Thesis Ref No

A STUDY ON MAJOR ENTEROPATHOGENS OF CALF DIARRHOEA IN DAIRY FARMS OF ASSELA AND ITS SURROUNDINGS ARSI ZONE OROMYA REGION, ETHIOPIA

MSc Thesis

By

YAEKOB BELAY KIDANE

Addis Ababa University, College Of Veterinary Medicine and Agriculture, Department Of Microbiology, Immunology and Veterinary Public Health

JUNE, 2014

BISHOFTU, ETHIOPIA

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A Thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University, in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology.

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Yaekob Belay Kidane

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Name: Yaekob Belay Kidane Signature

College of Veterinary medicine and Agriculture, Bishoftu

Date of submission: 9/6/2014

ACKNOWLEDGEMENTS

I would like to thank the almighty God who gave me strength and patience to finalize this paper.

I am highly indebted to my advisor Dr. Biruk Tesfaye and co-advisor Dr. Tesfaye Sisay without their encouragement, insight, guidance and professional expertise the completion of this work would not have been possible. My special appreciation also goes to Mr Endashaw Terefe who had advised me on matters related to the thesis/dissertation. A special word of thanks goes to W/rt Tsion Bilata who is a Laboratory Technologist in Assela Regional Laboratory, assisted me in the laboratory work including Saturday, Sunday and holydays.

LIST OF ABBREVIATIONS

AA	Aggregative adherence
AAF	Aggregative adherence fimbriae
AEEC	Attaching and Effacing *E. coli*
API	Analytical profile index
BDV	Border disease virus
BFP	Bundle-forming pilus
BVD	Bovine virus diarrhea
DAEC	Diffusely adherent *E. coli*
DEC	Diarrheagenic *E. coli*
DEM	Direct electron microscopy
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EAF	*E. coli* adherence factor
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbant assay
EMB	Eosin methylene blue
ETEC	Enterotoxigenic *E. coli*
HC	Hemorrhagic colitis
KB	Kilo base

LT	Thermolabile
NCD	Neonatal calf diarrhea
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SLT	Shiga like toxin
SMAC	Sorbitol-macconkey agar
SPV	Salmonella plasmid virulence
ST	Thermostable
STEC	Shiga toxin producing *E. coli*
Stx	Shiga toxin
TSI	Tripple sugar iron agar
UTR	Untranslated regions
VP	Voges-proskauer
VTEC	Vero toxin-producing *E. coli*

ABSTRACT

The proportion of major entheropathogens in diarrheic calves was investigated in 26 small, medium and large scale dairy farms of Assela during six months. A total of 108 fecal and sera samples were collected from diarrheic calves up to 4 months of age. Of these, the proportions of *E. coli*, Eimeria, Cryptosporidium and Giardia detected were 55(50.9%), 42(38.9%), 23(21.3%) and 8(7.4%), respectively. Salmonella Typhimurium was also detected in 3(2.8%) of the sera samples. The most abundant biotype was biotype V, at the rate of 9(56.2%), which fermented the sugars dulcitol, inositol and rhamnose which is possibly ETEC. Biotype VIII, at the rate of 4(3.7%), which fermented dulcitol, rhamnose and xylose might be EPEC. The result of individual and concurrent enteropathogen infections with associated risk factors showed differences in the rate of occurrences of diarrhoea. Considering the complexity of etiologies in calf diarrhoea, further studies are needed to design proper control and preventive measures relevant to the country.

Key words: Biotype, Calf diarrhea, Cryptosporidium, E. coli, Eimeria, Enteropathogens, Giardia, Salmonella, prevalence

1. INTRODUCTION

Newly born calves represent an important source of animal production for either meat or breeding worldwide. Diarrhoea in neonatal calves is a syndrome of great aetiological complexity that causes economic losses directly through mortality and need for treatment, and indirectly from poor growth. In addition to the influence of various environmental, managements, nutritional and physiological factors, the infectious agents capable of causing diarrhoea in the neonatal calf are numerous Schumann *et al*. (1990); Bendali *et al.* (1999).

Diarrhea in calves can be caused by a variety of pathogens including bacteria, viruses, protozoa and intestinal parasites. Important infectious agents are Rotavirus, Coronavirus, enterotoxigenic *E. coli. Salmonella species* and Cryptosporidia either singly or in combination Snodgrass *et al.* (1986); Waltner-Toews *et al.* (1986); Reynolds *et al.* (1986); Steiner *et al.* (1997); De la fuente *et al.* (1998). However other agents can play a role in enteric diseases, as Bovine Torovirus, Parvovirus, Pestivirus, Calicivirus, Astrovirus, Adenovirus, *Eimeria spp.,* Giardia, *Clostridium perfringens,* Campylobacter, Proteus and Klebsiella. Diarrhoea in farm animals, especially in neonatal calves is one of the most challenging clinical syndromes encountered by practicing large animal's veterinary practitioners. Diarrhoea is a leading cause of economic losses to the cattle industry and major cause of calf mortality and morbidity during first few weeks of life in most countries Radostits *et al*. (2000).

The economic losses occur not only from mortality but also from treatment costs and time spent on care as well as subsequent chronic ill thrift and impaired growth performance Bazeley (2003). 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 diarrhoea in order to devise preventive measures and reduce losses during the initial months of life Perez *et al.* (1998); Lorino *et al.* (2005).

In the study area, identification of major enthoropathogens that are involved in calf diarrhea has not been reported.

Therefore, this research was proposed to study on major enteropathogens of calf diarrhea in dairy farms of Asella and its surroundings with the following objectives:

- To isolate and characterize *E. coli* from diarrheic calf feaces
- To identify biotypes of *E. coli* isolates using standard sugar fermentation test
- To detect somatic (O) and flagellar (H) antigens of salmonella from serum of diarrheic calves
- To identify protozoan (Eimeria, Cryptosporidium and Giardia) enteropathogens from diarrheic calf feces
- To evaluate the associated risk factors for the occurrence of enteropathogens

2. LITERATURE REVIEW

Scours is a term for diarrhea; another term that may be applied to this disease is "enteritis," which means inflammation of the intestinal tract. While cattle of any age can develop diarrhea, most cases of scours occur under one month of age, with the majority occurring between roughly 3 and 16 days of life. There are a variety of causes of scours in calves. Most of these are infectious agents like viruses (rotavirus, coronavirus and bovine virus diarrhea (BVD)); Protozoans (Cryptosporidium and coccidian); and bacterial agents (*Escherichia coli*, Salmonella, and *Clostridium perfringens* Olson *et al.* (1997).

Giardia spps can frequently be isolated from calf feces by sucrose flotation and this protozoan may be pathogenic Xiao *et al.* (1993).

Scours is often caused by more than one of these infectious agents acting together. Overcrowding is a major contributing factor to calf scours and causes the number of these infectious agents in the environment to increase dramatically. Certain dietary items may result in diarrhea. These include excess milk production by the dam (the calf ingests more than it can digest), ingestion of foreign objects such as dirt and sand, and from people feeding things that calves can't digest, such as molasses or table sugar (sucrose) Olson *et al.* (1997).

The primary harm from scours is loss of water and electrolytes (body salts) in the diarrhea. This loss of water and salts creates dehydration and alteration of the acid-base balance of the bodily fluids. Inflammation of the intestinal lining impairs the calf's ability to digest nutrients, creating weight loss and the potential for hypoglycemia (low blood sugar). If untreated, these changes can be severe enough to result in death. Research has shown that a substantial proportion of normal, healthy-appearing adult cattle can shed many of the infectious agents that cause calf scours; the agents are mostly shed in faecal matter. This shedding is particularly common for rotavirus, coronavirus, and Cryptosporidium McAllister *et al.* (2005).

2.1. Viral scours

2.1.1. Coronavirus and rotavirus

A number of different viruses can be primary pathogens in the neonatal calf diarrhea complex. Rota and Coronaviruses are the most common identified viral causes of diarrhoea of neonatal food animals. These viruses have also been associated with diarrhoea in adult animals, but their disease incidence in adults is comparably low. However, clinically and sub clinically infected adults shed the virus and are a source of infection for young animals Garica *et al.* (2000).

