ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

INSTITUTE OF BIOTECHNOLOGY

Characterization of Diarrheagenic *Escherichia coli* strains Isolated from Dairy Calves in Fiche, Debretsige and Muketuri towns; North Shoa, Ethiopia

By

Abinet Endale

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Addis Ababa, Ethiopia

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By
Abinet Endale
Advisor
Tesfaye Sisay, DVM, MSc, PhD

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Addis Ababa, Ethiopia

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Abinet Endale** entitled: **Characterization of Pathogenic *Escherichia coli* strains Isolated from Dairy Calves in Fiche, Debretsige and Muketuri Towns North Shoa, Ethiopia** and recommend that it will be accepted to fulfill the thesis requirement for degree of Masters in **Biotechnology.**

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ACRONYMS

A/E	Attaching Effacing
aEPEC	Atypical Enteropathogenic *E. coli*
ANOVA	Analysis of variance
BFP	Bundle forming pilus
CF	Colonization factors
DAEC	Diffusely Adherent *E. coli*
DEC	Diarrheagenic *E. coli*
DNA	Deoxyribonucleic acid
EAE	Enterocyte Attaching Effacing
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *E. coli*
GIT	Gastrointestinal tract
HGT	Horizontal gene transfer
LEE	Locus of enterocyte effacement
LT	Heat labile toxin
MANOVA	Multivariate analysis of variance
PAI	Pathogenicity associated island
PCoA	Principal coordinates analysis
PCR	Polymerase Chain Reaction
PFGE	Pulse-field gel electrophoresis
SPSS	Statistical Package for Social Science
ST	Heat stable toxin
STEC	Shiga-like toxin producing *E. coli*
Stx	Shiga toxin
T3SS	Type III secretion system
TAE	Tris base, Acetic acid and EDTA
TBE	Tris, Borate and EDTA
tEPEC	Typical enteropathogenic *E. coli*
VG	Virulence gene
WHO	World Health Organization

ABSTRACT

Diarrheagenic *E. coli* (DEC) strains are associated with several outbreaks and sporadic cases worldwide. Understanding the nature of the organisms is important in order to tailor preventive and control strategies before any harm emanates. Thus, the distribution of these organisms among humans as well as animals should be studied, which was the major objective of the current study. In this study a total of 73 *E. coli* samples isolated from feces of dairy calves found in 56 farms located in Fiche, Debretsige and Muketuri areas were characterized. Isolates were assessed and characterized based on their virulence gene and plasmid content. Moreover, the study tried to show the relationship between virulence gene, plasmid content antibiotic resistance traits. The study identified the occurrence of virulence genes in 69.86% of isolates, carrying at least one of the six VGs screened. Among the VGs *eae* was most frequent, observed in 58.90% of isolates, followed by *stx1* 34.25 %, *stx2* 24.66%, *ehlyA* 23.29%, *bfpA* 12.33% and *aatA* 5.48% observed in the total sample isolates. A total of eleven distinct virulence profiles were identified based on combination of virulence genes and isolates were placed into five different pathotypes with a frequency of EHEC 30%, tEPEC 12%, aEPEC 16% and 6% of STEC and EAEC each. Plasmid analysis on the other hand revealed the occurrence of ten different kinds of plasmids ranging in size from 2.6 ± 0.14 Kbp to 98.2 ± 4.17 Kbp and exhibited 13 different kinds of distribution profile among isolates. In an effort made to observe any possible association among virulence factors, plasmid content and antibiotic resistance traits, a significant one to one correlation was observed. a Pearson product moment correlation in a range of $r = 0.17$ to $r = 0.56$. Accounting to the total dataset, isolates showed significant segregation in to the sample site, indicating spatial clustering of isolates based on overall pathogenic characteristics. The demonstration of pathogenic potential in substantial amount of *E. coli* isolates from dairy farms from all the three sites indicate the level of risk posed in humans as well as animals. Thus, identifying potential sources and route of transmission of DEC is vitally important in order to establish control and prevention strategies for DEC infection. Moreover, nationwide screening for virulence factors and antibiotic resistance genes and associated plasmids is recommended in order to prevent the spread of these pathogens among various sources.

Keywords: Calf, Diarrhea, *Escherichia coli*, Plasmid, Virulence factor, Virulence gene

1. INTRODUCTION

Escherichia coli used to be regarded as a harmless commensal to mammals ever since its first discovery in 1885 by Theodor Escherichia (Croxen *et al*., 2013). However, consecutive outbreaks in the developed world placed the organism under close scrutiny among public health authorities and researchers. Early in nineteen fifties' it was found that, *E. coli* has extremely diverse pathogenic varieties that cause diseases in humans as well as other mammals and birds (Nataro and Kaper, 1998; Croxen *et al*., 2013).

Currently pathogenic strains of *E. coli* are known to cause a wide variety of diseases in wide range of hosts, infecting sites such as; the urinary tract, blood stream, central nervous system and gastro intestinal tract (GIT) (Croxen *et al*., 2013; Nataro and Kaper, 1998). The transmission potential of these pathogens is extraordinary, given to their ability to survive in diverse habitat. Food, water, animals and contaminated environment are implicated as potential sources for the transmission of the organism (Bolton *et al*., 2009). These facts put pathogenic *E. coli* to be a major public health threat.

Among pathogenic strains, more than six pathotypes of intestinal pathogenic *E. coli* or diarrheagenic *E. coli* (DEC) were recognized so far. These are; Enteropathogenic *E. coli* (EPEC), Shigatoxigenic *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely Adherent *E. coli* (DAEC) O'Sulevan *et al*. (2007). The pathogenic potential of these strains is afforded by the presence of diverse virulence factors (VFs) encoded by specific virulence genes (VGs) which are generally not present in another *E. coli* (Bolton *et al*., 2008; de Sousa1, 2006). These virulence factors confer colonization, fitness and overall pathogenic potential to the organism (Kaper *et al*., 2004). Even though all pathotypes contribute to diarrhea; mechanism of colonization, symptoms and clinical outcomes vary considerably, exemplifying the diversity of the organism (Croxen and Finlay, 2010). The different pathotypes of DEC, were thoroughly reviewed in the comprehensive reviews by Nataro and Kaper (1998) and Croxen *et al*. (2013).

In general, diarrhea causes considerable morbidity and mortality worldwide, particularly in children under the age of five (Kotloff *et. al*., 2013). While there are many etiological agents responsible for diarrhea, DEC takes the major share as it is the most frequently isolated organism in diarrheal patients (Levine *et al*., 2012). Although DEC were found to be the most common cause of bacterial diarrhea in many parts of the world, still to date comprehensive picture of DEC incidence as well as bacterial and host factors that predispose to disease are largely unknown especially in the developing world (Butterton and Calderwood, 2005; Kotlof *et al.*, 2013). However, the disease burden is expected to be high in Africa, given that food-borne disease occurs commonly in developing countries because of inadequate food safety laws, weak regulatory system, poor food handling and sanitation practices (Kotlof *et al.*, 2013; Okeke, 2009).

Previous efforts by the scientific as well as clinical communities lead to great successes in understanding the biology, ecology and pathology of these organisms. These advances helped clinicians for quick and appropriate management of associated illness. Nonetheless, the high prevalence rate and the worrisome emergence of antimicrobial resistance and high asymptomatic carriage rates in diverse hosts and reservoirs, makes the clinical management difficult (Bryce *et al*., 2016; Chattaway *et al*., 2016; Goldwater and Bettelheim, 2012).

Like the rest of Africa, surveillance for various types of DEC is overlooked in Ethiopia. So far only limited studies were done on the prevalence and burden of associated illnesses in Ethiopia, indicating the need for local studies for understanding the distribution of various DEC types and associated health impact. Thus, this study is part of an effort that tries to map intestinal pathogenic *E. coli* circulating throughout the country. More particularly, this study was conducted to characterize DEC isolates sampled from cattle farms located in three towns of North Shoa Zone of Oromia region, Ethiopia.

The information that can be acquired through molecular characterization helps to identify pathogen-specific virulence factors, potential antibiotic resistance and occurrence of the different pathotypes that helps to monitor spread of these pathogens. Thus, presentation of the results of the study along with information gathered elsewhere help to fill the gaps in our knowledge and understanding related to these pathogens. The study would also contribute to the development and implementation of an effective management strategies against the pathogen and associated illness before causing significant health and economic damages. Furthermore, it can be used as a benchmark for further research.

2. OBJECTIVES

2.1. General objectives

The main objective of this study was to assess the occurrence of different diarrheagenic *E. coli* strains and characterize *E. coli* isolates based on various virulence features from diarrheic calves in Muketuri, Debretsige and Fiche towns of North Shoa Zone.

2.2. Specific objectives

The specific objectives of this research include;

- To characterize *E. coli* isolates based on major virulence factor genes and virulence phenotypes.
- To determine and describe the occurrence of different pathotypes of DEC in diarrheic calves of the sampled area
- To isolate and characterize plasmids carried by the *E. coli* isolates and identify possible association with drug resistance and virulence phenotype.
- To determine the association of the various factors with the distribution of phenotypic and genotypic characteristics.

3. LITERATURE REVIEW

3.1. Diarrheagenic *E. coli*

Infections of gastrointestinal tract by diarrheagenic *E. coli* (DEC) result in a mild to complicated diarrheal disease (Nataro & Kaper, 1998; Croxen *et al*., 2013). The disease-causing potential of DEC is conferred by the presence of specific virulence factors responsible for colonization and pathogenesis which are generally not present in another *E. coli* (Kaper *et al.*, 2004).

Based on their disease-causing potential, diarrheagenic strains of *E. coli* are categorized into different pathotypes. Currently, more than six diarrheagenic pathotypes are described (Croxen *et al*., 2013). While all DEC types induce diarrhea, the clinical symptoms, site and mechanism of colonization differ significantly exemplifying the diversity of the pathogen (Kaper *et al.*, 2004; Croxen and Finlay, 2010).

Infections by DEC are among the most common etiologic agents of diarrhea, making it a worldwide public health threat, with over two million deaths occurring each year (Kosek *et al*., 2003; Pupo *et al*., 2000). Currently the wealth of knowledge in the subject is immense, with countless articles published and extensive number of ongoing researches. Most of the previous researches were intensively reviewed by Nataro and Kaper (1998) and Croxen *et al*. (2013) and the following section summarizes most of the important findings covered in the above-mentioned comprehensive reviews together with latest findings the area.

3.2. The Different Pathotypes of Diarrheagenic *E. coli*

The different pathotypes of pathogenic *E. coli* are described based on pathogenic characteristics such as; site of infection, type of toxin secretion, histopathologic features or clinical manifestation and disease outcome. More recently, following advances in molecular biology pathotypes are described based on possession of specific virulence factor genes and/or their combination. The overlap in genetic makeup as well as pathologic behavior among DEC types made classification of the organisms in to distinct category challenging. Moreover, as the organisms are still evolving the emergence of new pathotypes makes the classification and nomenclature even more challenging. So far different classification of pathotypes had been published, however for the sake of simplicity only the well described six tier classification of DEC pathotypes according to O'Sulevan *et al*. (2007) will be discussed in detail below.

3.2.1. Enteropathogenic *E. coli*

Enteropathogenic *E. coli* (EPEC) were the first DEC type identified as diarrheagenic. EPEC was first isolated in 1945 from children with diarrhea in UK (Kaper *et al.*, 2004). EPEC is a noninvasive organism that does not produce any kind of toxin. It is distinguished from other diarrheagenic *E. coli* by its ability to cause attaching and effacing (A/E) lesions on the surfaces of intestinal epithelial cells, a characteristic afforded by genes of the LEE. This lesion is characterized by localized destruction (effacement) of brush border microvilli (Croxen and Finlay, 2010). LEE encodes intimin, which mediates the intimate attachment of EPEC to epithelial cells. In addition to intimin, it also encodes type III secretion system (T3SS) and the associated chaperones and effector proteins responsible for pathogenesis (Elliott *et al*., 1998).

There are two distinct subtypes of EPEC; typical (tEPEC) and atypical (aEPEC), where the previous possess *E. coli* adherence factor plasmid (pEAF), which carries *bfp* gene that codes for bundle-forming pili (O'Sulevan *et al*., 2007). Relatively, aEPEC is highly prevalent and heterogeneous group than tEPEC. aEPEC strains are more closely related to LEE-positive STEC strains in serotype, genetic, virulence, pathogenic characteristics and reservoirs (McDaniel *et al*., 1995). This can be demonstrated by the evolutionary relationship between the highly pathogenic STEC strain O157:H7 and aEPEC O55:H7 which is believed to be its ancestor (Ogura *et al*., 2009).

Unlike the other pathotypes of DEC, humans are the only known reservoir for tEPEC, with symptomatic and asymptomatic children and asymptomatic adults being the most likely sources. However, no specific environmental reservoir has been identified. In contrast, atypical strains have been isolated from both human and animal sources including; cattle, dogs, rabbits and monkeys same as with other DEC types (Croxen *et al.*, 2013).

EPEC is a significant cause of infectious diarrhea in children. EPEC strains cause a watery diarrhea often accompanied by vomiting, fever, malaise and dehydration that may contain mucus but no sign of colitis (de Sousa1, 2006). The exact mechanism of EPEC associated diarrhea is generally not fully understood and likely involves a combination of different mechanisms based on particular subtype. One possible mechanism is A/E lesion following growth of tEPEC in the small intestine. The effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane result in a marked cytoskeletal changes and accumulation of polymerized actin directly beneath the adherent bacteria resulting a complete loss of microvilli and consequently diarrhea as a result of malabsorption (Kaper *et* *al.*, 2004). Nonetheless the speed of diarrhea onset implies a secretory mechanism rather than malabsorption is more likely cause of diarrhea. In this regard a number of T3S effectors are implicated to impact various water and ion channels of the intestinal epithelia contributing to diarrhea (Croxen and Finlay, 2010). In contrast, aEPEC strains do not produce bundle forming pili resulting in the formation of loose clusters of bacteria on epithelial cells, a pattern of known as "localized adherence-like" (LAL) which is slower to establish (Bolton *et al.*, 2008)

tEPEC associated diarrhea is highly prevalent in children under age of two years compared to adults. Yet no outbreaks of tEPEC have been reported so far (Croxen *et al.*, 2013). Despite its relatively high prevalence in asymptomatic children, aEPEC is now thought to be important in sporadic diarrhea in children as well as in outbreaks (Ochoa and Contreras, 2011).

