JIMMA UNIVERSITY

COLLEGE OF AGRICULITURE AND VETERINARY MEDICINE

SCHOOL OF VETERINARY MEDICINE

ISOLATION AND MOLECULAR CHARACTERIZATION OF PATHOGENIC *Escherichia coli* STRAINS, THEIR ANTIMICROBIAL SUSCEPTIBILITY PATTERNS AND ASSOCIATED RISK FACTORS IN DIARRHEIC CALVES AND CHILDREN AT WOLAITA SODO TOWN, SOUTHERN ETHIOPIA

BY

AMANUEL WOLDE

OCTOBER, 2018

JIMMA, ETHIOPIA

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MSc Thesis

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

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October, 2018

Jimma, Ethiopia

DEDICATION

This thesis is dedicated to my beloved mother Adinke Eriso and my father Wolde Waniso for laying foundation of my education.

STATEMENTS OF THE AUTHOR

First, I declare that this thesis is my bona fide work and that all sources of materials used for this thesis have been duly acknowledged. It has been submitted in partial fulfillment of the requirements for MVSc degree at Jimma University and is deposited at the University Library to be made available to borrowers under rules of the Library. I solemnly 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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Place: Jimma University, College of Agriculture and Veterinary Medicine

Date of Submission: ----------

BIOGRAPHICAL SKETCH

Amanuel was born in April 04, 1989 from his father Wolde Waniso and his mother Adinke Eriso in Gibe woreda, Hadiya Zone, Southern Nation Nationalities and People Regional State (SNNPRs), Ethiopia. He attended his primary education (1-6) in Sade Elementary School in Sade district and junior education (7-8) in Shurmo Junior and Elementary School starting from 1997 years. He attended his secondary education (9-10) in Yekatit 25/67 Secondary School and he also attended his preparatory education (11-12) in Wachemo Senior Secondary School at Hossaena until 2008. After he completed his preparatory school he joined Jimma University College of Agriculture and Veterinary Medicine in 2009 and he awarded with Doctor of Veterinary Medicine (DVM) degree in Veterinary Medicine in June 2, 2014. After graduation, he was employed in Gibe Woreda Livestock and Fishery Resource Office in Hadiya Zone SNNPRs as Veterinarian from July, 2015-2016 for two consecutive years. He served as Veterinarian till he joined Jimma University; College of Agriculture and Veterinary Medicine to pursue Master of Science (MVSc) degree in Veterinary Microbiology in 2017.

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LIST OF ABBREVATIONS AND ACRONYMS

A/E	Attaching and Effacing
AOR	Adjusted odd ratio
CA	Colostrums awareness
CAM	Contact with either animals or manure
CFU	Colony forming unit
cGMP	Cyclic guanine monophosphate
CI	Confidence interval
COR	Crude odd ratio
DAEC	Diffusely adherent *E. coli*
DDW	Double distilled water
DEC	Diarrheagenic *Escherichia coli*
df	Degree of freedom
DNA	Deoxyribonucleic acid
EBMF6M	Exclusive breast milk feeding in six month.
E. coli	*Escherichia coli*
eae	*Escherichia coli* attaching and effacing gene
EDTA	Ethylene diamine tetraacetic acid
EIEC	Enteroinvasive *E. coli*
EPEC	*Enteropathogenic Escherichia coli*
et.al.	*et alii*/ *Alia* (and other people)
EtBr	Ethidium Bromide
ETEC	Enterotoxigenic *E. coli*
EVC	*Escherichia coli* verocytotoxin
GTPase	Guanine triphosphatase
H	Flagellar antigen
IMViC	Indole, Methylred, Vogaues prousker, citrate
L	Litre
LEE	Locus of enterocyte effacement

LT1	Heat labile toxin type one
M.a.s.l	Metre above sea level
MDR	Multi drug resistance
MDRI	Multi drug resistance index
MDa	Megadalton
MF	Method of feeding
NHWBM	Negligence to hand washing before meal
NT	Navel treatment
O	Somatic antigen
P	Probability
PCR	Polymerase chain reaction
Rpm	Revolution per minute
SFT	Supplementary feed type
STEC	Shiga toxin-producing *Escherichia coli*
SIM	Sulfide indole motility
SPSS	Stastical procedure for social science
St	Heat stable toxin
Stx	Shiga toxin
*Stx*1 and *Stx*2	Shiga toxin gene one and two
TBE	Tris base boric acid and EDTA
TD	Types of diarrhea
TFCF	Time of first colostrums feeding
Tir	Translocated intimin receptor
TSI	Triple sugar iron
$\chi 2$	Chi-square

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ABSTRACT

Pathogenic *E. coli* strains cause diarrheal infection in both calves and children due to their virulence factors.Their distribution is not well studied in Ethiopia, especially in current study area. Therefore, a cross sectional study was conducted from October 2017 to June 2018, with aim to isolate, molecularly detect pathogenic *E. coli* strains, to determine their antimicrobial susceptibility patterns and associated risk factors in diarrheic calves and children at Wolaita Sodo town. Purposive sampling technique was used to collect 88 feacal samples from diarrheic calves and 110 stool samples from diarrheic children. Semi-structured questionnaire, conventional culture, biochemical and molecular methods were conducted to achieve the objectives. Antimicrobial susceptibility patterns of the isolates were tested using the Kirby-Bauer disc diffusion method. All of the data were analysed using logistic regression in SPSS vertion 20. The overall isolation rate of *E. coli* in calves was 64.8% (57/88) (95% CI: 53.9-74.7%). The occurrence of the bacterium differed significantly by age and colostrum feeding time (*P<0.05*). The odds of being infected was highest in calves aged below two weeks (OR 4.3; 95% CI 34.8- 56.4%; *P*=0.001). The overall isolation rate of *E. coli* in children was 61.8% (68/110) (95% CI: 52.1-70.9%). The occurrence of the bacterium differed significantly by age, contact with either animals or manure, negligence to hand washing before meal and exclusive breast feeding in six month (*P<0.05*). The odds of being infected was highest in children whose caretakers had habit of negligence to hand washing before meal (OR 6; 95% CI 30.8- 49.8%; *P*=0.01). For eleven drugs, *E. coli* isolates were resistant to (62.5%, 61.9%), intermidiately resistant to (3.5%, 9.4%) and susceptible to (34%, 28.7%) in calves and children, respectively. The overall virulent genes detected in calves and children was 77.2% (44/57) (95% CI 53.9-74.7%); and 55.9% (38/68) (95% CI 52.1%-70.9%), respectively. Among these *stx1* [8 (14%), 6 (8.8%)] *stx2*, [5 (8.8%), 4 (5.9%)], *eaeA* [8 (14%), 5 (7.4%)], *eaat* [6 (10.5%), 14 (20.5%)] and St [5 (8.8%), 4 (5.9%)] were detected from calves and children, respectively. The MDR for *E. coli* isolates and their strains was 34 (59.7%) and 31 (70.5%), respectively in calves and 43 (63.2%) and 38 (100%) respectively in children.The MDR index value of DEC pathotypes was greater than or equal to 0.4 which indicates the high risk of resistance.The study demonstrated important pathogenic *E. coli* strains; and multi-drug resistance mostly in isolates containing more than one virulent gene. Wise use of antimicrobials and improving the hygienic practices in the farms and amongst parents of children reduces its occurrence.Therefore, awareness on importance of exclusive breast feeding to parents of children and colostrums feeding time for calves to farm owners should be maximized. Appropriate usage of antimicrobial agents should also be highly practiced among farms and hospitals.

Keywords: *Antibiotic susceptibility patterns, Gel electrophoresis, Molecular detection, Pathogenic E. coli strains, Polymerase Chain Reaction, Risk factors, virulent genes, Wolaita Sodo town*

1. INTRODUCTION

1.1. Background

Livestock is an integral part of the agricultural production system in any country and plays an important role in national economy as well as in socio-economic development of millions of rural household (Radostits *et al*., 2000). Despite the large livestock population of Ethiopia, the economic benefits remain marginal due to prevailing diseases, poor nutrition and animal production, reproductive in efficiency, management constraints, and general lack of veterinary care (Sissay *et al*., 2008). The future of any dairy and beef farms depends on the successful raising of calves and heifers for replacement. However new born animals suffer fairly higher mortality than their adult counterparts and it is one of major reprisal over economy in livestock industry (Radostits *et al*., 2000).

Diarrhea in farm animals, especially in neonatal calves is one of the most challenging clinical signs encountered by large animal veterinary practitioner and it has negative impacts on productivity and fertility of herds (CACC, 2003). It is also one of the major causes of morbidity and mortality among children less than 5 years of age in developing countries with global mortality rates estimated to be around 1.87 million (Kosek *et al*., 2003; Boschi-Pinto *et al*., 2008). Also diarrheal diseases are a leading cause of childhood morbidity and mortality in developing countries, particularly among infants and young children accounting for around 2 million deaths annually (Vilchez *et al*., 2009). It was estimated that in 2010 there were more than 1.7 billion episodes of diarrhea worldwide from which 700,000 led to death in children less than 5 years of age (Liu *et al*., 2012 and Walker *et al.*, 2013).

Diarrheagenic *Escherichia coli* (DEC) are reported to be a common cause of childhood diarrhea in developing countries, with a higher incidence during the first 2 years of life according to Presterl *et al*. (2003) and it is an important agent in both calves and children mostly in younger than 5 years, and it represents a major public health problem in these areas (Trevejo *et al*., 2005; Tobias and Vutukuru, 2012 and Taniuchi *et al*., 2012).

Among the etiological agents of diarrhea in humans and animals in developing countries DEC is predominant one. *Escherichia coli* are genetically the most versatile bacteria and are the

source of many plasmid and phage mediated genes. Even though its members are typically non pathogens that are a part of the normal microflora of the intestinal tract of humans and animals, certain subsets of this bacterial species have acquired genes that enable them to cause intestinal or extra intestinal disease (Abdella *et al.*, 2009 and Belanger *et al*., 2011).

Diarrheagenic *Escherichia coli* are identified and differentiated from *E. coli* of the intestinal flora by the presence of specific virulence markers (Moyo *et al.*, 2007). This bacterium which is classified under the family *Enterobacteriaceae*, is gram negative, rod shaped, flagellated, motile, oxidase negative, and facultative anaerobic. It produces septicemia and diarrhea in a wide range of hosts including man, avian and animals (Darsema, 2008; Mengesha *et al*., 2013).

Pathogenic *E. coli* strains are divided into intestinal pathogens causing diarrhea and extraintestinal *E. coli* (ExPEC) causing a variety of infections in both humans and animals including urinary tract infections, meningitis and septicemia (Kaper and Nataro, 2004). An altered movement of ions and water following an osmotic gradient is at the heart of diarrheal diseases. Under normal conditions, the capacity of gastrointestinal tract to absorb fluid and electrolytes is tremendous and from 8-9 liters of fluid presented to intestine daily, only 100-200 ml are excreted in the feaces. Enteric pathogens, however, can alter this balance towards net secretion leading to diarrheal disease (Hodges and Ravinder, 2010).

Pathogenic *E. coli* causing diarrhea in calves and children are also further divided into two types- Enteropathogenic *E. coli* and Uropathogenic *E. coli.* Uropathogenic *E. coli* cause 90% of the urinary tract infection. Based on their specific virulence factors, phenotypic traits and mechanisms by which they cause disease DEC strains are divided into six pathotypes as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Vero toxin-producing/Shiga toxin-producing *E.coli* (VTEC/STEC) which include its well-known subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Schmidt., 2010).

The identification of DEC types requires differentiation from non-pathogenic members of the animal and human normal intestinal flora. Molecular identification and classification of DEC is based on the presence of different chromosomal and/or plasmid-encoded virulence genes that are absent in commensal *E. coli*. Moreover, the prevalence and other epidemiological features of these pathogens as causative agents of diarrhea vary in the world from region to region, and even between and within countries in the same geographical area (Kaper *et al*., 2004; Nguyen *et al*., 2005; Nataro *et al*., 2006).

New strains of *E. coli* arise all the time from the natural biological process of genetic variability and hence monitoring the levels of *E. coli* contamination is important. The different virulence groups of DEC can be detected by culture, biochemical reactions, serotyping, and other phenotypic assays, as well as molecular methods (Nataro and Kaper, 1998). Polymerase chain reaction (PCR) has proved to be more sensitive and specific than most conventional techniques and has been used to identify DEC in a number of studies (Estrada-Garcia *et al*., 2009).

The risk factors for the occurrence of calf diarrhea due to *E. coli* includes environmental, nutritional, and management factors such as calves being born from a heifer, being born during the summer, suckling, low serum IgG concentrations, and large herd size (Svensson *et al.*, 2006 and Trotz *et al.*, 2008a). Current treatment option for the treatment of neonatal calf diarrhea due to pathogenic *E. coli* strains rely on antimicrobial agents and fluid therapy. Recently published studies described an increased incidence of resistance for different antimicrobials (Orden *et al.*, 2000).

1.2. Statement of the Problems

Calf diarrhea is the commonest disease in young calves which causes of calf morbidity and is the greatest single cause of death. It accounts for approximately 75% of the mortality of dairy calves with age below three weeks of age (Radostits *et al*., 2007). The incidence of diarrhea in calves under 30 days of age varies between 10% and 20% according to Svensson *et al.* (2003).

The high frequency and persistence of calf diarrhea in farms and childlhood diarrhea has gained the interest of many reasearchers. In central and south Eastern part of Ethiopia, limited researches had been conducted on calf mortality rate and supposed infectious agents. A few studies show mortality in the first six months of calf hood ranging 7 to 25% (Wudu *et al*., 2008; Bekele *et al*., 2009). *Escherichia coli* isolate in calf diarrhea accounts 36.8 % (Gebregiorgis and Tessema, 2006) in Kombolcha district, 50.9% (Yakob, 2014) in Arsi zone, 69.5% (Yimer, 2014) in North Shewa zone. In southern part of the country, particularly at Wolaita Sodo town the information on cause of diarrhea in calves and children with its associated risk factors are minimal.

The over all isolation rate of *E. coli* from children under five years of age was 48.3% in study conducted by Adugna *et al*. (2015). Other reports in Hawassa (Getamesay *et al*., 2014), Bahir Dar (Yemane *et al*., 2014), Jimma (Beyene and Tasew, 2014) and Ambo (Wagi, 2015) towns indicated *Campylobacter*, *Shigella* and *Salmonella* were isolated enteric bacterial pathogens as cause of diarrhea in children; however, the pathogenic *E. coli* strains causing diarrhea in calves and children are still not defined.Therefore it is important to identify the etiological and predisposing factors involved in calf diarrhea in order to increase the productivity per livestock unit without increasing livestock numbers, and devise preventive measures and reduce losses during the initial months of life (Lorino *et al*., 2005).

Understanding the population structure of pathogenic *E. coli* is important since it affects the effectiveness of molecular epidemiological studies. Molecular detection of the supposed pathogenic *E. coli* strains isolated from diarrheic calves and children in Ethiopia is limited. This hinders implementation of effective control and preventive measures (Nguyen *et al*., 2005).

Antibiotic resistance to bacteria is a serious and growing phenomenon and has emerged as one of the pre-eminent public health concerns of the 21st century. Due to the practice of under dosing, over dosing as well as indiscriminate usage of drugs, most pathogenic bacteria that are commonly involved in causing infection to human and animal have shown considerable degree of resistance to commonly used antimicrobials in this country (DACA, 2009).

1.3. Significance of the Study

Current study has significant role in improvement of management practices in dairy farms; hence it reduces the high level of calf diarrhea due to *E. coli* by taking account special emphasize on the time of colostrums feeding, the hygiene of calf house and isolation of sick calves. Similarly it provides information to parents of children about major transmission ways of DEC infection, which helps them to take necessary control measures; hence it reduces child morbidity and mortality due to DEC infection. Antimicrobial susceptibility patterns was studied in current work, which aids to choose an appropriate drugs in treatment of DEC infection in both calves and children; so that it minimizes the emergence and spread of resistant strains. Furthermore it provides information about type of pathogenic *E. coli* strains which are root causes of diarrhea in calf and children in the present study area.

Due to absence of previous work on pathogenic *E. coli* strains, associated risk factors and their antimicrobial susceptibility paterrns in child and calf diarrhea at Wolaita Sodo town, this research was proposed to study on this issue with the following objectives.

1.4. Objectives

1.4.1 General objective

To isolate and molecularly detect pathogenic *E. coli* strains and their antimicrobial susceptiblity patterns in diarrheic calves and children as well as determining their associated risk factors at Wolaita Sodo town.

1.4.2 Specific objectives

- ✓ To isolate, and molecularly detect pathogenic *E. coli* strains from diarrheic calves in dairy farms and diarrheic children admitted to Sodo Christian Hospital at Wolaita Sodo town.
- ✓ To determine risk factors associated with occurrence of diarrhea due to pathogenic *E. coli* in calves in dairy farms and children admitted to Sodo Christian Hospital.
- ✓ To determine antimicrobial susceptiblity patterns of the isolates from diarrheic calves and children.

2. LITERATURE REVIEW

2.1. General Overview of *Esherichia coli*

2.1.1. Historical background

A Bavarian pediatrician, Theodor Escherichia, first described *Escherichia coli* 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 (Kaper, 2005). It is 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 (Frydendahl, 2002).

Escherichia coli are an important member of the normal intestinal micro flora of humans and other mammals; it has also been widely exploited as a cloning host in recombinant DNA technology. However, *E. coli* is not a laboratory workhorse or harmless intestinal inhabitant; it can also be a highly versatile and frequently deadly, pathogen (Kaper *et al*., 2004). Moreover, *E. coli* is well known with the high genetic flexibility to acquire and/or transfer resistance or virulence genes from or to other strains of *E. coli* as well as other organisms (Hoyle *et al.*, 2005; Halawani, 2010).

2.1.2. Growth characteristics

Escherichia coli are a facultative anaerobe. Though most *E. coli* strains are capable of growing over a wide range in temperature (approximately 15-48°c), the growth rate is maximal in the narrow range of 37-42°c. It can grow within a PH range of approximately 5.5-8.0 with best growth occurring at neutrality. 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 (Fotadar *et al*., 2005).

Most *E. coli* strains can ferment D-mannitol, D-sorbitol, and Larabinose, maltose, D-xylose, trehalose and D-mannose. There are limited instances where pathogenic strains differ from the

commensals in their metabolic abilities. For example, commensal *E. coli* strains generally use sorbitol, but *E. coli* O157:H7 does not. Most diarrheagenic strains cannot utilize D-serine as a carbon and nitrogen source, but uropathogenic and commensal fecal strains can use this enantiomer of serine (Roesch *et al.*, 2003).

It is capable of reducing nitrates to nitrites. When growing fermentatively on glucose or other carbohydrates, it produces acid and gas (mainly H2 and CO2). By traditional clinical laboratory biochemical tests, *E. coli* is positive for indole production and the methyl red test. Most strains are oxidase, citrate, urease and hydrogen sulfide negative. The classic differential test to primarily separate *E. coli* from *Shigella* and *Salmonella* is the ability of *E. coli* to ferment lactose, which the latter two genera fail to do (Fotadar *et al*., 2005).

2.1.3. Mode of transmission

The primary mode of transmission for enteric pathogens is the fecal-oral route. *Escherichia coli* can also be transmitted by direct and indirect contact with infected animal's fur, hair, skin, and saliva, consumption of contaminated animal products, consumption of feeds and water contaminated by animal feacal material, or exposure to insect vectors such as fleas or ticks (Keen and Elder, 2002; Varma *et al*., 2003; Marler, 2016). Another route for acquiring the infection in human is direct transmission from cattle, especially calves, for instance on 'open farms' where groups of children are welcome to visit. As the bacterium survives well in the environment, drinking water, vegetables irrigated with contaminated water and public outdoor swimming pools have been mentioned as sources of community outbreaks (Gylers, 2007).

2.2. 4. Hosts and reservoirs of Escherichia coli

Escherichia coli is generally considered as commensal member of the normal intestinal micro flora however pathogenic strains can cause intestinal and extra intestinal infections in mammalian and avian hosts. Animal and farm environments are known reservoirs for *E. coli* O157 and other pathogenic *E. coli* serotypes across the world. Cattle is highly natural reservoir of *E. coli* besides other animal species such as sheep, pigs, deer and wide bird have also been implicated. The transmission of this pathogen to humans can occur through the

direct contact with infected animals, foods of animal origin such as meat or unpasteurized milk, or through water contamination. Because cattle are carriers of many different serotypes of *E. coli*, much emphasis has been placed on the public health and food safety concerns associated with the fecal shedding of this organism (Moxley and Smith, 2010).

Neonates and post weaned calves appear to be more frequently colonized by O157 with prevalence declining thereafter. Commensal *E. coli* inhabiting the gastrointestinal tract of farm animals or present on animal products are regarded as potential of resistance genes. Monitoring of genetic diversity of this bacterium derived from farm animals is important for many reasons. *Escherichia coli* can enter a dairy farm environment through new herd members and environmental media such as air, water, soil and wildlife or organic materials such as cattle feed and bedding. Once an *E. coli* strain has entered the herd, it can persist in the animals' intestines and can be excreted in the environment (Erikson *et al*., 2005; National Bio-Resource Project, 2007).

2.1.5. Pathogenesis and virulence factors of Pathogenic Escherichia coli

The virulence factors of pathogenic strains of *E. coli* include capsules, structures responsible for colonization, endotoxins and other secreted substances. Two types of enterotoxins, heat labile (LT) and heat stable (ST) have been identified and each type of enterotoxin has two subgroups. Many strains of Enterotoxigenic *E. coli* (ETEC) from pigs produce LT1 which induces hypersecretion of fluids into the intestine through stimulation of adenylate cyclase activity (Quinin *et al*., 2011). Most ETEC isolates which produce LT1 also possess K88 adhesins. *Escherichia coli* strains, containing virulence factors such as a heat-stable enterotoxin (STa) and fimbria such as F5 (K99) and F41, are mainly associated with diarrhea in newborn calves. The F17 fimbrial family was also detected on bovine pathogenic *E. coli* isolates implicated in diarrhea or septicemia in the infected animals (Mercado *et al*.**,** 2003 and Guler *et al*., 2008).

Pathogenicity of *E. coli* strains are due to the presence of one or more virulence factors including invasiveness factors like invasins, heat labile, heat stable enterotoxins, verotoxins and colonization factors or adhesions. General steps in the pathogenic process include

ingestion which is followed by bacterial attachment via pili fimbriae, or fibrils. This results in colonization of the ileal mucosa, then expression of bacterial mechanisms to allow evasion of host defenses and finally multiplication of bacteria and damage of host tissue (Kaper *et al*., 2004; Donnenberg, 2005).

All diarrheagenic strains can colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut. Pathogenic and nonpathogenic *E. coli* bacteria have surface adherence fimbriae, but diarrheagenic *E. coli* strains have specific fimbrial antigens that adhere to the small bowel mucosa, a site that is normally not colonized. The three general ways in which *E. coli* may cause diarrhoea is by enterotoxin production, invasion, and/or intimate adherence with membrane signaling (Nataro and kaper., 1998). Virulence attributes of enterotoxigenic *E. coli* include the adhesion of their pili or fimbriae to intestinal epithelial cells to prevent peristaltic elimination by the gut, and the production of STa and LT entrotoxins (Quinn *et al.*, 2001).

Three pathotypes of *E. coli* (EHEC, EPEC and EIEC) employ a T3SS to translocate bacterial proteins, known as effectors, directly into the eukaryotic host cell in order to subvert animal and human host cell processes. The non-T3SS dependent pathotypes of enteric *E. coli* (ETEC and EAEC) have comparatively simple and efficient molecular mechanisms of virulence requiring effective colonization factors followed by secretion of toxins that subsequently enter the host cell (Clements, 2012).

Mechanism of Enterotoxigenic Escherichia coli pathogenicity

On the tip of flagella, EtpA is located which attaches to host cells which is then degraded by the SPATE EatA. Adherence is maintained by colonization factors (CF) and intimate attachment achieved with Tia and the autotransporter TibB. Heat stable toxin (ST) is secreted by ETEC and binds to guanylate cyclase-C receptor increasing cGMP and cGMP-dependent protein kinase II. Heat labile toxin (LT) is contained in outer membrane vesicles, which are endocytosed after interaction with ganglioside receptors (GM1) (Vaandrager, 2002). Retrograde transport through the Golgi and ER leads to the A1 subunit being released in the

cytosol where it can ADP ribosylate mammalian guanine nucleotide binding protein a-subunit (Gsa) inhibiting the GTPase activity of Gsa and activating adenylate cyclase resulting in uncontrolled cAMP levels. Cyclic adenine mono phosphate (cAMP) and cGMP both contribute to phosphorylation of the cystic fibrosis transmembrane regulator chloride channel and modulation of other ion channels leading to osmotic diarrhea (Kesty, 2004).

Mechanism of Enteropathogenic Escherichia coli and Enteroheamorrhagic Escherichia coli pathogenicity

Intimate adherence (fig 1A): The translocated intimin receptor (Tir) binds intimin on the bacterial surface to initiate intimate attachment, actin accumulation and pedestal formation. Actin remodeling (fig 1B): Map, EspM and EspT activate Rho GTPases leading to filopodia, stress fibers and ruffles/lamellipodia respectively (Touze, 2004). (Fig 1C) Disruption of gut integrity: Tir, Map, EspF and EspB contribute to effacement of the normal absorptive microvilli. Map, EspF and EspI disrupt tight junction (TJ) integrity and epithelial barrier function. (Fig 1D) Manipulating immune responses: NleB, NleC, NleD NleE and NleH inhibit inflammatory responses through targeting NFkB, JNK and p38 pathways (Nadler *et al*., 2010). (Fig 1E) Balancing apoptosis and survival: Pro-apoptotic EspF causes mitochondrial dysfunction leading to activation of apoptotic pathways while Cif causes cell cycle disruption. (Fig 1F) Inhibiting phagocytosis: EspF, EspB, EspH and EspJ inhibit phagocytosis by macrophages through disruption of PI3K signaling, myosin-actin interactions, Rho GTPase signaling and an unknown mechanism respectively (Clements, 2012).