Rotaviruses can produce high-morbidity outbreaks of diarrhea in calves less than 10 days of age. Mortality is variable mainly owing to secondary bacterial infections and electrolyte imbalances. Rotavirus infection of the small intestinal mucosa leads to loss of enterocytes of the upper third of the intestinal villi with subsequent villous atrophy and malabsorption. There is growing evidence that different rotavirus serotypes of different pathogenicity exists Reynolds *et al.* (1984).

Coronavirus infections can produce high-morbidity outbreaks of diarrhea in calves less than 20 days of age, with variable mortality due to secondary complications. All coronaviruses associated with neonatal calf diarrhea appear to be of the same serotype Mebus *et al.* (1985).

Coronavirus has also been identified in feces of winter dysenteric cattle Saif *et al.* (1991).

Since calf diarrhea is a major health problem of young animals, it has been intensively investigated, and several methods have been used for the detection of enteropathogenic viruses However, each method has its limitations Dea and Garzon (1991).

Direct electron microscopy (DEM) of negatively stained particles is an indispensible tool in viral diagnosis Hyatt (1989).

The use of direct electron microscopy, enzyme-linked immunosorbent assay, and protein A-gold immunoelectronmicroscopy for the identification of bovine corona virus and type A rotavirus were examined. Two hundred and forty-nine samples from diarrheic calves and winter dysenteric cattle from seven geographic areas in Quebec were examined for the presence of viruses by direct electron microscopy of negatively stained preparations. The presence of corona virus and rotavirus in fecal samples obtained from neonatal calves and the presence of corona virus in samples from winter dysenteric adult cattle suggested their etiological roles in the respective diseases. Furthermore, results from protein A gold immunoelectron microscopy of corona virus like particles implied that a different corona virus or some other viruses might be involved in these diseases Saif *et al.* (1991).

2.1.2. Bovine virus diarrhea (BVD)

Bovine virus diarrhoea virus (BVDV) was first recognized in the United States in association with outbreaks of acute and often fatal disease characterized by diarrhoea and erosive lesions of the digestive tract Olafson *et al.* (1946).

BVD has a worldwide distribution. Infection with the virus is common, as indicated by the high prevalence of seropositive cattle and usually associated with disease in older animals; it has occasionally been incriminated as a cause of diarrhoea in neonatal calves Campbell (2004).

The virus of bovine virus diarrhea can cause diarrhea and death in young calves. Diarrhea begins 2 to 3 days after exposure and may persist for quite a long time. Ulcers on the tongue, lips, and in the mouth are the usual lesions that can be found in the live calf. These lesions are similar to those found in yearlings and adult animals affected with bovine virus diarrhea. Diagnosis is by history, lesions, and diagnostic laboratory assistance. Bovine virus diarrhea is controlled by vaccinating all replacement heifers 1 to 2 months before breeding Harkness *et al.* (1978).

Two distinct genotypes were existing, BVDV-I and BVDV-II, together with border disease virus (BDV) Fauquet *et al.* (2005).

BVDVs are genetically variable, containing a single positive-stranded RNA of approximately 12.5 kilo base (kb) in length. The viral genome contains a single large open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR) Carman and Hazlett (1992).

The ORF encodes a proximately 3988 amino acids and ultimately yields 11 to 12 final cleavage polyprotein products (NH2---N -C-E -E1-E2-p7-NS2-NS3-NS4Adefects NS4B-NS5A-NS5B---COOH) through co- and post- translational processing by cellular and viral proteases Rumenapf (1993).

Several European countries have initiated national and regional control-and-eradication campaigns for bovine viral diarrhea virus (BVDV). Most of these campaigns do not involve the use of vaccines; in Germany, vaccination is used only in states in which it is considered necessary because of high BVDV prevalence. In European countries without organized BVDV control programs, vaccination is commonly used to control BVDV. Diagnostic test strategies are fundamental to all control-and-eradication campaigns Van Kruiningen *et al.* (1985).

Laboratory techniques are available for BVDV diagnosis at the individual animal level and at the herd level. These are strategically used to achieve 3 main objectives: 1) initial tests to classify herd status, 2) follow-up tests to identify individual BVDV-infected animals in infected herds, and 3) continued monitoring to confirm BVDV-free status. For each objective or phase, the validity of the diagnostic tests depends on the mode of BVDV introduction and duration of infection in test-positive herds, and on how long non infected herds have been clear of BVDV. Therefore, the various herd-level diagnostic tools--such as antibody detection in bulk milk or in blood samples from young stock animals, or BVDV detection in bulk milk--need to be combined appropriately to obtain effective strategies at low cost. If the individual diagnostic tests are used with due

consideration of the objectives of a specific phase of a BVDV control program, they are effective tools for controlling and eradicating BVDV in regions not using vaccination and where vaccination is a part of the control or eradication program Houe *et al.* (2006).

2.2. Protozoal parasites

2.2.1. Cryptosporidium

Cryptosporidium is a protozoan parasite much smaller than coccidia. It has the ability to adhere to the cells which line the small intestine and to damage the microvilli. Cryptosporidia can be a primary pathogen, but they are often found to be part of a mixed infection in combination with coronavirus, rotavirus, and/or *E. coli*. Calves infected with Cryptosporidium range from one to three weeks in age Vaala (2002).

The first report on bovine cryptosporidiosis was published in 1971, when *C. parvum* parasites were identified in the faeces from an 8-month old heifer with chronic diarrhoea Panciera *et al.* (1971).

The infective stage of the life cycle of Crypto is the oocyst which is passed in the feces and which contains four sporozoites. When the oocyst is ingested the sporozoites are released. These sporozoites invade the cells in the intestine and go through several life stages and ultimately produce more oocysts Bowman (1999).

The most prominent finding in an infected calf is diarrhoea, sometimes accompanied by depression, inappetence, fever, dehydration and/or poor condition. The calf most often recovers spontaneously within 1-2 weeks even though there is a large variation between individuals in how they respond to and recover from infection. Concomitant infection with other enteric pathogens can aggravate the clinical signs and prolong the duration of disease O'Donoghue (1995).

C. parvum is transmitted as microscopic oocysts that are excreted in the faeces from infected animals. Finding oocysts in diarrhoeic faeces is indicative of *C. parvum* being the cause of the disease. During the first two weeks of infection a calf can shed millions of oocysts which ensure efficient dissemination of the parasite Uga *et al.* (2000).

A presumptive diagnosis of Cryptosporidiosis can be made based on history and clinical signs. Definitive diagnosis can be made by doing a fecal flotation and acid-fast stains. Infections in recently dead calves can be diagnosed via microscopic examination of ileal mucosal scrapings or fixed tissue secretions Moore (2002).

2.2.2. Coccidia

Coccidiosis is caused by protozoa of the genus Eimeria, results in health and economic problems to several classes of livestock. All domestic animal species are susceptible to coccidial infections. Although coccidia are host specific, each host may be infected with several species of coccidia at the same time Ernst and Benz (1986).

More than 20 bovine *Eimeria* species are known out of which in particular *E. bovis* and *E. zuernii* cause severe diarrhea in calves and significant economic losses. In contrast, infection with other *E. alabamensis* has been reported in outbreaks of watery diarrhoea in calves on pasture in northern Europe Bartels *et al.* (2010).

Infection with *Eimeria* spp. has a high prevalence in cattle, especially calves and yearlings and causes intestinal damage resulting in depressed growth and performance and greater susceptibility to other infections. Fortunately, where there is a known history of coccidial infection, outbreaks can be prevented with pro-active herd health planning. Most outbreaks are associated with stress, poor sanitation, overcrowding, or sudden changes in feed. Occasionally, affected calves may exhibit signs of brain damage but tarry or bloody scours are commonly observed Cornelissen *et al.* (2002).

The disease reduces feed consumption, body weight, and feed efficiency and may cause mortality of 24% in some cases Fitzgerald (1998).

Incubation periods for *E. zuernii* and *E. bovis* are usually 15 to 20 days. Immunity to coccidiosis persists only 3 to 4 months, and reinfection may occur in the absence of continuous challenge Georgi (1985).

Individual animal testing is of limited value because *Eimeria* spp are frequently found in the faeces of healthy calves Gulliksen *et al.* (2009).

In clinical coccidiosis, the development of diarrhoea is caused by the late stages of the life cycle (second merogony and especially gamogony Mundt *et al.* (2005).