3.2.2. Shiga-like toxin producing *E. coli*

Shiga-like toxin producing *E. coli* (STEC) represent a diverse group of DEC that cause illness ranging from mild diarrhea to hemorrhagic colitis (HC) and hemorrhagic uremic syndrome (HUS) (Muniesa *et al.*, 2012; Croxen and Finlay, 2010). These group of organisms were recognized as a distinct class of DEC following two key epidemiologic observations of HC and HUS by Riley and Karmali who described it as Verocytotoxigenic and Enterohemorrhagic *E. coli* respectively (Croxen *et al.,* 2013). But lately it was discovered that, both Verocytotoxin and Shiga toxin were the same pathogenic factor common between HC and HUS (Kaper *et al.*, 2004).

Usually a set of pathogenic characteristics are used as a signature in the description of pathotypes. Accordingly, the detection of either *Stx1* or *Stx2* genes coding Shiga-like toxin (Stx) in an *E. coli* isolate is considered as a standard to qualify an *E. coli* isolate as STEC (Feng *et.al.*, 2011). So far over 400 STEC serotypes were identified, yet only a subset of these has been associated with diarrheal illnesses (Wang *et al.,* 2003; DebRoy *et al.,* 2011). Among the various serotypes STEC O157:H7 is the most common and extremely pathogenic serotype indicated in diarrheal outbreaks worldwide (Bettelheim and Goldwater, 2014). Though STEC O157:H7 is considered as a major serotype of clinical importance, there are additional six serotypes, *i.e.*, O26, O45, O103, O111, O121 and O145 (also known as "Big 6"), are commonly found non O157 strain associated with sever human illnesses (Croxen *et al.* 2013*,* Bentancor *et al.*, 2012). Currently these pathogens become a major focus of public health authorities, as a result of high level of diarrheal diseases associated with foodborne sporadic cases and outbreaks (Scallan *et al.*, 2007).

STEC strains can be broadly classified based on their LEE carriage as; LEE negative and LEE positive STEC, where the latter one is viewed as a distinct subtype of STEC also known as Enterohemorrhagic *E. coli* (EHEC). This is because EHEC is usually associated with HC and HUS, a pathogenic characteristic conferred by virulence factors mainly encoded by genes on LEE (Kaper *et al.*, 2004; Croxen *et al.*, 2013).

Unlike the rest of DEC pathotypes, where humans are the major reservoirs, the major reservoirs for most STEC strains are ruminants. Predominantly cattle are implicated as major reservoirs of STEC (Croxen *et al.*, 2013, Bolton *et al.*, 2009). In addition to cattle; sheep, poultry and even game animals are also found to carry STEC strains of DEC (WHO, 1998; Bolton *et al.*, 2010, Miko *et. al.*, 2009). Moreover, a wide variety of other sources have been implicated as a source of infection, including fruits, vegetables, direct contact with animal fecal material, swimming in contaminated water and person to person contact (Bolton *et al.*, 2009).

3.2.3. Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) is a diverse pathotype that is defined by its ability to produce either a heat-labile (LT) or a heat-stable (ST) entero-toxin. In addition to the toxins the pathogens carry a diverse set of colonization factors (CFs) responsible for effective colonization in diverse mammalian hosts. ETEC strains might express only an LT, ST or both toxins (Kaper *et al.*, 2004).

On infection, ETEC first establishes itself by adhering to the epithelium of the small intestine via one or more colonization factors followed by the expression of ST or LT. These enterotoxins cause inhibition of sodium absorption and stimulation of chloride secretion, which give rise to intestinal secretion (Croxen and Finlay, 2010). These consequently leads to watery (secretory) diarrhea that range from mild, self-limiting disease to severe purging disease usually accompanied by abdominal cramps and sometimes nausea and headache. ETEC associated diarrhea typically lasts up to a week, but can be prolonged (Kaper *et al.*, 2004).

The infectious dose of ETEC is relatively high compared to the other DEC pathotypes. The bacteria can be isolated from both asymptomatic and symptomatic carriers and it is a major cause of traveler's diarrhea worldwide. ETEC is endemic in most underdeveloped countries causing significant mortality in children (Qadri *et al.*, 2005). It is estimated to cause around 600 million cases of human diarrhea and 800,000 deaths worldwide mainly in children under the age of 5 (Bolton *et al.*, 2009). In addition to the impact of on humans, ETEC is also an important cause of diarrhea in animals. Economically significant ETEC associated diarrheal disease in animals occur in neonatal calves, pigs and lambs (Bolton *et al*., 2009). A number of outbreaks of ETEC were reported worldwide and in most cases ETEC strains expressing only ST were found to be the major contributors to diarrhea than strains expressing only LT. the previous were also associated with an increased risk of death in children (Croxen *et al.*, 2013).

3.2.4. Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) are facultative intracellular pathotypes of DEC. EIEC are almost identical with *Shigella* in its biochemical, genetic and pathogenic characteristics. Both EIEC and *Shigella* are non-motile, lysine decarboxylase negative and unable to ferment lactose (O'Sulevan *et al*., 2007). Thus, both can be considered as a single pathotype of DEC.

The extensive virulence toolbox of EIEC and *Shigella* derived mainly from the acquisition of invasion plasmid pINV and multiple other virulence genes. The invasion plasmid encodes outer membrane proteins involved in cellular invasion that is localized on one pole of the bacterium and directs the actin microfilament polymerization necessary for invasive nature of the organisms (Kaper *et al.*, 2004). The gene encoding this invasion protein (*ipa*) is conserved and used to distinguish EIEC from another pathogenic *E. coli* (O'Sulevan *et al*., 2007).

EIEC/ *Shigella* infection occurs through contaminated food and water or direct person to person spread. Following ingestion, the organisms invade the epithelial cells of the intestine resulting an invasive inflammatory colitis and dysentery with a clinical presentation of blood and mucoidal stool accompanied by fever and severe cramps (Croxen and Finlay, 2010). In comparison, EIEC exhibits reduced virulence compared to that of *Shigella*, including reduced expression of virulence genes, less efficient macrophage killing, reduced invasiveness and decreased induction of a proinflammatory response relating to the less severe disease induced by the organism (Kaper *et al.*, 2004).

Incidence of EIEC is under represented as a result of limited surveillance and difficulty in detection and discrimination between EIEC and *Shigella* together with self-limited nature of its infection. However, few outbreaks of EIEC associated with food, water and milk have been reported so far. In contrast, *Shigella* has been shown associated 30 to 50% cases of bacillary dysentery worldwide (Croxen *et al*., 2013).

3.2.5. Enteroaggregative *E. coli*

Enteroaggregative *E. coli* (EAEC) are defined as *E. coli* that adhere to intestinal epithelial cells in a pattern known as auto-aggregative, in which bacteria adhere to each other in a 'stackedbrick' like configuration (Kaper *et al.*, 2004). EAEC are a heterogeneous group of bacteria that display a wide array of virulence factors and it is not clear that all the EAEC have any common factor that contribute to their shared adherence phenotype (Bolton *et al.*, 2008; Croxen *et al.*, 2013). Although no single virulence factor has been irrefutably associated with EAEC virulence, epidemiological studies implicate a number of plasmids and chromosomal DNA borne virulence factors. One of the most important is a fimbrial adhesin called aggregative adherence fimbriae (AAF), which confers characteristic adherence of the pathogen to hepatic epithelial cell type II (HEp-2) cells and intestinal mucosa (Kaper *et al.*, 2004). This virulence factor is mainly regulated by a single transcriptional activator called AggR encoded by *aggR*. EAEC strains are divided into 'typical EAEC' and 'atypical EAEC' based on the presence or absence of AggR respectively (Croxen *et al.*, 2013). EAEC also produce an enteroaggregative heat-stable toxin (EAST) encoded on a plasmid by *astA* genes (O'Sulevan *et al.*, 2007).

The pathogenesis of EAEC associated diarrhea is poorly understood. However, it seems to comprise colonization of the intestinal mucosa, predominantly the colon, followed by secretion of enterotoxins and cytotoxins such as *Shigella* enterotoxin and EAST (Kaper *et al.*, 2004; Bolton *et al.*, 2008). EAEC infection is typically followed by watery diarrhea, often with mucus and can be accompanied by a low-grade fever, nausea, vomiting, abdominal pain and occasionally bloody stool (Croxen and Finlay, 2010).

As the global surveillance for all strains of diarrheagenic *E. coli* was limited in the past, the overall prevalence and epidemiology of EAEC is unclear. However, EAEC has been identified in a number of endemic and epidemic diarrheal diseases worldwide, often associated with persistent diarrhea in children and adults (Croxen *et al.*, 2013). The pathogen has been found to be the most common bacterial pathogen identified in diarrheal stool samples and have been reported as the cause of several outbreaks worldwide (Bolton *et al.*, 2009).

Transmission of traveler's diarrhea, caused by EAEC mostly occurs through contaminated water and food. Infant foodstuffs, milk and water have all been implicated in EAEC outbreaks Although, atypical EAEC has been identified in calves and piglets, animals are not an important reservoir of typical EAEC strains pathogenic to humans (Croxen *et al.*, 2013).

3.2.6. Diffusely Adherent *E. coli*

Diffusely Adherent *E. coli* (DAEC) are comprised of heterogeneous groups of organisms with variable virulence factors. DAEC are defined by the presence of a characteristic diffuse pattern of adherence to HEp-2 cell monolayers where bacterial adherence occurs over the entire surface of the cell in a scattered pattern (Kaper *et al.*, 2004). DAEC are divided into two classes, those which harbor afimbrial adhesins (Afa)/Drori antigen (Dr) adhesins and those that express fimbrial adhesins involved in diffuse adherence. 75% of DAEC strains produce the F1845 fimbrial adhesin, encoded for by the *daaC* gene (O'Sulevan *et al*., 2007).

The pathogenesis of DAEC induced diarrhea is not fully elucidated yet, but it is characterized by the growth of long finger-like cellular projections that wrap around the adherent bacteria (O'Sulevan *et al*., 2007). Organisms that adhere to the epithelial cells of the intestine elicit a cytopathic phenotype and activation of signal transduction pathways, causing either watery or bloody diarrhea. The former is associated with physical alteration of the integrity of intestine. While bloody diarrhea is associated with acute tissue destruction process. Low grade fever and vomiting are also indicated as clinical signs of DEC infection (Kaper *et al.*, 2004).

The relative significance of DAEC as a pathogen await further study (Bolton *et al*., 2009). But whenever found DAEC is shown to cause persistent diarrhea in young children, while older adults become asymptomatic carriers and it has also been speculated that DAEC carriage may lead to chronic inflammatory intestinal diseases such as Chron's disease (Croxen *et al.*, 2013).

The epidemiology of DAEC is unclear given that there is no universal method to detect this strain in a clinical setting. However, DAEC have been implicated as a cause of diarrhea in several studies, particularly in children greater than 12 months of age (Kotlof *et al.*, 2013). Sources implicated in outbreaks of DAEC include contaminated food, especially undercooked ground beef, contaminated water and contact with livestock and other animals (Bolton *et al*., 2009).

3.3. Isolation, Detection and Typing of Diarrheagenic *E. coli*

Quick and reliable detection of the diverse DEC types is vitally important in surveillance of outbreaks and diagnosis of sporadic cases. It is especially important for the effective management of associated illnesses before causing significant damage in human as well as animal health. Clinically DEC are differentiated from one another on the basis of their biochemical and serological reaction, pathological features or their genomic composition (Riddle *et al*., 2016). On this regard several techniques have been developed for detection of different pathotypes of DEC. These methods vary from traditional culture-based techniques that are laborious and limited in their discrimination potential to molecular methods that offer quick and high throughput detection and identification of particular pathotype. Most of the current methods for isolation, detection and typing of DEC are covered by O'Sulevan *et al.* (2007) and Feng *et.al*. (2011). The following section discussed each of these techniques briefly.

3.3.1. Isolation

Generally, *E. coli* can be readily isolated from fecal specimens by growing on selective media at 37°C under aerobic condition. Selective medias take advantage of unique physiology of the organism. Commonly this can be done using chromogenic selective media such as MacConkey and eosin methylene-blue (EMB) that selectively grow members of the genus *Escherichia*. This traditional, culture technique primarily separates *E. coli* from other enteric pathogens such as *Shigella* and *Salmonella,* exploiting the ability of *E. coli* to ferment lactose, where the latter two fail to do. However, not all *E. coli* strains, particularly most EIEC and some STEC strains, ferment lactose so caution must be taken in using this technique (O'Sulevan *et al*., 2007).

3.3.2. Detection

Several detection methods have been developed for DEC or *E. coli* in general. These techniques fall under four major classes based on their approach. These are the classical culture-based detection method, immunoassay, phenotypic assay and genotypic assay (molecular method of detection) which are briefly discussed below.

Culture-based detection

In classic culture-based detection method *E. coli* is identified based on colony morphology by visual observation on agar plate. Further identification of *E. coli* is made using biochemical tests. The well-established biochemical test for this purpose is known by the acronym IMViC, which determine the ability of an isolate to produce: indole (I), acid below break point of methyl red (M), acetylmethyl carbinol (V) and the ability to utilize citrate (C). On the bases of biochemical reaction most *E. coli* strains are negative for oxidase, citrate, urease, and hydrogen sulfide, while positive for indol and methyl red (O'Sulevan *et al*., 2007).

Modifications made on the traditional culture-based techniques, enabled detection of certain DEC pathotypes taking advantage of unique biochemical characteristics of certain pathotypes. In this regard a number of chromogenic selective media were developed and applied in clinical diagnosis. For instance, most STEC strains are easily distinguished as clear colonies on sorbitol MacConkey (SMAC) plates (O'Sulevan *et al*., 2007). A number such medias are presented in Bacteriological Analytical Manual (BAM) of United State food and drug administration (FDA) and Pathogenic *Escherichia coli Network's* 'Methods for Detection and Molecular

Characterization of Pathogenic *Escherichia coli* (Feng *et.al.*, 2011; O'Sulevan *et al.*, 2007). Nonetheless, culture-based method of *E. coli* detection is limited and incapable to discriminate among the different pathotypes (Muniesa *et al.*, 2012). Moreover, the traditional culture-based techniques for isolation and detection of *E. coli* is time-consuming and laborious. These limitations lead to the development of more rapid and effective techniques based on physiological, serological, phenotypic and genotypic characteristics of the different pathotypes (DebRoy *et al.,* 2011; O'Sulevan *et al.*, 2007).