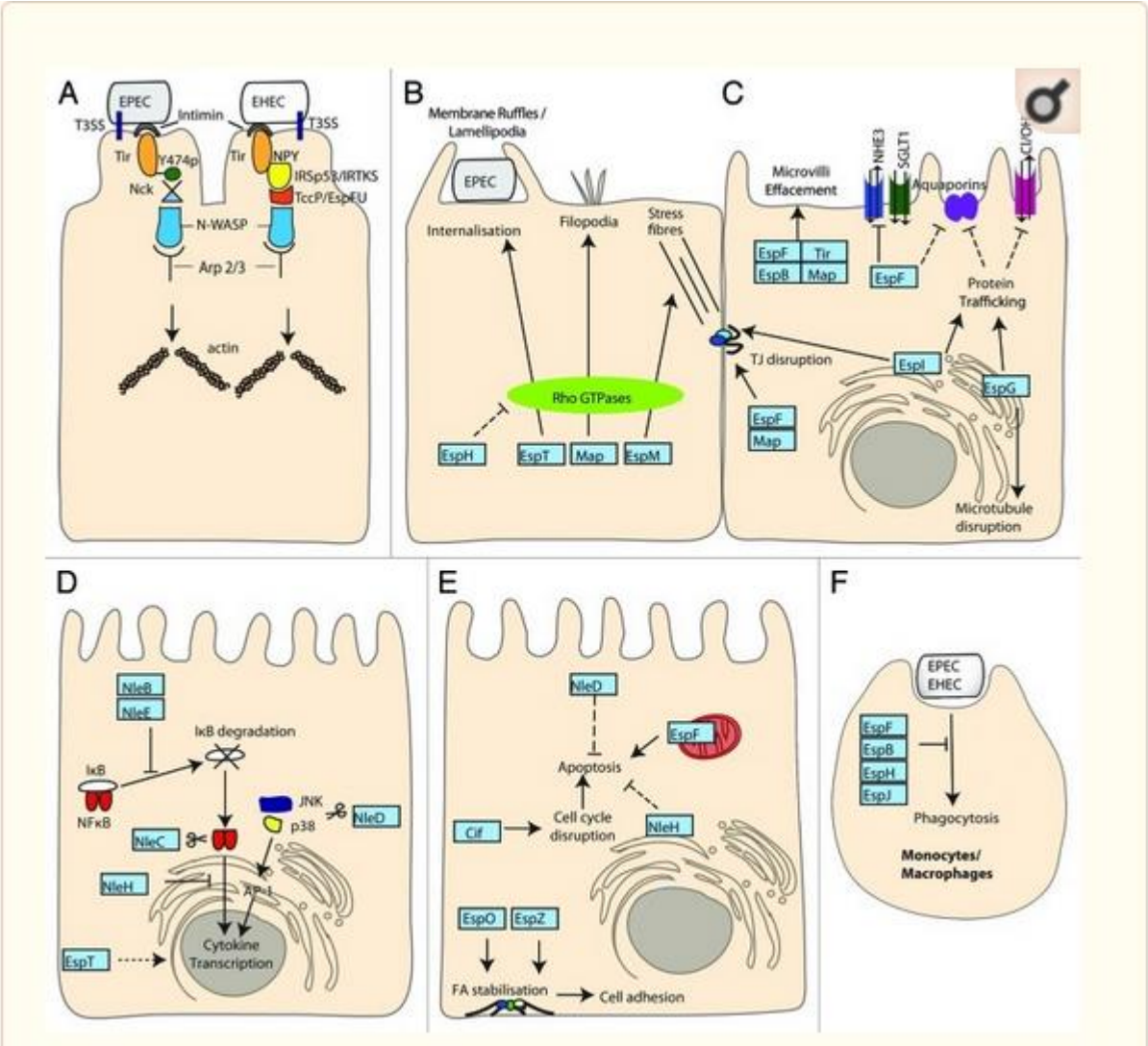

Figure 1. Mechanism of Enteropathogenic *Escherichia coli* and Enteroheamorrhagic *Escherichia coli* pathogenicity

Source. (Clements, 2012).

2.2. Diarrhoeagenic *Escherichia coli* pathotypes

2.2.1. Enterotoxigenic Eschericia coli (ETEC)

The Enterotoxigenic *E. coli* strains are known to produce two types of enterotoxis that causes diarrhea. One type is characterized by having larger molecular weight, heat-labile (LT) and possesses immunogenic quality. This type causes delayed onset of secretory response on intestinal mucosa which persists for a longer duration. The second type is of smaller

molecular weight, heat- stable (STa and STb) toxin that are apparently non immunogenic. The genes ST1and LT1 may be carried by ETEC strains on either plasmids or transposons that can be inserted into plasmids or the bacterial chromosome (Kaper *et al*., 2004).

Enterotoxigenic *E. coli* produces profuse watery diarrhea that are mainly a problem in calves up to 4 days old, although they can occasionally produce diarrhea in older calves too. Enterotoxigenic *E. coli* strains produce plasmid-mediated enterotoxins, which bind to their specific receptors on the gut epithelium and by a complex interplay of biological mediators cause diarrhea (Naylor, 2002).

The osmotic diarrhea is due to secretary effect at the crypt cells and inhibition of absorption by villous tip cells. Several types of enterotoxins are identified and a single ETEC may be capable of producing one or more enterotoxins. Both heat-labile and heat stable enterotoxins are found in ETEC. In calves, ETEC producing the low molecularweight STa cause the majority of neonatal diarrhea problems (Trabulsi *et al*., 2002). This is the mostrecognized diarrhoeagenic *E. coli*.It is the most common bacterial cause of diarrhoea in children below five years in developing countries. Although not so common in developed countries, ETEC is the most common pathotype isolated from children having diarrhoea cases in developed countries (WHO, 2013).

2.2.2. Enteropathogenic Escherichia coli (EPEC)

Enteropathogenic *Escherichia coli* (EPEC), is an important paediatric diarrheal pathogen, employs multiple adhesins to colonize the small bowel and produces characteristic ‘attaching and effacing’ (A/E) lesions on small intestinal enterocytes. It is characterized by the presence of intimin (*eae*) gene causing attachment and effacement on intestinal epithelial cells and the bundle forming pili (*bfp*) gene (Cleary *et al.*, 2004). Typical EPEC is characterized by the presence of *eae* and *bfp* genes, while atypical EPEC possess the *eae* gene alone. Current classification of EPEC however, is based on the presence of specific virulence genes, which the use of molecular techniques has shown to be present in serogroup/serotypes other than classical ones as well (Ochoa *et al*., 2008).

2.2.3. Shiga toxin-producing/ Enterohaemorrhagic/ Eschericia coli (STEC/EHEC)

Genes coding for shiga toxins (*stxl* and *stx2*), attaching and effacing mechanisms (*eaeA*) and haemolytic mechanisms (*ehly*) mainly mediate the pathogencity of shiga toxigenic *E. coli.* Although some (*stxl*) positive STEC isolates have previously been associated with severe disease, epidemiological studies have demonstrated that (*stx2*) is the most important virulence factor associated with severe human disease (Beutin *et al.*, 2004). The Enterohaemorrhagic genome contains the same locus of enterocyte effacement (LEE) as the EPECs and the intimate attachment of EHEC to host cells occur through interaction between an adhesin called intimin and Tir (translocated intimin receptor). This intimate attachment induces the characteristic attaching and effacing lesions (A/E), but the initial adherence of EHEC to colonocytes is not well defined (Johnson and Nolan, 2009).

The main virulence factor and the defining feature of STEC is a phage-encoded potent cytotoxin the effect of which was shown to be neutralizable by anti-Shiga toxin of *Shigella dysenteriae* (Boulianne *et al.*, 2011). Shiga toxin-producing *E. coli* are zoonotic *E. coli* pathotype and more than 380 different O: H serotypes have now been isolated from humans with gastrointestinal disease and many of these as well as others have been recovered from animals. The high virulence of STEC strains such as O157: H7 is not only dependent on the virulence factors but partially also on the pathogen's ability to survive (Karmali *et al.*, 2010).

2.2.4. Enteroinvasive Eschericia coli (EIEC)

Enteroinvasive *E. coli* are almost indistinguishable from *Shigella* species because they share the same essential virulence factors and EIEC infections result in the symptoms of Shigellosis, namely diarrhea and dysentery. The invasive property of EIEC is determined by the presence of a 140-MDa virulence plasmid (*pInv*), which encodes a number of genes for invasion that includes *virf , ipah, ipal* genes (Gupta *et al.*, 2004).

Enteroinvasive *E. coli* pathogenesis is characterized by invasion of the submucosal cells but no toxins are released. Invasion is facilitated by a type III secretion system. Among other functions, plasmid encoded proteins are responsible for pore formation leading to cellular

uptake and signaling which results in the cytoskeletal rearrangements which permit movement within and between cells. The invasion plasmid antigen H (*ipaH*) is a specific marker that has been used to identify EIEC (Song *et al.,* 2005).The serotypes associated with EIEC include O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, and O173 of which O112ac, O124, and O152 are identical to O antigens present in *Shigella* species making identification on the basis of serotyping alone is inadequate (Lan *et al.,* 2004).

2.2.5. Enteroaggregative Eschericia coli (EAEC)

Diarrhea caused by EAEC is often watery, but it can be accompanied by mucus or blood. Adherence of EAEC to the mucosa is characterized by the formation of a thick, aggregating mucus layer inside which they survive and this biofilm production has been attributed to the activity of *fis* and *yafK* genes (Sheikh *et al.*, 2002). The aggregating nature of this pathovar has made serotyping in many cases impractical and the fraction that can be serotyped belong to a wide range of O: H types, making serotyping of little use in EAEC diagnosis (Harrington *et al.*, 2006).

2.2.6. Diffusely adherent Eschericia coli (DAEC)

Diffusely adherent *E. coli* is so named for the pattern of diffuse adherence to the surface of epithelial cells. It is characterized by the presence of (*dae*) gene, which is essential for the expression of fimbriae, a putative virulence factor that mediates adherence of this pathotype. They do not invade cells or produce toxins, but they have been associated with diarrhea in a number of studies. Modes of transmission of DAEC have not been clarified (Kaper *et al.,* 2004).

Diffusely adherent *E. coli* is a heterogeneous group that generates a diffuse adherence pattern on HeLa and HEp-2 cells and has been associated with the watery diarrhea that can become persistent in young children in both developing and developed countries as well as recurring urinary tract infections (Servin, 2005).

2.3. Antimicrobial Resistance Patterns of *Escherichia coli*

Antimicrobial resistance is an increasingly serouse concern in both human and animal health world wide (WHO, 2016). Diarrheagenic *E. coli* strains are being recognized as important pediatric enteropathogens worldwide. In recent years, antibiotic resistance of diarrheagenic pathogens has reached alarming proportions worldwide. The inappropriate use of antibiotics has been found to be the most important selecting force in the generation of bacterial resistance to antimicrobial drugs (Sawant *et al.*, 2007; Yah and Eghafona, 2008). Antimicrobial resistance may arise either spontaneously by selective pressure or due to antimicrobial misuse in feeding or treatment of animals by farmers, unskilled practitioners and lack of confirmatory diagnostic result of the patients both in humans and animals (Schroeder *et al.*, 2002; Bok *et al*., 2015). Also inappropriate prescription of antibiotics prompted resistance and increased infectious disease mortality not only in developing countries but also in developed countries. Aging populations, changes in behavior and a decline in the development of new antibiotics exacerbated a deteriorating situation (Dandekar, 2010). The spread of antibiotic resistant bacteria in the environment is dependent on the presence and transfer of resistance genes among microorganisms, mutations and selection pressure to keep these genes in a population (Arya *et al*., 2008).

Antimicrobial resistance *E. coli* have been described in food producing domestic animals and these can spread to humans through the food chain to create a potential risk for human health (Johnson *et al*., 2007). Diseases caused by *E. coli* often require antimicrobial therapy; however, antibiotic resistant strains of this bacterium cause longer and more sever illnesses than their antibiotic susceptible counterparts. Several studies have shown that antibiotic resistance in *E. coli* has increased over time (Cortese *et al*., 2009; Tadesse *et al*., 2012); however, data on the distribution of serogroups, pathotypes, virulence genes and the antimicrobial resistance in feacal *E. coli* from cattle is age dependent and *E. coli* from calves is significantly more resistant and often multi drug resistant compared to that in older cattle. Calves may therefore serve as reservoirs for these bacteria and their resistance genes (Yamamoto *et al*., 2013).

The presence of virulence factors can be closely connected with antimicrobial resistance among commensal *E. coli* isolated from cattle. Thus, it is of major public health significance related to risk of introducing these bacteria to the food chain (Kaper *et al*., 2004; Moxley and Smith, 2010). Chromosomal and plasmid born interogons have been identified as one of the crucial factors for the development of multidrug resistance in *Enterobacteriaceae* as well as many other bacterial species by harboring and lateral gene transfer of gene cassettes. *Escherichia coli* is one of several pathogens for which elevated mutation frequencies for antibiotic resistance genes have been described among natural isolates (Munita and Arias, 2016). Currently, different standardantimicrobial susceptibility testing methods are developed to describe the resistant level of different pathogenic microbes (CLIS, 2015).

Disk diffusion (Kirby Buer) method

The disc diffusion method for antibiotic susceptibility testing is the Kirby Bauer method using Muller Hinton agar. This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values. There is also a zone of intermediate resistance indicating that some inhibition occurs using this antimicrobial but it may not be sufficient inhibition to eradicate the organism from the body. Many conditions can affect a disc diffusion susceptibility test and certain things are held constant from test to test include the depth of the agar used, the amount of organism used, the concentration ofchemical used and incubation conditions (time, temperature and atmosphere). The amount of organism used is standardized using a turbidity standard. This may be a visual approximation using a McFarland standard 0.5 or turbidity may be determined by using spectrophotometer (optical density of 1.0 at 600nm) (CLSI, 2015).

2.4. Calf and Child Diarrhea

Diarrhea in newborn farm animals, particularly calves under 30 days of age, is one of the most common disease complexes that the large-animal clinician encounters in practice. It is a significant cause of economic loss in cattle herds and continues to assume major importance as livestock production becomes more intensified. The incidence of diarrhea in calves under 30 days of age varies between 10% and 20% (Svensson *et al.*, 2003 and Guler *et al*., 2008). In order to increase the productivity per livestock unit without increasing livestock numbers,

it is important to identify the etiological and predisposing factors involved in calf diarrhea in order to devise preventive measures and reduce losses during the initial months of life (Lorino *et al.*, 2005).

In the early 1980s, diarrheal disorders were the biggest child killers, responsible for an estimated 4-6 million deaths worldwide every year. Despite widespread use of oral rehydration therapies and an increased understanding of the pathogenesis of diarrhea, 2-5 million children still die from these illnesses every year, almost all of them in developing countries (Thapar and Sanderson 2004).

Eschericia coli can be isolated and classified as a cause of diarrhea in calves and children using traditional methods, i.e. identifying its biochemical or antigenic characteristics. The pathogenic mechanisms may be studied in cell cultures and animal method assays, as well as more up to date molecular biology methods for study and diagnosis. The latter have proven that genes are involved in pathogenesis (Rodriquez, 2002).

2.5. Status of Calf Diarrhea in Ethiopia

Studies conducted in problems of calf morbidity and mortality particularly on market oriented small holder farms in Ethiopia is very low. Study of (Wudu *et al*., 2008) revealed that crude calf morbidity and crude mortality in the frist six months of calf hood were 62% and 22%, respectively. According to (Bekele *et al*., 2009), the over all crude calf morbidity and crude mortality rates were 29.3% and 9.3%, respectively in small holder dairy farms in hawassa.

There are very few recent studies done to identify specific agents involved in calf diarrhea syndroms in Ethiopian dairy calves. Regarding on the viral cause of calf diarrhea in Ethiopia, Abraham *et al*., 1992) tried to identify that bovine enteric *Coronavirus* and Group A rota virus in combination with K99 Enterotoxigenic *E. coli* agents associated with neonatal diarrhea. Also (Yakob, 2014) reported that bacterial agents particularly *E. coli* (50.9%) and protozoan of *Eimeria* (38.9) and *Crptosporidium* (21.3%) were highly associated with calf diarrhea in the frist week of age in Arsi zone of Oromia region. In another study, (Yimer, 2014) indicated that the over all occurrence of *E. coli, Salmonella, Cryptosporidium, Emeria, and Giardia*

found in calf diarrhea were 69.5%, 25.7%, 38.1% and 22.9%, respectively in northen Shewa zone. More recently, (Gebregiorgis and Tessema, 2016) reported that *E. coli* accounted 37% of calf diarrhea in Kombolcha district of Amhara region. In over all, the available reports indicated that bacterial and protozoal agents were tried to be isolated and assumed as major infectious causes of calf diarrhea. 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.

2.6. Status of Child Diarrhea in Ethiopia

Federal Ministry of Health report in 2011 showed that the death of children under five years of age due to diarrheal disease in Ethiopia was 20% (FMoH, 2011) The study done in Eastern Ethiopia showed that children who had diarrhea two weeks before the interview yielded a prevalence of diarrhea followed by the age groups 12-23 months (Mengistie *et al*., 2013). Another study conducted in Nekemte town among under five years children the prevalence of diarrheal morbidity over a period of two weeks preceding was about 28.9% (Regassa *et al*., 2008). The study done in Kotabe Health Center showed that occurrence of childhood diarrhea among children who started supplementary feeding before six months was around four times higher when compared with thos less than six months and not started supplementary food yet. In that study, an association has been found between hand washing after cleaning the Childs bottom and diarrhea among under five children (Tilahun and Adrew, 2014). Another study done in Gilgel Gibe Field Research Center, Southwest Ethiopia, also showed that out of the total causes of death in post neonatal period an acute diarrheal disease accounted for 30% (Deribew *et al*., 2007). In general there are considerable diarrheal diaease reports in childhood in different parts of the country however its definitive causes molecular epidemiology report is limited.

2.7. Risk Factors for the Occurrence of Calf and Child Diarrhea

Risk factors for the occurrence of diarrhea include the consumption of contaminated food (Frenzen *et al.*, 2005), drinking or swimming in contaminated water and contact with livestock and other animals (CDC, 2005). The infectious syndrome of pathogen associated with non infectious factors related to its poor immune capability, a new born calf is vulnerable

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

Resistance of the calf to enteric disease is closely related to the timely consumption of high quality colostrums in sufficient quantities within the frist six hour after birth. Special care is also required to reduce environmental risk factors closely associated with calving season including the provition of dry, draft free shelter. Domestic and environmental factors including direct and indirect contact with animals, inadequate safe water, sanitation and hygiene account for the disease burden in children, or factors related to the mothers hygienic practices and knowledge (Fernandez *et al*., 2009).

The impact of feeding practices, in particular weening practices and breast feeding has also been addressed whereas other studies have investigated anthropometric nutritional status as sick factor for diarrhea in children (USAID, 2010).

2.8. Economic and Public Health Importance

Diarrhea caused by multidrug-resistant bacteria is an important public health problem among children in developing countries. Global, regional and national estimates clearly place diarrheal diseases as a major public health problem worldwide as it is responsible for approximately 4 billion cases of diarrhea per annum, of which 2 billion cases result in death (UNICEF, 2012). Increasingly, food animals and their products are being identified as important sources of infectious pathogens for humans. Many studies also showed that both healthy and diarrheic calves harbor STEC in their intestine and shed the bacteria for several months and in great quantities (Roopnarine *et al*., 2007). Sporadic cases or large STEC outbreaks in humans are associated with the consumption of raw or undercooked meat of food animals and other foods contaminated by animal faces, and by contact with STEC-positive animals or with their environment (Widirasih *et al.*, 2004). In addition to economic losses,

diarrhea in livestock is important because of the public health implications. Numerous infectious agents causing diarrhea in animals are zoonotic and have been associated with food-borne diseases (Trevejo *et al.*, 2005).

2.9. Detection of *Escherichia coli*

2.9.1. Isolation and identification of Eschericia coli

Many standardized methods for pathogens are available are considerd the reference analytical methods for official controls and often involve conventional methods. An isolation method for all pathogenic *E. coli* is outlined in the Food and Drug Administration Bacteriological Analytical Manual. The combined method is a general procedure for the isolation of *E. coli* before subsequent testing for specific virulence traits of different pathotypes given by ISO standards (Quinn *et al*., 2002 and Feng and Xiao, 2011). The methods recommend pre enrichment of 25g feacal sample in 225 ml buffered peptone water (BPW) at 37^0c overnight to facilitate resuscitation of sub-lethally injured cells. A loop full volume of en-riched broth is then plated on to MacConkey agar plates (colonies are brick red in color) and Levines eosin-methylene blue (L-EMB); colonie produce a metallic sheen) agar by incubating at 37^0c hour. Colony morphology, color and fermentation ability of different sugars may vary among pathogenic *E. coli* strains. It is important to note that this method is useful for isolating all the pathotypes of *E. coli* but not certain for STEC O157:H7 which is phenotypically distinct from other *E. coli* as it can exhibit a delayed fermentation of D-sorbitol and glucuronidase activity Feng and Xiao, 2011).

Biochemical characterization of Escherichia coli isolates

Escherichia coli are preliminary characterized by biochemical tests of indole, methyl red, and voges proskaeur and citrate utilization. In these tests, indole production from tryptophan (indole test), production of acetone (voges- proskaeur) and use of citrate as the only carbon source (citrate) are conducted. The isolates which exhibited IMViC pattern (++- -) respectively presumed as *E. coli* isolates. Both Methyl red and voges proskaeur test are done in methyl red Voges Proskaeur (MR-VP) broth, the reagents that can be added differs (Quinn *et al*., 2002).

Polymerase chain reaction

In order to categorize *E. coli* pathotypes, the presence of virulence genes needs to be identified. The use of PCR based technology to identify virulence genes has become widely adopted to distinguish pathogenic *E. coli* strains from normal gut flora (Natalia, 2015).

Polymerase chain reaction operates by amplifying a specific target DNA sequence in a cyclic three steps process. Firstly, the target double stranded synthetic oligonucliotides (specific forward and revers primers) will anneal to the DNA strands. This is followed by the polymerization process whereby the primers complementary to the single stranded DNA are extended with the presence of deoxyribonucleotides and a thermostable DNA polymerase. Then the PCR amplification products are visualized on electrophoresis gel as bands by staining with ethidium bromide (Mandal *et al*., 2011; Zhao *et al*., 2014). The concentration of primers and other important factors including the PCR buffer concentrations, the balance between magnesium chloride and Tag polymerase need to be adjusted to ensure the production of reliable PCR products (Cheah *et al*., 2008; Khoo *et al*., 2009).

2.10. Control and Prevention of Calf and Child Diarrhea due to Diarrghenic *Escherichia coli*

Disease control and prevention in animal increases productivity and maximizes the animal health and welfare. Some of the management practices found to play an important role in how resistant the calves are to infection include the prevention of dystocia, the reception of adequate amounts of colostrums of good quality and an appropriate diet therafter (Booker *et al*., 2008; Uhde *et al*., 2008). When ever diarrhea occurred in animal and in human an oral rehydration therapy, most important management part, is highly required to restore the fluid balance in the host and to avoid acidosis. However multiple factors, both infectious and non-infectious, are involved in calf diarrhea outbreaks, which makes disease control on farms difficult (Svensson *et al*., 2003; Trotz-Williams *et al*., 2007).

Studies of dairy herds have indicated that improved environmental management of calving pens and calf housing reduces calf diarrhea incidence and the extent of outbreaks. A system of "all in all out" calf housing, cleaning, steaming and disinfection of calf housing and calving pens, regular disinfection of utensils and adequate straw were identified as important

management factors. In a study, calf diarrhea incidence was reduced from 36 to 11% within a year by the introduction of early colostrum feeding and improved housing hygiene (McGuirk, 2008). Therefore, a thorough investigation should include a study of dry cow management and calving practice as well as calf management (Andrews, 2004).

The bovine placenta does not permit the passive transfer of antibody to the fetus. As a result, the newborn calf does not receive any antibody from the dam and is very susceptible to environmental pathogens. Resistance of the calf to enteric disease is closely related to the timely consumption of high-quality colostrum in sufficient quantities (Barrington and Parish, 2001). The neonatal calf should ideally receive 2~3 L (for beef calves) or 3~4 L (in dairy calves) of colostrum within the first 6 hr after birth (Cortese, 2009).

The intervention for reducing the incidence of calf diarrhea are based on decreasing pathogen exposure by planning to breed and heifers first calving, which reduces the exposure of more susceptible newborn calves to the into loading pathogens, reducing pathogen environment by shortening the calving season through scheduling breeding, which reduces the period of pathogen entry into the environment; and keeping a clean area (or pathogen-free area) by grouping animals according to their calving date so that the calving area can be kept clean after occupation by the previous calving group (Cho and yoon., 2014).

The use of antibiotics in diarrheic cases has been shown to be contra indicated in many studies due to further disruption of gut flora and the establishment of carrier states and antimicrobial resistance factors of enteric flora. If confronted with the task to choose an optimal and rational antibiotic therapy, several factors have to be considerd: (i) the most likely pathogens to cause the specific infection, (ii) the local susceptibility profiles for these microorganism, (iii) specific host related risk factors, (iv) the specific environment where the infection occurred and (v) Previous antibiotic therapies of the patient (Lorenz *et al.*, 2011).

Vaccination of dams against specific pathogens increases the concentration of specific antibodies against targeted pathogens in the colostrums (Cortese, 2009). Scours vaccines are formulated to be given to pregnant cows and heifers late in gestation so they will make the correct antibodies as colostrums is being formed while the others are designed for calves. The main effect of the antibodies is presented in the lumen of the small intestine while antibodies

in the blood stream seem to have little effect on preventing the symptoms of diarrheal disease (Dhama *et al*., 2009). Currently, commercial multivalent vaccines are available and the most popular are ScourBos9®, ScourGuard 4KC®, Guardian® (Michelle, 2009). The level of antibodies is well increased in the colostrums for most of these vaccines but often the clinical efficiencies vary between studies (Lorenz *et al*., 2011).

Modified ETEC vaccine consisting of recombinant *E. coli* strains over expressing the major cytotoxic factors and a more labile toxin like hybrid toxoid are undergoing clinical testing (Svennerholm, 2011; Farar *et al*., 2013). More recently, oral inactivated vaccines consisting of toxin antigen and whole cells, licenced recombinant cholera B subunit (rCTB) cholera vaccine Dukoral have been developed and showed high (85-100%) short term protection (Bourgeois *et al*., 2016).

The mainstay of diarrhal disease treatment in childhood is the assessement of dehydration and replacement of fluid and electrolytes too. The control of diarrheal disease in childhood is keeping the hygienic practices of lifestyle and minimizing direct and indirect animal contacts besides the non specific antibiotic therapy. Hand washing thoroughly after contact with animals or their their environments (at farms, petting zoos, fairs, even own backyard) and after, using the bathroom or changing diapers before preparing or eating food is important as transmission occurs trough fecal contamination of food and water suppliers (Roopnarine *et al*., 2007).