A successful control of natural coccidiosis depends on profound information about incidence, extensity and course of infection under field conditions. Therapeutic intervention at this stage is of limited value and therefore emphasis should be given to metaphylactic treatment in outbreak situations or prophylactic treatment of at-risk groups Daugschies *et al.* (2007).

2.2.3. Giardia

The protozoan genus Giardia (Family Giardiidae, order Giardiida) contains at least six species that infect animals and/or humans. In most mammals, giardiasis is caused by *Giardia duodenalis,* which is also called *G. intestinalis* Adam (2001).

Additional species in animals include *G. agilis* in amphibians, *G. ardeae* and *G. psittaci* in birds, *G. muris* in rodents and *G. microti* in muskrats and voles. *G. varani,* which infects reptiles, is also thought to be a distinct species. Other species of *Giardia* probably also exist in animals, including fish. None of these species, other than *G. duodenalis,* is known to affect people Palm *et al.* (2005).

G. intestinalis, which was previously thought of as a single species, is now described as a group of different variants. This is due to a growing number of studies showing that *G. intestinalis* should be regarded as a species complex, whose genetically divergent members show little morphologic variation Prucca and Lujan (2009).

Genotypes of G. intestinalis and assemblage specific genes

The data supporting the discrimination of *G. intestinalis* into distinct genotypes relies on both, genomic and phenotypic analyses. Today eight distinct genotypes (A to H), also called assemblages, have been assigned. This A to H classification is mainly based on genotyping of several enzymes and highly expressed genes (glutamate dehydrogenase, beta-giardin, elongation factor-1 alpha, triose phosphate-isomerase and small sub-unit rRNA), without considering further genomic differences Caccio and Ryan (2008).

Almost all cases of human Giardia infections are associated with assemblage A or B and according to recent epidemiological statistics; assemblage B isolates seem to be most frequent in human infections worldwide Franzen *et al.* (2009).

Both assemblages are also found in other mammals. The remaining genotypes, grouped into assemblages C to G, are likely to be host-specific, as assemblages C and D have been identified in dogs, cats, coyotes and wolves, assemblage E in cattle, sheep, goats, pigs, water buffaloes and mouflons, assemblage F in cats, and isolates belonging to assemblages G and H in rats and aquatic animals, respectively. The genomes of three different isolates belonging to assemblage A (WB), B (GS), and E (P15) have been sequenced Ankarklev *et al.* (2010).

Using comparative genomics, a well-conserved core of genes coding for 4557 proteins could be identified for the *Giardia* species complex Jerlstrom-Hultqvist *et al.* (2010).

2.3. Bacterial scours

Among the bacterial causes of diarrhoea in neonatal calves, *E. coli* and *Salmonella spp.* are the most common and economically important, but *Clostridium perfringens, Bacteriodes fragilis, Campylobacter spp.* and *Yersinia enterocolitica* have also been identified as causes of enteric disease in calves Steiner *et al.* (1997).

2.3.1. Escherichia coli

Escherichia coli was first described by a Bavarian paediatrician, Theodor Escherich, in the late 19th century. In a series of pioneering studies of the intestinal flora of infants he described a normal microbial inhabitant of healthy individuals Kaper (2005).

E. coli is a gram-negative, motile or non-motile, facultative anaerobic, non-spore forming member of the *Enterobacteriaceae* family. Some strains express virulence genes that enhance organism ability to cause a variety of intestinal infections and diarrheal syndromes among animals and humans Nataro and Kaper (1998).

Diarrheagenic *E. coli* (DEC) strains are among the most common etiologic agents of diarrhea and based on their specific virulence factors and phenotypic traits are divided into 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).

Enteropathogenic Escherichia coli (EPEC)

ETEC produce profuse watery diarrhoea. They are mainly a problem in calves up to 4 days old, although they can occasionally produce diarrhoea in older calves too Naylor (2002).

The 12 serogroups originally recognized by the World Health Organization as EPEC or the classical EPEC are; O26, O55¸ O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 Hernandes *et al.* (2009).

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).

The *eae* is one of the genes currently used for the molecular diagnosis of EPEC. Originally, HEp-2 cell-adherence assay performed with serologically defined EPEC strains showed that 80% of these strains adhere to HEp-2 cells in vitro Cravioto *et al.* (1979).

The presence of the *E. coli* adherence factor (EAF) plasmid carrying *bfp* operon, encoding the type IV bundle-forming pilus (BFP), and *per* operons, a transcriptional activator called plasmid encoded regulator (Per) is the basis of typical and atypical classification of EPEC strains. All EPEC strains lack genes encoding Shiga toxin (*stx*) although they share A/E phenotype with some other strains of *E. coli*, therefore, strains that are *eae+ bfpA+ stx-* are classified as typical EPEC (tEPEC). Production of BFP protein induces the localized adherence pattern (LA) and most of tEPEC strains belong to classic O:H serotypes Trabulsi *et al.* (2002).

Most of the over 200 O-serogroups that have been identified among an EPEC strains, do not belong to classical EPEC serogroups and many have been designated nontypeable Schmidt (2010).

Enterotoxigenic E. coli (ETEC)

ETEC is the most important but under recognized bacterial cause of diarrhea or cholera like disease in all age groups in areas with poor sanitation and inadequate clean water. Diagnosis of ETEC is based on the production of LT and/or ST and the rabbit ileal loop and infant mouse physiological assays were initially used as gold standards for the identification of these enterotoxins respectively. These tests are difficult to perform and time consuming Qadri *et al.* (2005).

Enzyme-linked immunosorbant assay, passive latex agglutination and immunoprecipitation tests were developed subsequently and were found to be specific Donta and Smith (1994).

PCR has revolutionized clinical diagnosis of pathogenic microorganisms and was used in 1994 for detection of ETEC strains but prior to the advent of PCR methods radioactively and non-radioactively labeled probes were used for detection of enterotoxin genes and the method was shown to be both sensitive and specific. Besides determination of the toxins and serotyping, i.e. determination of O serogroups associated with the cell wall lipopolysaccharides and H serogroups of the flagella has been applied for identification and characterization of ETEC Orskov *et al.* (1978).

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

The main virulence factor and the defining feature of this group 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).

In 1983, an *E. coli* strain serotype O157:H7, was identified in association with outbreaks of a bloody diarrhea called hemorrhagic colitis (HC) leading to the recognition of EHEC as a new and increasingly important class of enteric pathogens causing intestinal and renal disease Nataro and Kaper (1998).

STECs are the only 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 Karmali *et al.* (2010).

The term enterohaemorrhagic *E. coli* (EHEC) is applied to those STEC serotypes that have the same clinical, epidemiological and pathogenetic features associated with the prototype strain *E. coli* O157:H7 Nataro and Kaper (1998).

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).

The EHEC 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 (*eaeA*), 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).

Laboratory confirmation of STEC infection can be achieved by isolation and confirmatory tests using culture media, immunoassays, cell toxicity assays and PCR Gould *et al.* (2009).

Screening of O157 relies on the strain's inability to utilize sorbitol rapidly, leading to the use of sorbitol-MacConkey agar (SMAC) as a differential medium with added cefixime and tellurite (CT-SMAC). More specific media have also been developed such as Rainbow agar, CHROMagar®, and O157:H ID agar that are able to recover O157 along with sorbitol-fermenting O157 and non-O157 strains Viazis *et al.* (2011).

Enteroinvasive E. coli (EIEC)

Colonic mucosa is the infection site of *Shigella* and EIEC where invasion of M cells, macrophages and epithelial cells occur resulting in a watery diarrhea, which in severe cases may be followed by the onset of scanty dysenteric stools containing blood and mucus Nataro *et al.* (1995).

There are very few biochemical characteristics that differentiate *Shigella* and EIEC from each other and the two most convenient are mucate and acetate tests. EIEC may be positive for either or both, whereas with rare exceptions *Shigella* strains are negative for both Lan *et al.* (2004).

Salicin fermentation and esculin hydrolysis have also been used to differentiate the two groups Van and Reubsaet (2012).

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).

Enteroaggregative E. coli (EAEC)

Diarrhea caused by EAEC is often watery, but it can be accompanied by mucus or blood Weintraub (2007). Examination of a collection of diarrheal *E. coli* strains that were not of EPEC serogroups showed that many of these strains also adhered to HEp-2 cells and the phenotype was different from that of EPEC Nataro *et al.* (1987).

