JIMMA UNIVERSTY
COLLEGE OF AGRICULTURE AND VETERINARY MEDICINE
SCHOOL OF VETERINARY MEDICINE

ISOLATION AND MOLECULAR CHARACTERIZATION OF PATHOGENIC STRAINS, THEIR ANTIBIOGRAM AND ASSOCIATED RISK FACTORS OF *Escherichia coli* ISOLATES FROM DIARRHEIC CALVES IN DAIRY FARMS BAHIR DAR CITY, NORTH WESTERN ETHIOPIA

MSc THESIS

BY

KEFALE MENGISTU

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JIMMA, ETHIOPIA

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Kefale Mengistu

MSc Thesis

Submitted to the School of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Jimma University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Veterinary Microbiology

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I have incorporated the suggestions and modifications during the internal thesis defense and got the approval of my advisors. Hence, I hereby kindly request the Department to allow me to submit my thesis for internal thesis defense.

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We the thesis advisors have verified that the student has incorporated the suggestions and modifications given during the internal thesis defense and the thesis is ready to be submitted. Hence, we recommended the thesis to be submitted for external defense

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DEDICATION

This MSc thesis work is dedicated to my beloved and kind father, Mengistu Tamire and mother, Yezina Alemu for their far sighted vision, dignified and disciplined personality, enhanced social relationship, positive attitude and hardworking spirit had shaped me and paved the way to my present situation.

STATEMENTS OF THE AUTHOR

First, I pronounce that this thesis is my original work and that all sources of material used for this thesis work have been duly recognized. This thesis has been submitted in partial fulfillment of the requirements for (MSc) degree at Jimma University College of Agriculture and Veterinary Medicine and is placed at the University/College library to be made accessible to debtors under rules of the Library. I seriously declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

Kefale Mengistu was born in August 1987 in Machakel wereda east Gojjam Zone, in Amhara regional state, Ethiopia. He attended his primary education from 1995 to 2002 at Medebebey elementary school. He pursued his secondary school education at Amanuel senior secondary school from 2003 to 2004. He joined Alage Technical &vocational Training college in 2005 and graduate with Diploma in Animal health in July 2007. After graduation Kefale worked at East Gojjam zone as Animal health worker from 2007- 2009. He joined Wollo University in 2010 and graduate with BVSc in 2012. From 2012- 2015 he worked at East Gojjam zone as Animal health worker. He employed by Jimma University College of Agriculture and Veterinary Medicine under School of Veterinary Medicine in October 2015. He joined the school of graduate studies of Jimma University College of Agriculture and Veterinary Medicine in September 2017 to pursue his Masters of Science (MVSc) degree study in Veterinary Microbiology.

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LIST OF ABRIVATIONS

aatA	Enteroaggregative Gene
AE	Attaching and Effacing Lesions (eaeA)
AOR	Adjusted Odd Ratio
Bfp	Bundle Forming Pillus
Bp	Base Pair
BPW	Buffered Pepton Water
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standards Institute
COR	Crude Odd Ratio
DAEC	Diffusely-Adherent *E. coli*
DEC	Diarrheagenic *E. coli*
dNTP	Deoxy Nucleotide Triphosphate
E. coli	*Escherichia coli*
eaeA	Enterocyte Attaching Effacing
EAEC	Entero Aggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosin Methylene Blue
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
hlyA	Entero Hemolysin Gene
IMViC	Indole,Methylred,Vocusproskaure&Citrat
LEE	Locus of Enterocyte Effacement
LT	Labile Enterotoxin
MCA	MacConkey Agar
MDR	Multi Drug Resistance
NCD	Neonatal Calf Diarrhea
NSAIDs	Nonstirodal Anti-inflammatory Drug
PCR	Polymerase Chain Reaction
STEC	Shiga Toxin-producing *E. coli*
tEPEC	Typical Enteropathogenic *E. coli*
TSB	Tryptose Soya Broth
TSI	Triple Sugar Iron
VTEC	Vero Toxin-producing *E. coli*
X^2	Chi-Squere

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ABSTRACT

Escherichia coli is recognized as one of the major causes of neonatal calf diarrhea with severe lethal outcome and major damage to the livestock industry worldwide. A cross-sectional study was conducted from January 2019 - Jun 2019 to isolate & identify pathogenic *Escherichia coli* strains, their antibiogram and associated risk factors from diarrheic calves in Bahir Dar city dairy farms. A total of 112 fecal samples were collected directly from the rectum of diarrheic calves and processed using standard microbiological procedures. Accordingly, 57 (50.89%) samples were positive for *E. coli*. The multivariable logistic regression analysis indicates that, the isolation rate of *E. coli* was significantly ($p < 0.05$) affected by factors such as age, breed, herd size and first colostrum feeding time. Genomic DNA extracted from the isolates was amplified using polymerase chain reactions to detect pathogenic strains. Furthermore, *stx2, stx1, eaeA* and *hlyA* genes were detected from the isolates at the rates of 10/57 (17.54%), 5/57 (8.77%), 3/57 (5.26%) and 3/57 (5.26%), respectively. Based on the genes detected three pathotypes/strains of *E. coli* were identified: STEC 11(19.3%), EHEC 4(7.02%) and aEPEC 1(1.75%). In vitro antimicrobial sensitivity testing showed susceptibility rates ranging from 0% up to 85.96% and resistance rates ranging 8.77% and 100%. All pathogenic *E. coli* strains were susceptible to chloramphenicol but all of the pathogenic strains showed resistance to Neomycin. In conclusion, the occurrence of *E. coli* isolates, their pathogenic strains and Frequent use of limited antibiotics were found at high frequency. Hence, Identifying pathogenic strains and strict control measures such as treatment of positive cases using effective drugs are vitally important as effective control and prevention strategies.

Keywords: *Antimicrobials, Bahir Dar, Diarrheic calves, Escherichia coli, Pathogenic Strains.*

1. INTRODUCTION

Ethiopia is one of the nations in Africa where livestock production has a long traditional practice with a huge and diverse livestock population (CSA, 2016/17). Even though, there is a large livestock population in Ethiopia, the economic benefits remain marginal due to low genetic quality of local breeds, prevailing diseases, poor nutrition, poor animal production systems, reproductive inefficiency, management constraint and general lack of veterinary care (Fentie *et al*., 2016).

Since the future of the dairy herd solely depends upon the successful raising of young calves, the health of replacement calves is an important component of total dairy operation profitability (Razzaque *et al.,* 2009). Calf diseases such as diarrhea in neonatal period, pneumonia in older calves and others that are the results of complex interaction of the management practices, environment and infectious agents are the prevailing problems in various dairy farms (Klein-Jöbstl *et al.*, 2014; Azizzadeh *et al.*, 2012; Windeyer *et al*., 2014; Wudu *et al*., 2008; Ferede *et al.*, 2014).

In Ethiopia, calf mortality due to diarrohea ranges from 7 to 30.7% (Wudu *et al*., 2008; Megersa *et al*., 2009; Ferede *et al*., 2014). Single primary pathogen or co-infection can predispose to the development of diarrhea. Diarrhea in calves could be attributed to both infectious and non-infectious factors. Thus, from infectious agents virus (Bovine viral diarrhea, rotavirus, coronavirus), bacteria (*Escherichia coli, Salmonella,* enterotoxaemia, cryptosporidia and Giardia) and protozoa (Ayana and Alemu, 2015) are a common problem in dairy farms. From non-infectious factor such as insufficient uptake of colostrum, poor sanitation, stress, and cold weather could cause diarrhea (Muktar *et al.,* 2015).

Among these organisms *Escherichia coli* is the main cause for the calf diarrhea as white scour. This bacterium is gram negative, rod shaped, flagellated, motile, Oxidize negative, facultative anaerobe and is classified under the family *Enterobacteriaceae*. It is genetically the most versatile bacteria and is the source of many plasmid and phage mediated genes. *E. coli* produces septicemia and diarrhea in a wide range of hosts including man, avian and animals (Hemashenpagam *et al.,* 2009).

Escherichia coli are the most important bacterial cause of diarrhea in young animals. Several pathogenic and nonpathogenic factors are predisposed to calf diarrhea (Bartels *et al.,* 2010; Izzo *et al*., 2011). Diarrheagenic *E. coli* (DEC) are recognized as the major cause of neonatal calf diarrhea (NCD) with severe lethal outcome and major damage to the livestock industry worldwide. Consequently, high mortality rate in calves under 3-weeks-old and up to 3-months-old has been reported (Windeyer *et al*., 2014).

Based on the molecular and pathological criteria, diarrheagenic *E. coli* (DEC) are classified into several pathotypes/strains such as: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) enterinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Diffusely-adherent *E. coli* (DAEC) and vero- or Shiga-like toxin producing *E. coli* (VTEC or STEC) (Nagy and Fekete, 2005; O'Sulevan *et al.* 2007).

Diarrheagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed (Nataro and Kaper, 1998). Although different approaches were attempted to diagnose pathogenic *E. coli*, polymerase chain reaction (PCR) is classically a sole and confirmatory nucleic acid-based method for detection of virulent genes. PCR exploits the thermo cyclic enzymatic amplification of specific DNA sequences of the target gene using a pair of oligo nucleotide primers (Cho and Yoon, 2014).

Domestic and wild animals are the sources of *E. coli*, but the major animal carriers are healthy domesticated ruminants, primarily cattle and, to lesser extent, sheep, and possibly goat. *E. coli* is transmitted by ingestion of contaminated food and water, direct contact with animals, faeces and contaminated soil and directly from one animal to another (CDC 2008).

Treatment includes fluid therapy for water and electrolyte replacement and correction of acid-base disturbances, alteration of the diet, and antimicrobial and anti-inflammatory therapy (Gruenberg, 2018b).

A number of studies conducted in Ethiopia from various clinical settings showed increments in the prevalence of antimicrobial resistance patterns of *E. coli* (Endalafer *et al.,* 2011 and Yismaw *et al.,* 2010). The prevalence, antibiogram and epidemiological features of *E. coli* as the causative agent of diarrhea vary from region to region around the world, and even between and within countries in the same geographical area (Kaper *et al.,* 2004).

Like the rest of Africa, surveillance for various types of DEC is overlooked in Ethiopia, only limited studies were done at the level of molecular based detection of pathogenic strains and their antimicrobial susceptibility. Furthermore, molecular based detection of pathogenic strains has never been studied at Bahir Dar. Thus, this study aimed to investigate studies for molecular characterization of pathogenic strains, antimicrobial susceptibility and risk factor of *E. coli* in diarrheic calves.

The information that can be acquired from this study helps to identify pathogenic *Escherichia coli* strains; antimicrobial susceptibility and risk factor are important information to use while planning and implementing control strategies to reduce diarrhea-based morbidity and mortality of calf in the study area. 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 design appropriate diagnostic and vaccine strategies. Appropriate drug of choice determined after performing antibiotic susceptibility test for each *E. coli* isolates so that it minimizes the emergence and spread of resistant strains. Considering the above questions this study was aimed to investigate the presence of pathogenic *Escherichia coli* strains, Antibiogram and associated risk factor from diarrheic Calves.

1.1. Objectives

1.1.1. General objective

The main objective of this study was to isolate and identify pathogenic *E. coli* strains, their antibiogram and associated risk factors from diarrheic calves in Bahir Dar city dairy farms.

1.1.2. Specific Objectives

- To isolate *E. coli* from diarrheic calves in Bahir Dar city dairy farms.
- To identify predisposing risk factors associated with presence of *E. coli*.
- To determine pathogenic *E. coli* strains among *E. coli* isolates using molecular tools.
- To determine the antimicrobial resistance pattern of *E. coli* isolates and pathogenic strains

2. LITERATURE REVIEW

2.1. Historical Background

Escherichia coli were first described by a Bavarian pediatrician, Theodor Escherich, in the late 19th century. In a series of pioneering studies of the intestinal flora of infants he described a normal microbial inhabitant of healthy individuals (Kidane, 2014). Although the organism was later described under multiple synonyms iterations by other researchers, the name of *E. coli* was not fully recognized until 1954 (Claeys *et al.,* 2013). Then after *E. coli* is known as a harmless commensal of a gastrointestinal tract in warm blooded animals and is used as the colloquial laboratory workhorse. However, there is an alternate side to *E. coli* afforded through gene gain and loss that enable it to become a highly divers and adapted pathogen (Croxen and Finlay, 2010).

The first confirmed isolation of *E. coli* O157:H7 in the United States of America was in 1975 from a Californian woman with bloody diarrhoea, while the first reported isolation of the pathogen from cattle was in Argentina in 1977, while the bacterium was first identified as a human pathogen in 1982 (Fernandez, 2008). Beyond single primary pathogen or co-infection, calf diarrhea can also be caused by other factors such as nutrition, hygienic conditions and environmentally related issues (Hashish *et al*., 2016). Until immunity is compromised and the epithelial integrity is breached off *E. coli* remains and coexists as part of the gut micro biota (Umpierrez *et al*., 2016).

2.2. Taxonomy and Classification

The comparative analysis of 5S and 16S ribosomal RNA sequences suggest that Escherichia and Salmonella diverged from a common ancestor between 120 and 160 million years ago, which coincides with the origin of mammals. Escherichia and Shigella have been historically separated into different genera within the Enterobacteriaceae family. DNA sequence analysis of their genomes reveals a high degree of sequence similarity and suggests that they should be considered a single species (Ochman & Wilson, 1987). The family of *E. coli* contains more than 28 genera and over 80 species. The major animal Enterobacteriaceae pathogens, *E. coli*, Salmonella and Yersinia species can cause both enteric and systemic disease (Quinn *et al*., 2011and Tassew, 2015).

Scientific classification of *E. coli*;

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Family: Enterobacteriaceae
Genus: Escherichia
Species: E. coli
Source: (Quinn *et al*., 2011)

2.3. The Organism and its Characteristics

Escherichia coli are considered as the normal bowel flora of different species of mammals and birds (Zinnah *et al*., 2007). For the most part, *E. coli* is a group of harmless bacteria that are most often used as indicator organisms for fecal contamination and breaches in hygiene. However, several *E. coli* clones have acquired virulence factors that have allowed them to adapt to new niches and in some cases to cause serious disease (Farrokh *et al*., 2012).

Escherichia coli commonly abbreviated as *E. coli*; is a gram negative rod-shaped motile or nonmotile, facultative anaerobic, non-spore forming member of the Enterobacteriaceae family found in the gastrointestinal tract of warm-blooded animals and humans. However, some pathogenic E. coli cause a wide range of illnesses that can be classified broadly as gastrointestinal and extra-intestinal. Pathogenic *E. coli* share a common pattern of mucosal colonization, evasion of host defenses multiplication and host cell damage ultimately leading to illness (Nataro *et al*., 1998).

2.3.1. Growth and Inactivation

Escherichia coli is a facultative anaerobe that can grow from 7ºC to 50ºC with an optimum temperature of 37ºC, although there have been reports of some ETEC strains growing at temperatures as low as 4ºC (Xia *et al*., 2010). A near neutral pH is optimal for its growth but growth is possible down to pH 4.4 under otherwise optimal conditions. Some diarrheagenic *E. coli* strains have the ability to tolerate exposure to pH 2.0. Such an acid shock mimics transit through the stomach and induces expression of sets of genes involved in survival and pathogenesis (Tassew, 2015).