3. MATERIALS AND METHODS

3.1. Study Area Descriptions

The current research work was carried out in dairy farms and Sodo Christian Hospital found in Woliata Sodo town, from October 2017 to June 2018. Wolaita Sodo town is located in the SNNPR about 383 km from Addis Ababa. The area is bounded with Damot gale woreda to the north, Humbo woreda to the south, Damot Woide woreda to east and Damot Sore woreda to the west (figure 2). Its altitude ranges from 1650 to 2980 (m.a.s.1) and receives an annual rain fall of 100-1200 mm and with an annual temperature of 25-35°c. The area is woinadega (mid altitude) with altitude below 1600 and livestock population found in the Wolaita Sodo town comprises about 128,919 cattle, 29191 sheep, 4606 equines and 55278 poultry in Wolaita Sodo zone. Small, medium and large scale dairy farms are found in this area that supply milk and milk products for human consumption. These dairy farms contain either local or cross breeds depending on the scale of production (CSA, 2015).

According to the result of housing and population census May, 2007 Wolaita Sodo town has population of 102,922, out of these 54,315 males and 48,617 females with the annual population growth rate about 5.3%. This makes Wolaita Sodo the second most populous city in South Region after Awassa. The predominant economic activities involve mixed farming, which broadly includes cultivation of cereal crops, cash crops including primarily coffee and production of livestock (CSA, 2015 projected from 2007 census).

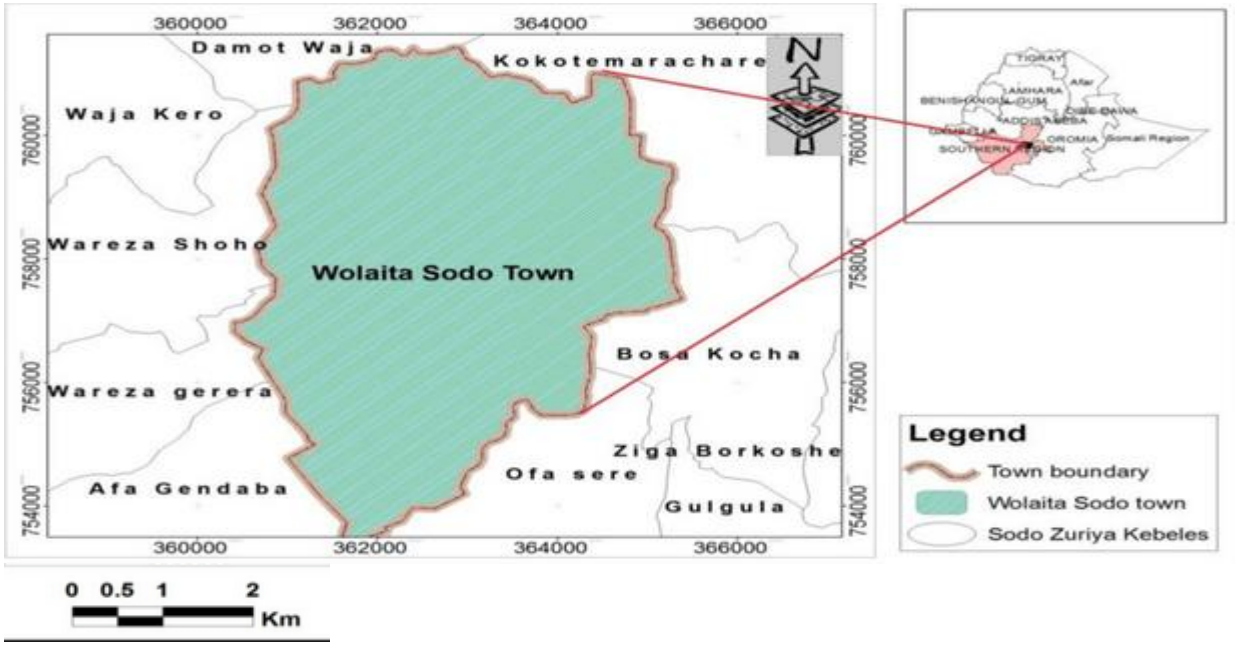

Figure 2. Map of the study area.

Source: (CSA, 2015)

3.2. Study Population

The study included both local and cross breeds of dairy calves of both sexes up to four months of age that are clinically affected with diarrhea and exhibiting signs of systemic disease like poor appetite, fever, dehydration, and reduced suckle reflex. In the study area there are few relatively large dairy farms, many were smallholder dairy farms. The majority small scale dairy farms were organized dairy cooperatives in their respective localities and the farms kept local breeds and crosses of both Holsein and Jersy breeds of calves. Farms involved in the study period were classified as small (<10 heads of dairy cow), medium sized (11-50 heads of dairy cow) and large (>50 heads of dairy cow) based on previous works in the urban and peri-urban production system (Morel *et al*, 2015.

Ages of diarrheic calves were categorized into four groups: less than 2 weeks, 2 to 4 weeks, 5 to 9 weeks and 10 to 16 weeks based on post-natal silent stress response coupled with lack of immune competence, preweaning and post weaning strategies in which calves are often susceptible to enteric disease (Morel *et al*, 2015; Lindsey and Sonia, 2016). Also children

under five years of age are highly vulnerable for diarrheic infection according to Black *et al*. (2010) and WHO, (2016). Therefore, children with diarrhea under five years of age admitted to Sodo Christian hospital at Wolaita Sodo town were included in the study.

3.3. Inclusion and Exclusion Criteria

3.3.1. Inclusion criteria

Diarrheic calves aged up to four months at dairy farms at Wolaita Sodo town and owners willing to provide sample from their calves were included. Wheras, children aged under five years with diarrhea admitted to Sodo Christian hospital at Wolaita Sodo town whose caretakers willing to participate in the study were included as population this study.

3.3.2. Exclusion criteria

Diarrheic calves and children aged above four months and five years respectively, that were on antibiotic therapy for two weeks and those whose owners and caretakers didn't show willingness to take sample were excluded from this study.

3.4. Ethical Considerations

The current study was ethically cleared (Appendix 10-I and 10-II) and the objectives of this research were explained to the parents of children visiting hospital and farm owners. All informations were kept confidentially. All of the stool samples from diarrheic children were collected by medical laboratory experts of the hospital. Verbal consent was also obtained from the managers or owners of the farms to take fecal samples from diarrheic calves.

3.5. Study Design and Sampling Methodology

3.5.1. Study type, type of sampling and source of samples

A cross sectional study starting from October 2017 to June 2018 was conducted in dairy farms and Sodo Christian Hospital found at Wolaita Sodo town. The selection of dairy farms and Sodo Christian hospital were done purposively based on availability of clinical cases

(diarrheic calves and children) and willingness of the owners of the calves and parents of children.

The health status of each calf was evaluated by clinical examination where as the status of the children were reported by the pediatrician. Calves and children free from diarrhea were classified as healthy where as sick calves and children that had showed abnormal stool consistency and /or signs of dehydration, sunken eye, diarrhea and weakness were classified as diarrheic. In addition, the type, characteristics and the color of diarrhea was recorded (Appendix 2-I and 2-II).

3.5.2. Questionnaire survey

The farm management practices and childhood informations were assessed by using questionnaire survey. Semi-structured questionnaire was administered to dairy farm owners or farm managers during the time of sample collection in the study period to assess relevant information on calf husbandry practices and the general farm management system in each farm. Other separately designed semi-structured questionnaire was also administrered to parents or caretakers of the children to assess the general family life standard, ways of handling the children and hygienic practices of the family during admission to the hospital.

The questionnaires were developed in accordance with the objectives of the study and designed in a way simple and easy to understand; to get accurate information from the dairy farm owners and the parents of the children. The questionnaire included all practices in the farm and in the family which can have impact on the proper rearing of calves and handling of the children associated risk factors responsible for calf and children diarrhea. These include age of calf, farm size, breed, knowledge on colostrum feeding, general health care, and sanitation of farms, animal housing, breast feeding, handwashing practice, disease preventive and control measures practiced in the farms (Appendix 1-I and 1-II).

3.5.3. Sample size determination

Non-probability purposive sampling was used for the selection of farms and Sodo Christian hospital as well as calf and child samples. The sample size was determined based on

availability of clinical cases (diarrheic calves and children) and on willingness of the owners in the farms and parents of the children. Based on that 88 and 110 fecal sampes were collected from diarrheic calves and children, respectively in current study.

3.5.4. Sample collection procedures

Fecal samples were collected directly from rectum of non treated diarrheic calves preferably soon after onset of diarrhea. The samples were collected from all diarrheic calves with age up to four months present in the farm at the time of the visit and on emergency calls from the farm owners. Sufficient amount (25-50gm) of fecal samples were collected directly from the rectum using sterile disposable latex gloves and transferred to 50ml sterile wide mouth screw capped universal bottles. Similar sample collection procedure was applied by medical laboratory technicians having the stool laboratory request of the pediatricians for diarrheic children under five years admitted to Sodo Christian hospital. The collected samples were labeled properly (Appendix 2-I and 2-II) and transported to the Wolaita Sodo regional veterinary laboratory in an ice box containing ice packs and then immediately processed for bacterial isolation.

3.6. Isolation and Identification of *Escherichia coli*

Isolation and identification of *E. coli* were conducted following standard procedures described in Quin *et al*. (2002) and the techniques recommended by the international Organization for standardization (IOS, 2001). Upon arrival at the laboratory, immediately or after overnight storage in refrigerator at 4^0c and thawing at room temperature, the samples were manually homogenized by using vortex mixer for approximately 40 seconds. Twenty five gram of fecal samples was stirred into 225 ml of sterile buffered peptone water (Himedia, India; appendix 4-I) or 1:9 ratio whenever there is little amount of sample present in a sterile flask. The pre-enriched samples were homogenized for two minutes in the flask and were incubated aerobically at 37^0c for 24 hour. All the media used for the study were prepared following the instruction of the manufacturers (Appendix 4).

Pre-enriched broth of 0.1ml (a loopfull) sample dilution was inoculated aseptically onto sterile MacConkey agar (Himedia, India; appendix 4-II) and incubated at 37 °c for 24 hrs.The

presence of growth on MacConkey agar was used as primary criteria to proceed for isolation and identification of *E. coli.* Suspected *E. coli* colonies, presumptively identified by their lactose fermenting character (pink colonies) and were further sub cultured on Eosin methyl blue (EMB) agar medium (Himedia, India; appendix 4-II) to selectively identify the isolate as *E. coli*. The characteristics *E. coli* colonies on EMB were identified based on their green metallic sheen color. All isolated colonies were preserved on nutrient agar slant (Oxoid, England, appendix 4II) for further biochemical testing. The cell morphology and purity of all isolates were checked by gram staining technique (Sagar, 2015 appendix 5) and then were subjected to the standard biochemical tests; Indole test, Methyl red (MR), Vogues Proskauer (VP) test, Citrate testand triple sugar iron (OIE, 2016). The isolates exhibited IMViC pattern (++- -) were presumed as *E. coli* isolates (Quinn *et al*., 2002 appendix 5-II). The same protocol was used for isolation and identification of *E. coli* isolates in both calf and children diarrhea. All the presumed *E. coli* isolates were subcultured on nutrient agar slant (Oxoid, England, appendix 4-II) for short term preservation or maintenance.

3.7. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed according to "Kirby and Bauer" method on Mueller- Hinton agar medium (Farshad *et al*., 2010), following the standard agar disk diffusion method according (CLSI, 2017) using antimicrobial disks of commonly used drugs for treatments (appendix 7-II). Each isolated bacterial colony from pure fresh culture was transferred into a test tube of 5 ml nutrient broth (Himedia, India; Appendix 4III) and incubated at 37°c for 18-24hrs. The turbidity of the culture broth was adjusted using sterile 0.9% saline solution and by adding more isolated colonies to obtain turbidity comparable with that of 0.5 McFarland standards, approximately $3x10^8$ CFU per ml, (Remel, USA).

Mueller-Hinton agar (Oxoid, England, appendix 4-IV) plates was prepared according to the manufacturer's guidelines. Thus, 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 were dried for 10 minutes, ten antibiotic discs with known concentration of antimicrobials were placed on the inoculated plates using sterile forceps and gently pressed onto the agar to ensure firm

contact with the agar surface; then, the plates were incubated at 37°c for 24 hrs. Following this the diameter of inhibition zone formed around each disc was measured using calibrated ruler. The results were classified as sensitive, intermediate, and resistant according to the standardized table supplied by the manufacturer of the discs (CLSIFAD, 2017; appendix 7-I). The same protocol was used for isolation and identification of *E. coli* isolates in both calf and children diarrhea.

3.8. Molecular Detection of *Eschericia coli* Isolates

3.8.1. DNA Extraction

Bacterial DNA extraction was perfomed using boiling method. *Escherichia coli* isolates were cultured in nutrient broth at 37^0c overnight. Exactly 1.5 ml of the young nutrient broth culture was harvested and spunned by centrifugation at 13,000 rpm for 10 minute in sterilized eppendorf tube. The supernatant was discarded; then, 50µl of nuclease free water was added on the pellet and boiled to lyse in a water bath at 95^0c for 10 minutes. It was then centrifuged again as before and aliquot of the supernatant was transferred to another autoclaved eppendorf tube as the test DNA template and stored at -20^0c for later use in PCR experiments. Three µl of the extracted DNA was used directly as template for PCR amplification (He, 2011).

3.8.2. Polymerase Chain Reaction

After extraction of the target DNA, all *E. coli* isolates was subjected to PCR in order to detect virulence genes. The optimized PCR protocol was carried out with PCR mix of a 25µl mixture containing 16 µl double distilled water, 2.5 µl 10X PCR buffer with MgCl2, 1 µl dNTP, 1 µl forward primer, 1 µl reverse primer, 0. 5 µl *Taq* DNA polymerase, (Himedia India) and 3 µl of template DNA. A negative control was performed by adding 3µl of sterile nuclease free water; a positive control was performed by adding 3 µl of known DNA sample. For those strains where positive control is not available in the laboratory, pooled target DNA samples was used as positive control. According to optimized PCR condition, the amplification of the reaction mixtures was performed in a PCR machine, Eppendorf thermal-cycler, Biometra, Geotting, Germany. The primer sequences and optimized PCR protocol used in this work and their features are indicated in table 1.

Table 1: Primer sequences specific for each virulent genes, their amplicon size and optimized PCR protocol.

Target gene	Primer Name	Sequence 5' to 3'	Amplicon size (bp)	Initial denaturation ^{0}c/min	Denaturation ^{0}c /sec.	Annealing ^{0}c /sec	Extension 0c /sec	Final extension ^{0}c /min	No. of cycle	Cooling temperature (0c)	*E. coli* strain detected	References
eaeA	EAE1	F:AAACAGGTGAAACTGTTGCC	490	95/3	95/40	55/60	72/60	72/10	35	25	EPEC/ EHEC	Khan *et al.*, 2002
	EAE2	R:CTCTGCAGATTAACCTCTGC										
Stx 1	EVS1	F:ATCAGTCGTCACTCACTGGT	110	95/3	95/40	57/40	72/30	72/8	30	25	STEC/ EHEC	Pal *et al*.,1999
	EVC2	R:CTGCTGTCACAGTGACAAA										
Stx 2	EVT1	F:CAACACTGGATGATCTCAG	350	95/3	95/40	57/40	72/30	72/8	30	25	STEC/ EHEC	Pal *et al.*,1999
	EVT2	R:CCCCCTCAACTGCTAATA										
Bfp	bfpA1	F:TTCTTGGTGCTTGCGTGTCTTTT	324	95/3	95/40	57/40	72/30	72/8	30	25	Typical EPEC	Khan *et al.*, 2002
	bfpA2	R:TTTTGTTTGTTGTATCTTTGTAA										
HlyA	hlyA-1	F: GGT GCA GCA GAA AAA GTT GTA G	190	95/3	95/40	45/60	72/60	72/10	30	25	EHEC	Gallien, 2003
	hlyA-2	R: TCT CGC CTG ATA GTG TTT GGT A										
aatA	aatA1	F: GGT GCA GAA CAC GAT GCA	590	95/3	95/40	45/60	72/60	72/10	30	25	EAEC	Khan *et al.*, 2002
	aatA2	R: CCA CGT CTT GTG CTA CGT										
Lt	LT-1	F: TCTCTATGTGCATACGGAGC	696	95/3	95/40	53/60	72/30	72/8	30	25	ETEC	Vidal *et al.*, 2005%
	LT-2	R: CCATACTGATTGCCGCAAT										
St	ST-1	F: TGCTAAACCAGTAGAGTCTTCAAAA	294	95/3	95/40	52/60	72/60	72/10	35	25	ETEC	Arif and Salih, 2010
	ST-2	R:GCAGGATTACAACACAATTCACAGCAG										
Dae	*dae*E 1	F: GAA CGT TGG TTA ATG TGG GGT AA	542	95/3	95/40	45/60	72/60	72/10	37	25	DAEC	Nishikawa *et al*., 2002
	*dae*E 2	R: TAT TCA CCG GTC GGT TAT CAG										

Keys: EPEC= enteropathogenic *E. coli*; ETEC= enterotoxigenic *E. coli*; STEC= shiga toxin-producing *E.coli*; EHEC= enterohaemorrhagic *E. coli*; EAEC=enteroaggregative *E. coli*; DAEC= diffusely adherent *E. coli*; EAE=effacing and attaching, Stx=shiga toxin, bp=Base pair

3.8.3. Agarose gel electrophoresis

Amplified PCR products were separated by agarose gel electrophoresis at 120 volt for 30 minutes in 0.8% agarose containing Ethidium Bromide in 1x in TBE buffer using a marker 100 bp DNA ladder (Himedia; India and Promega, Madison, Wisconsin, USA). Loading dye was used to load PCR product in each well in a gel. The products were then visualized by UV transilluminator (Hegde *et al*., 2012) and photographed under ultraviolet light using a digital Polaroid camera.

Molecular detection of virulent genes and antimicrobial susceptibility patterns were performed in microbial and molecular laboratory of Institiute of Biotechnology in Addis Ababa University.

3.9. Data Storage and Analysis

All the data that was generated from questionnaire survey and laboratory experiments was coded, filtered and recorded using Microsoft Excel spreadsheet 2007 (Microsoft Corporation) and analyzed using SPSS version 20.0 software (SPSS INC.Chigago, IL). Descriptive statistics was used to describe the study population in relation to risk factors. The proportion of *E. coli* occurrence was calculated as the number of positive samples divided by the total samples examined times 100. Also the association between *E. coli* isolates and different potential risk factors, as well as presence of different virulent gene detected and diarrheal infection due to *E. coli* in both diarrheal calves and children was analysed by using person's $\chi 2$ test. The strength of the association between risk factors and the outcome was also measured using odd ratio.

To control the possible effect of confounders, all variables with $P<0.25$ in univariable logistic regression were fitted into multivariable logistic regression model to observe the strength of the association between risk factors and the outcome.The suitability of the model was checked by multicollinearity diagnosis among independent variables by contingency coefficient. The goodness of fit of the model with the data was assessed by Hosmer and Lemeshow test. After selecting the final model of multivariable logistic regression, the beta (β) coefficients of each independent variable were observed to estimate odds ratio (OR) which

is used for assessing strength of association. Finally the results were reported as statistically significant if *p-value* less than 0.05 and not significant if *p-value* is greater than 0.05.

4. RESULTS

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

Escherichia coli were recovered in 57 (64.8%) out of 88 diarrheic calves that showed calf diarrhea (95% CI: 53.9-74.7%). In this study the occurrence of diarrhea due to *E. coli* differed significantly by age (P=0.008), farm size (P=0.004) and time of first colostrums feeding (P=0.02). Thus the isolation rate was highest, 40 (45.5%), in calves in age groups of less than two weeks and 26 (29.6%) in calves getting the first colostrums feeding after 24 hours. Its occurrence didn't show significant difference by sex, method of feeding, breed and navel treatment (Table 2).

Table 2: Overall occurrence of *Escherichia coli* with different risk factors in diarrheic calves.

Risk factors	Category	Number examined	Number positive	Category proportion (%)	Samples proportion (%)	(χ2)	*df*	*P-value*
Age /weeks/	< 2	51	40	78.4	45.5	43.2	3	0.008
	2 to 4	17	10	58.8	11.4			
	5 to 9	11	4	36.4	4.5			
	10 to 16	9	3	33.3	3.4			
Sex	Male	33	21	63.6	23.9	0.22	1	0.24
	Female	55	36	65.5	40.9			
CA	Yes	75	56	74.7	63.6	0.6	1	1
	No	13	1	7.7	1.2			
MF	Suckling	36	25	69.4	28.4	0.32	1	0.58
	Hand feeding	52	32	61.5	36.4			
NT	Yes	46	34	73.9	38.6	3.6	1	0.19
	No	42	23	54.8	26.1			
TFCF	<6 hour	21	12	57.1	13.6			
	6-24 hour	28	19	67.9	21.6			
	>24 hour	39	26	66.7	29.6	14.3	2	0.02
SFT	Hay	27	19	70.4	21.6			
	Concentrates	28	17	60.7	19.3			
	Grazing	33	21	63.6	23.9	5	2	0.86
Housing	Separate pen	48	32	66.7	36.4	2.4	1	0.12
	The same barn	40	25	62.5	28.4			
Breed	Local	19	17	89.5	19.3	0.08	2	0.96
	Cross	39	22	56.4	25			
	Exotic	30	18	60	20.5			
Farm size	Small	40	23	57.5	26.2	8.2	2	0.004
	Medium	29	19	65.5	21.6			
	Large	19	15	78.9	17			
TD	Watery	37	33	89.2	37.5	32.9	4	0.44
	Bloody	18	10	55.6	11.4			
	Yellowish	15	7	46.7	8			
	Greenish	12	4	33.3	4.5			
	Mucoid	6	3	50	3.4			

Keys: CA= Colostrums awareness; (χ2)= Chi-square; *df*= Degree of freedom; MF= Method of feeding; NT= Navel treatment; *P*= probability; TD= Types of diarrhea; TFCF=Time of first colostrums feeding; SFT= Supplementary feed type

Univariable and multivariable logistic regression analysis of risk factors associated with diarrhea in calves

Independent variables of age, time of first colostrum feeding and farm size in univariable analysis were significantly associated with occurrence of *E. coli* isolates in diarrheic calves ($P<0.05$). However, other variables like sex, method of feeding, breed and navel treatment didn't show significant difference with *E. coli* isolates (table 3). The variables with ($P<0.25$) in univariable logistic regression analysis were taken to multivariable logistic regression analysis to control confounders.

Multivariable logistic regression analysis was carried out to observe the independent effects of each risk factor in relation to occurrence of *E. coli* isolates in diarrheic calves. Accordingly, variables such as age ($P=0.008$), first colostrum feeding ($P=0.02$) were identified as a significant independent predictors for occurrence of *E. coli* isolates in diarrheic calves. The log odds ratio indicates that calves within age category less than two weeks (AOR=4.3; 95% CI: (34.8- 56.4%); $P=0.001$) were more susceptible to diarrhea due to *E. coli* compared to other age categories of diarrheic calves. Similarly calves that had get first colostrum feeding after 24 hours (AOR=2.8; 95% CI: (20.3- 40.2%); $P=0.005$) were more susceptible to diarrhea due to *E. coli* than those getting it before six hours (Table 3).

None of the variables found to be collinear and there was insignificant difference between the observed and the predicted values with ($\chi 2= 2.54$; $P=0.57$) which was fitted well with the data.

Table 3. Risk factors associated with *E. coli* isolates in diarrheic calves by univariable and multivariable logistic regression

Risk factors	Category	Number examined	Samples positive (%)	Univariable COR (95% CI)	*P-value*	Multivariable AOR (95% CI)	*P-value*
Age (weeks)	< 2	51	40 (45.5)	4.7 (35.0-56.3%)	0.008	4.3 (34.8- 56.4%)	0.001
	2 to 4	17	10 (11.4)	1 (6.0-20.8%)		1(6.3-19.7%)	
	5 to 9	11	4 (4.5)	0.4 (1.5- 13.0%)		0.2 (1.3- 11.2%)	
	10 to 16	9	3 (3.4)	**		**	
Sex	Male	33	21 (23.9)	0.8(27.2- 47.4%)	0.24	0.8 (27.4- 48.5%)	0.19
	Female	55	36 (40.9)	**		**	
Navel treatment	Yes	46	34 (38.6)	4.3 (28.0-52.5%)	0.19	3.8 (28.4-49.6%)	0.14
	No	42	23 (26.1)	**		**	
TFCF (hour)	<6	21	12 (13.6)	**		**	
	6-24	28	19 (21.6)	0.8 (12.4-30.4%)		0.9 (13.5- 31.6%)	
	>24	39	26 (29.6)	3.2 (19.6-43.7%)	0.02	2.8 (20.3- 40.2%)	0.005
Housing	Separate pen	48	32 (36.4)	2 (26.0- 48.2%)	0.12	2 (26.4-47.3%)	0.14
	The same barn	40	25 (28.4)	**		**	
Farm size	Small	40	23 (26.2)	0.9 (16.8- 38.0)	0.004	1.2 (17.3- 36.6%)	0.16
	Medium	29	19 (21.6)	0.56 (13.0-32.4%)		0.4 (13.5-31.6%)	
	Large	19	15 (17)	**		**	

Keys: AOR= Adjusted odd ratio; CI= Confidence interval; COR= Crude odd ratio; TFCF= Time of first colostrums feeding; *P*= Probability; ** = Reference point

4.2. Overall Isolation of *Escherichia coli* in Diarrheic Children

In total of 110 stool samples from children with diarrhea examined about 68 (61.8%) (95% CI: 52.1-70.9%) were *E. coli* positive. The occurrence of *E. coli* in child diarrhea showed significant difference by age (*P*=0.007), contact with either animals or manure (*P*=0.009) and exclusive breast feeding in six month (*P*=0.036). A higher isolates of *E. coli*, 47 (42.8%), was observed in children of age groups 25-60 months, 48 (43.7%) in those having history of contact with either animals or manure and 46 (41.8%) in children who didn't get only breast feed until six months of age. There was no significant difference in occurrence of *E. coli* by sex (table 4).

Table 4. Overall occurrence of *Escherichia coli* with different risk factors in diarrheic children.

Risk factors	Category	Number examined	Number positive	Category proportion (%)	Samples proportion (%)	(χ2)	*df*	*P-value*
Age	0 -6	22	10	45.5	9			0.007
/months/	7-24	27	11	40.7	10	10.2	2	
	25-60	61	47	77	42.8			
Sex	Male	62	38	61.3	34.5	0.13	1	0.96
	Female	48	30	62.5	27.3			
CAM	Yes	73	48	65.7	43.7	11.3	1	0.009
	No	37	20	54	18.1			
NHWBM	Yes	63	44	69.8	40	14.2	1	0.21
	No	47	24	51	21.8			
EBMF6M	Yes	39	22	56.4	20			
	No	71	46	64.8	41.8	13.5	1	0.036

Key: CAM= Contact with either animals or manure; (χ2) = Chi-square; CI= Confidence interval; *df*= Degree of freedom; EBMF6M=Exclusive breast milk feeding in six month; NHW= Negligence to hand washing before meal; *P*= probability

Univariable and multivariable logistic regression analysis of risk factors associated with diarrhea in children

In analysis of Risk factors concerning diarrhea in children independent variables like age, contact with either animals or manure and exclusive breast feeding in six month in univariable analysis were significantly associated with occurrence of *E. coli* isolates (P<0.05). On the other hand sex and negligence to hand washing before meal was not significantely associated with *E. coli* isolates (table 4). The variables with (P<0.25) in univariable logistic regression analysis were taken to multivariable logistic regression analysis to control confounders.