Immunoassay

The next development in the detection of DEC is "Immunoassay". Immunoassay occupies a central place in the history of *E. coli* detection. The classic serotyping technique is based on the Kauffman classification scheme designating a particular isolate in to O:H serotype, where the "O" stands for somatic polysaccharide antigen and "H" for flagellar surface antigens (O'Sulevan *et al.*, 2007).

This method utilizes specific antibodies targeting either surface antigens or toxins to detect specific DEC type, both of which require prior enrichment of the target cells to reach detectable levels. In situations where the pathogen cannot be cultured or when a rapid answer is required, alternative method of immunoassay targeting the detection of antibodies against surface antigens or toxins of DEC in the patients' sera can be utilized. In this respect, enzyme linked immunosorbent assay (ELISA) and immunoblotting techniques had been developed for detection of various serotypes (O'Sulevan *et al.*, 2007).

Currently, there are about 188 *E. coli* somatic (O), 53 flagellar (H), 60 fimbrial (F) and over 74 capsular (K) antigens were recognized (DebRoy *et al.,* 2011). However only a small subset of O:H combinations are associated with disease. While serotyping is informative for certain serotypes, it is not always useful for others due to isolates being non-typeable or cross-reactivity between antigens (Nataro and Kaper, 1998). In addition, as it is not in general the serologic antigens themselves that confer virulence, these markers are rarely suffice to reliably identify a strain as diarrheagenic. Thus, serotypes are only used as identifiable marker to correlate with specific virulent clones (Beutin *et al.*, 2007).

Given to limited specificity of serotyping, detection of diarrheagenic *E. coli* has increasingly focused on the identification of characteristics which themselves determine the virulence potential of the organisms. The US center for disease control (CDC) and US-FDA outlined methods for detection and diagnosis, which entails the detection of specific virulence factor gens (VGs) for confirmative assessment of different pathotypes, which can only be achieved either by *in vitro* phenotypic or genotypic assays (Gould *et al*., 2009; Riddle *et al*., 2016).

Phenotypic assay

Given to limited specificity of serotyping, detection of diarrheagenic *E. coli* has increasingly focused on the identification of characteristics which themselves determine the virulence potential of the organism. One of the most useful technique is *in vitro* phenotypic assays that identify the adherence pattern of a particular isolate. One of the most common method is HEp-2 adherence assay. Briefly, the HEp-2 adherence assay involves inoculation of test strain onto a semiconfluent HEp-2 monolayer and after appropriate incubation time the HEp-2 monolayer is washed, fixed, stained and examined under microscope, where the different types of adherence; localized adherence (LA), aggregative adherence (AA) and diffuse adherence (DA) can be identified (O'Sulevan *et al*., 2007).

There are also other phenotypic techniques developed to detect certain types DEC by taking advantage of cytotoxic effect of toxins such as LT/ST of ETEC or Stx of STEC. For example, the traditional bioassay for detection of LT/ST involves either Y1 adrenal cell assay or the Chinese hamster ovary (CHO) cell assay. In the Y1 assay, ETEC culture supernatants are added to Y1 cells and the cells are examined for rounding. While in CHO cell assay, elongation of the CHO cells is taken as a positive sign for the presence of the toxins (Nataro and Kaper, 1998). Similarly, cytopathic effect of Stx on vero-cell can be employed in phenotypic assay of STEC strains. Briefly, the supernatant of suspected STEC cultures or lysed bacteria are inoculated on Vero cell monolayers and the cytopathic effect will be scored after 24 hours under microscopic observation (Gould *et al*., 2009).

Phenotypic assay increased the sensitivity in detection of various DEC pathotypes compared to conventional serotyping. However, there are still few strains which yield equivocal results reproducibly in the HEp-2 assay (O'Sulevan *et al*., 2007). The need for additional techniques given rise to molecular methods that target specific molecular targets such as virulence factors such as toxins, which can only be achieved through molecular method of detection (Croxen *et al*., 2013).

Genotypic assay/ Molecular methods of detection

Diarrheagenic *E. coli* strains were among the first pathogens for which molecular methods of detection (genotypic assay) were developed (O'Sulevan *et al*., 2007). Indeed, molecular methods remain the most popular and most reliable technique for differentiating diarrheagenic strains from nonpathogenic members of the normal flora and distinguishing one pathotype from the other (Croxen *et al*., 2013). Currently the adoption of molecular techniques has allowed the rapid and precise detection and identification of the different pathotypes of DEC (Fujioka *et al*., 2013). Moreover, the technique has replaced cumbersome and costly animal models of phenotypic assay.

Molecular methods of DEC detection are based on detection of a particular gene or combination of genes coding for specific virulence or pathogenic factors. Following advance in molecular techniques and increase in the wealth of sequence data, most of the genes responsible for coding virulence factors are elucidated. These leads to substantial progress in the development of nucleic acid-based probes for blotting as well as PCR based detection techniques (Shields *et al*., 2007; Tobias and Vutukuru, 2012). These genetic markers are used to detect the presence of similar factors in the test specimen either in sporadic cases or outbreaks. These virulence markers can be detected either by hybridization or amplification of a genome with particular gene specific probe (Shields *et al*., 2007). Further advances in PCR; multiplex PCR and real-time PCR allowed simultaneous and quick detection of virulence factors, greatly increased the detection of various pathotypes (Fujioka *et al*., 2013). Comprehensive list of probes and PCR targets suitable for the detection of various pathotypes of *E. coli* are provided in the 'Bacteriological Analytical Manual' (BAM) of US food and drug administration and Pathogenic *Escherichia coli* Network's 'Methods for Detection and Molecular Characterization of Pathogenic *Escherichia coli'* (Feng *et.al*, 2011; O'Sullivan *et al*., 2007).

3.3.3. Typing DEC

Typing of pathogenic organisms is essential for phylogenetic analysis, surveillance and outbreak investigations. Typing elucidates the virulencepotential of an isolate in comparison to reference strains (Croxen *et al*., 2013). Several typing approaches were applied for DEC surveillance. These methods are either based on phenotypic trait "phenotyping" or genotypic trait "genotyping".

Phenotyping

Phenotyping techniques are based on detection of physically expressed traits of the organisms. The methods include biochemical typing, serotyping and phage typing. Biochemical typing involves the response of an isolate to a range of biochemical tests, for example fermentation of different carbon sources. The result is a metabolic fingerprint of the isolate. Though most DEC strains lack common biochemical characteristics, some metabolic fingerprints can be used for typing (O'Sulevan *et al*., 2007). The other Phenotyping technique is serologic typing. For the first time Kauffman proposed a scheme for the serologic classification of *E. coli* in 1944*,* a technique which is still used today with slight modification (Bettelheim and Goldwater, 2014). Finally, the susceptibility of isolates to lytic effect of bacteriophages is used in typing DEC strains. This technique is known as "Phage typing" as it utilizes a panel of bacteriophages to determine the lytic patterns of DEC by the bacteriophages. Bacteriophage typing does not usually provide the level of discrimination required for epidemiological and outbreak investigations, as the number of different types identified routinely may not be sufficient for confident interpretation of results.

Genotyping

Genotyping techniques are based on whole genome comparison or detection of genotypic pattern of the organisms in comparison to other isolates or reference strains. Principally the methods are based on either randomized analysis of whole genome or analysis of target DNA segment (Perez *et al*., 2013). Methods based on the previous technique comprise; pulse-field gel electrophoresis (PFGE) and restriction fragment length polymorphisms (RFLP). While based on the latter technique, methods such as amplified fragment length polymorphisms (AFLP), multilocus sequence typing (MLST) and multilocus variable-number tandem repeats (MVNTR) can be mentioned (Perez *et al*., 2013; Croxen *et al*., 2013).

RFLP/PFGE is an example of randomized genotyping approach based on pattern recognition of genomic DNA following digestion of genomic DNA by set of restriction enzymes and the result will be analyzed by comparison of PFGE results (Fujioka *et al*., 2013). Comparison of macrorestriction profiles from different isolates indicates whether they are potentially linked or not. The method has been applied successfully to discriminate outbreak-associated, sporadic, or unrelated infections since 1993 (O'Sulevan *et al*., 2007). This method has been standardized by CDC and considered as gold standard for DEC typing. Currently PulseNet, administrated by CDC allows rapid inter laboratory comparisons of generated data. Yet, PFGE is laborious, time consuming and the level of expertise required, makes it limited to be used in all diagnostic laboratories (Swaminathan *et al*., 2001).

There are also several typing techniques developed based on targeted DNA segment. The methods principally rely on identification of polymorphism among the target sequence. Genomic targets used as epidemiological markers including ribosomal RNA genes, insertion sequences, bacteriophage lambda or Stx-encoding genes (O'Sulevan *et al*., 2007; Perez *et al*., 2013). Single nucleotide polymorphism (SNP) is one of the foremost known approach. SNP detects nucleotide substitutions by sequencing, PCR or oligonucleotide hybridizations such as microarrays. The other approach is MLST, in this technique a small number of housekeeping genes are sequenced and assigned a unique allele, and the allelic profile of these genes is used to give an isolate a particular sequence type. The sequence types are further grouped into clonal complexes based on their similarity (Noller *et al*., 2003).

Another alternative for targeted DNA analysis is VNTR that identifies polymorphism in the number of tandem repeat short sequences*, i.e.* areas that evolve quickly in the bacterial genome. Closely related to it is, multilocus variable-number tandem repeat analysis (MVNTR) that determines the number of repeats at multiple variable-number tandem repeat loci. MVNTR is more informative than VNTR. These methods are fast, simple, excellent in discriminatory ability (equal or superior to that of PFGE) and can easily be standardized (O'Sulevan *et al*., 2007; Perez *et al*., 2013). However, in order to establish MVNTR on a new pathotype or serotype of *E. coli*, the fully sequenced genome has to be available otherwise, closely related groups of bacteria may not exhibit sufficient variation to allow effective discrimination (Perez *et al*., 2013).

3.4. Carriers, Sources of Infection & Mode of Transmission

Diarrheagenic *E. coli* strains can be found in humans and various animal reservoirs. Natural sources and reservoirs vary considerably among different pathotypes, the details of which is given under each pathotype section. However, in general the pathogens are transmitted to humans mainly through consumption of contaminated food and drinking water implying orofecal as a primary route of infection (Croxen and Finlay, 2010). Though consumption of contaminated food and water are the primary source of infection for most pathotypes of DEC, wide variety of non-source exposure such as human to human contact, human to animal contact, food and environmental contamination have been implicated as a secondary source of infection, where fecal matter contaminate food, irrigation water, or recreational and drinking water (Fig. 3.1) (Croxen *et al*., 2013; Bolton *et al*., 2009; Kaper *et al.,* 2004).

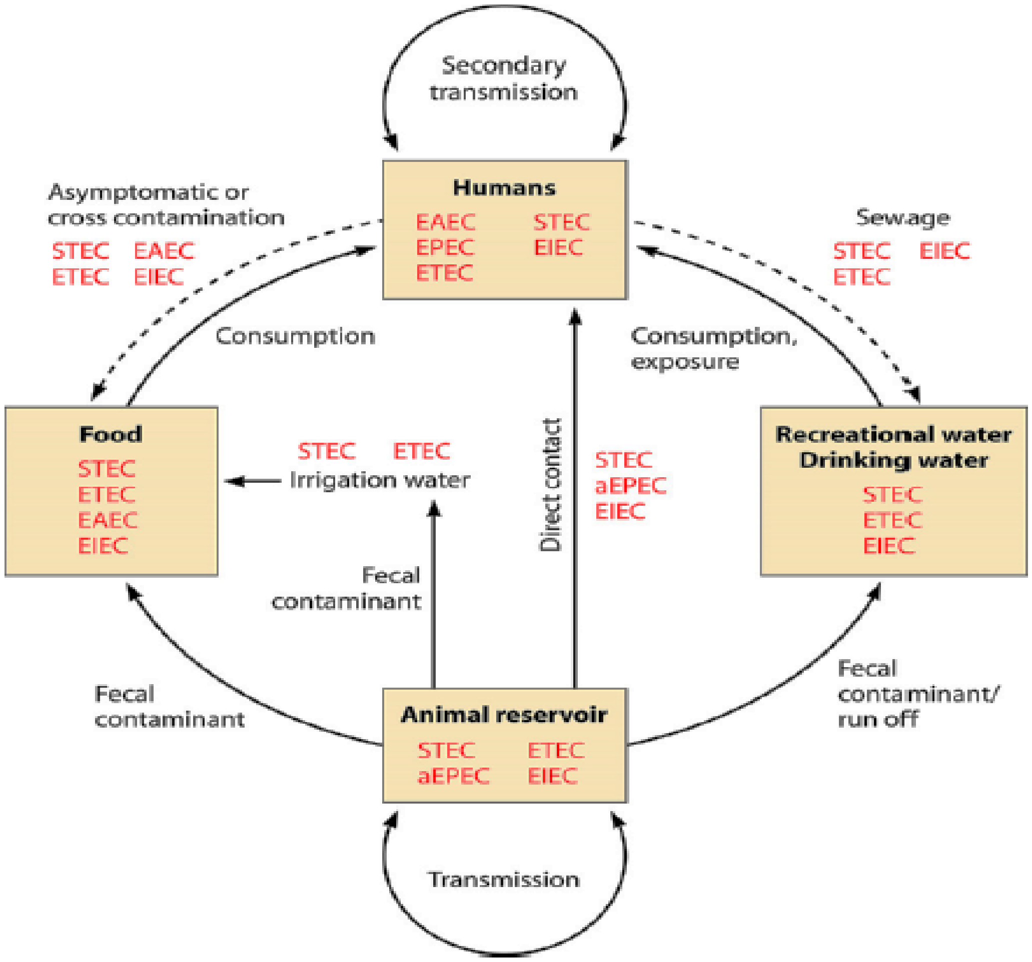

Figure 3.1. Overview of potential reservoirs and modes of transmission of diarrheagenic *E. coli* (Croxen *et al.*, 2013).

Food can become contaminated through poor cooking practice, where uncooked meat could come in contact with other food. Similarly, meat and milk can be contaminated through contact with animal feces during slaughter and processing of colonized animals. Whereas vegetables may contaminate through the use of manure as fertilizer or through contaminated irrigation water. Additionally, symptomatic or asymptomatic food handlers can contaminate food, particularly when hand hygiene is inadequate (Croxen *et al.*, 2013).

