# Prevalence and antibiotic resistance determinants of *Escherichia coli* pathotypes obtained from raw milk in two farms from the Eastern Cape, South Africa: Public health implications

**By**

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## SUMMARY

A large numbers of people in rural areas consume raw milk and its products. People believe that raw milk and its products have advantages or value over the pasteurized one. The presence of foodborne pathogens in unpasteurized raw milk either directly or indirectly increases the risk of ingestion and transmission of food-borne pathogens and potentially harmful toxins. Microbes may gain entry into raw milk directly from dairy cows experiencing sub-clinical or clinical mastitis. Mastitis in cattle is induced when pathogenic microorganisms enter the udder through the teat canal, overcome the cow's defense mechanisms, begin to multiply in the udder, and produce toxins that are harmful to the mammary gland. The objectives of this study were to determine the prevalence of *E. coli* in unpasteurized milk recovered from Middledrift and Fort Hare dairy farms, to characterize the identified isolates into different pathotypes, to determine the antibiogram of the isolates and to characterize the genes responsible for antibiotic resistance. In this study identification of these mastitic pathogens was done by molecular based methods (polymerase chain reaction) and antibiotic susceptibility testing was done using the disk diffusion method and the genes responsible for antibiotic resistance were detected by PCR. Target genes (*fliCH7*54 (63%), *eae* 28 (32%), *lt* 27 (31%), *eagg* 12 (14%), *ial* 6 (7%), *papC* 4 (5%) and *daaE* 2 (2%)) that encode pathogenicity for *E. coli* were successfully amplified by PCR confirming that the isolates were pathogenic strains. In this study multiple resistances of the pathogenic *E.coli* isolates were found in the tested antimicrobial agents. Penicillin G and erythromycin showed 100% resistance. The *strA* gene which encodes for streptomycin and the *tetA* gene which encodes for tetracycline were not present in the isolates using conventional PCR. The results in this study demonstrates that there is a widespread distribution of potentially virulent *E. coli* strains in the environment and in food that may be a cause of concern for human health and

the phenotypic showed total resistance to erythromycin and penicillin G. Monitoring of mastitis and antimicrobial resistance (AMR) in bacteria is of great importance and has clinical and public health significance.

**DECLARATION**

I, Lesley-Anne Caine, hereby declare that the work contained in this project is my own original work and that I have not previously, in it's entirely or in part, submitted it at any other university for a degree.

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Lesley-Anne Caine

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Date

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DR E Green

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Co-supervisor

**DEDICATION**

I dedicate this work to my precious daughter Lyndre' Yayama Caine whom I love dearly, who could not spend time with me because I had to do my research.

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# CHAPTER ONE

## 1.1 GENERAL INTRODUCTION

Raw unpasteurized milk is a white liquid produced by the mammary glands of cows and it is the primary source of nutrition to calves and some humans. Raw milk is a well-known good medium that supports the growth and multiplication of many kinds of microorganisms due to its complex biochemical composition and high water activity (Murinda *et al*., 2004; Oliver *et al*., 2005). It is well known that freshly obtained milk contain some bacteria and somatic cells, which constitute the biological constituents of the milk, which easily change depending on production conditions, such as the health status of the cattle and hygiene practices during milking as well as keeping and transportation of milk and milk products (Turner and Veary, 1990, Lues *et al*., 2010). Pasteurization of milk is a key component in control of milk-borne pathogens that threaten public health (Holsinger *et al*., 1997). It was originally developed for the control of infection due to brucellosis and tuberculosis from infected cattle, but also controls a wide range of other human pathogens (Tetra Pak Processing System AB, 1995). A large number of people in rural areas consume raw milk and raw milk products via consumption of several types of cheeses. People believe that raw milk and their products have advantages or value over the pasteurized one (Oliver *et al*., 2009; Guh *et al*., 2010). The presence of food-borne pathogens in unpasteurized raw milk either directly or indirectly increases the risk of ingestion and transmission of food-borne pathogens and ingestion of potentially harmful toxins. Microbes may gain entry into raw milk directly from dairy cows experiencing sub clinical or clinical mastitis (Rodojcic-Prodaova and Necev, 1991).

Mastitis is defined as an inflammation of the mammary gland (International Dairy Federation, 1987). It is induced when pathogenic microorganisms enter the udder through the teat canal, overcome the cow's defense mechanisms, begin to multiply in the udder, and produce toxins that are harmful to the mammary gland. Mammary tissue is then damaged, which causes increased vascular permeability. Mastitis can occur in all mammals, including humans (Hurley and Morrin, 1997, Oliveirra *et al.*, 2000).

Mastitis can either be clinical or subclinical. Clinical mastitis occurs when visible sign of inflammation is observed in the udder of the cow or the teats. The clinical case could be subacute, where the symptoms are very mild and may only be accompanied by slight swelling of the udder and the presence of the flakes in the milk. An acute or peracute case of clinical mastitis may occur where a sudden onset of symptoms such as severe inflammation of the teats, fever, loss of appetite, dehydration and even death occurs. Clinical mastitis will eventually lead to chronic mastitis if not treated. In cases of chronic mastitis, the cow will suffer from a constant infection oscillating between sub-acute and acute clinical mastitis. A permanent change in the udder may occur with the presence of scar tissue and a change in the shape and size of the glandular tissue (Giesecke *et al*., 1994; Philpot and Nickerson, 1999; Akers, 2002). Subclinical mastitis proceeds clinical mastitis and is more subtle in that no signs of infection are visible. However, special screening tests, such as California Mastitis Test (CMT), Wisconsin Mastitis Test (WMT) and the catalase test are utilized to show changes in the milk. This type of mastitis is referred to as "hidden". Most dairy herds will have cows with subclinical mastitis. The only way to detect the presence of infecting microorganisms invading the teat canal and udder tissue is to monitor the inflammatory response of the cows, i.e. quantification of the somatic cells in the milk (Giesecke *et al*., 1994; Philpot and Nickerson, 1999). Subclinical mastitis is considered more severe than

clinical mastitis, as early detection is impossible without regular monitoring. Cows with subclinical mastitis harbor a constant reservoir of pathogens that could lead to severe udder infection and spreading to other cows (Philpot and Nickerson, 1999). Many types of microbes can cause the infection and can be transmitted through both environmental sources (for example, contaminated water, soil, bedding) and from contagious sources (from other infected cows). Bacteria (pathogens) (or other dairy animals) can be a source of disease causing mastitis in cows and spoilage organisms in milk.

Microorganisms that are responsible for mastitis and spoilage of milk are among others *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, Mycoplasma species, *Streptococcus uberis* (Erskine, 2002), coliforms (*Escherichia coli*, (*E. coli*) Klebsiella species and *Enterobacter aerogenes*), Serratia, Pseudomonas, Proteus species, environmental Streptococci, Enterobacter species (Smith and Hogen, 1993; Quinn *et al*., 2002). Identification of theses pathogens could be done by phenotypic, genotypic or serotype based methods (Christen *et al*., 1993; Paton and Paton, 1998; Vidal *et al*., 2004; Todar, 2008). The consequences of a milk-borne infection may be limited to the common symptoms of diarrhoea, vomiting, nausea, fever, abdominal cramps etc., but a certain percentage of persons can develop more severe clinical symptoms such as Guillian-Barre' syndrome (*Camylobacter spp*.) and haemolytic uremic syndrome (HUS) (*E. coli* 0157:H7) or long term and sometimes chronic complications e.g. reactive arthritis or even death (Griffiths, 2010).

### 1.2 Statement of the problem

*E. coli* is an environmental pathogen found in the immediate surroundings of the cow such as the soil, grass, manure and the bedding of housed cows. It is therefore easy for the organism

to be found in the udder of cow thereby gaining entrance to the milk (Hogan and Smith, 2003). Its status as a true pathogen is of public health significance. It produces toxins that destroy cell membrane and can directly damage milk-producing tissues which can lead to bovine mastitis (Jones *et al*., 1998). The high prevalence of intramammary *E. coli* infection indicates the potential of a food hazard, which could have grave consequence to those who consume raw milk. Antimicrobials are used frequently for treatment and prevention of bovine mastitis. Therefore to successfully control mastitis and to circumvent potential problems associated with bacterial resistance and treatment failure, it is important to study antimicrobial resistance profiles of mastitis pathogens, especially so as there are seemingly no such studies reported in the environment of the Eastern Cape Province.

**1.3 Hypothesis-**

Dairy milk is a point source of pathogenic *E. coli* strains.

**1.4 Aim-**

The study aims at assessing the microbiological quality of *Escherichia coli* in raw unpasteurized milk and to detect the genes that mediate resistance.

**1.5 Objectives:**

- To determine the prevalence of *E. coli* in unpasteurized milk recovered from Middledrift and Fort Hare dairy.
- To characterize the identified isolates into different pathotypes.
- To determine the antibiogram of the pathotypes and to characterize the genes responsible for antibiotic resistance.

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# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 *Escherichia coli* (*E. coli*)

The genus *Escherichia* derives its name from Theodor Escherich, who first isolated this organism from feces in 1885. *E. coli* is a member of the *Enterobacteriaceae* (the intestinal bacteria) and belongs to the order *Enterobacteriales* and Kingdom Eubacteria (Berg, 1978). These bacteria are facultative anaerobes, Gram negative short rods that can grow under both aerobic and anaerobic conditions (Chapelle, 2001). If molecular oxygen is available, the bacteria rely on respiratory metabolism to survive, while the absence of molecular oxygen; the organisms use fermentation as an alternate means of survival (Berg, 2000; Chapelle, 2001). Bacteria belonging to the genus Escherichia are motile by means of peritrichous/multiple flagellum and are unable to form spores to survive unfavorable environmental conditions (Todar, 2008). *E. coli* typically colonizes gastrointestinal tract of humans and animals within a few hours after birth. Usually *E. coli* and its human host coexist in good health and with mutual benefit for decades. These commensal *E. coli* strains rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached (Kaper *et al*., 2004) *E. coli* is recognized as an important zoonotic pathogen, not because of the number of cases of human illness it causes, but by the serious and life threatening disease that a small number of *E. coli* strains may cause. Outbreaks of pathogenic *E. coli* like the German outbreak in 2011 and the outbreak related to Godstone Farm in Surrey in 2009 have a high profile in the media as a result of the serious illness

caused. The number of cases of foodborne disease associated with *E. coli* in the UK is around a thousand, dwarfed by an estimated 700,000 cases of *Campylobacter* infection. Although infection with pathogenic *E. coli* is rare in the UK, it is the serious and sometimes fatal diseases it may cause that give it a high importance (Pennington, 2010).

## 2.2 *E. coli* virulence factors

Pathogenic *E. coli* bacteria are typically specific to the type of disease they cause and to the animal species infected. They produce virulence factors involved in pathogenesis of specific diseases (Kaper *et al*., 2004). Bacterial virulence factors are required to colonize and infect the host and to fight host defense mechanisms. Major groups of *E. coli* virulence factors include toxins, adhesins, proteins secreted into host cells, polysaccharide capsules and O-antigens, and other mechanisms to resist killing by complement or to scavenge iron (Keller *et al*., 2002). Bacteria do not produce virulence factors constantly but only upon receiving certain signals from the host or environment (Keller *et al*., 2002). The genes for virulence factors may be present in the bacterial genome or may reside extrachromosomally on plasmids, even though the virulence factor is not produced (Harel and Martin, 1999; Kaper *et al*., 2004, Smith *et al*., 2007).

### 2.2.1 Adhesins

Adhesins are either hair-like appendages known as fimbriae or pili, or afimbrial adhesins associated with the cell surface (Soto and Hultgren, 1999; Kaper *et al*., 2004). Bacteria need adhesins to adhere to and colonize the host cell surface. Different adhesins have been detected in *E. coli* isolates associated with bovine diseases. The family of F17 fimbriae

includes F17a expressed by bovine enterotoxigenic *E.coli*, F17b expressed by *E. coli* isolated from septicaemic and diarrhoeic calves and lambs, F17c isolated from septicaemic *E. coli* strains and F17d described in bovine enterotoxigenic *E. coli* and *E. coli* isolated from diarrhoeic calves (Le Bouguenec and Bertin, 1999). The F17 fimbriae are often associated with other virulence factors in pathogenic *E. coli*. Genes encoding F17 fimbriae are located on the chromosome (Bertin *et al*., 1996; Treng *et al*., 2002). In the study of Pohl and Mainil (1995), almost half of the F17-positive *E. coli* strains were resistant to complement and produced aerobactin. S and P fimbriae are usually found in *E. coli* strains causing urinary tract infection (Soto and Hultgren, 1999). They are probably needed for adherence of bacteria to urinary tract epithelia and are suggested to be involved in recurrent urinary tract infections (Schilling *et al*., 2001). Genes encoding S and P fimbriae are located on the chromosome (Mainil *et al*., 1997). Afimbrial adhesins are encoded by different *afa* genes; afa-7 and afa-8 have been found in bovine isolates, afa-8 being more common than afa-7 (Lalioui *et al*., 1999). Afa-8 genes have been detected on plasmids, which also carried genes for F17c and cytotoxic necrotizing factor 2 (**CNF2**) (Le Bouguenec and Bertin, 1999). Another afimbrial adhesin found in bovine pathogenic *E. coli* strains is the capsule-like structure called CS31A adhesin (Le Bouguenec and Bertin, 1999). It has been described in *E. coli* strains from calves with septicaemia or diarrhoea (Contrepois *et al*., 1986; Mercado *et al*., 2003). The genes encoding CS31A have been detected on large plasmids carrying genes for other virulence factors, such as aerobactin and F17c fimbriae, as well as for antimicrobial resistance (Contrepois *et al*., 1986).

### 2.2.2 Toxins

Pathogenic *E. coli* strains may produce a variety of toxins with different activities: heat-labile (**LT**) and heat-stable (**ST**) enterotoxins, shiga toxins and cytotoxic necrotizing factors 1 and 2 (**CNF1** and **CNF2**). Shiga toxins are produced by enterohaemorrhagic *E. coli* strains (Riley *et al*., 1983; De Rycke and Oswald, 2001). Enterotoxigenic strains usually produce two enterotoxins, LT and ST, which have distinct roles in the pathogenesis. *E. coli* isolates producing CNF2 are common in cattle (Pohl *et al*., 1993). Many of these strains also produce F17b fimbriae and aerobactin and are resistant to the killing action of serum. The CNF1-positive strains are typically isolated from extraintestinal infections and are mainly positive for adhesins of the P and S fimbrial families or the Afa family (Mainil *et al*., 1999; Bertin *et al*., 1998). The production of CNF1 is chromosomally encoded, whereas CNF2 is encoded by genes located on a plasmid (Falbo *et al*., 1992; Mainil *et al*., 1999).

### 2.2.3 Aerobactin

Iron is an essential element for the survival and multiplication of *E. coli*. Aerobactin is one of the siderophores present in Gram-negative bacteria. It chelates iron, making it available for bacterial use. The gene for aerobactin is located either on a chromosome or on a plasmid, where many of the antimicrobial resistance genes are also located (Vidotto *et al*., 1991; Johnson *et al*., 2002).

### 2.2.4 Factors conferring serum resistance

Serum resistance is one of the most studied virulence properties of Gram-negative bacteria. Resistance to the killing action of serum is linked to structures at or near the bacterial surface. Capsule or outer membrane proteins, like OmpA and TraT, have been suggested to interfere with complement components and the membrane attack complex, preventing the killing action (Ward and Sebunya, 1981; Taylor, 1983; Weiser and Gotschich, 1991; Pramoonjago *et al*., 1992). TraT is a cell surface-exposed lipoprotein, which is assumed to inhibit the correct assembly or membrane insertion of the membrane attack complex of complement (Sukupolvi and O'Connors, 1990). TraT is encoded by the *traT* gene carried on large conjugative plasmids. The K1 capsular antigen protects *E. coli* from the killing effect of serum and has been thought to co-operate with TraT (Montenegro *et al*., 1985) However, in clinical studies (Nemeth *et al*., 1991; Wooley *et al*., 1993; Pfaff McDonough *et al*., 2000; Todar, 2008), no relation of K1 to serum resistance or the *traT* gene could be detected.

## 2.3 PATHOGENIC TYPES OF *E. coli*

Certain isolates of *E. coli* have been implicated in a wide range of diseases that affect either animals or humans worldwide. Pathogenic *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis) (Todar, 2008). The diseases caused by a particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defences. All diarrheagenic strains of *E. coli*

were initially termed enteropathogenic *E. coli*, but when more was learnt about their pathogenic mechanisms, they were grouped into eight pathotypes (Kaper *et al*., 2004).To date, eight pathovars and their mechanisms of disease have been extensively studied (Kaper *et al*., 2004). These pathovars can be broadly classified as either diarrheagenic *E. coli* or extraintestinal *E. coli* (ExPEC). Six pathovars - enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC; including *Shigella*), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) - are diarrhoeagenic, and two pathovars – Uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) - are the most common ExPEC isolates. Other pathovars (necrotoxigenic *E. coli*, cell detaching *E. coli* and adherent invasive *E. coli*) have been identified, but their mechanisms of pathogenesis are not as well defined (Croxen and Finlay, 2010).

### 2.3.1 ENTEROTOXIGENIC *E. coli*

Enterotoxigenic *E. coli* (ETEC) produce the *E. coli* heat-labile (LT) and/or heat-stable (ST) toxins. The ST are of low molecular size and resistant to boiling for 30 minutes (Todar, 2008; Croxen and Finaly, 2010). There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or STh is predominant in human isolates only. The ST enterotoxins are peptides of molecular weight about 4,000 daltons. Their small size explains why they are not inactivated by heat. The LTs are high-molecular-weight proteins similar to cholera toxin and consist of five enterocyte-binding B subunits and a biologically active A subunit. The internalized A subunit causes electrolyte imbalance and a net fluid loss to the gut lumen by activation of adenylatecyclase and accumulation of cyclic adenosine monophosphate. ETEC are important causes of diarrhoea among children in

developing countries and of traveler's diarrhoea (Kaper *et al*., 2004). Symptoms include acute watery diarrhoea that may be mild and of short duration and which in some cases is similar to cholera. ETEC strains are host specific, that is, some strains can cause diarrhoea in young animals (e.g., calves, lambs, and piglets), while other strains will specifically infect only piglets, and others only humans. The prevention of the spread of this strain of diarrheagenic *E. coli* depends on ensuring appropriate sanitary measures; hand-washing and proper preparation of food; chlorination of water supplies; and appropriate sewage treatment and disposal. Parenteral or oral fluid and electrolyte replacement is used to prevent dehydration, and broad-spectrum antibiotics are used in chronic or life-threatening cases, but in most cases, should be avoided because of severe side effects. The major virulence factors of ETEC are intestinal colonization factors, for example, fimbriae, and the enterotoxins (Todar, 2008).The host specificity of individual strains is determined by the type of colonization factors and fimbriae produced (Kaper and Nataro, 1998).

