Japan were an important reservoir for human STEC O157 infections.

The prevalence of this pathogen in faecal samples obtained from pigs had been evaluated in a study conducted in South Africa. Results obtained revealed a prevalence rate of 14.28% (Müller *et al.*, 2002). The prevalence of *E. coli* O157 in pigs in Chile was estimated at 16% (Borie *et al.*, 1997). In the study of pig population in the North-West Province, very high prevalence rates of *E. coli* O157 were detected in both communal and commercial pigs (44.2% and 50.0%, respectively). Prevalence rates were much higher than those previously reported (0.1% to 14.28%) (Borie *et al.*, 1997; Heuvelink *et al.*, 1999; Nakazawa *et al.*, 1999; Johnsen *et al.,* 2001; Müller *et al.*, 2002; Botteldoorn *et al.*, 2003). Furthermore, the present study was not designed to directly investigate the role pigs play in the transmission of *E. coli* O157 to humans. However, with the ever-increasing market demand for pork, pigs might be an important reservoir for human STEC disease in South Africa.

The prevalence of *E. coli* O157 in humans is usually linked to the level of exposure to contaminated animal products (Riley *et al.,* 1983). Prevalence rates of *E. coli* O157 in humans were in some cases as high as 30.2% (Asis *et al.*, 2002). The determination of prevalence rates in humans is also hampered by the fact that most infected persons rarely report their cases to hospitals (Müller *et al.,* 2002).

The prevalence data in animals and humans are based on isolation of *E. coli* O157 from faeces and stool, respectively (Tutenel *et al.,* 2003). The present study reveals a higher prevalence of *E. coli* O157 in animals than in humans. This could demonstrate the distribution of the pathogen and this strongly supports the route of transmission to humans. However, there is an urgent need for a more closer collaboration between the

medical and veterinary professions in order to provide a better understanding of the prevalence of *E. coli* O157 in cattle and the routes of transmission to humans (Sharp *et al.,* 1994).

Antibiotic resistance in bacterial isolates is a worldwide phenomenom and is becoming a problem especially in developing countries. Apart of being used in human and veterinary medicine, antimicrobials are routinely used for disease prevention and growth promotion in animal production (Schroeder *et al.*, 2002). This practise leads to the inevitable selection of antimicrobial resistance among commensal bacteria in the intestinal tracts of food animals (Witte, 1998). This could pose a public health threat. It also explains the need to determine the level of antibiotic resistance among bacterial isolates. This data could be useful to develop awareness campaigns and eventually help with controlling the development of bacterial antibiotic resistance within populations (O'Brien, 1997).

An objective of this study was to determine to what extent *E. coli* O157 isolated from cattle and pigs from selected populations in the North-West Province were resistant to antibiotics. A motivation for this was the fact that there has been increasing concern of the possible development of resistance to antimicrobial agents in *E. coli* O157 (Meng *et al.,* 1998; Galland *et al.,* 2001; Zhao *et al.,* 2001; Willis, 2000).

The intestinal flora of both humans and particularly animals may serve as a reservoir for resistant genes (Levy *et al.,* 1988; Levin *et al.*, 1997). Studies indicated that resistant genes are located either on the chromosome or on mobile genetic elements of

extraneous origins such as plasmids and/or transposons and integrons of microorganisms (Normark and Normark, 2002; Rice, 2002; Ferber, 2003). In the presence of antibiotics, such strains may have a selective advantage and therefore be more likely to cause an outbreak.

The present study reports on antibiotic resistance profiles of shiga toxin-producing, *E. coli* O157 strains isolated from human stool samples and faeces samples of pigs and cattle. Multiple antibiotic resistance (MAR), defined as resistance to three or more different classes of antibiotics, was observed in 71 (93.4%) of the 76 *E. coli* O157 isolated in the study. All 76 *E. coli* O157 isolates tested were resistant to at least one of the nine antimicrobials used in the study. Streptomycin, sulphamethoxazole, erythromycin and tetracycline were the four antimicrobial agents to which a large number of the *E. coli* O157 isolates were resistant. Furthermore, isolates that were resistant to tetracycline and sulphamethoxazole was more frequently identified. The results indicated that 36 (97.3%) and 23 (100%) of the resistant *E. coli* O157 from commercial and communal pigs, 5 (100%) and 4 (100%) from commercial and communal cattle from Lichtenburg and Mogosane and 3 (100%) human isolates were resistant to three or more antibiotics. In a study carried out in Nigeria that involved *E. coli* isolated from stool samples from students a similar observation was obtained in which more than half of the isolates were also multidrug resistant (Okeke *et al.*, 2000). This multi-drug resistance in developing countries such as South Africa and Nigeria is cause for concern. An outbreak of *E. coli* O157 related diarrhea could have catastrophic effects on the communities.

The results from this study also show that between 70% and 100% of *E. coli* O157 isolated from commercial and communal pig and cattle farms as well as human subjects were resistant to tetracycline. This drug is commonly used in veterinary and human health to treat a variety of infections. The drug is also relatively easily obtainable by commercial and subsistence farmers. The large number of isolates that were resistant to this drug thus did not come as a surprise. Similar observations had been reported from studies in developing and developed countries in which all the isolates from human stool samples were resistance to tetracycline (Okeke *et al.*, 2000; Wilkerson *et al.*, 2004). In another study involving *E. coli* isolated from faeces samples of pig faeces all the isolates were resistant to this antibiotic (Bischoff *et al.*, 2002). In the above-mentioned studies the actual causes of resistance were not determined but were assumed to have resulted through the misuse this antibiotic in the treatment of enteric infections in humans and animals. This practise results to the provision of selective pressure that is necessary for the expression of resistance.

Several processes within the bacterial cell may be responsible for resistance to tetracycline. These include, reduced uptake through the OmpC and OmpF porin channels (Chopra *et al.* 1992); active efflux of the drug (Franklin and Snow, 1998); ribosomal protection (Burdett, 1986) and enzymatic inactivation of the drug (Speer *et al.*, 1991). Resistance could also have been due to mutation in the 16SrRNA (Moine and Dahlberg, 1994). Although ribosomal protection seems to be the most common, all these mechanisms result from the acquisition of one or several tetracycline resistant genes (Schnappinger and Hillen, 1996). The frequency and distribution of tetracycline

resistance genes among *E. coli* populations within different species presents a picture of selection pressures in the various host animals (Bryan *et al.*, 2004).