So far, most sporadic cases and outbreaks were associated with contaminated water and food. Currently food products contaminated with bovine waste are found to be the most common sources of disease outbreaks worldwide for STEC and ETEC pathotypes (Kosek *et al.*, 2003). In few studies carried out in Ethiopia, the reported figures are staggering. For example, one study found out prevalence of EHEC O157: H7 in 10.2% of samples collected from 48 raw meat retail shops in Addis Ababa (Bekele *et al.,* 2014). Similarly, Aklilu *et. al.* (2013) and

Dulo, (2015) found 62.5 % and 2.55% prevalence of similar pathogen from beef sold around Mekelle and Dire Dawa. Likewise, *Shigella* has been isolated from a variety of street vended foods in Addis Ababa (Okeke, 2009) indicating the potential risk of food contamination in Ethiopia.

3.5. Pathogenesis and Clinical Manifestation of Diarrheagenic *E. coli* infection

3.5.1. Pathogenesis

The type of virulence factors or their combination in an isolate determine the clinical outcome of disease (Bolton *et al*., 2008). The most highly conserved feature of DEC strains is their ability to colonize the intestinal surface despite innate immune response and competition by the indigenous flora of the gut (including commensal *E. coli* strains). Different pathotypes of DEC share many virulence strategies in common to potentiate pathogenesis despite their remarkable diversity (Leimbach *et al*., 2013). Diarrheagenic strains of *E. coli* once ingested with food or water, use a multi-step scheme of pathogenesis that is similar to that used by other entericpathogens, which consist of bypassing gastric barrier, adherence to host cells, evasion of host defenses, multiplication and colonization of gastric mucosa and finally subversion of signaling pathway and induction of damage in the host cell (Russo and Johnson, 2003; Croxen and Finlay, 2010).

The acidic pH of the stomach is an important first line barrier to enteric pathogens. Therefore, following ingestion DEC must survive the low acidity of the stomach to colonize the lower intestinal mucosa. Most DEC strains have the ability to survive in extreme acidity of the gastric environment that is conferred by an array of acid resistance system (ARS). Although the ARS is shared among other *E. coli* strains, DEC strains have a higher basal level of ARS expression. The main mechanisms of ARS employed by acid resistance bacteria are well discussed in a review by Audia *et al.* (2001).

The mucosal immune system also constitutes the first line of defense against enteric pathogens. Given to the enormous number of microorganisms ingested with every meal, the normal host must combat a constant influx of potential enteric pathogens. Both cellular and antibody mediated immune responses play important role in protection against infections (Russo and Johnson, 2003). The binding of bacterial antigens to the luminal surface and the subsequent presentation of antigens to subepithelial lymphoid tissue by Microfold cells (M-cells), lead to the proliferation of sensitized lymphocytes. These lymphocytes circulate and evade all of the mucosal tissues, as plasma cells that produce immunoglobulins (IGs). Nonetheless, most DEC strains adapted for altering the host immune response (Mahajan and Gally, 2011).

In addition to the immune system, the normal flora of the gut provide protection against colonization pathogens. The enteric-system is colonized by large number of bacteria residing in the intestine with mutual communalism. The normal flora of the gut is mainly composed of anaerobic bacteria, which produce various metabolites like volatile fatty acids and even antibiotics such as colchicine that appears to be critical elements in resistance to colonization by entericpathogens (Kamada *et al*., 2013). However, most of these pathotypes harbor additional traits to outcompete over the commensal flora (Bolton *et al*., 2008).

Most enteric organisms adhere to the gastrointestinal mucosa as an initial step for colonization. The initial attachment of various DEC types varies considerably (Kaper *et al*., 2004). However, all pathotypes harbor a number of enterocytes specific surface adhesins, toxin co-regulated pilus and other accessory colonization factors that enhance adherence to the distal ileum of small bowel and large bowel mucosa, a site that is not normally colonized by other *E. coli* strains (Croxen and Finlay, 2010).

Following intimate attachment, DEC strains inject a large repertoire of diverse effector proteins and toxins that subvert cascade of host cell signaling pathways (Croxen and Finlay, 2010, Kaper *et al*., 2004). These effectors take part in deregulation of ion exchangers, leading to a concomitant decrease in water absorption and induction of diarrhea (Croxen *et al*., 2013). The secreted toxins and other effector proteins also result host cell damage by inducing a variety of fundamental changes in host cell processes such as; interfering with ribosomal RNA thereby disruption of protein synthesis, triggering a potent cytokine expression cascade and interfering with DNA replication, thereby blocking cell division (Kaper *et al*., 2004, Croxen and Finlay, 2010, Croxen *et al*., 2013).

3.5.2. Clinical Manifestation

Diarrhea is loosely defined as passage of abnormally soft or unformed stools at an increased frequency. Under normal conditions the gastrointestinal tract absorbs 8-9 liters of fluid presented to intestine daily and only 100-200 ml are excreted in the stool (Ahlquist and Camilleri, 2005). Enteric pathogens, however, alter this balance towards net secretion leading to diarrheal disease (Hodges and Ravinder, 2011). According to WHO guide line, the base line for diarrhea is the movement of soft bowel three or more times a day. Because of the fundamental importance of duration to diagnostic considerations, diarrhea may be further defined as acute if it lasts no more than 2 weeks, persistent if 2 to 4 weeks, and chronic if it lasts more than 4 weeks (WHO, 2012). With the variety of serotypes, virulence factors found among DEC strains, it is of little surprise that the severity of disease can differ considerably. Infection by various DEC pathotypes result from mild watery diarrhea to bloody diarrhea or hemorrhagic colitis (HC) and even life-threatening complications that may lead to death (Croxen and Finlay, 2010). The specific clinical manifestation of each pathotype is discussed under particular pathotypes section.

2.6. Impact of Diarrheagenic *E. Coli* Associated Illnesses in The Global Health Scenario

Due to the ease of access of pathogens to be ingested with food, the human gastro-intestinal tract is susceptible to DEC infections (Croxen and Finlay, 2010). The worldwide burden of diseases associated with DEC is staggering, with hundreds of millions of people affected annually (CDC, 2012). DEC associated diarrheal diseases are responsible particularly for infantile diarrhea in humans as well as livestock making the case complicated impacting wellbeing of both human as well as livestock (Scallan *et al.*, 2007; FAO, 2016). Several *E. coli* pathotypes have been implicated with diarrheal illness causing over two million deaths each year and putting DEC as a major public health problem worldwide especially in the case of an epidemic outbreaks (Kosek *et al.*, 2003).

Though it was in 1940s and 1950s, DEC was found to cause infantile diarrhea in the developed world, over the years it is found to be much more prevalent in developing countries (Kotloff *et. al*., 2013; WHO, 2012). This indicates the contribution of geographic and socioeconomic factors to the epidemiology of DEC induced diarrheal disease. However, it had not been considered as an important cause of diarrhea and there was no screening for *E. coli* in patients with diarrhea. Even the detection of specific diarrheagenic *E. coli* types has not been the focus of clinical and scientific research. The need for this was not recognized until recently, following outbreaks in the western world (Croxen *et al.*, 2013).

There have been a number of outbreaks of DEC reported around the world and their public impact has often been dramatic. Few examples to mention, six hundred people became ill and four children died in a major US outbreak in 1993 caused by undercooked beef hamburgers. In 1994 West Lothian outbreak, Scotland in contaminated milk resulted 71 cases, 11 with hemolytic uremic syndrome, one death. In 1995 outbreak of *E coli* O111 in South Australia, about 200 cases, 23 with hemolytic uremic syndrome, one death was recorded. A large outbreak in Scotland in 1996 had a similar impact, nearly 500 were affected and 20 elderly patients died.

The cause was thought to be cross-contamination of cooked meats (Adams and Moss, 2008). The more recent outbreak of EHEC (O104:H4) in Germany (2011), caused more than 30 fatalities and put over 100 people hospitalized due to severe diarrhea, HUS and HC (Brzuszkiewicz *et al.* 2011). These outbreaks proved the fact that *E. coli* is not only the cause of diarrhea in developing countries but also in developed countries. Putting surveillance and control of DEC a major focus of public health authorities.

Among small fraction of *E. coli* under surveillance, *E. coli* O157 is the most common member of a group implicated in foodborne illnesses and isolated with increasing frequency over the last two decades (Lim *et al*., 2010). While the primary focuses were placed on the detection of *E. coli* O157:H7, it has become apparent that non-O157 STEC are also major contributors of sporadic cases and outbreaks in North America, Australia, and Europe. Even some DEC types, such as EIEC might have been underrepresented in epidemiological surveys due to less severe clinical manifestations. In addition to very close biochemical, genetic and pathogenic similarity of EIEC to *Shigella* and commensal *E. coli* might put DEC to be misclassified and underrepresented in respective surveys. Although overall surveillance and clinical diagnostics are lacking, DEC is found to be responsible for a number of diarrheal illnesses in developing countries such as Argentina, and number of cases were also identified in other developing countries (Kotlof *et al*., 2013; Okeke *et al*., 2009).

In sub-Saharan Africa and South Asia, ETEC and *Shigella* were found to be two of the four main causative agents of moderate to severe diarrhea among children. Among the different pathotypes, increased fatality rates are associated with EPEC and certain ETEC, indicating the significant role of DECs in the global health burden of diarrheal disease (CDC, 2012; Croxen *et al*., 2013). DEC is also found indicated with moderate to severe diarrhea in children under 2 years of age in Kenya and associated with increased risk of death for patients aged 0 to 11 months (Kotloff *et al*., 2013).

3.7. Control, Prevention and Clinical Management of Diarrheagenic *E. Coli* Infection

3.7.1. Control and Prevention of DEC infection

Control of DEC transmission is the single most effective and least expensive means of infection management. Effective prevention of DEC transmission can be achieved by implementing control strategies to prevent shedding and spread of the pathogen in natural hosts, reservoirs and environmental sources. As DEC are zoonotic pathogens which have animal reservoirs, one of the best integrated interdisciplinary and cross sectoral approach is “One Health approach” which is designed to preventing epidemic or epizootic diseases and to maintain ecosystem integrity. In this regard twelve principles, known as the Manhattan principles, were outlined to deal with the unique dynamic interaction between the humans, animals, and pathogens (Calistri *et al*., 2013). Effective implementation of the approach demands combined efforts of physicians, veterinarians, epidemiologists, public health workers, urban planners and environmental, veterinary, food and health authorities for collaborative international surveillance, prompt reliable agent identification and optimization of the treatment regiments (Calistri *et al*., 2013). In this regard improvement in cattle management practices, utilization of inhibitory feeds, immunization, use of probiotic cultures and bacteriophage cocktail have been proposed as effective intervention measures at farm level (Bolton *et al*., 2010; Croxen *et al*., 2013; Glenn *et al*., 2013).

An effective prevention of DEC infection can be achieved by implementing strict strategies throughout the food supply chain. Thus, food handlers (suppliers, processors and ports of entry) should implement effective detection and identification of foodborne pathogens in raw food products, before distributed for human and animal consumption. Likewise, uncooked food products from animal origin must be kept isolated from other foods to prevent contamination (Bolton *et al*., 2010). Furthermore, improved environmental and personal hygiene practices help to prevent infection. These include promotion of hand washing with soap, improving quality of water supply and community wide sanitation are recommended preventive strategies (WHO, 2011). In the continued effort of developing preventive measures, various vaccines have been developed and still a lot more are under pipe line, targeting prevention of traveler’s diarrhea and complications like HUC. Some of the promising candidates are under different stage of clinical trial, yet there is no US FDA approved vaccine against any of the DEC pathotypes (Croxen *et al*. 2013).

3.7.2. Clinical Management of DEC Associated Diarrhea

In most cases DEC induced diarrhea is self-limiting, resolving long before patients seek medical attention (Russo and Johnson, 2003). Even though assays to identify all pathotypes of DEC are available, in many situations it is not necessary and also difficult to implicate a specific pathotype in a particular patient. Thus, it should only be applied in cases where severe complication is trending such as cases of persistent diarrhea or recurrent infections especially in; travelers, children and the immune compromised people as well as in outbreak situations

(Gould *et al*., 2009). In such cases identification of specific strain is important for effective clinical intervention (Russo and Johnson, 2003; Nicolle *et al*., 2005).

The clinical management of DEC infection is multi-dimensional. The first step is treatment of symptoms of diarrhea by limiting the loss of fluids and replacing electrolytes lost. This can be achieved by administration of oral rehydration solutions (ORS) or administration of intravenous fluids in critical condition (Russo and Johnson, 2003). Controlling the symptoms of diarrhea also helps to prevent associated complications such as HUS & HC (Goldwater and Bettelheim, 2012).

In the case of severe or persistent diarrhea the use of antibiotics is inevitable. When the use of antibiotics is eminent, the *status quo* in treatment of diarrhea associated with DEC is administration of broad-spectrum antibiotics, which is considered as golden standard. Antibiotics such as fluoroquinolones, azithromycin and rifampicin are commonly used to lessen the duration and progression of infection (Nicolle *et al*., 2005). Though it is at its infancy, there are also emerging treatments that are safer and effective in the treatment of DEC associated diarrhea. These include the use of bacteriophages and monoclonal antibodies that specifically target toxins and virulence factors of each pathotype (Croxen *et al.* 2013).

Though the clinical management of persistent infections requires the use of antibiotics, the use of antibiotics in DEC infections is strongly debated. The main problem is risk of increase in toxin production as a result of bacterial lysis via the induction of the bacterial SOS response following antibiotics administration (Goldwater and Bettelheim, 2012). Therefore, antibiotics cannot be taken as a first line treatment especially in ETEC and STEC infection. This holds true, especially in immune-compromised patients, children and elderly people, as these patient groups are more vulnerable to the effects of non-specific antibiotics and complications that may occur worsening the progression of the disease (Goldwater and Bettelheim, 2012; Croxen *et al*. 2013).

Furthermore, antibiotic resistance profile of most DEC is important factor that determine the success of treatment. Several clinical isolates of DEC exhibit high degree of resistance to standard antibiotics (Sa´enz *et al*., 2004; Szmolka and Nagy, 2016). This is mainly associated to the unique nature of the organism such as; multiple route of transmission, wide host range and enormous HGT which made the antibiotic resistance pattern complex (Zhang *et al*., 2015; van Hoek *et al*., 2011). Currently the prevalence of antibiotic resistance shows increasing trend both in the geographical coverage as well as range of antibiotics, making clinical management challenging (WHO, 2014; Bryce *et al*., 2016; Chattaway *et al*., 2016).