### 2.3.2 ENTEROPATHOGENIC *E. coli*

Enteropathogenic *E. coli* (EPEC) is a major cause of potentially fatal diarrhoea in infants in developing countries (Kaper *et al*., 2004). This pathovar belongs to a family of pathogens that form attaching and effacing (A/E) lesions on intestinal epithelial cells; other members of the family include EHEC, rabbit diarrhoeagenic *E. coli* (RDEC), the murine pathogen *Citrobacter rodentium* and the recently identified *Escherichia albertii* (formerly known as *Hafnia alvei*), a pathogen that is associated with diarrhoea in humans. EPEC induce a profuse watery, sometimes bloody, diarrhoea. Outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Pathogenesis of EPEC involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized

adherence of bacteria to intestinal cells and a non fimbrial adhesin designated intimin, which is an outer membrane protein that mediates the final stages of adherence. They do not produce ST or LT toxins (Todar, 2008). EPEC strains are said to be "moderately-invasive", meaning they are not as invasive as *Shigella*, and unlike ETEC or EAEC, they cause an inflammatory response. The diarrhoea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins (Todar, 2008).

### 2.3.3 ENTEROINVASIVE *E. coli*

It is generally accepted that EIEC and *Shigella* should form a single pathovar, because they have the same mechanisms of pathogenicity. However, the genus name *Shigella* is still used owing to its association with the disease shigellosis and is retained in this section. *Shigella* are highly infectious bacteria that cause bacillary dysentery and bloody diarrhoea (Kaper *et al*., 2004). This pathovar differs from the other *E. coli* pathovars, because it includes obligate intracellular bacteria that have neither flagella nor adherence factors. Virulence is largely due to a 220 kb plasmid that encodes a T3SS on the Mxi–Spa locus that is required for invasion, cell survival and apoptosis of macrophages (Vieira *et al*., 2007). Patients may first develop watery diarrhoea prior to onset of dysentery with a low volume of stools containing blood and mucus. Other symptoms are headache, fever, and cramping. Humans appear to be the main reservoir of EIEC, with little evidence to support EIEC carriage in animals or foods of animal origin. EIEC outbreaks are usually associated with water or food contaminated with human faeces or person-to-person transmission (Maurelli *et al*., 1998).

The incidence of the disease caused by EIEC is generally low in developed countries (Maurelli *et al*., 1998). Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose. Pathogenicity of EIEC is primarily due to its ability to invade and destroy colonic tissue. The invasion phenotype, encoded by a high molecular weight plasmid, can be detected by PCR and probes for specific for invasion genes (Todar, 2008).

### 2.3.4 ENTEROAGGREGATIVE *E. coli*

Enteroaggregative *Escherichia coli* (EAEC) was originally identified as the etiologic agent of persistent diarrhea in developing countries but is gaining increasing prominence for its role in a wider spectrum of diarrheal syndromes. EAEC strains have been implicated in acute as well as persistent diarrhoea among adults and children (Okeke and Nataro, 2001; Huang *et al*., 2004).A recent meta-analysis found that EAEC is significantly associated with disease in every group at high risk for diarrhea, including young children, human immunodeficiency virus-positive individuals, and visitors to developing countries (Haung *et al*., 2006). In addition to its association with disease in epidemiological studies in developing countries, EAEC has also been identified as a principal cause of diarrheal disease in Germany, the United Kingdom, and the United States (Huppertz *et al*., 1997; Tompkins *et al*., 1999; Cohen *et al*., 2005; Bhargava *et al*., 2009). Aggregative adherence is the defining characteristic of EAEC (Nataro *et al*., 1987). EAEC strains adhere to the intestinal epithelium, and to epithelial cells in culture, in a characteristic two-dimensional"stacked brick" fashion. The pattern features bacteria adhering to the eukaryotic surface, other bacteria, and the solid substratum. Four types of fimbriae have so far been documented as conferring aggregative adherence (Nataro *et al*., 1992; Czeczulin *et al*., 1997; Berneir *et al*., 2002; Dudley *et al*.,

2006). Two noncontiguous plasmid loci containing the complete complement of genes encoding aggregative adherence fimbriae I (AAF/I) or AAF/II are sufficient to confer aggregative adherence on non-adherent *E. coli* (Steiner *et al*., 2000). The plasmid bearing type IV pili found in Serbian EAEC outbreak strain C1096 are also sufficient to confer a weak aggregative adherence phenotype on *E. coli* K-12.Enteroaggregative *E. coli* (EAEC) is found mostly in humans. Although it is considered to be an emerging pathogen, EAEC is the second most common cause of travellers' diarrhoea after ETEC in both developed and developing countries. EAEC is also becoming commonly recognized as a cause of endemic and epidemic diarrhoea worldwide. Diarrhoea caused by EAEC is often watery, but it can be accompanied by mucus or blood. EAEC colonization can occur in the mucosa of both the small and large bowels, which can lead to mild inflammation in the colon. Much like the details of its transmission and epidemiology, the understanding of EAEC and its pathogenesis is limited, in part owing to the paucity of suitable animal models and the heterogeneity of virulence factors (Kaper *et al*., 2004). These strains are associated with persistent diarrhoea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhoea without invading or causing inflammation (Todar, 2008).

### 2.3.5 ENTEROHEMORRHAGIC *E. coli*

The enterohaemorrhagic *E. coli* (EHEC) group has a common ability to produce cytotoxins that are active on monkey kidney (Vero) tissue culture cells. These cytotoxins are mediated by genes carried by lysogenic bacteriophages and act in a similar manner by interfering with protein synthesis in eukaryotic cells (Todar, 2008). The toxins belong to two major antigenically distinct groups, stx1 and stx2, with various subgroups. Stx1 is similar to the

Shiga toxin produced by *Shigella dysenteriae*; consequently, Shiga toxin-producing *E. coli* are referred to as both STEC (Shiga toxin-producing *E. coli*) and VTEC (verotoxigenic *E. coli*) (Kaper and Nataro, 1998). EHEC is a term commonly used to refer to those STEC that cause enterohaemorrhagic disease. Since their recognition in the 1980s, EHEC have become the most notable of the enteropathogenic *E. coli* (EPEC) that are transmitted by food, including dairy products. They can cause illness in humans and diarrheal illness in young animals. Human EHEC infection can be asymptomatic or result in symptoms ranging from mild diarrhea to hemorrhagic colitis and life-threatening hemolytic–uremic syndrome (HUS) (Croxen and Finlay, 2010). Strains causing hemorrhagic syndromes frequently, but not exclusively, carry accessory virulence factors to the Shiga toxins located on a chromosomal pathogenicity island, the locus for enterocyte effacement (LEE). The LEE encodes genes that lead to the formation of attaching and effacing lesions typically seen in the intestinal epithelium in both EHEC and EPEC infections (Kaper *et al*., 2004). There are many serotypes of STEC, although a limited number are commonly associated with HUS. The most common is the O157:H7 serotype and others include O26, O111, O45, and O103. Clinical isolates of these types often carry the same virulence determinants, for example, Stx1 and/or Stx2, the LEE, and pEHEC. Transmission to humans usually occurs through contaminated food and water. In North America, Japan and parts of Europe, most outbreaks are due to EHEC serotype O157:H7, whereas other serotypes are important health concerns in other developed countries (Kaper *et al*., 2004). Almost all EHEC O157:H7 isolates harbour a 92 kb virulence plasmid called pO157, which has approximately 100 ORFs and encodes several virulence factors.

### 2.3.6 DIFFUSELY ADHERENT *E .coli*

Diffusely adherent *E. coli* (DAEC) are those that produce a more diffuse adherence on the Hep-2 cell surface (Scaletsky *et al*., 2002), and have been recognized as the sixth class of diarrheagenic *E. coli* (Lopes *et al*., 2005). DAEC is most commonly associated with age-dependent diarrhoea and in children less than 12 months of age (Scaletsky *et al*., 2002), however most case-control studies have demonstrated that the association of DAEC as a diarrhoea causing agent remains controversial. There are a limited number of studies regarding the virulence and pathogenic mechanisms of DAEC. It is presumed that the virulence factor of the organism is diffuse attachments to the intestinal lining of the infected host (Sarkar, 2008). In a study carried out by Meraz *et al*., 2008, the major clinical symptoms of the patients carrying DAEC strains were found to include watery diarrhoea in some instances with mucus and blood, vomiting, with vomiting being more prominent as compared to diarrhoea among children (Meraz *et al*., 2008).

### 2.3.7 UROPATHOGENIC *E. coli*

Uropathogenic *E. coli* (UPEC) infections account for roughly 80% of all UTIs, causing cystitis in the bladder and acute pyelonephritis in the kidneys. UPEC has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness (Alteri *et al*., 2009).The adhesin that has been most closely associated with Uropathogenic *E. coli* is the P fimbriae (or pyelonephritis-associated pilli [PAP]). The letter designation is derived from the ability of P fimbriae to bind specifically to the P blood group antigen which contains a D-galactose-D-

galactose residue. The fimbriae bind not only to red cells but to a specific galactose dissaccharide that is found on the surfaces uroepithelial cells in approximately 99% of the population (Todar, 2008).

### 2.3.8 NEONATAL MENINGITIS *E. coli*

NMEC is a common inhabitant of the gastrointestinal tract and it is the most frequent cause of Gram-negative-associated meningitis in newborns. Fatality rates can approach 40% (Kaper *et al*., 2004), and survivors are usually burdened with severe neurological sequelae. The pathogenesis of NMEC is complex, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood–brain barrier into the central nervous system, which leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid. The K-1 antigen is considered the major determinant of virulence among strains of *E. coli* that cause neonatal meningitis. K-1 is a homopolymer of sialic acid. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. K-1 may not be the only determinant of virulence, however, as siderophore production and endotoxin are also likely to be involved (Todar, 2008).

## 2.4 Epidemiology of *E. coli*

The epidemiology of foodborne pathogenic *E. coli* varies throughout the world. In communities with poor sanitation and hygiene, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enteropathogenic *E. coli* (EPEC) are prevalent (Wanke *et al*., 1991). In developing countries, enterotoxigenic *E. coli* (ETEC) is the most recognized cause of infectious diarrhoea. Worldwide, the incidence of ETEC infections is estimated to

result in 650 million cases of diarrhoea and 380,000 deaths in children under the age of 5 years. The pathogen is also an important cause of travelers'diarrhoea in persons traveling to endemic regions of the world (Okoh and Osode, 2008). The ease with which people move around the world has dramatically increased the frequency of traveler's diarrhoea, which now affects up to one-third of individuals who visit developing areas, such as Africa, South Asia, Latin America, and the Middle East. Because ETEC has endemic and epidemic potentials, the pathogen is a major cause of relatively serious disease during natural disasters. They are acquired by consumption of contaminated food and water and by cross-contamination through direct human contact. Foodborne pathogenic *E. coli* have emerged paradoxically in communities with better developed sanitation and hygiene. However, the pathotypes differ (e.g. STEC, EHEC and Enteroaggregative *E. coli* [EAggEC]) and the transmission pathways often include raw or inadequately processed animal or horticulture products, contact with animal manure, contaminated water and cross-contamination with raw food (Todar, 2008). Most *E. coli* strains pose no harm to human health, except for serotype O157:H7, which can cause food poisoning in humans and can become life-threatening (Karch *et al*., 2005). Other less common serotypes, such as O104:H4, O121, O26, O103, O111, O145, and O104:H21 can also cause serious infection. EAEC and persistent diarrhoea syndrome have been consistently associated (Lima *et al.*, 1992*;* Fang *et al.*, 1995). The increasing number of such reports and the rising proportion of diarrheal cases in which EAEC are implicated suggest that EAEC are important emerging agents of paediatric diarrhoea. Humans can be infected by ingesting contaminated water, even though tap water contains chlorine and has undergone ozone or ultraviolet treatment; some *E. coli* outbreaks have been caused by contaminated municipal water supplies (Okoh and Osode, 2008). Private wells can be a source of infection, as can some lakes and swimming pools. Ingesting contaminated food, examples include ground beef, unpasteurized milk, or fresh vegetables (Bielaszweka *et al*., 2011. Infected

people who work in restaurants and do not wash their hands properly after going to the toilet can spread the infection to customers and other members of staff (Buso *et al*., 2013; unpublished data). Having physical contact with an infected person is known as person-to-person contact. Good hand hygiene is important in stemming the spread of infection. Uropathogenic *E. coli* (UPEC) is responsible for approximately 90% of urinary tract infections (UTI) seen in individuals with ordinary anatomy (Todar, 2008). In ascending infections, fecal bacteria colonize the urethra and spread up the urinary tract to the bladder as well as to the kidneys (causing pyelonephritis) Nicolle (2008) or the prostate in males. Because women have a shorter urethra than men, they are 14 times more likely to suffer from an ascending UTI (Todar, 2008).

### 2.5 Clinical manifestations of different *E. coli* diseases

Patients with *E coli* traveler's diarrhea (i.e., watery nonbloody diarrhea; caused by enterotoxigenic *E coli* [ETEC] or enteroaggregative *E coli* [EAggEC]) may appear to be dehydrated (Kaper and Nataro, 1998; Todar, 2008). Traveler's diarrhea is observed in young healthy travelers to tropical countries and is watery diarrhea without polymorphonuclear (PMN) leukocytes. The differential diagnoses of *E coli* traveler's diarrhea include rotavirus infection, Norwalk virus infection, *Salmonella* infection, and *Campylobacter* diarrhoea (Okoh and Osode, 2008).

Patients with *E coli* childhood diarrhea (ie, watery nonbloody diarrhea; caused by EAggEC, enteroadherent *E coli* [EAEC], or enteropathogenic *E coli* [EPEC]) may also appear to be dehydrated. These infections produce a non-inflammatory watery diarrhea observed especially in children. The differential diagnoses of *E coli* childhood diarrhea include *Vibrio*

*cholerae* infection and Rotavirus infection. In May, June, and July, 2011 an outbreak of gastroenteritis caused by Shiga-toxin–producing *E coli* was seen in Germany (Bielaszweka *et al*., 2001). The majority of patients were adults and 22% of the cases developed hemolytic–uremic syndrome, the outbreak strain was typed as an enteroaggregative Shiga-toxin–producing *E coli* O104:H4, producing extended-spectrum beta-lactamase (Frank *et al*., 2011). The consumption of sprouts was identified as the most likely vehicle of infection. This outbreak was different as it was caused by EAggEC that produced a Shiga toxin and it exemplifies the threat posed by foodborne pathogens with their propensity to cause large common-source outbreaks (Frank *et al*., 2011; Buchholz *et al*., 2011).

Patients with *E coli* dysentery (caused by enteroinvasive *E coli* [EIEC] or enterohemorrhagic *E coli* [EHEC]) have fever, bloody diarrhea, and dehydration. Intestinal mucosa produces a significant inflammatory response (Pennington, 2010). Clinically, patients with *E coli* dysentery present with fever and have blood and PMN leukocytes in their stool. The differential diagnoses of *E coli* dysentery include shigellosis and amebic dysentery. Patients with *E coli* HUS (caused by EHEC) have fever, bloody diarrhea, dehydration, hemolysis, thrombocytopenia, and uremia requiring dialysis. Symptoms of *E coli* HUS range from asymptomatic to non-bloody diarrhea to bloody diarrhea, renal failure, microangiopathic hemolytic anemia, thrombocytopenia, and CNS manifestations. The differential diagnoses of *E coli* HUS include *Shigella* infections, *Clostridium difficile* enterocolitis, ulcerative colitis/Crohn disease, ischemic colitis, diverticulosis, and appendicitis (Croxen and Finlay, 2010).

## 2.6 Laboratory diagnosis

### 2.6.1 Morphology and identification

On MacConkey agar, deep red colonies are produced, as *E. coli* is lactose-positive, and fermentation of this sugar will cause the medium's pH to drop, leading to darkening of the medium. Growth on EMB agar produces black colonies with a greenish-black metallic sheen. This is diagnostic of *E. coli* (Okoh and Osode, 2008).On violet red bile mug agar selective medium a colour-change to red indicates lactose positive colonies like *E. coli* and other coliform organisms. *E. coli* can be demonstrated by fluorescence in the UV (Lues *et al*., 2010). Light microscope will show Gram-negative rods, with no particular cell arrangement. The organism is also lysine positive, and grows on TSI slant with a (A/A/g+/$H_2$S-) profile. IMViC is a battery of biochemical tests used in the clinical lab to distinguish between enteric microorganisms. The acronym IMViC stands for indole, methyl red, Voges- Proskauer and citrate. The "i" in the acronym is added for pronunciation purposes. IMViC is {+ + – -} for *E. coli*; as it is indole-positive (red ring) and methyl red-positive (bright red), but VP-negative (no change-colourless) and citrate-negative (no change-green colour) (Paton and Paton, 1998). Oxidase Test is used to demonstrate the ability of a bacterium to produce the enzyme cytochrome- c oxidase, capable of reducing oxygen. Only Aerobic bacteria have this enzyme. A positive test will show a colour change to blue, then to dark purple or black, within 10 to 30 seconds. *E. coli* is oxidase negative.

### 2.6.2 Serotyping

Somatic and flagellar antigen determinations help to classify organisms into primary phenotypes. Such antigen determination is called serotyping (Todar, 2008). Serotyping is a subtyping method based on the immunologic characteristics of two surface structures, the lipopolysaccharide (LPS), which contains the O-antigen, and the flagella, which contains the H-antigen. The O-antigen is the outermost component of the LPS, an immunoglycolipid contained in the outer membrane of gram-negative bacteria. O-antigens are composed of multiple repeats of an oligosaccharide unit typically composed of four to six sugars. Variations in the composition, arrangement, and linkages of these sugars all contribute to O-antigen diversity and are the basis for serotype diversity. The H-antigen is a part of the flagellar protein affecting motility. Identification of the H-antigen provides an additional tool for STEC surveillance and organism characterization, but H-antigen determination is not necessary for routine testing (Todar, 2008). Serotyping of *E. coli*, together with genome, virulence, and phage typing, is a useful epidemiological tool.