This pattern of adherence, which had been called "diffuse", was subsequently subdivided into aggregative and true diffuse adherence Bouzari *et al.* (1994).

E. coli showing aggregative adherence (AA) are auto agglutinating, but their hallmark is aggregative adhesion, which involves the formation of a stacked-brick pattern on HEp-2 cells. Colonization of intestinal mucosa by the EAEC occurs via aggregative adherence fimbriae (AAF) encoded by a 55-65 MDa plasmid named pAA. The first one of which, aggregative adherence fimbriae I (AAF/I), was cloned and characterized from EAEC prototype strain Czeczulin *et al.* (1997).

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).

Diffusely adherent E. coli (DAEC)

DAEC 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).

It has been shown that the relative risk of diarrhea associated with DAEC increases with age. Two types of adhesins mediating the DA pattern have so far been described dividing the DAEC strains into AIDA-I-dependent group and those that their adhesins is encoded by a family of related operons, which include both fimbrial and afimbrial adhesins. These groups of proteins are collectively designated Afa-Dr adhesins Labigne *et al.* (1984).

AIDA-I is a 100 kDa outer membrane protein which is associated with DA phenotype was not commonly encountered among DEAC isolates Bilge *et al.* (1989). The adhesion assay however, is not specific for Afa/Dr DAEC detection, since other pathogenic *E. coli* including EPEC strains may show this pattern of adhesion Labigne *et al.* (1984).

2.3.2. Salmonella

Bacteria of the genus Salmonella are Gram-negative, facultatively anaerobic, non-spore forming, usually motile rods belonging to the family *Enterobacteriaceae* and primarily associated with animals. The genus currently contains just two species, *Salmonella enterica* (including six subspecies) and *Salmonella bongori*. A third putative species, *S. subterranea*, has also been proposed following the isolation of a single unusual environmental strain but more recent unpublished data suggest that this organism does not actually belong in the genus *Salmonella* Grimont and Weill (2007).

Most of the Salmonella isolates from cases of human infection belong to *Salmonella enterica* subspecies *enterica*. The genus is also further subdivided into approximately 2,500 serovars (or serotypes), characterized on the basis of their somatic (O) and flagellar (H) antigens. Clinical signs associated with salmonella infection include diarrhea, blood and fibrin in the feces, depression, and elevated temperature Clegg *et al.* (1999).

The disease is more severe in young or debilitated calves. Finding a membrane-like coating in the intestine on necropsy is strong presumptive evidence that salmonella might be involved Clegg *et al.* (1999).

In cattle, *S. dublin* causes enteric fever in both calves and adults and also induces abortion by invading the fetal blood system; and the frequent development of a carrier state, including a condition in which cows shed bacteria in their milk provides the main reservoir of infection. An unusual feature of variation in *S. dublin* is that some strains express the virulence capsular antigen (Vi antigen), an acetylated polymer of galactosaminuronic acid that forms a coat on the external surface of the bacterial cell Spier *et al.* (1990).

The only other bacteria known to express this polysaccharide are the distantly related serovars *Salmonella typhi* and *Salmonella* paratyphi C and a few strains of Citrobacter freundii Selander *et al.* (1990).

For these bacteria, it has been established that the genes determining the structure of the Vi antigen are located in the Via B region of the chromosome Snellings *et al.* (1981).

Strains of *S*. dublin are normally motile and monophasic, expressing a phase 1 flagellin of antigenic type g,p; but in recent years, nonmotile strains of serotype have been recovered from cattle and other animals with increasing frequency both in North America and Europe Franklin *et al.* (1990).

The pathogenic role of the spv (Salmonella plasmid virulence) genes of *Salmonella Dublin* was determined in the natural, bovine host. Since the lack of overt signs of enteritis or enterocolitis due to Salmonella infections in mice has limited the development

of a convenient experimental system to study enteric disease, the contribution of the spv genes to *S*. dublin-induced salmonellosis was studied. Since the SpvR transcriptional regulator is required for expression of the spvABCD operon, the study constructed a spvR knockout mutation in a calf-virulent strain of *S*. dublin. Calves were infected with the wild-type strain, a spvR mutant, and a spvR mutant containing a complementing plasmid. Calves that were infected with the wild type or the complemented spvR mutant rapidly developed severe diarrhea and became moribund. Calves that were infected with the spvR mutant showed little or no clinical signs of systemic salmonellosis and developed only mild diarrhea. The survival and growth of the wild-type strain and the spvR mutant were determined by using blood-derived bovine monocytes. Wild-type *S*. dublin survived and grew inside cells, while the spvR mutant did not proliferate. These results suggest that the spv genes of *S*. dublin promote enhanced intracellular proliferation in intestinal tissues and at extraintestinal sites in the natural host Wray *et al.* (2005).

Serological tests can measure antibodies induced by a salmonella infection. At present there are tests that can detect antibodies against *Salmonella Dublin* and *Salmonella Typhimurium*, the two most common serotypes in cattle. Since some antigens are shared between different serotypes of salmonella, these may cross-react with *S. Dublin* and *S. Typhimurium*. Measurable levels of antibodies in the blood usually appear 1-2 weeks after infection and persist in young animals usually no longer than three months. In older cattle, especially if they have had numerous reinfections, antibodies are likely to persist during a longer period of time but it is not known for how long. In salmonella infection the excretion of bacteria in faeces is often intermittent and of shorter duration, a few days to a few weeks. This means that the period in which there are measurable levels of antibodies in the blood is considerably longer than the period there are bacteria in faeces Schiellerup *et al.* (2008).

Culture detects all serotypes and minimizes the risk of false positive results, but since the excretion of the bacterium in faeces is not constant, the method has a low sensitivity for

individual animals. The outcome of serological tests may vary between different animal populations in different countries Siorvanes *et al.* (2007).

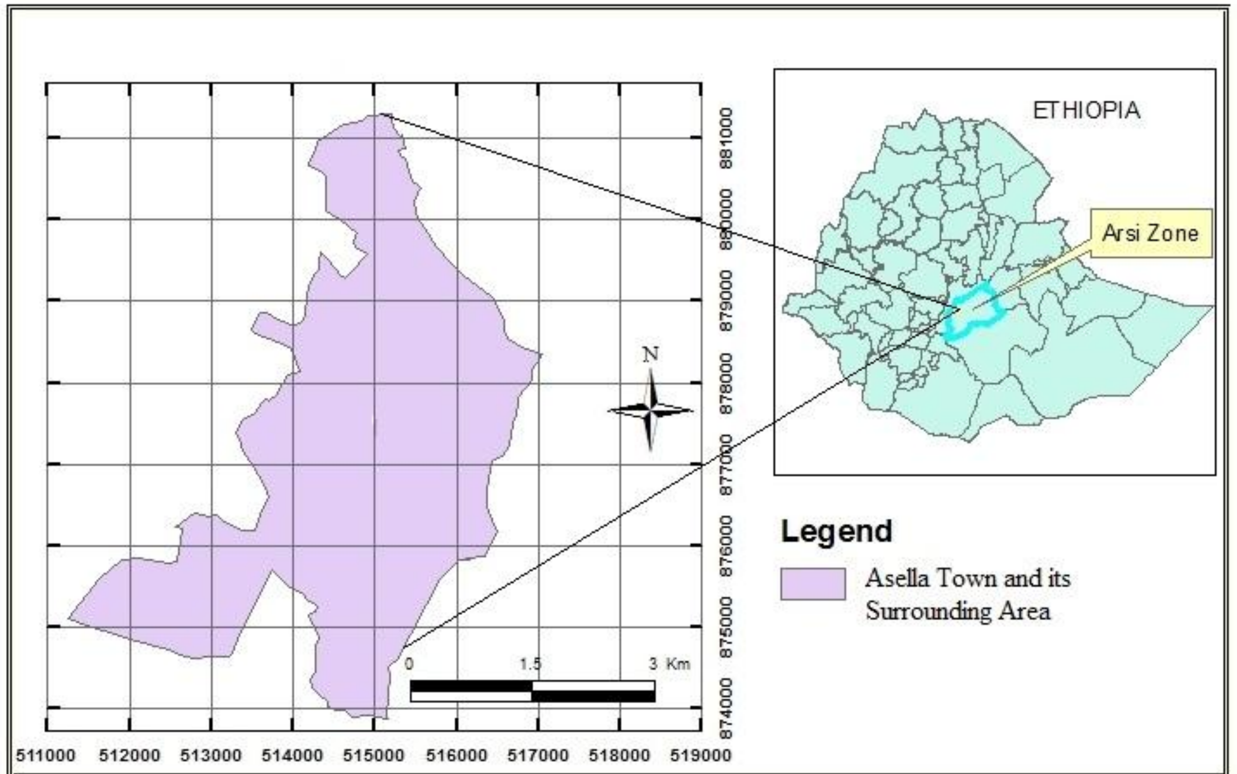
3. MATERIALS AND METHODS

3.1. Study area

The research work was carried out in and around Assela dairy farms during the period of November 2013 to April 2014. Assela is the capital of Arsi zone. Arsi Zone is found in the central part of the Oromiya National Regional State. The zone lies between 6^0 45' N to 8^0 58'N and 38^0 32' E to 40^0 50' E. Asella is found at 175 km Southeast of Addis Ababa. Arsi zone has a total area of 23,881 Km^2 and 22 districts OFEDB (2007).