Sulphamethoxazole and erythromycin were the other most common resistance phenotype observed. A high proportion of the *E. coli* O157 isolates were resistant to sulphamethoxazole and erythromycin. In a study involving *E. coli* O157 isolated from humans, cattle and beef a large proportion (93%) were resistant to sulphamethozaxole (Meng *et al.*, 1998) while in another study, 48% of *E. coli* O157 isolated from pigs were resistant to this drug (Sáenz *et al.*, 2001). Furthermore, in a related study, only 93 out of 220 (26%) of *E. coli* O157 isolated from humans, cattle, pigs and food were resistant to sulphamethoxazole (Schroeder *et al.*, 2002). The cause of resistance to this drug was generally attributed to selective pressure generated by overuse of this or closely related antimicrobials. The antimicrobial sulphamethoxazole has been used in veterinary and human medicine for decades and the genes that encode resistance to this antibiotic are commonly found on plasmids, thus making it possible for the organism to more readily acquire resistance to this antimicrobial.

The present study indicated that a large proportion (82.9%) of *E. coli* O157 isolated were resistant to erythromycin. Also, all the *E. coli* control strains were resistant to this antibiotic. Resistance to erythromycin may be the result of ribosomal protection (Champney and Burdine, 1998b) or through adenine methylation or mutations at the 23 rRNA (Tait-Kamradt *et al.*, 2000). Very little published data could be obtained for erythromycin resistance mechanisms in *E. coli*.

Results obtained revealed that a large proportion, (52.6% to 34.2%), of the *E. coli* O157 isolated from humans, pigs and cattle faeces samples were resistant to streptomycin and ampicillin, respectively. Of these, a large proportion of the *E. coli* O157 isolates from pig faeces (36.7%-63.3%) were resistant to these antibiotics compared to isolates from cattle faeces (7.7%-15.4%). All the isolates from human stool samples were resistant to ampicillin but none was resistance to streptomycin.

Several studies have been carried out to determine the proportion of *E. coli* O157 that are resistant to streptomycin. In a study involving *E. coli* isolated from students in Nigeria, a large proportion of the isolates (56%-100%) were resistant to streptomycin (Okeke *et al.,* 1998). In another study involving isolates from cattle and humans, 66% (29) and 45% (13) of the isolates were, respectively resistance to streptomycin (Wilkerson *et al.,* 2004). Data obtained in these studies, including the present one, indicated that *E. coli* O157 showed a generally higher level of tolerance to streptomycin. This aminoglycoside has been commonly used in the treatment of infections caused by gram-negative bacteria. A small proportion, 17 (22.4%) and 8 (10.5%), of the isolates in this study were resistant to the other aminoglycosides (neomycin and kanamycin). Interestingly, most of these were isolated from pig faeces.

Aminoglycoside carry out their bactericidal activity by binding to the 16S ribosomal subunit thus interfering with protein synthesis (Fluit *et al.,* 2001). Bacterial resistance to this class of antibiotics may result through modification of the amino and hydroxyl groups. This modification causes the antibiotic to lose its ribosome-binding ability and no longer inhibit protein synthesis (Paulsen *et al.* 1997). Resistance may also result

from mutations in rRNA (Fluit and Schmitz 1999). In a study by Sàenz *et al.,* (2001) it was shown that a relatively large percentage (38% and 40%) of *E. coli* isolated from broilers and food products, were resistant to kanamycin. In another study involving STEC isolated from diverse sources (humans and cow stool samples and beef samples), resistance to kanamycin and neomycin were observed in a very small proportion (0% to 3.2%) of the isolates (Khan *et al.,* 2002).

The results obtained in the present study also indicated that a low to moderate proportion (between 0 and 36.7%) of the *E. coli* O157 isolates from selected pig, cattle and human samples from the North-West Province were resistant to ampicillin. This finding is consistent with previously mentioned studies in which between 4 and 27% of *E. coli* isolated from bovines, pig and humans were resistant to ampicillin (Okeke *et al.,* 2000; Schroeder *et al.,* 2002; Wilkerson *et al.,* 2004). These authors suggested that the observed level of ampicillin resistance, resulted through overexposure to this antibiotic.

In the present study results obtained indicated that a low to moderate proportion (between 0 and 25.0%) of the *E. coli* O157 isolates from selected pig, cattle and human samples from the North-West Province were resistant to chloramphenicol. Studies by Nijsten *et al.* (1993) and Sunde *et al.* (1998) also demonstrated that in *E. coli* O157 isolated healthy pig, between 0 and 13% were resistant to chloramphenicol. Similar observations were made by Sáenz *et al.* (2001) who observed that between 15 and 17% of the *E. coli* O157 isolated from humans and pigs were resistant to this drug. Resistance to chloramphenicol is thus variable between species and prevalence is related to the usage of this drug.

Resistance to chloramphenicol has been reported to result from genetic linkage with other resistance determinant. This could mean that chloramphenicol resistance can persist even in the absence of direct chloramphenicol selection pressure (Aarestrup *et al.,* 1999). The question that arises is whether the detection of chloramphenicol resistant *E. coli* in pigs and cattle from the North-West Province is the result of such a linkage phenomenon.

Results obtained in the present study indicated that only a very small proportion of the *E. coli* O157 isolates, 2 (2.6%), were resistant to the quinolone (norfloxacin). In a related study involving *E. coli* isolated over a twelve-year period from stool samples of humans in Nigeria, only 1 of 578 (0.17%), was resistant to norfloxacin (Okeke *et al.,* 2000). Similarly, a low percentage *E. coli* (STEC) non-O157 strains were also resistant to norfloxacin (Asis *et al.,* 2002).

The results obtained from a previous study revealed that norfloxacin stimulates the production of shiga toxin and shiga toxin converting bacteriophages (Matsushiro *et al.,* 1999). This resulted in limited use of norfloxacin leading to a lack of selection pressure required for the development of antimicrobial resistance within this strain.

The antibiotic resistant data were used to determine predominant MAR phenotypes among the isolates and MAR indices of the isolates from various groups. The MAR index for *E. coli* O157 isolated from pig faces were higher than those of isolates obtained from humans and cattle faeces. Among the pig isolates, the MAR index was higher for *E. coli* O157 isolated from the commercial (Mareetsane) than the communal (Tlapeng) farm (Appendix D; Table 1D). The MAR indices for *E. coli* O157 isolated

from cattle were generally within the acceptable limits (Krumperman, 1983) indicating limited (responsible?) use of antibiotics. An exception was the *E. coli* O157 isolated from communal cattle from Mogosane. The MAR indices obtained in this study may possibly indicate that a very large proportion of the *E. coli* O157 may have been exposed to several antibiotics (Krumperman, 1983, Kaspar *et al.,* 1990). This suggests the need to always monitoring the susceptibilities of *E. coli* O157 to various antibiotics before the administration of treatment in order to reduce the spread of antibiotic resistant strains.