### 2.6.3 Molecular characterization

#### 2.6.3.1 Polymerase Chain Reaction (PCR)

PCR protocols targeting the genes of *Escherichia coli* provide a rapid and sensitive diagnostic tool to detect potentially virulent strains that have been isolated in culture from patient stool specimens (Vidal *et al*., 2004). Both conventional and real-time PCR methods provide a satisfactory detection limit for identifying Shiga toxin-producing organisms. The

selection of a specific methodology will depend on individual laboratory preference, acceptable timelines, available funding and the experience level of laboratory staff (Velusamy *et al*., 2007). Conventional PCR is a molecular testing option for laboratories that do not have real-time PCR instrumentation. This methodology requires the use of a conventional, or block, thermal cycler and resolution of the PCR products using agarose gel electrophoresis (Lang *et al*., 1994).

#### 2.6.3.2 Pulse Field Gel Electrophoresis (PFGE)

In recent years, the use of molecular "fingerprinting" methods has become standard practice in microbiology for evaluating the epidemiology of infectious diseases, investigating suspected outbreaks of bacterial infections, and typing bacteria (Mickelsen, 1997). Pulsed-field gel electrophoresis (PFGE) allows the generation of simplified chromosomal restriction fragment patterns without having to resort to probe hybridization methods. In this method, restriction enzymes that in frequently cut DNA are used for generating large fragments of chromosomal DNA, which are then separated by special electrophoresis (Swaminatham and Matar, 1993). PFGE has been applied to subtyping of several gram-positive and gram-negative bacteria. PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a gold standard in epidemiological studies of pathogenic organisms (Schwartz and Cantor, 1984). The preparation of genomic DNA suitable for PFGE begins by lysing bacteria that are encased in agarose blocks. After multiple washes, the DNA within the agarose is digested with restriction enzymes and electrophoresed using PFGE. PFGE differs from conventional agarose electrophoresis in that it takes longer because the orientation of the electric field across the gel is periodically changed in contrast to being unidirectional and

constant in standard electrophoresis. The variability in the electric field allows PFGE to resolve the very large fragments (>600 kb) associated with this analysis (Goering, 2004).

### 2.7 *E. coli* outbreaks from raw milk and dairy products

Raw milk has been implicated as the vehicle of sporadic cases and several outbreaks of EHEC-associated illness in North America, Europe, and Malaysia (Murinda *et al*., 2002; Chye *et al*., 2004 and Murphy *et al*., 2005). While cows' milk is the most common source, goats' and sheep milk has been implicated in *E. coli* O157 infection. Although EHEC and STEC are the most studied pathogenic *E. coli* in farm environments, other types (EAEC and EPEC) have been detected (Singh *et al*., 1970). Cheese has been the attributed vehicle in several EHEC outbreaks. Outbreaks of *E. coli* O157:H7 have been attributed to a variety of cheeses such as farm produced cheese, fresh cheese curds, a semi hard Lancashire cheese, and Gouda made from unpasteurized milk (Vivegnis *et al*., 1999). Other serotypes have included EHEC O103:H2 contaminating soft cheese made from a mixture of unpasteurized cows' and goats' milks; O119 EHEC in fromage frais; and O26 EHEC from a raw milk Camembert cheese in France (Vernozy-Rozand *et al*., 2005). The use of contaminated raw milk or postprocessing contact with raw milk and subsequent survival of the EHEC during the cheesemaking process have been the major contributing factors.

### 2.8 Common *E. coli* virulence genes isolated from raw milk

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin (*Stx*)-producing *E. coli* (STEC) strains that are isolated from human patients and are responsible for severe clinical symptoms, including diarrhoea, hemorrhagic colitis, and hemolytic-uremic

syndrome. These diseases are directly related to the virulence genes present in the causative agent (Betteheim, 2000; Elliot *et al*., 2001). Four of the most commonly assayed virulence factors of STEC are the two phage encoded cytotoxins, called Shiga toxin 1 and 2 (encoded by the *Stx1* and *Stx2* genes, respectively), the protein intimin (encoded by the chromosomal gene *eae*), which is responsible for the intimate attachment of the bacteria to intestinal epithelial cells, and the plasmid-encoded enterohaemolysin, also called enterohaemorrhagic *E. coli* haemolysin (EHEC-HlyA), encoded by the *ehxA* gene (Law, 2000; Gyles, 2007). Shiga toxin-producing *E. coli* can be transmitted through different routes, including food and water, person-to-person contact, and animal-to-person contact. Most human infections are caused by consumption of contaminated food. Domestic animals and wild ruminants, in particular cattle, are considered the main reservoir of STEC and the main source of contamination of the food supply (Caprioli *et al*., 2007; Erickson and Doyle, 2007). The presumed route of *E. coli* contamination of raw milk is via faecal contamination during milking (Hussein and Sakuma, 2005). However, direct excretion of the organisms from the infected udder has also been reported (Lira *et al*., 2004).

## 2.9 Antibiotic resistance in bacteria

### 2.9.1 Antibiotics

Antibiotics are a group of natural microbial products or synthetic chemical compounds that inhibit the growth of and even kill bacteria (Prescott *et al*., 1996). Natural antibiotics are produced by both bacteria or fungi and act by blocking some essential cell processes in other bacteria. The first natural antibiotic isolated in pure form was penicillin and it came into clinical practice in the 1940s.Synthetic chemical compounds are man-made and also target

some of the vital cell mechanisms in bacteria. In addition, another category of antibiotics, i.e. semi-synthetic antibiotics, are the chemically modified form of the natural antibiotics to enhance the effectiveness of natural antibiotics (e.g. ampicillin and methicillin). Antibiotics are grouped into different classes (Table 1) based on their chemical structures: such as ß-lactams, aminoglycosides, macrolides, ketolides, tetracyclines, glycopeptides and others, and based on their mode of actions: such as inhibitors of bacterial cell wall synthesis, inhibitors of protein synthesis, disruptors of cell membranes and inhibitors of nucleic acid synthesis (Walsh, 2003). Beta-lactams (penicillins, cephalosporins, cephamycins, monobactams, and carbapenems), glycopeptides and other antibiotics inhibit the biosynthesis of peptidoglycan, a rigid polymer in cell wall that resists a high osmotic pressure inside the cell. In contrast, aminoglycosides and tetracyclines complexes with the conserved sequences of the 16S rRNA of the 30S ribosomal subunit while macrolides, ketolides, lincosamides and chloramphenicol bind to the sequences of the 23S rRNA of the 50S ribosomal unit thus affecting the protein biosynthesis (Mascaretti, 2003). Antibiotics like fluroquinolones act on deoxyribonucleic acid (DNA) gyrase and topoisomerase IV and prevent synthesis of DNA and ribonucleic acid (RNA). On the other hand, sulfonamides (for example sulfamethoxazole) and trimethoprim interferes in the folic acid metabolism in bacteria that indirectly affects the nucleic acid synthesis. Sulfamethoxazole blocks the enzyme dihydropteroate synthase in folic acid synthesis while trimethoprim inhibits the enzyme dihydrofolate reductase that supplies the pyridime thymidylate for the DNA biosynthesis (Walsh, 2003).

**Table 2.1: Antibiotics used in the treatment of *E. coli* infection.**

| Mechanism of action | Antibiotic families |
|---|---|
| Inhibition of cell wall synthesis | Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides |
| Inhibition of protein synthesis | Tetracyclines; aminoglyocides; oxazolidonones; streptogramins; ketolides; macrolides; lincosamides |
| Inhibition of DNA synthesis | Fluoroquinolones |
| Competitive inhibition of folic acid synthesis | Sulfonamides; trimethoprim |
| Inhibition of RNA synthesis | Rifampin/ansamycins |
| Other | Metronidazole |

Major antibiotics grouped according to their mechanisms of action and their chemical structures (Levy and Marshall, 2004).

### 2.9.2 Antibiotic resistance in *E. coli*

Soon after the introduction of penicillin in clinical practices in the 1940s followed by other antibiotics thereafter, antibiotics were virtually effective against pathogenic bacteria. However, the effectiveness of the antibiotics was greatly reduced due to the use and misuse of antibiotics (Walsh, 2003; Aminov, 2009). Several new antibiotics, targeting essential physiological or metabolic functions of the bacterial cell, were introduced in the last several decades but bacteria responded each time by evolving themselves as resistant strains (Barbosa and Levy, 2000; Levy and Marshall, 2004).

In the context of food-animal production systems, mastitis is the most common and most economically significant disease affecting dairy cattle.

It is the leading cause of antimicrobial use on dairy farms (Saini *et al*., 2012). Antimicrobial therapy is commonly implemented for mastitis prevention and control. Unfortunately, despite the best possible antimicrobial treatments, failures of bacteriological cure are common, and antimicrobial resistance (AMR) is considered one of the reasons for low cure rates (Barkema *et al*., 2006). Bacterial infections are usually treated with antibiotics. However, the antibiotic sensitivities of different strains of *E. coli* vary widely. As Gram-negative organisms, *E. coli* are resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which may be used to treat *E. coli* infection include amoxicillin, as well as other semisynthetic penicillins, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides (Johnson *et al*., 2006). Several factors have been implicated for the emergence of the antibiotic-resistant bacteria and their spreading in the environment. In general, there is a consensus that the increased use of antibiotics is the prime factor for the increased resistance in pathogenic bacteria (Levy and Marshall, 2004). Not only the antibiotic use in human medicine has been linked to the elevated antibiotic resistance in bacteria, the use of antibiotics in veterinary medicine, agriculture, aquaculture, horticulture and other human activities have been identified as the other driving forces in escalating the problem (Aarestrup, 1999; Barbosa and Levy, 2000; Aminov, 2009). The antibiotics are used for food animal production in one of the following situations: treatment of infectious diseases, metaphylactics, prophylactis, and growth promotion (Aarestrup, 2005). Quantity as much as half of the total antibiotics produced worldwide is used for food animal production, primarily as growth promoter and prophylactic agents (Aarestrup, 1999). Although the use of antibiotics as growth promotion has been banned in the European Union (EU), however, it is being continuously used in other parts of the world (Levy and Marshall, 2004). This selective pressure on the environment is

not insignificant for the development of antibiotic resistance in enteric bacteria in animals, considering the fact that animals frequently harbor human pathogens in their intestinal tract such as *Salmonella*, *Campylobacter*, *Yersinia*, *Listeria* and enterohaemorrhagic *E. coli* (Aarestrup 1999; Levy and Marshall, 2004).

A bacterial strain is defined as resistant strain to an antibiotic when the strain is able to grow at the lowest concentration of the antibiotic that usually inhibits the growth of the wild strain belonging to the same species. This lowest concentration of the antibiotic that effectively prevents the growth of a particular strain is called minimum inhibitory concentration (MIC). The antibiotic resistance may be either intrinsic or acquired resistance. The intrinsic resistance is caused by structural or functional characteristics inherited to a bacterial group (that includes species, genus or even to a higher level) such as the low affinity of the antibiotics to the target, the inability of the antibiotics to enter the bacterial cell, removal of the antibiotics from the cell by chromosomally encoded active exporters, inactivation of antibiotics by innate enzymes and other mechanisms (Guardabassi and Courvalin, 2006). Unlike intrinsic resistance, the acquired resistance is inherited to only some strains of a particular genus or species. However, the acquired resistance possesses a serious problem as it causes the emergence and spread of resistance to the usually susceptible bacteria. This mobile nature of acquired resistance can easily disseminate among bacteria of morphologically and ecologically distinct groups (horizontal transfer) through mobile genetic elements such as bacteriophages, plasmids, naked DNA or transposons. In addition, mutation on genetic elements can also enable bacteria to acquire the resistance to a particular antibiotic (Levy and Marshall, 2004). The process of acquisition of resistance genes can be through transduction in which bacteriophages mediates the transfer of DNA having resistant determinants into the host cell or the transformation which involves the uptake of plasmids or

naked DNA from the environment. Another important process of acquiring resistance is the conjugation that is the transfer of plasmids or chromosomal DNA by cell-to-cell contact. The conjugation has been regarded as by far the most important mechanism in spreading the antibiotic resistance, even between distinct taxonomic and ecological groups of bacteria (Barbosa and Levy, 2000). The horizontally transferable plasmids that contain some specific genetic structures such as complex transposons or integrons enhance the distribution of resistance genes between bacteria (Walsh and Fanning, 2008).

### 2.9.3 Mechanisms of bacterial antibiotic resistance

Several mechanisms have been proposed by which bacteria evolved to overcome the detrimental effects of antibiotics in the surrounding environment. The bacteria may restrict the entry of antibiotics by modifying the cell membrane composition, by reducing the uptake or exporting the antibiotics from the cell by active-efflux. In addition, alteration of the antibiotics by cellular enzymes may be another way of getting resistance to antibiotics. The bacterial cell may also respond by altering the affinity of the antibiotics to the target or by overexpression of the target (Mascaretti, 2003). Inactivation of antibiotics by enzymes is the main mechanism of resistance to β-lactams, aminoglycosides, and phenicols. B-lactamases are the most important drug-inactivating enzymes that hydrolyze the β-lactam ring of penicillins, cephalosporins and carbapenems. On the other hand, the aminoglycoside-altering enzymes interfere catalyze the transfer reaction that restricts the binding of the antibiotic to ribosomes. However, enzymatic destruction of antibiotics has not been observed in the synthetic class of antibiotics: the sulfamethoxazole-trimethoprim, the fluroquinolones or the oxazolidines (Walsh, 2003). Specific-drug-resistance (SDR) efflux pumps are the primary mechanism of resistance against tetracyclines, other multiple-drug-resistance (MDR) pumps

play important roles for the multiple resistance against antimicrobials such as β-lactams, macrolides and fluoroquinolones (Walsh, 2003; Guardabassi and Courvalin, 2006). Multidrug resistance can be acquired by bacteria through accumulation of genes in R plasmids or transposons that encode resistance to specific antibiotics and/or by the MDR pumps that can export more than single drug type (Nikaido, 2009). This R plasmid with multidrug resistance genes may be transferred to another bacterial species quickly either by conjugation, transformation or transduction and hence the new species can be resistant to several antibiotics. In this way, the pathogenic bacteria can become multidrug resistance (Mascaretti, 2003).

### 2.9.4 Antibiotic resistance in food-related bacteria

The role of the food chain as a source of antimicrobial-resistant pathogens, including *Salmonella*, *E. coli* and *Campylobacter*, is of significant consequences to the public health (Mølbak, 2004). The use of antibiotics in food animals production, such as for growth promotion and prophylactic reason, is of significant importance for the increased resistance in zoonotic bacteria such as *Salmonella*, *Campylobacter*, *Listeria*, enterohaemorrhagic *E. coli* (Aarestrup, 1999; Sørum and L'Abée-Lund, 2002; Levy and Marshall, 2004). The antibiotics used in animal husbandry are also of great significance as many of these antibiotics are used in the human medicine and the resultant antibiotic-resistant bacteria can spread the resistance phenomenon worldwide through the global trade of animal foods (Aarestrup *et al*., 2008). Similarly, commensal bacteria that interact with zoonotic bacteria through the food chain are also of increased concern as these bacteria might act as reservoirs of antibiotic resistance genes that can be transferrable to pathogenic bacteria of humans (van den Bogaard *et al*., 2000; Wang *et al*., 2006; Hawkey and Jones, 2009). The agricultural practices provide a

significant impact on the dissemination of antibiotic resistance elements in the environment, including the use of antibiotics in the fruits and vegetables production. The application of antibiotics in animal husbandry and the subsequent isolation of the antibiotic-resistant bacteria in the production chain suggest, in many cases, a direct relationship while it may not be the case when the reduced use of antibiotics in organic farming brings the reduction of the antibiotic resistance on the pathogenic bacteria (Wright, 2010).

In the growing concern of antibiotic resistance in food-related bacteria, more and more studies were undertaken in recent years to assess the antibiotic resistance of bacteria isolated from food products such as raw milk (Citak *et al*., 2005; Straley *et al*., 2006; Munsch-Alatossava and Alatossava, 2007), cheese (Valenzuela *et al*., 2009), raw vegetables (Boehme *et al*., 2004), ground meat products (White *et al*., 2001) and poultry (Sackey *et al*., 2001). The majority of these studies were focused on assessing the prevalence of antibiotic resistance in enteric bacteria; however few of them also considered non-enteric bacteria. In a study on the prevalence of antibiotic-resistant bacteria in vegetables and seed sprouts, Boehme et al. (2004) reported that the antibiotic-resistant bacteria as high as 108 CFU/g, were observed in seed sprouts and many of these isolates were multidrug-resistant. Compared with seed sprouts the common vegetables were less contaminated with antibiotic-resistant bacteria. Like Sackey *et al*., (2001) reporting on the prevalence of multiresistant enteropathogenic bacteria in poultry in Ghana, a widespread multidrug resistance has been reported in *E. coli* and *Salmonella* isolated from retail raw chicken products in Japan; 40.6% of 69 *E. coli* isolates and all 10 *Salmonella* isolates studied were multidrug-resistant (Ahmed *et al*., 2009). These isolates showed multidrug resistance against antibiotics such as ampicillin, streptomycin, spectinomycin, kanamycin, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, cefoperazone, cephalothin, cefoxitin, and ciprofloxacin. The authors concluded

that the retail chicken meat could be of significant importance in spreading the multidrug-resistant bacteria.

