Studies on the prevalence of *Escherichia coli* O157:H7 in raw milk, milking machines, cattle udder and hand swabs collected from selected dairy farms in the Eastern Cape Province, South Africa.

By

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Declaration

I hereby declare that the dissertation I have submitted to the University of Fort Hare for the degree of Masters of Science in Microbiology, is original and has not been previously submitted for any degree at this institution or any other university. I also confirm that this dissertation has been subjected to antiplagiarism screening using Turnitin software and nowhere in this dissertation was there similarity index greater than 10%.

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Dedication

I dedicate this dissertation to my late mentor and above all; my friend, Mr SS Gusha, if it wasn't for your ever-felt presence from the onset, I wouldn't be where I am today. May your precious soul rest in peace.

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Abstract

Escherichia coli O157:H7 serotype has made its mark over the past decades as one of the common causes of gastrointestinal infections globally; responsible for a number of mortalities and hospilizations. Furthermore, the exploitative use of antimicrobials may support antimicrobial resistance in bacteria which is an alarming health concern in the world of medicine. In this study; the prevalence and antimicrobial susceptibility profiles of *Escherichia coli* O157:H7 serotype in raw milk and milking utensils, cattle udders and workers hands, in three commercial dairy farms in the Amathole District Municiplality, Eastern Cape Province of South Africa were evaluated. Raw milk samples were collected from bulk storage tanks and swab samples collected from milking machines, cattle udders and worker's hands fortnightly over a six month period (June to November 2014). Spread plate technique was used for the enumeration and isolation of *E. coli* O157:H7 from the samples using sorbitol MacConkey agar plates supplemented with cefixime and potassium tellurite. A serological confirmation of the presumptive *E. coli* O157:H7 isolates was done using the O157 Latex agglutination test kit. A total of 252 presumptive *E. coli* O157:H7 isolates obtained were further subjected to polymerase chain reaction (PCR) amplification of $rfbE_{O157}$ and $fliC_{H7}$ genes, out of which 27(11%) of the isolates were confirmed positive *E. coli* O157:H7. The phenotypic antibiotic susceptibility profiles revealed that the bacterial isolates were susceptible to the antimicrobials in the following proportions: amikacin (70%), Doxycline (66%), cefotaxime (66%) and gentamycin (48%). Nonetheless; multidrug resistance was obtained with as high as 85 and 81% of the isolates resistant against penicillin G and tetracycline antibiotics respectively. Our findings also showed about 70% of the isolates showed resistance against erythromycin, while 52% of the isolates were resistant against streptomycin.

These findings reveal that; the three selected dairy farms in the Eastern Cape Province, South Africa are reservoirs of the pathogenic and antimicrobial resistant *E. coli* O157:H7 serotype which is a cause for concern to public and environmental health.

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List of Abbreviations

AEMREG:	Applied and Environmental Microbiology Research Group
AIDS:	Acquired Immunodeficiency Syndrome
AMR:	Antimicrobial Resistance
ATCC:	American Type Culture Collection
A_w:	Water Activity
CDC:	Centre for Disease Control and Prevention
CFSPH:	Centre for Food Security and Public Health
CDPHE:	Colorado Department of Public Health and Environment
CLSI:	Clinical and Laboratory Standards Institute
DAEC:	Diffusely adherent *Escherichia coli*
DNA:	Deoxyribonucleic Acid
DEC:	Diarrhoeagenic *Escherichia coli*
EAEC:	Enteroaggregative *Escherichia coli*
EBLS's:	Extended Spectrum Beta-Lactamases
EDTA:	Ethylene diamine tetra acetic acid
EIEC:	Enteroinvasive *Escherichia. coli*
EHEC:	Enterohaemorrhagic *Escherichia coli*

EPEC:	Enteropathogenic *Escherichia coli*
ESC's:	Extended Spectrum Cephalosporins
HC:	Haemorrhagic Colitis
HPS:	Health Protection Scotland
HUS:	Haemolytic Uremic Syndrome
NICD:	National Institute Communicable Disease
PCR:	Polymerase Chain Reaction
STEC:	Shiga toxin-producing *Escherichia coli*
Stx:	Shiga toxin
TBE:	Tris-borate-EDTA
TSB:	Tryptone Soy Broth
TTP:	Thrombotic Thrombocytopenic Purpura
USA:	United States of America
UTI's:	Urinary Tract Infections
VTEC:	Verotoxin-producing *Escherichia coli*

CHAPTER ONE

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1.1 Background of the study

Escherichia coli O157:H7 serotype is a mesophilic, Gram positive, rod-shaped, non-spore forming motile bacterium which can thrive in both aerobic and anaerobic conditions (Berg, 2000, Chapelle, 2001). The bacterium has a simple genomic structure which is composed of only one circular chromosome and a copy or copies of circular plasmids (Hayashi *et al*., 2001). The serotype is an enterohaemorrhagic (EHEC) strain of the specie *E. coli* (Karch *et al.,* 2005), that is commonly found in the intestines of warm blooded animals; with cattle as major reservoirs (Musa *et al.,* 2010). The identification of *Escherichia coli* O157:H7 as a human pathogen in 1982 in the United States of America following an outbreak of bloody diarrhoea associated with contaminated hamburger meat (CDC, 2004); it has since been considered as the major food-borne pathogen (Musa *et al.,* 2010). There are several serotypes of enterohaemorrhagic *E. coli* with the O157:H7 serotype being the most prominent with respect to its severity and epidemiology of disease outbreaks worldwide (Bugarel *et al.,* 2011). This major serotype produces shiga-toxins and is well known for its worldwide reputation in human illnesses (Solomankos *et al.,* 2009). Although, there are other *E. coli* serotypes such as O26:H11 and O111: NM, which exhibit similar pathogenic potential as that of the O157:H7 serotype, most large outbreaks of EHEC infection have been caused by O157:H7 (Hayashi *et al.,* 2001).

There are several reports about this pathogen which is said to cause diseases like: haemolytic uremic syndrome (HUS) in children under 5 years old and elderly individuals, haemorrhagic colitis (HC) associated with bloody diarrhoea (Rahal *et al.,* 2012), and thrombotic thrombocytopenic purpura- a rare disorder of the blood coagulating system; causing extensive microscopic clots to form in the small blood vessels throughout the body (Moake, 2002; Brenjchi *et al.,* 2011). In most cases, *E. coli* O157:H7 infection of raw milk is via

faecal contamination during milking, however, direct excretion of this pathogen from an infected udder has been reported (Solomankos *et al.,* 2009). Previous studies indicate that the environmental niches for *E. coli* O157:H7 have not yet been clearly established. Nonetheless, dairy cattles appear to be the major reservoir for this pathogen (Lingathurai and Vellathurai, 2010). In addition, food safety epidemiologists have highlighted the emergence of antibiotic resistance amongst foodborne pathogens like *E. coli* O157:H7 as a worrisome subject with regards to food safety (Naser, 2007).

Raw milk is a white liquid that is usually produced by mammals (including cattle, goats, horses and humans) by their mammary glands to nourish and/or feed their offspring. Milk has always been known to be one of the most common sources of nutrients in its natural form for over centuries (Murinda *et al.,* 2004; Oliver *et al.,* 2005).

Due to its biochemical complexity and high water activity, raw milk also supports the growth of many kinds of microorganisms (Murinda *et al.,* 2004; Oliver *et al.,* 2005). Thus, pasteurization of milk is one of the major components in the control of milk-borne pathogens that threatens public health (Holsinger *et al.,* 1997). Statistics show that, a large number of people in rural areas consume raw unpasteurized milk and a much larger portion of the population often consume this type of milk indirectly via several types of raw milk products such as cheese, ice cream and yogurts (Rahal *et al.,* 2012). Raw milk consumption is not the solitary mode of transmission of *E. coli* O157:H7 to humans; ground beef is also one of the most common vehicles amongst foodborne outbreaks of *E. coli* O157:H7 (Josefa *et al.,* 2005). Direct contact with infected animals and person to person transmission are the other modes of transmission (Goldwater and Bettelheim, 2012).

1.2 Problem Statement

E. coli O157:H7 has been identified as a pathogen that causes severe and life-threatening diarrhoea (Momba *et al.,* 2008). This makes the consumption of *E. coli* O157:H7-contaminated raw milk a potential high health-risk aspect which can lead to morbidity and mortality (Lye *et al*., 2013). However, the trend of illness resulting from consuming contaminated raw milk is increasing on a daily basis in rural areas as people prefer consuming raw milk rather than pasteurized milk due to their ethical beliefs (Lingathurai and Vellathurai, 2010).

Clinical antibiotic treatment does not appear to alleviate the severity of illness or prevent the development of disease associated with *E. coli* O157:H7 pathogen. The possible explanations highlighted by Gerrish *et al*. (2007) for this lack of benefit for antibiotic treatment, are the elimination of competing bowel flora by the antibiotic, giving a competitive advantage to *E. coli* O157:H7 and lysis of *E. coli* O157:H7 leading to increased release of verotoxins.

The prevalence of *E. coli* O157:H7 has been previously reported in a number of studies in different countries including Argentina, Austria, Brazil, Colombia, Denmark, Egypt, France, Greece, Netherlands, Switzerland, United Kingdom and the United States of America (Dontorou *et al.*, 2003). Brenjchi *et al.* (2011) reported low incidence of *E. coli* O157:H7 in bulk milk tanks, however, when considering the very low infective dose of this pathogen, which is about 100 to 200 or even less than 10 cells in susceptible consumers which is hazardous, it suggests a very high health-risk to humans, regardless of how low the incidences are (CFSPH, 2009, Grant *et al.,* 2011).

Poor sanitation in dairy farms, especially those located in rural areas ignites the presence of a number of microbial pathogens in milk especially *E. coli* O157:H7 which is mostly found in faeces and animal wastes (Lye *et al.,* 2013). In South Africa, there is dearth of documented information with regards to the epidemiology of this pathogen, more especially in the Eastern Cape Province (Ateba and Bezuidenhout, 2008). As a result of this, there is need to evaluate the prevalence of *Escherichia coli* O157:H7 in raw milk, milking machines, udder and hand swabs collected from selected dairy farms within the Eastern Cape Province, South Africa.

1.3 Hypothesis

This study is based on the hypothesis that:

- ✓ The Eastern Cape dairy farms do not harbour *E. coli* O157:H7 serotype.
- ✓ The Eastern Cape dairy farms harbour *E. coli* O157:H7 serotype.