It is not appropriate to treat *E. coli* O157 infections, such as HUS, with antibiotics (Riley *et al.*, 1983; Carter *et al.,* 1987; Butler *et al.,* 1987; Cimolai *et al.,* 1990; Pavia *et al.*, 1990; Slutsker *et al.,* 1998; Wong *et al.,* 2000; Zimmerhackl, 2000; Dundas *et al.*, 2001) as these agents may cause bacterial cell lysis and result in the release of free shiga toxins in the gut lumen. However, the development of antibiotic resistance among *E. coli* O157 results in significant disadvantages on the hosts if infections are caused by multiple antibiotic resistant strains.

Another objective of the study was to evaluate PCR for the detection of STEC virulence genes (*eae* and *hylA*) in *Escherichia coli* O157 isolated from faeces of cattle and pigs and human stool samples in the North-West Province of South Africa. A motivation for this was the fact that such a study had not been conducted in the area and the prevalence of these genes are unknown. A previous study had shown that there is significant diversity and great variability in virulence among *E. coli* O157 strains that had the same known virulence determinants (Kim *et al.,* 1999). A comparison of the *E.*

coli O157 isolates from the latter study demonstrated that there are two distinct lineages and suggested that one of these lineages may be less virulent for humans or may not be efficiently transmitted to humans (Kim *et al.,* 1999).

In the present study, the prevalence of *hlyA* was higher than that of *eae* (Table 4.7). The *eae* is one of the constituent genes of the locus for enterocyte effacement (LEE) and codes for intimin that is responsible for attaching and effacing lesions (Oswald, 2000). The *eae* gene has been found to be located on the bacterial chromosome while *hlyA* is a plasmid-encoded virulence marker (Schmidt *et al.,* 1999). Gyles *et al.*, (1998) demonstrated that the *eae* was the single most important accessory factor correlated with severe disease. However, *eae* negative strains are normally associated with animals (Hornitzky *et al.,* 2002). This may have bearing on the validity of the results of the present study in which a low percentage of *E. coli* O157 isolates from animal and human faecal samples sources in the North-West Province possessed this gene.

In humans the presence of *eae* in *E. coli* O157 isolates was shown to be age related (Beutin *et al.,* 1997). In that study it was demonstrated that *eae* positive strains were generally isolated from young individuals and *eae* negative ones from adults. A study by Hornitzky *et al.,* (2002) showed that the reason why adult individuals contain *eae* negative strains was due to frequent contact with animals.

One of the controversies about HUS development is that the absence of *eae* does not exclude the development of the disease (Beutin *et al.,* 2004). There are several studies dealing with these controversies (Beutin *et al.,* 1997; Gyles *et al.*, 1998; Paton *et al.*, 1999; Tarr *et al.,* 2002; Frydendahl, 2002; Hornitzky *et al.,* 2002; Beutin *et al.,* 2004).

However, one of these clearly demonstrated the association of *eae* and *hlyA* genes in the development of disease (Tarr *et al.,* 2002). The *hlyA* gene is also generally more prevalent in *E. coli* isolated from pigs than cattle and humans (Frydendahl, 2002; Hornitzky *et al.,* 2002). This plasmid borne gene has a greater opportunity for spreading amongst enteric bacteria than the chromosomally borne *eae* gene (Hideki *et al.,* 2003). This explains why the prevalence of *hlyA* was higher among the *E. coli* O157 isolated from the faeces of animals and humans in the North-West Province. The *hlyA* may also not be an effective marker in the identification of pathogenic *E. coli* strains (Frydendahl, 2002; Hornitzky *et al.,* 2002).

Another goal of the study was to genotypically characterise antibiotic resistant *E. coli* O157 strains isolated from animal species and humans. A motivation for this was the reports indicating the development of resistance to antibiotics by *E. coli* O157 (Schroeder *et al.,* 2002). Resistance phenotypes arise from different genetic determinants that present specific epidemiological features (Lanz *et al.,* 2003). An assessment of the resistance situation at genetic level would be of importance in controlling antimicrobial resistance.

A large proportion of the *E. coli* O157 isolated from both humans and animals in the North-West Province were resistant to multiple antibiotics tested (Appendix B, Tables 1B and 2B). Most of these were resistant to tetracycline. Different tetracycline resistant genes are responsible for resistance of *E. coli* strains to this drug (Chopra and Roberts, 2001). Little is known about the distribution of these genes in *E. coli* O157 isolated from humans and animals in the North-West Province. This is similar to a study carried

out on *E. coli* isolated from humans and animals in which the *tetB* gene was detected in 63% of the isolates (Bryan *et al.*, 2004). Of the four *tet* genes that were surveyed, *tetB* was present in two of the isolates. At least 34 *tet* genes are associated with tetracycline resistance. In a previous study, *tetA* was most prevalent among *E. coli* isolates (Sengelov *et al.*, 2003). It is possible that some of the other *tet* genes could be widely distributed among *E. coli* O157 in the North-West Province. This is an aspect that needs further investigation i.e. the prevalence of other *tet* genes present in *E. coli* O157 in the North-West Province need to be determined.

5.2 CONCLUSION AND PROSPECTS

The present study examined the prevalence of *E. coli* O157 from faeces samples of pigs and cattle and human stool samples. Results indicated a generally higher prevalence of *E. coli* O157 in pigs, than in cattle and as well as in humans. A follow-up study to determine the prevalence of *E. coli* O157 in meat, especially pork and beef would be of value to the rural communities of the North-West Province. Such a study should be a seasonal one and include the summer periods that coincides with the festive season. Furthermore, results obtained from such studies would be of high value if the prevalence levels are determined in meat and faecal matter simultaneously. Moreover, the findings of such studies should be communicated to the community, especially the rural communities in the North-West Province. However, results presented here also suggest that proper hygiene management be implemented in order to reduce the prevalence of *E. coli* O157 in farm animals.

An evaluation of the antibiotic resistant patterns and genetic determinants conferring resistance of *E. coli* O157 strains isolated from pigs, cattle and human stool samples were made. Results indicated a high level of multiple-antibiotic resistant (MAR) bacteria in all samples. The highest prevalence of antimicrobial resistance occurred among *E. coli* O157 isolated from pigs and humans. More than 60% of *E. coli* O157 isolated from humans and pigs were resistant to tetracycline, sulphamethoxazole or erythromycin and more than 30% were resistant to ampicillin and streptomycin. Although the data do not demonstrate a direct link between the consumption of contaminated meat products and development of antimicrobial resistance in *E. coli* O157 isolated from humans, the high prevalence of MAR *E. coli* O157 in pigs may suggest that these animals are a source of the emergence of antimicrobial resistance in *E. coli* O157 in the North-West Province. Furthermore, antimicrobial agents are used for treating enteric infections in animals and animals serve as food for humans. Results obtained indicated that *E. coli* O157 isolated from pigs in the communal setting were more resistant to antimicrobials than the commercial farm. This may have resulted from the fact that the animals in the communal setting were exposed to poor sanitation. In such situations pigs may feed on human stool and drink water from rainfall runoffs.