In multivariable logistic regression analysis the occurrence of *E. coli* isolates in diarrheic children were more likely higher in age groups 25-60 months (AOR=2.4; 95% CI: 2.4 (33-52.5%); P=0.003) and in children having history of contact with either animals or manure (AOR=3.5; 95% CI: 3.5 (34.2- 53.4%); P=0.001) than other age categories and in those having no history of contact with either animals or manure respectively. Similarly children experiencing habit of negligence to hand washing before meal (AOR=6; 95% CI: 6 (30.8-49.8%); P=0.01) and those who did not get only breast feed in six months of age (AOR=5.6;

95% CI: 5.6 (32.5- 51.6%); *P*=0.02) were more likely susceptible to *E. coli* infection than those who washes their hands and those who get only breast feed in six months of age respectively (Table 5).

None of the variables found to be collinear and there was insignificant difference between the observed and the predicted values with (χ2= 1.24; *P*=0.64) which was fitted well with the data.

Table 5: Risk factors associated with *E. coli* isolates in diarrheic children by univariable and multivariable logistic regression

				Univariable		Multivariable	
Risk factors	Category	Number examined	Samples positive (%)	*P-value*	COR (95% CI)	*P-value*	AOR (95% CI)
Age /months/	0 -6	22	10 (9)		®		®
	7-24	27	11 (10)		1 (5- 18.4%)		1 (5.1- 17.2%)
	25-60	61	47 (42.8)	0.007	2.9 (34.2- 53.8%)	0.003	2.4 (33- 52.5%)
CAM	Yes	73	48 (43.7)	0.009	3.8 (33.6- 55.3%)	0.001	3.5 (34.2- 53.4%)
	No	37	20 (18.1)		®		®
NHWBM	Yes	63	44 (40)	0.21	4 (32.0- 52.4%)	0.01	6 (30.8- 49.8%)
	No	47	24 (21.8)		®		®
EBMF6M	Yes	39	22 (20)		®		®
	No	71	46 (41.8)	0.036	6.8 (31.5- 49%)	0.02	5.6 (32.5- 51.6%)

Keys: CAM= Contact with either animals or manure; NHWBM= Negligence to hand washing before meal; EBMF6M= Exclusive breast milk feeding in six month. ®= Reference point

4.3. Description of Dairy Farms and Socio-Demographic Situation Pattern Based on Questionnaire Survey Findings

A total of 34 questions comprised five areas of interest: farm descriptions, farm management data, awereness about importance of colostrum feeding to neonates and experience on calf health problem, prevention and control of the problems. Owners of 46 farms used for sample collection were interviewed. The farms kept 76% Holstein Fresian breed, 21% Jersey breed and 3% Zebu breed animals under intensive system of production with an average number of 7 calves per farm. Eighty seven (98.9%) of the dairy farmers had knowledge of the advantage of colostrum feeding. Thirty nine (44.3%) out of 88 diarrheic calves fed first colostrum after

24 hours and 48 (54.5%) of calves were kept in separated calving pen that were often not cleaned and disinfected regularly.

Navel treatment during birth of calves was practiced in 46 (52.3%) of the visited farms. The practice of providing bedding was limited in large dairy farms and non in small holders. Fourty (86.9%) of farms weaned calves at 3 months of age where as 6(13.1%) of them weaned at 4 months. In general, the weaning age was lower for male calves, mostly under 3 months. In all large dairy farms, there were veterinary personnel employed to deal with aspects of the farms. Owners in smallholder dairy farms call private veterinary practitioners whenever their animals face health problems. Farm managers or owners mentioned calf diarrhea which accounted 58% of calf health problems in the farms.

Similarly one hundred ten diarrheic children with age below five years admitted to Sodo Christian hospital were considered. Twenty six interview based questionnaire which comprised areas of interest like presence of contacts of manure or domestic animals in house, breast milk feeding, treatment history of the child and so on was administered. Fifty two (47.3%) out of 110 diarrheic children were females and 58 (52.7%) were females to male ratio of 1.2: 1. The age level is up to 60 months of which the highest number of participants 54 (49%) were found between the age category of 25-60 months. From all parents of the sampled children 102 (92.7%) used toilet for defecation and the remained were not in which children were susceptible to diarrheal infection because they did not pay attention to hygiene. Fifty three (48.3%) of the parents reared domestic animals in home and 46 (41.8%) used animal products as supplement feed source.

4.4. Antimicrobial Susceptibility Patterns of *Escherichia coli* Isolates

4.4.1 Mono-drug resistance

Antimicrobial drugs used, their employed concentrations, the percentage of resistant, intermediate and susceptible values for each antimicrobial disc is shown in appendix 7 as standard for interpretation of our results. The antimicrobial susceptibility patterns of 57 and 68 *E. coli* isolates from diarrheic calves and children respectively, were shown in table 6. Accordingly, the highest sensitivity to ciprofloxacin (89.5%) followed by norfloxacillin

(80.7%) was observed in *E. coli* isolates from diarrheic calves whereas (91.2%) followed by (86.8%) sensitivity, respectively was observed in *E. coli* isolates from child diarrhea. Chloramphenicol and clindamycin for all isolates and oxytetracycline and trimethoprim in isolates of diarreic calves and neomycin, streptomycin and norfloxacillin in isolates of diarreic children did not show intermediate resistance while the other tested drugs showed intermediate resistance in one or more of the tested isolates. Similarly the highest resistance to clindamycin (100%) followed by neomycin (94.7%) was observed in *E. coli* isolates from diarrheic calves; and (97.1%) neomycin followed by (95.6%) streptomycin resistance was observed in *E. coli* isolates from child diarrhea.

Table 6. Antimicrobial susceptiblity patterns in *Escherichia coli* isolates from diarrheic calves in dairy farms and diarrheic children in Sodo Christian hospital at Wolaita Sodo town.

	Calves			Children		
Antimicrobial agents	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Ampicillin	13 (22.8)	5 (8.8)	39 (68.4)	8 (11.8)	12 (17.6)	48 (70.6)
Chloramphenicol	26 (45.4)	-	31 (54.4)	30 (44.1)	-	38 (55.9
Ciprofloxacin	51 (89.5)	4 (7)	2 (3.5)	62 (91.2	2 (2.9)	4 (5.9)
Clindamycin	-	-	57 (100)	5 (7.4)	-	63 (92.6)
Neomycin	2 (3.5)	1 (1.7)	54 (94.8)	2 (2.9)	-	66 (97.1)
Norfloxacillin	46 (80.7)	3 (5.3)	8 (14)	59 (86.8)	-	9 (13.2)
Oxytetracycline	22 (38.6)	-	35 (61.4)	13 (19)	15 (22)	40 (58.8)
Streptomycin	9 (15.8)	4 (7)	44 (77.2	3 (4.4)	-	65 (95.6)
Sulphonamides	6 (10.5)	3 (5.3)	48 (84.2)	7 (10.3)	21 (30.9)	40 (58.8)
Tetracycline	23 (40.5)	2 (3.5)	32 (56)	10 (14.7)	14 (20.6)	44 (64.7)
Trimethoprim	16 (28.1)	-	41 (71.9)	16 (23.5)	6 (8.8)	46 (67.6)
Total (%)	213 (34)	22 (3.5)	392 (62.5)	215 (28.7)	70 (9.4)	463 (61.9)

As shown in table 6 resistance for sulphonamides in *E. coli* isolates from calf diarrhea is 84.2%; but it is 58.8% those isolates from samples of diarrheic children. Also resistance for clindamycin in *E. coli* isolates from calf diarrhea is 100%; but it is 92.6% in those isolates from samples of diarrheic children. On the other hand sensitivity towards norfloxacillin in *E. coli* isolates from calf diarrhea is 80.7%; compared to 86.8% in those isolates from diarrheic children.

4.4.2 Multi-drug resistance

Multi- drug resistance patterns of the *E. coli* isolates are shown table 7. Out of 57 isolates from calves and 68 from children isolates, 34 (59.7%) and 43 (63.2%) were resistant to two or more (up to ten) antimicrobials, respectively. The highest proportion of resistant isolates is observed to five drugs 7 (12.3%) followed by to seven drugs 6 (10.5%) in isolates from diarrheic calves; whereas the highest to seven drugs 8 (11.8%), followed by 7 (10.3%) to eight drugs in isolates from diarrheic children.

Table 7: Multi drug resistance patterns of *Escherichia coli* isolates

Number of antimicrobial agents	Isolates from calves (n=57) Multidrug resistance patterns/number of isolates	Number of isolates /%	Isolates from children (n=68) Multidrug resistance patterns/number of isolates	Number of isolates (%)
Two	CHL,NE (1) AMP,TE (1)	2 (3.5)	SNM- OT (1); NOR- CLN (1); STM- NE (1)	3 (4.4)
Three	STM, CLN, OT (1); NE, CLN, OT (1); TRM, NOR, CLN (2)	4 (7)	TE-NE-CHL (1); TRM-CLN-AMP (1); STM, CLN, OT (2)	4 (5.9)
Four	AMP, NE, CHL, CLN (1)OT, NE, CHL, CLN (2); CLN,TRM, TE, SNM (2)	5 (8.8)	AMP-OT- NE- CLN (1);CHL, CLN, TRM, NE (1); CLN, TRM, TE, AMP (1)	3 (4.4)
Five	TRM- AMP- NOR- CHL- CLN (3); OT- NE- AMP – CLN-STM (1);TRM- NOR- CLN- SNM- OT (2); STM- NE- TE-CIP- CHL (1)	7 (12.3)	NOR- CLN- SNM-CHL-STM (1); OT- NE- -CHL-NE-TRM (1) TRM- NOR- CLN- SNM- TE (1); STM- NOR TE- CIP-CHL (1)	4 (5.9)
Six	AMP-NE-CIP-TRM-CLN-OT (2); AMP-NE-CIP-TRM-CLN-OT (1); NE- TE- NE -NOR- CLN- SNM (1).	4 (7)	NE- TE- CIP- CHL-TRM- NOR (2);NOR- CLN- SNM- OT AMP- STM (1); CHL-TRM- NOR-AMP- STM- TE (3).	6 (8.8)
Seven	AMP- STM- NE-CIP- CHL-TRM-CLN (1); CLN-SNM- OT-NE - CHL-TRM-STM (2); CHL-TRM- NOR- CLN STM- NE- TE (3)	6 (10.5)	TE-STM-CLN- NOR-CHL-NE-TRM (3); SNM- OT-CHL-TRM- NOR-STM- NE- TE (3); CLN- SNM-CHL-STM- NE-TE-AMP (2)	8 (11.8)
Eight	TE-STM-CLN-OT- NOR-CHL-NE-TRM (1); SNM- OT-CHL-TRM- NOR-STM- NE- TE (2); NOR- CLN- SNM-CHL-STM- NE- TE-AMP (2)	5 (8.8)	CIP-TRM- NOR-SNM- OT-AMP- STM- NE (2); TE- CIP-CHL-TRM- NOR- CLN- SNM- OT (3); AMP- STM- NE- TE-CIP- CHL-TRM- NOR (2);	7 (10.3)
Nine	AMP -STM-CLN-OT- NOR-CHL-NE- NOR (1)	1 (1.8)	OT -TE -AMP- STM- NE-CIP- CHL-TRM-CLN (2); NOR -AMP- CLN-SNM- OT- NE - CHL-TRM-STM (1); CHL-TRM-NOR- CLN-STM- NE- TE- SNM- OT (3)	6 (8.8)
Ten	-	-	TRM -AMP -STM-CLN-OT- NOR-CHL-NE- NOR- TE (1); TRM - CLN- SNM-CHL-STM- NE- CIP - TE- OT- NOR (1)	2 (2.9)
Total		34 (59.7%)		43 (63.2%)

Keys: AMP- Ampicillin; CLN- Clindamycin; CHL- chloramphenicol; CIP- ciprofloxacillin; STM- Streptomycin; NE- Neomycin; NOR- Norfloxacillin; OT- Oxytetracycline; TE- tetracycline; TRM- Trimethoprim; SNM- Sulphonamides; n=number

4.5. Pathogenic *Escherichia coli* strains Identified from Diarrheic Calves

Out of 88 fecal samples collected from diarrheic calves, 57 (64.8%) of *E. coli* isolates were detected (95% CI: 53.9-74.7%). Among those *E. coli* isolates, 44 (77.2%) were detected as harbouring one or more virulent genes (Figures 3-8). Out of these, 32 (72.7%) isolates contained one virulent gene and rest 12 (27.3%) isolates contained more than one virulent genes. As shown in table 8, out of 44 (77.2%) detected virulent genes, 22 (38.6%) is contained in STEC which is followed by Atypical EPEC 8 (14%) which is also followed by EAEC 6 (10.5%), then by ST (ETEC) 5 (8.8%) and lastly 3(5.3%) EHEC.

Out of 22 (38.6%) virulent genes detected in STEC, [8 (14%) positive for *stx1* gene only, 5 (8.8%) for *stx2* gene only, 2 (3.5%) for *stx1* + *HlyA* genes, and 7 (12.3%) for both *stx1* and *stx2* genes)]. Similarly out of 3 (5.3%) virulent genes detected in EHEC, 2 (3.6%) and 1 (1.7%) harboured (*eae* + *Stx1* and *HlyA*) and (*eae* + *Stx1* and *stx2*) genes respectively. All isolates showed a negative result for diffusely adherent gene and (Lt) toxin. All virulent genes detected were significantly associated ($P<0.05$) with diarrheal infection due to *E. coli* in calves.

4.6. Pathogenic *Escherichia coli* strains Identified from Diarrheic Children

In current investigation 110 stool samples from diarrheic children aged 0 to 60 months, *E. coli* was isolated in 68 (61.8%) (95% CI: 52.1-70.9%). Among those *E. coli* isolates, 38 (55.8%) were detected as harbouring one or more virulent genes (Figures 9-14). Out of these 35 (92.1%) isolates harbored one virulent gene and rest 3 (7.9%) isolates harbored more than one virulent gene. As illustrated in table 8, out of the 38 (55.8%) detected virulent genes in diarrheic children, 14 (20.5%) were detected in EAEC, 10 (14.7%) in STEC [which harboured 6(8.8%) *stx1* gene and 4 (5.9%) *stx2* gene], 1(1.5%) as (*stx 2 + eaeA),* 2(2.9%) as (*stx 1+ eaeA),* 5 (7.4%) as atypical EPEC, 4 (5.9%) as (St) toxin, 4.4% in EHEC [which harboured 2 (2.9%) is *stx 1+ eaeA* genes) and 1 (1.5%) is *stx 2 + eaeA* genes] and 2 (2.9%) in DAEC. All isolates were checked for the presence of *bfp* gene but none of them contained this gene. Also neither EHEC nor STEC harboured *hlyA* gene. All virulent genes detected were significantly associated ($P<0.05$) with diarrheal infection due to *E. coli* in children.

Table 8: Frequency of virulent genes and corresponding pathogenic *Escherichia coli* strains in diarrheic calves from dairy farms and diarrheic children from Sodo Christian hospital at Wolaita Sodo town.

Sample source	Virulence genes detected	Frequency among *E. coli* isolates (N=57)	Frequency among diarrheic calves (N=88)	Pathogenic *E. coli* strains	$\chi 2$	*P-value* (95% CI)
Calf samples	*stx 1*	8(14%)	8(9%)	STEC	13.5	0.004 (26.0-52.4%)
	stx 2	5(8.8%)	5(5.7%)	STEC		
	stx 1 + *HlyA*	2(3.5%)	2(2.3%)	STEC		
	stx1 and *stx2*	7(12.3%)	7(8%)	STEC		
	eae + *Stx1* and *HlyA*	2(3.6%)	2(2.4%)	EHEC	9.6	0.023 (1.1-14.6%)
	eae +*Stx1* and *stx2*	1(1.7%)	1(1.1%)	EHEC		
	eaeA $^{+}$ only	8(14%)	8(9%)	Atypical EPEC	11.2	0.035 (6.3-25.8%)
	St	5 (8.8%)	5 (5.7%)	ETEC	8.7	0.04 (2.9-19.3%)
	Eaat	6 (10.5%)	6 (6.8%)	EAEC	6.5	0.001 (4.0-21.5%)
	Total	44 (77.2%)	44 (50%)			
Child samples	*stx 1*	6 (8.8%)	6 (5.5%)	STEC	15.1	0.02 (7.3-25.4%)
	stx 2	4 (5.9%)	4 (3.6%)	STEC		
	stx 2 + *eaeA*	1 (1.5%)	1 (0.9%)	EHEC	6.8	0.041 (0.9-12.4%)
	stx 1+ *eaeA*	2 (2.9%)	2 (1.8%)	EHEC		
	eaeA $^{+}$ only	5 (7.4%)	5 (4.5%)	Atypical EPEC	7.4	0.003 (2.4- 16.3%)
	St	4 (5.9%)	4 (3.6%)	ETEC	10	0.002 (1.6- 14.4%)
	daat	2 (2.9%)	2 (1.8%)	DAEC	9.4	0.01 (0.4- 10.2%)
	Eaat	14 (20.5%)	14 (12.7%)	EAEC	13.9	0.01 (17.7-32.1%)
	Total	38 (55.9%)	38 (34.4%)			

Key: ($\chi 2$) - Chie squre; CI - Confidence interval; *P*- probability

Typical gel pictures for the specific virulence genes investigated are indicated in figures 3-14.

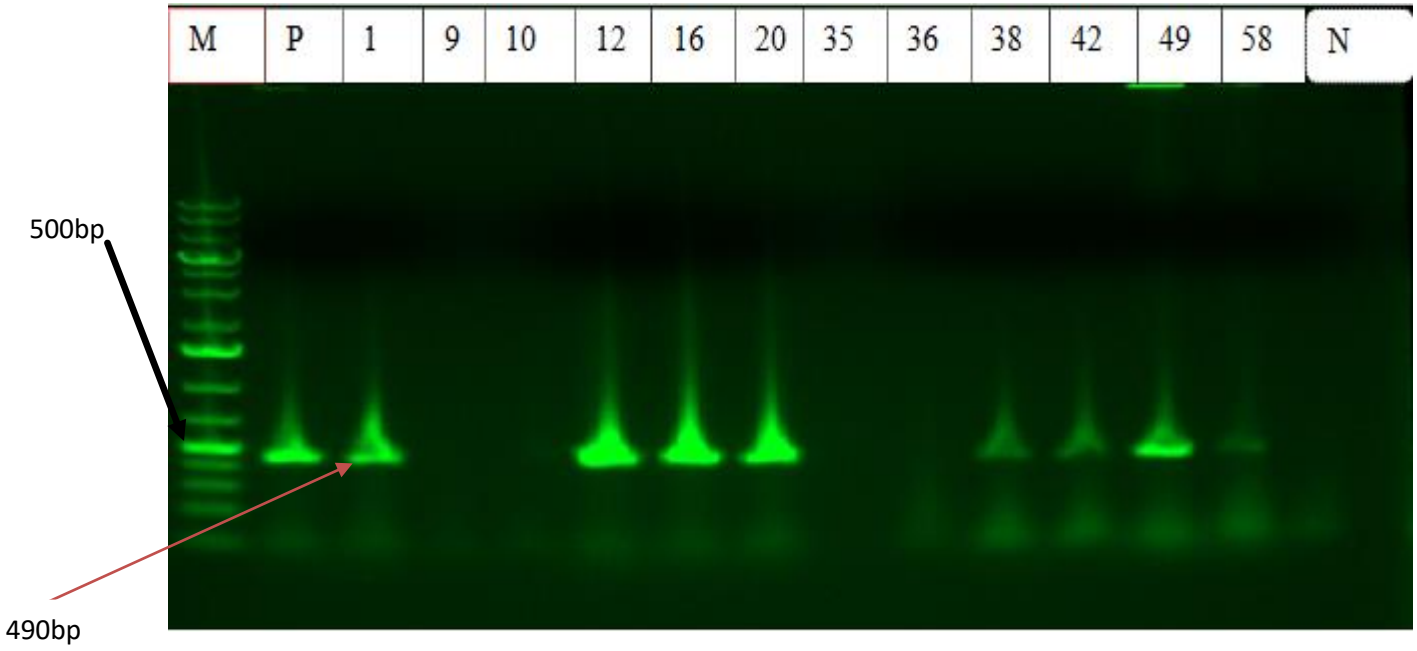

Figure 3: Amplification of *eaeA* gene in *E. coli* isolates from diarrheic calves: M, Marker (100 bp DNA ladder); P, positive control;Lane No 1, 12, 16, 20, 38,42, 49 and 58 indicates positive samples;Lane No 9,10, 35,36 indicates negative samples and N, negative control.

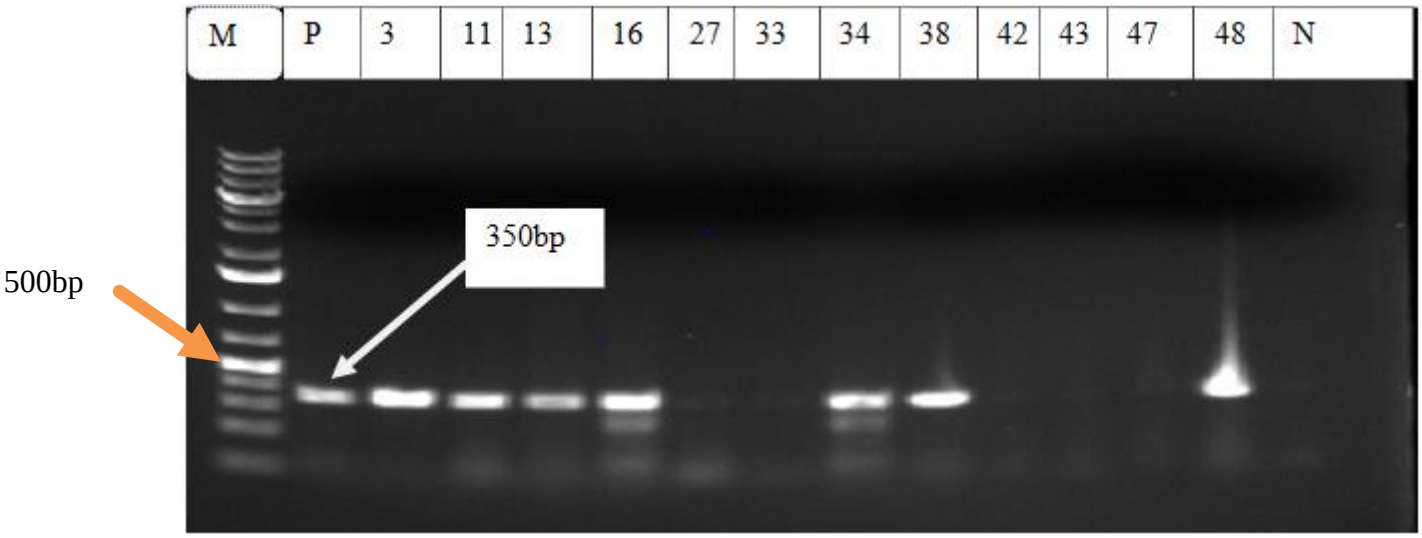

Figure 4: Amplification of *stx1* gene in *E. coli* isolates from diarrheic calves: M, Marker (100 bp DNA ladder); P, positive control; Lane No 3, 11, 13, 16,34, 38 and 48 indicates positive samples; Lane No27, 33,42, 43 and 47 indicates negative samples and N, negative control.

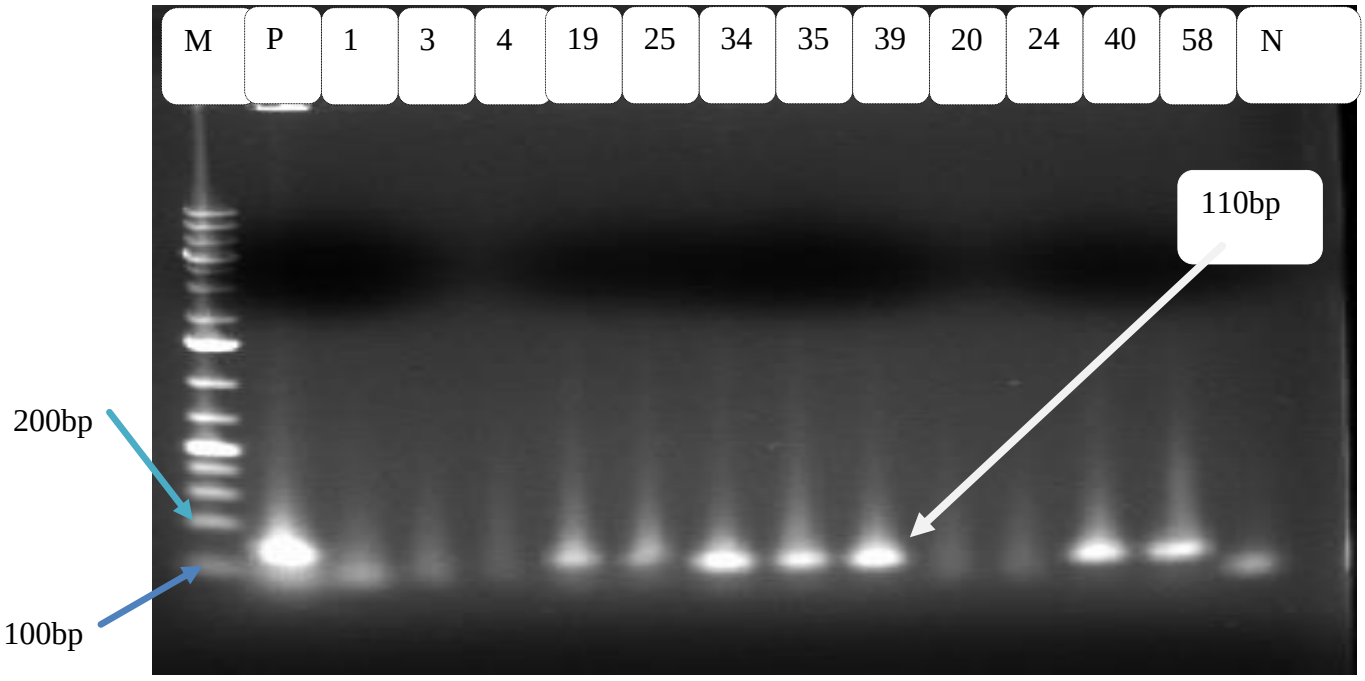

Figure 5: Amplification of *stx2* gene in *E. coli* isolates from diarrheic calves: M, Marker (100 bp DNA ladder); P, positive control; Lane No 19, 25, 34, 35,39, 40 and 44 indicates positive samples; Lane No 1,3, 4,20 and 24 indicates negative samples and N, negative control.