1.4 Aim and Objectives

1.4.1 Aim

This study aimed at assessing the prevalence of *Escherichia coli* O157:H7 in raw milk, milking machines, cattle udder and hand swabs collected from three selected dairy farms in the Eastern Cape Province.

1.4.2 Specific objectives

1. To collect, isolate and identify *Escherichia coli* O157:H7 from raw milk, milking machines, cattle udder and hand swab samples from three selected dairy farms in Nkonkobe Municipality of the Eastern Cape Province of South Africa.
2. To evaluate the incidence of *E. coli* O157:H7 from the collected samples.
3. To elucidate the antimicrobial susceptibility profiles of the confirmed *E. coli* O157:H7 isolates.

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CHAPTER TWO

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Literature Review

2.1 Verocytotoxin-producing *Escherichia coli* pathotype

Verocytotoxin-producing *Escherichia coli* (VTEC), also described as shiga-toxin producing *E. coli* (STEC), are pathogens that are associated with pandemic food and water-borne illnesses across the globe (Karmali *et al.,* 2009), with the serotype O157:H7 among the other serotypes being the major food poisoning source with outbreaks reported worldwide and thus raises serious public health concerns (Nguyen and Sperandio, 2012).

According to literature, the first discovery of VTEC dates back to the late 1970's by the work of Karmali and colleagues in Canada (Karmali *et al.,* 2009). Since then, an estimated 380 different OH serotypes have been isolated in both humans and animals and only a smaller proportion (including the O157:H7 serotype) is linked to human illnesses (Nguyen and Sperandio, 2012).

E. coli O157:H7 is the most important serotype with clinical infections, although other infections triggered by non-O157s serogroups including O103, O26, O111, O45, O121, O118, and O145 have been reported (Dikici *et al*., 2015). These VTEC's may also be referred to as Shiga-toxin producing *E. coli* (STEC) or Enterohaemorrhagic *E. coli* (EHEC) because of the ability to induce Haemorrhagic colitis (HC) and Haemolytic Uraemic Syndrome (HUS) which are fatal human infections and can result in death (Orden *et al.,* 2008). *Escherichia coli* O157:H7 is one of the most severe *E. coli* serotypes with the ability to survive in very low temperatures (refrigeration temperatures) (Chang *et al.,* 2013). Also, it has been reported in a number of studies that *E. coli* O157:H7 can tolerate pH's as low as 4.4 and can thrive in foods with a minimum water activity of 0.95 (WHO**,** 2012).

2.2 Other *Escherichia coli* Diarrhoeagenic Pathotypes

E. coli among other bacterial pathogens (Shigella, Salmonella, Klebsiella Pneumonae etc) belongs to the family Enterobacteriaceae which are basically Gram negative bacteria (Berg, 2000; Chapelle, 2001) that thrive commensally in most animal and/or human gastrointestinal tracts (Karmali *et al.,* 2009).There are about six known diarrhoeagenic *E. coli* (DEC) pathotypes that are linked with severe diarrhoeal infections in humans (Gomez-Aldapha *et al.,* 2014) and these include Shiga-toxin producing *E. coli* Verocytotoxin producing *E. coli* (STEC/VTEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Diffusely adherent *E. coli* (DAEC) and Enteroinvasive *E. coli* (EIEC) (Alizade *et al.*, 2014).

2.2.1 Enteropathogenic *Escherichia coli* (EPEC)

Typical EPEC strains possess the *eae*A and *bfp*A genes, which induce the production of intimin and promote bundle forming pili respectively which are responsible for attaching and effacing lesions of intestinal microvilli (Alizade *et al.,* 2014) and has been mostly isolated in humans. An atypical EPEC strain lacks the bundle forming pili as compared to the typical EPEC strain and has been isolated in different animals (Cortes *et al.,* 2005). EPEC infections are often hard to distinguish from those caused by other pathogens; its symptoms include bloody and/or watery diarrhoea, vomiting and to a lesser extent and fever (Lee *et al.*, 2012).

2.2.2 Enteroaggregative *E. coli (EAEC)*

Enteroaggregative *E. coli* (EAEC) is a diarrhoeagenic *E. coli* pathotype which is often associated with serious and persistent diarrhoeal illnesses mostly affecting children in most developing countries (Dow *et al.,* 2006). The EAEC and EIEC pathotypes are mainly studied in human-associated infections (Puno-Sarmiento *et al.,* 2013). The roles and virulence factors

of these pathotypes with respect to diarrhoea are not well-identified in literatures and thus vital research is required (Puno-Sarmiento *et al.,* 2013).

2.2.3 Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) are known to produce enterotoxins which stimulate the lining of the gastro-intestines causing them to secrete excessive fluid, thus producing diarrhoea (CDC, 2005). ETEC is one of the leading enteric infections that cause death in infants reportedly in under-resource countries in the world (WHO, 2008). In most cases, ETEC strains possess the *st and lt* genes which characterise the heat-stable and the heat-liable enterotoxins, respectively (Cerna-Cortes *et al.,* 2012). The most important vehicles of ETEC are food and water that are contaminated with animal or human faeces (CDC, 2005).

Secondary immune response system often initiates immunity against ETEC infections in children older than 5 years and adults due to several encounters with the disease (Fisher-Walker and Black, 2010).

2.2.4 Enteroinvasive *E. coli (EIEC)*

Enteroinvasive *E. coli* pathotype is commonly known for inducing a mild form of diarrhoea or dysentery (Liu *et al.,* 2012). The biochemical similarities and modes of infections between EIEC and shigella suggest their evolutional common ancestry (Aribam *et al.,* 2013). Its invasiveness is initiated by a large plasmid which codes for the production of several outer membrane proteins (Prats and Llovet, 1995). EIEC do not produce toxins, but with the aid of such proteins, they bind and invade the intestinal cells causing severe damage to the intestinal walls (Rolland *et al.,* 1998; Lan *et al.,* 2004).

2.3 Epidemiology and clinical manifestations of Enterohaemorrhagic *Escherichia coli* pathotype

EHEC has been the common cause of most cases of gastrointestinal infections, which led to major children morbidity and mortality rates in developing countries, hence raising a worrisome public health concern (Alizade *et al.,* 2014).

Clinical manifestations of infections by this pathotype range from asymptomatic carriage to diarrhoea to haemorrhagic colitis. HUS is reportedly being the common complication in children, whereas, the thrombotic thrombocytopenic purpura (TTP) is a sporadic complication in adults. Furthermore, treatment with antibiotics also aids in the development of HUS (Kehl, 2002; CDC, 2015).

Almost everywhere in the world, *E. coli* O157:H7 illnesses have been reported with a very few unreported cases (Centre for Food Security and Public Health, 2009). Reports show that in the United States of America (USA) an estimate of over 265 000 illnesses are caused by VTEC strains with 96.534 of those associated with VTEC O157 and 168.698 non-O157; more than 3600 hospitalisations and 30 deaths each year (Dikici *et al.,* 2015). Literature reveals that, there have been reports of VTEC O157 outbreaks in North America, Japan and Scotland; in which an estimation of about Hundreds to Thousands of people in these regions were extensively victimised by the pathogen (Nielsen *et al.*, 2002).

In another study conducted by Ateba and Mbewe (2013) in the North West Province, South Africa, where a total of 94 confirmed *E. coli* O157:H7 isolates were obtained from pigs, cattle, pork, beef and human stools which are somewhat primary food sources to many, suggests a great deal of problem to human health when consumed undercooked (Ateba and Mbewe 2014). It is therefore crucial to practise excellent hygiene in farms, piggeries, abattoirs, food markets and in handling any food product as an attempt to limit transmission of this pathogen.

A number of sources have been linked to be a common cause of a variety of case-fatalities and hospitalizations with respect to the *E. coli* O157:H7 infection in different parts of the world (Saxena *et al.,* 2015). Typical examples include the following:

- Undercooked hamburgers in Washington State: an outbreak which took place between 1992 and 1993 with a panel of 233 *E. coli* O157:H7 isolates (Saxena *et al.,* 2015);
- Unpasteurized Gouda cheese where 13 cases were reported during the 2002-2003 period in Edmont, Alberta state in Canada (Honish *et al.,* 2005);
- Leaf lettuce with 13 hospitalizations and 1 HUS case in 1995 in Western Montana in the United States (Saxena *et al.,* 2015);
- An outbreak; which was traced back to a water distribution system in New York, U.S; that resulted in 128 patients in 1999 (Bopp *et al.,* 2003). Another case of Strawberries where; 6 hospitalizations, 4 HUS cases and 2 deaths were reported in the Oregon State, U.S in 2011between July and August (Saxena *et al.,* 2015);
- In 2013; 9 cases resulting from contaminated cucumber were reported in the State of Colorado (Colorado Department of Public Health and Environment (CDPHE), 2014);
- Incidences were reported in Sakai, Japan in 1996 which had about 7,470 school children infected, 1,000 hospitalizations for gastrointestinal symptoms, 100 HUS cases and 3 deaths;
- In Xuzhou, China, in 1999 where about 195 hospitalized patients who had clinically diagnosed HUS and 177 deaths, with these cases only reported in Chinese journals (Xiong et al., 2012); The Health Protection Scotland (HPS) identified 18 incidences of verotoxin-producing Sorbitol Fermenting (SF) -O157 infections in Scotland, about 13 of those incidences were linked with a nursery. HUS was associated with 8 of the 18 incidences (CDC, 2010).

Several researchers have identified *E. coli* O157:H7 serotype to be associated with a number of diseases, to name a few, these include: diarrhoea, septicemia, bladder and kidney complications, pneumonia, neonatal meningitis and bacteremia in children and grown-ups with AIDS, pyelonephritis; and the well-known HUS, HC and TTP which are linked to renal failures in our urinary systems (Ateba and Mbewe, 2014). In the past; Haemorrhagic colitis incidences have been reported in Kwa Zulu Natal and Mpumalanga provinces in South Africa and the nearby Swaziland (Muller *et al.,* 2000).Also, the National Institute for Communicable Diseases (NICD) published some of their *E. coli* EHEC cases in the provinces of South Africa from 2011 to 2012 (NCID, 2012) and 2012 to 2013 (NICD, 2013) (Table 1). Also, failure of affected individuals in seeking medical attention was reported to results in some outbreaks and/ or incidences being ignored. Scallan *et al,* (2006).

Table 2.1: Annual reports on *Escherichia coli* (EHEC) surveillance in South Africa from year 2011 to 2013.

Year(s)	Month(s)	No. of cases by age group (0 to 9 years)	No of cases by month	Province	No. of cases by Province
2011	January	2	1	Gauteng	2
	April		1	Gauteng	
2012	February	2	1	Gauteng	2
	November		2		
2013	July	3	1	Gauteng	2
	November		1	Mpumalanga	1

(Adapted from NICD, 2012; 2013).