The detection of large numbers of MAR isolates in animals especially pigs is of concern as this could lead to the dissemination of antimicrobial resistant *E. coli* O157 strains with negative clinical implications. The results presented herein demonstrate the need for a continued surveillance of antimicrobial resistance among zoonotic food-borne pathogens, and especially *E. coli* O157. This might improve public health standards.

Results indicated that a large proportion of the *E. coli* O157 isolated in the present study were resistant to three or more antibiotics. However, there was no significant difference in the antibiotic inhibition zone diameter for some antibiotics. Most of the *E. coli* O157 isolates were highly resistant to tetracycline, an antibiotic commonly used to treat enteric diseases.

Multiple antibiotic resistances in STEC have been reported to arise partly from the spread of genetic elements including plasmids, transposons and integrons (Zhao *et al.*, 2001). While all attempts to isolate plasmids failed, characterisation of tetracycline resistant strains by PCR analysis revealed the presence of the *tetB* gene among some of the *E. coli* O157 isolated from pigs.

Phenotypic resistance exhibited by bacterial isolates may result through different mechanisms (Zhao *et al.*, 2001; Walsh *et al.*, 2006). Further research to characterise the resistant phenotypes observed is thus needed. An investigation on the transfer of resistance genes among these bacteria and other pathogens inhabiting food animals is also required. Conjugation experiments between the *E. coli* O157 isolates obtained in this study and other pathogens should thus be conducted. This would evaluate the frequency in which these resistant genes are transferred within enteric bacterial populations in the North-West Province. Such studies would be of high value if carried out to simulate typical situations from communal settings.

The PCR analysis for the presence of STEC O157 virulence genes (*eae* and *hlyA*) showed that these genes were present in *E. coli* O157 strains of human, pig and cattle

origin. Studies to evaluate and identify specific properties that may distinguish between these virulence genes of pathogenic *E. coli* O157 of animal and human origin would be of great value.

E. coli O157 is indeed an important pathogen of humans in developed and developing countries. Carefully planed studies should be conducted to demonstrate the potential risk of transfer of these microbes from asymptomatic animal reservoirs to human subjects that come into close contact with such animals. These include farm and abattoir workers as well as herds men and those individuals that slaughter these animals during traditional rituals especially in rural communities.

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APPENDICES

APPENDIX A

Appendix A outlines all the culture media, enzymes, antibiotics and reagents used in the study. It also outlines some of the procedures that were attempted.

A.1 SOLID MEDIA

The following solid media were used for bacterial cultivation. *E. coli* isolates were grown on Sorbitol-MacConkey Agar (SMAC Agar) [9Peptone 2%, Sodium chloride 0.5%, Bile salts 0.15%, D (-) Sorbitol 1%, Crystal violet 0.0001%, neutral red 0.003%, lactose 1.0%, agar 1.5%9], Mueller-Hinton agar (MH) [9beef infusion 30%, casamino acids 1.75%, starch 0.15% and Bacto agar 1.7%9], Blood agar supplemented with 5% defibrinated sheep blood [(Meat extract 1.0%, peptone 1.0%, Sodium chloride 0.5%, agar 1.0%)].

A.2 LIQUID MEDIA

The following liquid media were used for bacterial cultivation. MacConkey broth [(Oxgall 0.5%, peptone 2%, lactose 1% and bromcresol purple 0.001%)], Luria – Bertani broth [(Tryptone 1.2%, Yeast extract 0.6%, Sodium chloride 1.2%,)] and saline [(0.85% sodium chloride)].

A.3 ANTIBIOTICS

The antibiotics tetracycline (10µl/ml) and chloramphenicol (10µl/ml) were added into broth to select for plasmids from resistant strains while norfloxacin (50µl/ml) was used for induction of bacteriophages from *E. coli* O157 strains. Antibiotic discs (ampicillin 10µg, kanamycin 30µg, tetracycline 30µg, streptomycin 10µg, sulphamethoxazole 10µg, neomycin 30µg, norfloxacin 10µg and erythromycin 15µg) were purchased from Roche Daignostics, Randburg – South Africa.

A.4 CHEMICALS AND REAGENTS

The following chemicals and reagents were used; $MgCl_2$, ethanolmethanol, tetramethyl-p-phenylenediamine dihydrochloride, 1% Kovac's reagent, safranin O, crystal violet dye, Gram's iodine solution, 95% alcohol, 3% hydrogen peroxide, ethidium bromide, ethylene-diamine tetra-acetic acid (EDTA), glacial acetic acid, hydrochloric acid, chloroform, phenol, sodium dodecyl sulphate (SDS), potassium acetate, Tris (hydroxymethyl) aminomethane, sodium chloride, sodium acetate, caesium chloride, TE buffer (Tris –HCl, p.H 8.0; 1mM EDTA p.H 8.0) were purchased from Roche Diagnostics, Randburg – South Africa. Electrophoresis grade reagents were obtained from Roche Diagnostics, Randburg-South Africa, which include, agarose and bromophenol blue.

A.5 ENZYMES

AmpliTag DNA polymerase and associated supplied buffers: Buffer II (100mM Tris and 100mM KCl) and 25mM $MgCl_2$, Proteinase K, Lysozyme, RNase-Dnase free water

and PCR master mix (2X) were purchased from Inqaba Biotechnical Pty – Sunnyside – Pretoria.

A.6 OLIGONUCLEOTIDE PRIMERS

Synthetic oligonucleotides (primers) were purchased from Inqaba Biotechnical Pty – Sunnyside - Pretoria, South Africa that was responsible for synthesising them. All primer stocks were prepared as directed by the synthesizers and were stored at – 4°C.The following section outlines some of the methods used during the study but failed to give positive results.

A.7 INDUCTION OF BACTERIOPHAGES

Induction of phages was performed using the method of Matsushiro *et al.,* (1999). Overnight cultures of *E. coli* O157 were prepared by inoculating the isolate into 5ml of Luria –Bertani broth and incubating aerobically at 37°C for 18 to 24 hours while shaking vigorously. After incubation 0.5 ml of overnight cultures were subcultured into 4.5ml of fresh Luria-Bertani and incubated aerobically at 37°C while shaking for 1 hour to obtain early logarithmic growth phase cells. They were treated with various subinhibitory concentrations of norfloxacin (provided by Lehlabile Scientific services) for prophage induction for 30 minutes, centrifuged, resuspended in drug-free Luria-Bertani broth, and subsequently cultured for phage growth and toxin production for a further 2 h. Infective-center assays were carried out by plating cells within a few minutes after induction together with the indicator *E. coli* JM109, which is nonlysogenic and on the following day, the plates were observed for the presence of plaques. Unfortunately, there were no plaques.