### 2.9.5 Antibiotic resistance of raw milk-associated bacteria

The raw milk-associated bacteria have shown various levels of resistance against many antibiotics of clinical significance. Although many studies focused on pathogens in raw milk of public health significance, however, few studies also considered antibiotic resistance on raw milk associated other bacteria. Bulk tank milk (BTM) was used to monitor the antimicrobial resistance in dairy farms and suggested that the monitoring of the antimicrobial resistance of *E. coli* in bulk milk can reasonably provide the overview of antimicrobials use in the farm (Berge *et al*., 2007). The prevalence of multidrug resistant *E. coli* and *Salmonella* in BTM was reported being 23%; the frequency of isolating *E. coli* was more frequent than *Salmonella*. Citak et al. (2005) studied the antibiotic resistance of enterococci isolated from raw milk and reported that the predominant enterococci were *E. faecalis* (54.2%) and *E. faecium* (29.0%). These isolates demonstrated extensive resistance to antibiotics such as ampicillin, ciprofloxacin, erythromycin, gentamicin, imipenem, streptomycin, tetracycline and vancomycin. Similarly, enterococci isolated from the raw cow's milk and cheeses derived from such milk showed resistance against many antibiotics such as ciprofloxacin, levofloxacin, erythromycin, tetracycline but all were sensitive to ampicillin, chloramphenicol and gentamicin (Valenzuela *et al*., 2009).

The prevalence of Gram-negative bacteria in bulk tank milk was studied by Straley et al. (2006). Among 54 bulk tank milk samples (obtained from six farms for a period of nine months) analysed, 46 (85%) of the samples were positive for Gram-negative bacteria. The

amount of these bacteria in bulk tank milk varied widely among farms (12-1310 CFU/mL) and *Pseudomonas* spp. were the dominant non-coliform Gram-negative bacteria that showed extensive resistance to antibiotics considered in the study. The antibiotic-resistant *Pseudomonas* spp. are of significant importance in dairy industries because they grow at low temperature and have the potential of forming biofilms in the bulk tank. In addition, other Gram-negative bacteria detected in this study were belonging to the genera *Acinetobacter*, *Citrobacter*, *Enterobacter*, and to *E. coli*. These bacteria were resistant to first, second and third generation of cephalosporins but were susceptible to fourth generation of cephalosporins, fluoroquinolones (enroflaxacin) and aminoglycosides (gentamicin). This study suggested that bulk tank milk could be a major source of antimicrobial-resistant Gram-negative bacteria (Straley *et al*., 2006).

## 2.10 Prevention of mastitis

### 2.10.1 Management aspects

The key elements in the control of mastitis include sound husbandry practices and sanitation, post-milking, teat dipping, treatment of mastitis during non-lactating period, and culling of chronically infected animals. Dry animal therapy can eliminate 70% of environmental streptococcal infections. The fundamental principle of mastitis control is that the disease is controlled by either decreasing the exposure of the teat to potential pathogens or by increasing resistance of dairy animals to infection. Jones (2006) has suggested approaching the treatment in the same way a surgeon approaches surgery. Wash hands with soap and water, wash teats and udder in sanitizing solution, thoroughly dry teats and udder with individual towels, dip teats in an effective germicidal teat dip. Allow 30 seconds of contact

time before wiping off teat dip with an individual towel; thoroughly scrub the teat end with a cotton swab soaked in alcohol. If all four quarters are being treated, start by cleaning the teat farthest from you and work toward the closest teat, use commercial antibiotic products in single dose containers formulated for intramammary infusion. Treat teats nearest to you first, then those farthest away to prevent contaminating clean teat ends. Dip teats in an effective germicidal teat dip after treatment (Sharif and Muhhamad, 2009).Before permitting supply of products made from unpasteurized milk, it is necessary to investigate potential hazards to public health and recommend control strategies to mitigate them. Nationwide control programmes have successfully eliminated or greatly reduced the risk of historic pathogens such as brucellosis or tuberculosis entering human milk supply. But product from cows, sheep or goats still cannot be guaranteed free from other pathogens, even when produced under hygienic conditions, because of fecal contamination or direct excretion into the milk (Rampling, 1996). Additionally potential risk factors for bacteria in milk and on-farm control measures to reduce risk must be reviewed and recommendations given to minimize these risks.

### 2.10.2 Vaccination

Prophylactic immunization is used in some countries to prevent coliform mastitis. Vaccines developed using the common core antigen of coliform bacteria, administered systemically at drying-off and again 3 weeks before the calving date, have been in use in North America for many years (Hogan *et al*., 1992; Wilson and González, 2003; Wilson *et al*., 2007a). Vaccination is able to reduce the severity of clinical signs and protect cows from culling or death and to reduce the milk loss. However, the incidence of clinical mastitis has not been reduced (Wilson *et al*., 2007a; Wilson *et al*., 2007b). In some earlier studies, a reduced risk

for clinical mastitis in vaccinated cows was reported (Cullor, 1991; Hogan *et al*., 1992). In addition to the common core antigen, iron regulated outer-membrane protein vaccines against coliform mastitis have also been investigated (Lin *et al*., 1999).

### 2.10.3 Genetic engineering in improving mastitis

Genetic engineering is one tool suggested to increase host defense against mastitis (Bramley *et al*., 2001; Maga *et al*., 2005; Pyörälä, 2002 ). Increasing mastitis resistance by improving the immune response through modifying activity of genes or incorporating beneficial genes from other organisms has been proposed to have a positive impact on cow welfare and economics of milk production (Oliver, 2005). The transgenic approach to enhance mastitis resistance was studied in a mouse model (Kerr *et al*., 2001). In the first published cow model (Wall *et al*., 2005), cows carrying a gene coding for an anti-staphylococcal peptide, lysostaphin, were shown to resist *S. aureus* intramammary infection. The first transgenic cows with the hLf gene were reported to express Lf in their milk at a concentration of 0.3 to 2.8 mg/ml (Brink *et al*., 2000; Van Berkel *et al*., 2002). A gene encoding human lactoferrin (hLf) in the bovine mammary gland could be a good candidate to increase resistance of dairy cows to coliform mastitis by transgenesis.

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## CHAPTER THREE

## Prevalence of *Escherichia coli* in raw milk from two dairy farms of the Eastern Cape, South Africa


### ABSTRACT

Milk quality continues to be a topic of intense debate in the dairy industry, medical and public health communities. Production of maximum quantities of high-quality milk is an important goal of every dairy operation. High-quality milk must contain a low number of somatic cells and low bacteria count, and must be free of human pathogens and antibiotic residues. The objective of this study was to determine the prevalence of *E. coli* in unpasteurized milk recovered from Middledrift and Fort Hare dairy. In this study 400 milk samples were collected from two commercial farms (Middledrift and Fort Hare) in the Eastern Cape, South Africa, 200 raw milk samples from each farm. Samples were cultured on violet red bile mug-agar (VRB-MUG Agar) and incubated at 37ºC for 24 hours and preliminary identified by Gram stain and catalase test. Isolates that were Gram negative and catalase positive were screened for a marker of *E. coli uid*A gene using PCR assays. Middledrift dairy farm had 50 (25%) *E. coli* isolated from raw milk and Fort Hare farm showed 37 (18.5%) *E. coli* present in the milk samples. The presence of *E. coli* found in the milk samples points to the fact that fecal contamination was unavoidable and traditional practices are likely to contribute to the contamination of the milk and proliferation of the microorganisms.



**Keywords**: Raw Milk, *Escherichia coli*, Polymerase Chain Reaction (PCR).

## 3.1 INTRODUCTION

Milk being a major constituent of the diet, quality control of milk is considered essential to the health and welfare of a community. However, people from rural areas show very little interest as to whether food and drink are good or detrimental to their health; their only concern is on buying enough food to keep them from starvation (Fox and Cameron, 1995; Lues *et al*., 2010). In developed societies, practices generally used to curb microbial proliferation in milk include pasteurization and refrigeration. People from rural areas, however, do not utilize such practices mainly due to lack of infrastructure and funds (Rohde, 1985; Collins *et al*., 1995). Milk has distinct physical, chemical and biological characteristics and its colour, odour, taste, consistency, freezing point (- 0.55°C), pH (6.6) and specific gravity (1,032) are characteristics that remain particularly constant (Abdullah *et al*., 2009). These characteristics present a favourable environment for the multiplication of several bacteria of various genera. Generally, bacteria in the milk can occur through colonization of the teat canal or an infected udder (clinical and subclinical mastitis) or gets contaminated at various stages be it from the animal, milker (manual as well as automated), extraneous dirt or unclean process water (Stewart, 1978; Rohde, 1985; Banwart, 1989; Philips & Griffiths, 1990; Gruetzmacher & Bradley, 1999; Hayes *et al*., 2001; Among all microorganisms *Escherichia coli* is the frequently contaminating organism, and is a reliable indicator of fecal pollution generally in insanitary conditions of water, food, milk and other dairy products (Diliello, 1982; Asmahan and Warda, 2011). *E. coli* is an environmental pathogen found in the immediate surroundings of the cow such as the soil, grass, manure and the bedding of housed cows. It is therefore easy for the organism to be found in the udder of cow thereby

gaining entrance to the milk. Its status as a true pathogen is of public health significance. It produces toxins that destroy cell membrane and can directly damage milk-producing tissues which can lead to bovine mastitis (Jones *et al*., 1998). It therefore became the objective of this study to determine the prevalence of *E. coli* in unpasteurized milk recovered from Middledrift and Fort Hare dairy farms.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Study Sites

Eastern Cape Province is one of the poorest and second largest provinces in South Africa and mainly comprised of rural settlements, it is also the "livestock" province of the country and is home to 21% of South Africa's cattle, 28% of sheep and 46% of goats. Fort Hare dairy has been in operation since 2007, it has 800 dairy cows and produces about 10 000 L of milk per day which is bought by Clover SA, one of the biggest milk companies in South Africa. While Middledrift dairy farm has been in operation since 2008, it has 600 dairy cows and produces about 8 000 L of milk per day (http://www.amadlelo.co.za). Fort Hare dairy has a rotary milking parlour whereas Middledrift dairy has an in line milking system. Fort Hare dairy is located in the Eastern Cape Province in Alice with geographic coordinates of 32°46′59″ South and 26°49′59″ East. Middledrift dairy is also located in the Eastern Cape Province with geographic coordinates of 32° 49' 0" South, 26° 59' 0" East.

### 3.2.2 Sampling and pH measurement

Four hundred samples were collected; 200 samples from each of the two commercial farms, Middledrift dairy and Fort Hare dairy Trust. Samples were collected in sterile 50 ml

containers and transported to the University of Fort Hare microbiology laboratory on ice in a cooler bag. The pH of the milk was immediately measured using a pH meter in the laboratory.

### 3.2.3 Isolation and identification

For the isolation and identification of *E. coli*, the samples were cultured on selective medium Violet red bile - mug agar (VRBMA, Merck, RSA) and incubated at 37 °C for 24 hours (Chirsten *et al*., 1993; Houghtby *et al*., 1993). The plates were checked under UV light at about 360-370 nm after 24 hours of incubation. Light blue fluorescence indicates the presence of *E. coli*. Biochemical tests including Gram staining, catalase test and API 20E were performed to preliminary identify *E. coli*.

Gram stain was done according to the method of Gram, (1884) by heat fixing a single colony on a microscopic slide and covering the slide with crystal violet for 20 seconds and then rinsing it off with water. After rinsing with water, Gram's iodine was flooded for 1 minute and then poured off with 95% ethanol. The slide was then rinsed with water and covered with safranin for 20 seconds. Lastly it was rinsed with water and air dried and afterwards it was ready for observation under the light microscope.

Catalase test was done by placing a single colony onto a microscope slide and 3% hydrogen peroxide was added. After 30 seconds if bubbles formed the test was positive and if not the test was negative (WHO, 1998). API 20 E was done following the manufacturer's instructions.

### 3.2.4 DNA extraction

DNA was extracted from identified *E. coli* isolates and from a positive control strains for *E. coli* (ATCC 8739) (SABS No ESC 20) purchased from the South African Bureau of Standards (SABS), Pretoria, South Africa. The extraction was done following the method of Maugeri *et al.* (2004) and Torres *et al.* (2003). Single colonies of presumptive *E. coli* grown overnight at 37 $^{o}$C on EMBA plates were picked, suspended in 200 µl of sterile distilled water, vortexed using a Minishaker (Digisystem laboratory instruments INC, Taiwan) and the cells were lysed using a Dri-Block DB.2A (Techne, SA) for 15 min at 100 ºC. The cell debris was removed by centrifugation at 10 000 rpm for 5 minutes using a MiniSpin microcentrifuge (ThermoFisher Scientific, Germany) to remove any particulate material that might still be present after processing. The lysate supernatant was placed on ice for 5 min. The supernatant was used as a template in the PCR assay immediately after extraction.

### 3.2.5 Molecular characterization of *E. coli*

Oligonucleotide primers targeting the *uidA* gene (147 bp) were used in the polymerase chain reaction with the conditions as shown in Table 3.1. The reaction mixture for running PCR contained 12.5 µl of 2X PCR master mix (0.05 units/µl Taq DNA polymerase in reaction buffer, 4 mM MgCl2, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP) (Thermo Scientific , SA), Nuclease free water (6.5 µl) (Thermo Scientific, SA), the cell lysates (5 µl) and 0.5 µl of the forward and reverse primer. PCR assay was carried out in a 25 µl reaction volume.

### 3.2.6 Gel electrophoresis

The PCR products (10 µl aliquots) were resolved in 1.8 % agarose gel containing 5 µl Ethidium bromide in 1X TBE buffer (40 mM Tris–HCl, 20 mM Naacetate, 1 mM EDTA, pH 8) (Cagney *et al*., 2004; Wang *et al*., 2002) before being visualized and photographed under the Alliance System. A 100-bp DNA ladder was included on each gel as a molecular size standard. The electrophoresis was carried out at 100 V for 1 hour.

**Table 3.1-Primer sequences and expected size of PCR-amplified gene targets of the pathogenic strains of *Escherichia coli*.**

| Target strain | Target gene | Primer sequence (5'-3') | Conditions | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| *E.coli* | *uidA* | AAAACGGCAAGAAA AAGCAG<br><br>ACGCGTGGTTAACAG TCTTGCG | 2minutes denaturation at $94^0$C followed by 25 cycles of denaturation $94^0$C for 1 minute, $58^0$C annealing for 1 minute and $72^0$C extension for 2 minutes. Amplified products were held at $4^0$C after amplification of cycles | 147 | Tsai *et al*., 1993 |

## 3.3 RESULTS

### 3.3.1 Biochemical characterization of *E. coli*

Gram negative rods in Middledrift dairy samples were 100 (50%) and 88 (44%) in Fort Hare farm. One hundred (50%) of the samples were catalase positive from both farms. In Middledfift dairy 72 (36%) *E. coli* were identified by API 20E and in Fort Hare dairy 68

(34%) *E. coli* were identified by API 20E. Species identity by API ranged from 76.8% to 99.9% in Middledrift and 76.8% to 100% in Fort Hare dairy (Appendix).

### 3.3.2 Molecular identification of *E. coli*

Isolates that were Gram negative and catalase positive were screened for a marker of *E. coli uid*A gene using PCR assays. Middledrift dairy farm had 50 (25%) *E. coli* isolated from raw milk and Fort Hare farm showed 37 (18.5%) *E. coli* present in the milk samples (Figure 3.1).

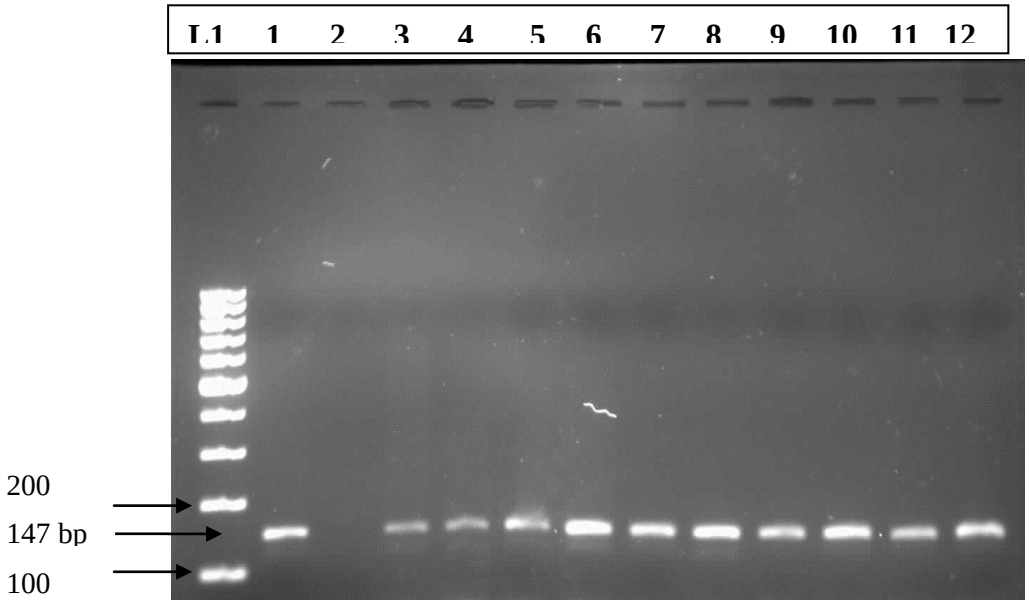


**Figure 3.1-Amplification product from the primer pair of the *uidA* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024 and Lane 12: 1415. The expected molecular size of *uidA* fragments was 147 bp.

### 3.4 Discussion

Nowadays, public health concern associated with microbial food safety has risen. The bacterium *E. coli* is one of the best and most thoroughly studied free-living organisms. It is also a remarkably diverse species because some *E. coli* strains live as harmless commensals in animal intestines. *E. coli* is a widely used indicator of fecal contamination in water bodies. External contact and subsequent ingestion of bacteria from fecal contamination can cause detrimental health effects (Meng *et al*., 2007). As *E. coli* is an indicator organism its recovery from milk suggests that other organisms of faecal origin including pathogens may also be present (Christen *et al*., 1992). Stomach cramps, nausea and vomiting are the symptoms caused by *E. coli*; however serious complications can also occur (Todar, 2008). *E. coli* is also a known causative agent of diarrhea and other foodborne-related illnesses through the ingestion of contaminated foodstuffs. Dairy cattle are considered the primary reservoir of pathogenic *E .coli* and the main route of *E. coli* infections in humans is via consumption of contaminated food (Hussein and Sakuma, 2005; Meng *et al*., 2007).