Tiyo district is one of the administrative units located in Northwestern central part of Arsi zone. The total area of the district is 665 km^2 and it has 21 administrative units of which 18 are Peasant associations and 3 urban administrative units. Undulating plain, hills, rift valley escarpment, Welkesa valley and mountain peak of Chilalo characterize the topography of the district. The altitude of the district ranges from 1500-4105 m. The highest peak in the district is Chilalo Mountain at an elevation of 4005 m above sea level. The district has a tropical high land climate characterized by heavy and erosive rainfall with long wet season. The district is divided into four ecological zones, namely high land (31.7%), Mid-altitude (42.5%), the temperate highland (20.1%) and the low land (5.7%). The annual mean temperature ranges from 15-22^0c and the mean annual rainfall ranges from 900-1100 mm OFEDB (2007). According to Arsi zone livestock development and health agency, the current animal population of the district is: 70, 967 cattle; 55,237 sheep, 14,157 goats, 8,884 horses, 15,730 donkeys and 303 camels.

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 and cross breeds depending on the scale of production.



Source: Arsi Zone Livestock Development and Health Agency

Figure 1: Map showing location of the study area

3.2. Study population

Animals that were included in this study are cross breed calves which are under four months old that were clinically affected with diarrhea.

3.3. Study design and Sampling Methodology

The study was conducted in large, medium and small scale dairy farms of Assela and its surroundings in the period of six months from November 2013 up to April 2014.

Purposive type of sampling was employed, i.e., calves showing diarrhea in the study area during the study period was used as source of sampling.

A total number of 108 loose faeces samples were aseptically collected from 26 small, medium and large scale dairy farms. Approximately 10 ml of faecal sample were collected from non-treated diarrheic calves by direct digital stimulation using a disposable latex glove and submitted to Assela Regional parasitology and microbiology laboratories in sterile plastic bottles cooled on ice packs for isolation and characterization of *E. coli* and protozoas (coccidia, cryptosporidium and giardia). Samples were processed on the same day or stored at 4^0C and cultured within 2 days. Isolation and identification of *E. coli* was performed as per procedures described by Marchant and Packer (1967). Blood samples were also collected from diarrheic calves and Sera samples were extracted and stored at -20^0C for almost six months. Samples were then transported to Arsho Medical Laboratories Plc for detection of antisera of salmonella.

3.4. Data Collection, Management and Analysis

Structured questionnaire format was designed and used to collect information from responsible personnel was interviewed after collecting blood and diarrheic faeces. Data describing diarrhogenic conditions suggestive of *E. coli*, Cryptosporidium, Giardia and Coccidia infection observed on calves along with age (the age of the calves were categorized into four groups 18(14.8%) were from 1-7 days, 18(14.8%), from 8-15 days, 17(15.7%), 16-30 days and 57(54.7%). sex, herd size, calving facility, colostrum feeding method, colostrum feeding duration, time of first feeding, calf house floor bedding and floor type were classified, filtered and coded using Microsoft Excel®2007. The data were exported to SPSS windows version 20 (SPSS INC. Chicago, IL) for appropriate statistical analysis. The proportion of enteropathogens from the total diarrhogenic calves were determined by using descriptive statistics. Chi square (X^2) was used to measure the association among different variables and the occurrence of diarrhea SPSS (2010). Effects were reported as statistically significant if P-value was less than 0.05.

3.5. Isolation of *E. coli*

Primary culture was performed in Eosin-Methylene Blue (EMB) agar, after overnight incubation at 37°C, the characteristics of metallic sheen samples were selected as typical *E. coli* then isolates were preserved in nutrient broth. The representative bacterial pathogens were isolated from diarrheic samples then stained with Gram's staining techniques for the purity of the isolates. The isolated bacterial pathogens were subjected to biochemical reaction such as Triple sugar iron (TSI) agar slant reaction, Methyl- Red (MR), Voges– Proskauer (VP) test, and Indole test Marchant and Packer (1967) (Appendix-II).

3.5.1. Biotypes of E. coli isolates

The pure isolates of *E. coli* were used for fermentation reaction of nine sugars Viz. Dulcitol, inositiol, lactose; maltose, raffinose, rhamnose, salicin, sucrose and xylose were studied. One percent of each sugar in peptone water base with Andradi's indicator was used. Each isolates were inoculated into each sugar medium to be tested. Test tubes were incubated at 37^0C together with control tubes for the consecutive seven days and the results were recorded in every 24 hours. Production of pink color was considered as positive reaction and proper controls were kept for each of the biochemical tests performed. Isolates showing similar fermentation reaction patterns on the nine sugars were considered as belonging to one biotype (Appendix-III).

3.6. Serological Examination of Salmonella

The CROMA TEST stained antigens are standardized suspensions of killed bacteria prepared for the detection and semi-quantitation by agglutination in slide tests of serum agglutinins. The assay was performed by testing the stained *salmonella Typhimurium* antigens-somatic blue; falgellar, red against suspected Serra. After the test reagent and

Sera samples were brought to room temperature, the antigen vial was gently resuspended and aspirated by dropper several times to obtain a through mixture. One drop of the appropriate well-shaken suspension was placed to each circle on glass test card next to the samples to be tested and the contents were mixed by a disposable stirrer then rocked gently by hand. Visible agglutination was observed in positive samples and the positive samples were further analyzed by semi-quantitative test using serial dilutions and the titer was reported as the highest dilution that showed agglutination (Appendix-V).

3.7. Parasitological Examination of protozoans

Faecal samples were tested for the presence of Eimeria, Giardia and Cryptosporidium. Laboratory analysis of the faecal samples was conducted using a flotation technique. For each sample, 5 ml of faeces were processed using sugar solution as the flotation medium to recover Cryptosporidium oocysts, Eimeria oocysts and Giardia cysts. In addition, Modified Ziehl-Neelson Staining technique was conducted for Cryptposporidum oocyst. Microscopic examination was realized using bright field and phase contrast microscopy. The sample was considered positive for the respective parasite when Giardia cysts, Cryptosporidium oocysts and Eimeria ocysts were detected in the specimen (Appendix-IV).

4. RESULTS

Faecal and Sera samples were collected from 108 diarrheic calves in 26 small, medium and large scale dairy farms.

4.1. Occurrence of enteropathogens in calf diarrhoea

Out of the 108 diarrheic samples examined, the proportions of *E. coli*, Eimeria, Cryptosporidium and Giardia were 55(50.9%), 42(38.9%), 23(21.3%) and 8(7.4%), respectively. In addition, anti-*Salmonella Typhimurium* antibodies were found in 3(2.8%) of the sera samples tested. Eimeria is the most dominantly detected enteropathogen 23 (24.73%) next to *E. coli* 25 (26.88%) as a single etiology whereas Giardia 4 (4.3%) was found to be the least of all examined samples. None of infections were detected in 25 (26.88%) of all examined samples (Table 1).