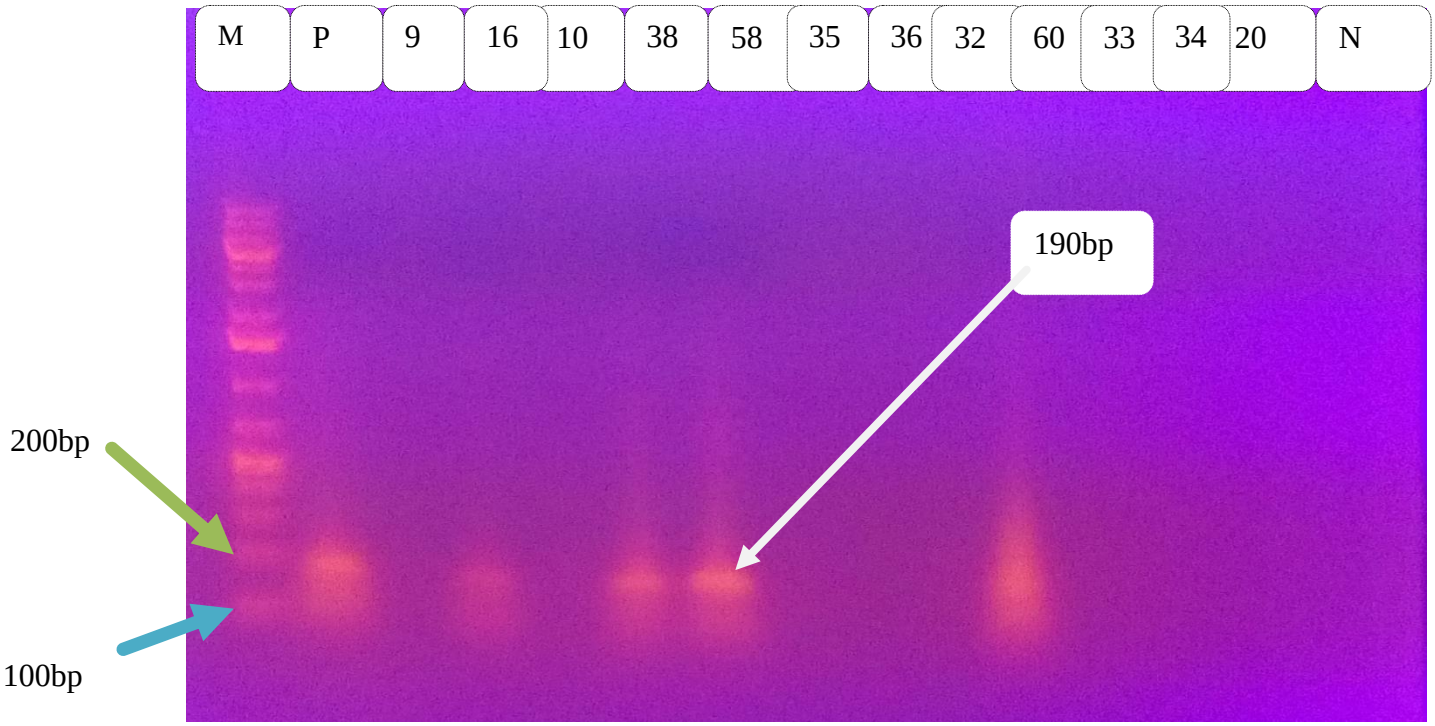

Figure 6: Amplification of *hlyA* gene in *E. coli* isolates from diarrheic calves: M, Marker (100 bp DNA ladder); P, positive control; Lane No 16, 38 and 58 indicates positive samples; Lane No 9,10, 35,36,32,60,33, 34, and 20 indicates negative samples and N, negative control.

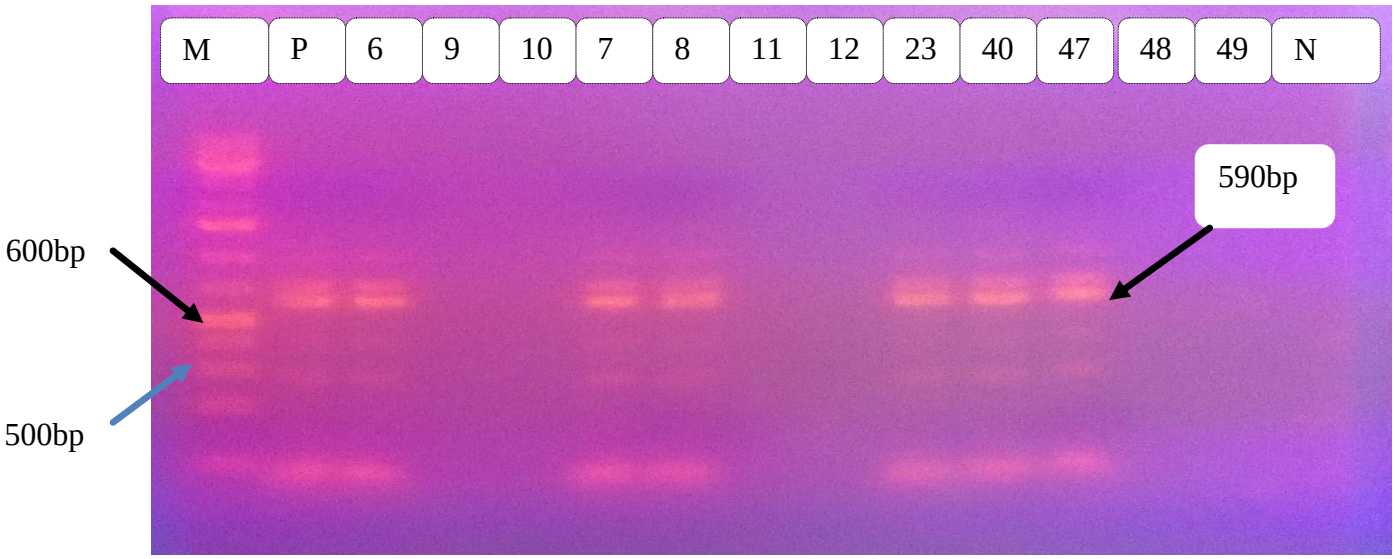

Figure 7: Amplification of *eat* gene in *E. coli* isolates from diarrheic calves: M, Marker (100 bp DNA ladder); P, positive control; Lane No 6, 7,8,23,40 and 47 indicates positive samples; Lane No 9,10, 11, 12, 48, and 49 indicates negative samples and N, negative control.

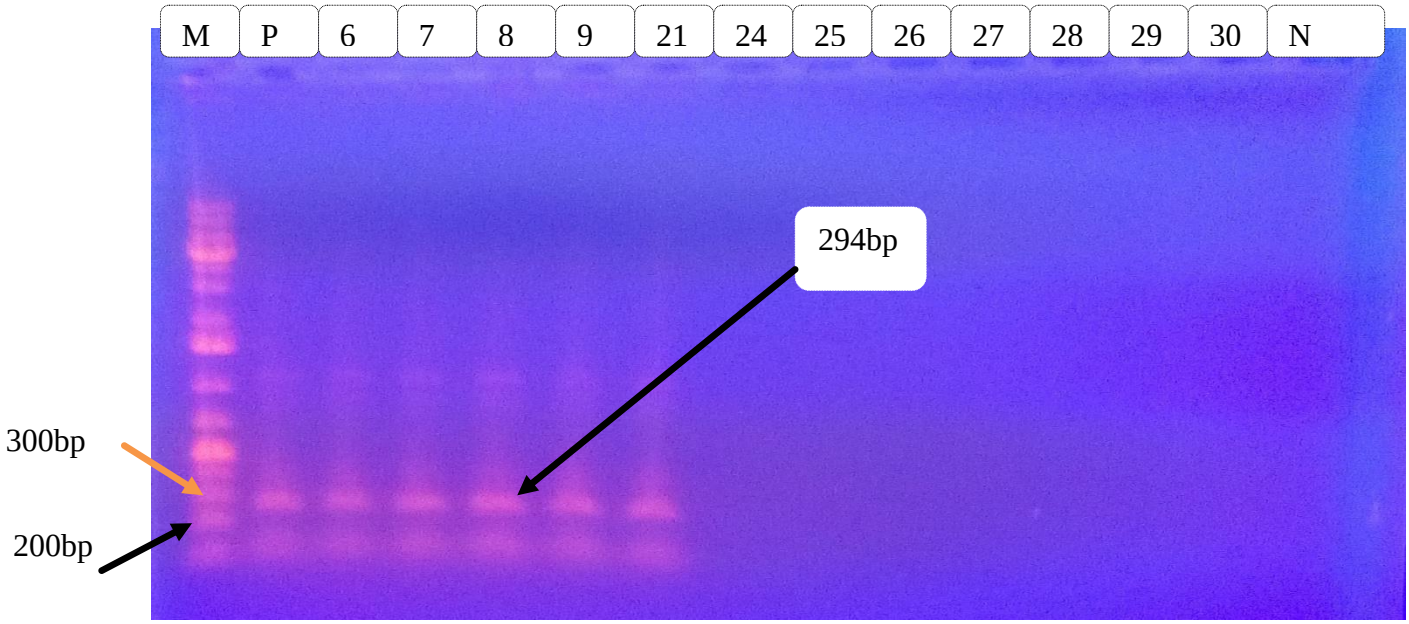

Figure 8: Amplification of St gene in *E. coli* isolates from diarrheic calves: M, Marker (100 bp DNA ladder); P, positive control; Lane No 6, 7, 8, 9 and 21 indicates positive samples; Lane No 24, 25, 26, 27, 28, 29, and 30 indicates negative samples and N, negative control.

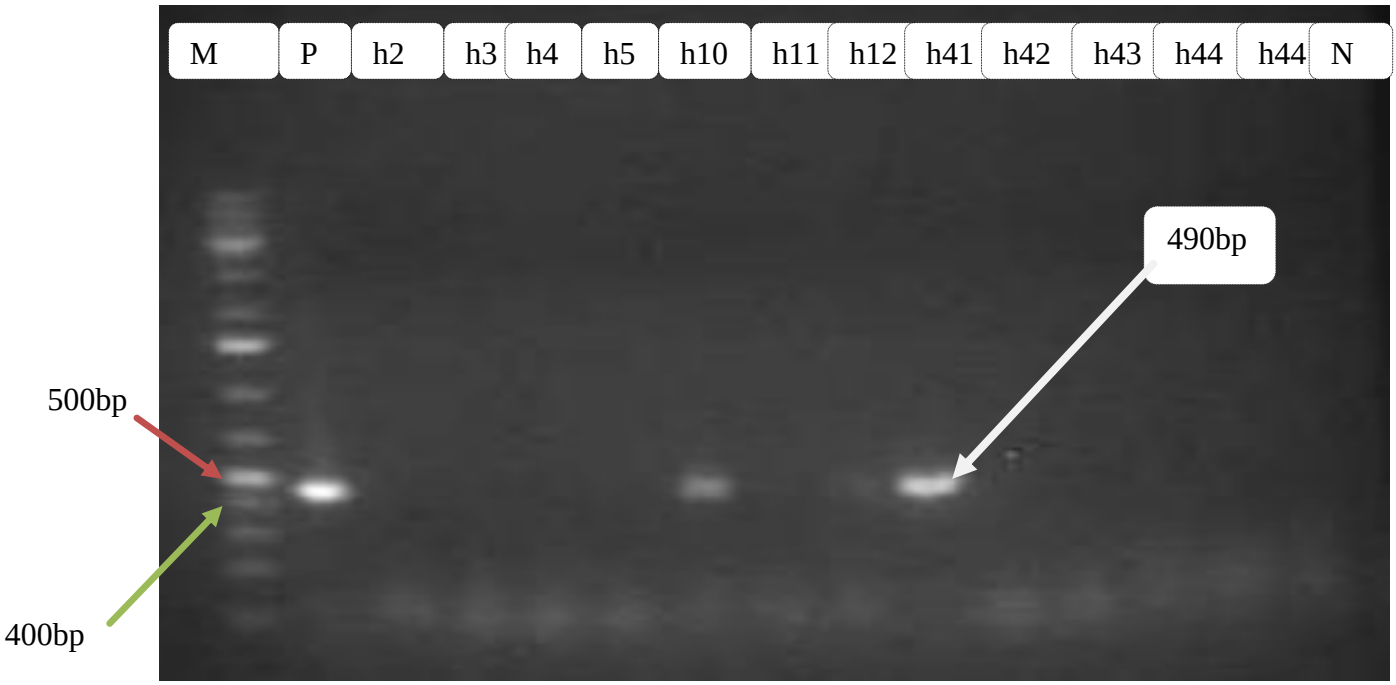

Figure 9: Amplification of *eae* gene in *E. coli* isolates from diarrheic children: M, Marker (100 bp DNA ladder) P, positive control, Lane Noh10 and h41 indicates positive samples, Lane Noh2-h5, and h43-h44 indicates negative samples and N, negative control.

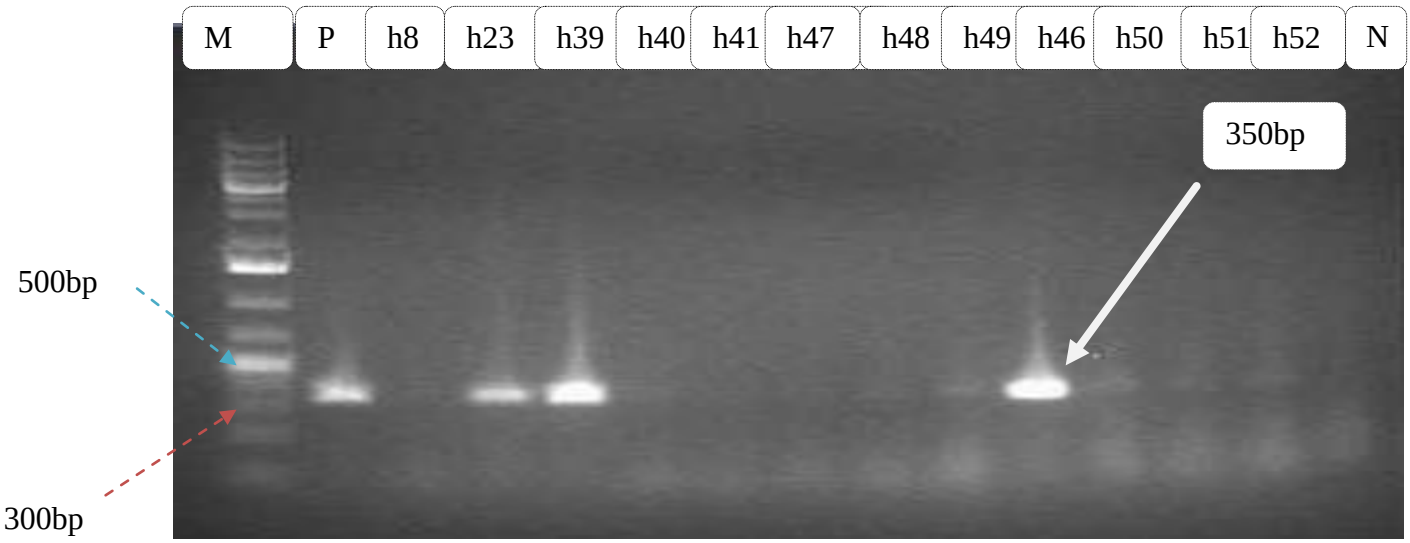

Figure 10: Amplification of *stx1* gene in *E. coli* isolates from diarrheic children: M, Marker (100 bp DNA ladder) P, positive control, Lane No h23, h39 and h49 indicates positive samples, Lane No h8, h40, h41, h47, h48, h49, h51,and h52 indicates negative samples and N, negative control

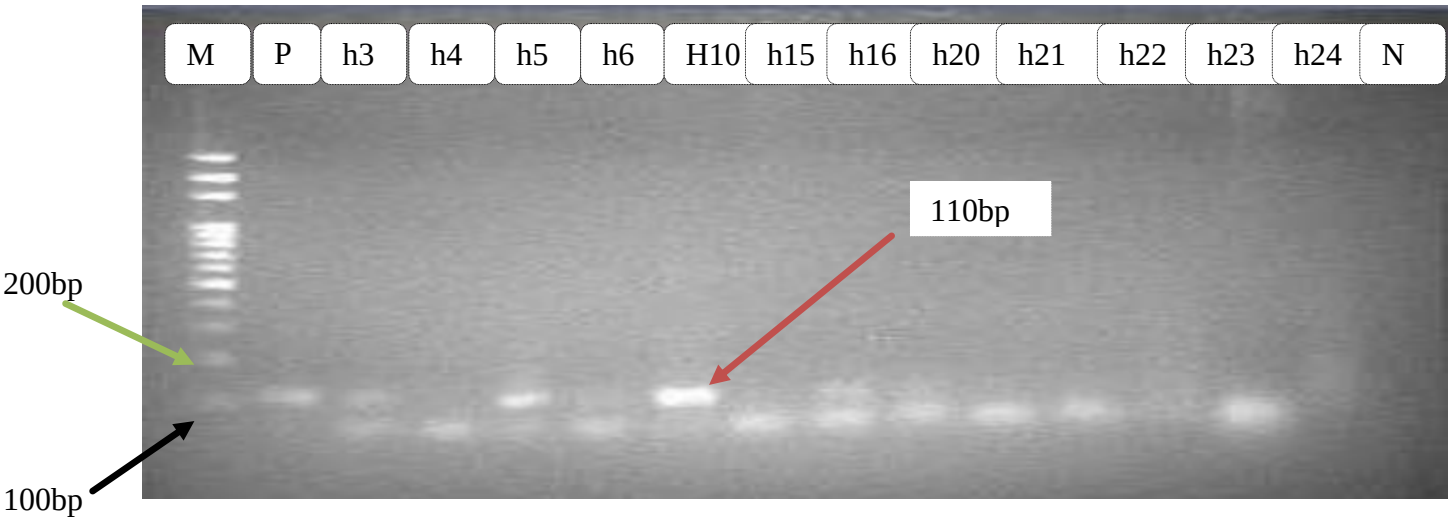

Figure 11: Amplification of *stx2* gene in *E. coli* isolates from diarrheic children: M, Marker (100 bp DNA ladder) P, positive control, Lane No h5 and h10 indicates positive samples, Lane No h3, h4, h6, h15, h16 and h20-h25 indicates negative samples and N, negative control.

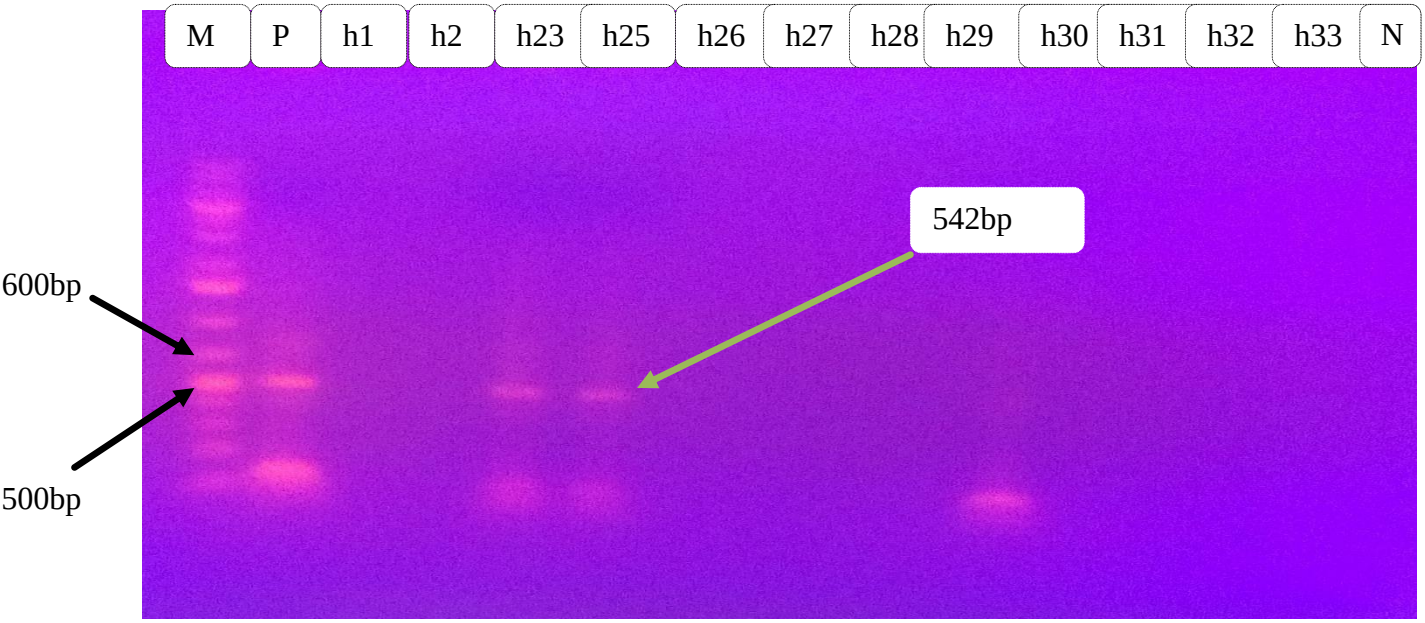

Figure 12: Amplification of *dae* gene in *E. coli* isolates from diarrheic children: M, Marker (100 bp DNA ladder) P, positive control, Lane No h23 and h25 indicates positive samples, Lane No h1, h2 and h26-h33 indicates negative samples and N, negative control.

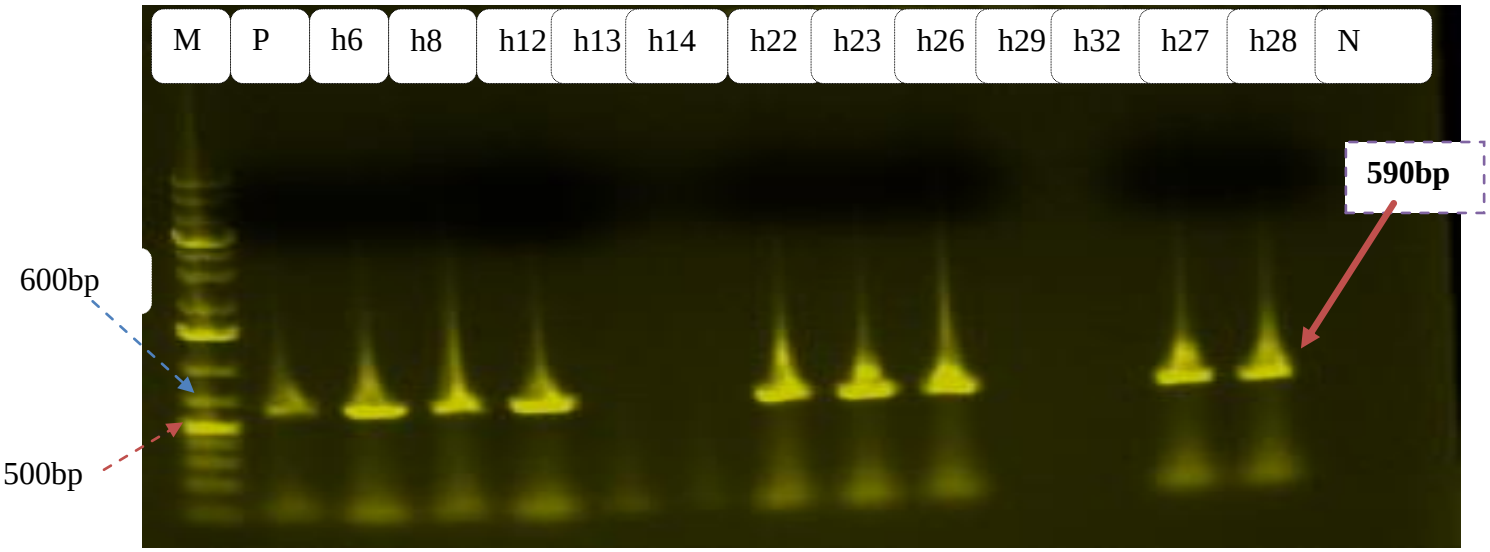

Figure 13: Amplification of *eat* gene in *E. coli* isolates from diarrheic children: M, Marker (100 bp DNA ladder) P, positive control, Lane No h6, h8, h12, h22,h23, h26,h27 and h28 indicates positive samples, Lane No h13,h14, h29, and h32 indicates negative samples and N, negative control.\';\'

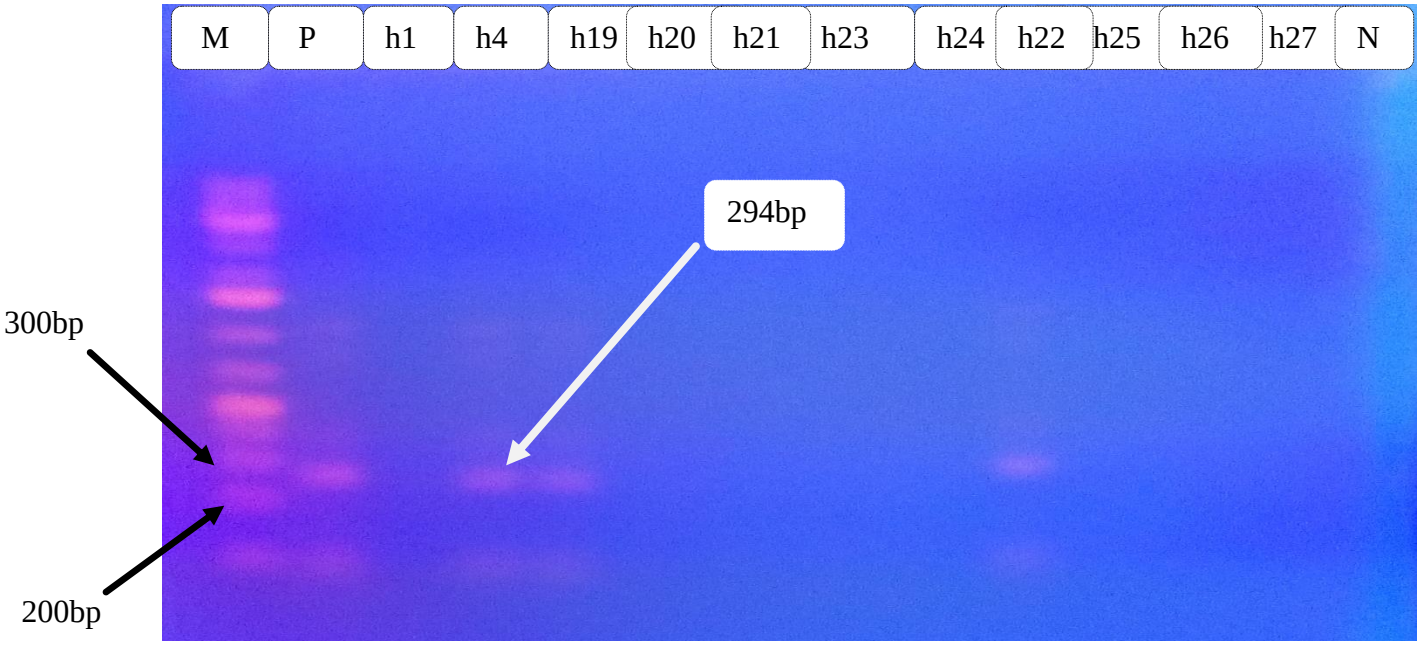

Figure 14: Amplification of St gene in *E. coli* isolates from diarrheic children: M, Marker (100 bp DNA ladder) P, positive control, Lane No h4, h19 and h22 indicates positive samples, Lane No h1, h20- h24 and h25-h27 indicates negative samples and N, negative control.