A.8 PLASMID DNA EXTRACTION

A.8.1 Rapid Plasmid DNA Extraction

The rapid plasmid extraction protocol, which was an adaptation from the method of Birnboim and Doly (1979). This method was employed as a screening technique for the presence of plasmids. Overnight cultures were prepared by inoculating the isolates into 5ml of Luria-Bertani broth that contains the appropriate antibiotic (5µl for tetracycline) required for selection of the plasmids. The broth was incubated at 37°C overnight while shaking vigorously. After incubation 10µl of chloramphenicol was added to the culture and incubated again for 1 hour. The drug is known to interfere with protein synthesis hence increasing plasmid yield. Following incubation, 1ml of sample was transferred into 1.5ml microfuge tubes and centrifuged for 5 minutes at 13500 rpm. Pellets were resuspended in 100µl of TE buffer and centrifuged at 13500 rpm for 5 minutes. The pellets were resuspended in 50µl of TE buffer. 10µl of lysozyme was added and the contents of the tube were incubated for 30 minutes at 37°C. 50µl of proteinase K was added and incubated for 30 minutes at 37°C. The contents of the tube were boiled for 1 minute and cooled rapidly on ice. The extracted DNA was run on a 1% agarose gel and viewed under UV light.

A.8.2 Small–Scale Plasmid DNA Extraction

The extraction of plasmid DNA from STEC on a small scale was also performed by the alkali lysis method using a modification of the procedure of (Birnboim and Doly, 1979). A single pure colony of an STEC isolate was inoculated into 5 ml LB containing either 5 µl of tetracycline (100 µg/ml) and incubated aerobically at 37°C for 18 to 24 hours with shaking. After incubation, 5µl of chloramphenicol (50 µg/ml) was added

and incubated at 37°C with shaking for three hours. This was mainly to increase plasmid yield. Bacterial pellet was harvested by dispensing 1.5 ml of the overnight culture into a sterile microfuge tube, and centrifuged for 1 minute at 13 000 rpm using Heraeus biofuge pico (Kendro, Germany) microfuge machine. The supernatant was discarded and the latter procedure repeated to increase the amount of pellets obtained. To the pellet, added 100 µl of solution 1 (glucose 50 mM, tris 25 mM, EDTA, 10 mM) and kept at room temperature for 5 minutes. Two hundred microlitres of solution 2 (0.2M NoaH, 1% SDS) was added and gently mixed by inverting the tube, and kept on ice for 5 minutes. One hundred and fifty microlitres of solution 3 (5M CH3COOK, 11.5µl glacial acetic acid and 28.5µl water) was added, the mixture vortexed gently and kept in ice for 5 minutes. The mixture was centrifuged at 13 000 rpm for 5 minutes and supernatant fluid transferred into a sterile microfuge tube. Four-hundered and fifty microlitres of 1:1 phenol:cholrofom:isoamyl alcohol (24:1) was added and vortexed for 30 seconds then centrifuged for 5 minutes at 13 000 rpm. The aqueous supernatant fluid was then transferred into a sterile microfuge tube. One millilitre of absolute ethanol was added and incubated for 30 minutes at –20°C. The mixture was centrifuged for 10 minutes at 13 000 rpm and supernatant fluid discarded. The pellet was washed in 70% ethanol and centrifuged for 1 minute at 13 000 rpm and supernatant fluid carefully discarded. Pellet was vacuum-dried for 10 minutes using Tomy micro Vac$_{TM}$ mv-100 (Tomy Medico, Japan) vacuum dryer and 30µl of TE added. The extracted extrachromosomal DNA was stored at –20°C.

APPENDIX B

Appendix B outlines an overview of the antibiotic resistant phenotypes obtained from *E. coli* O157 isolated during the study.

Table 1B shows by percentages the antibiotic resistant phenotypes for *E. coli* O157 isolated from communal pigs at Tlapeng.

Table 1B: Antibiotic resistant phenotypes for *E. coli* O157 communal pig isolates

Phenotype	Number observed	Percentage
S-Smx-T-E-Ap	1	4.3
S-Smx-T-E-C	1	4.3
K-S-Smx-T-E-C	1	4.3
Smx-T-E-Ap	2	8.7
Smx-T-Ap	5	21.7
Smx-T-E	4	17.4
S-Smx-T	1	4.3
S-Smx-T-E	7	30.4
K-S-T-Ne	1	4.3

Ap (ampicillin), C (chloramphenicol), T (tetracycline), Nor (norfloxacin), S (streptomycin), E (erythromycin), Ne (neomycin), K (kanamycin), Smx (sulphamethoxazole

Table 2B shows by percentages the antibiotic resistant phenotypes for *E. coli* O157 isolated from commercial pigs at Mareetsane.

Table 2B: Antibiotic resistant phenotypes for *E. coli* O157 commercial isolates from pigs at Mareetsane

Phenotype	Number observed	Percentage
K-S-Smx-T-E-C-Ap-Nor-Ne	1	2.7
K-S-Smx-T-E-Ap-Nor-Ne	1	2.7
K-Smx-T-E-Ap-Ne	1	2.7
K-S-Smx-T-E-Ap-Ne	2	5.4
K-S-T-E-Ne	1	2.7
S-Smx-T-E-Ne	4	10.8
S-Smx-T-E-C-Ne	1	2.7
S-Smx-T-E-C-Ap-Ne	1	2.7
S-Smx-T-E-Ap-Ne	2	5.4
Smx-T-E-Ap	1	2.7
Smx-E	1	2.7
Smx-T-E	6	16.2
S-Smx-T	2	5.4
S-Smx-T-E	3	8.1
Smx-T-Ap	1	2.7
S-Smx-E-Ne	1	2.7
S-Smx-T-E-Ap	2	5.4
S-Smx-T-C	1	2.7
S-Smx-T-E-C	1	2.7
S-Smx-T-Ap	2	5.4
Smx-T	1	2.7
S-T-E-Ne	1	2.7

Ap (ampicillin), C (chloramphenicol), T (tetracycline), Nor (norfloxacin), S (streptomycin), E (erythromycin), Ne (neomycin), K (kanamycin), Smx (sulphamethoxazole).

APPENDIX C

Table 1C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Tlapeng

Characteristic/Biochemical test		Sample number							
		PT13	PT25	PT20	PT29	PT40	PT47	PT24	PT22
Gram stain reaction		-	-	-	-	-	-	-	-
Cell morphology		Rod	Rod	rod	rod	Rod	rod	rod	rod
Oxidase test		-	-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	6	6	15	22	17	17	6	19
	-Tetracycline (30)	6	6	6	6	6	6	6	6
	-Chloramphenicol (30)	24	23	20	29	12	24	23	25
	-Kanamycin (30)	6	20	20	22	19	21	20	22
	-Erythromycin (15)	12	15	11	18	11	12	12	13
	-Norfloxacin (10)	42	39	41	40	44	40	39	47
	-Neomycin (30)	12	26	22	25	25	21	27	30
	Streptomycin (10)	8	17	17	6	6	6	19	6
	Sulphamethoxazole (25)	6	6	6	6	6	6	10	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+	+	+	+

- = Negative; + = Positive.