Table 1**:** Frequency and proportion of various enteropathogens, and their occurrence as a single form

Enteropathogen(s)	**Frequency & %**	**Single etiology**		**Total infected**		**Non infected**	
	+Ve & (%)	(N)	(%)	(N)	(%)	(N)	**(%)**
E. coli	55(50.9)	25	26.88				
Eimeria	42(38.9)	23	24.73	60	55.6	25	26.88
Cryptosporidium	23(21.3)	8	8.6				
Giardia	8(7.4)	4	4.30				

Table 2: Frequency and proportion of various entheropathogens with *E. coli* and their occurrence as concurrent form

Concurrent infections of protozoan enteropathogens and *E. coli* with different age groups of calves and sex revealed no significant association in all of the episodes (P-value >0.05) (Table 2).

***E. coli* with Giardia**						***E. coli* with Eimeria**				***E. coli* with Crypto.**				**Eimeria with Crypto.**				***E.coli**Crypto*Eimeria**			
Variable	Level	N	+Isolate & (%)	χ^2	P-Val	N	+&%	$\chi2$	P-Val	N	+&%	$\chi2$	P-Val	N	+&%	$\chi2$	P-Val	N	+&%	$\chi2$	P-Val
Age	0	0	0(0)	-	-	0	0(0)	0.183	0.913	3	2(22.2)	1.757	0.624	1	0(0.0)	2.188	0.534	0	0(0.0)	2.00	0.368
	1	0	0(0)	-	-	2	1(7.7)			4	1(11.1)			4	1(50)			1	0(0.0)		
	2	0	0(0)	-	-	3	1(7.7)			7	4(44.4)			3	0(0.0)			1	1(50)		
	3	6	2(100%)	-	-	31	11(84.6)			3	2(22.2)			2	1(50)			2	1(50)		
Sex	0	3	1(50%)	0.00	1.00	13	5(38.5)	0.049	0.825	5	2(22.2)	0.476	0.490	5	2(100)	2.500	0.114	2	0(0.0)	4.00	0.056
	1	3	1(50%)			23	8(61.5)			12	7(77.8)			5	0(0)			2	2(100)		

Different risk factors that may contribute to the occurrence diarrhea in *E. coli* infections were analyzed and all of the variables were found to be non-significant in this study (P>0.05) (Table 3).

Table 3: Analysis of *E. coli* isolates with different variables

Variables	**Level**	**No of +ve isolate (%)**	**$\chi 2$**	**P-Value**
Calf age	0 (1-7 days)	9(16.4)	3.956	0.266
	1(8-15 days)	9(16.4)		
	2(16-30 days)	12(21.8)		
	3(>30 days)	25(45.5)		
Calf sex	0(Female)	21(38.2)	1.780	0.182
	1(Male)	34(61.8)		
Calving facility	0(Calving pen)	37(67.3)	0.297	0.586
	1(Same barn)	18(32.7)		
Colostrum feeding method	0(Hand feeding)	41(74.5)	0.111	0.739
	1(Suckling)	14(25.5)		
Colostrum feeding duration	0(24-48 hr)	12(21.8)	0.809	0.369
	1(>24 hr)	43(78.2)		
Time of first colostrum feeding	0(<6 hr)	51 (92.7)	0.526	0.468
	1(6-24 hr)	4(7.3)		
Calf house bedding	0(Present)	32(58.2)	0.313	0.576
	1(Absent)	23(41.8)		
Calf house floor type	0(Concrete)	42(76.4)	0.111	0.739
	1(Soil)	13(23.6)		
Herd size	0(<10)	11(20.0)	7.228	0.057
	1(10-20)	7(12.7)		
	2(>20)	37(67.3)		

4.2. Distribution of *E. coli* biotypes in calf diarrhea

All (55) positive *E. coli* isolates were studied for their fermentation activities on nine sugars and all of them (100%) showed the ability to utilize one or more sugars. Accordingly, the most abundant *E. coli* biotypes from calf diarrhea was biotype V whereas the least were biotype III and XII. Biotype I & VIII, biotype II, III & X, biotype XI, IV, & VI and biotype XII & XIII were fermented the sugars in the following proportion 4(3.7%), 3(2.8%), 2(1.9%) and 1(0.9%), respectively. 1(0.9%) of the isolates 30 and 63 collected from two calves fermented only a single sugar (xylose and inositol), respectively (Table 4).

Table 4: Pattern of biotypes of *E. coli* isolates on the basis of fermentation reactions of nine sugars

Biotype No.	**Isolates**	**Total No. of isolates& Proportion**	**Positive sugars**
I	31,93,105 and 107	4 (3.7)	Dulcitol and sucrose
II	18,38 and 83	3 (2.8)	Dulcitol and inositol
III	1,62 and 75	3 (2.8)	Maltose, sucrose and lactose
IV	5,7,26,37,89 and 104	6 (5.6)	Dulcitol, raffinose, Rhamnose and Sucrose
V	3,6,14,15,17,21,22,32,40,54,61,67, 77,86,94,99 and 106	17 (15.7)	Dulcitol, inositol and Rhamnose
VI	10 and 23	2 (1.9)	Dulicitol, raffinose and xylose
VII	16,43,45,64,90,95 and 97	7 (6.5)	Dulcitol, rhamnose and salicin

VIII	20,24,39 and 91	4 (3.7)	Dulcitol, rhamnose and xylose
IX	33 and 42	2 (1.9)	Dulcitol, sucrose and raffinose
X	34,56 and 82	3 (2.8)	Inositol
XI	29 and 52	2 (1.9)	Rhamnose and lactose
XII	30	1(0.9)	Xylose
XIII	63	1(0.9)	Dulcitol and xylose
Total		55 (100)	

Furthermore, based on the result (Table 5) age has great association with the occurrence of *E. coli* biotypes (p-<0.05). Biotype V was most dominantly occurring in 1-7 days of age groups. As age increased the occurrence of *E. coli* decreased (Table 5).

Table 5: Distribution of *E. coli* biotypes among different age groups of diarrheic calves

Biotype	**Level (Age groups)**				**Total**
	0 (1-7 days)	**1(8-15 days)**	**2(16-30 days)**	**3(>30 days)**	
I	0(0.0%)	2(11.1%)	2(11.8%)	0(0.0%)	4(3.7%)
II	0(0.0%)	0(0.0%)	0(0.0%)	3(5.3%)	3(2.8%)
III	0(0.0%)	0(0.0%)	1(5.9%)	2(3.5%)	3(2.8%)
IV	0(0.0%)	1(5.6%)	1(5.9%)	4(7.0%)	6(5.6%)
V	9(56.2%)	3(16.7%)	3(17.6%)	2(3.5%)	17(15.7%)
VI	0(0.0%)	0(0.0%)	1(5.9%)	1(1.8%)	2(1.9%)

VII	0(0.0%)	0(0.0%)	1(5.9%)	6(10.5%)	7(6.5%)
VIII	0(0.0%)	1(5.6%)	0(0.0%)	3(5.3%)	4(3.7%)
IX	0(0.0%)	0(0.0%)	0(0.0%)	2(3.5%)	2(1.9%)
X	0(0.0%)	2(11.1%)	1(5.9%)	0(0.0%)	3(2.8%)
XI	0(0.0%)	0(0.0%)	1(5.9%)	1(1.8%)	2(1.9%)
XII	0(0.0%)	0(0.0%)	0(0.0%)	1(1.8%)	1(0.9%)
XIII	0(0.0%)	0(0.0%)	1(5.9%)	0(0.0%)	1(0.9%)
Count within age	16	18	17	57	108
%within age	100.0%	100.0%	100.0%	100.0%	100.0%

X^2= 62.77 df= 39 P-value= 0.008

4.3 Calf management systems practiced and their association with occurrence of enteropathogens

Management of the calves in the study farms with variations depending on the conditions of the farms was in general as follows: 29(26.9%) calves were left to suckle their dams and 79(73.1%) of them were used hand feeding practice after birth. Thirty (27.8%) of the dairy farms had a herd size of less than 10; the herd size of 19(17.6%) of them was between 10 and 20, and that of 59(54.6%) was greater than 20. The durations of colostrum feeding were for 24-48 hrs and >48hrs in 20(18.5%) of calves and 88(81.8%) of the calves, respectively. Time of first feeding of colostrum were <6hrs and 6-24hrs after birth in 98(90.7%) and 10(9.3%) of the calves, respectively. Calving pens were used for 70 (64.8%) of the calves; whereas 38 (35.2%) of the calves were born in the same

barn with that of their dams. After calving 83(76.9%) calves were housed in separate pen and 25(23.1%) of them were housed together with their dam in the same barn. Calf bedding was used for 60(55.6%) of the calves, but absent for 48(44.4%) calves. The floor types were concrete and soil in pens of 81(75%) and 27(25%) calves, respectively. All of the farms fed on or allowed to suckle colostrum two times per day (morning and evening).