Table 9: Antimicrobial resistance percentage of pathogenic *Escherichia coli* strains in diarrheic calves at Wolaita Sodo town.

Antimicrobial agents	Detected virulent genes/strains in diarrheic calves (n=44)											
	Resistant Pathogenic *E. coli* strains or genes / (%)											
	STEC (n=22)		aTypical EPEC (*only eae*$^{+}$) (n=8)		EAEC (*eat gene*) (n=6)		St (ETEC) (n=5)		EHEC (n=3)		Total	
	R	S	R	S	R	S	R	S	R	S	R	S
Ampicillin	15(68.2)	7(31.8)	6(75)	2 (25)	6(100)	-	5(100)	-	3(100)	-	35 (79.5)	9 (20.5)
Streptomycin	22(100)	-	7(87.5)	1 (12.5)	5(83.3)	1 (16.7)	5(100)	-	3(100)	-	42 (95.5)	2 (4.5)
Neomycin	22(100)	-	8(100)	-	6(100)	-	5(100)	-	3(100)	-	44 (100)	-
Tetracycline	7(31.8)	15(68.2)	3(37.5)	5(62.5)	4(66.6)	2 (33.3)	3(60)	2 (40)	2(66.7)	1(33.3)	19 (43.2)	25 (56.8)
Ciprofloxacillin	1(4.5)	21 (95.6)	-	8(100)	-	6(100)	-	5(100)	-	3(100)	1 (2.3)	43 (97.7)
Chloramphenicol	5(22.7)	17(77.3)	5(62.5)	3(37.5)	4(66.6)	2 (33.3)	3(60)	2 (40)	1(33.3)	2 (66.7)	18 (40.9)	26 (59.1)
Trimethoprim	9(40.9)	13 (59.1)	6(75)	2 (25)	3(50)	3(50)	4(80)	1 (20)	1(33.3)	2 (66.7)	23 (52.3)	21 (47.7)
Norfloxacillin	5(22.7)	17(77.3)	3(37.5)	5(62.5)	2(33.3)	4(66.6)	-	5(100)	-	3(100)	10 (22.7)	34 (77.3)
Clindamycin	22(100)	-	8(100)	-	6(100)	-	5(100)	-	3(100)	-	44 (100)	-
Sulphonamides	17(77.3)	5 (22.7)	7(87.5)	1 (12.5)	4(66.6)	2 (33.3)	5(100)	-	3(100)	-	36 (81.8)	8 (18.2)

Keys: N= Number of strains; R= resistant; S= Susceptible

Table 10: Antimicrobial resistance percentage of pathogenic *Escherichia coli* strains/virulent genes in diarrheic children in Sodo Christian hospital at Wolaita Sodo town

Antimicrobial agents	Detected virulent genes/strains in children (N=38)													
	Resistant Pathogenic *E. coli* strains or genes / (%)													
	STEC (n= 10)		atypical EPEC (only *eae*$^{+}$) (n=5)		EAEC (*eat* gene) (n=14)		EHEC (n=3)		DAEC (*dae* gene) (n=2)		St (ETEC) (n=4)		Total	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Ampicillin	10(100)	-	5(100)	-	14 (100)	-	3(100)	-	2(100)	-	4(100)	-	38 (100)	-
Streptomycin	9(90)	1 (10)	4(80)	1 (20)	14 (100)	-	3(100)	-	2(100)	-	4(100)	-	36 (94.7)	2 (5.3)
Neomycin	10(100)	-	5(100)	-	14 (100)	-	3(100)	-	2(100)	-	4(100)	-	38 (100)	-
Tetracycline	8(80)	2 (20)	4(80)	1 (20)	12 (85.7)	2 (14.3)	2(66.7)	1(33.3)	2(100)	-	4(100)	-	32 (84.2)	6 (15.8)
Ciprofloxacillin	-	10 (100)	-	5 (100)	-	14 (100)	-	3(100)	-	2 (100)	2(50)	2 (50)	2 (5.3%)	36 (94.7)
Chloramphenicol	6(60)	4 (40)	3(60)	2 (40)	9 (64.3)	5 (35.7)	1(33.3)	2(66.7)	2(100)	2 (100)	3(75)	1 (25)	24 (63.2)	14 (36.8)
Trimethoprim	6(60)	4 (40)	4(80)	1 (20)	11 (78.6)	3 (21.4)	1(33.3)	2(66.7)	2(100)	-	4(100)	-	28 (73.7)	10 (26.3)
Norfloxacillin	3(30)	7 (70)	3(60)	2 (40)	8 (57.1)	6 (42.9)	-	3(100)	1(50)	1 (50)	3(75)	1 (25)	18 (47.4)	20 (52.6)
Clindamycin	10(100)	-	4(80)	1 (20)	13 (92.9)	1 (7.1)	3(100)	-	2(100)	-	4(100)	-	36 (94.7)	2 (5.3)
Sulphonamides	5(50)	5(50)	4(80)		12 (85.7)	2 (14.3)	2(66.7)	1(33.3)	2(100)	-	4(100)	-	29 (76.3)	9 (23.7)

Keys: N= Number of strains; R= resistant; S= Susceptible

The EHEC, atypical EPEC, EAEC, DAEC and St (ETEC) strains in diarrheic calves were highly resistant to neomycin 44 (100%), clindamycin 44 (100%), streptomycin 42 (95.5%), sulphonamides 36 (81.8%), and ampicillin 35 (79.5%). Moderately high resistance was detected towards trimethoprim 23 (52.3%), tetracycline 19 (43.2%), chloramphenicol 18 (40.9%), norfloxacillin 10 (22.7%). The DEC pathotypes were least resistant to ciprofloxacillin 1 (2.3%) (Table 9).

Similarly STEC, EPEC, EAEC, EHEC, DAEC and St (ETEC) strains in diarrheic children were highly resistant to neomycin and ampicillin 38 (100%) and to clindamycin 36 (94.7%). Moderately high resistance was detected towards norfloxacillin 18% (47.4) and chloramphenicol 24 (63.2%). The DEC pathotypes were least resistant to ciprofloxacillin, 2 (5.3%) (Table 10).

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Table 11: Multi drug resistance of pathogenic *Eschericia coli* strains in fecal/stool samples from diarrheic calves and children in dairy farms and in Sodo Christian hospital respectively at Wolaita Sodo town.

	Isolates from calves (n=44)		Isolates from children (n=38)	
Number of drug resisted	Multidrug resistance patterns/number of strains`	Number of strains/ %	Multidrug resistance patterns/number of strains	Number of strains/ %
Three	NOR-CHL-NE (2); CLN-SNM- OT (1); CHL-TRM-NOR (1)	4 (9.1)	-	-
Four	CLN -NE- CHL- AMP (2) AMP-OT- NE- TRM (1);AMP, CLN, TRM, NE (1)	5 (11.4)	CLN- SNM-CHL- TRM (1); AMP - CHL-NE- STM (2)	3 (7.9)
Five	OT - CLN- SNM-CHL- TRM (1); NOR - NE- -CHL-NE- STM (2); CHL - NOR- CLN- SNM- TE (1); STM- NOR TE- CIP- TRM (2)	7 (15.9)	AMP-NE-CHL-NOR-SNM-(1); CLN-TRM-OT-STM-AMP-(2) STM- NOR- CLN- SNM- TE (3)	6 (15.9)
Six	STM-CLN- NOR-CHL-NE-TRM (1); SNM -CHL-TRM- NOR-STM- NE- TE (1); CLN- SNM-CHL-STM- NE- TE (2)	4 (9.1)	NE-OT-CHL-TRM-NOR-CLN (1);AMP-STM-NE-OT-CHL-TRM (1); NOR-CHL-NE-SNM-CLN-AMP-(2)	4 (10.5)
Seven	AMP-CLN -NE -SNM - CHL-TRM- NOR (2); NE-NOR- CLN-SNM- OT-AMP- STM (1); CHL-TRM-NOR-AMP- STM- TE-CLN (1); STM-NE-OT-CHL-TRM-NOR-CLN(2)	6 (13.6)	STM -CLN -NE -SNM - CHL-TRM- NOR (2); NE-NOR- CLN-SNM- OT-AMP- STM (1); CHL-TRM-NE-OT - STM- TE-CLN (1)	4 (10.5)
Eight	TRM- NOR -TE- CIP - OT-AMP- STM- NE (2); AMP -SNM - CHL-TRM- CLN- TE - NOR- OT (1); AMP- STM- NE- TE- CIP- CHL- CLN -TRM (1)	4 (9.1)	SNM -TRM- NOR -TE- CIP - OT-AMP- NE (4); STM -AMP- CHL-TRM- CLN- TE - NOR- OT (3); AMP-STM- NE- TE- CIP- CHL- CLN -TRM (3)	10 (26.4)
Nine	TRM- NOR- CLN - STM- NE-CIP- CHL-AMP - TE (1)	1 (2.3)	CLN - NOR- TRM - STM- NE-CIP- CHL-AMP - TE (2); NOR -AMP- CLN-SNM- OT- NE - CHL-TRM-STM (3); AMP -STM- NE- TE- SNM- OT -TE - CHL-CIP - TRM(3)	8 (21)
Ten	-	-	CLN -NE- CHL- AMP-OT-SNM-TRM-STM-NOR-TE (2) AMP-OT- CIP - TRM- TE- CHL- CLN-TRM-NE-SNM (1)	3 (7.9)
Total		31 (70.5%)		38 (100%)

Keys: AMP- Ampicillin; CLN- Clindamycin; CHL- chloramphenicol; CIP- ciprofloxacillin; STM- Streptomycin; NE- Neomycin; NOR-Norfloxacillin; OT- Oxytetracycline; TE- tetracycline; TRM- Trimethoprim; SNM- Sulphonamides

Thirty one (70.5%) of the isolates in calves showed multi drug resistance; and 4 (9.1%) of isolates showed resistance to at least three drugs and 1 (2.3%) of isolates resisted to nine drugs. Similarly 38 (100%) of the isolates in children showed multi drug resistance; and 3 (7.9%) of isolates were showed resistance to at least four drugs and at most to ten drugs (Table 11).

Multi drug resistance index (MDRI) of individual isolates is calculated by dividing the number of antimicrobial agents to which the isolate was resistant by the total number of drugs to which the isolate was exposed. Values lower than 0.2 are considerd as low risk as well as value higher than 0.2 considerd as high risk (Oladoja and Onifade, 2015). Accordingly MDRI was calculated for fecal/stool samples from both diarrheic calves and children. Four (9.1%) of Pathogenic *E. coli* strains showed maximum (0.8) and minimum (0.3) MDR index value in calves while 4 (10.5%) and 3 (7.9%) of strains showed maximum (0.9) and minimum (0.4) MDR index value respectively, in children (Table 12). The calculated MDR index value which is greater than or equal to 0.3 and 0.4 in diarrheic calves and children respectively indicates high risk of resistance.

Table 12: Multi drug resistance index of pathogenic *Escherichia coli* strains isolated from diarrheic calves and children under 5 years in Sodo Christian hospital at Wolaita Sodo town.

	For calves (n=44)	For children (n=38)
MDR index value	Number of strains /%	Number of strains /%
0.3	4 (9.1)	-
0.4	5 (11.4)	3 (7.9)
0.45	7 (15.9)	6 (15.9)
0.54	4 (9.1)	3 (7.9)
0.6	6 (13.6)	4 (10.5)
0.7	4 (9.1)	10 (26.4)
0.8	4 (9.1)	8 (21)
0.9	-	4 (10.5)
Total	31 (70.5%)	38 (100%)

Key; (n = number of strains)

5. DISCUSSION

This study was conducted to assess the overall isolation rate, to detect virulence genes of pathogenic *E. coli* strains and their antimicrobial susceptibility patterns and associated risk factors in diarrheic calves and children at Wolaita Sodo town. Diarrhea in the neonatal calf is a serious welfare problem and a cause of economic loss due to mortality, treatment costs and poor growth. It is also a complex or multifactorial disease, resulting as it does from an interaction between the host, its environment, nutrition and infectious agents (Scott *et al.*, 2004; Stipp *et al.*, 2009). The detection of pathogenic *E. coli* from diarrheic calves and children has great importance and diarrheic calves are also potential source of the bacterium for human infection. Although, the present study focused on isolation of *E. coli,* the involvement of other microbial, parasitic or management factors 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 (64.8%) out of 88 feacal samples from diarrheic calves. This isolation rate is higher than finding of Bekele *et al.* (2009) 37% in Hawasa, Masud *et al.* (2012) 44% (22/50), Dereje**,** (2012) 43.1% (25/58), Hossain *et al*. (2013) (49%) in Bangladesh, Ghada *et al*. (2013) (50%) in Egypt, Yakob, (2014) (50.9%) in Arsi zone, Abdisa and Minda, (2016) 12.5% in Holeta, Gebregiorgis and Tessema, (2016) 36.8% in Kombolcha . This result obtained was lower when compared with findings of (Paul *et al.* (2010) 76% (76/100), Dawit, (2012) 64% in Addis Ababa and Debrezeit, Yimer, (2014) 69.5% in North Shewa, Sunday *et al*. (2016) 63.2% in Nigeria, and 70.7% (53/75) by Tarekegn and Molla, (2017). This variation in isolation rate could be due to the difference in climatic conditions, age of the calves, farm size and its management practices, sample size, *E. coli* isolation methods used (LeJeune *et al*., 2006).

5.1.1. Risk factors associated with calf diarrhea

In the present study isolation rate of *E. coli* from calf diarrhea was significantly differed by age of calves ($P<0.05$). The occurrence of *E. coli* in calf diarrhea was isolated from all age groups examined, but the odd of being infected was high among age category of less than two

weeks. Accordingly, young calves (age group below 2 weeks) were at high risk of being affected with diarrhea due to *E. coli.* This is compared well with the findings of Temesgen, (2004), Aggernesh, (2010) and Dereje, (2012) who founded the calves aged between 0-30 days were at great risk of diarrhea due to *E. coli*, particularly during the first week of their life and risk decreases with age. This could be due to variation in gut absorption and the efficiency of colostrums intake of calves in different farm management practices. Also the association between the age level and diarrhea was in consistence with study conducted by Yimer, (2014; Islam *et al*. (2015; 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). Neonatal calves under one week of age are particularly susceptible because of incomplete establishment of the normal flora of the intestine, the presence of naive immune system and access of receptors for the adhesions of *E. coli* on the first week of life of the calves.The isolation rates of the bacterium decreases in the subsquent age groups (Villarroel, 2009).

Calving managements and care of newborn are important for the health of the calves. The occurrence of *E. coli* in diarrheic calves due to the time of first colostrums feeding was considered and significantly differed ($P<0.05$). The isolation rate of *E. coli* isolates was significantly higher in calves that fed first colostrums in more than 24 hours than that fed before six hours early. 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 Yimer, (2014) in North Showa. The importance of early colostrums management concerning the diarrheal infection in neonates was also indicated by Klein *et al*. (2015) in Austria. According to Mellor and Stafferd, (2004) and Godden, (2007) the reason for this variation explained as; the structure of the bovine placenta impedes easy acquision of immune globulins by unborn calves during pregnancy and those calves that are born without circulating protective antibodies will become more susceptible to different pathogens. Matte *et al.* (1982) also found that 61% of colostral immunoglobulin containing 80mg/ml of 43 IgG is absorbed in six hours and decreases sharply thereafter. By 24 hours, the gut can absorb only 11% of what it originally could have absorbed at birth and digestive enzymes breakdown and digest all of the antibodies (Donna *et al*., 2006). Again Olsson *et al.* (1993) reported as each hour of delay in

colostrums ingestion in the first 12 hours of age increased the chance of a calf becoming ill by 10%. This indicates that the first six hours are the period in which maximum absorbation of colostral immunoglobulin takes place. For adequate passive of immunoglobulin, in addition to feeding time, quantity, and quality of the immunoglobulin fed to the calf plays an important role (Klein *et al*., 2015).

There was significant association between types of diarrhea and *E. coli* isolation rate in study of Aggernesh, (2010), Dereje, (2012) and Tarekegn and Molla, (2017) in which *E. coli* was recovered at highest rate from watery diarrhea. Current study was in contrast to their study in which no significant association was observed. However in agreement to their work proportion of *E. coli* isolates were higher in watery diarrhea and lower in mucoid diarrhea.

Further more, the occurrence of this bacterium was higher in calves that did not get navel treatment during the birth. This could be due to the contamination of umbilicus during the treatment provides the bacterium with an easy route of entry to the bloodstream and body of neonatal calf. When a calf has septicemia due to *E. coli*, as the bacterium or its toxins presented in blood, the infection disseminates and damages many different organs and develops disease including diarrhea as reported by Walter, (2014). However, it wasn't significantly associated (P>0.05). In current investigation sex, breed, and calving facility were not significantly associated with the occurrence of *E. coli* in calf diarrhea which is similar with finding of Yimer, (2014 and Klein *et al*. (2015).

5.2. Overall Isolation of *Escherichia coli* in Child Diarrhea

The overall isolation rate of *E. coli* isolates was 68 (61.8%) out of 110 stool samples collected from diarrheic children. This result is higher when compared with findings of Rang *et al*. (2006) 31% in Austria, Adugna *et al*. (2007) 48.3% in Bahir Dar, Mandomando *et al*. (2007 22.6 % in Mozambique and Ansari *et al.* (2012) 2.3% (12/525) in diarrheic children under five years old. However it was found to be lower when compared to the findings of Shah *et al*. (2016) 86.5% in Kenya and Ugwu *et al.* (2017) 88% (23/26) in Nigeria. The difference in isolation rate might be attributed to different sociodemographic characteristics, sample size variation and the season when the studies were carried out (Shetty *et al*., 2012).

5.2.1. Risk factors associated with children diarrhea

In this study occurrence of *E. coli* in diarrheic children were observed in all age groups. Its occurrence was differed statistically by age ($P<0.05$). The odd of being diarrheic due to *E. coli* was more likely occur in children 25-60 months age groups than other groups. This finding agrees with study conducted by Moyo *et al*. (2011) in Tanzania and by Ansari, *et al.* (2012) in Nepal. This may be due to protection of infants with age below 6 months against severe diarrhea initially to some extent by maternal antibodies and they seem to have acquired adequate immunity between 12 and 16 months of age. It can also be explained according to Sherchand, (2009) as the children at this age group are more actively involved in playing outdoors, careless about their feeding and drinking habits, poor knowledge about healthcare and personal hygiene that may led them to great risk. Therefore this relatively higher chance of contact to either animals or their manure among higher children age groups than lower age groups could be a possible reason.

There was statistically significant association ($P<0.05$) between diarrheic children with history of contact with either animals or manure and occurrence of *E. coli* in child diarrhea. The odds of dirrheic children who had history of contact with either animals or manure were more likely to be diarrheic than those did not have contact. According to study conducted in Kenya by Edward *et al*. (2006), children having contact with manure was independently associated with the occurrence of *E. coli* infectious diarrhea; farm resident and manure contacted children were significantly more likely to have diarrhea compared with non farm resident children. Similarly according to Radostits *et al*. (2007) small number of *E. coli* pathogens in feaces from cattle has the potential to infect humans; since children are vulnerable to be infected with *E. coli* through direct contact of calves, consumption of feacal contaminated food or water and indirectily by contact with objects contaminated with animal feaces.

According to Aillo *et al*. (2008) hand washing with soap could protect one out of three young children suffered with diarrhea and proper hand washing before meals and after defecation can lower exposure of children to enteric pathogens. The habit of negligence to washing

hands before feeding the child was significantly associated with the occurrence of *E. coli* in diarrheic children. The odds of being infected with *E. coli* was observed more likely in children whose care takers did not wash hands before feeding their hands than those wash their hands before feeding. This is in agreement with the findings of George *et al*. (2014) in Bolivia and Adugna *et al*. (2015) in Bahir Dar that indicates the likely of children being diarrheic whose their caretakers having the habit of negligience to wash their hands.

In present study the statistically significant association ($P<0.05$) between exclusive breast feeding practice of the mothers within the first six months and the isolation rate of *E. coli* in diarrheic children was observed. Children who did not get exclusive breast milk feeding in six months of age were more likely to be susceptible for diarrhea due to *E. coli* than those children who fed exclusive breast milk. This finding is in consistent with study conducted in Kenya by Kageni, (2011) in which the number of diarrheic children was the least when complimentary feeding was initiated after six months of age. In addition National Institiute of Child Health and Development report, exclusive breast milk feeding supplies oligosaccharides and lactoferin bioactive component for effective diarrhea prevention along with the nutritional components (NICHD, 2012). Therfore mothers are adviced to feed their babies with breast milk only and not to give their babies supplementary food items until the age of six months (Abdullahi, 2010).

In this study significant association was not observed between sexes of child of the child and diarrhea due to *E. coli* which is in agreement with study of Adugna *et al*. (2015).

5.3. Antimicrobial Susceptiblity Patterns of *Escherichia coli* Isolates in Both Calves and Children Diarrhea

5.3.1. Antimicrobial suseptiblity patterns of Escherichia coli isolates in diarrheic calves

Antimicrobial resistance has been recognized as an emerging worldwide problem in both human and veterinary medicine, and antimicrobial agents misuse is considered as the most important factor for the emergence, selection, and dissemination of drug resistant bacteria (Sayah *et al.*, 2005). The emergence and dissemination of antimicrobial resistance is an important issue in public health, animal health, and food safety. In the present study antibiotic

susceptibility testing to *E. coli* isolates was performed using eleven different antimicrobial agents, which are commonly used for treatment of bacterial disease in animals.

In current study highly susceptibility of *E. coli* isolates in diarrheic calves to ciprofloxacin (CIP5µg) is in agreement with work of Hiko *et al.* (2008; Masud et *al.* (2012 and Osaili *et al.* (2013). However, the study conducted in Saudi Arabia by (Naser and Wabel, 2007); revealed that there was resistant isolates to ciprofloxacin (CIP5µg). This variation may be probably attributed to the expression of resistant gene code by the pathogen which associated with emerging and re emerging aspects of the isolates (Reuben and Owuna, 2013).

In current study neomycin and sulphonamides which were 54 (94.7%) and 48 (84.2%) 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 work of Abd-Elrahman, (2011). Similarly 46 (80.7%) susceptible to norfloxacillin is in agreement with work of Hiko *et al*. (2008) and (Hossain *et al.* (2013) who reported mostly all isolates highly susceptible to it.

In our investigation, *E. coli* isolates are highly resistance to streptomycin 44 (77.2%), chloramphinicol 31 (54.4%) and trimethoprim 41 (71.9%). This finding agrees with the study conducted by Ali, (2000), Naser and Wabel, (2007) in Saudi Arabia, Aksoy *et al.* (2007), Yenehewot, (2008), Hiko *et al.* (2008) and Abd-Elrahman, (2011) who found streptomycin, trimethoprim and chloramphinicol highly resistant to all isolates. However it is incontrast with the findings of Nazir, (2007)**,** Hiko *et al.* (2008), Taye *et al*. (2013), Osaili *et al.* (2013), Woynshet, (2014) and Dulo, (2014) who stated that all *E. coli* isolates were susceptible to those mentioned antimicrobial agents. Also in study conducted by Shahrani et *al.* (2014), all *E. coli* isolates resisted to streptomycin (98.25%), chloramphenicol (73.8%), and trimethoprim (62.22%) which is in agreement with our investigation. In contrast to this study all *E. coli* isolates were sensitive to choramphenicol in report of Mohamed, (2009), (Masud *et al.* (2012) and Hossain *et al.* (2013). In line with our study Masud *et al.* (2012) also reported streptomycin, highly resistant to all *E. coli* isolates.

In current investigation, 39 (68.4%) *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. Also 100% of *E. coli* isolates resisted ampicillin in study conducted by Sato *et al*. (2005), Hiko *et al.* (2008), Nasir, (2009), Taye *et al.* (2013), Hossain *et al.* (2013) and Dulo, (2014).

The high antimicrobial resistance observed in our study raises a broad discussion on the indiscriminate or improper use of antimicrobials which becoming an alarming situation in drug resistance. The high resistance of these 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).

Multi drug resistance was considerd when an isolate is resistant simultaneously to two or more drugs (Tavares, 2001). Accordingly 59.7% (34/57) *E. coli* isolates in current study were resistant to two or more (up to nine) antimicrobials agent. The number by of isolates resistant to five drugs was higher followed by seven drugs resistant in calves. Multi drug resistance of *E. coli* isolates in calves were observed in study were conducted by Osman *et al*. (2013), Hossein *et al*. (2013; Yimer, (2014), Islam *et al*. (2015).

5.3.2. Antimicrobial susceptiblity patterns of Escherichia coli isolates in diarrheic children

The problem of antimicrobial resistance to *E. coli* isolates in diarrheic children is not unique to Sodo Christian hospital. According to Nguyen *et al.* (2005), it is also critical problem in many developing countries.

In present study resistance of *E. coli* isolates to ampicillin 48 (70.6%) in diarrheic children was observed. This result agrees with findings in Vietnam (86%) by Nguyen *et al*. (2005), in México (73%) by Estrada-García *et al*. (2005), and in Mozambique (72%) by Mandomando,

(2007). Furthermore Akingbade *et al*. (2014) reported (55.6%) isolates resistance to ampicillin, which is lower resistance level when compared to our finding. The prevalence of *E. coli* resistance to ampicillin may be due to widespread and indiscriminate use of antibiotics and production of beta-lactamases enzymes (WHO, 2001).