A study looking at the changing antibiotic resistance profiles of ETEC from 2001 to 2004, showed resistant to trimethoprim-sulfamethoxazole, tetracycline and ampicillin in more than 50% of isolates. Similarly, worrisome increase in ciprofloxacin resistance from 1% in the period of 1994 to 1997 to 8% between 2001 and 2004 was reported (Croxen *et al*., 2013). The trend is also similar in other pathotypes, for instance Sa´enz *et al*. (2004) demonstrated the level of multiple-antibiotic-resistant strains of *E. coli* in human, animal, and food origins. A comprehensive analysis of both *Shigella* and EIEC strains collected from North and South America, Africa and Asia also showed tetracycline resistance in more than 70% of *Shigella* and 48% of EIEC isolates (Qadri *et al*., 2005). High prevalence of resistance was also observed among all serovars from Sudan, Kenya, Nigeria and Ethiopia (Okeke *et al*., 2009).

4. MATERIALS AND METHODS

4.1. Study Design

Experimental study was conducted to characterize *E. coli* isolates based on their virulence factors and plasmid contents. The samples were provided by Dr. Tesfaye Sisay (Institute of Biotechnology, AAU). Samples were collected from diarrheic calves that were exhibiting signs of systemic disease and clinically identified as diarrheic at the time of sampling. The calves were located in 56 dairy farms, composed of three large and 53 smallholder dairy farms found in Muketuri, Debretsige and Fiche towns of North Shewa Zone, Ethiopia. These towns are located in North-West of Addis Ababa. Fitche town is 147km away from Addis Ababa, while Muketuri and Debretsige are 78 km and 89 km North-West of Addis Ababa respectively. In 2010 there have been 3.3 million cattle in North Shewa Zone; where cattle take the larger proportion (53%) followed by Shoat and Equines (25.56%) and (15.91) respectively. These areas are the major suppliers of milk to the capital, where 13000 liters of milk is collected from Debretsige town only.

The samples for this study were 84 isolates, previously collected and identified as *E. coli* and stored in glycerol stocks at -20^{0}C. These samples originated from 27 (36.98%) cross breed calves and 46 (63.01%) exotic breed calves. The age distribution of calves ranged from 1-16 weeks where; 40 (54.79 %) were between 1 - 4 weeks old, while 29 (39.73 %) were 4 - 8 weeks old and 4 (5.48%) were more than 8 weeks of age. Male calves account only 26.00%, while females account 74.00% hence male calves were sold soon after birth and females were kept for replacement stock in all dairy farms.

Glycerol stocks of *E. coli* isolates were thawed and sub-cultured overnight at 37^{0}C on MacConkey agar (Himedia, India) and further confirmed for purity by sub-culturing on EMB agar (Himedia, India) as previously described in O'Sullivan *et al.* (2007). All viable samples at the time of the experiment (n=73) were screened for virulence genes (VGs). All of the samples were previously tested for their susceptibility against a panel of eleven antibiotics. The antibiotic resistance of each isolate is given on annex 1. Accordingly, all of the isolates were found to be resistant to at least one of the antibiotics tested and 27 different types of resistance profiles were obtained (Annex 2).

4.2. Virulence Gene Detection

Chromosomal DNA was extracted from single colony of bacterial isolate grown on EMB medium taken and grown in nutrient broth at 37°C overnight. Culture (1.5 ml) was spun by centrifugation at 5000 g for 10 min and the bacterial pellet was mixed with 50 µl of sterile distilled water and lysed by boiling in a water bath at 95^0C. The lysate was centrifuged again as mentioned earlier. The supernatant was then directly used as a template for PCR.

For all isolates, PCR based screening of DEC VGs were performed as previously described by Pal *et al*. (1999) and Khan *et al.* (2002) with minor modifications. Briefly, PCR reaction mix of 25 µl containing 2.5 ml of tenfold reaction buffer (Himedia, India), 1 µl 0.25 mM of each of the four dNTPs, 0-1.5 mM $MgCl_2$, 1 U of Taq. polymerase enzyme (Himedia, India), 3 µl of 100-400 ng/ml extracted DNA template and 0.5µmol $m1^{-1}$ of respective primers (Table 4.1) balanced to 25 µl with ultrapure water. Ultrapure water, instead of template DNA, was used as a negative control.

Table 4.1. List of primers used for screening VGs in *E. coli* isolates.

Primer code	Primer sequence 5'– 3'	Target gene	Product size (bp)	Reference
EAE-f	AAACAGGTGAAACTGTTGCC	*eaeA*	454	Khan *et al.* (2002)
EAE-r	CTCTGCAGATTAACCTCTGC			
EVC-f	ATCAGTCGTCACTCACTGGT	*stx1*	349	Pal *et al*. (1999)
EVC-r	CTGCTGTCACAGTGACAAA			
EVT-f	CAACACTGGATGATCTCAG	*stx2*	112	Pal *et al.* (1999)
EVT-r	CCCCCTCAACTGCTAATA			
BFPF	AATGGTGCTTGCGCTTGCTGC	*bfpA*	324	Gunzburg *et al.* (1995)
BFPR	GCCGCTTTATCCAACCTGGTA			
EAEC F	CTGGCGAAAGACTGTATCAT	*aatA*	630	Schmidt *et al.* (1995)
EAEC R	CAATGTATAGAAATCCGCTGTT			
EHEC-F	ACGATGTGGTTTATTCTGGA	*hlyA*	167	
EHEC-R	CTTCACGTCACCATACATAT			

Key: *eaeA, E. coli* attaching effacing gene; *stx1,* Shiga-like toxin I gene; *stx2,* Shiga-like toxin II gene; *ehlyA, E. coli* hemolysin gene; *bfpA,* bundle forming pili gene.

The PCR reaction mixtures were incubated in a thermo cycler programmed with the thermal profile described by Paton and Paton (1998) with slight modification. Briefly initial denaturation at 95 ^{0}C for three minutes was followed by 35 cycles of each consisting 1 min of denaturation, annealing and extension. Denaturation and extension temperatures were 95 ^{0}C and 72 ^{0}C, respectively. While the annealing temperatures were 55 ^{0}C, 45 ^{0}C, 55 ^{0}C, 54^{0}C, 46^{0}C, 48^{0}C for *stx1, stx2, eaeA, bfpA, ehlyA* and *aatA*, respectively. Following the 35 PCR cycles, each sample run was subjected to final extension at 72 ^{0}C for 10 min.

Amplified PCR products were separated by gel electrophoresis at 10 volts per cm for 45 minutes in 1x TBE buffer, in 1% agarose gel (Himedia, India) containing 0.5 µg ml^{-1} ethidium bromide (Himedia, India) using a DNA marker of 100 bp (Himedia; India). The gels were visualized, photographed and documented in UV illuminator (Bio-Rad; Germany).

4.3. Plasmid Profiling

Isolates were examined for their plasmid content as previously described by Soleimani *et al.* (2016) with slight modification. Briefly, plasmid DNAs were extracted from *E. coli* isolates by alkaline lysis method (Birnboim and Doly, 1979). Briefly, 1.5ml of peptone water broth culture of single bacterial colony grown overnight at 37^{o}C were centrifuged at room temperature. Collected cells pellets were then resuspend glucose tris EDTA solution and placed on ice after adding NaOH/SDS solution and sodium acetate solution consecutively. The preparations were then centrifuged at 13000rpm for 3 minutes and the supernatant was precipitated with ethanol to get the plasmid DNA. This plasmid DNA was washed and resuspended in TE buffer. Plasmid extracts were then separated by gel electrophoresis on 0.7% agarose gel alongside 1 kbp marker in 1X TAE buffer at 10 volts per cm for 3 hours. The gels were then stained with ethidium bromide, visualized and documented by Gel-documentation system (BioRad; Germany).

4.4. Statistical Methods

Statistical analysis was made to generate descriptive statistics and look for associations among the different variables. The statistical evaluation was done using the SPSS for Windows package version 21.5.1 (SPSS Inc., Chicago, USA). Initially, the occurrence of each virulence factors and plasmids was described using descriptive statistics based on their frequency and average distribution. Moreover, any potential association between plasmid profile, resistance phenotype and virulence phenotype were assessed by applying multivariable logistic regression, as previously applied by Johnson *et al.* (2012) and Chandran and Mazumder (2014). level of association between respective variables were considered significant at $P < 0.05$. Finally, principal coordinates analysis (PCoA), a multivariate technique related to correspondence analysis that enables plotting the major patterns within a dataset, was used to visualize the level of similarity of isolates based on all variables (consisting; each of virulence factors, plasmids and antibiotic resistance traits) and the values for pairwise combinations of the first two axes were plotted as a series of Cartesian grids, to show the relationship among the variables in two-dimensional space according to Johnson *et al*. (2012). Multivariate analysis of variance (MANOVA) was also conducted to determine whether the groups differed significantly according to the three PCoA axes as previously applied Johnson *et al*. (2012).

5. RESULTS

5.1. Virulence factor genes

Virulence gene specific bands (Fig. 5.1) were totaled for each isolate under each category. Overall results showed 51 isolates, about 70% of the total 73 *E. coli* samples, were found to carry at least one of the targeted virulence gene. This figure is significantly high (p=0.001, One-Sample Binomial test compared to 22 isolates (30%) that carried no VGs, which indicate high level of pathogenic potential in the sample population.

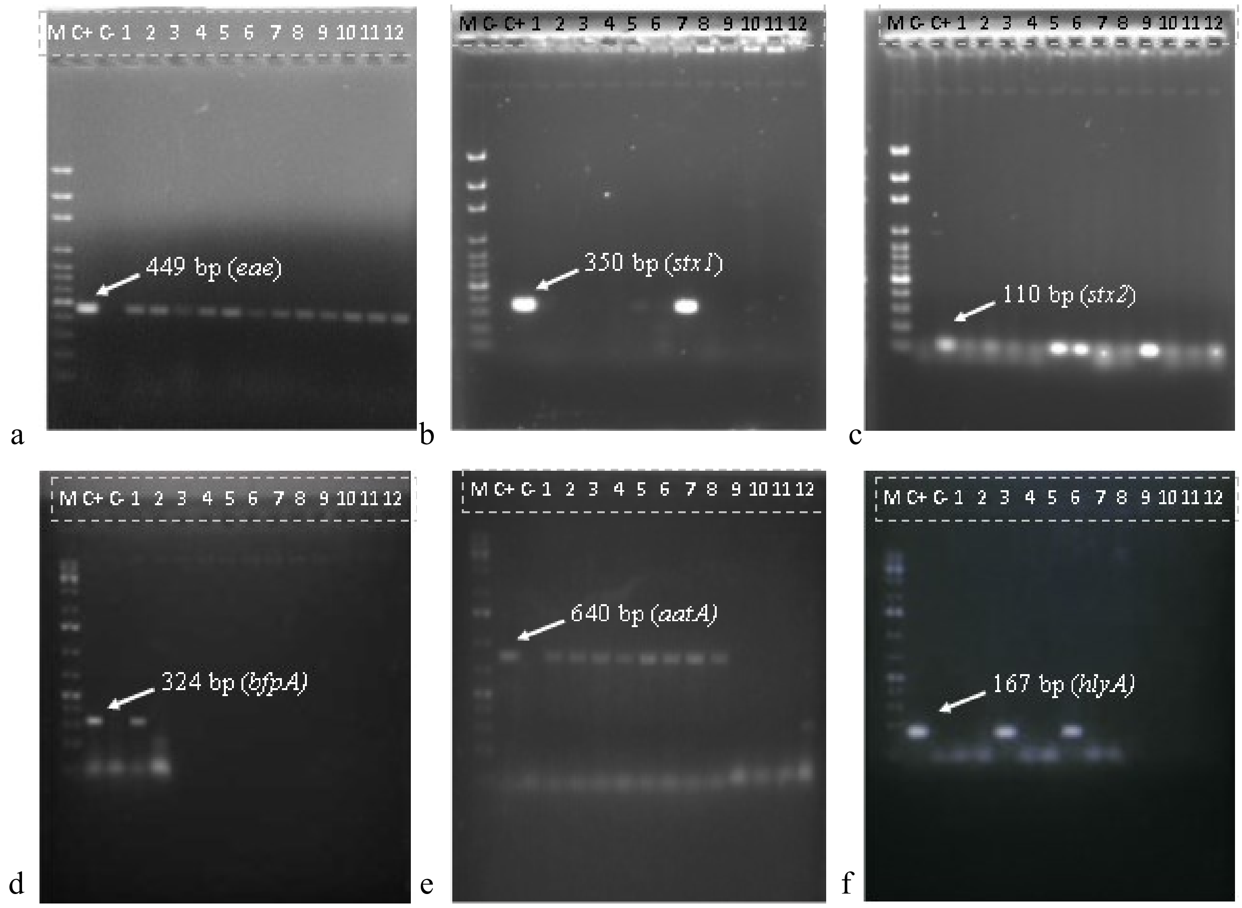

Figure 5.1. Representative gels for PCR product of *E. coli* virulence factor genes.
a) *eaeA* (449 bp), b) *stx1* (350 bp), c) *stx2* (110 bp), d) *bfpA* (324), e) *aatA* (640) and f) *hlyA* (167). Lane M; DNA size marker (100 bp), lane; 1–12 PCR product of isolates, lane C+ positive control and C– negative control. The relative position of each PCR products is indicated by arrow heads.

Results revealed *eaeA*, which codes for intimin in both EHEC and EPEC was found to be the single most frequent VG, detected in 59% of the isolates (Table 5.1). While the *stx1* primarily carried by STEC and also by EHEC was the second most common VG, detected in 25 (34%) of isolates followed by *stx2* and *ehlyA* which were detected in 25 and 23 percent of the total sample isolates respectively. Whereas, EPEC bundle-forming pili gene (*bfp)* and the EAEC transcriptional regulator gene (*aatA)* were detected in 12% and 5% of isolates, respectively (Table 5.1).