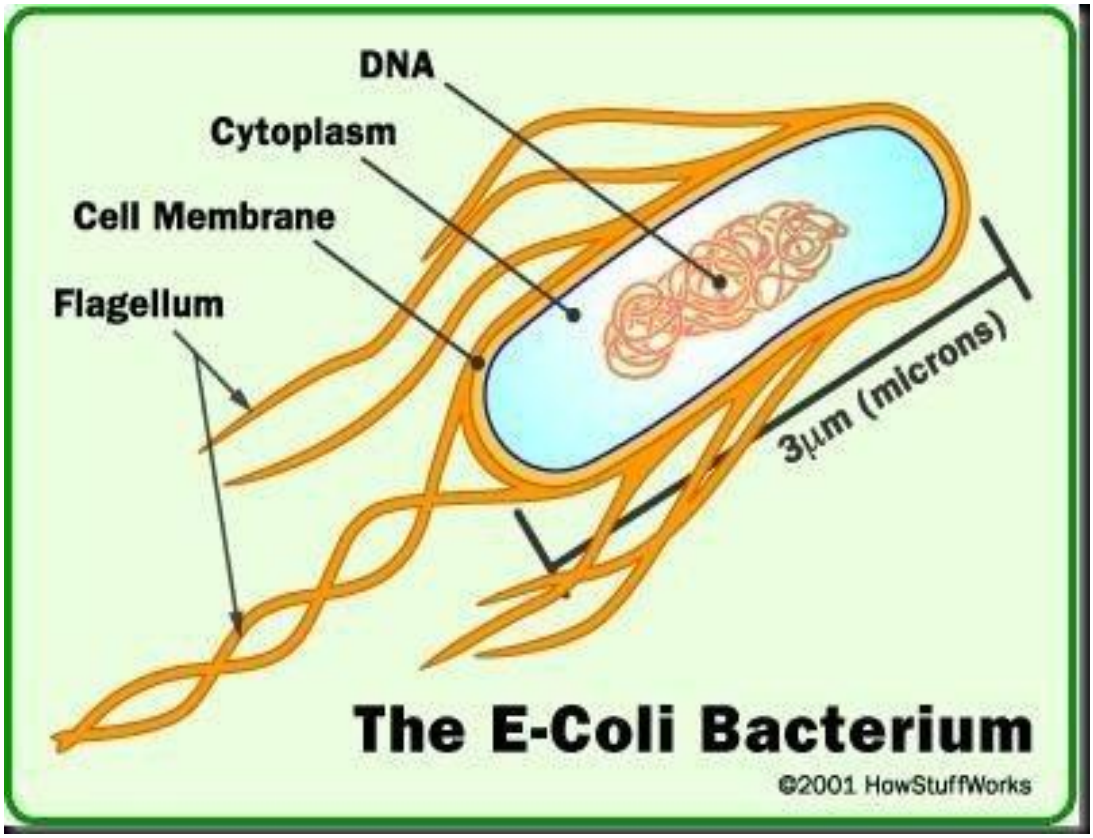


Fig 1: Structure of *E. coli*

Source: http://www.nature-education.org/water-ecoli.html

2.3.2. Biochemical Properties

Escherichia coli can be differentiated from other members of the Enterobacteriaceae on the basis of a number of sugar-fermentation and other biochemical tests. Classically an important group of tests used for this purpose are known by IMViC. They are indole (I) positive, methyl red(M) positive, Voges Proskaur (V) negative, simmon's citrate (C) negative, catalase positive and urease negative. *E. coli* are ferment most sugars producing gas but do not produce H2S on TSI agar slants (A/A with gas) (Wesonga, 2011). Despite *E. coli* can be identified with a variety of biochemical reactions, the indole test remains the most useful method to differentiate lack of production of β- glucuronidase and from other members of the Enterobacteriaceae (Xia *et al*., 2010 and Ashraf, 2016).

2.3.3. Acid and Salt Tolerance

Escherichia coli are an acid resistant food borne pathogen that survives in the acidic environment of stomach and colonies the gastrointestinal tract (Price *et al*., 2004). Furthermore, it also increases the survival of *E. coli* particularly STEC O157:H7 in acidic foods, enabling survival for extended periods, especially at refrigeration temperature (Meng *et al*., 2007). It grows well on MacConkay agar because they are not inhibited by the bile salts in the medium (Nakazato *et al*., 2009). Hence, contaminated cultured and fermented foods such as yoghurt and cheese have also been implicated in sporadic cases and the disease outbreaks (Farrokh *et al.*, 2012).

2.4. Epidemiology

2.4.1. Geographical distribution

Pathogenic *E. coli* strains have been reported through the worldwide geographic distribution in Canada, United Kingdom , China, Argentina, Japan, Swaziland, Malawi, Kenya, Central African Republic, Cameroon, Nigeria, Ivory Coast, Kenya (Wesonga, 2011). The differences of the prevalence rates among different studies may be due to the ecological differences and management practice as well as hygienic measures (Cho and Yoon, 2014).

2.5. Sources and Mode of Transmission

It is assumed that the primary source of the infection is the feces of infected animals, including the healthy dams and neonates, and diarrheic newborn animals, which act as multipliers of the organisms. Invasion occurs primarily through the nasal and oropharyngeal mucosa but can also occur across the intestine or via the umbilicus and umbilical veins. The organism is excreted in nasal and oral secretions, urine and feces excretion begins during the preclinical bacteremic stage. Initial infection can be acquired from a contaminated environment. In groups of calves, transmission is by direct nose-to-nose contact, urinary and respiratory aerosols, or as the result of navel sucking or fecal-oral contact (Gruenberg, 2014).

Transmission is through faecal-oral route including the healthy dams and neonates and diarrheic newborn animals, which act as multipliers of the organisms (Pugh and Baird, 2012; Ashraf, 2016) although infection via the umbilical vessels and nasopharyngeal mucosa can occur. Infected animals are the source of the organisms; they excrete them and infect other animals, directly or indirectly by contaminant of the environment, primarily feed and water supplies (Radostits *et al*., 2007; Gruenberg, 2018b).

2.6. Risk Factors for Infection

The risk factors increase the exposure to infection and further lowering the defenses mechanism within the calf in early life. Host immunological and nutritional status due to insufficient uptake of colostrum and milk giving, poor sanitation, environmental stress, overcrowding, inadequate ventilation and general poor management practice involved have vital roles in the occurrence of the disease (Izzo *et al.*, 2011; Cho and Yoon, 2014).

Resistance of the calf to enteric diseases is closely related to the timely consumption of high quality colostrum insufficient quantities within the first 6 hour after birth. Special care required to reduce environmental risk factors closely associated with calving season including the provision of dry, draft free shelter (Fernandez *et al.*, 2009).

2.7. Reservoir Host and Susceptibility

Domestic and wild animals are sources of *E. coli* from these ruminants primarily sheep, goats and cattle have been identified as major reservoirs and source for human infection (Kiranmayi *et al.,* 2010). Cattle are generally regarded as the main natural reservoir of EHEC. All ages of cattle are susceptible to colonization with EHEC, although peak shedding is observed in sub adult cattle from weaning to 24 months of age (Joris *et al*., 2012).

2.8. Pathogenesis and Virulent Factors of *Escherichia coli*

The primary harm from scours is loss of water and electrolytes (body salts) in the diarrhea. This loss of water and salts creates dehydration and alteration of the acid base balance of the bodily fluids. Inflammation of the intestinal lining impairs the calf's ability to digest nutrients, creating weight loss and the potential for hypoglycemia (low blood sugar). If untreated, these changes can be severe enough to result in death. *E. coli* causes a watery diarrhea and weakness in 1 to 4 days old newborn calves. Death usually occurred within 24 hours due to severe dehydration (Cho *et al*., 2010).

Based on the molecular, and pathological criteria, intestinal pathogenic *E. coli* or diarrheagenic *E. coli* (DEC) are classified into several pathotypes such as: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) enterinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Diffusely-adherent *E. coli* (DAEC) and vero- or Shiga-like toxin producing *E. coli* (VTEC or STEC) (Nagy and Fekete, 2005; O'Sulevan *et al.,* 2007).These Virulent Factors are detail as follow;

2.8.1. Enteropathogenic *E. coli*

Enteropathogenic *E. coli* 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). The gene *eae* encode a protein called intimin, a 94-kDa outer membrane protein involved in the intimate adherence between bacteria and enterocyte membranes (Croxen *et al*., 2013). 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). 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).

Intimin gene is principally amplified in various works and can be found alone or together with other virulent genes in isolates of *E. coli* giving it EPEC or EHEC. The presence of this gene is therefore informative to cause of calf diarrhea. Lots of works have been documented on this virulent gene from various parts of the world at various times. For instance, in the work of Umpierrez *et al*., (2016), PCR amplification of *eae* showed an important component in the prevalence of EHEC and EPEC.

In Iran *E. coli* were isolated from diarrheic calves to detect few virulent genes to which *eae* prevalence was reported to be 2.6% (Badouei *et al.,* 2014), 1.3% (Picco *et al*., 2015), 2.1% (Umpierrez *et al.,* 2016) and 2% (Rehman *et al.,* 2014) from Brazil, Argentina, Uruguay and Kashmir respectively.

2.8.2. Shiga Like Toxin producing *E. coli*

Both human and bovine derived STEC produces two phage encoded potent cytotoxins (Pizarro *et al.,* 2013). These toxic proteins produced can be classified into Shiga toxin one (*Stx1*) and two (*Stx2*) encoded by *stx1* and *stx2* genes respectively on prophages integrated into the chromosome. *stx1* and *stx2* variants on STEC can be found either alone or in combination and the toxin is released from lysed bacterial cells during the lytic cycle of the phage while bacteria gets stressed. Loss of the stx containing phage has been reported several days after isolation (Croxen *et al.,* 2013).

It is hypothesized that STEC-induced inflammation may provide the toxin an opportunity to breach the epithelial barrier and other study proposed that STEC is able to cross the intestinal epithelium through microfold cells (Mcells) and survive in macrophages, and this may be a way for the Shiga toxin to be released into the bloodstream, where it can target other organs (Etienne-Mesmin *et al.,* 2011). *Stx* binds to Gb3 on the surface of endothelial cells and is internalized and trafficked through the retrograde pathway from the Golgi apparatus and endoplasmic reticulum (ER) and eventually to the host cell cytoplasm (Tesh, 2012).

Toxicity with STEC is asymptomatic in adult ruminants (Sperandio and Nguyen, 2012) and diarheagenic in calves, if the dose of toxin is higher (Kuyucuoglu *et al*., 2011). Recent outputs indicated for binding difference between *Stx1* and *Stx2* that might involve more than one glycan (Kolenda *et al.,* 2015).

Genes for Shiga toxin were the most frequently detected among virulent genes in *E. coli* isolates from diarrheic calves. Likewise mentioned above for *eae, stx* genes are also found either solely or in combination with other virulent genes rendering the isolate EHEC or STEC. In most of the works *stx1* identified more than its counterpart *stx2. stx1* and *stx2* genes were identified at 12.2% and 7.8% respectively (Herrera-Luna *et al.,* 2009). Hashish *et al.,* (2016) found *stx1* and *stx2* at 86.67% and 26.67%, respectively. *stx1* was also amplified at 41.3% in the work of De Moura *et al*., (2012) in Brazil and 4.8% were found from Uruguay (Umpierrez *et al.,* 2017). In contrast, a finding found in Iran showed higher *stx2* (30%) than *stx1* gene (10%) (Dastmalchi and Ayremlou, 2012). Likewise, STEC harboring *stx2* isolate was significantly more (53.42%) than *stx1* carried STEC (10.27%) (Tahamtan *et al*., 2010).

2.8.3. Enteroaggregative *E. coli* (EAEC**)**

Gene *aatA* is one of the virulent genes of enteroaggregative *E. coli* (EAEC), a pathogenic strain in the digestive tract that causes a severe and stable diarrhea. Like wise to other virulence genes of this strain, *aatA* gene also mostly located on the 55,989 bp plasmid. This gene produces a membrane protein necessary for translocation of pathogenic proteins (Nazemi *et al.,* 2011). However, never found *aatA* gene from various works of *E. coli* isolated from samples of diarrhea calves, For instance 35.4% and 12.5% from UPEC in human(Nazemi *et al*., 2011) and malnourished stool sample of children (Havt *et al*., 2017).

2.8.4. Entero-Haemolysin

Hemolysin is a spore-forming toxin encoded by plasmid bearing *hlyA* gene of *E. coli* that has a potential to lyse erythrocytes (Croxen *et al.,* 2013). Although the role of entero-haemolysin in an intestinal disease is unclear, it has been suggested that entero-haemolysins may enhance the effects of Shiga toxins (Herrera-Luna *et al.,* 2009). The toxin is produced by many strains of *E. coli* (Lorenz *et al.,* 2013) that can be isolated from both healthy/diseased animals and humans and their specific physiological or pathophysiological role remains unclear (Kolenda *et al.,* 2015). These toxins have been shown to be involved in endothelial cytotoxicity (Aldick *et al.,* 2007), cargo with outer membrane vesicles (Aldick *et al.,* 2009) and was shown to be inactivated by another STEC virulence factor, EspP (Brockmeyer *et al.,* 2011).

This virulent gene is found with STEC/EHEC in most of the assessed documents. On the other hand 7% of the isolates carried *hlyA* gene of which 3.5% were from STEC strain and the remaining 3.5% from EHEC. This gene was also found in 2.5% (Taghadosi *et al.,* 2018) and 60% in Iran (Dastmalchi and Ayremlou, 2012).

2.8.5. Bundle Forming Pilli

Bundle forming pilli gene is the one among the cluster of genes encompassed on, a large ~80 kb, EAF plasmid that is responsible to encode a protein called bundle forming pili (*BFP*)(Cleary *et al.,* 2004; Trabulsi *et al.,* 2002). *BFP* are involved in bacteria–bacteria interaction and micro colony formation. Thus, it promotes their stabilization and produces a localized adherence (LA) pattern in the form of compact three-dimensional micro colonies that can be seen within 3 hours of infection(Cleary *et al.,* 2004). This plasmid encoded protein is the predominant factor that mediates initial attachment of tEPEC to the surface of the host intestinal epithelium (Croxen *et al.,* 2013).

Published documents and relevant alternatives were assessed on status of *bfpA* for typical EPEC and from diarrheic calves. However, little was found about *bfpA* to which no isolates found carried it from the works of Sweden (De Verdier *et al.,* 2012). Therefore, use of plasmid genes as indicative for pathogenic *E. coli* prevalence have its own limitation such as, its variable gene content, it may lost on sub-culture and the plasmid may transfer and be

detected in entirely unrelated bacteria which are not actually able to cause diarrhea (Chattaway *et al.,* 2011).

2.8.6. Enterotoxin (ETEC)

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).

Colonization factors (CF) and one or more enterotoxins that induce a secretory diarrhea are the major determinants of ETEC virulence. CFs is proteinaceous fimbrial and afimbrial structures that enable bacteria to attach to intestinal mucosa. More than 20 CFs have been identified and characterized in ETEC (Torres *et al.,* 2005). 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 lead 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).

Epidemiologic studies found that contaminated food and water serves as the most common vehicles for ETEC infections, and fecal contamination of water and food is the primary reason for high incidence of ETEC infection in the developing countries (King *et al.,* 2003).

2.8.7. Diffuse Adherent (DAEC)

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). 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).

2.9. Isolation and Detection of pathogenic *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; Feng *et al.,* 2011).

2.9.1. 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).

2.9.2. 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). The following section briefly discussed about genotypic assay (molecular method of detection) which is the most popular and most reliable technique.

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). 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).

2.10. Antimicrobial Resistant Patterns of *Escherichia coli*

Extensive and uncontrolled treatment of farm animals plays a major role in an emergence of antimicrobial resistant strains and has become a serious issue this day. Resistance develops due to mechanisms incorporating acquisition of gene-encoding enzymes (e.g. β-lactamases), increased activity of efflux pumps, acquisition of several genes encoding bacterial cell walls lacking binding sites for antimicrobials, and mutations leading to decreased permeability. If those resistance genes are on plasmids, it can be transferred rapidly among a number of bacterial species (Chirila *et al.,* 2017).