2.4 Toxicity of *Escherichia coli* O157:H7

The toxicity of this pathogen results from a number of virulence factors that it possesses, and of the virulence factors, the Shiga Toxins, *stx*1 and *stx*2 with their variants are the most significant virulence genes responsible for disease-infections in humans (Law, 2000; Ateba and Mbewe, 2014).The *eae*A and *hly*A genes also form part of the virulence genes of *E. coli* O157:H7 which codes for intimin and haemolysin respectively which enables the organism to exploit or confuse host-cell signalling pathways in the nervous system of the host resulting to altered cellular responses (Olsen *et al.,* 2002). Haemolysin is a cytotoxic protein that is able to induce osmotic lysis to erythrocytes and *E. coli* tends to produce several types of this hemolysin inclucing α-hemolysin (extracellular proteins), β-hemolysin (cell bound proteins) and γ-hemolysin which are a product of nalidixic acid resistant mutants (Wyborn *et al.,* 2004; Rebecca and Elizabeth, 2005).

Escherichia coli and its variants have contributed to most cases of Urinary Tract Infections (UTI's), recording about 80% of acquired UTI's (Rawa'a Al-Chalabi *et al.,* 2010). The pathogenicity causing UTI's have been traced back to a number of virulence factors such as haemolysin, cytotoxic necrotising factors, aerobactin, biofilm and a number of different adhesion types (Usein *et al.,* 2001; Rawa'a Al-Chalabi *et al.,* 2010).

2.5 Sources of transmission of *E. coli* O157:H7

Reported studies across the globe have shown the source of transmission of *E. coli* O157:H7 from 1998 to 2006 to be food, dairy products, animal contact and water and a small percentage from those reports was stated as unknown. However, with food and dairy products exceeded the other sources of transmission (Pennington, 2010). Cattles are still regarded as the major reservoirs of *E. coli* O157:H7 but other domestic and wild animals have also been reported (Rangel *et al.,* 2005; Karmali *et al.,* 2009; Qiong Meng *et al.,* 2013).

Lye *et al.* (2013) in his study revealed several factors in the farming industry that contribute in milk contamination, and these include poor hygienic milking conditions, contaminated equipment, milking utensils and milk handlers' poor personal hygiene.

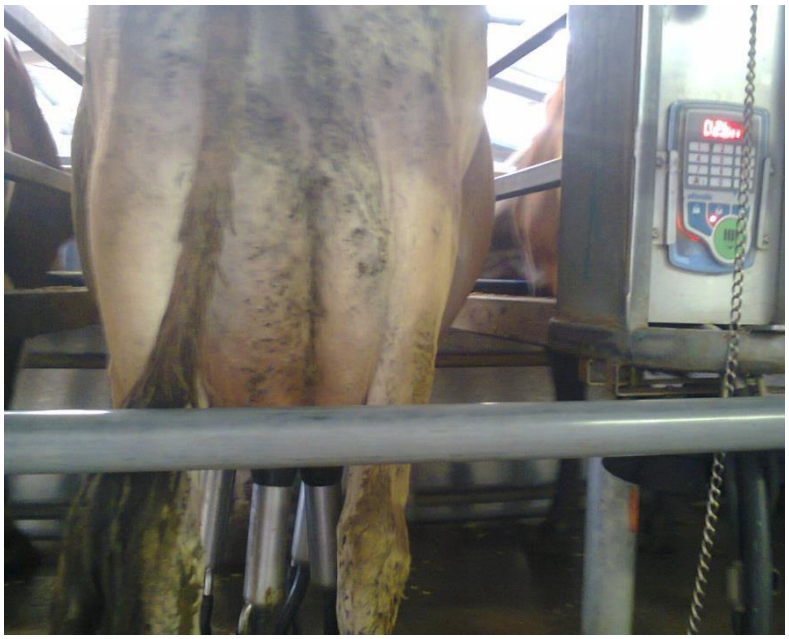

Figure 2.1: Cattle udder and back limbs covered with faeces during a milking process in some commercial dairy farm in the Eastern Cape Province, South Africa.

2.6 Diagnosis, Treatment and Prevention of VTEC – associated infections

VTEC infections may differ from each person, they may include symptoms like stomach cramps, bloody diarrhoea and constant vomiting, fever is often not very high (<38.5 %) which most people tend to recover within 5 to 7 days of infection. Though some infections are very mild, others are severe and to a greater extent; deadly (CDC, 2015).The bowel inflammation referred to as the Prodrome often occurs prior the onset of HUS, which within a week of ingestion, the colon becomes severely swollen and alternatively bloody diarrhoea takes over (CDC, 2015). Re-hydration (drinking a lot of clean water) is therefore necessary to infected individuals to avoid dehydration and should seek medical attention immediately. Also, dialysis (for inducing kidney functioning), blood transfusion and/or plasma therapy and renal transplantation may be highly considerable to individuals with severe HUS (Scheiring *et al.,* 2008).

The use of antibiotics in the treating VTEC infections is heavily discouraged due to the fact that there is a lack of evidence that substantiate treatment with antibiotics as helpful. Furthermore, the use of these antibiotics and Antidiarrheal agents like Imodium® may also elevate this risk of HUS in some patients (CDC, 2015).

Generally, personal hygiene (washing of hands thoroughly with soap after using the toilet), consuming of pasteurized milk and milk products, sufficiently cooked foods including clean-fresh fruits and vegetables and use sufficiently treated water for any purpose, can play a vital role in preventing EHEC infections (Kulkarni *et al.,* 2002; CFSPH, 2009; WHO, 2012; Mustafa *et al.,* 2013).

2.7 Trends in antimicrobial resistance of *E. coli* O157:H7

Abong'o and Momba (2009) in their study in Amathole District, Eastern Cape Province, South Africa reported that some of their *E. coli* O157:H7 isolates collected from meat and meat products were resistant to five of the eight antibiotics. Resistance to antibiotics by bacteria (including *E. coli* O157:H7) may be triggered by the selective pressure and/or inappropriate use of these antibiotics either clinically or by farmers on their everyday farming practises (Schroeder *et al.,* 2002; Abong'o and Momba, 2009).

Antimicrobial resistance exhibited by *E. coli* isolated from zoonotic sources including human stools may compromise the effectiveness of treatment agents such as aminoglycosides, cephalosporins, fluoroquinolones and sulphonamides since they are frequently used (Frye and Jackson, 2013).

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CHAPTER THREE

Prevalence of *Escherichia coli* O157:H7 in raw milk and milking utensils from selected commercial dairy farms in Amathole District Municipality, Eastern Cape Province, South Africa: public health implications

CHAPTER THREE

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Abstract

The prevalence of *Escherichia coli* O157:H7 serotype in raw milk and milking utensils, cattle udders and workers hands, in three commercial dairy farms in the Amathole District Municiplality, Eastern Cape Province of South Africa was evaluated. Raw milk samples were collected from bulk storage tanks and swab samples collected from milking machines, cattle udders and worker's hands fortnightly over a six month period (June to November 2014). Spread plate technique was used for the enumeration and isolate of *E. coli* O157:H7 from the samples using sorbitol MacConkey agar plates supplemented with cefixime and potassium tellurite. A preliminary molecular confirmation of the presumptive *E. coli* O157:H7 isolates was done using the O157 Latex agglutination test kit. A total of 252 presumptive *E. coli* O157:H7 isolates obtained were further subjected to polymerase chain reaction amplification of $rfbE_{O157}$ and $fliC_{H7}$ genes. Results showed only 27(11%) of the isolates positive for *E. coli* O157:H7. The highest number of positive isolates was obtained from Farm A (5%) while 3% and 2% of the positive isolates were from Farms B and C respectively. The detection of *E. coli* O157:H7 pathogen from these dairy farms is a cause for concern to public health.

Keywords: Prevalence, Raw milk, milking utensils, *Escherichia coli* O157:H7, gene markers, Verotoxins.

3.1 Introduction

E. coli O157:H7 succeeds as one of the most prominent causes of diarrhoeal diseases in humans and warm blooded animals. The ever-escalating *E. coli* O157:H7 infections have been reported in over 30 countries from six continents over the years (Saari *et al.,* 2001). These include haemolytic uremic syndrome in infants and immunocompromised adults, haemorrhagic colitis which are associated with bloody diarrhoea (Rahal *et al.,* 2012). *E. coli* O157:H7 serotypes also known as verotoxin producing *E. coli* (VTEC's); Shiga-toxin producing *E. coli* (STEC) or enterohaemorrhagic *E. coli* (EHEC) are known to produce verotoxins or shiga-like toxins which possess the sole ability to kill vero cells (Chapman *et al.,* 2001; Bean *et al.,* 2004; Nazemi *et al.,* 2011; Benjamin *et al.,* 2013). The significance of this pathogen from others is characterised by the ability to infect individuals at very low infectious doses; their unusual acid tolerance and their association with animals that are mostly primary food sources to humans (Isibor *et al.,* 2013).

Several sources and reservoirs have accounted for presence of *E. coli* O157:H7 in the environment; with cattle as the most recognised reservoir of this pathogen (Avery *et al.,* 2008; Lingathurai and Vellathurai, 2010). It is more likely that cattle excrete these pathogens via its faeces (Fitzgerald *et al.,* 2003; Espie *et al.,* 2006; Brenjchi *et al.,* 2011; Chauret 2011; Regua-Mangia *et al.,* 2012). This however provides a significant route of milk contamination directly during milking or indirectly through poor hygienic milking conditions, contaminated equipment and/or utensils and milk handler's poor personal hygiene (Lye *et al.,* 2013; CDC, 2007).

Undercooked ground beef, raw milk and other dairy product intake have been associated with several global incidences of foodborne illnesses; reportedly caused by VTEC strains (Effler *et al.,* 2001; Bouvet *et al.,* 2001; Evins, 2004; Fusco *et al.,* 2012). Raw milk is one of the most important food sources and thereby an important source of food-borne pathogens with its biochemical complexity which enables it to support a wide range of microorganisms, therefore; possible milk contamination from bulk storage tanks by direct udder excretions of infected cattle and other forms of contamination brings a worrisome concern with regards to public health (Murinda *et al.,* 2004; Oliver *et al.,* 2005; Caine *et al.,* 2014). In addition; direct transmission from infected animal host to humans or by interpersonal contact have also been implicated in the accumulation of VTEC infections (Bouvet *et al.,* 2001).To the best of my knowledge, there is scarcity of information on the prevalence of *Escherichia coli* O157:H7 in dairy farm environments in South Africa, hence this study is aimed at investigating the prevalence of *Escherichia coli* O157:H7 in raw milk and milking utensils from some commercial dairy farms in the Amathole District Municipality in the Eastern Cape Province, South Africa.