Table 5.1. Distribution of virulence factors in *E. coli* isolated from diarrheic calves (n=73)

Virulence factor	Samples	
	№ of positive isolates *(n)*	Frequency (%)
eaeA	43	58.9
stx1	25	34.25
stx2	18	24.66
ehlyA	17	23.29
bfpA	9	12.33
aatA	4	5.48

Key: *eaeA, E. coli* attaching effacing gene; *stx1,* Shiga-like toxin I gene; *stx2,* Shiga-like toxin II gene; *ehlyA, E. coli* hemolysin gene; *bfpA,* bundle forming pili gene.

Furthermore, occurrence of *E. coli* virulence genes was described in terms of spatial distribution. It was found that, Muketuri site has the lowest overall frequency of VGs. In contrast, Debretsige has the highest occurrence of VGs. Among the VGs screened in this study, *eaeA* was most frequently detected across all the sites, occurring in Debretsige in 86%, Fiche in 64% and Muketuri in 46% of the sample population. Whereas, *stx1* gene was the second most commonly detected genes across all sites after the *eaeA*. Among the three sites, Debretsige has the highest occurrence of *bfp*A (43%) compared to the other two sites which had occurrence of the gene in around 5% of isolates. Among the VGs *aatA,* which is the list frequent, was detected only in the two of the sample sites (Muketuri and Fiche) detected in 8% and 5% of isolates, respectively. Comparative analysis of the distribution of VGs across all the three sites is presented in Fig. 5.2. below.

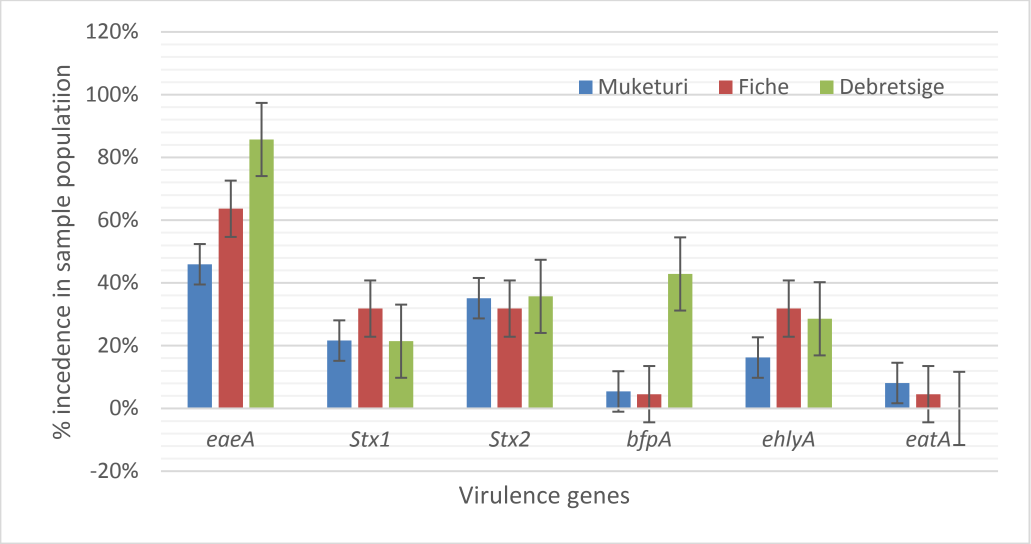

Figure 5.2. Spatial distribution of *E. coli* virulence genes among the three sample sites.

Key: *eaeA, E. coli* attaching effacing gene; *stx1,* Shiga-like toxin I gene; *stx2,* Shiga-like toxin II gene; *ehlyA, E. coli* hemolysin gene; *bfpA,* bundle forming pili gene.

The average number of virulence genes per sample isolate was found to be 1 ± 0.34 (media ± standard deviation). Out of the total isolates carrying virulence genes, 16 (31%) of the isolates were found to carry two VGs, while 39% of isolates carried 3 or more VGs. Among the isolates that had a single virulence factor, *eaeA* was the most frequent which is observed in 12 of the 15 isolates. While two of the remaining isolates had either of *aatA* or *Stx1* as a single VG. The remaining VGs were detected in combination with other VGs, out of which 31 (61%) had *eaeA* in combination with one of the other VGs. While the other combinations were *Stx1* with *Stx2* 3 isolates (6%) representing typical STEC and two isolates (4%) showing peculiar combination of *Stx1* with *aatA* (Table 5.1).

Based on the difference in combination of the six virulence genes, eleven virulence profiles (VPs) have been identified (Table 5.2). Isolates carrying a single VG, any one of *eaeA*, *Stx1* or *aatA* were categorized in to three different VPs (VP-I, VP-II and VP-III). While isolates carrying two VGs showed 4 different VPs carrying two of the six virulence factors in different combination. Similarly, three VPs were identified in isolates carrying 3 VGs.

Table 5.2. Virulence profiles identified based on combination of virulence factor gens in *E. coli* isolates.

No. of Isolates	Proportion (%)	Virulence genes						Virulence profile (VP)
		eaeA	*stx2*	*stx1*	*bfpA*	*ehlyA*	*aatA*	
2	3.92						+	I
1	1.96			+				II
12	23.53	+						III
2	3.92			+			+	IV
3	5.88		+	+				V
9	17.65	+			+			VI
2	3.92	+		+				VII
3	5.88	+	+			+		VIII
5	9.80	+		+		+		IX
3	5.88	+	+	+				X
9	17.65	+	+	+		+		XI

Key: *eaeA, E. coli* attaching effacing gene; *stx1,* Shiga-like toxin I gene; *stx2,* Shiga-like toxin II gene; *ehlyA, E. coli* hemolysin gene; *bfpA,* bundle forming pili gene.

On the basis of combinations of VGs, the *E. coli* isolates were further placed into one of four main pathotypes (STEC, EPEC, EHEC and EAEC) and two subtypes of EPEC, tEPEC and aEPEC. Whereas isolates that were found negative for the presence of VGs investigated in this study were designated as un-identified (UI), hence the markers used in the study were not exclusive to detect all previously identify pathotypes of DEC.

Accordingly, approximately 70% of isolates were classified as distinct pathotype and the remaining 30% were not placed under defined pathotype, thus designated as un-typeable (UI). Isolates belonging to the EHEC subtype were most commonly detected representing 22 isolates (30%), followed by EPEC (28%) where, aEPEC and tEPEC subtypes constituting 16% and 12% respectively. Whereas STEC and EAEC showed equal prevalence of 6% represented by 4 isolates each. The percentage distribution of *E. coli* isolates with defined pathotypes are shown in Fig. 5.3.

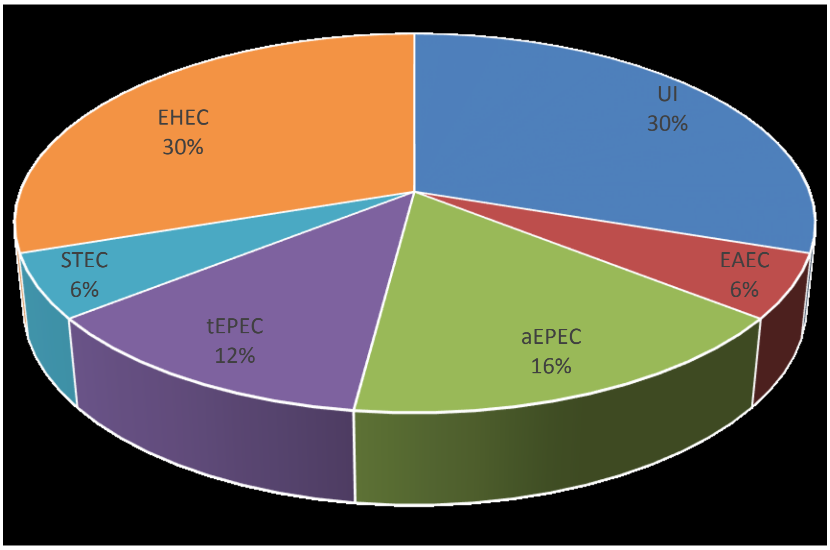

Figure 5.3. Frequency of different diarrheagenic *E. coli* pathotypes in sample isolates

Key: EAEC, Enteroaggregative *E. coli*; EHEC, Enterohemorrhagic *E. coli*; aEPEC, Atypical enteropathogenic *E. coli*; tEPEC, typical enteropathogenic *E. coli*; STEC, Shiga-like toxin producing *E. coli;* UI, unidentified *E. coli*

Furthermore, the prevalence of *E. coli* pathotypes was described in terms of spatial distribution. The distribution of *E. coli* isolates with defined pathotypes from the three sampling locations is shown in Fig. 5.4 below. From the isolates that were designated as distinct pathotype, EHEC was most frequently detected in Fiche and Debretsige represented by 45% and 36 % of samples isolates collected from the sites respectively. Similarly, Debretsige has the highest prevalence of tEPEC representing 43% of sample isolates. Whereas STEC was detected only in Muketuri site and EAEC has not been detected in Debretsige.

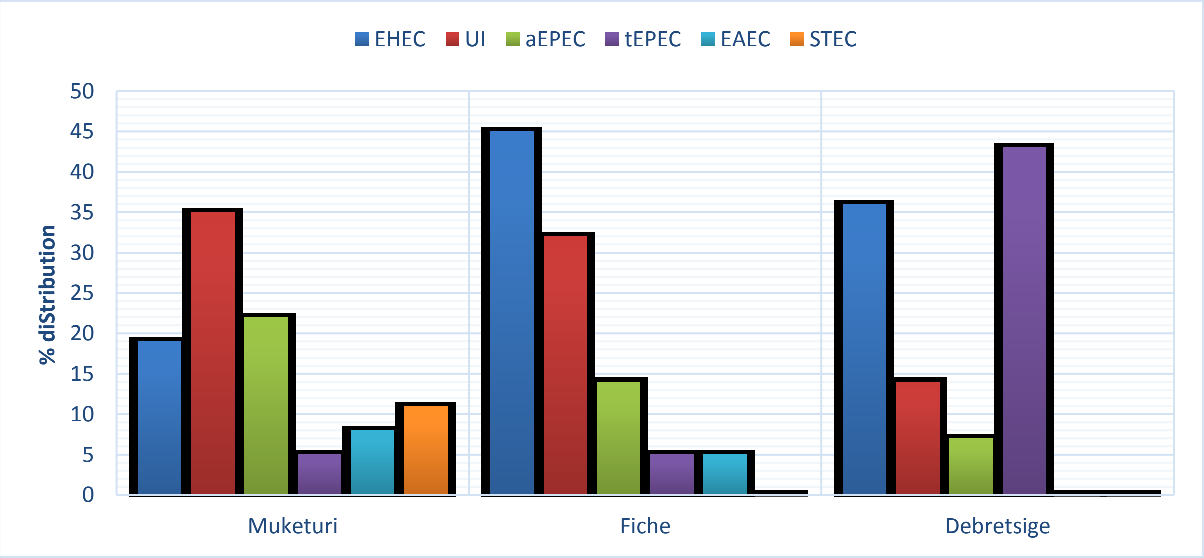

Figure 5.4. Distribution of *E. coli* pathotypes among the three sampling sites.

5.2. Plasmid Profile

In order to further characterize the DEC isolates, 30 samples were randomly selected from isolates that were found to carry at least one of VGs and investigated for their plasmid content. Plasmid DNA were extracted from sample isolates and classified according to their size by electrophoresis on 0.7% agarose gel alongside 1 Kbp DNA marker. Analysis of isolate's plasmid content revealed the presence of ten different kinds of plasmids, with sizes ranging from 2.5±0.13 Kbp up to around 98.2±4.17 Kbp as measured by an open access software GelQuant® (Fig. 5.5).

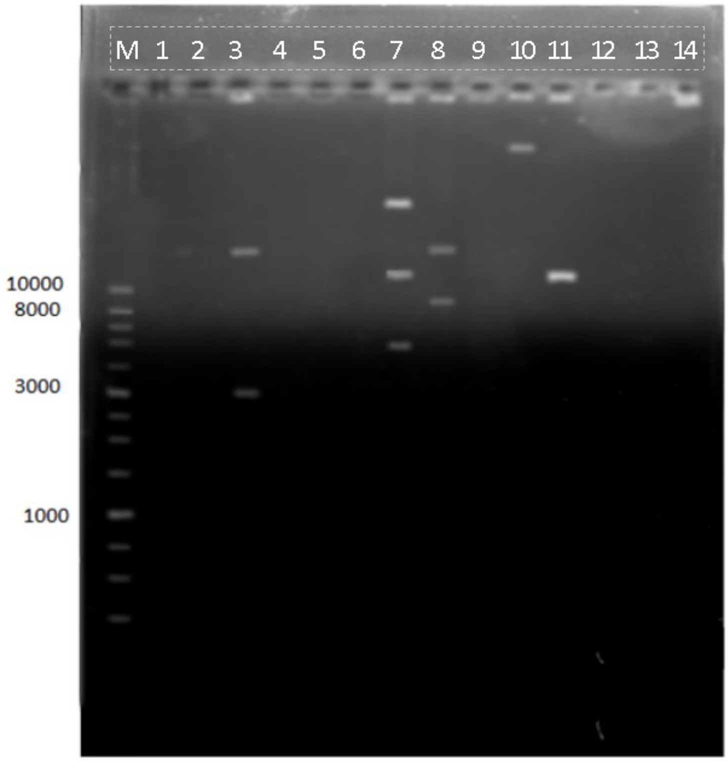

Figure 5.5. Representative plasmids following electrophoresis on 0.7% agarose gel. Lane M: DNA size marker (1 Kbp), lane 1–14: isolated plasmids.

The number of plasmids per sample isolate varied from 1 to 5 and all of the isolates were found to carry at least one plasmid, were the largest plasmid 98.2±4.17 Kbp in size were observed in all isolates that were screened for their plasmid content. Whereas the smaller plasmids showed inconsistent distribution among sample isolates therefore, regarded as putative plasmids. Among the putative plasmids, plasmid size of 16±1.4Kbp was most frequently observed, detected in 14 (47%) isolates. While plasmid size of 35±1.7Kbp was the least frequently detected, occurring in 2 of the isolates (7%). The distribution of plasmids and their frequency is summarized in table 5.3 below.