Because resistance genes can be found in clusters, the recipient can obtain it together, and dissemination of multi-drug resistance will develop through the horizontal genes transfer. In addition, these resistance genes may be transferred by conjugation, transformation, or transduction (Chirila *et al.,* 2017). As a consequence presence of resistance traits and horizontal gene transfer due to indiscriminate use of antibiotics may favoring inter-species resistance transmission and hindering methods to treat bacterial infections and generates important public health issue (Umpierrez *et al.,* 2017).

Antimicrobial resistance of *E. coli* in developing countries including Ethiopia is reported to be one major reason for failure of treatment of infectious diseases (Erb *et al.,* 2007). A number of studies conducted in Ethiopia from various clinical settings show increments in the prevalence of antimicrobial resistance patterns of *E. coli* (Endalafer *et al.,* 2011and Yismaw *et al.,* 2010). Factors responsible for an increase in rates of antimicrobial resistance include misuse/overuse of antibiotics by healthcare professionals and general public and inadequate surveillance systems due to lack of reliable microbiological techniques leading to the inappropriate prescription of antibiotics (Eshetie,*et al*., 2016).

The lowest and highest proportions of *E. coli* resistance were reported, respectively, from Bahir Dar (55.20%) and Mekelle (27.50%) cities. The average prevalence of *E. coli* resistance was also noted in different regions of Ethiopia; Addis Ababa region was ranked first (62.55%, 95% CI: 38.28–6.83%), followed by Southern Nations, Nationalities, and Peoples of Ethiopia (58.14%, 95% CI: 48.69–67.58%), Amhara (47.83%, 95% CI: 39.77–55.89%), and Oromia

(42.86%, 95% CI: 32.77–52.95%), whereas relatively low magnitude of *E. coli* resistance was reported from Tigray region (27.51%, 95% CI: 16.14–38.88%) (Tuem *et al*., 2018).

2.11. Status of Calf Diarrhea in Ethiopia

Diarrhea is a complex multifactorial disease in which numerous infectious and noninfectious factors are involved. Diarrhea or scouring is the commonest disease and the greatest single cause of neonatal mortality during the first week of life and this risk decreases with age (Wudu *et al*., 2008). As documented by Cho *et al.*, (2013), calf diarrhea has been commonly attributed to bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), Salmonella spp. *Escherichia coli* (*E. coli*) K99, *Clostridium perfringens* type C and *Cryptosporidium parvum* (*C. parvum*). However, various authors indicated that bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), Salmonella spp. *Escherichia coli* (*E. coli*) K99 and *Cryptosporidium parvum* were the most commonly reported causes of neonatal calf diarrhea (Hussain, 2011).

Diarrhea (21.4%), were reported in pre-weaned crossbred calves in Bahir Dar Zuria and Gozamen Districts (Ferede, *et al.,* 2014). Calf scour (19.0%) was the main causes of calf mortality at Andassa ranch (Amuamuta, 2006). overall 76.9% diarrheic calves were identified by Asmare and Kiros, *et al*., 2016) in Sodo Town and Its Suburbs, Wolaita Zone, Ethiopia. Survey study by Tsegaw, (2016) young stock mortality in major livestock production systems of Ethiopia also reported calf diarrhea (48.7%) was the most causes of calf mortality in Oromia region. According to Gebremedhin, (2014) among the causes of death recorded, calf diarrhea was the leading cause of calf mortality with case specific mortality rate of 5.8% in intensive dairy farms of Bishoftu town. Minda *et al*., (2016) in Holleta Agricultural Research Center Dairy Farm, Holleta, Ethiopia, reported the predominant calf health problem, with incidence rate of 12.5% was calf diarrhea.

Calf diarrhea was the most frequently reported causes of dairy calf morbidity and mortality in Ethiopia (Hussain, 2011 and Gitau *et al.,* 2010). However this did tell little about the significance of the presumptive isolated agents alone to be the cause of the disease for proper control and preventive measures to be implemented in the country.

2.12. Economic Importance of the Disease

Neonatal calf diarrhea is one of the most common diseases in young animals, causing huge economic and productivity losses to bovine industry worldwide (Cho and Yoon, 2014). (Gunn, 2003) reported that the losses due to an occurrence of the disease include calf death. which is effectively the loss of income from a cow for the year, cost of treatment of the calf, including the time taken, which can be significant in a paddock situation, impact on growth rate and possible lower weaning weight, culling cost of the dam, loss of genetic potential from the calf and the dam and decreases capacity to improve and maintain the herd. In general, the published data shown that, diseases of the new born calf mortality are the major causes of economic losses in livestock production (Singh *et al*., 2009).

2.13. Treatment

Treatment requires aggressive antimicrobial, fluid and anti-inflammatory therapy. Although blood cultures are recommended to retrospectively confirm the diagnosis, antimicrobial therapy must be initiated immediately in any animal suspected of being septic. Because there is no time for sensitivity testing, the initial choice should be a bactericidal drug that has a high probability of efficacy against gram-negative organisms. Administration IV of large volumes of balanced electrolyte solutions over several hours is essential to correct hypovolemia and assure adequate peripheral tissue perfusion; fluids should include glucose to correct hypoglycemia. The beneficial effect of NSAIDs has been attributed to their anti-inflammatory, antipyretic, and analgesic properties. Glucocorticoids have also been proposed to treat septicemia, although their benefits for treatment of sepsis are less well established (Gruenberg, 2014).

2.14. Control and Prevention

Calves that acquire adequate concentrations of immunoglobulin from colostrum are resistant to colisepticemia. Therefore, prevention depends primarily on management practices that ensure an adequate and early intake of colostrum. The adequacy of the farm's practice of feeding colostrum should be monitored, and corrective strategies applied as required. In North American Holstein dairy herds, natural sucking does not guarantee adequate concentrations of

circulating immunoglobulins, and calves should be fed 2 to 4 L of first-milking colostrum containing a minimal total mass of 100 g of IgG, using a nipple bottle or an esophageal feeder, within 2 hrs of birth; this is followed by a second feeding at 12 hrs. A cow-side immunoassay test can assist in selection of colostrum with adequate immunoglobulin concentration. Although the circulating concentration of immunoglobulin required to protect against coli septicemia is low, high concentrations are desirable to decrease susceptibility to other neonatal infectious diseases (Gruenberg, 2014).

When natural colostrum is not available for a newborn calf, commercial colostrum substitutes containing 25g of IgG will provide sufficient immunoglobulin for protection against coli septicemia if fed early in the absorptive period. Plasma containing at least 4 g and preferably 8 g of IgG, administered parenterally, will provide some protection for older calves that have not been fed colostrum and are unable to absorb immunoglobulin from the intestine. Small-volume hyper immune serum is of benefit only when it contains antibody specific to the particular serotype associated with an outbreak. The risk of early infection should be minimized by hygiene in the calving area and disinfection of the navel at birth. To minimize transmission, calves reared indoors should be in separate pens (without contact) or reared in calf hutches (Gruenberg, 2014).

Ensuring a clean environment for calving minimizes exposure to potential pathogens such as *E. coli*. The passive immunity acquired from the colostrum and absorbed into the circulation from the gut is the calf's main defense mechanism against *E. coli* diarrhea. Inadequate amounts of antibodies in the colostrum, inadequate intake of the colostrum and inadequate absorption of antibodies from the gut render very young calves susceptible to infection (Groutides and Michell, 1990).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted from January 2019 to end of Jun 2019 for a period of six months in dairy farms at Bahir Dar city which is the capital city of the Amhara National Regional State (ANRS) in northern part of Ethiopia 565 km away from Addis Ababa. It is located near Lake Tana, the headwaters of the Blue Nile, and is a major tourist destination. Bahir Dar city has a flat plateau earth structure which is located at 11°36" North latitudes and 37°23" East longitudes. Bahir Dar city has 5144 bovines, 390 ovine, 440 caprine, 1058 equines and 36870 avian species (Wudneh, 2018).

The altitude of this city rages from 1810 to 1850 meters above sea level and temperature ranges from 10 to 38°c. The area receives mean annual rainfall of 750mm. The naming of the city as Bahir Dar is connection with its proximity to the two water bodies of Lake Tana and River Abay (Nile). Hence, literally Bahir Dar means a city situated on or very close to the shore of Lake Tana and Blue Nile. Today, it is one of the fast growing and largest cities in the country. In line with its growth, different service sectors such as education, health, transport and communication have grown. According to 2007 population census, 220,344 inhabitants are living in Bahir Dar city administration (CSA, 2009) (Figure 2).

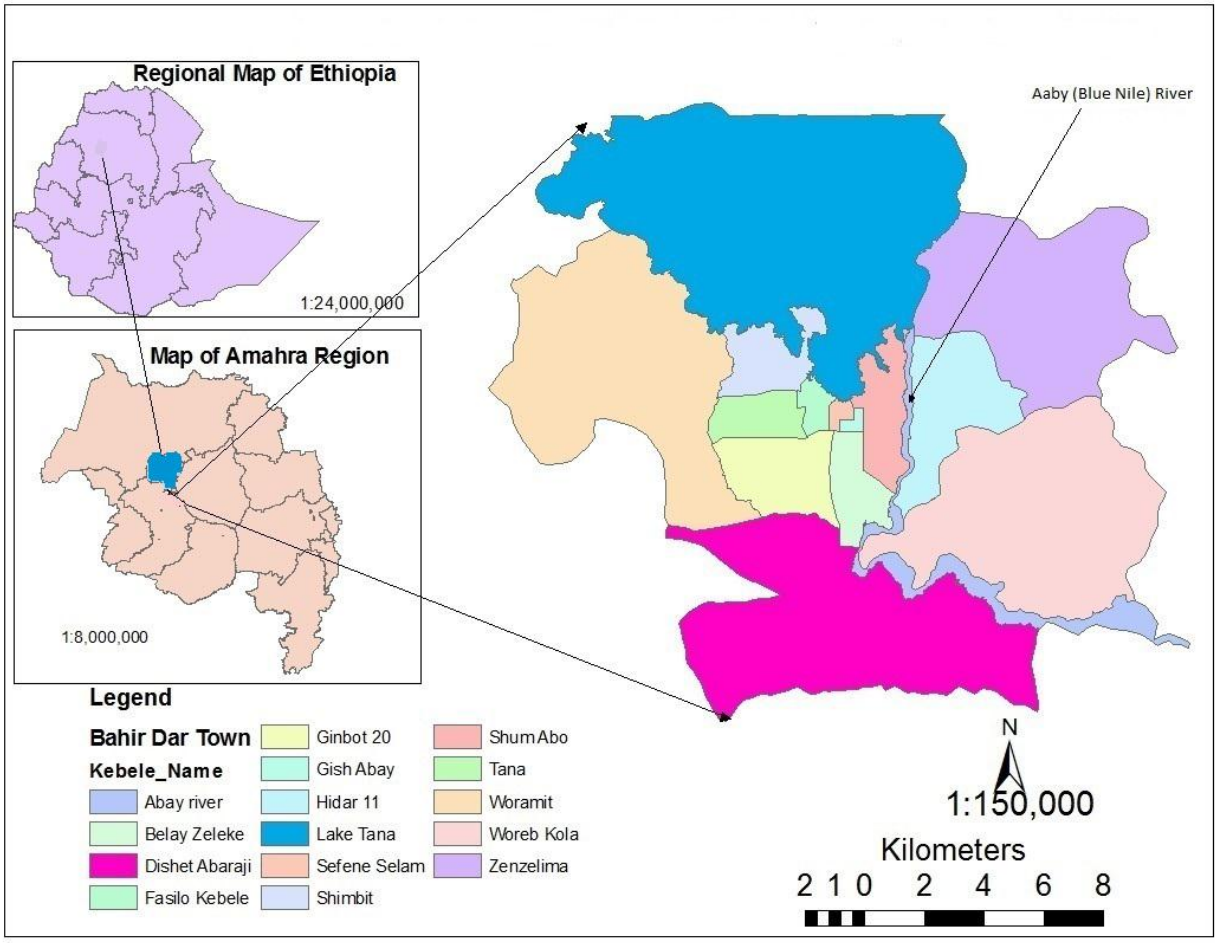


Fig 2: Map of the study area

Source: Fikirte (2015).

3.2. Study Farms

For this study, Dairy farms located in Bahir Dar city, both peri urban and urban, which encompass from smallholder to highly specialized dairy farms, and engaged in market oriented dairy production were used. This classification was made based on location, spatial land use and integration with crop production (Yitaye, 2008). Farms were classified as small (<5 heads of dairy cow), medium sized (6-50 heads of dairy cow) and large (>50 heads of dairy cow) based on previous works in the urban and peri-urban production system (Lemma *et al.*, 2001).

3.3. Study Population

The study animals were both local and crossbreed dairy calves of both sexes reared under small-holder and commercial dairy farms aged between births to 4 months that are clinically affected with diarrhea exhibiting signs of systemic diseases (Nolan, 2013). Age of diarrheic calves were categorized in to three groups: 0-2 weeks, 3-8 weeks and 9-16 weeks of based on post natal silent stress response coupled with lack of immune competence, pre- weaning and post weaning strategies in which calves are often susceptible to enteric disease (Lindsey and Sonia, 2016), age at first colostrum feeding (less than 6hrs and above 6hrs) (Aklilu *et al.*, 2013).

3.4. Inclusion and Exclusion Criteria

3.4.1. Inclusion Criteria

Diarrheic calves aged between birth to 4 months at dairy farms Bahir Dar city that are clinically affected with diarrhea and exhibiting signs of systemic disease and willingness of the farm owners were include as the population of this study.

3.4.2. Exclusion Criteria

Calves aged above 4 months, that were on antibiotic therapy for two weeks and those whose owners not voluntary to allow samples taken were excluded from the study farm.

3.5. Ethical Consideration

When collecting the sample in each selected dairy farm the purpose of the study were explained to the farm owner. In addition to that, there is a letter from Jimma University College of Agriculture and Veterinary Medicine from School of Veterinary medicine for the approval and credibility of the work. Verbal consent was also obtained from the owners or attendants of the farms to take fecal samples from diarrheic calves.

3.6. Study Design and Sampling Methodology

A cross sectional study was conducted in 43 dairy farms (small, medium and large sized) in Bahir Dar city from January 2019 to end of June 2019 and the study sites were selected by convenient sampling method. A purposive sampling method used to collect samples from calves that showed major clinical sign of diarrhea (elevated temperature, depression, dehydration, reduced suckling reflex, rough hair coat, loss of weight, weakness and soiling of hind quarter and tail with diarrheic feces (Lemma *et. al.*, 2001). Fecal samples were obtained after digital stimulation of the rectal mucosa using disposable latex glove. Structured questionnaire was used to collect risk factors associated with the occurrence of *E. coli* in diarrheic calves.

3.6.1. Sample Size determination

Sample size was determined based on the availability of the clinical cases and willingness of the farm owners. Based on those 112 diarrheic calves were included in this study.

3.6.2. Samples Collection Procedures.

Approximately 30 grams of fecal materials were collected aseptically directly from the rectum of diarrheic feces using disposable latex glove. A total of 112 fecal samples were collected aseptically for isolation of *E. coli* pathogens from calf diarrhea. Specimens were placed in dry, leak proof, sterile plastic bag and placed into ice box containing ice packs and transported to Amhara Public Health institute of microbiology laboratory, for processing. Feces were stored at 4 °C until the time of processing. At the time of sampling the name of the farm, date of sampling, consistency of feces, age, breed, sample ID, feeding management practices as well as past history of diarrhea were recorded for each calf on a recording format (Annex 2).