3.2 Materials and Methods

3.2.1 Description of the study areas

Three commercial dairy farms under the Amathole District Municipality in the Eastern Cape Province, South Africa were used for this study and for confidentiality purpose are identified as farms A, B and C respectively.

Dairy farm A is surrounded by a number of villages and peri urban settlements. It is located on the geographical coordinates 32°37′ 0”S and 27°07′ 0” E. This Dairy covers about 700 hectares of land with about 400 cows, a production capacity of 2000 litres of milk a day and 36 workers. Dairy farm B is located on the geographical coordinates of 32° 49' 0" S and 26° 59' 0" E and covers a terrain of about 280 hectares of land with 600 cows producing 2 000 litres of milk per day and has 16 permanent workers.

Dairy farm C is situated along the geographical coordinates of 32° 47' 0" S, 26° 50' 0" E. About 800 cows are milked daily in the farm which produces an estimate of 10 000 litres milk per day and has a total of 10 full time workers. It supports both the local region and other regions abroad the Amathole District Municipality borders with its produce.

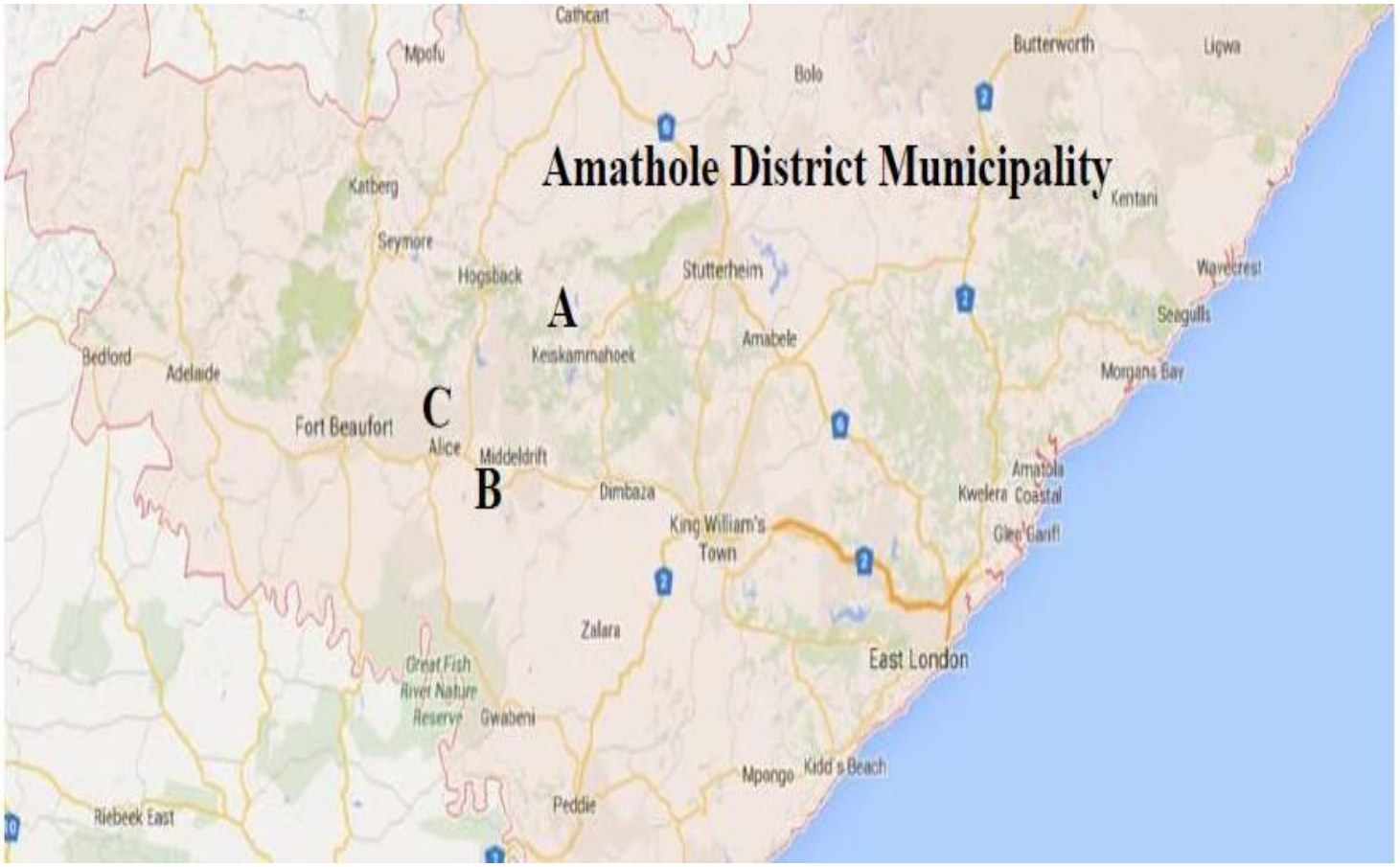

Figure 3.2.1: Map of the three sampling locations (A, B and C) in the Amathole District Municipality, Eastern Cape Province, South Africa.

3.2.2 Sample collection

Samples were collected forthnightly over a period of six months (June to November 2014). Samples included raw bovine milk samples from farm bulk storage tanks and were collected using pre-sterilized 50 ml centrifuge tubes (3 tubes for each farm), while sterile swabs-sticks (COPAN GROUP: COPAN, Italia) were used to collect samples from milking machines, udder and hands of workers, and all samples were appropriately labelling (Figure 3.3.1). Samples were then transported on ice to the Applied and Environmental Microbiology Research Group (AEMREG) Laboratory at the University of Fort Hare and analysed within 6 hours of collection.

Figure 3.2.2: Sample collection from the milking machines in one of the dairy farms.

3.2.3 Isolation of *Escherichia coli* O157:H7

Isolation of *E. coli* O157:H7 from raw milk samples was done following the protocol of Ateba and Mbewe (2011) with some modifications. For raw milk samples, tenfold dilutions (from 10^{-1} to 10^{-3}) of milk were made using sterile saline water, where 1 ml of raw milk sample was transferred into 9 ml of sterile saline water (10^{-1} first dilution) and another 1 ml from the 10^{-1} dilution was transferred into another 9 ml of sterile saline water, and the process repeated until 10^{-4} dilution is reached. One hundred microliter from each dilution was immediately spread-plated (in triplicates) on sorbitol- MacConkey agar plates supplemented with cefixime (50µg/l) and potassium tellurite (25mg/l) for the detection of *E. coli* O157:H7 and then incubated at 37 ^{o}C overnight. Colonies that appear colourless or exhibited a beige colour on the agar were considered as presumptive *E. coli* O157:H7 positive isolates.

In the same vein, swab samples from milking machines, cattle udders and from the hands of workers collected across the three farms were inoculated into 10 ml of Tryptone Soy Broth and incubated on a shaker at 37 ^{o}C overnight at 150 rpm. At the end of the incubation period, 100 µl of each turbid culture was plated on sorbitol McConkey agar plates supplemented with cefixime (50 µg/l) and potassium tellurite (25 mg/l) using spread plate technique and incubated at 37 ^{o}C overnight. Colourless or beige colonies were picked as presumptive *E. coli* O157:H7 isolates. The presumptive *E. coli* O157:H7 were purified by repeated aseptic transfer onto fresh nutrient agar plates to obtain pure isolates and stored them on sterile 25% glycerol until use.

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3.2.4 Serological test for confirmation of *E. coli* O157:H7 isolates

Serological test for *E. coli* O157:H7 was done for further confirmation, where, presumptive *E. coli* O157:H7 isolates from glycerol stocks were resuscitated by inoculating them on sterile Tryptone Soy Broth tubes and incubated on a shaker at 37 ^{o}C overnight at 150 rpm. After incubation, they were further triple-streaked on sterile Nutrient agar plates and incubated at 37 ^{o}C overnight. About 3 pure colonies were picked and tested for agglutination of O157 antigen using the latex agglutination test (*E. coli* O157 Latex Test Kit, Oxoid), following the manufacturer's instructions. Briefly, a single drop of the latex test was dispensed onto a circle on the reaction card, placing the drop next to the edge of the circle. A pasteur pipette drop of saline was then added to the same circle, ensuring that the latex test and saline do not mix. A loopful of the colony to be tested was emulsified in the saline ensuring a smooth resultant suspension. The suspension was then mixed with the latex test and spread to cover the reaction area using a loop. After mixing up the card was shaken in a circular motion for 1 minute and observed for agglutination.

3.2.5 DNA Extraction

DNA extraction from the bacterial isolates was done using the boiling method of Gugliandolo *et al.* (2010). Briefly, pure isolates were inoculated into sterile Tryptone Soy Broth and incubated at 37 ^{o}C for overnight. At the end of the incubation, about 2 ml from the previously grown culture was transferred into sterile 2 ml Eppendorf tubes and centrifuged at 11 000 g for 10 mins, the obtained pellet was washed twice using sterile distilled water before re-suspending into 200 µl of sterile distilled water. The mixture was then boiled for 10 minutes

at 100 ^{o}C. The boiled cell lysate was immediately cooled at -20 ^{o}C for 10 minutes, followed by centrifugation at 12 000 g for 5 minutes. The supernatant was then carefully transferred into new sterile microfuge tubes and used as template DNA for PCR amplification. *E. coli* O157:H7 ATCC 35150 was used as reference strain.

3.2.6 Polymerase chain reaction amplification of *E. coli* O157:H7 isolates

PCR amplification was performed in a 25 µl reaction cocktail in a 200 µl tube with 12.5 µl of Master Mix, 0.25 µl each of forward and reverse primers, 2 µl of nuclease free water and 10µl of template DNA. The amplification was performed using thermal cycler (BIORAD, Mycycler^{TM} thermal cycler). Primer pairs used included in the amplification is listed in Table 3.3.1. The thermal conditions for the PCR were as follows: initial denaturation at 95 ^{o}C for 5 minutes, denaturation at 94 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 90 seconds, extension at 72 ^{o}C for 90 seconds, and initial extension at 72 ^{o}C for 5 minutes and the amplicons were held at 4 ^{o}C until ready for electrophoresis (Lye *et al.,* 2013).The PCR products were then subjected to gel electrophoresis using 2% (w/v) agarose gel with 0.5X Tris-borate-EDTA (TBE) buffer at 100 V for 60 minutes. The gel was stained with 5 µl ethidium bromide and 100 base pair ladder was used as the DNA size marker and the gel was visualized under UV Transiluminator (Alliance 4.7).