Table 1C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Tlapeng

Characteristic/Biochemical test		Sample number							
		PT32	PT15	PT39	PT23	PT36	PT9	PT50	PT42
Gram stain reaction		-	-	-	-	-	-	-	-
Cell morphology		Rod	Rod	rod	rod	Rod	rod	rod	rod
Oxidase test		-	-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	6	17	6	15	6	17	16	19
	-Tetracycline (30)	6	6	6	6	6	6	6	6
	-Chloramphenicol (30)	25	25	24	23	22	22	23	13
	-Kanamycin (30)	20	20	20	20	20	20	21	21
	-Erythromycin (15)	16	13	12	12	12	13	13	12
	-Norfloxacin (10)	43	36	37	40	38	38	48	40
	-Neomycin (30)	24	24	19	21	25	24	25	24
	Streptomycin (10)	15	6	18	6	6	8	18	11
	Sulphamethoxazole (25)	6	6	6	6	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+	+	+	+

- = Negative; + = Positive

Table 1C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Tlapeng

Characteristic/Biochemical test		Sample number						
		PT18	PT27	PT46	PT43	PT41	PT33	PT3
Gram stain reaction		-	-	-	-	-	-	-
Cell morphology		Rod	Rod	rod	rod	Rod	rod	rod
Oxidase test		-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	20	6	19	18	6	6	16
	-Tetracycline (30)	6	6	6	6	6	6	6
	-Chloramphenicol (30)	26	24	25	16	24	25	24
	-Kanamycin (30)	18	20	24	22	22	21	6
	-Erythromycin (15)	13	14	10	10	14	14	14
	-Norfloxacin (10)	40	42	45	38	43	40	39
	-Neomycin (30)	24	26	27	22	24	24	10
	Streptomycin (10)	18	18	19	11	15	18	6
	Sulphamethoxazole (25)	6	6	8	6	6	6	13
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+	+	+

- = Negative; + = Positive

Table 2C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Mareetsane

Characteristic/Biochemical test		Sample number							
		PMA40	PMA44	PMA42	PMA66	PMA11	PMA10	PMA8	PMA60
Gram stain reaction		-	-	-	-	-	-	-	-
Cell morphology		Rod	Rod	rod	rod	Rod	rod	rod	rod
Oxidase test		-	-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	17	18	18	18	6	17	19	6
	-Tetracycline (30)	6	6	21	6	6	14	6	6
	Chloramphenicol(30)	23	23	23	24	27	21	24	6
	-Kanamycin (30)	20	23	20	21	19	18	19	6
	-Erythromycin (15)	11	11	11	11	13	9	15	12
	-Norfloxacin (10)	36	35	41	35	41	35	35	6
	-Neomycin (30)	6	8	25	6	24	25	25	6
	Streptomycin (10)	6	9	18	6	14	15	6	6
	Sulphamethoxazole (25)	7	6	8	6	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+	+	+	+

= Negative; + = Positive

Table 2C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Mareetsane

Characteristic/Biochemical test		Sample number							
		PMA5	PMA12	PMA20	PMA52	PMA50	PMA64	PMA31	PMA71
Gram stain reaction		-	-	-	-	-	-	-	-
Cell morphology		rod	rod	rod	rod	rod	rod	rod	rod
Oxidase test		-	-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	20	14	6	17	15	15	6	10
	-Tetracycline (30)	6	6	6	18	6	13	6	6
	Chloramphenicol(30)	19	21	26	21	24	18	18	25
	-Kanamycin (30)	19	20	18	19	20	17	20	6
	-Erythromycin (15)	13	12	15	11	12	8	12	10
	-Norfloxacin (10)	39	37	41	36	36	39	40	6
	-Neomycin (30)	24	26	24	9	6	23	24	8
	Streptomycin (10)	10	15	15	9	10	17	6	6
	Sulphamethoxazole (25)	6	6	6	7	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haenolysis on Blood Agar		+	+	+	+	+	+	+	+

- = Negative; + = Positive

Table 2C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Mareetsane

Characteristic/Biochemical test		Sample number							
		PMA55	PMA70	PMA49	PMA47	PMA28	PMA57	PMA3	PMA18
Gram stain reaction		-	-	-	-	-	-	-	-
Cell morphology		Rod	Rod	rod	rod	Rod	rod	rod	rod
Oxidase test		-	-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	14	16	19	17	6	6	6	17
	-Tetracycline (30)	6	6	6	6	6	6	6	6
	Chloramphenicol(30)	22	14	10	9	20	18	22	25
	-Kanamycin (30)	18	20	20	19	18	6	19	20
	-Erythromycin (15)	11	11	16	10	11	12	14	12
	-Norfloxacin (10)	38	40	40	39	37	38	41	40
	-Neomycin (30)	22	14	27	15	20	6	24	24
	Streptomycin (10)	20	6	7	6	11	7	6	16
	Sulphamethoxazole (25)	6	6	6	6	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+	+	+	+

- = Negative; + = Positive

Table 2C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Mareetsane

Characteristic/Biochemical test		Sample number							
		PMA4	PMA14	PMA1	PMA48	PMA37	PMA45	PMA41	PMA38
Gram stain reaction		-	-	-	-	-	-	-	-
Cell morphology		rod	rod	rod	rod	rod	rod	rod	rod
Oxidase test		-	-	-	-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+	+	+	+
	-Sucrose	+	+	+	+	+	+	+	+
	-Lactose	+	+	+	+	+	+	+	+
	-Gas formation	+	+	+	+	+	+	+	+
	-H_2S production	-	-	-	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	18	6	6	14	15	17	16	6
	-Tetracycline (30)	6	6	6	6	6	6	6	6
	Chloramphenicol(30)	25	23	18	24	15	24	6	6
	-Kanamycin (30)	20	20	6	20	19	20	22	19
	-Erythromycin (15)	17	14	11	11	10	13	10	10
	-Norfloxacin (10)	41	38	39	40	37	36	37	37
	-Neomycin (30)	22	23	10	38	25	6	12	6
	Streptomycin (10)	17	11	20	12	6	8	6	6
	Sulphamethoxazole (25)	6	6	6	6	6	17	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+	+	+	+

- = Negative; + = Positive

Table 2C: Characteristics of *E. coli* O157 strains isolated from pig faecal samples
Sampling site: Mareetsane