3.7. Questionnaire Survey

Structured questionnaire was administered to dairy farm owners or farm managers to assess the relevant information on calf husbandry practice and the general farm management systems. The questionnaires were developed in accordance with the objectives of the study and designed in a simple manner to get accurate information from the dairy farm owners. Generally the questionnaire includes all practices in the farm that performed about calf health care, colostrum feeding time and method, duration as well as types of diarrhea that affect the growth of calves, preventive and control measures practiced in the farms (Annex 1). All 43 farm owners were interviewed through face to face conversation by local language in Amharic.

3.8. Laboratory works

3.8.1. Isolation and Identification of *E. coli*

Stage 1: Liquid enrichment media

The bacteriological media was prepared according to manufacturer recommendations (Annex 4). Twenty five (25) gram of each fecal sample were enriched with 225ml BPW and placed in separate plastic bags in a laminar flow hood, and then the bags were vigorously shaken. The inoculated BPW with fecal material Incubated at 37^0c for 24 hours to increase recovery of the organisms followed by selective plating (OIE, 2016).

Stage 2: Culturing of extracted enrichment

A loopful of the incubated sample was cultured on MaCconkey agar for 24-48hrs at 37 °C. MaCconkey (MC) agar medium was used for the selective and differentiation of the bacteria. The presence of lactose fermenter colonies on MaCconkey agar was used as primary criteria to proceed for isolation and identification of *E. coli*.

Lactose fermenting character (pink colonies) colonies were transferred to Eosin methyl blue (EMB) agar medium to identify selectively. The characteristic colonies on EMB were identified based on green metallic sheen or blue-black to brown color. The purified cultures of *E. coli* were stored temporarily as nutrient broth cultures for further identification by biochemical tests.

Stage 3: Biochemical tests

The purified colonies were tested by using Triple Sugar Iron agar (TSI) slant (Oxoid) and IMViC tests. The isolates showed a result of, yellow slant and butt with gas but no hydrogen sulfide (Y/Y/ H2S -) production on TSI slant agar after incubation of the media at 37°C for 24 hours as stated at (annex 5). Finally IMViC tests, viz., indole, methyl red, Voges- Proskauer, and citrate utilization exhibited the IMViC pattern of +, +, −, and −, respectively, were presumed as *E. coli* isolates (Quinn *et al*., 2002) (Annex 9).

3.9. Virulent Gene Detection of *Escherichia coli* Isolates

All diarrheagenic *E. coli* isolates identified in the study were transported to Institute of Biotechnology, Addis Ababa University, for further molecular based characterization of pathogenic strains and antimicrobial resistance patterns of the *E. coli* isolates.

3.9.1. DNA Extraction

A single bacterial colony grown on EMB agar was inoculated into nutrient broth and incubated at 37ºC overnight. DNA of each isolate was extracted using boiling method. Exactly 1.5 ml of the culture was taken in eppendorf tube and spun by centrifugation at 13000 rpm for 10 minutes. The supernatant was discarded. The bacterial pellet was lysed by boiling in 50 µl of nuclease free water in a water bath at 95°C for 10 minutes. Then, the lysate was centrifuged again as before and an aliquot (50µl) of supernatant to be used as template for PCR amplification (He *et al*., 2011) was stored in -20^{0}c until use for PCR.

3.9.2. PCR Based Detection of Virulent Genes

DNA extract of each *E. coli* isolate was subjected to PCR for the presence of target genes. According to the annealing temperatures of the different primers used, nine PCR assays were performed. The PCR experiments were carried out using the following protocols; details of primer gene sequences and the different reaction temperatures that were carried out in the PCR assays are indicated (Table1).

Table 1: Primer, Gene sequence and PCR conditions

Primer	Nucleotide sequence	Target gene	Pathogenic *E. coli* strain	Denaturing	Annealing	Extension	Bp	Cycles	Reference
EAE1 EAE2	F:5'-AAACAGGTGAAACTGTTGCC3' R:5'-CTCTGCAGATTAACCTCTGC-3'	*eaeA*	EPEC/ EHEC	94^0c,2 min.	55^0c,60s	72^0c,60s	490	35	Khan *et al.* (2002)
EVT1 EVT2	5'-CAACACTGGATGATCTCAG-3' 5'-CCCCCTCAACTGCTAATA-3'	*Stx2*	STEC/EHEC	95°C,3min.	57^0c,40s	72^0c,30s	350	30	Pal *et al*., (1999)
EVS1 EVC2	F:5'-ATCAGTCGTCACTCACTGGT-3' R:5'-CTGCTGTCACAGTGACAAA-3'	*Stx1*	STEC/EHEC	95^0c,3min.	57^0c,40s	72^0c,30s	110	30	Pal *et al*., (1999)
EHEC	F: 5'-ACGATGTGGTTTATTCTGGA-3' R:5'-CTTCACGTCACCATACATAT-3'	*hlyA*	EHEC	95^0c,3 min.	45^0c,40s	72^0c,30s	165	30	Sheng *et al.* (2005)
EAEC	F:5'CTGGCGAAAGACTGTATCTAT-3' R:5'CAATGTATAGAAATCCGCTGTT-3'	*aatA*	EAEC	95^0c,3 min.	45^0c,40s	72^0c,30s	630	30	Schmidt *et al.* ,(1995)
BFP	F:5'AATGGTGCTTGCGCTTGCTGC-3 R:5'GCCGCTTTATCCAACCTGGTA-3'	*bfpA*	EPEC	95^0c,3 min.	57^0c,40s	72^0c,30s	324	30	Hinenoya *et al.*, (2009)
St	F:TTTATTTCTGTATTGTCTT R:GCAGGATTACAACACAATTCA	*St*	ETEC	950c,3 min.	520c,1 min	720c,10 min	294	35	Arif and Salih,(2010)
Lt	F:TCTCTATGTGCATACCGAGC R;CCATACTTGATTGCCGCAAT	*Lt*	ETEC	950c,3 min.	530c,30 s	720c,8 min	696	30	Vidal *et al.,* (2005)
daaE	F:GAACGTTGGTTAATGTGGGGT R:TATTCACCGGTCGGTTATCAG	*daaE*	DAEC	950c,3 min.	470c,1 min	720c,10 min	542	38	Kruger *et al.*, (2005)

Keys: EPEC = Enteropathogenic *E. coli*; EHEC= Enterohemorrhagic *E. coli*; STEC= Shiga-like toxin producing *E. coli*; EAEC= Enteroaggregative *E. coli.*

3.10. Agarose Gel Electrophoresis

Amplified PCR products were analyzed by gel electrophoresis at 120 voltage for 30 minutes in 1.5% agarose made in 1x TAE buffer containing ethidium bromide (0.5 µg ml-1) using a marker DNA ladder of 100 base pairs (bp) (Himedia MBT049). Known positive control and negative controls of the strains STEC, EPEC, EHEC, EAEC. ETEC, DAEC and tEPEC were also placed along with the samples. The gel was visualized through ultraviolet transilluminator (Bio-Rad) and imaged with gel documentation system.

3.11. Antimicrobial Susceptibility Testing of *E. coli* Isolates.

The antimicrobial susceptibility testing of the *E. coli* isolates was performed using a panel of eight antimicrobial discs using the Kirby-Bauer disk diffusion test according to the Clinical and Laboratory Standard Institute guideline (CLSI, 2017). The selection criteria of antibiotics testing discs depended on regularly use of antimicrobials in the ruminants and potential public and animal health importance. Antimicrobial disks used in the study were oxy tetracycline (30µg), chloramphenicol (30µg), sulphonamide (300µg), streptomycin (25µg), ampicillin (10µg), neomycin (10µg), ceftriaxone (30µg) and trimethoprim (5µg).

Isolates were revived on EMB agar and colonies were transferred to a test tube of 5 ml tryptone soya broth (TSB) (Oxid, England) and incubated at 37^0C for 6 hours. The turbidity of the culture broth was adjusted using sterile saline solution usually comparable with that of 0.5 McFarland standards (approximately 3x108 CFU per ml). Mueller-Hinton agar (M 173 Hi Media) plates were prepared according the manufacturer. A sterile cotton swab was immersed into the suspension and rotated against the side of the tube to remove the excess fluid and then swabbed in three directions uniformly on the surface of Mueller-Hinton agar plates. After the plates dried, antibiotic disks were placed on the inoculated plates using sterile forceps. The antibiotic disks were gently pressed onto the agar to ensure firm contact with the agar surface, and incubated at 37^0C for 24 hours. Following this the diameter of inhibition zone formed around each disk was measured using a black surface, reflected light and transparent ruler by lying it over the plates as described on (Annex 6).

For the susceptibility testing, interpretations of the results were classified as sensitive, intermediate and resistant according to the standardized table supplied by the manufacturer CLSI (2017) as shown on (Annex 7).

3.12. Data Analysis

Data entry and validation were done using Microsoft Excel (2007), and were analyzed using SPSS software version 20.0 for appropriate statistical analysis. Descriptive statistics (determination of proportions) were used to describe the study population in relation to risk factors. The number of positive samples were divided by the total number of samples examined multiplied by 100. The association between occurrence of *E. coli* isolates and the risk factors analyzed using person's x^2 test. Univariable and multivariable logistic regression analysis were performed to quantify crude and adjusted effect of the risk factors on the occurrence of *E. coli.* variables with p- value <0.25 for controlling the possible effect of confounder, in univariable logistic regression were fitted in to multivariable Logistic regression model to observe the strength of the association between risk factors and the outcome. The goodness of fit of the model with the data was assessed by Hosmer and Lemeshow test. After selecting the final model of multivariable logistics regression, the beta (β) coefficients of each independent variable were observed to estimate odds ratio (OR) which is used for assessing the strength of association. Effects were reported as statistically significant as p-value was less than 0.05.

4. RESULTS

4.1. Overall Isolation of *Escherichia coli* in Diarrheic Calves

A total of 57 from 112 (50.89%) diarrheic samples studied showed metallic sheen on EMB agar medium from which forty (40) were non lactose fermenters (pale colony on macConkey agar). On a sequential biochemical assay fifteen (15) colonies that exhibited metallic sheen on EMB agar were found to be non-*E. coli* based on IMViC test.

Thus, Out of 112 samples collected and processed, 57 were positive for *E. coli*. The overall isolation rate of *E. coli* was 50.89%. Once the Occurrence of *E. coli* was determined and the risk factors were considered; Age, Breed, colostrum feeding time and herd size had a statistically significant impact on the occurrence of *E. coli* ($P< 0.05$). On the contrary factors such as, amount of colostrum feed/time, type of supplements and method of feeding have impact but did not show significant difference ($p > 0.05$) (table 2).

Table 2: Overall *E. coli* isolates obtained from diarrheic calves with different factors

Risk factors	category	No. examined	No. of positive (%)	X^2	P-value
Sex	Male	59	29 (50.89)	0.15	0.901
	Female	53	28 (49.12)		
Age	0-2 weeks	37	25(67.57)	9.357	0.001
	3-8 weeks	45	23 (51.11)		
	9-16 weeks	30	9 (30)		
Breed	Local	38	12(10.75%)	8.584	0.004
	Cross	74	45(40.18%)		
Herd size	Small	20	3 (15)	13.494	0.001
	Medium	60	33 (55)		
	Large	32	21 (65.63)		
ACF/T	1-2Litter	95	49(51.58)	0.118	0.674
	>2Litter	17	8(47.06)		
TFCF	≤ 6 hour	52	19 (36.54)	8.003	0.000
	>6 hour	60	38 (63.33)		
SFT	Grazing	19	11 (57.89)	0.449	0.421
	Concentration	93	46(49.46)		
MCF	Suckling	53	26 (49.06)	0.136	0.687
	Hand Feeding	59	31 (52.54)		
Overall		112	57(50.89)		

Keys: ACF/T= Amount of colostrum feeding per time, MCF= Method of colostrum feeding, P= Probability, SFT= Supplementary feed type, TFCF=Time of first colostrum feeding and (X2) = Chi- square

4.2. Univariable Logistic Regression Analysis of Risk Factors Associated with Diarrheic Calves

In this study the occurrence of diarrhea due to *E. coli* differed significantly by breed (*P*=0.004), age (*P*=0.001) herd size (*P*=0.001) and time of first colostrum feeding (*P*=0.000). Its occurrence didn't show significant difference by sex, method of feeding, Amount of colostrum/time and Type of supplements as presented table (table 3). Variables with p-value <0.25 in univariable logistic regression were taken to multivariable logistic regression analysis to control confounders.

Table 3: Univariable logistic regression analysis of *E. coli* occurrence with risk factor

Risk factors	category	No. examined	No. of positive (%)	P-value	COR	95% CI
Sex	Male	59	29 (50.89)	0.901	1.063	.403-2.806
	Female	53	28 (49.12)		*	
Age	0-2week	37	25(67.57)	0.001	10.954	2.642-45.419
	3-8week	45	23 (51.11)	0.090	2.892	0.849-9.855
	9-16week	30	9 (30)	0.004	*	
Breed	Local	38	12(10.75)	0.004	.179	0.055-0.581
	Cross	74	45(40.18)		*	
Herd size	Small	20	3 (15)	0.001	0.040	0.006-0.267
	Medium	60	33 (55)	0.552	0.660	0.168-2.593
	Large	32	21 (65.63)	0.002	*	
ACF/T	1-2Litter	95	49(51.58)	0.674	1.414	0.282-7.086
	>2Litter	17	8(47.06)		*	
TFCF	≤ 6 hour	52	19 (36.54)	0.000	0.107	0.033-0.0351
	>6hour	60	38 (63.33)		*	
SFT	Grazing	19	11 (57.89)	0.421	1.754	0.446-6.901
	Concentration	93	46(49.46)		*	
MCF	Suckling	53	26 (49.06)	0.687	0.799	0.269-2.375
	Hand feeding	59	31 (52.54)		*	

Keys: ACF/T= Amount of colostrum feeding per time, CI= Confidence Interval, COR= Crud odd ratio, MCF= Method of colostrum feeding, P= Probability, SFT= Supplementary feed type, TFCF=Time of first colostrum feeding and *= Reference,

4.3. Multivariable Logistic Regression Analysis of Risk Factors Associated with Diarrheic Calves

Multivariable logistic regression analysis was carried out to observe the independence of each risk factor in relation to the occurrence of *E. coli* isolates in diarrheic calves. Variables such as breed (P=0.002), first colostrum feeding time (P= 0.000), age (P= 0.001) and herd size (P= 0.000) were identified as significant independent predictors for occurrence of *E. coli* isolates in diarrheic calves. Calves of 0-2 weeks of age had a 9.950 times more likely probability of being infected by *E .coli* as compared with other age categories of diarrheic calves (AOR=9.950, 95% CI: 2.483-39.870, P: =0.001). Calves that were fed first colostrum, in less than six hours had a 0.121 times less likely probability of being infected by *E. coli* as compared with those fed after six hours (AOR=0.121, 95%CI: 0.040-0.361, P= 0.000). Calves that found in small herd size had a 0.041 times less likely probability of being infected by *E. coli* as compared with those found in large herd size (AOR=0.041, 95%C.I: 0.007-0.240, P=0.000). Calves of local breed had a 0.173 times less likely probability of being infected by *E. coli* as compared with calves of cross breeds (AOR=0.173, 95%CI =0.056-0.537, P= 0.002) as summarized in table 4.