3.3 Results and Discussion

A total of 252 presumptive *E. coli* O157:H7 isolates were subjected to serological test for the presence of the O antigen using the O157 latex agglutination test kit. Of these, 27(~11%) were positive for the O antigen suggesting them to be *E. coli* O157:H7. The 27 isolates were further confirmed by PCR technique using two sets of primers; *RfbE* and *FlicH7* (Table 3.3.1) which targeted the $RfbE_{O157}$ and $FliC_{H7}$ genes, respectively. PCR products were then subjected to 2% agarose gel for electrophoresis which was observed at 327 and 247 base pairs respectively. Representative PCR products of some of the isolates are as shown in Figures 3.3.1 and 3.3.2.

The highest incidence of *E. coli* O157:H7 was obtained from cattle udders in Farm A with about 55% of the presumptive isolates positive for the strain (Table 3.3.2). Indeed frequency of detection of the organism is generally highest at this farm and ranged between 33.3% and 54.5% (Average 47%). In Farm B, the frequency of detection ranged from 5.9% to 17.2% (average 9.3%), while in Farm C, the frequency of detection varied from 0 to 13.3% (Average 7.5%) . Over the years several reports have shown an increased prevalence of *E. coli* O157:H7, more prominently in faecal samples of bovine, caprine and porcine origin (Johnsen *et al.,* 2001; Bouvet *et al.,* 2002; Feder *et al.,* 2003; Ezawa *et al.,* 2004; Chase-Topping *et al.,* 2007; Solomankos *et al.,* 2009; Masana *et al.,* 2010)

The above results also corroborate the findings of Mersha *et al*. (2010) and Iweriebor et al. (2015) reports. In this current study, it was observed that; the cattle udders were mostly covered in faeces and there was no form of sterilization done to them prior to milking, hence, contamination of milking machines and consequently raw milk, was probably as a result of the dirty udders and the milking machine parlours (rotary system) (Figure 3.2.2). The

recovery of *E. coli* O157:H7 from workers hands suggests an unhygienic handling of milking utensils and cattle udders by the workers. Considering the pathogenicity and the low infection dose ability of this pathogen, its presence becomes a crucial public health risk. Poor hygienic practises observed in these farms might be a contributory factor in the dissemination of this pathogen as faeces were sighted within and around the milking parlour floor, cattle udders and limbs, which pose a great deal of public health risk. A certain portion of the population in rural areas still use and consume unpasteurised milk either directly or indirectly through milk products i.e cheese, ice creams and yogurts (Rahal *et al.,* 2012). Since milk supports wide range of microbial growth (Murinda *et al.,* 2004; Oliver *et al.,* 2005); pasteurisation is thus an important alternative in the control of milk-borne pathogens that threatens public health (Holsinger *et al.,* 1997).

Table 3.3.1: Primer sequences and expected size of PCR amplified genes targeted in the isolates.

Primer	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
*FliC*H7	TACCATCGCAAAAGCAACTCC GTCGGCAACGTTAGTGATACC	$fliC_{H7}$	247	Wang *et al.,* 2002
*Rfb*E	CTACAGGTGAAGGTGGAATGG ATTCCTCTCTTTCCTCTGCGG	$rfbE_{O157}$	327	Nazemi *et al.,* 2012

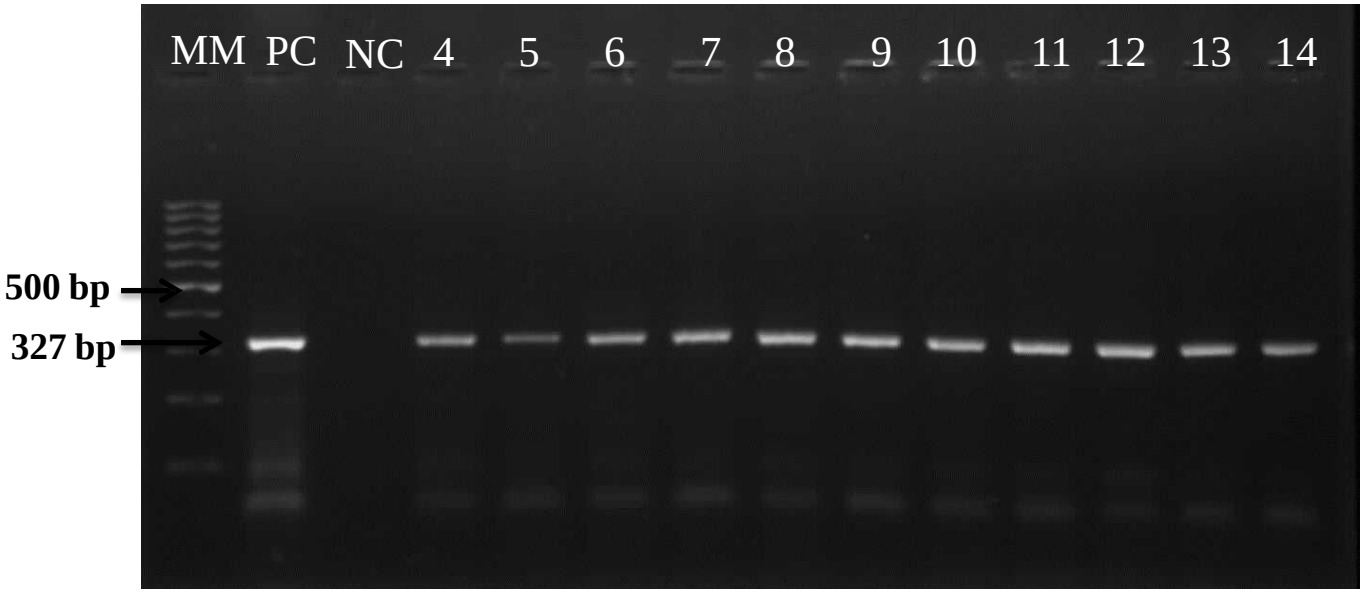

Figure 3.3.1: The amplified RfbE genes of *E. coli* O157:H7 isolated from the three dairy farms. Lane 1: **MM**= 100 bp Molecular Marker; Lane 2: **PC**= Positive Control; Lane 3: **NC**= Negative Control; Lanes **4** to **14**= some of the positive *E. coli* O157:H7 isolates obtained from raw milk, milking machines, cattle udder and worker's hand swabs across the three farms.

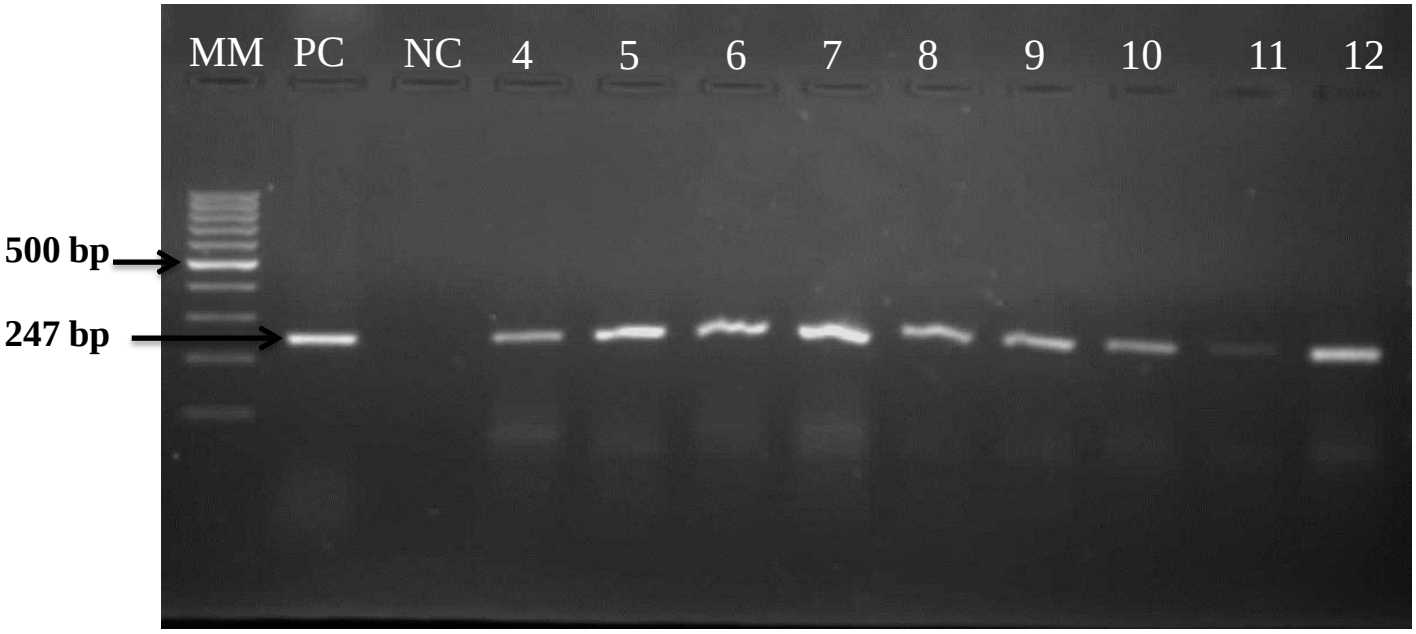

Figure 3.3.2: The amplified fliCH7 genes of *E. coli* O157:H7 isolated from the three dairy farms. Lane 1: **MM**= 100 bp Molecular Marker; Lane 2: **PC**= Positive Control; Lane 3: **NC**= Negative Control; Lanes **4** to **12**= some of the positive *E. coli* O157:H7 isolates obtained from raw milk, milking machines, cattle udder and worker's hand swabs

Table 3.3.2: Frequency of occurrence of *E. coli* O157:H7 in samples collected from the different farms.

Farm (s)	**Frequency (%) of *E. coli* O157:H7 based on population of screened presumptive isolates**				
	Raw milk	Milking machine	Udder	Hand	Average
A	50	50	54.4	33.3	47
B	7.7	6.3	17.4	5.9	9.3
C	3.6	13.3	12.9	0	7.5

3.4 Conclusion

It is apparent that the three commercial dairy farms studied are reservoirs of *E. coli* O157:H7 pathogen through the raw milk, udder, utensils and workers hands. The necessity for best hygienic practices in the three farms cannot be overemphasized. Consequently, we recommend the consumption of properly pasteurised milk and milk products rather than the unpasteurized products, as well as ensure proper sanitation in the dairy industries to safeguard public health.