In this study resistant level of ampicillin, chloramphenicol, tetracycline and streptomycin which was 48 (70.6%), 38 (55.9%), 44(64.7%), 65 (95.6%) respectively. However report of Kipkorir *et al*. (2016) showed slight similarity with resistant level of ampicillin which 84% and showed disagreement, in which isolates showed moderate resistant level to chloramphenicol (27%), tetracycline (16%) and streptomycin (9%). Moderate resistance level to chloramphenicol 9.3% and 29 (17%) was also reported by Amaya *et al*. (2011) and Estrada-Garcia et *al*. (2005), respectively. In study conducted by Manikandan and Amsath, (2013) and in Iran by Heidary *et al*. (2014) (93.3%) and 89% of *E. coli* isolates, respectively were showed resistant to ampicillin. Also higher resistant to ampicillin was observed in study of Alikhani *et al.* (2013).

Further more 44 (64.7%) of *E. coli* isolates in current investigation were resistant to tetracycline which was slightly in agreement with report in Mexico by Estrada-Garcia *et al*. (2005) and in Iran by Heidary *et al*. (2014) who founded 145 (85%) and 83%, respetively resistant isolates to tetracycline. Higher resistance to tetracycline was also reported by Alikhani *et al.* (2013) in Iran. On the other hand, *E. coli* isolates in child diarrhea was highly susceptible tociprofloxacillin 62 (91.2%) in current study which is inconsistence with findings of Manikandan and Amsath, (2013) and Estrada-Garcia *et al.* (2005) who reported 97.2% and 100 % susceptibility to ciprofloxacin respectively. The relatively higher resistance level of *E. coli* isolates to tetracycline in this study may probably relate to the indiscriminate use of these antibiotics (Usein *et al*., 2009).

In current investigation 63.2% (43/68) of *E. coli* isolates in children showed multi drug resistance. Multi drug resistance of isolates was observed higher to seven drugs followed by resistance to eight drugs. In study of Adugna *et al*. (2015) and Shah *et al*. (2016) reported presence multi drug resistance of isolates in their study. The increasing development of

multidrug resistant bacteria is signalling a serious alarm from treatment point of view or the possible transfer of resistance genes to other related pathogens (Osaili *et al*., 2013).

5.4. Pathogenic *Escherichia coli* Strains in Both Calves and Children Diarrhea

5.4.1. Pathogenic Escherichia coli strains in diarrheic calves

In current study out of 57 *E. coli* isolates 44 isolates was confirmed by PCR which contains at least one virulent gene. In agreement to this Badouei *et al*. (2010) also reported 13 (8.2%) carried strains positive for one or more of the virulence factors. Out of the 44 detected DEC in this study, 22 (38.6%) are identified as STEC, 8 (14%) as atypical EPEC, 6 (13.6%) as EAEC, 5 (11.4%) as heat stable ETEC and lastly 3(5.3%) as EHEC. All virulent genes detected in each pathogenic *E. coli* strains in current investigation were significantly associated with diarrheal infection due to *E. coli* in calves. In agreement to our investigation, Shahrani et *al.* (2014) reported (5.07%) of EHEC and in contrast (28.41%) of ETEC which is higher than our findings.

Shiga toxin *Escherichia coli* (STEC) has been implicated as an etiological factor of calf diarrhea and these animals form a principal reservoir of STEC that is pathogenic for humans (Sandhu and Gyles, 2002)**.** In this study STEC was the first most prevalent *E. coli* pathotype which was detected in 22 (38.6%) out of 57 *E. coli* isolates. In agreement with this finding, a similar percentages (40% or more) of *stx* gene positive *E. coli* strains have been reported in Brazil by Salvadori *et al*. (2003) and India by Arya *et al*. (2008). In contrast to our study Nguyen *et al.* (2011) found a total of 177 isolates (51%) positive for the *stx* genes which is higher prevalence than our findings. Other authors in Austria (Herrera-Luna *et al*., 2009) and Poland (Osek *et al*., 2000) have reported a lower rate 15.2% of STEC and 15 (7.6%) of STEC out of 192 *E. coli* isolates, respectively in diarrheic calves.

In our study out of 22 (38.6%) detected STEC, 8 (14%) and 5 (8.8%) were *stx1* and *stx2* genes respectively and 7 (12.3%) harboured both genes. Slightely similar findings were reported in German by Awad-Masalmeh, (2004) who identified 10.1% and 17.8%, of *stx1* and *stx2* respectively and Ok *et al*. (2009) who detected 13.5% and 5.4% of *Stx1* and *Stx2* genes respectively out of 37 *E. coli* isolates from diarrheic calves. Also in study conducted by

Badouei *et al*. (2010), 8 (5%) and 3 (1.9%) harboured *stx1* and *stx2* gene respectively and 11 (6.9%) harboured both genes from diarrheic calves. Shiga toxin production is essential but not sufficient for EHEC virulence. However, epidemiological studies have revealed that *stx2* is more associated with severe human disease like hemorrhagic colitis (HC) and haemolytic–uraemic syndrome (HUS) than *stx1* (Beutin *et al*., 2004; Caprioli *et al*., 2005).

The second most prevalent DEC identified in our study was atypical EPEC 8 (14%) out of 57 *E. coli* isolates from diarrheic calves. This pathotype is classified as typical when it carries both intimin (*eae* gene), and *bfp*A, whereas atypical strains when it only encode *eae* gene but not *bfp*A (Vidal *et al*., 2007). Most EPEC strains have both bundle-forming pilus gene (*bfpA*) and *eaeA* gene, but in this study, all of EPEC strains isolated were atypical (only contained the *eaeA* gene but not *bfpA*) which is in agreement with report of Islam, (2015) who identified atypical EPEC from all isolates. Study conducted in Bangladesh by Islam, (2015) and in Korea by Hur *et al*., (2013 who detected 12.50% and 13-17% respectively, is slightely higher than our finding. Similarly in study conducted in Poland by Osek *et al*. (2000) and in Austria by Herrera-Luna *et al.* (2009), *eaeA* gene was detected in 7 (46.7%) and 57.1% of *E. coli* isolates in diarrheic calves respectively, which is higher than our finding.

Also study conducted in Japan by Yuluo *et al*. (2010), in Vietnam by Nguyen *et al*. (2010) and Iran by Salehi *et al*. (2011) showed a prevalence of *eaeA* gene at range of 1.2% to 9.8% which is lower than our findings. Similar to this findings, Badouei *et al*. (2010) and Ok *et al*. (2009) identified (6.3%) and (8.1%) out of 37 *E. coli* isolates respectively was detected from diarrheic calves which was lower than our finding. Also the *eaeA* genes were detected in approximately between 13 and 17% of the isolates, out of 130 darrheic calves by Hur *et al*. (2013).

In present study 3 (5.3%) EHEC out of 57 *E. coli* isolates, was detected which is lower than finding of Kang *et al*. (2004) who reported 24 (9.8%) of EHEC out of 244 fecal samples respectively in diarrheic calves. About 2 (3.6%) of EHEC and 2 (3.5%) of STEC harboured *hlyA* gene in our investigation is contrast with study study of De Verdier *et al*. (2012) who did not detect this gene in any of *E. coli* isolates. Again in contrast to our study higher prevalence was observed in study conducted in Poland by Osek *et al*. (2000) and in Austria by Herrera-

Luna *et al*. (2009) who founded (46.7%) and (50%) of *hlyA* gene respectively in the diarrheic calves. Also Badouei *et al.* (2010) identified (6.3%) of *hlyA* gene in his study.

In current study 5 (8.8%) of (St) toxin was detected out of 57 *E. coli* isolates. This result is very low when compared with with the report of Ok*et al*. (2009) and Hur *et al*. (2013) who identified 18.9% (St) toxin out of 37 *E. coli* isolates and 13% (St) toxin out of 130 fecal samples respectively in darrheic calves. In study of Rigobelo *et al.* (2006) 85 (49.1%) of the *E. coli* strains produced toxins and out of this 53 isolates were detected as producing (St) toxin. Also from one week old diarrheic calves 12 (16%) isolates out of 75 diarreheic calves were detected as enterotoxigenic *E. coli* in study conducted by Guler *et al*. (2008).

In this study *bfpA* and (Lt) toxin were not detected in any isolates which is in agreement with study of De Verdier *et al*. (2012) who did not detect these genes in all isolates. However study conducted by Rigobelo *et al*. (2006) 85 (49.1%) of the *E. coli* strains produced toxins and out of this 9 isolates produced Lt toxin and also 23 isolates were shown to harbor only the LT-II gene.

In the present study, 13 (22.8%) of *E. coli* isolates that were isolated from faecal samples of diarrheic calves didn't harbor any virulent genes. This result agrees with finding of Islam, (2015) who reported 30 (35.3%) of *E. coli* strains without any virulenct genes. This may be due to either these strains were non-pathogenic *E. coli* or the animals have diarrhea caused by some other infectious agent.

All detected virulent genes in this study showed variation in prevalence when compared to other research works conducted in different places by different authors. This variation in prevalence of virulence genes might be due to season, farm size or number of animals on the farm, sample size or number of experimented isolates, hygienic status, and farm management practices, variation in sampling time, and way of *E. coli* isolate preservation and age of the animals.

5.4.2. Pathogenic Escherchia coli strains in diarrheic children

In current study, five distinct pathotypes of DEC were detected in children with diarrhea at Sodo Christian hospital at Wolaita Sodo town. The diarrheagenic *E. coli* as a group was responsible for 30-40% of acute diarrhea episodes in children (Haghi *at al*., 2014). All virulent genes detected in each pathogenic *E. coli* strains in current investigation were significantly associated with diarrheal infection due to *E. coli* in children. In this study 38 (55.9%) DEC categories out of 68 *E. coli* isolates were detected. This finding is higher when compared to the findings of Bueris *et al.* (2007, Moyo *et al.* (2007, Alikhani *et al.* (2013 and Canizalez *et al*. (2016) who reported 23% frequency of DEC categories in their study. Also slightely in agreement to our study, it was detected in Iran by Haghi *et al*. (2014) and in India by Rajendran *et al*. (2010) who founded DEC pathotypes as 30.4% out of 137 cases and 203 (52%) out of 394 cases respectively from diarrheic children aged below five years. In other reports in Nigeria by Onanuga *et al.* (2014) and in Sweden by Saeed *et al.* (2015) DEC strains were recovered as 61 (18.4%) out of 201 cases and 211 (48 %) out of 437 cases respectively from diarrheic children.

In present study the first most isolated *E. coli* pathotype is EAEC with prevalence of 14 (20.5%) out of 68 *E. coli* isolates, which is followed by EHEC 10 (14.7%), EPEC 8 (11.8%), ST 4 (5.9%) finally DAEC 2 (2.9%). Similarly in report of Canizalez *et al*. (2016), EAEC was the most commonly isolated category, followed by EPEC and ETEC. These three pathotypes of diarrheagenic *E. coli* were also detected most frequently in children with acute diarrhea in Brazil by Bueris *et al*. (2007), in Tanzania by Moyo *et al*. (2007) and in Lybia by Ali *et al*. (2012), and Similarly Bueris *et al.* (2007), Theresa *et al*. (2009), Makobe *et al.* (2012), Haghi *et al.* (2014) and Canizalez *et al*. (2016) also reported (10.7%), 14% (78/557), 3.86% (8/207), 8% and 12.2% (126/242) of EAEC pathotype respectively from diarrheal children.

Enteroaggregative *Escherichia coli* is an emerging enteric pathogen associated with acute and persistent diarrhea ($\geq$14 days) and may cause malnutrition and growth defects in children. EAEC strains have been associated with traveler's diarrhea in both developing and industrialized countries (Kaper *et al*., 2004), (Nguyen *et al*., 2005) and (Kaur *et al*., 2010).

The high frequency of EAEC described in our study, along with the high resistance to antibiotics, support the need for follow-up epidemiological studies, pathogenesis, and its role in the different forms of diarrhea (Nakhjavani *et al*., 2013). An increasing number of studies support the association of EAEC with diarrhea in populations in developing countries (Nataro *et al*., 2006). It has also been shown that EAEC is a heterogeneous group of *E. coli* and that not all strains are capable of causing diarrhea (Vilchez *et al*., 2009).

In the present study, STEC were second most prevalent strains which was detected in 10 (14.7%) out of 68 isolates in children with diarrhea. In contrast to our investigation lower result 1% (5/557) was reported in Peru by Theresa *et al*. (2009). Also in another study conducted by Haghi *et al.* (2014), 7 (1.6 %) were positive for *stx1* only, 6 (1.3 %) were positive for *stx2* only.

In current study 8 (11.76%) of atypical EPEC (*eaeA* $^{+}$ and *bfp* $^{-}$*)* was third most prevalent strain which was detected out of 68 isolates. However 39 (17.7%) and 45 (40.5%) *E. coli* isolates in Thailand reported by Ratchtrachenchai *et al.* (2004) and in Iran by Graves *et al.* (2006) respectively are possessing *eaeA* gene. Also (Chen *et al.*, 2005) found that all EPEC isolates harboured *eaeA* gene. Theresa *et al*. 2009) reported the prevalence of EPEC as 7% (37/557) in stool samples from diarrheal children. This pathotype was also isolated with prevance of 19.3% (40/207) in Kenya by Makobe *et al*. (2012) and 5.1% (53/242) in Mexico by Canizalez *et al*. (2016). Atypical and Typical EPEC was detected 9.4% and only in one sample respectively in Brazil which was reported by Bueris *et al*. (2007).

On the other hand, a lower percentage of *eaeA* gene distribution among EPEC was reported in Japan by Fukuda *et al.* (2002) who showed all EPEC strains isolated from children with diarrhea did not react with *eaeA* specific primer. Also in report of Vila *et al*. (1999), from 21 EPEC strains 13 had only the *eaeA* gene, only one strain harboured *bfp* gene and 5 strains were positive for *eae* and *bfp*. None of 77 serological identified EPEC strain contained *eaeA* gene in study conducted by Phantouamath *et al*. (2003).

Atypical EPEC is an emergent enteric pathogen that has only recently begun to attract the attention of investigators (Afset *et al*., 2004; Vidal *et al*., 2007; Nakhjavani *et al*., 2013). In our study all isolates were identified as atypical EPEC. However typical EPEC were more prevalent than atypical EPEC in children less than 2 years old in study conducted in Mexico by Estrada-Garcia *et al*. (2009), an epidemiological studies in several countries showed that atypical EPEC strains have become a more frequent cause of diarrhea than typical EPEC (Trabulsi *et al.*, 2002; Afset *et al*., 2004; Franzolin *et al.*, 2005; Vidal *et al.*, 2005 and (Canizalez *et al*., 2016). These observations have been linked to the duration of the diarrheal disease (Afset *et al.*, 2004; Nguyen *et al*., 2006).

Among children less than 5 years of age, Enterotoxigenic *E. coli* (ETEC) is strongly associated with disease and in many studies is the most common cause of acute diarrhea among children in developing and developed countries (Okeke, 2009). Similar to studies conducted in Brazil and other countries, ETEC was the most commonly isolated pathotype in children with diarrhea (Regua-Mangia *et al.*, 2004; Vilchez *et al*., 2009). In this study all *E. coli* isolates were checked for the presence of heat labile and heat stable toxin but all isolates were negative for heat labile toxin (Lt) and but only 4 (5.8%) of heat-stable toxin (St) was detected. This finding was lower than previous studies on ETEC who reported it as 21.6% by Nweze, (2009), 20.5% by Vilchez *et al*. (2009) and 14.4% by Haghi, *et al*. (2014).

Also in report of Vila *et al*. (1999), ETEC strains were isolated from 44 children (12.7%) and the distribution of these strains according to the type of enterotoxin synthesized was as follows: 33 strains (75%) produced the heat-stable toxin (St), 6 strains (14%) synthesized the heat-labile toxin (Lt), and 5 strains (11%) produced both toxins. Enterotoxigenic *E. coli* was also reported with prevalence of 4% (20/557) in Peru by (Theresa *et al*., 2009) and (4.1%) in South India by (Rajendran *et al.*, 2010) from stool samples of diarrheal children. In another study ETEC was also isolated with prevalence of 7.25% (15/207), in Kenya by Makobe *et al*. (2012), 4.3% (43/242) in Mexico by Canizalez *et al*. (2016) and 3.7% in Brazil by Bueris *et al*. (2007).

In the current investigation, EHEC were detected in 3 (4.4%) out of 68 isolates in children affected with diarrhea. This finding was slightly higher than other studies performed in different parts of the world reported as 1.6 % (Pabst *et al*., 2003), 0.6% by (Bueris *et al*., 2007), 2.1% by (Vilchez *et al*., 2009), 1.3% by (Nweze, 2009), 2.0% by (Rajendran *et al.*, 2010), 3.8% by (Haghi, *et al*., 2014). It was also detected in frequency of 0.3% (3/242) by Canizalez *et al*., (2016) in Mexico, 0.97% (2/207) by Makobe *et al*. (2012) in Kenya. Also in another study conducted by Haghi *at al.* (2014), among the 17 EHEC strains isolated, 1 (0.2 %) was positive for *stx1/eaeA* and 3 (0.7 %) were positive for *stx2/eaeA*. However EHEC strains were not detected in study conducted by Canizalez *et al*. (2016).

The frequency of DAEC strains in our study 2 (2.9%) out of 68 *E. coli* isolates was in general low which is in agreement withwork of Canizalez *et al*. (2016) in Mexico, who reported low frequency 1.4% (15/242) of DAEC strains. However it has been recovered frequently from adults with diarrhea in Brazil Mansan-Almeida *et al.* (2013). Furthermore, in Brazilian children aged 2-5 years living in low socio-economic level, DAEC strains were the second most frequently isolated pathotype associated with diarrhea (Lozer *et al.*, 2013). It was also reported as 4% (21/557) in Peru by Theresa *et al*. (2009) and (0.5%) in South India by Rajendran et *al*. (2010).

In present study all isolates were checked for the presence of *bfp* gene but none of them contained this gene. In contrast to this in study conducted in Tanzania by Vila *et al*. (1999), from 21 EPEC strains 1 strain had only the *bfp* gene, 5 strains were *eae* and *bfp* positive. Also neither EHEC nor STEC harboured *hlyA* gene.

5.5. Multidrug Resistance of Pathogenic *Escherichia coli* Strains in Diarrheic Calves and Children

In the present study Thirty one (70.5%) of the pathogenic *E. coli* strains showed multidrug resistance (MDR) in diarrheic calves. In agreement with our study, MDR was observed in studies of Khan *et al*. (2002), Rigobelo *et al*. (2006), Ynehiwot, (2008) in Ethiopia and Nasir, (2009) in Bangladesh. In contrast other authers Zhao *et al*. (2005; Salehi and Bonab, (2006); Guerra *et al*. (2007); Akond *et al*. (2009) reported MDR in almost all pathogenic *E. coli* strains in their study. In present study, sample containing more than one pathogenic strain of

DEC, were resistant to more than one antimicrobial drug. This might be related to exchange of resistance factors between related bacteria (Tenover, 2006). Multidrug resistance might occur due to lack of proper knowledge, negligence and indiscriminate use of antibiotics that leads to ultimately replacement of the drug sensitive microorganisms from antibiotic saturated environment (Aksoy *et al*., 2007).

All pathogenic *E. coli* strains, 38 (100), in diarrheic children in this study showed MDR (resistance of one isolate to more than four drugs). In contrast to this, other authors Estrada-Garcia *et al.* (2005), Alikhani *et al*., (2013) in Iran and Akingbade *et al*. (2014) in Nigeria reported the MDR of diarrgenic *E. coli* 105 (62%), 67% and 21 (25.9%) respectively. Also in contrast to this study, only 63% of pathogenic *E. coli* strains showed MDR in study of Theresa *et al*. (2009). More than 80% of DEC isolates were also identified as multi-drug resistant in the study conducted in Northwestern Mexico by Canizalez *et al*. (2016).

Multidrug resistance of pathogenic *E. coli* strains in this study could be largely due to acquired antimicrobial resistance phenotypes most often developed via conjugative transfer of plasmid genes (Di Conza *et al*., 2002). Seventy five percent of MDR in pathogenic *E. coli strains* have been reported by Vaishnavi and Kaur, (2003) in India.

Plasmid genes may carry class I integrons, which are mobile DNA elements that are important in the proliferation of bacterial MDR, especially among the gram- negative enteric species.

In present study, 5 (100%) of atypical EPEC, 4 (100%) St (ETEC) and 14 (100%) to EAEC resisted to ampicillin in diarrheic children. In consistence to this in report of Roy *et al*. (2013), resistance of ampicillin was shown as 96% to EPEC, 73% to ETEC and 100% to EAEC. Similarly, in Thailand isolated pathogenic *E. coli* strains showed high resistance to ampicillin in report of Kalnauwakul, (2007). Also EPEC and DAEC strains presented the highest rates of resistance to ampicillin and chloramphenicol in study conducted by Canizalez *et al*. (2016).

In this study all of STEC, EPEC, EAEC and EHEC are highly susceptible to ciprofloxacin. In contrast to these findings, resistance to ciprofloxacin was indicated as 39% to EPEC, 36% to ETEC and 38% to EAEC in study conducted by Roy *et al*. (2013). Also in contrast to our

finding EPEC and DAEC strains presented the highest rates of resistance to ciprofloxacin in study of Canizalez *et al*. (2016). In a study carried out in diarrheic children in Mexico by Estrada-Garcia *et al*. (2005) 70% of DEC strains were resistant to ampicillin whereas most strains were found to be sensitive to ciprofloxacin. The reason for varying rates of resistance against ciprofloxacin according to Yang *et al*. (2009), can be explained as the high prescription rate of this drug in study area as a treatment for enteric infections caused by Gram-negative bacteria.

In this study resistance patterns were strikingly different among the individual DEC groups. Likely DAEC and EAEC had higher resistance levels than STEC. In line with this EPEC and DAEC were the categories with major resistance proportion in study conducted by Canizalez *et al*. (2016). It is possible that DAEC and EAEC are more resistant because they are exposed to antimicrobials more often, which may be because they cause persistent diarrhea and/or are often carried asymptomatically. Thus the long time within human hosts increases the chance that they will be exposed to antimicrobials and/or acquires resistant genes from the resident flora. These data could also reflect an association of resistance genes with plasmid-associated virulence genes, such as adherence factors present in DAEC (i.e. Dr Adhesins) and EAEC (i.e. AAF or aggregative adherence fimbria) according to Lopes *et al*. (2005) and Harrington *et al.* (2006). It is known that resistance to multiple antibiotics can be due to a variety of mobile genetic elements such as plasmids, transposons, and gene cassettes in integrons (Brueggemann, 2006) or to alteration in the *E. coli* multiple antibiotic resistance operon (*mar*) (Barbosa and Levy, 2000).

The high level of resistance is most likely due to selective pressure resulting from uncontrolled and inappropriate use of these agents in hospitals and in the country as a whole. Inappropraite use of antimicrobial agents could lead to resistance in pathogenic bacteria as well as the development of resistance strains in flora bacteria. Historically, much of the interest has focused on pathogenic bacteria; more recently, the role of commensal organisms as a reservoir or vehicle to transfer resistance genes to more harmful, pathogenic bacteria has been postulated according to Sharada *et al*. (2010 and Majalija *et al*. (2010).

6. CONCLUSION AND RECOMMENDATIONS

The study was the first conducted to determine pathogenic *E. coli* strains and their antimicrobial susceptibility patterns as well as associated risk factors from diarrheic calves and children at Wolaita Sodo town. Calf diarrhea is major problem for farm productivity and affected calves could be potential reservoirs of resistant genes carrying pathogenic *E. coli* strains to humans. The overall high proportion of *E. coli* isolates and their significant pathogenic strains indicates the widely distribution of the infection in calves and children in the study area. Age and colostrums feeding time in calves and age, breast milk feeding, hand washing habit and contacts to manure in children were identified as independent predictors for the occurrence of *E. coli*. *Escherichia coli* isolate showed susceptibility mostly to ciprofloxacin and norfloxacillin; therefore, they are considered as best choice of treatment. Higher proportion of *eaat* gene positive *E. coli* strains was detected in children than in calves. Multiple drug resistance was mostly observed in pathogenic *E. coli* strains harboring one or more virulent genes. The calculated MDR index value for all *E. coli* pathotypes in both calves and children indicates the high risk of resistance. Inline with this conclution the following points are recommended.

- Farm owners should focus on appropriate dairy managements especially on time of colostrums feeding and care of the neonates to reduce associated risk factors for occurrence of diarrhea due to *E. coli.*
- Awareness to parents or caretakers of children on importance of exclusive breast feeding and personal hygiene should be maximized.
- To minimize drug resistance appropraite prescription of antibiotics in the farms and hospitals along with perception of customers to use it accordingly; and continouse monitoring of the resistance patterns is highly required.

- Further researches on virulence genes and serotypes of pathogenic *E. coli* strains in diarrheic calves and children in different parts of our country should be done to determine their epidemiological status and to undergo specific vaccine trial.

- Whole genome DNA sequencing, Phylogenetic tree analysis, detection of variant genes and emerging multidrug resistance encoding genes should be done for all molecularly detected *E. coli* strains detected in this study.