Not all the isolates that produced a blue fluorescence colour under 300nm UV light on VRB-MUG agar and were Gram-negative rods were identified as *E. coli*. This implies that VRB-MUG agar is not a good selective media as other organisms producing a blue fluorescent colour like *Enterobacter cloacae* were also identified. Gram negative rods in Middledrift dairy samples were 100 (50%) and 88 (44%) in Fort Hare farm. One hundred (50%) of the samples were catalase positive from both farms. In Middledfift dairy 72 (36%) *E. coli* were identified by API 20E and in Fort Hare dairy 68 (34%) *E. coli* were identified by API 20E. Species identity by API ranged from 76.8% to 99.9% in Middledrift and 76.8% to 100% in

Fort Hare dairy. The presence of *Enterobacter* spp, *Klebsiella* spp, *Citrobacter* spp and *Salmonella* spp identified by API 20E from the raw milk samples could render milk unfit for human consumption, since sufficient number of these organisms could cause infection and intoxication (Stephan *et al*., 2008).

Raw milk can be easily contaminated by infected food handlers who practice poor personal hygiene or by water containing human discharges. Globally, higher percentage of *E. coli* was reported by many authors including Egypt where the presence of coliform bacteria in raw milk was shown (Aly and Galal, 2002), India the raw milk and products were heavily contaminated by *E. coli* (Soomro *et al*., 2002), South Africa where a higher percentage (51.3%) of *E. coli* in raw milk was detected (Lues *et al*., 2010), however Malaysia indicated that 90% of the examined raw milk was contaminated by coliform bacteria and 65% were *E. coli* positive (Chye *et al*., 2004) a figure higher than in our study. In Saudi Arabia, Altahali and Hassan (2009) recorded 60% of coliforms as the main contaminants of raw milk.

### 3.5 Conclusion and Recommendations

The results obtained in this study suggest that high and strict preventive measures like regular washing and sterilization of dairy equipment, utensils, milker's hands, and animal udders, and eradication of diseased animals from the herd are highly recommended.

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## CHAPTER FOUR

## Occurrence of virulence genes associated with diarrheagenic *Escherichia coli* isolates from raw milk: Public Health Implications


### ABSTRACT

*Escherichia coli* remains a major threat in many places around the globe as the causative agent of diarrhea, and its reservoir in raw milk may play an important role in the survival and transport of pathogenic strains. Diarrhoegenic *E. coli* (DEC) strains are a diverse group of food-borne pathogens and diarrhea causing pathogens with various levels of virulence for humans. The main objective of this present study was to determine the prevalence of pathogenic *E. coli* in raw milk. The raw milk samples of two dairy farms were screened for the presence of pathogenic *E. coli* strains using conventional PCR, targeting genes such as *fliCH7*, *eagg*, *ial*, *eagg*, *lt*, *papC*, *ibeA* and *daaE*. In samples from Middledrift dairy the virulent genes *fliCH7* 538 (44%), *eae* 19 (22%), *lt* 20 (23%), *eagg* 5 (7%), *ial* 4 (5%), *papC* 3 (4%) and *daaE* 2 (2%) were present in higher amounts compared to those obtained from Fort Hare farm with *fliCH7* 16 (18%), *eae* 9 (10%), *lt* 7 (8%), *eagg* 7 (8%), *ial* 2 (2%), and the least was *papC* 1 (1%), while *daaE* 0 (0%) did not amplify. All the targeted genes except for *ibeA* that encode pathogenicity for *E. coli* were successfully amplified by PCR confirming that the isolates were pathogenic strains. This demonstrates that there is a widespread distribution of potentially virulent *E. coli* strains in the environment and in food that may be a cause of concern for human health.



**Keywords:** *Escherichia coli*, free-living, virulence markers, raw milk.

## 4.1 INTRODUCTION

Despite the fact that *Escherichia coli* as a commensal bacteria can be found as intestinal microflora of a variety of animals including man, not all the strains are harmless, and some can cause debilitating and sometimes fatal diseases in humans as well as mammals and birds (Belanger *et al*., 2011). Pathogenic strains are divided into intestinal pathogens causing diarrhea (diarrhoeagenic *E. coli*) and extraintestinal *E. coli* (ExPEC). Three main types of clinical syndrome can result from infection with one of these pathotypes: enteric and diarrheal diseases, urinary tract infections, and sepsis/meningitis (Croxen and Finlay, 2010). The *E. coli* pathotypes responsible for intestinal infections include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli*, necrotoxic *E. coli*, and cell-detaching *E. coli* (Kaper *et al*., 2004). Two additional *E. coli* pathotypes, collectively called extraintestinal pathogenic *E. coli* (Russo and Johnson, 2000) are responsible for extraintestinal infections. Extraintestinal pathogenic *E. coli* is composed of uropathogenic *E. coli* (UPEC) isolates that cause urinary tract infections, neonatal meningitis-associated *E. coli* (MNEC), and *E. coli* strains that cause septicaemia (Bekal *et al*., 2003). Each pathotype has distinguishing characteristics related to its epidemiology, pathogenesis, clinical manifestations, and treatment. EPEC, DAEC, EIEC and ETEC are the most important of these groups in terms of total diarrheal episodes on a global scale, but in recent years verocytotoxin-producing *E. coli* (VTEC) has gained increasing interest due to the occurrence of numerous cases of clinical syndromes associated with the consumption of meat and meat products, and raw milk and dairy products (http://www.who.int/mediacentre/factsheets accessed Apr. 25, 2008; Anonymous, 2007).

Among the VTEC particular attention is paid to the enterohaemorrhagic *E. coli* (EHEC) subgroup, whose serotype O157:H7 has been recognized as cause of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans (Botteldoorn *et al*., 2003). The virulence factors of VTEC are related to Vt1 and Vt2 genes (Kaper *et al*., 1998). Other virulence factors have been identified in EHEC: a membrane protein intimin, encoded by the *eae* gene, and an enterohaemolysin, encoded by the *HlyA* gene (Stephan *et al*., 2008). VTEC strains are commonly isolated from healthy cattle, and the presence of haemolysis may be used as a marker to differentiate virulent VTEC from other less virulent strains (Gyles *et al*., 1998). The study of the techniques to discriminate the various *E. coli* strains has received great attention during the last few years. In this field, biomolecular techniques are largely applied as they proved to be the most sensitive and specific (Rappelli *et al*., 2001; Gilgen *et al*., 1998; Fagan *et al*., 1999; Chapman *et al*., 2001; Belanger *et al*., 2003). In this chapter, we describe a conventional PCR assay for the simultaneous identification of strains of *E. coli* belonging to the eight principal virulence groups (VTEC (EHEC), EPEC, ETEC, EIEC, EAEC, DAEC, UPEC and NMEC) in raw milk.

## 4.2 MATERIALS AND METHODS

### 4.2.1 DNA extraction

DNA was extracted from *E. coli* isolates identified in chapter 3 and from a positive control strains for *E. coli* (ATCC 8739) (SABS No ESC 20) purchased from the South African Bureau of Standards (SABS), Pretoria, South Africa. The extraction was done as described in section 3.2.4 of chapter 3.

### 4.2.2 Pathotyping of *E. coli*

Oligonucleotide primers targeting the *fliC*H7 gene encoding for Enterohemorrhagic *E. coli* structural flagella antigen H7, *eagg* gene encoding for antiaggregation protein (dispersin) of Enteroaggregative *E. coli* ,*ial* gene encoding for invasion-associated locus of Enteroinvasive *E.coli, eae* gene encoding for Enteropathogenic *E. coli* intimin, *Stx1*and *stx2* gene encoding for shigatoxin producing Enterohemorrhagic *E. coli, lt* gene encoding for heat-labile Enterotoxigenic *E.coli, papC* gene characterized by uropathogenic *E. coli* and *ibe10* gene characterized by neonatal meningitis *E. coli* were used in the polymerase chain reaction following the conditions in Table 4.1. The reaction mixture for running PCR contained 12.5 µl of 2X PCR master mix (0.05 units/µl Taq DNA polymerase in reaction buffer, 4 mM MgCl2, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP) (Thermo Scientific , SA), Nuclease free water (6.5 µl) (Thermo Scientific, SA), the cell lysates (5 µl) and 0.5 µl of the forward and reverse primer. PCR assay was carried out in a 25 µl reaction volume.

**Table 4.1-Primer sequences and expected size of PCR amplified gene targets of the pathogenic strains of *Escherichia coli*.**

| Target strain | Target gene | Primer sequence (5'-3') | Conditions | Amplicon size (bp) | References |
|---|---|---|---|---|---|
| NMEC | *IbeA* | TGGAACCCCCTCGTAATATAC<br>CTGCCTGTTCAAGCATTGCA | An initial denaturation step at $94^0$C for 5min, followed by 36 cycles of $94^0$C for 35 seconds, annealing at $62^0$C for 30 seconds and elongation at $72^0$C for 1min. A final elongation step at $72^0$C for 5min. | 342 | Celuba *et al*., 2005 |
| EHEC | *flicH7* | TACCATCGCAAAAGCAAC TCC<br>GTCGGCAACGTTAGTGATACC | Initial denaturation at $95^0$C for 15 minutes followed by 35 cycles of heat denaturation at $94^0$C for 45 seconds, primer annealing at $55^0$C for 45 seconds and DNA extension at $68^0$C for 2 minutes. After the last cycle the samples were kept at $72^0$C for 5 minutes to complete synthesis of all strands. | 230 | Wang *et al*., 2000 |
| EIEC | *ial* | CTGGATGGTATGGTGAGG<br>GGAGGCCAATTATTTCC | 1 cycle for 2 minutes at $50^0$C, 1 cycle for 5 minutes at $95^0$C, 40 cycles for 45 seconds at $95^0$C, 45 seconds at $55^0$C and 45 seconds at $72^0$C and a final extension step for 10 minutes at $72^0$C to complete synthesis of all strands. | 700 | Presterl *et al*., 2003 |
| EAEC | *eagg* | AGACTCTGGCGAAAGACTGTATC<br>ATGGCTGTCTGTAATAGATGAGAAC | Initial denaturation at $95^0$C for 15 minutes followed by 35 cycles of heat denaturation at $94^0$C for 45 seconds, primer annealing at $55^0$C for 45 seconds and DNA extension at $68^0$C for 2 minutes. A final elongation step at 720C for 5min. | 194 | Kong *et al*., 2002 |

| | | | | | |
|---|---|---|---|---|---|
| EPEC | *eae* | TCAATGCAGTTCCGTTATCAGT<br>GTAAAGTCCGTTACCCCAACC TG | An initial denaturation step at 94$^0$C for 15 min, followed by 35 cycles of 94$^0$C for 45 seconds, annealing at 55$^0$C for 45 seconds and elongation at 68$^0$C for 2 min. A final elongation step at 72$^0$C for 5min. | 482 | Stacy-Phipps *et al*., 1995 |
| ETEC | *lt* | GCACACGGA GCTCCTCAGTC<br>TCCTTCATCCTTTCAATGGCTT | An initial denaturation step at 95$^0$C for 15 min, followed by 35 cycles of 94$^0$C for 45 seconds, annealing at 55$^0$C for 45 seconds and elongation at 68$^0$C for 2 min. A final elongation step at 72$^0$C for 5min. | 218 | Stacy-Phipps *et al*., 1995 |
| DAEC | *daaE* | GAACGTTGGTTAATGTGGGGTAA<br>TATTCACCGGTCGGTTATCAGT | An initial denaturation step at 94$^0$C for 2 min, followed by 40 cycles of 92$^0$C for 30 seconds, annealing at 59$^0$C for 30 seconds and elongation at 72$^0$C for 30 secomds. A final elongation step at 72$^0$C for 5min | 300 | Vidal *et al*., 2005 |
| UPEC | *papC* | GACGGCTGTACTGCAGGGTGTGGC G<br>ATATCCTTTCTGCAGGGATGCAATA | An initial denaturation step at 94$^0$C for 2 min, followed by 30 cycles of 94$^0$C for 1 min, annealing at 55$^0$C for 1 min and elongation at 72$^0$C for 1min. A final elongation step at 72$^0$C for 5min | 328 | Stacy-Phipps et al., 1995 |
| EHEC | *stx1* | CAGTTAATGTGGTGGCGAAGG<br>CACCAGACAATGTAACCGCTG | An initial denaturation step at 94$^0$C for 5min, followed by 36 cycles of 94$^0$C for 35 seconds, annealing at 62$^0$C for 30 seconds and elongation at 72$^0$C for 1min. A final elongation step at 72$^0$C for 5min. | 348 | Celuba *et al*., 2005 |
| EHEC | *stx2* | ATCCTATTCCCGGGAGTTTAC G<br>GCGTCATCGTATACACAGGAGC | An initial denaturation step at 94$^0$C for 5min, followed by 36 cycles of 94$^0$C for 35 seconds, annealing at 62$^0$C for 30 seconds and elongation at 72$^0$C for 1min. A final elongation step at 72$^0$C for 5min | 584 | Celuba *et al*., 2005 |

### 4.2.3 Gel electrophoresis

The PCR products (10 µl aliquots) were resolved in 1.8 % agarose gel containing 5 µl Ethidium bromide in 1X TBE buffer (40 mM Tris–HCl, 20 mM Naacetate, 1 mM EDTA, pH 8) (Cagney *et al*., 2004; Wang *et al*., 2002) before being visualized and photographed under the Alliance System. A 100-bp DNA ladder was included on each gel as a molecular size standard. The electrophoresis was carried out at 100 V for 1 hour.

## 4.3 RESULTS

### 4.3.1 Molecular Characterization of *E. coli*

Several genes representing *E. coli* pathotypes (EHEC, ETEC, EPEC, EAEC, UPEC, DAEC and EIEC) were amplified except for *ibeA* (NMEC) using PCR. Representative gel electrophoresis profiles of amplified products of target genes for pathogenic *E. coli* strains are illustrated in Figures 4.1 to 4.8.

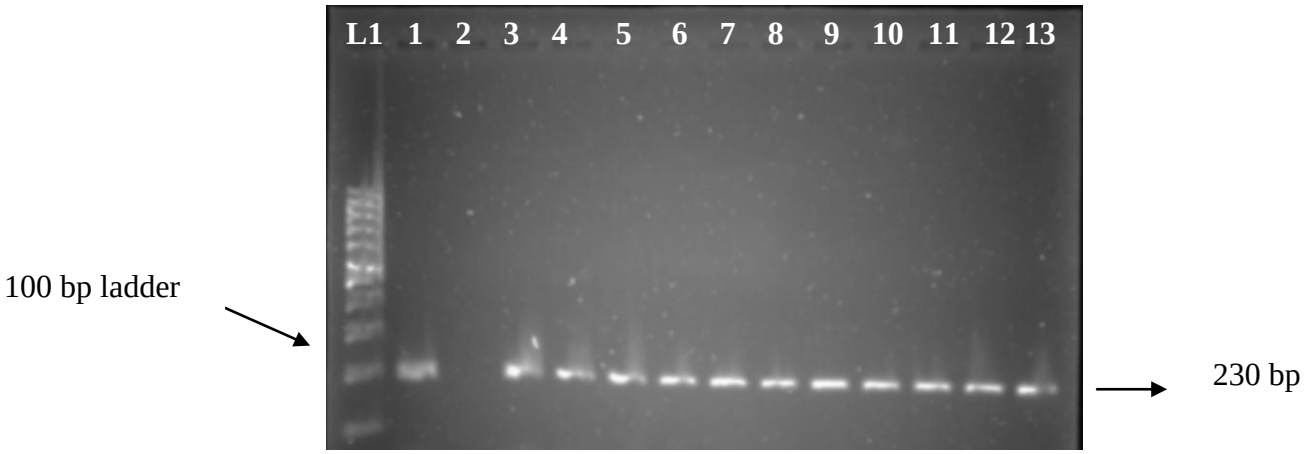


**Figure 4.1- Amplification product from the primer pair of the *fliCH7* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970 and Lane 13:1836. The expected molecular size of *fliCH7* fragments was 230 bp.

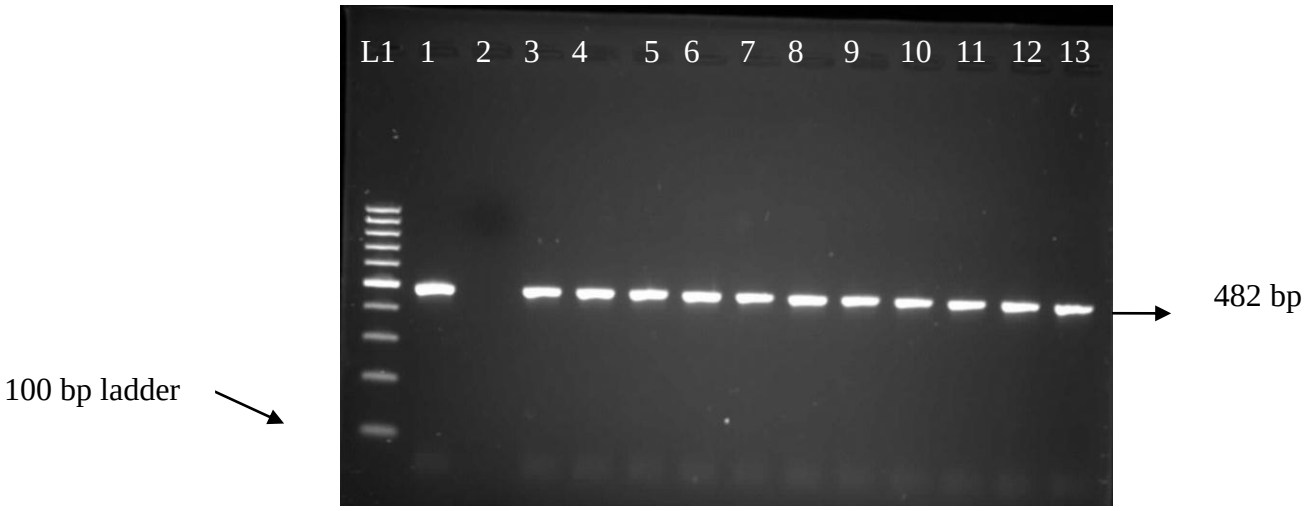


**Figure 4.2- Amplification product from the primer pair of the *eae* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970 and Lane 13:1836. The expected molecular size of *eae* fragments was 482 bp.