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CHAPTER FOUR

Antimicrobial susceptibility profiles of *Escherichia coli* 0157:H7 isolates recovered from three selected dairy farms in the Eastern Cape Province, South Africa.

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Abstract

E. coli O157:H7 is one of the most imperious foodborne pathogens liable for a number of mortalities and hospitalizations worldwide. In this study; we elucidated the antimicrobial susceptibility profiles of the *E. coli* O157:H7 isolates recovered from raw milk samples, cattle udder, milking machines and worker's hand swabs from three selected commercial dairy farms using disc diffusion method of Kirby-Bauer recommended by the Clinical and Laboratory Standards Institute. A total of 27 *E. coli* O157:H7 isolates obtained from three dairy farms; were subjected to susceptibility test. The phenotypic antibiotic susceptibility profiles revealed that the bacterial isolates were susceptible to the antimicrobials in the following proportions: amikacin (70%), Doxycline (66%), cefotaxime (66%) and gentamycin (48%). Nonetheless; multidrug resistance was obtained with as high as 85 and 81% of the isolates resistant against penicillin G and tetracycline antibiotics respectively. Our findings also showed about 70% of the isolates showed resistance against erythromycin, while 52% of the isolates were resistant against streptomycin. We conclude that *E. coli* O157:H7 exhibits multidrug resistance and consequently a challenge topublic health issue and a burden to clinical medicine.

Keywords: Antimicrobial susceptibilty, *E. coli* O157:H7, Multidrug-resistance, Dairy farms

4.1 Introduction

The emergence of *E. coli* O157:H7 serotype dates back to 1982 where it was first discovered in an outbreak traced to contaminated Hamburgers (Igwe *et al.,* 2015). Ever since its discovery to date; *E. coli* O157:H7 remains one of the most imperious foodborne pathogens, known to cause bloody diarrhoea, haemolytic uremic syndrome (HUS) and hemorrhagic colitis in humans almost everywhere in the world (Mersha *et al.,* 2010).

Antimicrobial resistance has developed as an alarming health concern over time (Popowska *et al.,* 2012). The Enterobacteriaceae, such as *E. coli* (with its variants) and some *Klebsiella* spp., produce different β-lactamase enzymes, some of which have activity against penicillin as well as 2nd and 3rd generation cephalosporins. They however; have been reported to have improved their β-lactamases activity in recent years with the capability to hydrolyze the extended spectrum cephalosporin (ESCs) which led to the rapid evolution of extended spectrum β-lactamases (EBLSs) with a capacity to confer resistance towards β-lactamase and non-penicillin antibiotics (Paterson and Bonomo, 2005; Thenmozhi *et al.,* 2014; Trivedi *et al.,* 2015). It is quite apparent that resistant bacteria evolves naturally when these bacterial strains self-replicate spontaneously or horizontally through genetic transfer mechanisms by microorganisms with resistant characteristics in conjunction with those that do not (Vadhana *et al.,* 2015). The multi-drug resistant (MDR) isolates, particularly *E. coli* have shown an alarming increase and wide resistance capability to broad-spectrum antimicrobials which are consequent causes of treatment failures, resulting to high mortality rates (Trivedi *et al.,* 2015; Coates *et al.,* 2002).

To catalyse production among dairy farms, it has been established that, most farmers tend to use antibiotics as growth promoters which may have a different intercourse in the animal

somehow enabling the development of resistance to some bacteria (Reuben *et al.,* 2013). Agricultural practises and the abnormal use of antimicrobials in veterinary medicine often promote the antimicrobial resistant bacteria and their positive selective pressure (Popowska *et al.,* 2012). Inadequate clinical waste treatment may contribute in the prevalence and persistence of antimicrobial resistant bacteria and antibiotic residues in the environment which is a major concern (Kümmerer, 2008).

A number of studies suggest that the exploitative use of antimicrobial agents in humans and animals may support the increased resistance patterns by *E. coli* strains including O157:H7, to antimicrobials (Iweriebor *et al.,* 2015). Antimicrobial resistance (AMR) is reported as a massive setback towards effective prevention and treatment of the ever-increasing infections by bacteria, parasites, fungi and viruses which is a global threat and a worrisome concern to the world of medicine (WHO, 2014). The use of antibiotics for growth promotion, and antimicrobial agents in dairy farms solely as treatment against *E. coli* is common in the Eastern Cape Province with the ever-increasing development of antimicrobial resistance by this bacteria (Iweriebor *et al.,* 2015), hence this study is aimed at elucidating the antimicrobial susceptibility profiles of the confirmed *E. coli* O157:H7 isolates recovered from three selected dairy farms in the Eastern Cape Province, South Africa.

4.2 Materials and Methods

4.2.1 Description of the study areas

Three commercial dairy farms under the Amathole District Municipality in the Eastern Cape Province, South Africa were used for this study and for confidentiality purpose are identified as farms A, B and C respectively.

Dairy farm A is surrounded by a number of villages and peri urban settlements. It is located on the geographical coordinates 32°37′ 0”S and 27°07′ 0” E. This Dairy covers about 700 hectares of land with about 400 cows, a production capacity of 2000 litres of milk a day and 36 workers. Dairy farm B is located on the geographical coordinates of 32° 49' 0" S and 26° 59' 0" E and covers a terrain of about 280 hectares of land with 600 cows producing 2 000 litres of milk per day and has 16 permanent workers.

Dairy farm C is situated along the geographical coordinates of 32° 47' 0" S, 26° 50' 0" E. About 800 cows are milked daily in the farm which produces an estimate of 10 000 litres milk per day and has a total of 10 full time workers. It supports both the local region and other regions abroad the Amathole District Municipality borders with its produce.

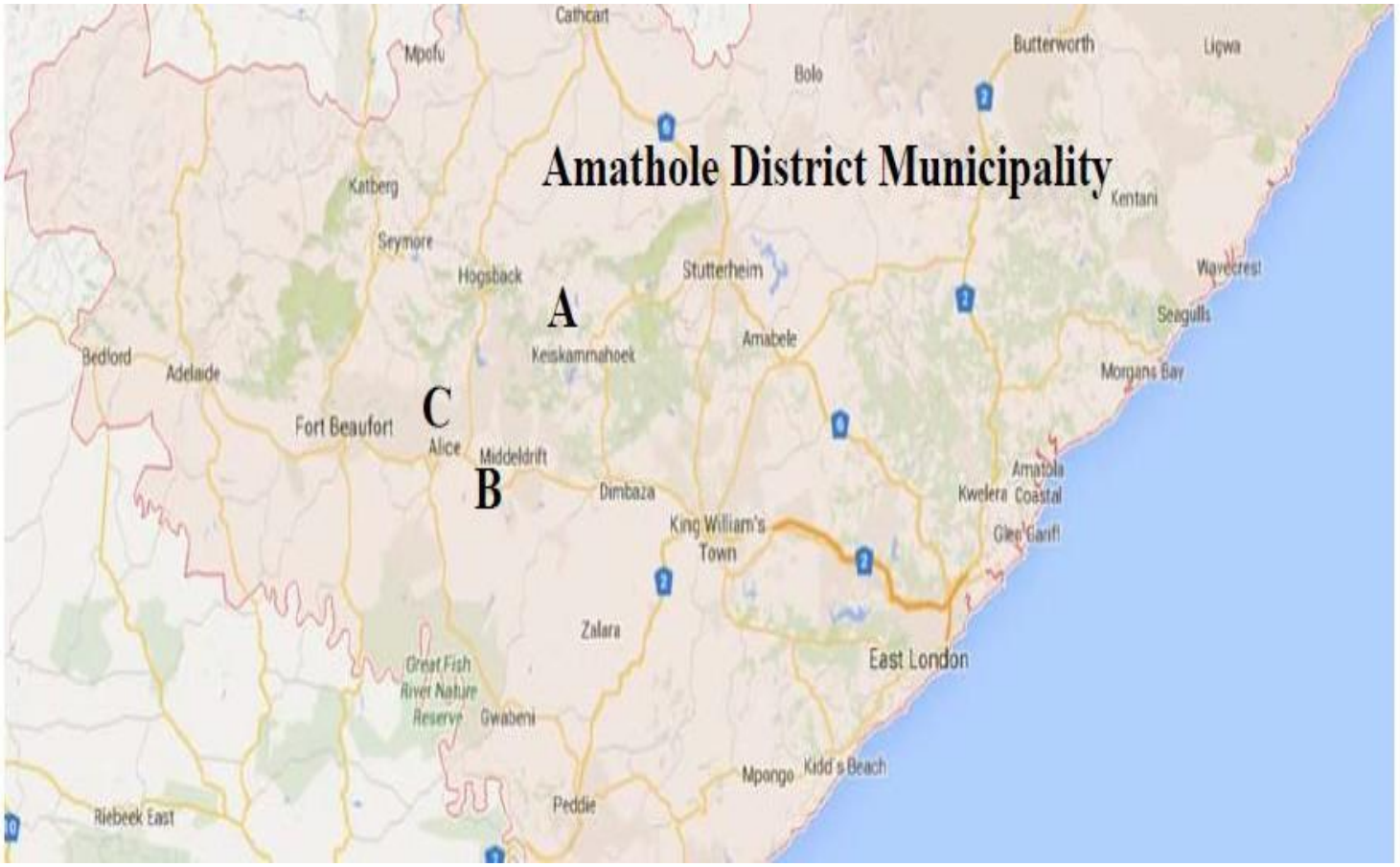

Figure 4.2.1: Map of the three sampling locations (A, B and C) in the Amathole District Municipality, Eastern Cape Province, South Africa.

4.2.2 Sample collection

Samples were collected forthnightly over a period of six months (June to November 2014). Samples included raw bovine milk samples from farm bulk storage tanks and were collected using pre-sterilized 50 ml centrifuge tubes (3 tubes for each farm), while sterile swabs-sticks (COPAN GROUP: COPAN, Italia) were used to collect samples from milking machines, udder and hands of workers, and all samples were appropriately labelling. Samples were then transported on ice to the Applied and Environmental Microbiology Research Group (AEMREG) Laboratory at the University of Fort Hare and analysed within 6 hours of collection.