Table 4: Multivariable logistic regression analysis of *E. coli* occurrence with risk factors

Risk factors	Category	No. examined	Sample positive (%)	p-value	AOR	95% CI OR
Breed	Local	38	12(10.75)	0.002	0.173	0.056-0.537
	Cross	74	45(40.18)		*	
Age	0-2 weeks	37	25 (67.57)	0.001	9.950	2.483-39.870
	3-8 weeks	45	23(51.11)	0.103	2.739	0.817-9.187
	9-16 weeks	30	9(30)	0.005	*	
TFCF	≤6hrs	52	19 (36.54)	0.000	0.121	0.040-0.361
	>6hrs	60	38 (63.33)		*	
Herd size	Small	20	3 (15)	0.000	0.041	0.007-0.240
	Medium	60	55 (41.98)	0.739	0.827	0.271-2.527
	Large	32	21(65.63)	0.001	*	

Keys: AOR= adjusted odd ratio, CI= Confidence Interval, P= Probability, TFCF= Time of first colostrum feeding and *=reference

4.4. Description of Socio demographic situation pattern

A total of 36 questions comprised six area of interest; farm identification, farm description, farm management, feeding and knowledge of the owners for the antimicrobial and treatment practice. Totally 43 farm owners or attendants were interviewed during sample collection. Most of the farm that studied followed similar management practice. Due to this there are no statistical comparisons were done for most of the farm related factors and no relevant association were found to various variables. While, some of the potential risk factors mentioned are used in comparison for occurrence of *E. coli*.

However, all of the farms had knowledge for immunological importance of colostrum fed to their calves, Based on their feeding time, 46.43% neonates were to feed colostrum within 6 hours of calving and 53.57% were practiced it after 6 hours. Ninety five (84.82 %) out of 112 diarrheic calves fed 1-2 litters of colostrum per time and the remaining 17 (15.18 %) of Calves fed more than two litters. Among the respondents 47.32% allowed their calves freely to suckle their dam after birth and 52.68% were used hand (bottle) feeding of colostrum. Based on their feeding supplement 16.96% calves feed by Grazing and 83.03% calves feed concentration. Among the questioner survey, herd size, age, colostrum feeding time and animal breed were the most important risk factors for the occurrence of *E. coli* in calves as stated in (Table 2).

In most large dairy farms, there were veterinary personnel employed to deal with health aspects of the farms. Smallholder dairy farms call private veterinary professional whenever their animals were faced health problem and to treat their animal commonly used some drugs like, sulphonamides, oxy tetracycline and penstrip (compound of streptomycin and penicillin). As the attendant mentioned that, from majority of calves' health problems, diarrhea and lump skin disease were the most frequent disease encounter in calves.

4.5. PCR Based Detection of Virulent Gene from Pathogenic Strains

All (57) isolates were screened for 7 different virulence genes (*stx1, stx2, eae, lt, st, daaE* and *aatA*) by PCR. Positive isolates for *stx1, stx2* and *eae* were then further examined for hlyA gene and those only *eae* positive isolates were tested for *bfpA* gene. Out of 57 *E .coli* isolates 16 isolates (28.07%) revealed to carry at least one of the targeted virulence genes.

Among positive isolates, 12 (75%) harbored a single virulent gene from which 8 of them (66.67%) carried stx2, 3 (25%) carried *stx1* and 1(8.33%) carried *eaeA* gene.

On the other hand 4 isolates (25%) were confirmed to have two or more virulent genes on which one isolates carried both *stx1* and *eae* genes (25%). Likewise, two isolate carried both *stx2* and *hlyA* genes (50%), and only one isolates carried all *stx1, eae* and *hlyA* genes (25%) were detected. However none of the isolate found positive for all *aatA, bfpA, st, lt and daaE* genes in all processed sample.

Based on the different virulence genes detected, *E. coli* pathotypes/strains were identified as follows. Enteropathogenic *E. coli* (EPEC) strains were identified as those positive for eaeA (intimin) gene. Enteropathogenic *E. coli* are further classified into typical (positive for additional bundle forming pillus, gene with *eaeA*) and atypical (negative for *bfp*) strains, but in this study, the EPEC strains isolated were atypical in that they only contained the *eaeA* gene. Shigatoxin producing *E. coli* (STEC) strains were identified as those positive for *stx1* and *stx2* genes. Enterohemorrhagic *E. coli* strains were identified as those positive for *stx1+eaeA*, *stx2 + hlyA* and *stx1 + hlyA + eaeA* genes (EHEC strains may or may not be *hlyA* positive).

Overall Results revealed that, *stx2* primarily carried by STEC and also by EHEC strains were found the most frequent virulent gene, detected in 10 (17.5%) and the *stx1* primarily carried by STEC and also by EHEC strains were found the second most common VG, detected in 5 (8.7%) of the isolates. Whereas, the *hlyA* which codes for entero hemolysin gene entirely found in EHEC strain and intimin (*eaeA*) carried in both EHEC and aEPEC strains were detected in 3(5.3%) and 3(5.3%) of isolates, respectively. Summary of these findings is presented in table 5.

Table 5: Distribution of virulence genes in *E. coli* isolates and diarrheic calves

Virulent gene Detected	Isolates examined	Frequency among *E. coli* isolates (%)	Frequency among diarrheic calves (%)	Strains
Stx2	57	8(14.04)	8(7.14)	STEC
Stx1	57	3(5.26)	3(2.68)	STEC
eaeA	57	1(1.75)	1(0.89	aEPEC
Stx1+eaeA	57	1(1.75)	1(0.89)	EHEC
Stx2 +hlyA	57	2(3.51)	2(1.79)	EHEC
stx1+eaeA +hlyA	57	1(1.75)	1(0.89)	EHEC
bfpA	57	__	__	tEPEC –ve
aatA	57	__	__	EAEC –VE
st	57	__	__	ETEC –ve
lt	57	__	__	ETEC –ve
daaE	57	__	__	DAEC –ve
Total	57	16(28.07)	16(14.29)	

Keys: *eaeA*= intimin gene, *stx1* and *stx2*=shiga toxin producing gene 1 and 2, *hlyA*=hemolysin gene, *aatA*=enteroaggregative gene, *bfp*= bundle forming pillus gene, *st*= heat stable gene, *lt*= heat labile gene, aEPEC= atypical Enteropathogenic *E. coli*, STEC=shiga like toxin producing *E. coli*, EHEC= enterohemorrhagic *E. coli*, EAEC –ve = negative for Enteroaggregative *E. coli*, tEPEC –ve = negative for typical Enteropathogenic *E. coli*, ETEC –ve = negative for Enterotoxigenic *E. coli* gene and DAEC –ve= negative for Diffusely adherent *E. coli* strain.

Typical agarose gel images generated from the different PCR runs according to specific base pairs of the virulence genes were presented in figures 3-6.

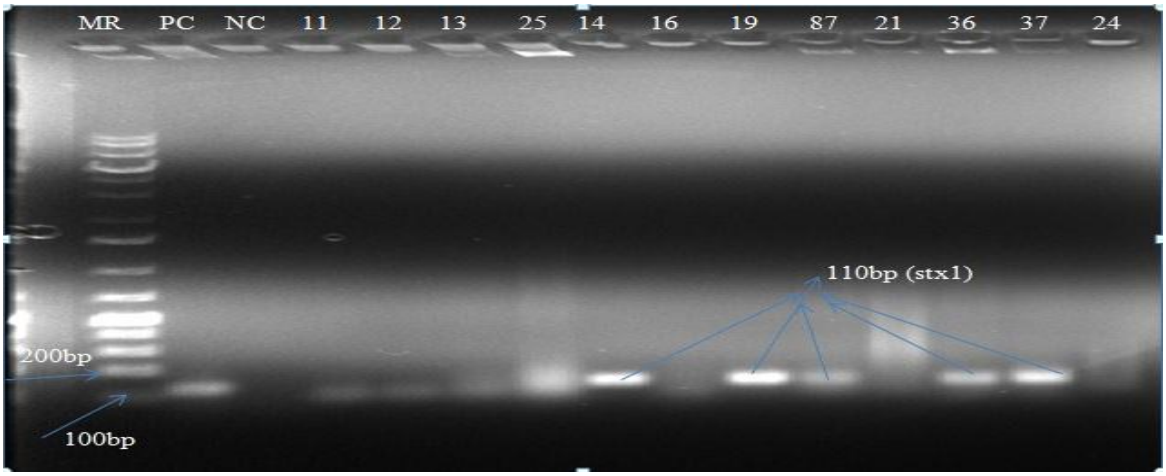


Fig 3: Agarose gel electrophoresis of amplified *stx1* gene generating 110 base pair amplicon

The PCR products were separated on 1% agarose gel electrophoresis: MR= Marker (100 bp DNA ladder), Lane 1 and 2 are PC= positive and NC=negative control respectively. Lane no 11 12 13 25 14 16 19 87 21 36 37 and 24 indicates sample number (PCR products prepared from *E. coli* isolates) and Lane 7 9 10 12 and 13 are positive results of sample codes 14 19 87 36 and 37 respectively.

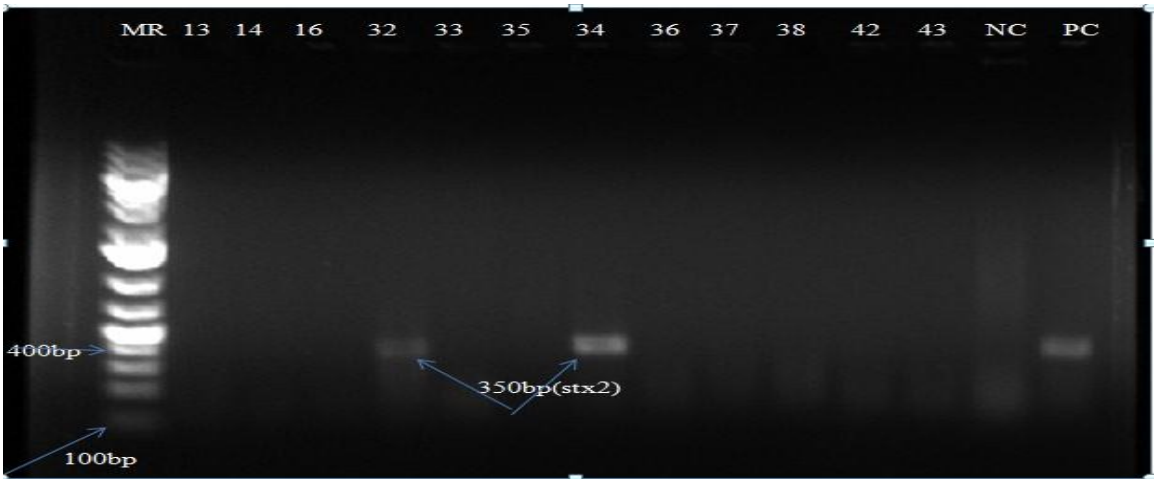


Fig 4: Agarose gel electrophoresis of amplified *stx2* gene generating 350 base pair amplicon

The PCR products were separated on 1% agarose gel electrophoresis: MR= Marker (100 bp DNA ladder), Lane 13 and 14 are NC=negative and PC= positive control respectively.

Lane No. 13 14 16 32 33 35 34 36 37 38 42 and 43 indicates sample number (PCR products prepared from *E. coli* isolates) and Lane 4 and 7 are positive results of sample codes 32And 34 respectively.

Fig 5: Agarose gel electrophoresis of amplified *eae* gene generating 490 base pair amplicon

The PCR products were separated on 1% agarose gel electrophoresis: M= Marker (100 bp DNA ladder), Lane 1 and 2 are PC= positive and NC=negative control respectively. Lane no 86 85 11 87 37 057 069 21 23 and24 indicates sample no. (PCR products prepared from *E. coli* isolates) and Lane 3, 6 and 7 are positive results of sample codes 86, 87 and 37 respectively.

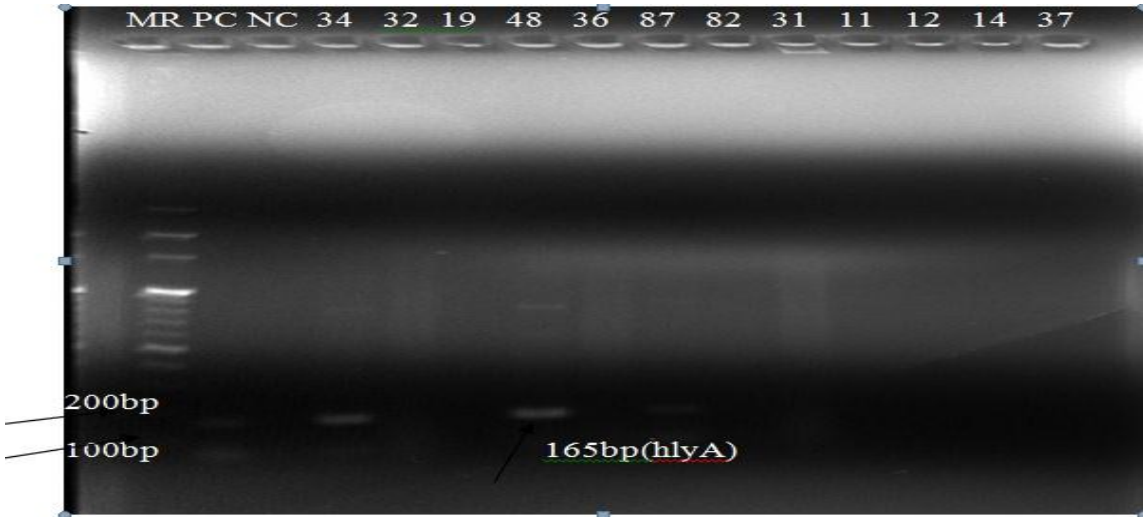


Fig 6: Agarose gel electrophoresis of amplified *hlyA* gene generating 165 base pair amplicon

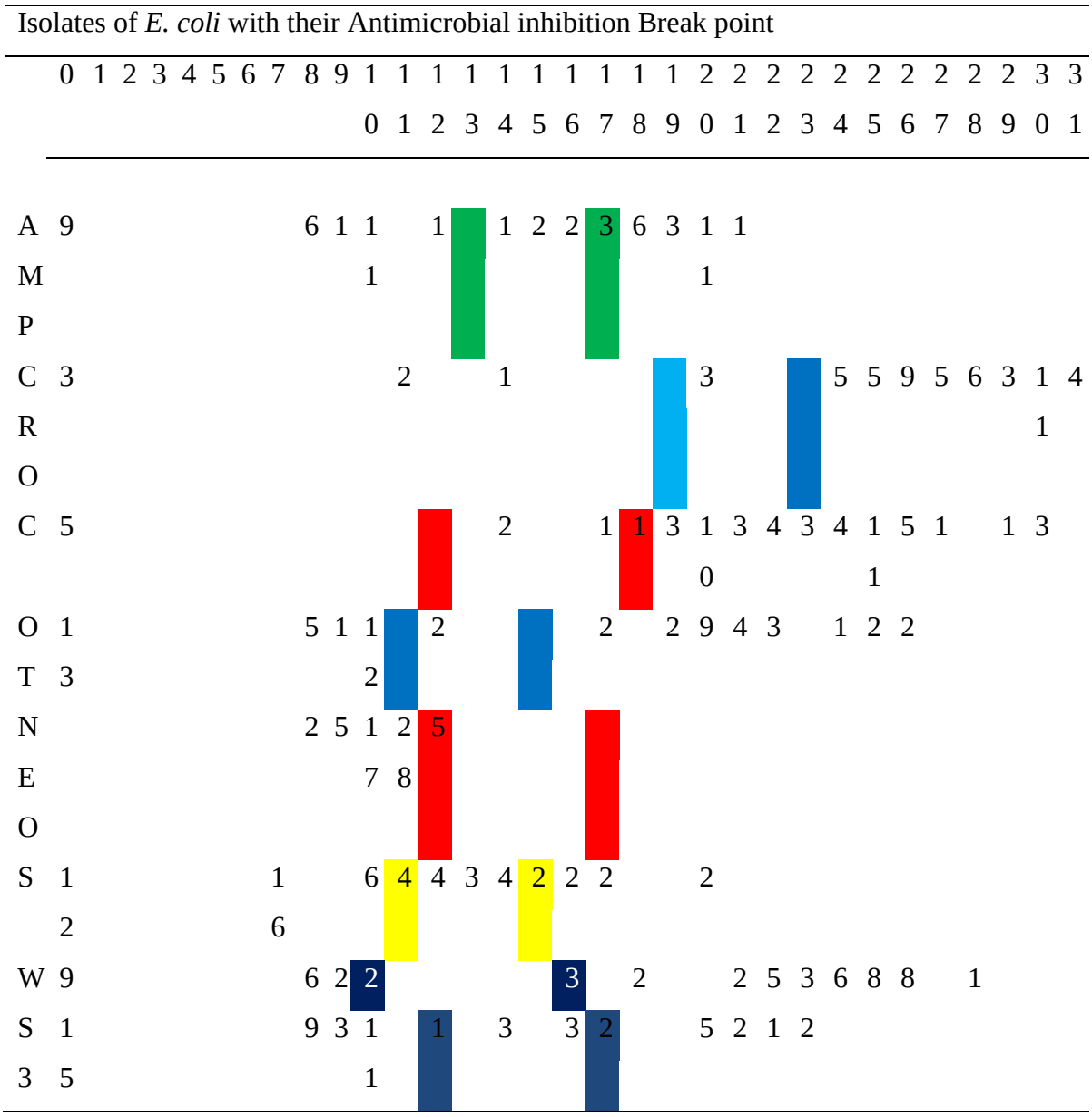
The PCR products were separated on 1% agarose gel electrophoresis: M= Marker (100 bp DNA ladder), Lane 1 and 2 are PC= positive and NC=negative control respectively. Lane no 34 32 19 48 36 87 82 31 11 12 14 and37 indicates sample no. (PCR products prepared from *E. coli* isolates) and Lane 3, 6and 8 are positive results of sample codes 34, 48 and 87 respectively.