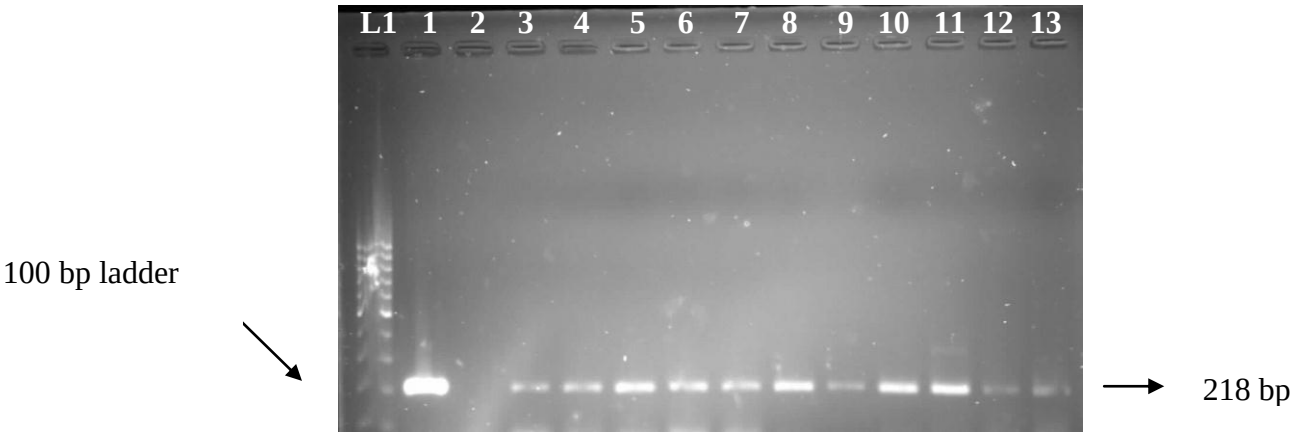


**Figure 4.3 - Amplification product from the primer pair of the *lt* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970, Lane 13:1836 and Lane 14: 1415.The expected molecular size of *lt* fragments was 218 bp.

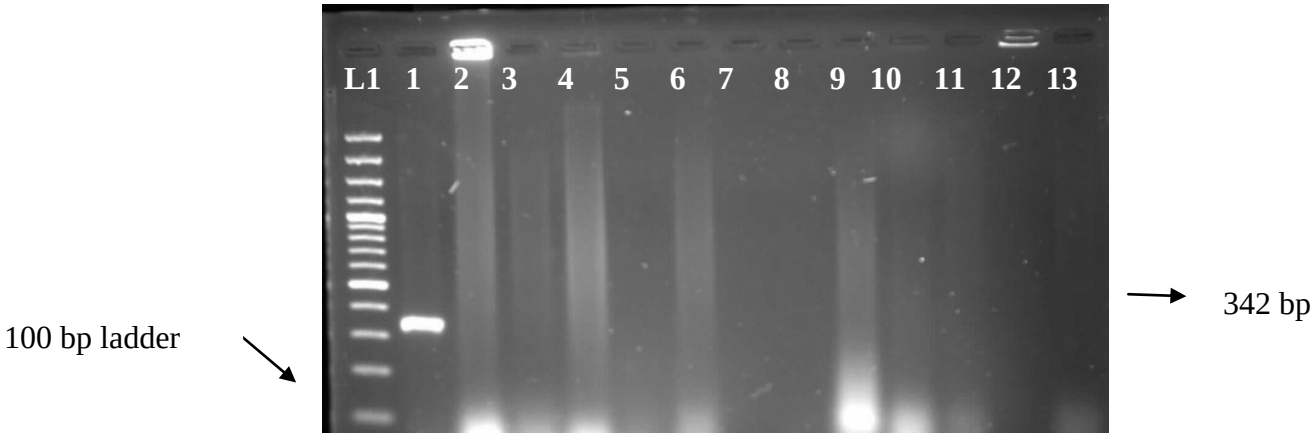


**Figure 4.4 - Amplification product from the primer pair of the *ibeA* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970 and Lane 13:1836. The expected molecular size of *ibeA* fragments was 342 bp. There was no isolate identified with the *ibeA* gene.


**Figure 4.5 - Amplification product from the primer pair of the *papC* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024 and Lane 12: 1415. The expected molecular size of *papC* fragments was 328 bp.

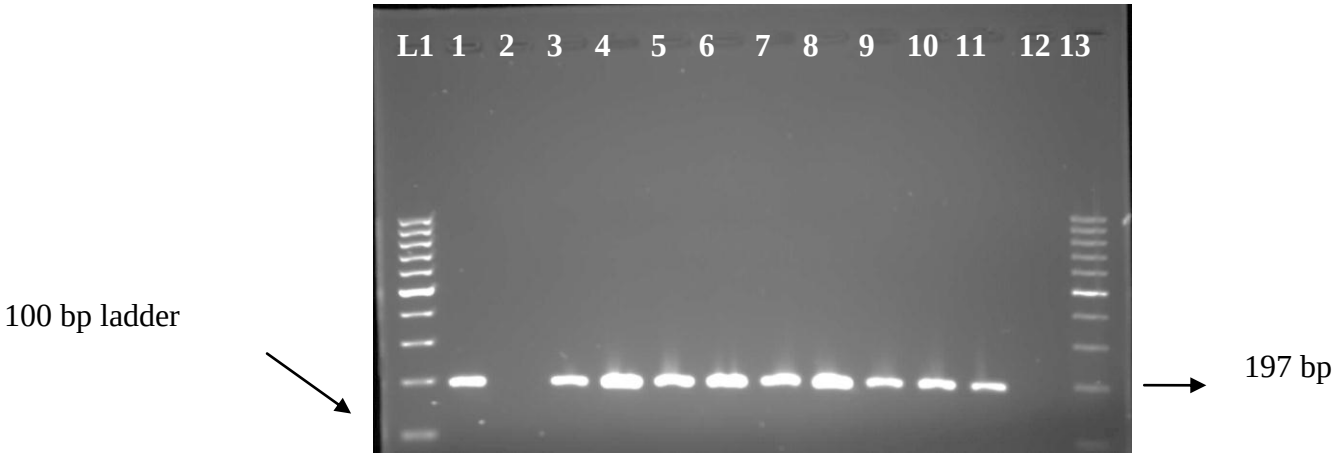


**Figure 4.6 - Amplification product from the primer pair of the *eagg* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970, Lane 13: 100 bp ladder.The expected molecular size of *eagg* fragments was 197 bp.

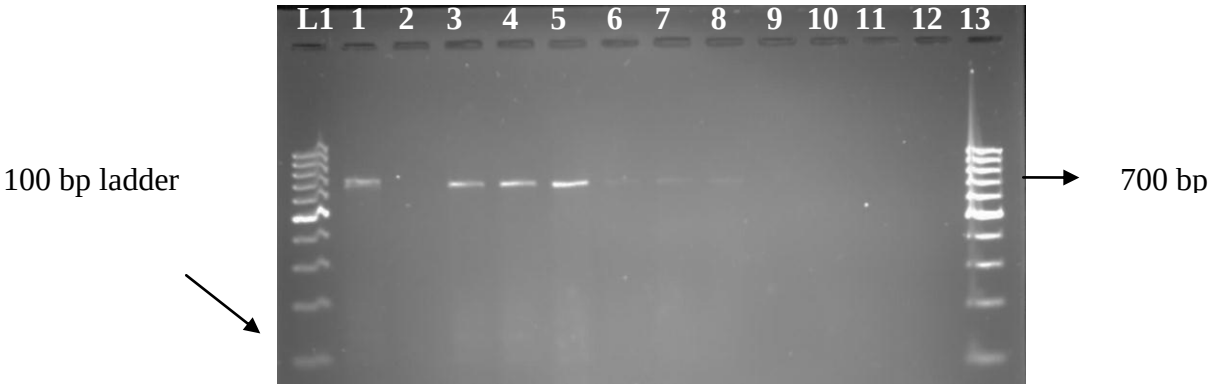


**Figure 4.7 - Amplification product from the primer pair of the *ial* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970, Lane 13:100 bp ladder. The expected molecular size of *ial* fragments was 700 bp.

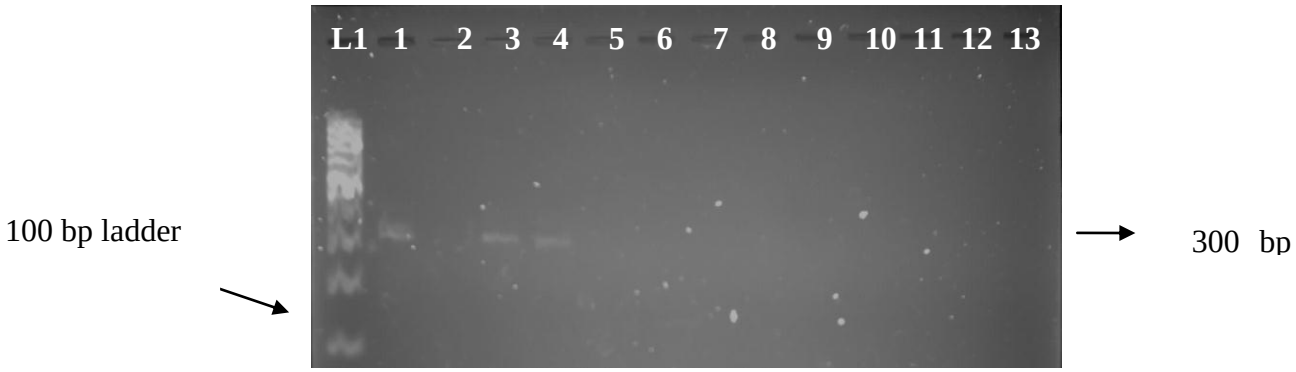


**Figure 4.8 - Amplification product from the primer pair of the *daaE* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: positive control, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970 and Lane 13:1836. The expected molecular size of *daaE* fragments was 300 bp.

The number of *E. coli* isolates that were positive by PCR for *fliCH7, eae, lt, papC, ial, eagg* and *daaE* genes are summarized in Table 4.2. Middledrift dairy had the highest amount of virulence genes (42%) compared to Fort Hare dairy (38%) except for *eagg* gene which encodes for EAEC. The target gene of *E. coli* O157 (*fliCH7*) was noticed in the isolates obtained from both dairy farms in high amounts in comparison to other virulence genes.

Prevalence rate was calculated by dividing the number of occurrences of the health indicator during the specified time period by the size of the population in which the health indicator occurs. The result is expressed as a percentage. The formula for calculating prevalence in PedNSS (Pediatric Nutrition Suveillance System) and PNSS is shown below.

$$\text{Prevalence} = \frac{\text{persons with a given health indicator during a specified time period}}{\text{population during the same time period}} \times 100$$

Prevalence of *E. coli* = number of pathogenic *E. coli* isolated from raw milk X 100

population during the same period

= 87÷400×100

= 21.75%

**Table 4.2- Occurrence of pathogenic *E. coli* isolates from two dairy farms as indicated by presence of the target gene marker.**

| Location | Amplified genes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *fliCH7* | *eae* | *Lt* | *ibeA* | *papC* | *ial* | *eagg* | *daaE* |
| **Middledrift dairy** | 38 (44%) | 19 (22%) | 20 (23%) | 0 (0%) | 3 (4%) | 4 (5%) | 5 (6%) | 2 (2%) |
| **Fort Hare dairy** | 16 (18%) | 9 (10%) | 7 (8%) | 0 (0%) | 1 (1%) | 2 (2%) | 7 (8%) | 0 (0%) |
| **Total** | 54 (62%) | 28 (32%) | 27 (31%) | 0 (0%) | 4 (5%) | 6 (7%) | 12 (14%) | 2 (2%) |

## 4.4 Discussion

Raw unpasteurized milk can harbor a variety of microorganisms and can be an important source of food-borne pathogens. The presence of foodborne pathogens in raw milk is due to direct contact with contaminated sources in the dairy farm environment and to udder excretion of an infected animal (Oliver *et al*., 2005). There are several reasons to be concerned about the microbial quality of raw unpasteurized milk and dairy products: first, outbreaks of disease in humans have been traced back to the consumption of unpasteurized milk, and also to pasteurized milk; second, unpasteurized milk is consumed directly by dairy producers, farm employees, their families and neighbours, and raw milk advocates; third, unpasteurized milk is consumed directly by a large segment of population via consumption of several types of cheese produced from unpasteurized milk (Collins *et al*., 1995; Oliver *et al*., 2005). Nowadays, public health concern associated with microbial food safety is on the

increase since *E. coli* is not only regarded as an indicator of faecal contamination but more likely as an indicator of poor hygiene and insufficient sanitary practices during milking. Detection of pathogenic bacteria in raw milk samples is still important, especially in more rural areas as it is easily available and economical.

The results of this study confirm the poor microbiological quality of raw milk obtained from two dairy farms in the Eastern Cape Province of South Africa. The PCR assays successfully amplified the target gene *fliCH7* from 38 (44%) samples from Middledrift and 16 (18%) from Fort Hare Dairy which is characteristic of the Enterohaemorrhagic *Escherichia coli* O157:H. Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin (*Stx*)-producing *E. coli* (STEC) strains that are isolated from human patients and are responsible for severe clinical symptoms, including diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (Bettelheim, 2001; Elliot *et al*., 2001). An outbreak of *E. coli* O157:H7, which affected 16 people and caused 5 HUS cases among children, was linked to a yogurt made on a farm from pasteurized milk. Post-pasteurization contamination of milk was likely due to either inadequate cleaning or contamination from farmyard material (Murinda *et al*., 2004). This gene has also been isolated in *E. coli* from water by Okoh and Osode (2010).

Target gene *ibeA* which is also characteristic of the Neonatal Meningitis *Escherichia coli* was not present in all the isolates from both dairy farms. Neonatal Meningitis *E. coli* is usually present in new borne and since this gene was not present in the isolates it proves that this gene cannot be present since new borne don't drink raw milk. NMEC is a common inhabitant of the gastrointestinal tract and it is the most frequent cause of Gram-negative-associated meningitis in new borne. Fatality rates can approach 40% (Kaper *et al*., 2004), and survivors are usually burdened with severe neurological sequelae.

The *lt* gene which encodes for heat-labile Enterotoxigenic *E. coli* was amplified from 20 (22 %) isolates in Middledrift and 7 (8%) from Fort Hare dairy farms. Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhea in infants and in travelers from developed to underdeveloped countries, especially in regions of poor sanitation (Ericsson, 2003). The ETEC are acquired by the ingestion of contaminated food and water, and adults living in endemic areas develop immunity. The disease condition manifests as a minor discomfort to a severe cholera-like syndrome and requires colonization by the microorganism and the elaboration of one or more enterotoxins (Evans *et al*., 1990; WHO, 2007). This gene has also been detected from water (Okoh and Osode, 2008).

The *ial* gene which is found in Enteroinvasive *Escherichia coli* was also amplified from 4 (5%) isolates in Middledrift dairy and 2 (2%) isolates from Fort Hare dairy. EIEC has principally been associated with contaminated food and water (Gordillo *et al*., 1992; Maier, *et al*., 1973) although cases of person-to-person transmission of EIEC have been noted (Harris *et al*., 1985). After ingesting the organisms of EIEC, there is an invasion and adhesion of the epithelial cells of the intestine. The invasion of the cells can trigger a mild form of diarrhea or dysentery, often mistaken for dysentery caused by Shigella species. The illness is characterized by the appearance of blood and mucus in the stools of infected individuals or a condition called colitis (Todar, 2008). The illness is characterized by abdominal cramps, diarrhea, vomiting, fever, chills, and a generalized malaise. Outbreaks have been associated with hamburger meat and unpasteurized milk (Lan *et al*., 2004). This is the first report where EIEC has been isolated from raw milk in the Eastern Cape Province of South Africa.

Enteroaggregative *Escherichia coli* characterized by *eagg* gene was amplified from 5 (6%) isolates in Middledrift and 7 (8%) isolates from Fort Hare dairy. Enteroaggregative *Escherichia coli* is an emerging diarrheagenic pathogen associated with diarrheal illnesses among patients in developed and developing countries. This organism has been increasingly isolated and characterized around the world from human clinical, animal, and environmental samples (Yamamoto and Nakazawa, 1997; Kahali *et al*., 2004; Falcao *et al*., 2004). However, frequencies of Enteroaggregative *Escherichia coli* among patients with diarrhea in the Eastern Cape Province, South Africa, are not known.

Enteropathogenic *E. coli* characterized by the presence of the *eae* gene was amplified from 19 (22%) isolates in Middledrift and 9 (10%) isolates in Fort Hare dairy farm. EPEC strains are the oldest recognized category of diarrheogenic *E. coli* (Zuber, 1999) and are well known prominent cause of diarrhea, particularly in children in less developed countries (Tennant *et al*., 2009; Trabulsi *et al*., 2002).

Uropathogenic *E. coli* which is characterized by *papC* gene was amplified from 3 (4%) isolates rom Middledrift and from 1 (1%) isolate in Fort Hare farm. If UPEC is ingested it can be capable of causing cystitis in the bladder and acute pyelonephritis in the kidneys (Alteri *et al*., 2009; Croxen and Finlay, 2010).

The *daaE* gene which encodes diffusely adherent *E. coli* was only amplified from 2 (2%) isolates in Middledrift dairy. It is presumed that the virulence factor of the organism (DAEC) is diffuse attachment to the intestinal lining of the infected host (Sarkar, 2008). In a study carried out by Meraz *et al*., 2008, the major clinical symptoms of the patients carrying DAEC strains were found to include watery diarrhea in some instances with mucus and blood,

vomiting, with vomiting being more prominent as compared to diarrhea among children. The pathotypes identified in this study are all from feaces and could mean that the staff does not wash their hands after visiting the mens or ladies rooms

The Act in South Africa which governs the safety of milk per se, and which sets standards to which milk and dairy products must confirm to is the foodstuffs, cosmetics and disinfection Act, No. 54 0f 1972. According to this Act milk may not contain pathogenic organisms, extraneous matter or any inflammatory product or any substance which may render it unfit for consumption. Bacteriologically it may not contain more than 20 coliforms or any *E. coli* per ml. This study indicates that raw milk samples from both Middledrift and Fort Hare dairy were not satisfactory in course of public health standard as some pathogenic *E. coli* strains were detected from the milk samples.