4.2.3 Isolation and identification of *E. coli* O157:H7 isolates

Isolation of *E. coli* O157:H7 from raw milk samples was done following the protocol of Ateba and Mbewe (2011) with some modifications. For raw milk samples, tenfold dilutions (from 10^{-1} to 10^{-3}) of milk were made using sterile saline water, where 1 ml of raw milk sample was transferred into 9 ml of sterile saline water (10^{-1} first dilution) and another 1 ml from the 10^{-1} dilution was transferred into another 9 ml of sterile saline water, and the process repeated until 10^{-4} dilution is reached. One hundred microliter from each dilution was immediately spread-plated (in triplicates) on sorbitol- MacConkey agar plates supplemented with cefixime (50 µg/l) and potassium tellurite (25 mg/l) for the detection of *E. coli* O157:H7 and then incubated at 37 ^{o}C overnight. Colonies that appear colourless or exhibited a beige colour on the agar were considered as presumptive *E. coli* O157:H7 positive isolates.

In the same vein, swab samples from milking machines, cattle udders and from the hands of workers collected across the three farms were inoculated into 10 ml of Tryptone Soy Broth

and incubated on a shaker at 37 ^{o}C overnight at 150 rpm. At the end of the incubation period, 100 µl of each turbid culture was plated on sorbitol McConkey agar plates supplemented with cefixime (50 µg/l) and potassium tellurite (25 mg/l) using spread plate technique and incubated at 37 ^{o}C overnight. Colourless or beige colonies were picked as presumptive *E. coli* O157:H7 isolates. The presumptive *E. coli* O157:H7 were purified by repeated aseptic transfer onto fresh nutrient agar plates to obtain pure isolates and stored them on sterile 25% glycerol stock.

Polymerase chain reaction (PCR) amplification target the two specific genes *rfb*E and *fliC*H7 and latex agglutination test (*E. coli* O157 Latex Test Kit, Oxoid) were conducted for the confirmation of the presumptive *E. coli* O157:H7 isolates.

4.2.4 Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed on Mueller-Hinton agar (MHA) using the standard disc diffusion method of Kirby-Bauer recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014). Briefly, fresh isolates from sorbitol-MacConkey agar plates were plated on nutrient agar and incubated at 37 ^{o}C for 18 to 24 h. After incubation, a loopful of colonies was inoculated on normal saline to make up a bacterial suspension adjusted to 0.5 McFarland standards. A sterile swab-stick was then deepened into the prepared bacterial suspension and spread evenly on the entire surface of MHA plates and allowed to stand for about 15 minutes. Thereafter, different antibiotic discs (Table 4.4.1) were placed equidistance on the lawn of bacteria and the plates incubated at 37 ^{o}C for 18 to 24 h. After incubation the plates were examined for zones of inhibition and interpreted based on the interpretation standard of the Clinical and Laboratory Standard Institute (CLSI, 2014). These antibiotics are

frequently used in the treatment of *E. coli* O157:H7 related illnesses, thus they were chosen for this study design.

Table 4.2.1: Antimicrobial discs used for *E. coli* O157:H7 susceptibility test.

Antimicrobial agent	**Code**	**Disk Content (µg)**
Amikacin	AK	30
Streptomycin	S	10
Gentamycin	GM	10
Tetracycline	T	30
Doxycycline	DO	30
Oxytetracycline	OT	30
Cephalothin	KF	30
Cefotaxime	CTX	30
Cefoperazone	CFP	75
Chloramphenicol	C	30
Ampicillin	AMP	10
Penicillin G	PG	10
Polymoxin B	PB	300 (units)
Erythromycin	E	15
Sulfamethazole/Trimethoprim	TS	25
Trimethoprim	TM	25
Sulfamethaxozole	SMX	25

4.3 Results and Discussion

A total of 252 presumptive *Escherichia coli* O157:H7 isolates were obtained from three dairy farms (A, B and C); only 27 (~11%) isolates were confirmed as positive *Escherichia coli* O157:H7 and these were further assessed for their antimicrobial susceptibility profiles. The results showed multidrug resistance against penicillin (85%), tetracycline (81%), erythromycin (70%), streptomycin (52%) and chloramphenicol (45%) (Table 4.3.1). Highest resistances were obtained against penicillin (85%) and tetracycline (81%) respectively (Table 4.4.2). Information gathered from the three dairy farms had that, these farms use both penicillin and tetracycline excessively; hence the high resistances obtained in our study which suggests an urgent intervention as to improve general well-being and diminish public health risks. According to Popowska *et al.* (2012), most antibiotics are partially degraded in waste-treatment plants with tetracycline and erythromycin not degraded which suggests the accessibility of such residues in the environment and hence high resistances were exhibited.

However; with the isolates were susceptible to some of the antibiotics in the following proportions: amikacin approximately (70%), doxycycline (66%), cefotaxime (66%) and gentamycin (48%) (Table 4.4.2). A similar study conducted by Iweriebor *et al.* (2015) reported high prevalence of multidrug resistance to various antimicrobial agents among 95 *E. coli* O157:H7 isolates obtained from dairy cattle faeces in the Eastern Cape Province, South Africa. According to the report; resistance was observed in the following propotions: chloramphenicol (90 %), ampicillin (95%), tetracycline (97) %, oxytetracycline (95 %), cefuroxime (82 %), cephalothin (95%), streptomycin (84. %) and trimethoprim/sulfamethazole (84%). A phenomenon referred to as horizontal gene transfer could be responsible for dispensing resistance genes to susceptible bacteria via bacterial

plasmid, which may elevate the tenacity of antimicrobial resistant bacteria in the environment and thus a great public health risk factor (Nontongana *et al.,* 2014; Vadhana *et al.,* 2015; Trivedi *et al.,* 2015).

Table 4.3.1: Antimicrobial susceptibility patterns of *E. coli* O157:H7 isolates collected from the three dairy farms (n=27)**.**

Antibiotic class	**Antimicrobial agent**	**Code**	**Concentration (µg)**	**Isolates n=27 (%)**		
				S	**I**	**R**
Aminoglycosides	Amikacin	AK	30	19(70)	5(19)	3(11)
	Streptomycin	S	10	12(44)	1(4)	14(52)
	Gentamycin	GM	10	13(48)	5(19)	9(33)
Tetracyclines	Tetracycline	T	30	5(19)	0(0)	22(81)
	Doxycycline	DO	30	18(66)	5(19)	4(15)
	Oxytetracycline	OT	30	14(52)	7(26)	6(22)
Cephalospirines	Cephalothin	KF	30	17(63)	6(22)	4(15)
	Cefotaxime	CTX	30	20(74)	1(4)	6(22)
	Cefoperazone	CFP	75	16(59)	5(19)	6(22)
Phenicols	Chloramphenicol	C	30	9(33)	6(22)	12(45)
Penicillins	Ampicillin	AMP	10	3(12)	12(44)	12(44)
	Penicillin G	PG	10	0(0)	4(15)	23(85)
Polymoxins	Polymoxin B	PB	300 (units)	14(51)	5(19)	8(30)
Macrolides	Erythromycin	E	15	8(30)	0(0)	19(70)
Foliate pathway inhibitor	Sulfamethazole/Trimethoprim	TS	25	21(77)	1(4)	5(19)
	Trimethoprim	TM	25	19(70)	3(11)	5(19)
	Sulfamethaxozole	SMX	25	5(19)	2(7)	20(74)

R: Resistant, I: Intermediate, S: Susceptible,

4.4 Conclusion

This study has clearly established that Eastern Cape dairy farms harbour the multidrug resistant *E. coli* O157:H7 bacteria and potential antimicrobial resistant determinants in its environments. As these dairy farms support a huge rural and peri urban populace of the Amathole region, these findings indicate a worrisome concern with regards to public health and well-being, and suggest the necessity of urgent intervention; improved awareness, and effective communication and education implemented.

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CHAPTER FIVE

General Discussion

Illnesses associated with the pathogenic *E. coli* O157:H7 serotype has been reported almost everywhere in the world with a very few unreported cases (CDC, 2004). This study was aimed at assessing the prevalence of *E. coli* O157:H7 in raw milk, milking machines, cattle udder and worker's hand swabs collected from three selected dairy farms in the Eastern Cape Province, South Africa. About 252 presumptive isolates were obtained from these farms, only 27 isolates which is about 11% were confirmed as *E. coli* O157:H7 using two sets of primers; *RfbE* and *FlicH7*. Previous studies have reported low prevalence of *E. coli* O157:H7 in raw milk from storage bulk tanks (Brenjchi *et al.*, 2011), however, when taking into careful consideration, the low infection dose of this pathogen (about 100 to 200 or even less than 10 cells in susceptible consumers) is still a major public health-risk concern (CFSPH, 2009; Grant *et al.*, 2011).

In this study, about 5 (19%) isolates were obtained from raw milk, another 5 (19%) from milking machines and 2 (7%) from milk handler's or workers hands compared to 15 (55%) which were isolated from cattle udders through the six month period; these findings suggest that cross-contamination may be possible during the milking process. Several authors have also highlighted that there are various factors that contribute greatly in milk contamination in dairy industries and these factors include; poor hygienic milking conditions, contaminated equipment, milking utensils and milk handlers' poor personal hygiene (Lye *et al.*, 2013), this is in line with the findings of this study as isolates were recovered from the milking utensils;

suggesting poor hygienic practises. In a similar study by Caine *et al*. (2014), about 54% prevalence of *E. coli* O157:H7 isolates was reported from raw cattle milk samples collected from selected Eastern Cape commercial dairy farms, signifying cattle as important carriers of this pathogen. A certain portion of people from rural areas still consume unpasteurised milk either directly or indirectly through milk products i.e cheese, ice creams and yogurts (Rahal *et al.,* 2012). Since milk supports wide range of microbial growth (Murinda *et al.,* 2004; Oliver *et al.,* 2005); pasteurisation is thus an important alternative in the control of milk-borne pathogens that threatens public health (Holsinger *et al.,* 1997).

Antimicrobial resistance among enteric bacteria has become a global burden over the past years, playing a fundamental role in restricting treatment options in sickness control and treatment therapy, with evidence of transmission of resistant pathogenic strains to humans through food (Reuben et al., 2013 Nontongana *et al.,* 2014).

E. coli O157:H7 isolates showed multi-drug resistance with penicillin, tetracycline, erythromycin, streptomycin and chloramphenicol in the current study, maximum reaction percentages of 85% and 81% towards penicillin and tetracycline antibiotics were observed, respectively. In a study of the same nature; Reuben *et al*. 2013 obtained similar patterns of resistance to penicillin and tetracyline with the latter being commonly used as first line drug by humans, growth promoter and routine chemoprophylaxis by various farmers among their livestock in Nigeria, which may be an explanation to the high resistance level of this antibiotic (Reuben *et al.,* 2013). In a similar study by Iweriebor *et al.* (2015) in the Eastern Cape Province, South Africa; their findings deduced that *E. coli* O157:H7 isolates from cattle faeces exhibited multi-drug resistance. This is a troublesome discovery with regards to public health and human well-being.