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8. APPENDICES

Appendix 1. Questionnaires survey formats

I. Management and Description of dairy farms

1. Farm Identification

Animal code: _____ Farm name: _____

Owners name _____

Address: Kebele _____

When it established _____

2. Farm descriptions

2.1. Owner/manager educations status

a) Illiterate b) Read and write c) Elementary school c) Highschool d) graduate e) professional If professional: Related to animal production ☐ Unrelated to animal production ☐

2.2. Herd size: Cow ☐ Male calf ☐ Heifers ☐ Female calf ☐ Bulls ☐

2.3. Sex: Male ☐ Female ☐

2.4. Age: Day 0-2 Weeks ☐ 3-5 Weeks ☐ 6-9 Weeks ☐ ≥10 Weeks ☐

Breed: Local ☐ Cross ☐ Exotic ☐

2.5. The farms a source of income: Primary income ☐ Secondary income ☐

2.6. Organization of farms: Family farm ☐ Partnership ☐ State ☐

3. Management data

3.1. Calf caretakers (attendants): Owner (family member) ☐ red help ☐

3.1.1. Sex of attendants': Male ☐ Female ☐

3.1.2. Experience of calf caretakers: Less than 5years ☐ reater 5 years ☐

3.1.3. Education of calf care taker: Elementary school ☐ High School ☐

College graduate ☐ Professional ☐

3.2. Perparturient care:

3.2.1. Calving facilities: Calving pen ☐ the same barn ☐

3.2.2. Navel treatment: Practiced ☐ not practiced ☐

4. Awareness of attendants

4.1. About importance of colostrum feeding to neonates: Yes ☐ o ☐

4.2. If yes method of feeding: Suckling ☐ Hand feeding ☐

4.3. Time of first feeding: Less than 6hour ☐ 6-24hours ☐ Greater 24hours ☐

4.4. Duration of feeding: For 24 hrs ☐ For 24 hrs to 4 days ☐ > 24hrs ☐

4.5. If hand feeding - source of feeding: from dam ☐ om another cow ☐

4.6. Types of feeding: Milk ☐ Milk replaces ☐

5. Amount of milk/milk replaces given daily per unit of body weight

5.1. Frequency of feeding: Once per day ☐ Twice/day ☐ Three times/day ☐

5.2. Types of supplementary feed and quality per unit of body weight:

Grazing ☐ Concentrates ☐ hay ☐ ☐

5.3. Weaning age: 4-6 wks ☐ 6-8 wks ☐ 8-18 wks ☐ 2-16 wks ☐

5.4. Housing: Separate pen ☐ ogether with cows in the same born ☐ thers ☐

If separate pen: Individual ☐ Group pen ☐

5.4 Bedding: Present ☐ Absent ☐

If present what is the bedding material and how frequently it is changed

> Once a week ☐ Once a week ☐ < One a week ☐ Daily ☐

5.5 Types of floor: Concrete ☐ Soil ☐ Other ☐

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

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

Diarrhea as important calf health problem----------

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

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

Number of calves that the farm lost due to diarrhea during the last one year----------

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

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

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

II. General information for children under five years

Date: ----------; Code: ----------

1. Childhood and family identification

1.1. Diarrhea Status: Yes ☐ No ☐

1.2. Address: _____; Date of Birth: _____

1.3. Age Category (months): < 5 ☐ 6-12 ☐ 13-36 ☐ 37-60 ☐

1.4. Gender : Male ☐ Female ☐

1.5. Number of household member: Total: _____; No. <5 years old:_____

1.6. Number of rooms in House: _____

1.7. Number of household workers: none ☐ ≥1 ☐

2. Domestic animals in house

2.1. Type: Poultry ☐ Goat ☐ Sheep ☐ Cattle ☐ Donkey ☐

2.2. Have contact with any cows or cattle: Yes ☐ No ☐

2.3. Where any of these animals ill with diarrhea in 7 days before illnesses: Yes ☐ No ☐

2.4. Touch any cow manure: Yes ☐ No ☐

3. Hygienic practice and usual sources of feed and water

3.1. Feed: Cereals ☐ Animal products ☐ Vegetables ☐

3.2. Drinking water: Tap ☐ Filtered ☐ Boiled ☐ Bottled ☐ Well ☐

3.3. Did you have raw meat and milk (unpasteurized): Yes ☐ No ☐

3.4. Hand wash before and after meal:Yes ☐ No ☐

3.5. Toilet: Present ☐ Absent ☐

3.6. Hand wash after defecation: Yes ☐ No ☐

4. Type of diarrhea and observed clinical signs during sampling

4.1. Duration of diarrhea: _____ / days; other :_____

4.2. Number of stools in the last 24h: _____ / per day

4.3. Number of household members reported:_____

4.4. Diarrhea within 10 days before patient's illness: _____times; _____ /days

4.5. Diarrhea nature: Watery ☐ Blood ☐ Mucous ☐ Yellowish ☐ Greenish ☐

4.6. Clinical: Fever ☐ Chills ☐ Vomiting ☐ Loss of weight ☐

5. Antibiotic practices:

5.1. Received antibiotic within 4 weeks before the beginning of diarrhea: Yes ☐ No ☐

5.2. Name of antibiotic:_____ ; Duration of antibiotic therapy:____/ days

6. Results Obtained

6.1. Microscopic examinations result:_____

6.2. Stool culture results:_____

Appendix 2. Generic data Record sheet formats

I. Generic data record sheet format for diarrheic calves.

No	Date	Code given	Calf ID	Name of the farm	Age	Sex	Breed	Type of diarrhea

II. Generic data record sheet format for diarrheic children under five years of age.

No	Date	Code	Address	Age /month/	Sex	Clinical sign	Diarrhea type
1							
2							
3							
4							
5							

Appendix 3. Laboratory record formats

I. Plate isolation and biochemical test result record sheet

No	Mac (Pc/Pac)	EMB (GMSC/NGMSC)	TSI	IMVCi Tests			
				I	MR	VP	Ci
1							
2							
3							
4							
5							

Keys: Mac=MaCconkey agar, EMB=Eosine methylen blue agar, I= Indole, MR=methyl Red, Vp=Vagouse prouskure, Ci= citrate, GMS= Green metallic sheen colony, NGMS=None green metallic sheen colony, Pc=pink colon,pac=pale colony,TSI = Triple sugar iron agar

II. Record sheet for antibiomicrobial suseptibility patterns and molecular detection result.

No	Antibiotic susceptibility test pattern				Type of *E. coli* strain or pathogenic gene was detected
	DIZ that was measured	S	I	R	
1					
2					

Keys: DIZ= diameter of inhibition zone, S=Susceptible, I= intermediate, R= Resistant

Appendix 4. Composition and method of preparation of the media used for isolation of *Eschericia coli.*

I. Non selective enrichment

Buffered peptone water (Himedia, India)

Compostion (g/l): Peptone 10.0, Sodium chloride 5.0, Disodium hydrogen phosphatedoxdecahydrate 9.0 (Na2HPO4.12H2O) Potassium dihydrogen phosphate (KH2PO4)1.5, distilled Water 1000 ml.

Preparation: Dissolve 20 gram the peptone in 1000ml water, adjust pH to 7.0 after sterilization. Dispense into suitable flasks and autoclave at 121^{o}c for 15 min.

II. Isolation on selective plate agar (Himedia, India)

MacConkey agar (MCA) (HiMedia, India)

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

Preparation: Suspended 55.07 gm of dehydrated MCA in 1000 ml distilled water andsterilized by autoclaving at 121 ^{o}c for 15 minutes. The molten mediumwill be cooled to about 50 ^{o}c temperature and poured into sterile Petri plates.

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 (at 25 ^{o}c) 7.2

Preparation: Suspended 36.00 gm of dehydrated EMB in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121 ^{o}c for 15 minutes. The molten medium will be cooled to about 50 ^{o}c temperature and poured into sterile petri plates.

Subculturing / Maintanance

Nutrient agar (Oxoid, Bsingstone, England)

Ingredients (g/l): protease peptone 9.0, sodium chloride 5.0, disodium hydrogen phosphate dodecahydrate 9.0, potassium dihydrogen phosphate 1.5

Preparation: Dissolve 28.09gm in 1000ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121^{0}c) for 15 minutes.

III. Biochemical Tests

Nutrient broth media

Ingredients (g/l) Peptic digest of animal tissue 10.00, Sodium chloride 5.00

Preparation: Suspend 15.0 grams in 1000 ml distilled water. Heat if necessaryto dissolve the medium completely. The pH will be adjusted to 7.4±0.2 and the preparation will be distributed in 5 ml amount into test tubes and sterilized by autoclaving at 121°C for 15 minutes.

Sulphide indole motility (SIM) medium

SIM Medium is recommended for determination of hydrogen sulphide production, indole formation and motility of enteric bacilli.

Ingredients g/l: Beef extract 3.000 g, Peptic digest of animal tissue 30.000g, Peptonized iron 0.200g, Sodium thiosulphate 0.025g, Agar 3.000g Final pH (at 25°C) 7.3±0.2

Preparation: Suspend 36.23 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the tubes to cool in an upright position.

Methyl red-Voges proskauer 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: Suspend 17 grams in 1000 ml of distilled water. Heat if necessary todissolve the medium completely. Distribute in test tubes in 10 ml amounts and sterilizeby autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Simmon's citrate agar.

Ingredients g/l: Sodium Chloride 5.0, Sodium Citrate (dehydrate) 2.0, Ammonium Dihydrogen Phosphate 1.0 Dipotassium Phosphate 1.0 Magnesium Sulfate (heptahydrate) 0.2 Bromothymol Blue 0.08 Agar 15 Final pH 6.9 +/- 0.2 at 25 ^{o}c

Preparation: Twenty three grams of the powder will be suspended in 1 L of distilled water andbrought to boil to dissolve completely. The pH will be adjusted to 6.8. It will be distributed in sterile bottles and sterilized by autoclaving at 121°C for 15 minutes. After sterilization the bottles will be left to solidify in inclined position.

Triple sugar-iron agar.

Ingredients g/l: Beef extract 3.0, yeast extract 3.0, peptone 15.00, protease peptone 5.00, lactose 10.0, saccharose 10.0, glucose 1.0, ferrous sulphate 0.2, sodium chloride 5.0, sodium thiosulphate 0.3, phenol red 0.024 and agar 12

Preparation: Suspended 64.524 gm of the powder will be suspended in 1 L of distilled water and brought to boil to dissolve completely. The pH will be adjusted to 7.4. It will be distributed in sterile bottles and sterilized by autoclaving at 121°C for 15 minutes. After sterilization the bottles will be left to solidify in inclined position.

IV Antimicrobial susceptibility tesing media

Mueller hinton agar (Oxoid, England)

Ingredients g/l: Casein acid hydrolysate 17.50, Beef heart infusion 2.00, Starch, soluble

1.5, Agar 17.00 gm, Final pH (at 25°C) 7.3 +0.2.

Preparation: Suspended 38 gm in 1000 ml distilled water. Sterilized by autoclaving at15 psi pressure, 121 ^{o}c for 20 minutes. The molten medium will be cooled to about 50 ^{o}ctemperature and poured into sterile Petri plates.

0.5 McFarland turbidity standards (Remel, USA)

Composition: 0.05ml of 1% barium chloride (BaCl2); 9.95ml of 1% Sulfuric acid (H2SO4); Approx. Cell density (approximately $3x10^{8}$ CFU per ml); and distilled water

Appendix 5. Gram staining and IMViC tests

I. Gram staining test

Principle: It is used to differentiate members of the bacteria based on the comparative biochemistry of their cell envelopes into Gram positive and Gram negative bacteria (Beveridge, 2001).

Reagents: Crystal violet, Gram's iodine (mordant), Ethanol 95%, Counter – stain (carbon fuchsine / safranin)

Preparation of smears: Small inoculums of bacterium colony will be emulsified in a drop of a sterile normal saline and spreaded on a clean slide. The prepared smears willthen allowedto dry in air and thenfixed by passing over Bunsen burner flame.

Staining Procedure (Carter, 1984)

Make a thin smear or film. Allow the film to dry in air. Fix the film by passing through the Bunsen flame several times. Flood the slide with crystal violent for 30 to 60 seconds. Pour of the stain and wash the remaining stain with iodine solution. Wash off the iodine and shake the excess water from the slide. Decolorize with acetone alcohol. Counter stain with safranin for 30 to 60 seconds and wash with water.

II. IMViC test

Is a set of four useful reactions that are commonly designed for the differentiation of enterics (members of family Enterobacteriaceae). The IMViC series includes following four tests: Indole test, Methyl red test, Voges-Proskauer test and Citrate test.

Indole test (Quinn *et al.,* 1999)

Principle: indole positive bacteria possess an enzyme tryptophanase which converts tryptophan to indole.

Procedure: stab inoculate SIM medium with test bacterium and incubate at 37^0c for 18 to 24 hours. Then add kovac's reagent (0.2ml) to tube and stand for 10 minutes.

Interpretation: the formation of dark red ring indicates positive reaction while in negative reaction a yellow ring is formed.

Methyl red (MR) test (Quinn *et al.,* 1999)

Principle: it is quantitative test for acid production, requiring positive organism to produce strong acids (lactic, acetic, and formic).

Procedure: inoculate MR-VP broth with pure culture of test organism and incubate at 37^oc for two days, then add 5 drops of MR solution in to the media.

Interpretation: production of red color indicates a positive result and yellow color negative in methyl red test.

Voges-proskauer (VP) *test* (Quinn *et al.,* 1999)

Principle: some organisms produce acetone as the chief end product of glucose metabolism and form less quantity of mixed acids.

Procedure: inoculate MR-VP broth with pure culture of the test organism and incubate at 37^0c for 2 days. Then aliquot 1 ml of broth to a clean test tube and add 0.6ml of 5% alpha-naphthol followed by 0.2ml of 40% KOH. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Interpretation: a pink color indicates a positive reaction.

Citrate Utilization Test

Procedure: Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony. Incubate aerobically at 35 to 37^{o}c for up to 4-7 days. Observe a color change from green to blue along the slant.

Materials: Simmonds Citrate Agar, Bunsen burner, Incubator, Loop, Bacteria, Disinfectant, Paper towels.

Interpretation: Positive Reaction shows growth with color change from green to intense blue along the slant. E.g. *Salmonella* negative reaction shows no growth and color change; Slant remains green. E.g *E.coli*

Appendix 6. Reagents that were used isolate *Escherichia coli*

Alpha- naphthol solution: This was obtained from Hopkin and Williams, London; and it was Prepared as 5% solution for Voges-Proskauer (VP) test.

Potassium hydroxide: This was obtained from Hopkin and Williams, London; it was preparedas 40% solution for Voges-Proskauer (VP) test.

Methyl red solution: This solution was prepared by dissolving 0.04 g of methyl red in 40 ml ethanol and the volume were made up to 100 ml with distilled water.

Kovac's reagent: This reagent was composed of 5 g of para dimethyl aminobenzaldehyde, 75 ml amyl alcohol and 25 ml concentrated hydrochlorideacid. The aldehyde was first dissolved in alcohol at 50-55°C, then cooledand the acid was added carefully. It was stored at 4°C for later use in indoletest.

Gram's stain reagents

Crystal violet: Ingredients g/l Crystal violet 20, Ammonium oxalate 9, Ethanol 95ml

Lugol's iodine: Ingredients g/l Potassium iodide 20 g, Iodine 10 g, Distilled water 1L

Decolorize (acetone-alcohol) Ingredients ml/lAcetone 500 ml**,** Ethanol or methanol absolute 475 ml, Distilled water 25 ml

Strong carbol fuchsin: Strong carbol fuchsin consists of two solutions:1-Solution A: Ten grams of basic fuchsin mixed with 10 ml of ethanol(95%), dissolved in stopper bottle and

kept at 37°C overnight. 2- Solution B: Five grams of phenol were mixed with 100 ml of distilled water and shacked to dissolve.Strong carbol fuchsin was prepared by pouring 10 ml of solution Ainto 100 ml of solution B.

Appendix 7. The antimicrobial agent discs used in the present study, their respective disc concentrations and zone diameter interpretive standard chart for *Enterobacteriacea*

I. Antimicrobial agents with respective disc concentrations and their zone diameter interpretive standard chart for *Enterobacteriacea.*

Antimicrobial agents	Disc contents(μg)	Zone diameter, nearest whole mm		
		Resistance	Intermediates	Susceptible
Ampicillin	10	≤ 13	14-16	>17
Norfloxacillin	10	≤ 12	13-16	≥ 17
Sulphonamides	300	≤ 12	13-16	≥ 17
Oxytetracycline	25	≤ 11	12-14	≥ 15
Streptomycin	25	≤ 11	12-14	≥ 15
Trimethoprim	5	≤ 10	11-15	≥ 16
Neomycin	10	≤ 12	13-17	≥ 17
Tetracyclin	30	≤ 11	12-14	≥ 15
Chloramphenicol	30	≤ 12	13-17	≥ 18
Ciprofloxacin	5	≤ 14	15-17	≥ 21
Clindamycin	10	≤ 12	15-20	≥ 17

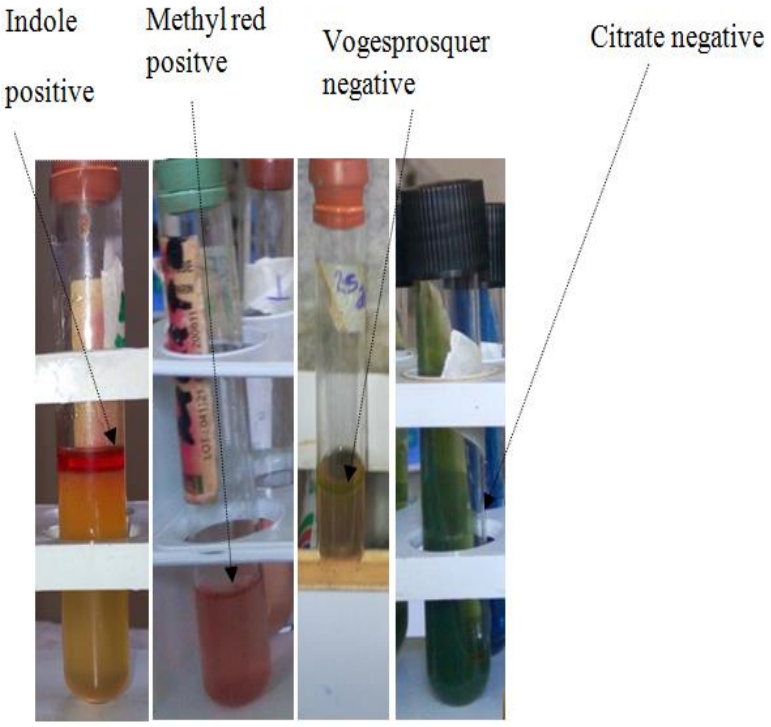
Source: CLIS, 2017.

II. The antimicrobial agent discs used in the present study

Ampicilline 10μg, Norfloxacillin 10 μg, Sulphonamides 300 μg, Oxytetracycline 25 μg, Streptomycin 25 μg, Trimethoprim 5 μg, Neomycin 10 μg, Tetracyclin(TE) 30 μg, Chloramphenicol 30 μg, Ciprofloxacin 5 μg, Clindamycin 10 μg

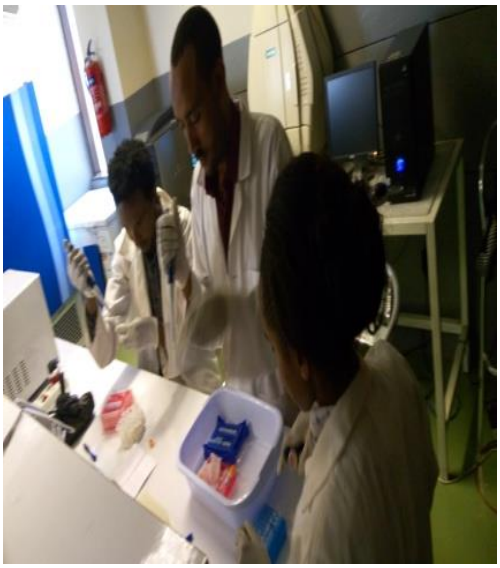
Appendix 8. Some of the photos of entire work

Isolation of *E. coli*

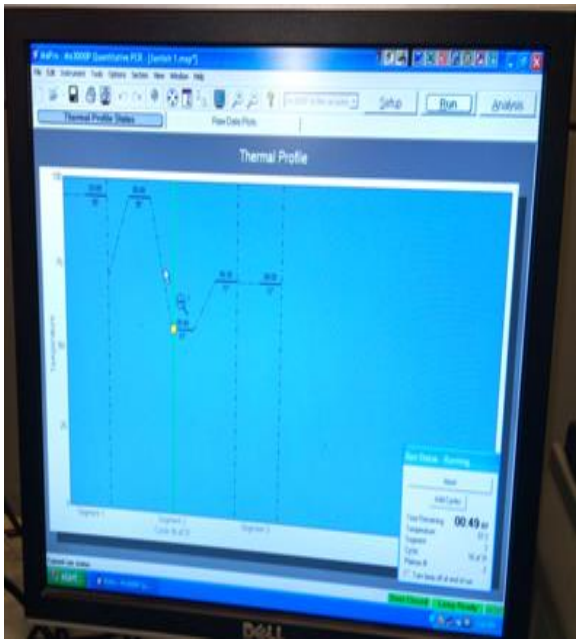


The

Picture showing zone of inhibition of *E. coli* isolates.



PCR master mixing



PCR Optimization

Primers and DNTP

RT –PCR and gel doc system

Appendix 9. Polymerase Chain Reaction protocol and gel preparation and run protocol

I. Materials, equipments and reagents for PCR

a) For DNA extraction and Amplication: Cultures of *E.coli* isolated on nutrient broth, Double distilled water (DDW), Vortex, water bath, pipette tips, Centrifuge (Centurion Scientific Ltd Co) and Pipettors to cover range of volumes (0.25µl-1000µl) with sterile plugs.Template DNA, Primers, Buffer (MgCl2),Tag DNA polymerase, Deoxynucleoside Triphosphate (dNTPs), ice box, gloves, aerosol tips, Sterile PCR tubes, epindorf tubes and PCR machine (Thermocycler) [Biometra, Geotting, Germany]

b) PCR protocol that was used to detect virulent genes in 22µl master mix reaction volume and 3µl template DNA.

The PCR mix contained:

16µl of nucleus free water: 1µl of 0.5µmol of each primer : 2.5µl of PCR buffer with 2µl of 1.5 mmol $MgCl_2$: 1µl of 0.35mmol of dNTPs (dATP, dCTP, dGTP, dTTP): 0.5µl of 1U Taq polymerase enzyme (Solis Biodine), and 3µl of template DNA.

c) For PCR product analysis: Standard power pack, Mini gel electrophoresis and Gel Doc 2000 documentation system (Bio-Rad).

d) PCR reagents

Agarose gel: Two grams of agarose (SIGMA) will be dissolved in 100 ml TAEbuffer. The mixture will be melted in a microwave oven for 45 seconds, left tocool. Then 0.7 µl ethedium bromideswill be added swirled and poured on aplate. The comb will be placed and the gel left to solidify.

Loading dye: Ingredients Bromophenol blue 11% (10 µl), Glycerol (40 µl), DDW (50 µl)

Ethedium bromide (PROMEGA, Madison): Stock solution 10 mg/ml (protected from light).

Running buffer (TAE buffer) Ingredients Tris-HCL 40 ml, Sodium acetate 20 ml, EDTA 584 ml, Distilled water 456 ml.

DNA molecular marker (Ladder) (INVITROGEN): 100 base pair ladder (1.0 µg/µl) 20 µl, Blue dye 80 µl.

II. Gel preparation and run protocol

a) Equipments

Two 1L orange cap bottles: 250 mL flask: Volumetric cylinders: Spatula: Gel casting tray: Gel combs: Tape: Electrophoresis tank: Power supply and cables.

The first six items are used to pour the gel, and the last three are required for running the gel.

b) Gel preparation

- ❖ Both sides of the casting tray were taped so that it will hold the liquid gel.
- ❖ Pour 100 mL of 1x TBE into a clean 250 ml flask (or any other container that is >>100mL and fits well into the microwave) and save the other 900 ml for running buffer.
- ❖ Add 0.8g of Agarose to make for a 0.8% gel (w/v).
- ❖ Microwave until solution is clear and just starting to bubble. Insulated mitts, a paper towel folded several times over, or the like will be used to transport the flask.
- ❖ If the solution is clear and fully liquefied, bring it to your bench and let it cool to about 60ºC.
- ❖ Add Ethidium Bromide (EtBr) to a final concentration of 0.05 μg/mL.
- ❖ Pour the warm liquid agarose. Place the comb into the casting tray by placing the sides into the notches. Wait about an hour until the gel polymerizes and look opaque and uniform. Finally carefully remove the comb to expose the sample wells.

c) Gel running

- ✓ Place the gel into the tank.
- ✓ Add EtBr to the ~900 mL 1x TAE to make it 0.05 μg/mL (45 μL of a 10 mg/mL stock solution). Pour the buffer into the tank high enough to cover the gel.
- ✓ Add 1 μL loading dye per 5 μL sample (because the dye is 6x).
- ✓ Add samples: -Loading 100-500 ng of DNA per lane is usually sufficient
    - -One of the samples should be a marker that contains DNA fragments of

- Known lengths that are in the range of your samples.

✓ Connect the tank to the power supply:

✓ Run for about an hour or until the faster dye migrates most of the way through the gel. To visualize the DNA bands, you must look at them under UVlight.

Appendix 10. Formal letters written to dairy farms and Sodo Christian hospital

I. Formal letters written to dairy farms

ቁጥር: Vet//med/1160/2010

ቀን 22/05/2010

ለ ወላይታ ሶዶ ግብርና ኮሌጅ የወተት ላሞች ፋርም

ጉዳዩ፣ ትብብር ስለመጠየቅ ይሆናል

ከላይ በርዕሱ ለመጥቀስ እንደተሞከረው ሁሉ ዶ/ረ አማኑኤል ወልዴ በጂማ ዩንቨርሲቲ ግብርናና እንስሳት ሕክምና ኮሌጅ በ veterinary microbiology ድህረ ምረቃ ተማሪ ሲሆኑ isolation, identification, antibiogram profile study and detection of pathogenic E. coli strains በሚል ረዕስ ጥናትና ምርምሩን ያካሄዳሉ። ስለሆነም ወደ [illegible] /ለሕክምና ከምመጡት/ [illegible] ናሙና/ stool sample/ እንድወስዱ እንድፈቀድላቸው ስንጠይቅ ለሚታደርጉላቸው ትብብር እናመሰግናለን።

ከሠላምታ ጋር!

T/C ታደለ ቶሎሣ
የእንስሳት ህክምና ት/ቤት
ኃላፊ

II. Formal letters written to Sodo Christian hospital

ቀን 25/07/2010

ለ ሶዶ ክርስቲያን ሆስፒታል

ሶዶ

ጉዳዩ፡ ትብብር ስለመጠየቅ ይሆናል

ከላይ በርዕሱ ለመጥቀስ እንደተሞከረው ሁሉ በጂማ ዩንቨርሲቲ ግብርናና እንስሳት ህክምና ኮሌጅ በ veterinary microbiology ድህረ- መረቃ ተማሪ የሆኑ ዶ/ር አማኑኤል ወሰደ። በመላይታ ሶዶ ሪጂናል ላብራቶሪ Isolation, Identification, Antibiogram Profile Study, Molecular Detection of Pathogenic E. *coli* Strains and Their Associated Risk Factors from Diarrheic Calves in Dairy Farms and Diarrheic Children Admitted to Health Institutions in Wolaita Sodo Town በሚል ርዕስ ጥናትና ምርምሩን እያካሄዱ ይገኛሉ። ስለሆነም ወደ ሶዶ ክርስቲያን ሆስፒታል ለህክምና ከሚመጡ ተቅማጥ ካለባቸው ሕፃናት ናሙና /stool sample/ እንዲወስዱ እንድፈቀድላቸው ስንጠይቅ ለሚታደርጉላቸው ትብብር እናመሰግናለን።

ከሠላምታ ጋር፣

ሳምሶን አበበ ቦይዛ (ዶ/ር)
Samson Abebe Boyiza (Dr)
የላቦራቶሪ ኃላፊ
Laboratory Head