### 4.5 Conclusion and recommendations

The results obtained in this study concluded that raw cow's milk available to consumers in the Eastern Cape, South Africa was contaminated with the opportunistic pathogen *E. coli*. High and strict preventive measures like regular washing and sterilization of dairy equipment, utensils, milker's hands, and animal udders, and eradication of diseased animals from the herd are highly recommended with the milker’s hands taking priority. Teaching and training programs, for those working at the dairies, can possibly improve the situation

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# CHAPTER FIVE

## Frequency of antibiotic resistance determinants obtained from raw milk in two farms of the Eastern Cape Province


## ABSTRACT

Monitoring of antimicrobial resistance (AMR) in bacteria has clinical and public health significance. This study was carried out to evaluate the antibiogram of the *E. coli* strains and to determine the genes that mediate antibiotic resistance. A total of 87 *E. coli* isolates, 50 (57%) from Middledrift and 37 (43%) from Fort Hare dairy were subjected to antibiotic susceptibility testing. The genes implicated in antibiotic resistance (*ermA*, *bla-Z*, *strA* and *tetA*) were amplified using conventional PCR method. All of the isolates were 100% resistant to penicillin G and erythromycin and contained the erythromycin *ermA* gene and the penicillin G *bla-Z* gene. Middlefrift dairy farm showed intermediate resistance to streptomycin 30 (81.1%) and 32 (86.5%) for tetracycline while Fort Hare farm showed 48 (96%) and 40 (40%) for streptomycin and tetracycline respectively. However, *strA* gene which encodes for streptomycin and the *tetA* gene which encodes for tetracycline could not be amplified. The present results showed that the phenotypic antibiotic susceptibility patterns were similar to those obtained by genotyping done by conventional PCR for erythromycin and penicillin G but not in the case of streptomycin and tetracycline. Rapid and reliable methods for antibiotic susceptibility are important to determine the appropriate therapy decisions.



**Keywords**: Antibiotic resistance, *Escherichia coli*, Erythromycin, Tetracycline, Penicillin G and Streptomycin.

## 5.1 Introduction

The progressive emergence and rapid dissemination of antibiotic resistance in *E. coli* and its association with the use and consumption of antibiotics constitute a major health concern and have been considered a global crisis (Barkema *et al*., 2006; Velusamy *et al*., 2007). *E. coli* is the causative agent of most diarrheal diseases and is associated with serious diseases such as hemolytic uremic syndrome (HUS), meningitis and bloody diarrhea. (Kaper *et al*., 2004). Serious complications occur because of multiple-antibiotic resistant *E. coli*. As *E. coli* is representative of many drug resistant bacteria (Barbosa and Levy, 2000), it is necessary to study the ways to control this bacterium. Penicillin G the first antibiotic used in the control of *E. coli* developed resistance within approximately 2 years of its introduction and has increased gradually over the past 50 years (Barbosa and Levy, 2000). Other antibiotics thereafter also developed resistance to *E. coli* such as erythromycin, tetracycline, streptomycin, etc. Antibiotics used in animal husbandry, have led to the increasing concerns with regard to their contribution to the abundance and persistence of antibiotic resistance in populations of pathogenic, commensal, and nonpathogenic microorganisms (Auerbach *et al*., 2007, Yang and Carlson, 2003). The overuse of antibiotics, chemicals such as disinfectants, antiseptics, pesticides together in control of mastitis and on farm maintenance, has resulted in a significant increase of antibiotic resistant bacteria in dairy farms (Schwartz *et al.,* 2003). Bacteria may be defined as resistant when they are not susceptible to a concentration of antimicrobial agent such as antibiotics and this is indicative of the selection pressure exerted on bacteria (Cloete, 2003). The occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater have been reported in other parts of the world (Brown *et al.*, 2006; Miao *et al.*, 2004; McArdell *et al.*, 2003; Alder *et al.*, 2001). Reinthaler and co-workers (2003) reported antibiotic resistance of *E. coli* in sewage and sludge. There are no reports (to my knowledge) available on antibiotics susceptibility patterns in pathogenic *Escherichia coli*

isolated from raw milk in the Eastern Cape Province of South Africa. This study therefore hypothesize that the raw milk from two dairy farms in this Province are potential reservoirs of multi resistant antibiotics.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Antimicrobial Susceptibility Determination

The identified *E. coli* isolates from Chapter 3 were subjected to antibiotic sensitivity testing by the disc diffusion method (Bauer *et al*., 1966). The inocula of the *E. coli* isolates were prepared using the colony suspension method (EUCAST, 2003). Briefly, colonies were picked from 18-24 hour old cultures grown on Nutrient Agar (NA) were used to make suspension of the test organisms in saline solution to give an optical density of approximately 0.1 at 600nm. The suspension was then diluted 1:100 by transfer of 0.1 ml of the bacterial suspension to 9.9 ml of sterile Nutrient Broth (NB) before use. Isolates were sub-cultured onto Mueller-Hinton agar (MHA) (BD Bioscience, Sparks, MD) and screened for susceptibility to locally produced commercial antimicrobial discs (Davies Diagnostics Pty Ltd) by the disk diffusion method (NCCLS 2000) using 10 antibiotics (Table 5.1). *E. coli* strain 8739 (American Type Culture Collection) was used as a reference control strain. Plates were incubated at 35 $^{o}$C for 18-24 hours and zones of inhibition were interpreted as resistant or sensitive using the interpretative chart of the zone sizes of the Kirby – Bauer sensitivity test method (Cheesbrough, 2000).

**Table 5.1-Antibiotics used for antibiotic susceptibility test**

| Antibiotic | Concentration |
|---|---|
| Florfenicol | 20 µg |
| Neomycin | 30 µg |
| Erythromycin | 15 µg |
| Streptomycin | 10 µg |
| Penicillin G | 10 µg |
| Thrimethropim | 5 µg |
| Gentamicin | 10 µg |
| Tetracycline | 30 µg |
| Kanamycin | 30 µg |
| Chloramphenicol | 30 µg |

### 5.2.2 Detection of resistant genes by molecular characterization

Firstly DNA was extracted from *E. coli* isolates identified in chapter 3 and 4 and from a positive control strains for *E. coli* (ATCC 8739) (SABS No ESC 20) purchased from the South African Bureau of Standards (SABS), Pretoria, South Africa. The extraction was done

as described in section 3.2.4 of chapter 3. The presence of relevant antimicrobial resistance genes encoding for the streptomycin phosphotransferases (*strA*); the *ermA* gene which encodes for erythromycin, the *tetA* gene which encodes for tetracycline and the *bla-Z* gene which encodes for beta-lactamase enzyme (penicillin G) were determined by PCR (Velusamy *et al*., 2007). The ingredients for running PCR contained 12.5 µl of (2X) PCR Master Mix (0.05 units/µl Taq DNA polymerase in reaction buffer, 4mM MgCl2, 0.4mM dATP, 0.4 mM dCTP, 0.4mM dGTP and 0.4mM dTTP (Thermo Scientific, SA), 6.5 µl of nuclease free water (Thermo Scientific, SA), 0.5 µl of each primer and 5 µl of DNA . The PCR assays will be carried out in a 25 µl reaction volume (Table 5.2).

**Table 5.2-Oligonucleotide sequences and predicted sizes for PCR amplification of different antimicrobial resistance genes from *E. coli*.**

| Target gene | Nucleotide sequence (5'-3') | Conditions | Amplicon size (bp) | References |
|---|---|---|---|---|
| *strA* | CTTGGTGATAACGGCAATTC<br>CCAATCGCAGATAGAAGGC | Pre-incubation at 94°C for 4 minutes. Thirty PCR cycles will run under the following conditions: denaturation at 94°C for 45s, primer annealing at optimum temperature for 45s and DNA extension at 72°C for 45s in each cycle. After the last cycle PCR tubes will be incubated for 7 minutes at 72°C and held at 4°C (Velusamy *et al*., 2007). | 548 | Gebreyes and Altier, 2002 |
| *ermA* | AAGCGGTAAACCCCTCTGA<br>TTCGCAAATCCCTTCTCAAC | Reactions were hot started for 5 min at 94°C. The reaction mixtures were then subjected to 35 PCR cycles (94°C for 2 min, 2 min at 55°C, and 1 min at 72°C). A final elongation step at 72°C for 7 min was also applied. | 190 | Duran *et al*., 2012 |
| *bla-Z* | ACTTCAACACCTGCTGCTTC<br>TGACCACTTTTATCAGCAACC | Reactions were hot started for 5 min at 94°C. The eaction mixtures were then subjected to 35 PCR cycles (94°C for 2 min, 2 min at 55°C, and 1 min at 72°C). A final elongation step at 72°C for 7 min was also applied. | 173 | Duran *et al*., 2012 |

| | | | | |
|---|---|---|---|---|
| *tetA* | GGCCTCAATTTCCTGACG<br><br>AAGCAGGATGTAGCCTGTGC | Pre-incubation at 94°C for 4 minutes. Thirty PCR cycles will run under the following conditions: denaturation at 94°C for 45s, primer annealing at optimum temperature for 45s and DNA extension at 72°C for 45s in each cycle. After the last cycle PCR tubes will be incubated for 7 minutes at 72°C and held at 4°C (Velusamy *et al*., 2007). | 372 | Guillame *et al*. (2000) |

### 5.2.3 Gel electrophoresis

The PCR products (10 µl aliquots) were resolved in 1.8 % agarose gel containing 5 µl Ethidium bromide in 1X TBE buffer (40 mM Tris–HCl, 20 mM NaAcetate, 1 mM EDTA, pH 8) (Cagney *et al*., 2004; Wang *et al*., 2002) before being visualized and photographed under the Alliance System. A 100-bp DNA ladder was included on each gel as a molecular size standard. The electrophoresis was carried out at 100 V for 1 hour.

## 5.3 RESULTS

### 5.3.1 Antibiotic susceptibility test

*E. coli* from both farms showed 100% resistance to both erythromycin and Penicillin G and intermediate resistance to streptomycin and tetracycline (Table 5.3 and 5.4).


**Figure 5.1- Kirby Bauer susceptibility assay results**

**Table 5.3-Antibiotic susceptibility testing of *E. coli* isolates from Fort Hare farm**

| | *E. coli* (n=37) | | |
|---|---|---|---|
| Antibiotics | S | I | R |
| Streptomycin (10 µg) | 7 (18.92%) | 30 (81.08%) | 0 (0%) |
| Neomycin (30 µg) | 37 (100%) | 0 (0%) | 0 (0%) |
| Gentamicin (10 µg ) | 37 (100%) | 0 (0%) | 0 (0%) |
| Penicillin G (10 µg) | 0 (0%) | 0 (0%) | 37 (100%) |
| Thrimethropim (5 µg) | 37 (100%) | 0 (0%) | 0 (0%) |
| Tetracycline (30 µg) | 5 (13.50%) | 32 (86.50%) | 0 (0%) |
| Florfenicol (20 µg) | 37 (100%) | 0(0%) | 0 (0%) |
| Kanamycin (30 µg) | 37 (100%) | 0 (0%) | 0 (0%) |
| Erythromycin (15 µg) | 0 (0%) | 0 (0%) | 37 (100%) |
| Chloramphenicol (30µg) | 37 (100%) | 0 (0%) | 0 (0%) |

S= susceptible, I= intermediate, R= resistant

**Table 5.4- Antibiotic susceptibility testing of *E. coli* isolates from Middledrift farm**

| | *E. coli* (n=50 ) | | |
|---|---|---|---|
| Antibiotics | S | I | R |
| Streptomycin (10 µg) | 2 (4%) | 48 (96%) | 0 (0%) |
| Neomycin (30 µg) | 50 (100%) | 0 (0%) | 0 (0%) |
| Gentamicin (10 µg ) | 50 (100%) | 0 (0%) | 0 (0%) |
| Penicillin G (10 µg) | 0 (0%) | 0 (0%) | 50 (100%) |
| Thrimethropim (5 µg) | 50 (100%) | 0 (0%) | 0 (0%) |
| Tetracycline (30 µg) | 10 (20%) | 40 (80%) | 0 (0%) |
| Florfenicol (20 µg) | 50 (100%) | 0(0%) | 0 (0%) |
| Kanamycin (30 µg) | 50 (100%) | 0 (0%) | 0 (0%) |
| Erythromycin (15 µg) | 0 (0%) | 0 (0%) | 50 (100%) |
| Chloramphenicol(30 µg) | 50 (100%) | 0 (0%) | 0 (0%) |

S= susceptible, I= intermediate, R= resistant

### 5.3.2 Molecular characterization of resistance genes

Amplification of resistance genes was attained on *bla-Z* and *ermA* genes but not on *tetA* and strA genes. Representative gel electrophoresis profiles of the target genes for antibiotic resistance are illustrated in Figures 5.1 and 5.2.

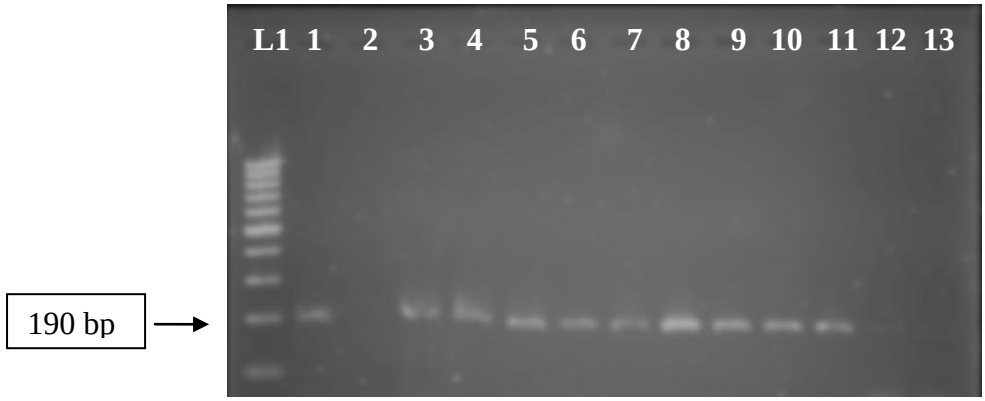


**Figure 5.2- Amplification product from the primer pair of the *ermA* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: H248, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 12:1970 and Lane 13:1836. The expected molecular size of *ermA* fragments was 190 bp.

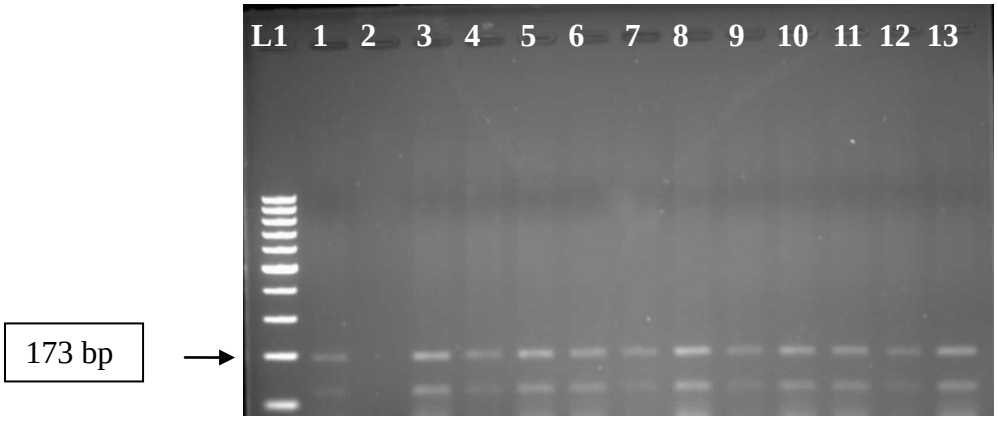


**Figure 5.3- Amplification product from the primer pair of the *bla-Z* gene.**

Lane L1: Low Range 100 bp DNA Ladder (Fermentas Life Sciences, SA); Lane 1: 9004, Lane 2: negative control, Lane 3: 1970, Lane 4: 8195, Lane 5: 1836, lane 6: 1415, Lane 7: 9010, Lane 8: H248, Lane 9: 8111 Lane 10: 8073, Lane 11: 9024, Lane 11:1970 and Lane 13:1836.The expected molecular size of *bla-Z* fragments was 173 bp.

All the isolates resistant to Penicillin G and erythromycin contained amplicons for *bla-Z* and *ermA* respectively. However there was no amplification for *strA* and *tetA* (Table 5.5).

**Table 5.5- Occurrence of resistant determinants of penicillin G and erythromycin isolated from raw milk in two dairy farms as indicated by presence of the target gene marker.**

| **Location** | **Amplified genes** | | | |
|---|---|---|---|---|
| | *ermA* | *bla-Z* | *strA* | *tetA* |
| Middledrift dairy | 50 (100%) | 50 (100%) | 0 (0%) | 0 (0%) |
| Fort hare dairy | 37 (100%) | 37 (100%) | 0 (0%) | 0 (0%) |

## 5.4 DISCUSSION

Many bacteria develop resistance mechanisms enabling them to inactivate antimicrobial compounds in their environment. Genetic exchange between similar or different bacterial species may result in the spread of resistant genes (Prescott *et al*., 1996). Pathogens in animals that are used for food products pose one of the greatest risks for human health, as this is a major route for the transfer of bacteria from animals to humans (Mevius *et al*., 2005). The incorrect use of antibiotics in the treatment of diseases such as mastitis and for use in feed as growth promoters has led to the assumption that antibiotic resistance in bacteria could become widespread because of the transmission of resistant zoonotic and non-zoonotic

bacteria via food (Mevius *et al*., 2005). This could have serious impact on the treatment of bacterial pathogens causing disease in humans if similar antibiotics are used (Shyrock, 2004). Veterinarians are ethically required to administer antibiotics in such a manner as to protect animals and prevent spread of disease. In addition, the spread of zoonoses to humans must also be prevented and the human safety must be ensured (Passantino, 2007).