There is a lack of substantive treatment for *E. coli* O157:H7 infection and acute diarrheal illnesses have proven antibiotics useless and some reports suggest that antimicrobials somehow increase HUS development, by the release of enterotoxins by damaged bacteria (WHO, 2014).

One of the limitations in the incidences of *E. coli* O157:H7 is that, infected individuals tend to not seek medical attention or health care especially those in rural areas, which results in undetected or rather unknown or ignored cases. Generally, personal hygiene, consuming of pasteurized milk and milk products, sufficiently cooked foods including clean-fresh fruits and vegetables and use of sufficiently treated water for any purpose, can play a vital role in preventing EHEC infections (Kulkarni *et al.,* 2002; CFSPH, 2009; WHO, 2012; Mustafa *et al.,* 2013).

The three studied dairy farms support both rural and peri-urban communities in the Eastern Cape region, and remain as one of the major affiliates towards the province's economic growth and social welfare; however it is very troubling to enumerate such disturbing findings presented in this research which evidently highlight the amount of public and environmental health risk among other things; that the province is confronted with.

5.1 Potential for future study

Although the prevalence and the antimicrobial profiles of *E. coli* O157:H7 isolates have been thoroughly investigated in this study, yet a future research can be designed towards elucidating the antimicrobial resistance determinants in order to detect and establish the presence and distribution of resistance genetic marker(s) in the phenotypically resistant *E. coli* 0157:H7 isolates using polymerase chain reaction technique..

5.2 Conclusion and Recommendations

The findings of this research suggest that three dairy farms in the Eastern Cape Province, South Africa are reservoirs of the pathogenic and antimicrobial resistant *E. coli* O157:H7 serotype which can cause mild to severe diarrheal disease such as haemolytic uremic syndrome, haemolytic colitis, thrombotic thrombocytopenic purpura and urinary tract infections, which can eventually lead to death and/or hospitalisations.

Exploitative use of antimicrobials should be discouraged at any level of application as to ease selective pressure presented either clinically or by farmers. Continuous surveillance, extreme good quality and intensive hygiene practises among dairy farms, especially in handling of milk, milk products and utensils is highly recommended as primary preventive measures sat to public health risk.

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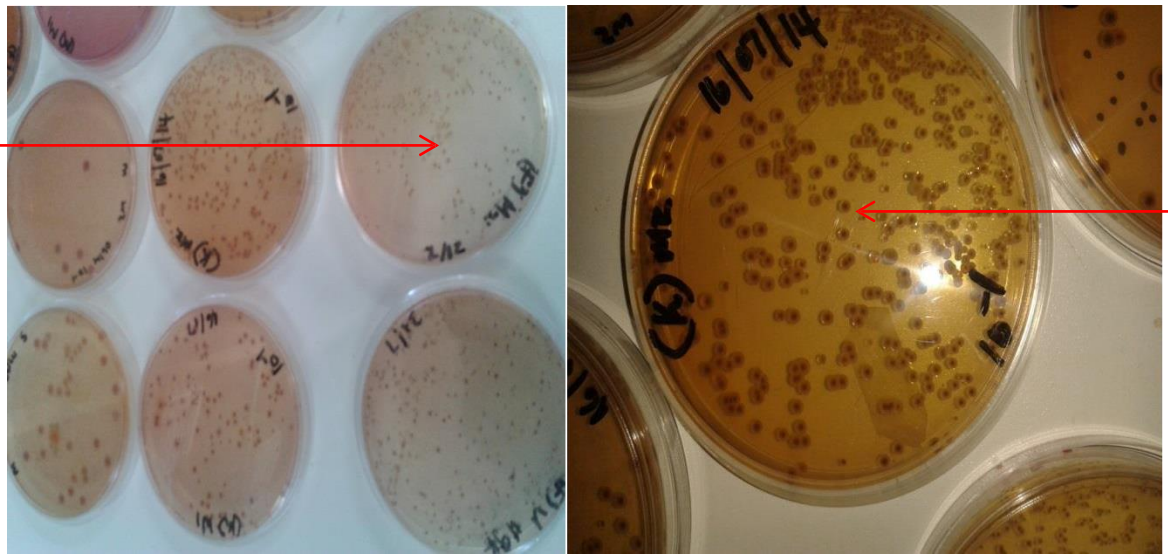
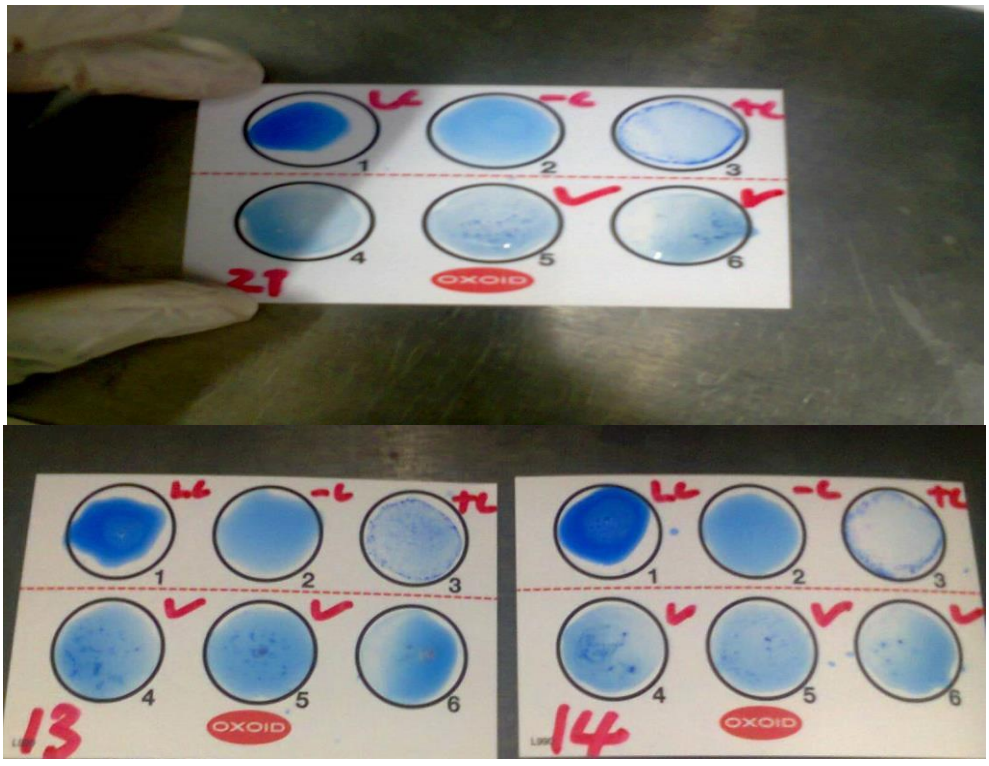
Appendices

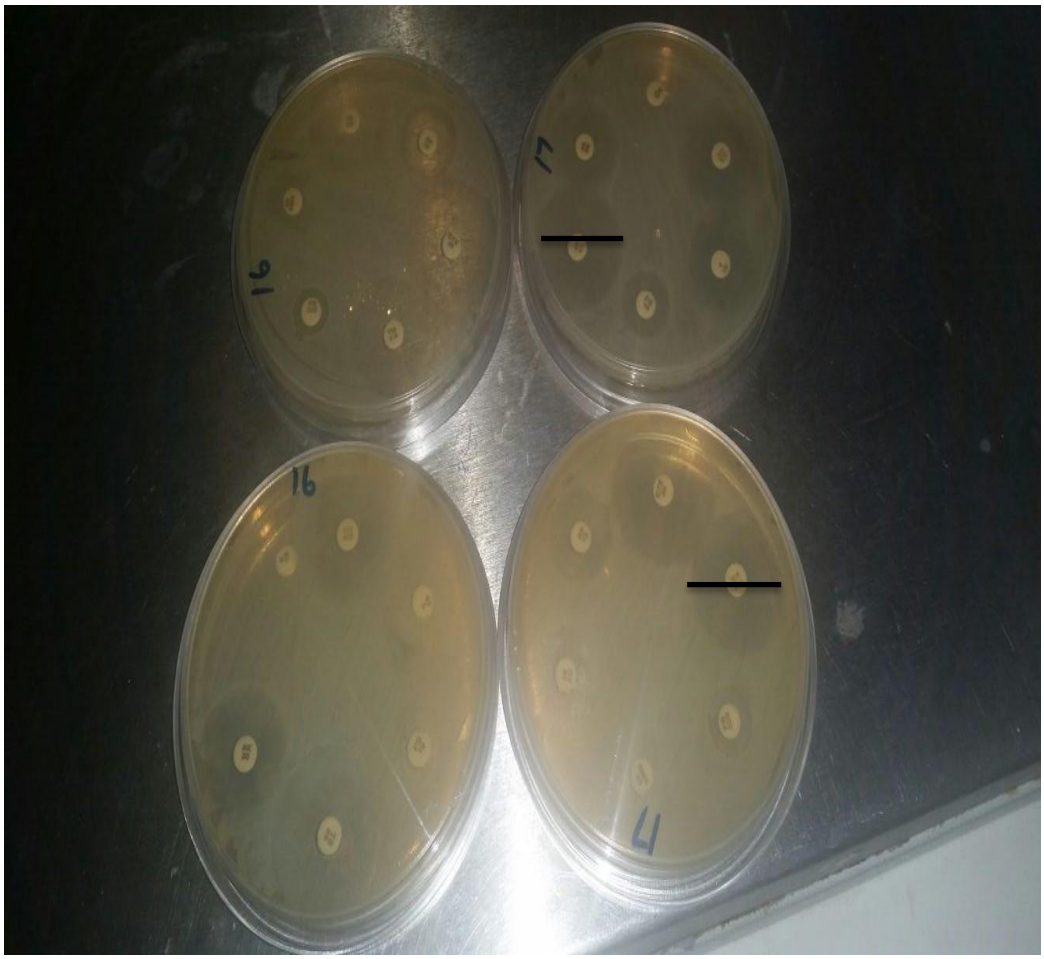


Appendex 1: Presumptive E. coli O157:H7 colonies on supplemented Sorbitol- McConkey agar.



Appendex 2 : Some of the positive results for latex agglutination test.

LC= Latex control**; -C=** Negative Control**; +C=** Positive Control



Appendex 3: Antimicrobial susceptibility testing by disk diffusion method, zone of inhibition indicated with a line.

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