Table 5.3. Plasmid distribution frequency in *E. coli* isolates (N=30)

Plasmid Size ± SD (Kbp)	Incidence	% frequency
2.6 ± 0.14	3	10
3.1 ± 0.26	10	33
5.0 ± 0.31	5	17
7.8 ± 0.59	10	33
11.8 ± 0.82	7	23
14.3 ± 1.1	7	23
16.2 ± 1.4	14	47
34.6 ± 1.7	2	7
42.5 ± 2.1	6	20
98.2 ± 4.17	30	100

Plasmid distribution show considerable variation among the three sample sites (Table 5.5). Among the identified plasmids, plasmid size 11.8 Kbp and 34.6 Kbp were detected only in Fiche, while plasmid size 2.5 Kbp, 14.3 Kbp and 42.5 Kbp were not observed in this locality. Similarly, plasmid size around 5 Kbp was unique for Muketuri and Fiche, while the remaining plasmids were detected in common among the three sample sites.

Table 5.4. Distribution of plasmids in *E. coli* isolates among the three sample sites.

Sample Area (N)	Plasmid size (Kbp)										
	2.5	3.1	5.0	7.8	11.8	14.3	16.2	34.6	42.5	98.2	Freq. (N)
MT (11)	1	3	1	2	0	4	2	0	3	11	8
F (9)	0	1	1	2	2	0	2	1	0	9	16
DT (10)	1	4	0	4	0	1	6	0	2	10	36

Key. MT: Muketuri, F: Fiche, DT: Debretsige

Based on the difference in the distribution of identified plasmids, twelve distinct plasmid profiles (PPs) have been identified. The most commonly occurring plasmid profile was plasmid profile-I observed in 13 (43.33%) isolates followed by plasmid profile IV and VIII each occurring in 3 (10%) isolates (Table 5.4).

Table 5.5. Plasmid distribution profile in *E. coli* isolates (N=30).

No. of Plasmid	Plasmid profile	ẍ Plasmid Size (Kbp)	Number of Isolates
1	I	98.2	13
2	II	98.2, 11.8	1
	III	98.2, 16.2	1
3	IV	98.2, 16.2, 7.8	3
	V	98.2, 14.3, 3.1	1
	VI	98.2, 16.2, 3.1	2
4	VII	98.2, 42.5, 14.3,2.5	2
	VIII	98.2, 16.2, 7.8, 3.1	3
	IX	98.2, 42.5, 14.3, 5	1
	X	98.2, 34.6, 11.8, 5	1
	XI	98.2, 42.5, 14.3, 7.8, 3.1	1
5	XII	98.2, 42.5, 16.2, 7.8, 3.1	1

Key. ẍ Plasmid Size, mean plasmid size

5.3. Association Between Plasmids, Antimicrobial Resistance and Virulence Factors.

In previous work on the isolates, the samples used in this study were tested for antibiotic resistance against eleven antibiotics and all isolates showed resistance against one or more antibiotics (Annex 1). Based on combination of antibiotic resistance traits, the sample isolates exhibited 27 different resistance profiles (RT-1 to RT-27) (Annex 2). These data were used together with the data generated in this study to investigate any possible association between plasmid content, antimicrobial resistance and virulence factors in test isolates.

Correlation between antimicrobial resistance and plasmid distribution revealed a positive correlation ($r = 0.32$; $p < 0.01$) between the two variables (Fig. 5.6.a). These can be observed by significantly high frequency of plasmids in resistant strains ($p <0.05$) than susceptible strains. It is also found that, isolates resistant to multiple antibiotics (4 or more drugs) were found to carry 3 or more plasmids on average. Chi-squared distribution was also employed to identify the level of significance between a plasmid profile and antibiotic resistance trait. In this regard, several plasmid profile types occurred in a significantly higher proportion of resistant isolates than their susceptible counterparts. The analysis identified plasmid profiles associated with the greatest numbers of resistance traits, include PP-II (resistance to E, S, K, and AMP), PP-VIII (resistance to E, S, and AMX) and PP-IX (resistance to E, S, FOX and TET). However, multivariable logistic regression applied to identify any significant associations between a particular plasmid type and resistance trait, showed no significant association, as no particular plasmid can be directly associated to specific drug resistance trait.

Moreover, strong correlation was observed between virulence factor content and both plasmid profile and drug resistance trait. Results show the number of plasmids in an isolate were positively correlated with number of virulence gene content (r = 0.56; p $\leq$ 0.044). This is depicted in Fig. 5.6.b. Similarly, virulence gene content of an isolate was positively correlated with antibiotic resistance trait, even though it is found to be statistically insignificant. This can be explained by a 20% higher level of antibiotic resistance observed among isolates carrying one or more virulence gene compared to isolates that doesn't have any VG or 60% increase in antibiotic resistance from isolates with single VG to isolates carrying 4 VGs (Fig. 5.6.c).

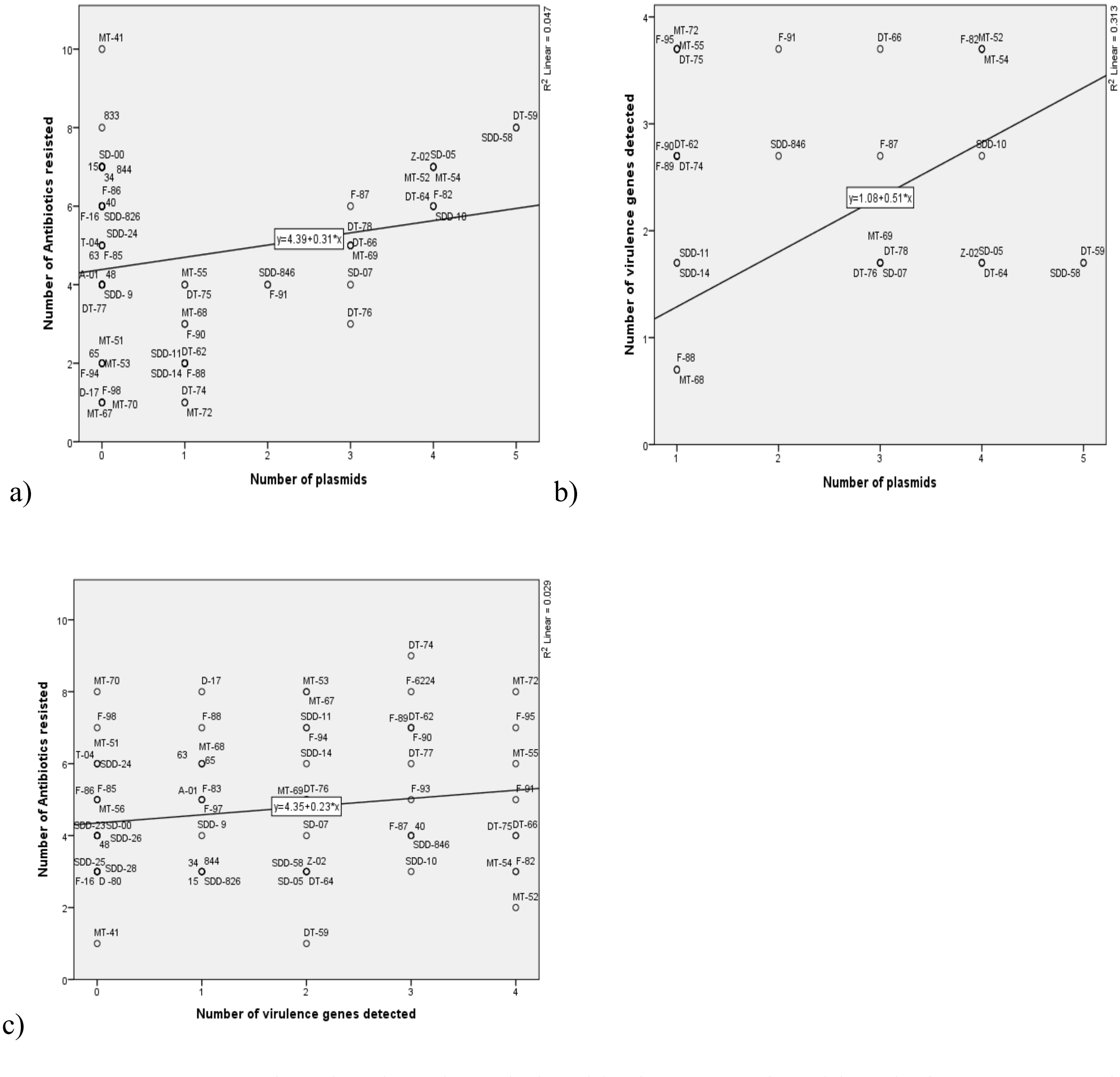

Figure 5.6. Scatter Plot showing the relationship between plasmids, virulence gene and antibiotic resistance. a) number of plasmids vs number of virulence factor gens detected b)

number of plasmids vs level of antimicrobial resistance c) number of virulence factor gens detected vs level of antimicrobial resistance.

The values for each pairwise combinations of the first three axes plotted as a series of Cartesian grids show the relationship among the three variables in two-dimensional space (Fig. 5.7).

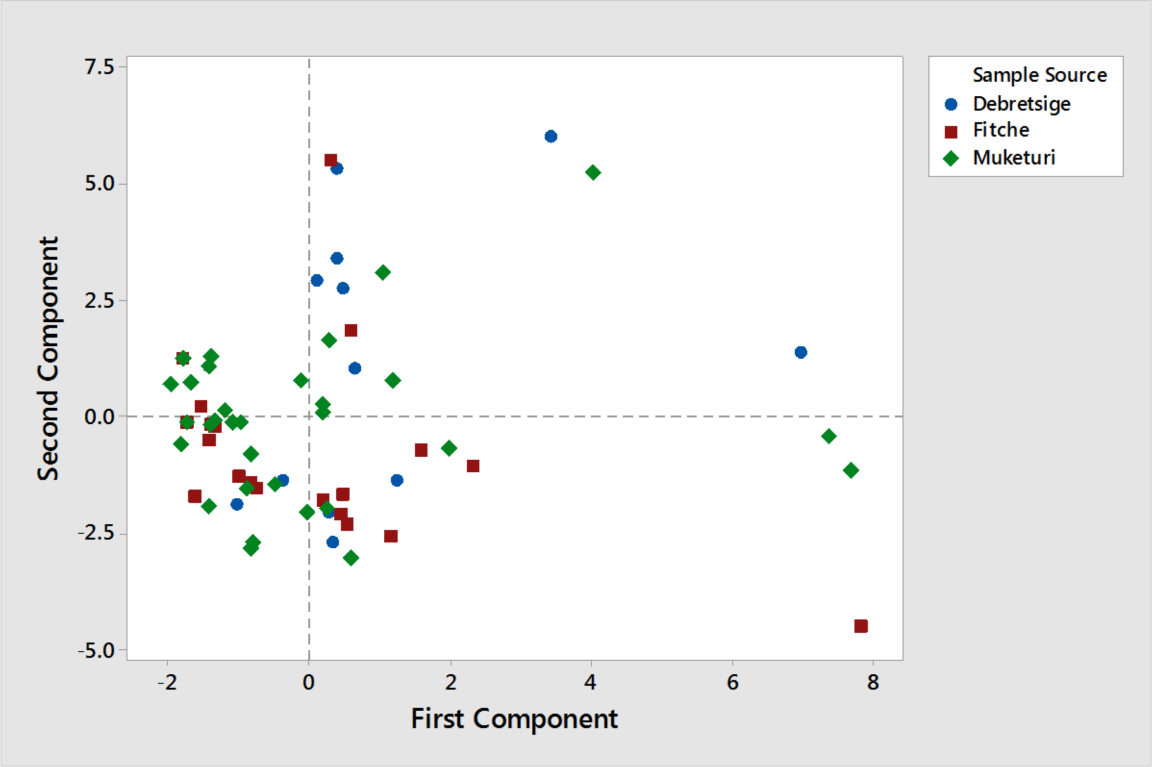

Figure 5.7. Score plot showing the first two components based on PCoA of isolates based on distribution of plasmids, virulence gene and antibiotic resistance traits.

Multivariate analysis of variance (MANOVA) conducted to determine whether the groups differed significantly according to the first three PCoA axes and significant difference was observed. In order to identify the factor responsible to the difference, univariate ANOVA was used by grouping the three arises against sample site and sample source variables such as age, sex and breed type of calve. The pairwise comparisons of individual groups according to each PCoA axis revealed significant difference ($p<0.05$) only across sample sites.

6. DISCUSSION

The overall occurrence of virulence genes observed in this study is significantly high compared to 22 isolates (30%) that carried no virulence genes. The result is comparable with what is reported by Shahrani *et al.* (2014), occurrence of virulence genes in 76.45% of fecal samples collected from 824 diarrheic calves in Iran. But it is comparatively high compared to 30.1 % occurrence of isolates carrying at least one of the eight virulence genes from 620 calves investigated by Piccoa *et al.* (2015) in Cordoba province, Argentina and 15.2% prevalence of virulence genes reported in Australia (Luna *et al.,* 2009). This indicates high level of virulence factor gene carrying *E. coli* in the sample area, indicating high level of pathogenic potential in the sample population

The high frequency of virulence genes observed in the current study is mainly attributed to high occurrence of *eaeA* (59%), that cods for an adhesin protein intimin in both EPEC and EHEC. This result is in agreement with previously reported occurrence of this gene in 38% of diarrheic calve fecal samples in Iran by Shahrani *et al.* (2014) and 57.1% from Australian calves by Luna *et al*. (2009). But it is by far more than 6% prevalence of *eae* in Mediterranean water buffalo calves in Italy Borriello (2012). Intimin is a 94-kDa protein encoded by the *eae* gene on locus of enterocyte effacement and it is found in all strains capable of inducing A/E histopathology (Kaper *et al*., 2004).

The locus of enterocyte effacement also harbors additional virulence factors such as T3SS which provide the pathogen leverage over the normal intestinal flora in the competition for intestinal site by intimate adhesion and biofilm formation, favoring overgrowth of pathogen at low infection dose (Deng *et. al*., 2004; Schroeder *et. al*., 2017). Thus, the relative abundance of A/E *E. coli;* EHEC (28%) and EPEC (30 %) in isolates of the current study can be explained by the presence of LEE in this isolates and combined effect of different virulence factors encoded on it.