Characteristic/Biochemical test		Sample number				
		PMA65	PMA15	PMA56	PMA67	PMA29
Gram stain reaction		-	-	-	-	-
Cell morphology		Rod	rod	Rod	rod	rod
Oxidase test		-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+
	-Sucrose	+	+	+	+	+
	-Lactose	+	+	+	+	+
	-Gas formation	+	+	+	+	+
	-H_2S production	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	8	16	6	6	19
	-Tetracycline (30)	8	6	6	6	6
	-Chloramphenicol (30)	25	22	21	18	27
	-Kanamycin (30)	19	6	19	6	19
	-Erythromycin (15)	10	12	10	12	19
	-Norfloxacin (10)	36	35	32	40	39
	-Neomycin (30)	6	6	6	6	26
	Streptomycin (10)	6	6	6	6	6
	Sulphamethoxazole (25)	8	18	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+

-=Negative; + = Positive

Table 3C: Characteristics of *E. coli* O157 strains isolated from cattle faecal samples. Sampling site: Lichtenburg

Characteristic/Biochemical test		Sample number				
		LC50	LC36	LC27	LC66	LC60
Gram stain reaction		-	-	-	-	-
Cell morphology		rod	rod	Rod	rod	rod
Oxidase test		-	-	-	-	-
TSI test	-Glucose	+	+	+	+	+
	-Sucrose	+	+	+	+	+
	-Lactose	+	+	+	+	+
	-Gas formation	+	+	+	+	+
	-H_2S production	-	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	15	17	18	16	16
	-Tetracycline (30)	6	6	6	6	6
	-Chloramphenicol (30)	20	21	24	26	23
	-Kanamycin (30)	22	23	22	21	22
	-Erythromycin (15)	6	6	6	8	6
	-Norfloxacin (10)	41	42	35	40	40
	-Neomycin (30)	26	29	25	21	25
	Streptomycin (10)	20	18	17	18	18
	Sulphamethoxazole (25)	6	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+	+

- = Negative; + = Positive

Table 4C: Characteristics of *E. coli* O157 strains isolated from cattle faecal samples
Sampling site: Rustenburg

Characteristic/Biochemical test		Sample number			
		RC67	RC35	RC90	RC65
Gram stain reaction		-	-	-	-
Cell morphology		rod	Rod	rod	rod
Oxidase test		-	-	-	-
TSI test	-Glucose	+	+	+	+
	-Sucrose	+	+	+	+
	-Lactose	+	+	+	+
	-Gas formation	+	+	+	+
	-H_2S production	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	15	18	16	20
	-Tetracycline (30)	17	19	19	22
	-Chloramphenicol (30)	21	21	23	25
	-Kanamycin (30)	22	29	25	20
	-Erythromycin (15)	6	9	11	8
	-Norfloxacin (10)	40	41	43	41
	-Neomycin (30)	25	22	27	22
	Streptomycin (10)	19	23	20	25
	Sulphamethoxazole (25)	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+

- = Negative; + = Positive;

Table 5C: Characteristics of *E. coli* O157 strains isolated from human faecal samples
Sampling site: Mafikeng Provincial Hospital

Characteristic/Biochemical test		Sample number		
		HB6F	HB9B	HB6A
Gram stain reaction		-	-	-
Cell morphology		rod	rod	rod
Oxidase test		-	-	-
TSI test	-Glucose	+	+	+
	-Sucrose	+	+	+
	-Lactose	+	+	+
	-Gas formation	+	+	+
	-H_2S production	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	6	6	6
	-Tetracycline (30)	6	6	6
	-Chloramphenicol (30)	6	23	19
	-Kanamycin (30)	23	19	16
	-Erythromycin (15)	6	6	6
	-Norfloxacin (10)	36	32	41
	-Neomycin (30)	23	20	26
	Streptomycin (10)	22	23	18
	Sulphamethoxazole (25)	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+

- = Negative; + = Positive

Table 6C: Characteristics of *E. coli* reference strains used in the study

Characteristic/Biochemical test		Bacterial Strains			
		ATCC 43888	ATCC 43889	E. coli 0145	E. coli JM109
Gram stain reaction		-	-	-	-
Cell morphology		rod	rod	rod	rod
Oxidase test		-	-	-	-
TSI test	-Glucose	+	+	+	+
	-Sucrose	+	+	+	+
	-Lactose	+	+	+	+
	-Gas formation	+	+	+	+
	-H_2S production	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	25	20	19	12
	-Tetracycline (30)	19	17	16	15
	-Chloramphenicol (30)	29	22	22	18
	-Kanamycin (30)	29	22	22	24
	-Erythromycin (15)	12	12	9	10
	-Norfloxacin (10)	41	38	36	28
	-Neomycin (30)	29	23	25	26
	Streptomycin (10)	29	23	21	21
	Sulphamethoxazole (25)	11	8	7	6
Serotype		*E. coli* O157:H7	*E. coli* O157:H7	*E. coli* O145	*E. coli* JM 109
Haemolysis on Blood Agar		+	+	+	+

- = Negative; + = Positive

Table 7C: Characteristics of *E. coli* O157 strains isolated from cattle faecal samples
Sampling site: Mogosane

Characteristic/Biochemical test		Sample number			
		MOG7	MOG13	MOG24	MOG46
Gram stain reaction		-	-	-	-
Cell morphology		rod	rod	rod	rod
Oxidase test		-	-	-	-
TSI test	-Glucose	+	+	+	+
	-Sucrose	+	+	+	+
	-Lactose	+	+	+	+
	-Gas formation	+	+	+	+
	-H_2S production	-	-	-	-
API 20 E results		*E. coli*	*E. coli*	*E. coli*	*E. coli*
Response to antibiotics (µg/disc).	-Ampicillin (10)	17	12	15	6
	-Tetracycline (30)	6	6	6	6
	-Chloramphenicol (30)	6	20	24	21
	-Kanamycin (30)	21	6	21	19
	-Erythromycin (15)	11	10	10	8
	-Norfloxacin (10)	45	42	40	33
	-Neomycin (30)	26	26	25	31
	Streptomycin (10)	12	6	6	15
	Sulphamethoxazole (25)	6	6	6	6
Serotype		*E. coli* O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Haemolysis on Blood Agar		+	+	+	+

- = Negative; + = Positive

APPENDIX D

Table 1D: Number of isolates with identical MAR index and resistance profiles from each sample area