4.6. Antimicrobial Susceptibility Profiles of *Escherichia coli* Isolates

4.6.1. Mono- drug resistance

Antimicrobial susceptibility tests of 57 *E. coli* isolates against 8 commercially available antimicrobial disks and commonly used for treatment of bacterial disease in animals were analyzed by standard disc diffusion technique (Annex 6). Among these results revealed that Chloramphenicol was found most potent drugs with 85.96% sensitivity followed by Ceftriaxone, Trimethoprim, Oxy tetracycline, Ampicillin, Sulphonamides, Streptomycin and Neomycin with 84.21%, 66.67%, 43.86%, 42.11%, 21.05%, 14.04% and 0% sensitivity respectively. On the other hand, 100% of *E. coli* isolates were resistant to Neomycin followed by Sulphonamides, Streptomycin, Oxy tetracycline, Ampicillin, Trimethoprim, Ceftriaxone and Chloramphenicol with 68.42%, 66.67%, 54.39%, 49.12%, 33.33%, 10.53% and 8.77% resistant respectively. Also 11(19.3%) of isolates shown intermediate sensitivity to Streptomycin, 6(10.53%) to Sulphonamides, 5(8.77%) to Ampicillin, 3(5.26%) to both Ceftriaxone and Chloramphenicol, 2(3.51%) to Oxy tetracycline and 0% to both Neomycin and Trimethoprime was found. However, there is no *E. coli* isolate was found to be susceptible to all eight antimicrobial agents, entirely *E. coli* isolates were found resistant against for Neomycin. Data on the Antimicrobial resistance pattern of the isolates are summarized in table 6.

Table 6: Antimicrobial sensitivity test inhibition break point on *E. coli* isolates



Keys: AMP; Ampicillin, CRO; Ceftriaxone, C; Chloramphenicol, OT; Oxy tetracycline, NEO; Neomycin, S; Streptomycin, W; Trimethoprim, S3; Sulphonamides

On the above table, lines between numbers shows antimicrobial inhibition break point in which the resistance isolate is/ are occurs to the left side of the first lines of each antimicrobial inhibition break point, the intermediate isolate is/are occurs between the line of each antimicrobial inhibition break point and while the susceptible isolate is/are occurs to the right side of the second lines of each antimicrobial inhibition break point.

4.6.2. Multi- drug resistance

Multidrug resistance refers to resistance of single isolate against two and more than two drugs). Regarding the multidrug resistance profile, forty (70.18%) of *E. coli* isolates were found to be multidrug resistant. The highest proportion of resistant isolates is observed to four drugs (Oxy tetracycline, Neomycin, Streptomycin, and Sulphonamides) with a resistance of 8 (14.04%) *E. coli* isolates. This is followed by six drugs with a resistance of seven (12.28%) *E. coli* isolate from diarrheic calves. Data on the multidrug resistance pattern of the isolates are summarized in table 7.

Table 7: Multidrug resistance distribution among *E. coli* isolates

No. of Antimicrobials disks	Multidrug resistance pattern	No. of isolates	Percentage (%) (n=57)
Two	NEO, S	3	5.26
	NEO, S3	3	5.26
	NEO, OT	1	1.75
3	NEO, OT, S	1	1.75
	NEO, S, S3	3	5.26
	NEO, AMP,OT	1	1.75
4	NEO, S, OT, S3	8	14.04
5	NEO, S, AMP,OT, S3	4	7.01
	NEO, S, AMP, W, S3	2	3.51
	NEO, S, OT, S3,C	1	1.75
6	NEO,S, AMP, OT, W, S3,	7	12.28
	NEO,S, AMP, CRO, W, S3	1	1.75
7	NEO, S, AMP, CRO, OT, W, S3	3	5.26
	NEO, S, AMP, C, OT, W, S3	2	3.51
Total		40	70.18

Keys: AMP; Ampicillin, CRO; Ceftriaxone, C; Chloramphenicol, OT; Oxy tetracycline, NEO; Neomycin, S; Streptomycin, W; Trimethoprim, S3; Sulphonamides

4.7. Antimicrobial Resistance profiles of pathogenic *E. coli* strain's genes

Out of the 16 isolates that carried virulent genes, eleven (68.75%) developed multidrug resistance. These all isolates are completely resistant to Neomycin followed by Oxy tetracycline, all (Ampicillin, Streptomycin and Sulphonamides), Trimethoprim, Ceftriaxone and Chloramphenicol. On the other extreme, all virulent gen isolates revealed entire susceptibility to Chloramphenicol followed by Ceftriaxone, Trimethoprim, all (Ampicillin, Streptomycin and Sulphonamides), Oxy tetracycline and Neomycin which is summarized in table 8.

Table 8: Antimicrobial resistance profiles of pathogenic *E. coli* strain's genes

Antimicrobials agents	Resistance bearing specific virulence genes (%) N=21				
	Stx2(n=10)	*Stx1*(n=5)	*eaeA*(n=3)	*hlyA*(n=3)	Total(n=21)
AMP	5(50%)	4(80%)	2(66.67%)	2(66.67%)	13(61.9%)
CRO	1(10%)	1(20%)	-	1(33.33%)	3(14.2%)
C	-	-	-	-	-
OT	8(80%)	4(80%)	2(66.67%)	3(100%)	17(80.95%)
NEO	10(100%)	5(100%)	3(100%)	3(100%)	21(100%)
S	5(50%)	4(80%)	2(66.67%)	2(66.67%)	13(61.9%)
W	3(30%)	3(60%)	1(33.33%)	1(33.33%)	8(38.1%)
S3	5(50%)	3(60%)	3(100%)	3(100%)	13(61.9%)

Keys: AMP; Ampicillin, CRO; Ceftriaxone, C; Chloramphenicol, OT; Oxy tetracycline, NEO; Neomycin, S; Streptomycin, W; Trimethoprim, S3; Sulphonamides.

5. DISCUSSION

Diarrheagenic *E. coli* (DEC) are recognized as the major cause of neonatal calf diarrhea (NCD) with severe lethal outcome and major damage to the livestock industry worldwide. Consequently, high mortality rate in calves up to 3-months-old has been reported (Windeyer *et al.,* 2014). This study also indicates *E. coli* to be the major dairy development challenge in the study area. With this concern in the present work detailed studies of the virulence factors produced by *E. coli* strains in farm animals and their antimicrobial sensitivity pattern are needed. Although, the present study focused on isolation and determination of *E. coli*, the involvement of other microbial, parasitic and protozoa as a cause of calf diarrhea could not be ruled out.

5.1. Overall Isolation of *Escherichia coli* in Calf Diarrhea

The overall isolation rate of *E. coli* in this study is 57(50.89%) out of 112 fecal samples from diarrheic calves. This result is in line with the result reported by Yaekob (2014) (50.9%) from Arsi Zone ormia, Ethiopia. Hassan (2014) (50%) and Hossain *et al.,* (2012) (49%) in Bangladesh. These biochemically confirmed isolates were proportionally higher than the study of Dereje (2012) 25 (43.1%) in and around Addis Ababa, Ethiopia, Mailk *et al.,* (2013) 37.61% and 36.8% of (Gebregiorgis and Tesfaye, 2016) from Kombolcha, Ethiopia. Svensson *et al.,* (2017) in India also reported (46.51%) samples were found positive for *E. coli*. But, this result is lower isolation rate than El-Seedy *et al.,* (2016) (75.6%), Tarekegn and Molla, (2017) (70.7%) from Debre Zeit Ethiopia and muktar, (2014) (69.6%) in North Shewa. In contrast to this study much higher detection of *E. coli* was reported by Adesiyun *et al*., (2001) 84.3% and Pourtaghi *et al.,* (2013) 86.7% in Iran from diarrheic calves. These differences might be attributed to the differences in farm management systems, climatic conditions, sample size, personal hygiene, breed, and isolation method and handling practices among different farms in different countries and also within countries. Kaper *et al.,* (2004) stated that, the prevalence and epidemiological features of *E. coli* as the causative agent of diarrhea vary from region to region around the world, and even between and within countries in the same geographical area.

5.2. Risk Factors Associated with Calf Diarrhea

Regarding the risk factors associated with the isolation rate of *E. coli*, many factors were tested, while, relatively few were significant in the final model. In this study isolation rate of *E. coli* from calf diarrhea was significantly differed by age of calves (P<0.05). Among different age groups, higher prevalence of *E. coli* was observed in 0-2 weeks age (67.57%) of *E. coli* in diarrhoeic calves than 3-8 and 9-16 weeks of calves. Similar results were reported by Islam *et al.,* (2015) from Bangladesh, who observed highest prevalence (66.7%) of *E. coli* in diarrhoeic calves of up to 6 days age compared to the calves of above 6 days to 2 months. Davoodi and Nourmohammadzade (2013) also reported highest prevalence of *E. coli* (68.81%) in faecal samples of diarrhoeic calves of 1 week age, while lower prevalence (31.37%) was observed in older calves (4 weeks age). Tarekegn and Molla (2017) from Debre Zeit, Ethiopia reported 56% highest being in calves of <2 week old. Who concluded that *E. coli* is one of the most common diseases of newborn calves (9–10 days of age) characterized by watery diarrhea and the affected calves die within 2–3 days.

Also the association between the age level and diarrhea was being curvilinear as observed elsewhere (Islam *et al*., 2015 and Gebregiorgis and Tessema, 2016). This could also be related to the beginning of stress due to environmental exposure and infection pressure when the immune system of the calves is still developing (Radiostat *et al*., 2007). Most newborn calves are exposed to *E. coli* from the environment, particularly when sanitation is marginal (Charles *et al.,* 2003) in North Dakota. The other author stated that young neonates under 1 week of age are particularly susceptible because the normal flora of the intestine is not fully established. In addition to that, they have a naive immune system and also receptors for the adhesions of *E. coli* are present on the first week of life of the calves (Villarroel, 2009).

The occurrence of *E. coli* in diarrheic calves due to the time of first colostrum feeding and amount of colostrum per time was considered and significantly differed (P<0.05). Occurrence of *E. coli* in calves that were given colostrum lately and small amount were at high risk of being affected with diarrhea due to *E. coli* than feds colostrum early (before 6 hours) and high amount (greater than 2 litters). In comparable to this isolation rate of *E. coli* was detected as 100% out of 23 diarrheic calves that fed colostrums before six hours in study of (Muktar, 2014) in North Showa. This is due to, an inadequate, in quality and

quantity supply of colostrum and delay in first colostrum feeding, which leads to failure of transfer of passive immunity is an important reason. Calves with inadequate colostral immunoglobulin concentration within 24 h of birth were at greater risk of neonatal morbidity and mortality. Colostrum feeding practices also have effect in that allowing calves to nurse their dam may predispose them to failure of transfer of passive since they consume late and small amount (Meganck *et al.,* 2014).

Among herd size, highest prevalence of *E. coli* was also observed in higher herd size than medium and small dairy farms. This result also supports previous evidence of Calf diarrhea was apparently higher in medium and large sized dairy farms than small dairy farms (Tarekegn and Molla, 2017) from Debre Zeit Ethiopia. This might be due to when the number of Calves in the same barns is higher, the contaminations also increase, and therefore, microorganism can easily transmit from infected to the health calves.

The questionnaire survey indicated that the prevalence of *E. coli* was found higher in hand colostrum feeding than suckling by itself. This due to during bottle feed the colostrum might be contaminated with environmental pathogens due to poor management systems. The prevalence of *E. coli* among animal breed showed significant difference ($P<0.05$) in which *E. coli* was recovered at higher occurrence in cross breed (40.18%) than local breed (10.75%). Calf diarrhea is result from multifactorial: incorrect management of calves, feeding, age, and animal breed were the most important risk factors of death rate (Muluken *et al*., 2017).

Interaction between several management and environmental factors commonly associated with DEC. Therefore, screening of the DEC virulence genes is needed. Persistence of the problem of NCD might associate with the poor environmental hygiene, failure to clearly understand the disease ecology, and biased epidemiological data (Younis *et al.,* 2009). Although medications, and herd management have been implemented to minimize the economic loss, the NCD economic impact is still significant (Cho and Yoon, 2014). Several approaches should be considered for future control such as vaccination of the pregnant dams, and fluid therapy (Younis *et al.,* 2009).

5.3. Pathogenic *Escherichia coli* Strains Gene in Calves Diarrhea

Polymerase chain reaction is a rapid and reliable tool for the molecular based diagnosis of a variety of infectious diseases (Fredricks and Relman, 1999) and due to its sensitive and specific nature of the assay; it should be applied for further confirmation of the isolated *E. coli* species. PCR and agarose gel electrophoresis was used to detect virulence genes of *E. coli* and their respective pathogenic strains.

In the present study, from the 57 *E. coli* isolates, 4 different *E. coli* virulence genes were identified. Thus, found 16 (28.07%) isolates to be positive for at least one of the virulence genes giving the type of strain EPEC, EHEC and STEC. This prevalence is in accordance with the work of Herrera Luna *et al*., (2009) from Austria reported as 28.9% for similar virulent genes. This finding is greater than the work (6.9%) of (Badouei *et al.,* 2014) from Iran. These differences of prevalence of virulence genes might be due to season, farm size, hygienic status, farm management practices, variation in sampling, differences in detection methods, age of the animals, and number of virulence genes investigated.

Shiga toxin producing *E. coli* (STEC) is important for the herd since shiga toxin can be responsible for economic losses and a threat to human health as animals can be carriers to humans. Cattle are considered to be the major reservoir of STEC worldwide (Aidar-Ugrinovkch *et al.,* 2007). The *stx2* is an important virulent gene detected in this study most importantly incriminated as diarrheagenic in calves with *stx1*.