The associations among different risk factors that may contribute to the occurrence of Eimeria were assessed. Based on the result shown in table 6 there was no any association among different risk factors except age. Age has very strong association for the occurrence of Eimeria ($p<0.05$) was considered at statistically significant. None infection was detected in calves less than 7 days of ages in this study (Table 6).

Table 6: Association of Eimeria detection along with different variables

Variables	**Level**	**keys**	**+ve Isolate (%)**	**$\chi 2$**	**P-Value**
Calf age	0	1-7 days	0 (0)	27.768	0.000
	1	8-15 days	3(7.1)		
	2	16-30 days	4(9.5)		
	3	>30 days	35(83.3)		
Calf sex	0	Female	21(38.2)	1.78	0.182
	1	Male	34(61.8)		
Calving facility	0	Calving pen	27(64.3)	0.008	0.927
	1	Same barn	15(35.7)		

Colostrum feeding method	0	Hand feeding	30(71.4)	0.103	0.748
	1	Suckling	12(28.6)		
Colostrum feeding duration	0	24-48 hr	9(21.4)	0.386	0.535
	1	>24 hr	33(78.6)		
Time of first Colostrum feeding	0	<6 hr	39(92.9)	0.366	0.545
	1	6-24 hr	3(7.1)		
Calf house floor Bedding	0	Present	25(59.5)	0.438	0.508
	1	Absent	17(40.5)		
Calf house floor type	0	Concrete	34(81.0)	1.299	0.254
	1	Soil	8(19.0)		
Herd size	0	<10	11(26.2)	3.569	0.168
	1	10-20	11(26.2)		
	2	>20	20(47.6)		

Similarly, the associations among different risk factors that may contribute to the occurrence of Cryptosporidium were assessed. Based on the result shown in table 7 age has great association for the occurrence of Cryptosporidium ($p<0.05$). As age increased the occurrence of cryptosporidium also increased. Other risk factors didn't show any significant association (Table 7).

Table 7**:** Association of Cryptosporidium detection along with different variables

Variable	Level	N	+ve isolate (%)	$\chi2$	P-Value
Calf age	0	16	3(13.0)	12.294	0.006
	1	18	6(26.1)		
	2	17	8(34.8)		
	3	57	6(26.1)		
Calf sex	0	48	8(34.8)	1.105	0.293
	1	60	15(65.5)		
Calving facility	0	70	14(60.9)	0.199	0.655
	1	38	9(39.1)		
Colostrum feeding method	0	79	16(69.6)	0.191	0.662
	1	29	7(30.4)		
Colostrum feeding duration	0	20	2(8.7)	1.869	0.172
	1	88	21(91.3)		
Time of first colostrum feeding	0	98	22(95.7)	0.839	0.360
	1	10	1(4.3)		

Calf house floor bedding	0	60	12(52.2)	0.135	0.713
	1	48	11(47.8)		
Calf house floor type	0	81	17(73.9)	0.018	0.892
	1	27	6(26.1)		
Herd size	0	30	7(30.4)	1.938	0.379
	1	19	6(26.1)		
	2	59	10(43.5)		

The associations among different risk factors that may contribute to the occurrence of Giardia were assessed. Based on the result shown table 8, age and calf house floor bedding has association for the occurrence of Giardia ($p \leq 0.05$). As age increased the occurrence of being exposed to Giardia also increased. Calves using floor bedding were highly affected than those with no bedding. No calves were affected with less than one month of age and all 8(100%) of calves affected with Giardia were over one month of age (table 8).

Table 8: Association of Giardia detection with different variables

Variable	**Level**	**N**	**+ve Isolate (%)**	**$\chi 2$**	**P-Value**
Calf age	0	16	0(0)	7.731	0.050
	1	18	0(0)		
	2	17	0(0)		
	3	57	8(100)		

Calf sex	0	48	3(37.5)	0.169	0.681
	1	60	5(62.5)		
Calving facility	0	70	3(37.5)	2.827	0.093
	1	38	5(62.5)		
Colostrum feeding method	0	79	6(75.0)	0.015	0.902
	1	29	2(25.0)		
Colostrum feeding duration	0	20	5(62.5)	11.076	0.051
	1	88	3(37.5)		
Calf house floor bedding	0	60	1(12.5)	6.487	0.011
	1	48	7(87.5)		
Calf house floor type	0	81	4(50.0)	2.880	0.090
	1	27	4(50.0)		
Herd size	0	30	4(50.0)	3.090	0.213
	1	19	0(0.0)		
	2	59	4(50.0)		

Out of 108 sera samples tested against *Salmonella Typhimurium* somatic (O) and flagellar (H) antigens, only 3(2.8%) were found to be positive. The result of the titration showed recent infection in all of the three samples (Tabale 9).

Table 9: Indirect of serodiagnostic test results of Salmonella from sera samples of diarrheic calves

Age		Salmonella		Total	Titer	Detected Ag
		0	1			
0		1593.8%	1(6.2%)	16(100.0%)	1/160	O
					1/320	H
1		17(94.4%)	1(5.6%)	18(100%)	1/320	O
2		17(100.0%)	0(0.0%)	17(100.0%)		
3		56(98.2%)	1(1.8%)	57(1000%)	1/320	H
	Count	105	3	108		
Total	% within age	97.2%	2.8%	100.0%		

X^2= 1.935 df = 3 P-value= 0.586

5. DISCUSSION

Diarrhea is a leading cause of economic losses to the cattle industry and major cause of calf mortality and morbidity during first few weeks of life in most countries Radostits *et al*. (2000). Diarrhoea in calves can be caused by a variety of pathogens including bacteria, viruses, protozoa and intestinal parasites. Rotavirus, coronavirus, enterotoxigenic *E. coli* and *Cryptosporidium parvum* are the four major pathogens associated with neonatal calf diarrhoea worldwide. These organisms are responsible for the vast majority (75%-95%) of enteric infections in neonatal calves worldwide (Tzipori, 2005).

The overall prevalence of *E. coli*, Eimeria, Cryptosporidium and Giardia in descending order were 55(50.9%), 42(38.9%), 23(21.3%) and 8(7.4%), respectively. The final sum percent of positive calves is over 100% due to concurrent infection (infection with two or more pathogens). *E. coli* as a single cause of diarrhea is the most dominantly detected enteropathogens (Table1).

E. coli was detected in more than half of the faecal samples concurrently in the presence of one or more other protozoan etnteropathogens as compared to single cause of the diarrhoiec samples in the present study. But since this organism is regarded as a normal member of the intestinal flora of warm blooded animals, the finding dissimilarity in concurrent and single infection might be considered as indicative of a normal flora. Diarrhoea due to the enterotoxigenic *Escherichia coli* is one of the most frequent bacterial diseases in neonatal calves and the predominant pathogen cultured from calves with septicemia Lofstedt *et al.* (1999). The detection of *E. coli* with greatest frequency from diarrhoeic calves in this study is consistent with results of Bendali *et al.* (1999); Garcia *et al.* (2000) and Bekele *et al.* (2009) but much more lower than reports by Acha *et al.* (2004) and Qais *et al.* (2011) who have demonstrated the prevalence of *E. coli* in the faeces of young calves in 76% and 64%, respectively in Debrezeit and Addis ababa and much more higher than the results reported by Dersema (2008), and Radostits *et al.*

(2000), 13.5%, and 22%, respectively. The reported results variation in Ethiopia as well as in other countries as compared to the present study might be due to variations in the management practices, including hygienic conditions, age groups examined and housing system of the farms and so on. Charles *et al.* (2003) indicated that gaps in management includes inadequate nutrition, exposure to severe environment, insufficient attention to the new born calf, or a combination of these are often involved in scours outbreaks. There was no statistically significant association between *E. coli* and calves with practice of hand feeding and suckling of colostrum.