Multiple resistances of the pathogenic *E. coli* isolates were found in the tested antimicrobial agents. The isolates showed a 100% resistance to Penicillin G and erythromycin. In this study all *E. coli* isolates were found to be resistant to penicillin G and erythromycin, our results are similar to what Osode (2008) have reported. The resistance of *E. coli* to β-lactams antibiotics (penicillin G) could be associated with the predominant use of penicillin for treatment of animal diseases; this result agrees with other results regarding the increase in incidence of β-lactam antibiotics resistance (Alekshun and Levy, 2000; Jovetic, *et al*., 2010; Al-Thani and Al-Ali, 2012). Erythromycin is a macrolide containing large cyclic molecule. Erythromycin in *E. coli* is predominantly mediated by erythromycin resistant methylases encoded by the *erm* gene. ErmA has been reported as having the most prevalent resistance in Staphyloccocci and is usually associated with inducible resistance. *ErmA* and *ErmC* are the most prevalent genes in human infection (Nicola *et al*., 1998). The resistance of organisms to macrolides is mediated by chloramphenicol acetyl transferase enzyme in some organisms (Scalet *et al*., 2010). All *E. coli* isolates showed high susceptibility to the antibiotics gentamicin and neomycin in our study a result also similar to Ibitsam and El Owni (2009) and Osode (2008). High susceptibility was revealed in the antibiotic kanamycin and thrimethropim a similar result to Obi *et al*., (2008). The *strA* gene which encodes for streptomycin and the *tetA* gene which encodes for tetracycline were not present in the isolates using conventional PCR. The results in our study showed that the phenotypic antibiotic susceptibility patterns were similar

to those obtained by genotyping done by conventional PCR for erythromycin and penicillin G but not in the case of streptomycin and tetracycline. Tetracycline and streptomycin are not only coded in the plasmid that carries the *tetA* and *strA* genes, other genes may also be involved. Streptomycin and tetracycline resistance genes *strA* and *tetA* respectively were also found positive by PCR method in 2 (4%) isolates and 10 (20%) isolates in Middledrift farm, whereas in Fort Hare dairy they were found to be positive in 7 (18.92%) isolates and 5 (13.50%) isolates. The accurate and rapid diagnosis of antibiotic resistance genes in the treatment of *Esherichia coli* infections is extremely important in preventing the spread of infections. PCR-based molecular methods are often preferred for determination of antibiotic resistance genes (Woodfrord and Sundsfjord, 2005). An explanation for the extra bands in some of the lanes is that the primers are hybridized to secondary sites of template and as a result of miss priming (Titus, 1991).

Antibiotics have been used for many years to eliminate bacterial pathogens causing disease. In the case of mastitis, it is important to note that antibiotic therapy cannot be relied upon to reduce the incidence of mastitis as a stand-alone anti-mastitis action. Isolation of bacterial agents and antimicrobial susceptibility test are important in reducing the occurrence of drug resistance and increase the production of milk and milk products. Failure to do so may cause the emergence of multidrug resistance in these organisms.

### 5.5 Conclusion and recommendations

The degree of antibiotic resistance exhibited by most of the isolates in this study is satisfactory. Given the growing number of reports of multidrug resistance to pathogenic *E. coli* isolates, it is evident that further research is still needed in this area. Given the severity of

the clinical manifestations of the diseases in humans and the inability and/or the potential risks of antibiotic administration for treatment, it appears that the most direct and effective measure towards ensuring public health is the prevention of *E. coli* infections in humans. Although finding effective pre-harvest control measures is not easy, approaches should focus on reducing the pathogens' incidence in foods and water.

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# CHAPTER SIX

## GENERAL DISCUSSION

*Escherichia coli* remains a major threat in many places around the globe as the causative agent of diarrhea, and its reservoir in raw milk may play an important role in the survival and transport of pathogenic strains. This study showed that target genes of *fliCH7, eagg, ial, eagg, lt, papC* and *daaE* that encode pathogenicity for *E. coli* were successfully amplified by PCR confirming that the isolates were pathogenic strains. Contamination of unpasteurized raw milk is a major human health issue. Certain types of *Escherichia coli* are among the pathogens commonly found in unpasteurized raw milk which can cause gastrointestinal illness. Since a lot of people still drink raw milk, especially in rural areas, this will emphasize the need for educational efforts on health risks associated with consumption of raw unpasteurized milk.

Resistance is common where antibiotics are heavily used, and additionally antibiotic resistant bacteria are present in raw milk due to the use of antibiotics in the treatment of mastitis (Barkema *et al*., 2006). The antibiotic resistance patterns of the isolates in this study corroborate results from previous investigations (Obi *et al.,* 2004). All isolates were resistant to penicillin G and erythromycin. High susceptibility patterns were seen in Florfenicol, chloramphenicol, neomycin, and gentamicin. All *E. coli* isolates showed high susceptibility to the antibiotics gentamicin and neomycin in our study a result also similar to Ibitsam and El Owni (2009) and Okoh and Osode (2010). A low level of resistance was seen amongst streptomycin and tetracycline by the disk diffusion method. The *strA* gene which encodes for streptomycin and the *tetA* gene which encodes for tetracycline were not present in the isolates

using conventional PCR. The results in our study showed that the phenotypic antibiotic susceptibility patterns were similar to those obtained by genotyping done by conventional PCR for erythromycin and penicillin G but not in the case of streptomycin and tetracycline. Tetracycline and streptomycin are not only coded in the plasmid that carries the *tetA* and *strA* genes, other genes may also be involved. Streptomycin and tetracycline resistance genes *strA* and *tetA* respectively were also found positive by PCR method in 2 (4%) isolates and 10 (20%) isolates in Middledrift farm, whereas in Fort Hare dairy they were found to be positive in 7 (18.92%) isolates and 5 (13.50%) isolates. The accurate and rapid diagnosis of antibiotic resistance genes in the treatment of *Esherichia coli* infections is extremely important in preventing the spread of infections. PCR-based molecular methods are often preferred for determination of antibiotic resistance genes (Woodfrord and Sundsfjord, 2005).

In summary this chapter presents a series of concluding remarks regarding the set objectives. The prevalence of pathogenic *E. coli* strains was ascertained. The antibiotic susceptibility profiles of the isolated *E. coli* strains were elucidated and the genes responsible for antibiotic resistance were determined.

**Conclusion and Recommendations**

The results obtained in this study concluded that raw cow's milk available to farmers and their families, dairy workers and neighbours in two dairy farms of the Eastern Cape Province of South Africa was contaminated with pathogenic *E.coli*. High and strict preventive measures like regular washing and sterilization of dairy equipment, utensils, milker's hands, and animal udders, and eradication of diseased animals from the herd are highly

recommended. Teaching and training programs, for those working at the dairies, can possibly improve the situation. Also, the degree of antibiotic resistance exhibited by most of the isolates in this study is satisfactory. Given the growing number of reports of multidrug resistant to pathogenic *E.coli* isolates, it is evident that further research is still needed in this area. Given the severity of the clinical manifestations of the diseases in humans and the inability and/or the potential risks of antibiotic administration for treatment, it appears that the most direct and effective measure towards ensuring public health is the prevention of *E.coli* infections in humans. Although finding effective pre-harvest control measures is not easy, approaches should focus on reducing the pathogens' incidence in foods and water and on consumer education. Major and/or minor limitations of the study: Farm managers do not want to be disturbed when milking because sample collection disturbs the process; they are concerned about time and money since these are commercial dairy farms.

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**APPENDIX**

**Table 1-API 20E results Middledrift dairy (n=72)**

| Cow tag | Identity (%) |
|---|---|
| 1. 9005 | *Escherichia coli* 96,6 |
| 2. 2055 | *Esherichia coli* 98.5 |
| 3. 7021 | *Esherichia coli* 65.5 |
| 4. 9090 | *Esherichia coli* 96,6 |
| 5. 2881 | *Escherichia coli* 76.5 |
| 6. 7074 | *Esherichia coli* 96,6 |
| 7. 1420 | *Esherichia coli* 99.9 |
| 8. 7050 | *Esherichia coli* 64.4 |
| 9. 8131 | *Esherichia coli* 93.3 |
| 10. H248 | *Esherichia coli* 94.5 |
| 11. 8111 | *Esherichia coli* 95,5 |
| 12. 0002 | *Esherichia coli* 100 |
| 13. 100028 | *Esherichia coli 98.8* |
| 14. 10064 | *Esherichia coli* 98.4 |
| 15. 7003 | *Esherichia coli 94.3* |
| 16. 9069 | *Esherichia coli* 76,8 |
| 17. 2249 | *Esherichia coli* 96,6 |
| 18. 2334 | *Esherichia coli* 98.5 |
| 19. 9088 | *Esherichia coli* 100 |
| 20. 2327 | *Esherichia coli* 99.8 |
| 21. 0083 | *Esherichia coli* 99.9 |
| 22. 2470 | *Kluyvera ssp* 76.5 |
| 23. 2105 | *Esherichia coli* 98.5 |

24. 2124 *Enterobacter cloacae* 65.5

25. 8254 *Esherichia coli* 96,6

26. 2043 *Escherichia coli* 76.5

27. 0147 *Esherichia coli* 93.3

28. 2361 *Esherichia coli* 94.5

29. 9043 *Esherichia coli* 95,5

30. 2592 *Esherichia coli* 100

31. 2283 *Esherichia coli 98.8*

32. 0026 *Esherichia coli* 98.4

33. 2098 *Enterobacter cloacae 94.3*

34. 2266 *Esherichia coli* 76,8

35. 2331 *Esherichia coli* 96,6

36. 2503 *Esherichia coli* 93.3

37. 9056 *Esherichia coli* 94.5

38. 2437 *Esherichia coli* 95,5

39. 7048 *Esherichia coli* 100

40. 2461 *Esherichia coli 98.8*

41. 09103 *Esherichia coli* 98.4

42. 8136 *Escherichia coli* 76.5

43. 011 *Esherichia coli* 93.3

44. 2073 *Esherichia coli* 100

45. 2352 *Esherichia coli 98.8*

46. 096 *Esherichia coli* 94.5

47. 2261 *Esherichia coli* 95,5

48. 9024 *Esherichia coli* 98.8

49. 2503 *Esherichia coli* 76.8

50. 8073 *Esherichia coli* 99.0

51. 2091 *Esherichia coli* 98.4

52. 2613 *Esherichia coli* 97.9

53. 2079 *Esherichia coli* 99.9

54. 8244 *Esherichia coli* 76.9

55. 2077 *Esherichia coli* 76.8

56. 9112 *Esherichia coli* 94.3

57. 0946 *Enterobacter cloacae* 99.9

58. 8128 *Esherichia coli 99.9*

59. 6596 *Esherichia coli* 96,8

60. 8050 *Esherichia coli* 99.9

61. 2592 *Esherichia coli 98.9*

62. 8313 *Esherichia coli* 99.9

63. 2011 *Esherichia coli* 76.4

64. 0012 *Esherichia coli* 99.9

65. 8405 *Esherichia coli* 99.8

66. 2588 *Esherichia coli* 98.9

67. 8361 *Esherichia coli* 76.4

68. 0026 *Esherichia coli* 98.9

69. 8171 *Enterobacter cloacae* 98.5

70. 8131 *Esherichia coli 76.4*

71. 2598 *Esherichia coli* 75.9

72. 7001 *Esherichia coli* 78.9

**Table 2-API 20E results Fort Hare dairy (n=68)**

| Cow tag | Identity (%) |
|---|---|
| 1. 7022 | *Esherichia coli* 100 |
| 2. 7060 | *Esherichia coli* 98.8 |
| 3. 9021 | *Esherichia coli* 98.4 |
| 4. 7041 | *Enterobacter cloacae* 94.3 |
| 5. 8195 | *Esherichia coli* 76,8 |
| 6. 1960 | *Esherichia coli* 96,6 |
| 7. 8110 | *Esherichia coli* 98.5 |
| 8. 7053 | *Serratia liquefaziens* 100 |
| 9. 8031 | *Esherichia coli* 99.8 |
| 10. 9816 | *Esherichia coli* 99.9 |
| 11. 1760 | *Esherichia coli* 76.5 |
| 12. 1415 | *Esherichia coli* 76.8 |
| 13. 8195 | *Esherichia coli* 99.0 |
| 14. 1836 | *Esherichia coli* 98.4 |
| 15. 1970 | *Esherichia coli* 97.9 |
| 16. 1544 | *Esherichia coli* 94.3 |
| 17. 1194 | *Esherichia coli* 99.9 |
| 18. 1254 | *Esherichia coli* 76.9 |
| 19. 8122 | *Esherichia coli* 76.8 |
| 20. 8100 | *Esherichia coli* 94.3 |
| 21. 1041 | *Enterobacter cloacae* 99.9 |
| 22. 1170 | *Esherichia coli 99.9* |
| 23. 7029 | *Esherichia coli* 96,8 |
| 24. 8088 | *Esherichia coli* 100 |
| 25. 1376 | *Esherichia coli 98.8* |

26. 1051 *Esherichia coli* 94.5

27. 1797 *Esherichia coli* 95,5

28. 1772 *Esherichia coli* 98.8

29. 10276 *Esherichia coli* 76.8

30. 7053 *Esherichia coli* 99.0

31. 1859 *Esherichia coli* 98.4

32. 1400 *Esherichia coli* 97.9

33. 6375 *Esherichia coli* 99.9

34. 1890 *Esherichia coli* 76.9

35. 1757 *Esherichia coli* 76.8

36. 6346 *Esherichia coli* 94.3

37. 1121 *Esherichia coli 99.9*

38. 1618 *Esherichia coli* 96,8

39. 1881 *Esherichia coli* 99.9

40. 1636 *Esherichia coli 98.9*

41. 1942 *Esherichia coli* 99.9

42. 10122 *Esherichia coli* 76.4

43. 8045 *Esherichia coli* 99.9

44. 1611 *Esherichia coli* 99.8

45. 7023 *Esherichia coli* 98.9

46. 1900 *Esherichia coli* 76.4

47. 1960 *Esherichia coli* 98.9

48. 1961 *Esherichia coli 99.9*

49. 1749 *Esherichia coli* 96,8

50. 10040 *Esherichia coli* 99.9

51. 1008 *Esherichia coli 98.9*

| | |
|---|---|
| 52. 1194 | *Esherichia coli* 99.9 |
| 53. 1915 | *Esherichia coli* 76.4 |
| 54. 1396 | *Esherichia coli* 99.9 |
| 55. 1002 | *Esherichia coli* 99.8 |
| 56. 1317 | *Esherichia coli* 98.9 |
| 57. 1174 | *Esherichia coli* 76.4 |
| 58. 1544 | *Esherichia coli* 98.9 |
| 59. 8132 | *Esherichia coli* 76,8 |
| 60. 1671 | *Esherichia coli* 96,6 |
| 61. 1618 | *Esherichia coli* 93.3 |
| 62. 1586 | *Esherichia coli* 94.5 |
| 63. 9210 | *Esherichia coli* 95,5 |
| 64. 1037 | *Esherichia coli* 100 |
| 65. 1835 | *Esherichia coli 98.8* |
| 66. 1316 | *Esherichia coli* 98.4 |
| 67. 7044 | *Escherichia coli* 76.5 |
| 68. 1829 | *Esherichia coli* 93.3 |

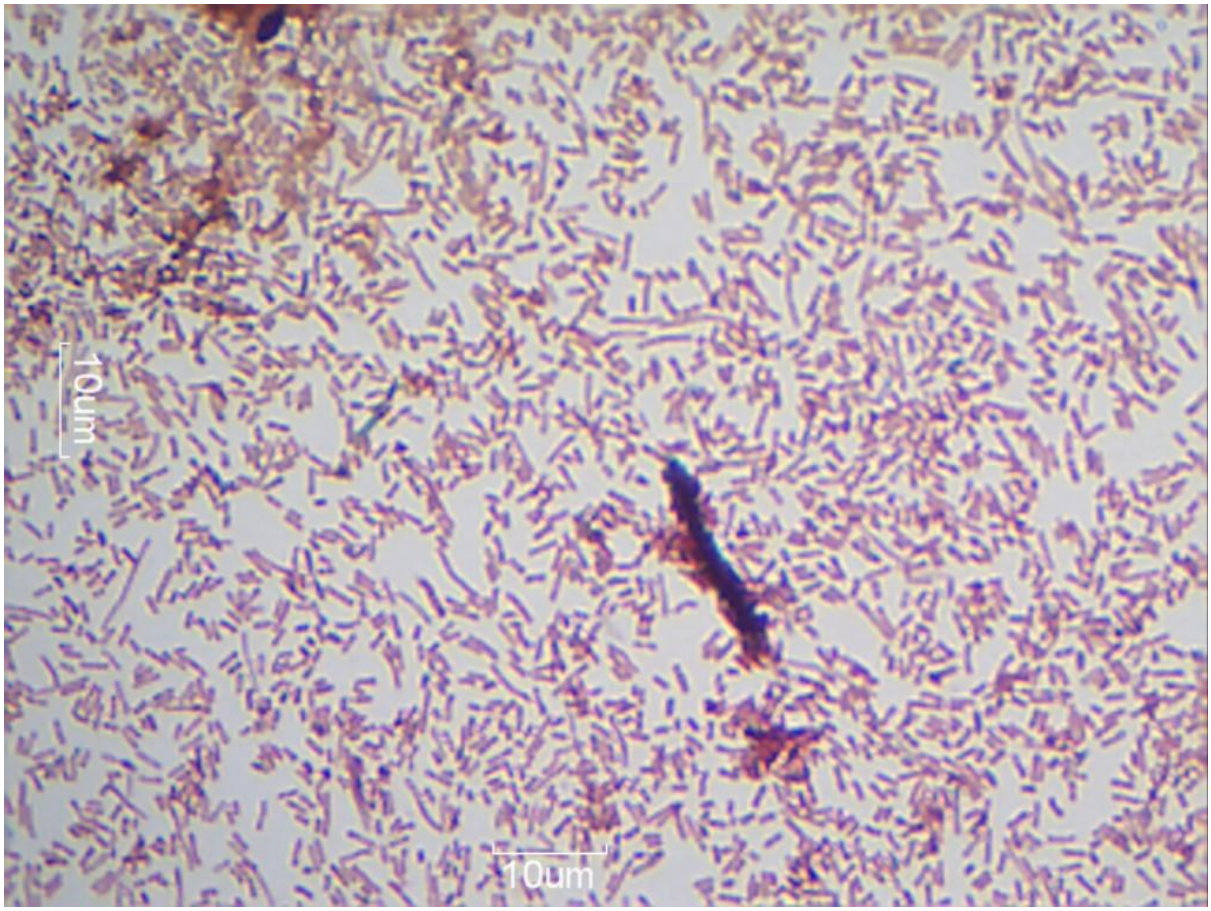


**Figure 1**-Illustration of Gram negative rods isolated from raw milk observed under a light microscope.