In this finding, *stx2* genes 62.5% (10/16) were found either alone per isolate or in combination with other virulent genes of an isolate. Of these, eight *stx2* (50%) genes were found exclusively in eight isolates, the remaining two isolates (12.5%) combined with *hlyA* gene. This Prevalence of *stx2* gene were higher than which reported by Tahamtan *et al.,* (2010), Dastmalchi and Ayremlou, (2012) from Iran and Hashish *et al.,* (2016) from Pakistan, who recorded 53.42%, 30% and 26.67% respectively.

The *stx1* is another important virulent gene in STEC and EHEC strains to which five isolates 31.25% (5/16). And constitutes 3(16) 18.75% of detected genes carried it exclusively. The remaining two isolates (12.5%) combined as a single *stx1* gene with *eae* gene (6.67%). and as mentioned above this gene is also a component of triple gene carried in one isolate with *eaeA* and *hlyA* (6.25%). This result is lower than reported by Hashish *et al.,* (2016) from Pakistan found this gene with frequency of 86.67% and 41.3% also

reported from De Moura *et al.,* (2012) in Brazil to which both are much higher than our finding. While lower percentage 10.27%, 9.75% and 3.8% were recorded by Tahamtan *et al*., (2010), Salvadori *et al.,* (2003) from Brazil and Mohammed *et al.,* (2019) from Saudarebia are respectively.

Therefore, frequency of *stx2* in this study is found higher than other detected virulent genes. The predominant occurrence of *stx2* was also detected in Iran showed that STEC harboring *stx2* isolate was significantly more (53.42%) than harboring *stx1* (10.27%) (Tahamtan *et al.,* 2010). Similarly, 30% *stx2* harboring isolates were reported which is higher than 10% stx1 gene (Dastmalchi and Ayremlou, 2012) from Vietnam (Nguyen *et al.,* 2011). In contrast, a finding from Iran stx1 gene was the most prevalent variant among the isolates (Taghadosi *et al.,* 2018). Additionally, Mohammed *et al.,* 2019) from Saudarebia reported that from the total isolates, stx2 gene were not found. And from Uruguay were found carrying no stx2 but 4.8% of stx1 (Umpierrez *et al.,* 2017) reported.

Most EPEC strains have both bundle-forming pilus gene (*bfpA*) and *eaeA* gene, but in this study, the EPEC strains isolated were atypical in that they only contained the *eaeA* gene which is in agreement with work of (Islam, 2015) that identified atypical EPEC from all isolates. In this study, the detection rate of *eaeA* genes was (3/16) 18.75%. From this constitutes (1/16) 6.25% of detected genes carried it exclusively. A single *eaeA* genes carried isolates co-existed with *stx1* in an isolate 1(6.25%). and the remaining one (6.25%) amplified products of this gene were obtained in combination with *stx1* and *hlyA*. Our finding of *eaeA* gene is higher than (12.5%) reported by Islam *et al.,* (2015) from *Bangladesh* and extremely higher prevalence than 1.3% (Picco *et al.,* 2015) from Argentina, 2.1% (Umpierrez *et al.,* 2017) from Uruguay and 2% (Rehman *et al*., 2014) from Argentina, Uruguay and Kashmir respectively. However, this finding (18.75%) is in agreement with 19% from Saudi by Mohammed *et al.,* (2019) but other studies conducted in Brazil by De Moura *et al.,* (2012) and in Austria by (Herrera-Luna *et al.,* 2009), the distribution of (*eaeA*) intimin genes in diarrheic calves was (60.3%) and 57.1% respectively, which is higher than our finding.

One of the detected virulence genes observed in the current study is the occurrence of 18.75% (3/16) *E. coli* isolate revealed hemolysis genes in combination with other virulent genes of an isolate. Of these, two *hlyA* (12.5%) genes were found combined with *stx2* gene. the remaining one isolates (6.25%) combined found with *eaeA* and *stx1*gene. Our

result is strongly lower than 60% and 50% which is reported by Dastmalchi and Ayremlou, (2012) and in Austria by Herrera *et al.*, (2009). While this result is higher than (Badouei *et al*., 2010) who identified (6.3%) of *hlyA* gene in his study. In most detected virulent genes in this study showed variation in prevalence when compared to other research works conducted in different place by different authors. This variation in prevalence of virulence genes might be due to different study area, season, farm size, and number of animals on the farm, hygienic status, farm management practices, variation in sampling, variation in types of samples evaluated, and differences in detection methods.

Forty two (42) *E. coli* isolates that were isolated from diarrheic samples did not carry any of the virulence factors assessed in this study. One possible explanation could be that these isolates were non-pathogenic *E. coli* strains and the animals have diarrhea caused by some other infectious agent; alternatively, that the isolates may carry other virulent genes, which was not investigated during this work. Furthermore, the result from the current study is consistent with the previous reports where virulence genes were detected in only few *E. coli* strains that may be due to the fact that there are occasional strains that have the genes but do not express the toxins (Lenahan *et al.,* 2007).

5.4. Antimicrobial Resistance Patterns of *Escherichia coli* Isolates

The emergence and dissemination of antimicrobial resistance is an important issue in public health, animal health, and food safety. With regard to the antibiogram of *E. coli* in the current study, 8 different commercially available antimicrobial discs and commonly used for treatment of bacterial disease in animals were used. All the 57 *E. coli* isolates subjected to antimicrobial sensitivity test. This study were found to be most of the bacterial isolates were susceptible to Chloramphenicol (85.96%), Ceftriaxone(84.21%) and Trimethoprime (66.67%).The degree of susceptibility for *E. coli* isolates ranges from 0% up to 85.96% . however the resistance ranges were from 8.77% up to 100% in this *E. coli* isolates.

In this study 100% of *E. coli* isolates were resistant to Neomycin followed by Sulphonamides (68.42%), Streptomycin (66.62%), Oxy tetracycline (54.39%), Ampicillin (49.12%), Trimethoprim (33.33%), Chloramphenicol (10.53%) and Ceftriaxone (8.77%). these resistant were lower than Hossain *et al.* (2012) (100%) from Bangladesh ,who reported 100% resistant against ampicillin and streptomycin. Higher resistance of *E. coli* isolates than our work was reported by Shahrani and his colleague (2014) from Iran. These

have descending resistance level of streptomycin (98.25%), tetracycline (98.09%), sulfonamides (90.31%), chloramphenicol (73.8%), ampicillin (71.11%), trimethoprim (62.22%). In current study neomycin and sulphonamides which were (100%) and (68.42%) resistant respectively to *E. coli* isolates in diarrheic calves, was in contrast with study conducted by (Herrera-Luna *et al*., 2009) who reported neomycin and sulphonamides 13 (33.3%) and 17 (43.5%) resistant isolates respectively. Highly resistance to neomycin in this study is also in agreement with the work of (Abd-Elrahman, 2011). In current investigation, (49.12%) *E. coli* isolates were showed resistance to ampicillin in diarrheic calves, which is in agreement with findings of (Shahrani *et al*., 2014) and (Herrera-Luna *et al*., 2009) who reported (71.11%) and 25 (64.1%) isolates resistant to ampicillin respectively.

This variation may be probably attributed to the expression of resistant gene code by the pathogen which associated with emerging and reemerging aspects of the isolates (Reuben and Owuna, 2013).The high resistance of these all drugs in gram-negative bacteria might also be due to the transfer of resistance genes from gram-positive bacteria of β-lactamase genes. The extensive use of antibiotics often without prescription from qualified veterinarians and wide spread and erratic use of broad spectrum antibiotics without proper isolation of the causative agent and without performing antiboitic sensitivity testing are real causes of high resistance of drugs (Mokhtar, 2008).

This result indicates the use of antibiotics in cases of diarrhea into question. Constable (2004) recommended that in calves with diarrhea without signs of systemic disease (normal appetite for milk, no fever), oral as well as parenteral antimicrobials should not be administered. Khachatryan *et al*., (2004) suggested that high prevalence of antimicrobial drug-resistant strains in younger animals might be related to better adaptation of bacteria to neonatal intestines.

Multi Drug Resistance is defined as resistance of an isolate two or more than 2 antimicrobials tested (Dominic *et al*., 2005). In this study, forty (70.18%) of *E. coli* isolates were developing multidrug resistance to different antibiotics. In agreement with our study, MDR was observed in studies of (Rigobelo *et al*., 2006). Khachatryan *et al.* (2004) and Donaldson *et al.* (2006) detected high rates of multi-drug resistance in *E. coli* isolated from healthy animals. In the case of pathogenic *E. coli* strains, eleven (68.75%) develop multidrug resistance. This could be the consequence of indiscriminate use of

antimicrobial in clinical practice, lack of proper knowledge that leads to ultimately replacement of the drug sensitive microorganisms from antibiotic saturated environment.

5.5. Antimicrobial Resistance of Pathogenic *Escherichia coli* Strains

In the case of pathogenic *E. coli* strains, however, all pathogenic strains were developing resistance to Neomycin but the entire pathogenic *E. coli* strains were susceptible to chloramphenicol. So the present study suggested that the development of resistance pathogenic *E. coli* strains to chloramphenicol was limited. those encoding for Neomycin. tetracycline, sulphonamide, and streptomycine resistance. De Verdier and his co-worker (2012) indicated possible factor in a linkage between resistance genes and genes conferring selective advantage to colonize the intestinal lumen of calves. Streptomycin - sulfonamide – tetracycline has a selective advantage to colonize the intestine of calves given a dietary milk supplement also in absence of antimicrobials.

Additionally, antimicrobial residues taken within colostrum or milk from treated cow in the dry period could select for resistance in the enteric flora of calves. Epidemiology of antimicrobial resistance reportedly occurred through HGT and chromosomal mutation. Both resistance and virulence genes transferred together but HGT was found more effective than chromosomal mutation did (Giedraitienė *et al.,* 2011). This all indicates, Occurrence of resistant isolates in the environment revealed that the direct or indirect contamination with resistant gene carried *E. coli* strain developed through continious exposure to the respective antimicrobials.

6. CONCLUSION AND RECOMMENDATIONS

The present study was the first conducted to isolate and determine pathogenic *E. coli* strains and their antimicrobial susceptibility patterns as well as associated risk factors from diarrheic calves at Bahir Dar city dairy farms. The overall high proportion of *E. coli* isolates and their significant pathogenic strains indicates, *E. coli* to be the major dairy development challenge in the study area. The occurrence of *E. coli* among animas having different age groups, breed, colostrum feeding time and herd size had significantly associated with the occurrence of *E. coli* contamination. This study also tried to identify the higher cumulative occurrence of pathogenic *E. coli* strains STEC strains 11 (19.3%), EHEC strains 4 (7.02%) and aEPEC strains 1(1.75%) was reported. The antimicrobial susceptibility patterns of the isolates showed that Ceftriaxone, Chloramphenicol and Trimethoprime should be considered as first choice of drugs as the isolates are susceptible to these drugs. The multidrug resistant profile of our study showed that, forty (70.18%) of *E. coli* isolates were developing multidrug resistance to different antibiotics. Even though, antimicrobial resistance is an emerging worldwide problem in human and veterinary medicine both in developed and developing countries, frequent and improper use of antimicrobials were observed in our study areas. Taking into consideration the fact that the period and the scope of our study were limited further study. Continued surveillance of *E. coli* on large number of animals should be carried out to investigate microbial causes of calf diarrhea and to identify emerging antimicrobial-resistant phenotypes.

Based on the finding of the study and the above conclusion of the following recommendations, trusted to be constructive for concerned authorities are forwarded;

- Further investigation about the remaining pathogenic *E. coli* strains should be conducted to have a complete picture about the prevalent strains in the study area.
- Awareness creation should be given to the farm owners about the most important potential risk factors through agriculture extension service.
- To minimize Antimicrobial resistance, appropriate prescription of antibiotics in the farm and continuous monitoring of the resistance patterns is highly required.
- Recommend antimicrobial sensitivity test before treatment.

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8. ANNEXES

ANNEX 1: questionnaire survey forms

1. Farm (household) identification;

Animal cod: _____ date: _____

Owners' name (optional): _____ Farm name_____

Address (kebele): _____When is established_____

2. Farm descriptions

2.1. Herd size: Cow_____ Calves_____ Heifer_____ Bulls _____

2.2. The farm as a source of incomes: a) Primary income, b) secondary income

2.3. Organization of farms: a) Family farms, b) Partnership, c) Institutional

2.4. Sex: a) Male b) Female

2.5. Breed: a) Local, b) Cross,c) Exotic

2.6. Age of the Calf: a) 1day-2month, b) 61day -4months, c) 121days-6months

2.7. Type of diarrhea: a)Yellowish, b) Mucousy, C) Greenish, d) Watery, e) Bloody

2.8. Production systems: a) urban b) pri-urban

3. Management Data:

3.1. Owner/manager education status:

a) Illiterate, b) Read and write, c) Elementary school, d) High school, e) professional

If professional, a) related to Animal production, b) unrelated to Animal production

3.2. Calf caretakers (attendants): a) owner (family members) b) Hired help

3.3. Sex of attendants: a) Male b) Female

3.4. Experience of calf caretakers: a) Less than 5 year b) Greater than 5 year

3.5. Education of calf caretakers: a) Elementary school b) High school c) Collage d) graduate e) professional

3.6. Per parturient care: Calving facility: a) Calving pen b) the same barn

4. Feeding

4.1. Colostrum feeding: a) yes, b) no

4.2. Colostrum is fed by: a) suckling, b) hand fed, c) not fed

4.3. If colostrum fed; the time was: a) before 6hrs, b) after 6hrs

4.4. Amount of colostrum/time given; a) 1-1.5 litter b) 2 litter c) more than 2 litter d) unknown

4.5. Frequency of feeding: a) once/day b) Twice/day c) thought the day

4.6. Types of feed supplementary; a) Grazing b) concentrates c) hay

4.7. Weaning age; a) 2 weeks b) 3 weeks c) 4 weeks and above

5. Knowledge of the owners for the antimicrobial and Treatment practice

5.1 Antibiotic therapy for diarrhea: Yes ☐ No ☐

5.2. Received antibiotic within 4 weeks before diarrhea: Yes ☐ No ☐

5.3. Where animals are treated: Vet clinic ☐ At home ☐

5.4. Duration of antibiotic therapy: __ /day for ____days

5.5. Source of drugs: Vet clinic ☐ Market ☐

5.6. Drug storage: Present ☐ Absent ☐

5.7. Commonly used drugs: _____; _____; _____

Experience on calf health problem, prevention and control of the problems

Major health problems for the farm----------

Diarrhea as important lamb health problem----------

Age group affected by diarrhea----------

Types of diarrhea often encountered----------

Measures taken to isolate and treat sick calves----------

Response of sick calves to treatment----------

Measures taken to prevent disease problem----------

Thank you for your cooperation

Principal investigator: Kefale Mengistu

Supervisors: Dr. Yosef Deneke (phD) & Dr. Tesfaye Sisay(phD)

ANNEX 2: Data recording format for sampling

Date_____farm_____Enumerator_____

No.	Calf ID	Age (week)	Sex	Breed	Clinical sign	Diarrhea Type
1						
2						
3						
4						
5						
6						
7						

ANNEX 3: Laboratory record sheet format

Plate isolation and biochemical test result record sheet

No.	Farm	Calf ID	Age (wk/s)	Sex	Mac (pc/pac	EMB (GMSC/NGMSC	TSI	IMVCi Tests			
								I	MR	VP	C

Keys: Mac = MaCconkey agar, EMB = Eosinemethylenblueagar, I = Indole , MR = methyl Red, Vp = Vagouse prouskure, Ci = citrate, GMSC=Green metallic sheen colony, NGMSC=None green metallic sheen colony, PC= Pink colony, PAC= Pale colony and TSI=Triple suger iron agar

ANNEX 4: List of Media and it's preparations for the isolation of *E. coli.*

1. Non-selective enrichment

1.1. Buffered peptone water (BPW) (Oxoid, England)

Composition (g/l): Peptone 10.0 g Sodium chloride 5.0 g, Disodium hydrogen phosphate

doxdecahydrate 9.0 g (Na2HPO4.12H2O) Potassium dihydrogen phosphate (KH2PO4) 1.5 g, Water 1000 ml.