In the current study comparably, higher frequency of *stx1* harboring STEC strain were observed in sample area. As shiga like toxin I is reported to be more often detected than shiga like toxin II in STEC strains, the relative abundance *stx1* in current investigation is in agreement with most reports of studies conducted around the world. For instance, Seyda *et al.* 2014, showed that all STEC isolates from cow with mastitis harbor *stx1* gene and identified *stx2* only in 20% of the STEC isolates. Similarly, high frequency of *E. coli* isolates carrying the *stx1* was observed in 86.67%, 40.7%, 12.2% *E. coli* isolates from diarrheic calves compared to 26.67%, 28.3%, 7.8% of *stx2* in Egypt, Iran and Australia respectively (Hashish *et al.,* 2016; Shahrani *et al.,* 2014; Luna *et al.,* 2009). In contrast higher prevalence of 33% *stx2* gene compared to 17% s*tx1*gene in cattle has been reported in Iran (Badouei *et al.,* 2016)

Epidemiological studies revealed that *stx2* is more associated with severe human disease such as; HC and HUS than *stx1* (Croxen *et al*. 2013). In one study for instance, *stx*2 and a variant, *stx*2c, were the only subtypes found from HUS cases (Friedrich *et al.,* 2002). Thus, even though *stx2* is less frequently detected than *stx1*, the overall occurrence of *stx2* among sample isolates is 25%, indicating a potential risk for severe illness following DEC infection in the areas.

In addition to Shiga-like toxin, virulence potential of STEC strains is also determined by additional virulence factors. Among others, virulence factors encoded on LEE had been well characterized so far and it is associated with to A/E lesion in the host resulting sever illnesses such as HUS and HC (Schmidt and Hensel, 2004; Deng *et. al*., 2004). In this regard considerable amount of STEC strains 22 of the 28 STEC isolates (78.57%) were found to contain *eae* and additional virulence factors that are harbored in locus of enterocyte effacement, a pathogenic island that harbored the gene, indicating the associated potential risk following DEC infection in the area. This result is in agreement with results obtained by Seyda *et al*. (2014) who identified *eae* in 40% of STEC isolates.

LEE harboring STEC strains stem as distinct subtype EHEC Nataro and Kaper, (1998), 22 of the isolates in the current investigation can be regarded as EHEC. Among the EHEC isolates 18 (50%) harbored only *stx1* and 2 (5.6%) isolates carried *stx2* as a single Stx gene. While the remaining 16 isolates (44.4%) contained both *stx1/stx2* Virulence genes. This result is inconsistent with the findings of Badouei *et al*. (2016) who identified 15.4% isolates to harbored *stx1* and 56.4% carried *stx2* as a single Stx gene. While the remaining contain both *stx1*and *stx2* from 39 EHEC isolates collected from cattle in Iran. Similarly, Borriello (2012) identified STEC isolates from fecal samples of water buffalo calves, were all Stx and intimin-positive, with Stx1 (80%) more frequent than Stx2 (27%).

In Addition to shiga-like toxins, EHEC strains may carry the EHEC hemolysin toxin encoded by *hlyA* or *ehx*. In the current investigation *ehlyA* was detected in 23 percent of the total isolates. In similar study, Luna *et al*. (2009) identified *Ehly* genes in 56% of isolates from diarrheic calves in Australia. This is a pore-forming toxin that lyses erythrocytes and found to be cytotoxic to endothelial cells contributing to the development of HC and HUS (Badouei *et al., 2016*) indicating a great potential risk posed by these isolates.

The other virulence gene, EAEC transcriptional regulator gene (*aatA)* were detected in 5.5% of isolates. In contrast the previously mentioned study in Australia, showed the occurrence of this VG in 29% of isolates (Luna *et al*., 2009). This result would be an evidence for the presence the said pathotype in the study area.

On the basis of combinations of Virulence genes, the *E. coli* isolates characterized in this study were further placed into one of the three pathotypes of DEC; STEC, EPEC, EAEC and two subtypes of STEC and EPEC each.

Approximately 70% of *E. coli* investigated in this study were classified as distinct pathotype based on combinations of virulence genes detected. The recognized pathotypes, 6% STEC, 30% EHEC, 12% tEPEC, 16% aEPEC, 6% EAEC were found to be comparable with similar studies such as; 9%STEC, 26% EHEC, 12% EPEC in Iran (Shahrani *et al.,* 2014) and 2.2% tEPEC, 6.7% aEPEC, 48.9% STEC, 6.7% EAEC and 2.2 % of both ETEC and EIEC in Egypt (Shabani *et al*., 2014). Similarly, studies made in Egypt that tried to characterize diarrheagenic *E. coli* virulence genes in newborn calves, identified different set of virulence genes; ST; 33.3%, LT; 30%, Stx1; 86.67%, Stx2; 26.67% and two ETEC adhesions (F5; 13.3%) and (F41; 16.67%) (Hashish *et al*., 2016).

The overall occurrence of the different virulence factors in *E. coli* isolates of the current investigation show the potential risk posed by these organisms not only to humans and domestic animal but also for wild animals. The potential transmission among different sources has been demonstrated by Miko *et al*. (2009) who isolated 140 STEC strains from game animals in Germany between 1998 and 2006 and compared with 101 STEC isolates from farm animals, their feed products and human patients. The result showed genes linked to high-level virulence for humans (*stx*2, *stx*2, and *eae*) belong to the same genotypes and other virulence attributes, regardless of origin.

Moreover, the current study tried to investigate plasmid content of study isolates as plasmids within DEC have been shown to carry both antibiotic resistance genes as well as virulence factor genes among others (Da Silva and Mendonça, 2012). The study has identified ten different kinds of plasmids, with size ranging from 2.6 ± 0.14 Kbp to 98.2 ± 4.17 Kbp. In agreement with the current study, Nsofor and Iroegbu (2013) isolated *E. coli* plasmids from domestic animals, that harbor one or more plasmids with molecular size in the range of 1 to 120 Kbp. Similarly, 7 different plasmids 2.4 kbp to 65 kbp in size were isolated from 1,266 fecal specimens derived from environmental, human, animal and food sources (Wells *et al.,* 1991).

Among the various plasmids identified, a large plasmid with slight variation in size has been observed in all isolates examined for their plasmid content. DECs are known to harbor large conserved plasmids associated with virulence potential as well as resistance trait (Lim *et al*., 2010; Brunder *et al*., 2006). For instance, the presence of large conserved plasmid is established in DEC pathotypes EAEC, EHEC and EPEC which are pAA, pO157 and pEAF respectively (Croxen *et al*. 2013). The size variation in the large conserved plasmids is also likely. For instance, the large plasmids of STEC were shown to vary between 115Kbp and 65Kbp in different studies (Venturini *et. al*., 2013; Ostroff *et al.,* 1989; Wells *et al*., 1991).

The smaller plasmids identified in the current investigation were regarded as putative due to their inconsistent distribution, even among isolates with similar characteristics in terms of virulence factors as well as antibiotic resistance profile. However, the study identified high level of antibiotic resistance in isolates containing these plasmids. Though results cannot be directly compared, as different methods employed, Bello *et al*. (2013) and Kalantar *et al*. (2011) detected plasmids more frequently in resistant strains than susceptible strains. Bello *et al*. (2013) for instance isolated a total of 48 different plasmids occurring in various combinations from 50 *E. coli* isolates that showed MDR. Similarly, resistance genes for MDR was reported in *E. coli* plasmids by Kalantar *et al*. (2011).

Even though plasmids were found to be strongly correlated with resistance phenotype, no particular plasmid was found to be significantly associated with a particular drug resistance phenotype. This result confirmed that there was no a linear correlation between the plasmids content and the antibiogram studies of the isolates, which suggests resistance genes might be located in various plasmids.

Antibiotic resistance is not only conferred by plasmids, studies also show inherent resistance to antibiotics by chromosomally encoded resistance gens (van Hoek *et. al*., 2011). Moreover, the large conserved plasmid of STEC strains has been shown to harbor genes resistance for antibiotics (Da Silva and Mendonça, 2012). These facts potentially explain the observed antibiotic resistance despite the absence of putative plasmids in PP-I isolates.

Studies made in understanding the immense antibiotic resistance in *E. coli* indicated plasticity of *E. coli* genome and the role of HGT by mobile genetic elements such as plasmids in conferring MDR and the possibility for the transmission of these plasmids among distantly related microbial population (van Hoek *et. al*., 2011). Thus, the results of the current study indicate the need for early intervention to prevent further spread of DEC in the sample areas in order to, as the occurrence of such high level of mobile genetic elements in highly resistant bacteria presents a serious challenge in transmission of antibiotic resistance among other pathogens.

Similarly, the significant positive correlation between antimicrobial resistance and virulence gene content showed in this study agrees with what is reported by Zhang *et al.* (2015) who showed the prevalence of resistance to single and multiple antibiotics to be significantly higher for pathogenic isolates than for commensal isolates. In contrast, Seyda *et al*. (2014), identified no significant differences in virulence genes between antibiotic-resistant and antibiotic-susceptible strains. However, the observed significant correlation between virulence and antibiotic resistance in this study can be explained by the effects of selection pressure and genetic association between these factors according to Zhang *et al*. (2015) and Schroeder *et. al*. (2017). This study can also be taken as additional evidence for the positive correlation between antibiotic resistance and virulence factor carriage similar to the results obtained by Zhang *et al*. (2015).

Furthermore, this study showed significant correlation between plasmid carriage and virulence factor, even though all the virulence genes used in the study are not plasmid encoded, where 80% of the putative plasmids were detected in intimin containing isolates. This finding may explain the abundance of EHEC among test samples, as extra virulence factors like additional adhesins can be conferred by plasmid encoded Virulence genes. DEC plasmids are known to be associated with virulence potential (Lim *et al*., 2010; Brunder *et al*., 2006). Such strong association between plasmids and virulence factors may be explained based on pathogenomics and concurrent evolution according to Johnson and Nolan (2009).

Based on all the study variables *i.e.* each of virulence factors, plasmids and antibiotic resistance traits, clear clustering of isolates across sample sites was observed indicating significant phenotypic difference across sampling sites. Such clustering of isolates in spatial and seasonal difference has also been reported on the incidence of *E. coli* strains in diarrheic calves by Shahrani *et al*. (2014) in Egypt and Sidhu *et al.* (2012) in Australia. This clustering of isolates among sites may be explained by similar sources of infection in the area as a result of environmental contamination by contaminated litters and asymptomatic carriers or as a result of difference in geography, climate and antibiotic consumption trend among sample sites.

Despite the valuable information generated in this study, the study had limitations in showing all the relevant information to get the clear picture of the different DEC pathotypes, their biology and distribution as a single host can be infected by multiple pathotypes of DEC. This is mainly the result of limited resources available to conduct the research.

7. CONCLUSION AND RECOMMENDATIONS

Despite the inherent limitations, this work provides valuable information on the occurrences of major DEC virulence factors in significantly high level in diarrheic calves from all 56 farms of three sampling sites, which is the major source of dairy products supplied to Addis Ababa, indicating the potential risk posed by these pathogens. Furthermore, the study demonstrated significant association between antibiotic resistance, plasmid content and virulence gene content of DEC indicating potential risk of associated infections in the areas. The role of calves as a reservoir and their feces as a source of contamination for food, environment and humans suggests, these hosts have high potential as for transmission of antibiotic resistance in addition to be source of infection.

It is recommended that nationwide isolation and characterization of DEC is vital for early detection of potentially fatal strains before causing considerable impact on public health. Thus, further studies should be made in order to establish effective intervention strategies that address all the sources of infection, reservoirs and hosts control DEC transmission. More over studies that investigate the clonal complex of these organisms would help in identifying the real source of this organisms and determining their potential to cause disease in humans and the wild life. Similarly, the level of antibiotic resistance elucidated is a strong evidence for the need of rational use of antibiotics and further researches in this area that encompass identifying trends in antibiotic resistance and identifying particular antibiotic resistance genes. This is especially important to control the rapidly increasing antimicrobial resistance. It is also recommended to initiate a control and prevention strategy based on One-Health approach.

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APPENDICES

Annex 1. Antimicrobial response of study samples N (%)

Antibiotic Tested	Isolates		
	Resistant	Intermediate	Susceptible
Gentamycin	7 (10)	0 (0)	66 (90)
Erythromycin	70 (96)	1 (1)	2 (3)
Streptomycin	56 (77)	11 (15)	6 (8)
Kanamycin	17 (23)	25 (34)	31 (42)
Ampicillin	42 (58)	24 (33)	7 (10)
Cefoxitin	34 (47)	6 (8)	33 (45)
Chloramphenicol	21 (29)	9 (12)	43 (59)
Tetracycline	53 (73)	0 (0)	20 (27)
Cotrimoxazole	10 (14)	1 (1)	62 (85)
Amoxicillin	31 (42)	28 (38)	14 (19)
Ciprofloxacin	2 (3)	11 (15)	60 (82)

Annex 2. Antibiotic resistance profiles identified in *E. coli* isolates.

Resistance type	frequency	Antibiotics Tested									
I	6	E									
II	3	E	AMX								
III	4	E	S								
IV	2	TET	AMP								
V	1	E	TET								
VI	1	E	K								
VII	2	E	TET	AMP							
VIII	1	E	S	AMX							
IX	13	E	S	TET	FOX						
X	1	E	S	AMP	K						
XI	1	S	TET	AMP	FOX	AMX					
XII	1	E	S	TET	AMX						
XIII	2	E	S	TET	AMP	K					
XIV	1	E	TET	AMP	AMX	K					
XV	1	E	S	TET	FOX	CAF					
XVI	2	E	S	AMP	AMX	K					
XVII	6	E	S	TET	AMP	SXT	GN				
XVIII	2	E	S	TET	AMP	FOX	K				
XIX	2	E	S	TET	AMP	FOX	AMX				
XX	2	E	S	AMP	FOX	AMX	CAF				
XXI	11	E	S	TET	AMP	FOX	AMX	CAF			
XXII	1	E	TET	AMP	FOX	AMX	CAF	K			
XXIII	1	E	S	TET	AMP	K	SXT	GN			
XXIV	2	E	S	TET	AMP	AMX	CAF	K			
XXV	2	E	S	TET	AMP	AMX	CAF	K	SXT		
XXVI	1	E	S	TET	AMP	AMX	CAF	K	CIP		
XXVII	1	E	S	TET	AMP	FOX	AMX	CAF	K	SXT	CIP

Key: AMP, Ampicillin; AMX, Amoxycillin; FOX, Aefoxitin; CAF, Ahloramphenicol; E, Erythromycin; SXT, Sulfamethoxazole-Trimethoprim; S, Streptomycin; K, Kanamycin; CIP, Ciprofloxacin; GN, Gentamicin; TET, Tetracycline.