Sample No. of Area Isolates[b]	MAR index[a]	Isolate	Antibiotics[c]								
			K	S	Smx	T	E	C	Ap	Nor	Ne
Commercial (Mareetsane) Pigs	1.0	1	+	+	+	+	+	+	+	+	+
	0.889	1	+	+	+	+	+	-	+	+	+
	0.778	2	+	+	+	+	+	-	+	-	+
	0.778	1	-	+	+	+	+	+	+	-	+
	0.667	3	-	+	+	+	+	-	+	-	+
	0.667	1		-	+	+	+	-	+	-	+
	0.556	4	-	+	+	+	+	-	-	-	+
	0.556	2	-	+	+	+	+	-	+	-	-
	0.556	1	-	+	+	+	+	+	-	-	I
	0.556	1		+	-	+	+	-	-	-	+
	0.444	4	-	+	+	+	+	-	-	-	-
	0.444	1	-	+	+	+	I	+	-	-	-
	0.444	1	-	+	+	+	I	-	+	-	-
	0.444	1	-	+	-	+	+	-	-	-	+
	0.444	1	-	I	+	+	+	-	+	-	-
	0.444	1	-	+	+	I	+	-	-	-	+
	0.333	2	-	-	+	+	+	-	-	-	-
	0.333	2	-	-	+	+	+	-	I	-	-
	0.333	2	-		+	+	I	-	-	-	-
	0.333	1	-	I	+	+	+	I	-	-	-
	0.333	1	I	-	+	+	+	-	-	-	-
	0.333	1	-	-	-	+	+	I	+	-	-
	0.222	1	-	+	-	+	+	-	-	-	-
	0.222	1	-	+	+	+	I	-	-	-	-
Communal (Tlapeng) Pigs	0.667	1	+	+	+	+	+	+	-	-	-
	0.556	1	-	+	+	+	+	+	-	-	-
	0.556	1	-	+	+	+	+	-	+	-	-
	0.444	2	-	+	+	+	+	I	-	-	-
	0.444	5	-	+	+	+	+	-	-	-	-
	0.444	1	+	+	I	+	I	-	-	-	+
	0.444	2	-	-	+	+	+	-	-	-	-
	0.333	4	-	-	+	+	+	-	-	-	-
	0.333	5	-	-	+	+	I	-	+	-	-
	0.333	1	-	+	+	+	+	-	-	-	-
Commercial (Lichtenburg) Cattle	0.333	5	-	-	+	+	+	-	-	-	-

Commercial	0.222	1	-	-	+	I	+	-	-	-	-
(Rustenburg)	0.222	3	-	-	+	-	+	-	-	-	-
Cattle											
Communal	0.556	1	+	+	+	+	+	-	I	-	-
(Mogosane)	0.444	1	-	I	+	+	+	+	-	-	-
Cattle	0.444	1	-	+	+	+	+	-	-	-	-
	0.444	1	-	-	+	+	+	-	+	-	-
Humans (MPH)	0.556	1	-	-	+	+	+	+	+	-	-
	0.444	2	-	-	+	+	+	-	+	-	-
E. coli O157:H7 ATCC 43889	0.222	1	-	-	+	I	+	-	-	-	-
E. coli O157:H7 ATCC 43888	0.111	1	-	-	I	-	+	-	-	-	-
E. coli O145	0.222	1	-	-	+	I	+	-	-	-	-

[a]See Materials and Methods (Section 3.7) for calculation.
[b]Number of *E. coli* O157 from each area with an identical MAR index and resistant profile.
[c]Antibiotic abbreviations as indicated in Section 3.9 (Table 3.2).
- = Sensitive, + = Resistant, I= Intermediate.

APPENDIX E

Table 1E: Summary of the antibiotic resistant data obtained for isolates from the different species. The number and percentages of isolates that were resistant to each of the antibiotics tested are given. Percentages were obtained from a fraction of the number of isolates resistant to particular antibiotics and total number of isolates from the specie and / or sample source.

		K	S	T	E	Ne	Nor	C	Ap	Smx
Communal pigs	No. Resistant	2	12	23	16	1	0	2	8	22
	% resistant	8.7	52.2	100	69.6	4.3	0	8.7	34.8	95.7
Commercial pigs	No. Resistant	5.0	26.0	35.0	31.0	16.0	2.0	5.0	14.0	35.0
	% resistant	13.5	70.3	94.6	83.8	43.2	5.4	13.5	37.8	94.6
Cattle Commercial (Lichtenburg	No. Resistant	0	0	5	5	0	0	0	0	5
	% resistant	0	0	100	100	0	0	0	0	100
Cattle Commercial (Rustenburg)	No. Resistant	0	0	0	4	0	0	0	0	4
	% resistant	0	0	0	100	0	0	0	0	100
Cattle Communal (Mogosane)	No. Resistant	1	2	4	4	0	0	1	1	4
	% resistant	25.0	50	100	100	0	0	25	25	100
Human	No. Resistant	0	0	3	3	0	0	1	3	3
	% resistant	0	0	100	100	0	0	33.3	100	100
Control Strains	No. Resistant	0	0	1	3	0	0	0	0	2
	% resistant	0	0	33.3	100	0	0	0	0	66.7

Ap (ampicillin), C (chloramphenicol), T (tetracycline), Nor (norfloxacin), S (streptomycin), E (erythromycin), Ne (neomycin), K (kanamycin), Smx (sulphamethoxazole

Table 2E: Summary of the antibiotic resistant data obtained for isolates from the different species, depicting multiple antibiotic resistance (MAR). The number of isolates that were resistant to different numbers of antibiotics tested is given.

	No. of Antibiotics			1	2	3	4	5	6	7	8	9
Pigs	Communal (Tlapeng)	R	N	0	0	10	10	2	1	0	0	0
			%	0	0	43.5	43.5	8.7	4.3	0	0	0
	Commercial (Mareetsane)		N	0	2	9	10	8	4	3	1	1
			%	0	5.4	24.3	27.0	21.6	10.8	8.1	2.7	2.7
Cattle	Commercial (Lichtenburg)		N	0	0	5	0	0	0	0	0	0
		R	%	0	0	100	0	0	0	0	0	0
	Commercial) (Rustenburg)		N	0	4	0	0	0	0	0	0	0
			%	0	100	0	0	0	0	0	0	0
	Communal (Mogosane)		N	0	0	0	3	1	0	0	0	0
			%	0	0	0	75	25	0	0	0	0
Human	MPH	R	N	0	0	0	2	1	0	0	0	0
			%	0	0	0	66.7	33.3	0	0	0	0
Control Strains	DMVUP	R	N	0	0	0	0	0	2	1	0	0
			%	0	0	0	0	0	66.7	33.3	0	0

N; Number resistant: %; Percentage resistant: MPH=Mafikeng Provincial Hospital, DMVUP=Department of Medical Virology, University of Pretoria.

APPENDIX F

Figure 1F: A graphical map showing the nucleotide sequences of an *E. coli* O157: H7 strain isolated in the study. Sequencing was performed in the forward direction within the 16S rRNA gene fragments and was employed as a tool to determine the identity of the isolates.