Preparation: 20g of this media was dissolved in 1000ml of distilled water sterilized by autoclaving at 121^0c for 15 minutes.

2. Isolation on selective and differential plate agars

2.1. MacConkey Agar (MCA) (HiMedia, India)

Ingredients g/l: Peptic digests of animal tissue 20.00 gm, Lactose 10.00 gm, Bile salt 5.00 gm, sodium chloride 5.00 gm, neutral red .07 gm, Agar 15.00 gm, distilled water 1000.00 ml, Final pH 7.5 + 0.2 at 25°C

Preparation: 55.07grams of this media was suspended in 1000ml of distilled water,Boil to dissolve the medium completely and sterilized by autoclaving at 121^0C for 15minutes and then Poured into Petri dishes.

2.2. Eosin Methylene Blue (EMB) Agar (HiMedia, India)

Ingredients (g/l) Peptone 10.00, Lactose 10.00, Dipotassium hydrogen phosphate 2.00, Eosin Yellow 4.00, Methylene blue 0.065, Agar 25.00, Final pH 7.2 at 25^0c.

Preparation:

36 grams of this media was suspended in one liter of distilled water.

Boil to dissolve the medium completely

Sterilized by autoclaving at 121^0C for 15minutes and then

Poured into Petri dishes.

2.3. Nutrient agar (Oxiod, England)

Ingredients (g/ l): Peptone, 10.000, Beef extract 10.000, Sodium chloride 5.000 Agar, 12.000, pH, after sterilization 7.3±0.1, Final pH 7.3 ±0.1 at 25oc.

Preparation: 28g of this media was suspended in 1000ml of distilled water, boil to dissolve completely, sterilized by autoclaving at 121^OC for 15minutes and then

Pour into Petridishes.

3. Biochemical tests

3.1. Indol test:

Reagent required for Indole reaction

Kovacs reagent (HiMedia, Nashik, India)

Ingredients(g/l):4-Dimethylaminobenzaldehyde5gm,Ethanolalcohol 75ml,Hydrochloric acid 25ml.

Preparation: Mix the components with constant stirring. The final reagent should be stored in brown bottle, Two to five pure colonies was inoculated using a sterile wire loop in 2 ml of peptone water in bijous bottles, Incubate overnight at 35^0C, 0.5 ml of Kovac's reagent was added and then Examined after 1minute.

Presence of rose red colour on upper layer was considered positive (+), while absence of rose red or pale colour will be considered negative (-) (Quinn *et al*., 1994).

3.2. MR-VP Medium (HiMedia, India)

Ingredients g/l: Buffered peptone, 7.000, Dextrose, 5.000, Dipotassium phosphate, 5.000 Final pH (at 25°C) 6.9±0.2.

Preparation: 17g of this medium was dissolved in 1000ml of distilled water, Mix thoroughly, Autoclaved for 15 minutes at 121^0 C.

3.3. BBL TM Simmons citrate agar (Logo and BBL, sparks, USA):

Ingredients g/l: Magnesium sulfate 0.2, Ammonium dihydrogen phosphate 1.0, Dipotassium phosphate 1.0, Sodium citrate 2.0, Sodium chloride 5.0, Agar 15.0 and Bromo thymol blue 0.08 Final pH 6.9± 0.2. at 25^0c.

Preparation: 24.2g of Simmons citrate agar was suspended in 1000l distilled water, Mixed thoroughly.

Heat with frequent agitation and boiled for 1 minute to completely dissolved powder Autoclaved at 121^0C for 15 minutes. Test samples of the finished product for performance using stable, typical control cultures.

Simmons Citrate agar slants in test tubes were stabbed use a sterile wire loop and incubate for 48hrs at 35^0C. Positive (+) growth for example citrate utilization produce an alkaline reaction and the medium change color from green to blue, while no color change (no citrate utilization) was considered negative (-) (Quinn *et al.*, 1994).

3.4. Difco TM Triple sugar iron agar (Difco, sparks, USA):

Ingredients g/l: Beef extract 3, Yeast extract 3, Pancreatic digestion of casein 15, Proteose peptone 5, Dextrose 1, Lactose 10, Sucrose 10, Ferrous sulphate 0.2, Sodium thiosulfate 0.3, Sodium chloride 5, Agar 12 and Phenol red 0.024.

Preparation: 65g of this media was dissolved in 1000ml of distilled water, Heating in a boiling water bath thenAutoclaved at 1210C for 15 minutes.

Cool in a slanted position so that deep butts were formed. Test samples of the finished product for performance using stable, typical control cultures.

The TSI agar tube was inoculated in the middle of the agar to within 5mm from the bottom of the tube with a straight inoculating wire. On the withdrawal of the straight wire, the entire slant was streaked (right to the top) and incubated at 370C for 16 to 24hrs.

Production of acid (yellow) slant and acid (yellow) butt, gas, without production of H2S (hydrogen disulfide) (blackening of agar) was considered positive for E. coli. While an alkaline (red) slant and yellow butt (acid), gas, with or without H2S gas (blackening) was considered positive for Salmonella (Quinn et al., 1994).

4. Antibiotic susceptibility test

4.1. Muller Hinton Agar (M 173 Hi Media)

Ingredients g/l: Beef infusion 300, Casein acid hydrolysate 17.50, Starch 1.5 and Agar 17.0

Preparation: 38.00 grams will be add in 1000 ml of distilled water, Boiling to dissolve the medium completely and Sterilized by autoclaving at 15 lbs pressure (121°C) for 15

ANNEX 5: biochemical test procedure

Indole test

Trypton broth prepared according to manufacturer's prescription

Autoclaved at 1210c for 15 minutes

Poured 2 ml of broth to test tubes

E. coli isolates inoculated

It has incubated at 370c for 48 hrs

1 ml of KOVAC's added and shaken gently

Allowed to stand for 1-2 minutes

Observe formation of cherry red ring at the top.

Methyl red test

MR – VP broth prepared

Inoculate E. coli and incubate at 370c for 48 hrs

PH indicator Methyl red was added

Red color observed for positive result and orange color for negative one

Voges-proskauer (Vp)

MR – VP broth prepared(Himedia)

Inoculate *E. coli* and incubate at 370c for 48 hrs

Alpha naphtol added followed by potassium hydroxide

Color change observed red (positive) or yellow (negative)

Citrate utilization test

Citrate slant prepared (Himedia)

Inoculate *E. coli* and incubate at 370c for 48 hrs

Test tubes observed for color change from green to blue

Source; (Hemraj *et al*., 2013)

ANNEX 6: Antibiotic susceptibility test procedure

The disk diffusion method is more suitable for routine testing in a clinical laboratory where a large number of isolates are tested for susceptibility to numerous antibiotics.
Each isolated bacterial colony from pure fresh culture was transferred in to a test tube of 5 ml tryptone soya broth (TSB) (Oxid, England)
Incubated at 37^0C for 6 hours.
The turbidity of the culture broth was adjusted using sterile saline solution or added more Isolated colonies to obtain turbidity usually comparable with that of 0.5 McFarl and Standards (approximately 3x108 CFU per ml).
Mueller-Hinton agar (M 173 Hi Media) plates was
prepared according the manufacturer.
A sterile cotton swab was immersed into the suspension and rotated against the side of the tube to remove the excess fluid and then swabbed in three directions uniformly on the Surface of Mueller-Hinton agar plates
After the plates dried, antibiotic disks were placed on the inoculated plates using sterile forceps.
The antibiotic disks were gently pressed onto the agar to ensure firm contact with the agar Surface, and incubated at 37oC for 24 hours.
Following this the diameter of inhibition zone formed around each disk was measured using a black surface, reflected light and transparent ruler by lying it over the plates.

The results were classified as sensitive, intermediate, and resistant according to the Standardized table supplied by the manufacturer (CLIS, 2017)

ANNEX 7: Antibiotic susceptibility test result recording sheet

	Plate 1		Plate 2		Plate 3	
Antimicrobial disk	Zone diameter	category	Zone diameter	category	Zone diameter	Category
Ampicillin						
Ceftriaxone						
Chlorampnicol						
Nyomycin						
Oxy tetraciclin						
Streptomyocin						
Sulphonamid						
Trimethoprime						

ANNEX 8: Antimicrobial susceptibility test interpretive criteria for Enterobacteriaceae

No.	Antimicrobial Agents	Disk concentration (-g)	zone diameter (nearest whole mm)		
			Resistant	Intermediate	Susceptible
1.	Ampicilin (AMP)	10μg	≤ 13	14-16	≥ 17
2.	Ceftriaxone (CRO)	30 μg	≤ 19	20-22	≥ 23
3	Chloromphenicol (C)	30 μg	≤ 12	13-17	≥ 18
4.	Nyomycin (NEO)	10 μg	≤ 12	12-16	≥ 17
5.	Oxy teteracyclin (OT)	30 μg	≤ 14	15-16	≥ 17
6.	Streptomycine (S)	25 μg	≤ 11	12-14	≥15
7.	Sulphonamides (S3)	300 μg	≤ 12	13-16	≥ 17
8.	Trimeethoprime (W)	5 μg	≤ 10	11-15	≥16

Source: Clinical Laboratory Institute Standards (CLIS, 2017)

ANNEX 9: flow diagram of the entire study

Buffered Peptone Water for enrichment of fecal sample

25g of fecal sample in 225ml BPW or at 1:9 ratios incubated (37^0c for 24hrs)

⇩

Isolation

A loop full of enriched culture broths on MCA with an inoculation loop incubated (370c for 24-48hrs)

Supposed isolates from MCA to EMB plate with an inoculation loop incubated (370c for 24-48hrs)

Isolated colonies to nutrient agar incubated (370c for 24hrs)

⇩

Biochemical taste

Triple sugar iron agar test

Take one isolate colony from MacConkey and inoculated in the middle of the agar with a Straight inoculating Wire incubated at 37^0c for 18 hrs.

IMVIC test

Indole test: *E. coli* isolates inoculated to Trypton broth and incubated at 37^0c for 48 hrs.

Methyl red test: *E. coli* isolates inoculated to MR – VP broth and incubate at 370c for 48 hrs

Voges-proskauer (Vp) test: *E. coli* isolates inoculated to MR –VP broth and incubate at 370c for 48 hrs

Citrate utilization test: *E. coli* isolates inoculated to citrate slant and incubate at 370c for 48 hrs

⇩

Antimicrobial sensitivity test

Pure fresh cultures were transferred in to a test tube of 5 ml tryptone soya broth (TSB) and incubate at 370c for 6 hrs

Swabbed the culture on the Surface of Mueller-Hinton agar plates and placed the Antibiotic disk and incubate at 370c for 24 hrs

⇩

Virulent Gene Detection

DNA extraction (boiling method)

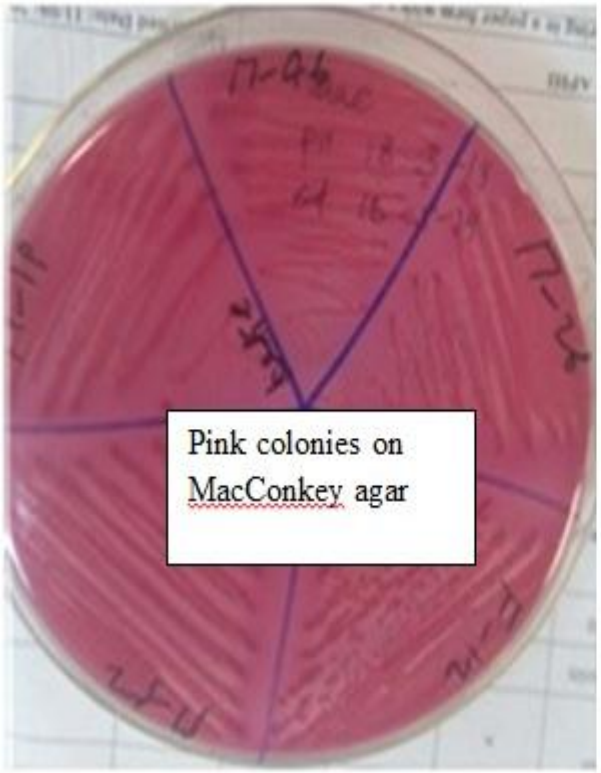
Detection of Virulent Gene Sequences by PCR

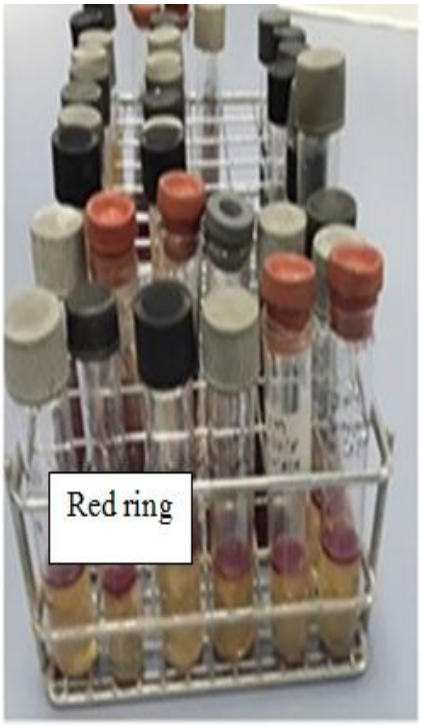
Analyzed by gel electrophoresis

ANNEX 10: List of photos captured during the entire work

Photo during fecal sample collection

during Culturing of extracted enrichment

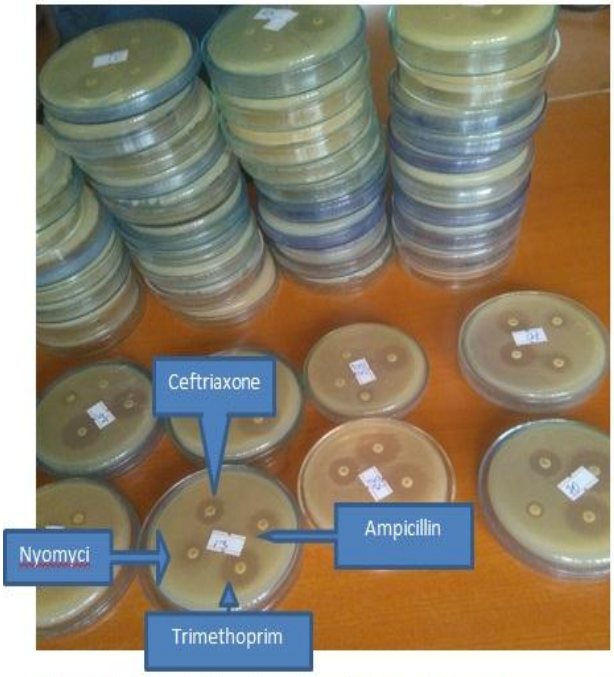




E. coli isolates with Indol positives

Methyl red positives and Voges-proskauer negative

Citrate negative



Disc diffusion test of *E. coli* isolate on Mueller Hinton agar

Swabbing of *E. coli* isolates on Mueller-Hinton agar plates

Water bath during DNA Extraction

During PCR Master mixing at hood

Prima- 96 plus PCR machine

Amplified PCR products to gel electrophoresis

Visualizing through (Bio-Rad) and imaged with gel documentation system