All positive *E. coli* isolates were studied for their fermentation activities of nine sugars and all of them (100%) were showed the ability to utilize one or more sugars. Among fermented sugars, the most commonly occurring *E. coli* biotype from calf diarrhea was biotype V with a prevalence of 17(15.7%) fermented dulcitol, inositol and rhamnose. This result was consistent with a report by Gargan *et al*. (2013) who performed on same type of sugars to determine biotyping of *E. coli* and adjacently O-serotyping from rabbit diarrhea. He assigned as biotype I for an isolate fermenting the above three sugars, which accounted 66.6% of the isolates, and based on the API 20 E system he concluded that the identified biotype I confirmed with sero-typing were *ETEC* containing O-antigen. So according to him the most abundant *E. coli* biotype V of the present study might be *ETEC*.

Limited information suggests that EPEC strains can cause diarrhea in calves from 2 days to 4 months of age Janke *et al.* (1990). During their first month of life, calves are exposed to many infectious agents for the first time, which alone or in combination with other infectious agents can cause diarrhea. The pathogen or pathogens encountered in addition to environmental, nutritional and management practices, all influences the duration, severity and outcome of the disease Luzoan *et al.* (1999). Analysis of the relationship among different *E. coli* biotypes with different age groups of the calves was statistically significant. The biotype test result showed age group 1-7 days was affected at high rate (9, 56.2%), followed by 8-15 days old (3, 16.7%), 16-30 days old (3, 17.6%) and >30

days old (2, 3.5%). This result is consistent with Lofstedt *et al.* (1999) who stated that young neonates under one week of age are particularly susceptible because the normal flora of the intestine is not fully established. In addition they have a naive immune system and also possess receptor for the adhesions of *E. coli* Villarroel (2009).

Levine *et al.* (1987) stated that a given strain from diarrheic rabbit (that fermented dulcitol, rhamnose and xylose) and assigned as biotype VI isolated at the rate of 17.6%, was able to induce watery diarrhea and high mortality after experimental infection. This was in consistent with biotype VIII at the rate of 4(3.7%) of the present study. Similar observations have been made with other strains in England Varga *et al*. (1982), Belgium and the Netherlands Peeters *et al*. (1984) and France Camguilhem *et al.* (1986). None of these strains produced heat-labile or heat-stable enterotoxins, nor were they enteroinvasive. So they are considered to be enteropathogenic *E. coli*, according to the definition of Levine *et al*. (1987). Histology and electron microscopy showed these strains to be tightly adherent to the brush border of intestinal epithelial cells after experimental infection and to cause effacement of microvilli, followed by epithelial desquamation, villous atrophy, and malabsorption Levine *et al*. (1987). So it could be considered that biotype VIII of the present study might be EPEC.

The total infection as a single form was 60(55.5%) and none infections were detected in 25(26.88%) of all examined samples (Table 1). Concurrent infections of protozoan enteropathogens with *E. coli* in different age groups of calves and sex were also assessed and the result revealed no significant association in all of the episodes (P-value >0.05) (Table 2).

Bovine coccidiosis is an important protozoan disease of genus Eimeria affecting calves all over the world resulting in considerable economic losses each year to the beef and dairy industries Daugschies and Najdrowski (2005). The prevalence of Eimeria was 38.9%, which is lower than previous findings reported in Addis Ababa and Debre Zeit by Abebe *et al.* (2008) (68.1%) and in South Africa by Matjila and Penzhorn (2002) (70%) and higher than Kebadu (1998) (20%) in Debre Zeit, and Bekele *et al.* (2012) (22.7%) in

Dire Dawa, Pfukenyi *et al.* (2012) (19.8 %) from Zimbabwe and Gillhuber *et al.* (2014) (13.3%), from Southern Germany. This variation is most likely attributed to the differences in agro-ecology, and husbandry practices of the study animals in different countries Radostits *et al.* (2007). There was a strongly significant association ($P<0.05$) between the age of the calves with the risk of infection in Eimeriosis. This finding agrees with the report by Alemayehu *et al*. (2013) and Bekele *et al.* (2012).

Cryptosporidium has been identified in calves worldwide, with infection rates up to 100%. Infection can occur in calves as young as 4 days of age. However the parasite is most commonly identified in calves between 8 and 21 days of age Fayer *et al.* (1997). Cryptosporidium oocyst was detected in 23(21.3%) of the diarrheic calves in this study. This result is comparable to the findings reported in previous studies from other countries: Naciri *et al.* (1999); 25.6% by Kvac *et al.* (2006); Al-alousi and Mahmood (2012) 17.6%, in Ethiopia by Abebe *et al*. (2008); 27.8% by Alemayehu *et al.* (2013). Lower prevalence of Cryptosporidium oocyst has been reported in western Canada (Gow and Waldner 2006). The association between the occurrence of diarrhoea and Cryptosporidium infection analysis revealed a ($P<0.006$), indicating strong association between the infection with Cryptosporidium and occurrence of diarrhoea in calves. This is consistent to the results of Gillhuber *et al*. (2014) in Southern Germany. Cryptosporidium was the only pathogen identified concurrently with Eimeria and *E. coli* in the present study. The result from this study suggest that Cryptosporidium is one of the major etiological agents of neonatal diarrhoea in calves, this might be due to several factors like early contamination soon after birth by contact with their dams, contaminated litters, asymptomatic carriers and contaminated environment Castro-Hermida *et al.* (2002).

Another protozoan parasite, Giardia, has recently emerged as potentially important parasite of cattle. Many studies have identified Giardia in domestic livestock with prevalence up to 89% reported in calves Xiao (1994). Giardia-infection in cattle is often subclinical or asymptomatic, but this infection can also cause symptoms including acute

or chronic diarrhoea, reduced weight gain and ill thrift in young calves Geurden *et al.* (2010). Giardia-cysts were detected in 4(7.4%) in the present study. It is significant, that the age had a nonlinear influence on the probability of being infected with Giardia spp.

The presence of calf house floor bedding has significant association (P<0.011) with giardia occurrence; this may be attributed to the explanation that removal of bedding would also be expected to decrease faecal material harboring Giardia Waltner-toews *et al.* (1986); and previous studies have concluded that cleaning is an important management factor in preventing high levels of Giardia-cysts Mohammed *et al*. (1999).

The sex and other management practices of the calves were analyzed and not significantly associated (P>0.05) with the infection by Giardia (Table 8). In addition, calves were classified in age groups and sex to realize the association between occurrence of diarrhea with four enteropathogens (*E. coli,* Eimeria, Crypto and Giardia) but no statistical significant association was observed in all of the events (P>0.05) (Table 2).

Salmonella infections in calves continue to be a major problem worldwide. Substantial economic losses were manifested through mortality and poor growth of infected animals as well as the hazard of transmitting food poisoning to humans. Many outbreaks of salmonella infections has been reported worldwide, the most frequently isolated serovars being *S. typhimurium*, *S. enteritidis, S. anatum S. newport, S. cerro, S. montevideo, S. agona* and *S. dublin* which was considered the major host-adapted *salmonella* for cattle Mitz *et al.* (1981); Konrad *et al.* (1994). *Salmonella Typhimurium* is the most common spps to infect calves. Generally, calves over two weeks of age are most likely to become infected by salmonella Radke *et al.* (2002).

Out of 108 sera samples collected from diarrheic calves, 3(2.8%) of the analysed samples in the present study were positive against *S. Typhimurium* both O (somatic) and H (flagellar) antigens and the titration results revealed recent infection in all of the three positive calves. This result was lower as compared to the result reported for *S. Typhimurium* by Siorvanes *et al.* (2007).); (7.3%). The variation of the results with the

present study might be attributed to the diagnostic techniques, management, and environmental conditions of the calves because the infection was always aggravated by poor hygienic conditions and inadequate nutrition and young calves are most susceptible to infection due to their immature immune responses, undeveloped micro flora in their gastrointestinal echo-system and the permanent exposure to the source of infection from the environment and their dams Radke *et al.* (2002).

6. CONCLUSION AND RECOMMENDATIONS

This study tried to show major aetiological agents of calf diarrhea in dairy farms of Assela. The prevalence of diarrhea was considerably variable among different enteropathogens. ETEC is the most prevalent in diarrheic calves in the first week of age followed by EPEC using biotypes of nine fermented sugars. This work also demonstrated that Eimeria and Cryptosporidium were highly associated with calf diarrhea. Even though, the role of giardia as a cause of calf diarrhea was less prevalent in this study, the detection of giardia in calves may pose a risk to public health. Anti- Salmonella antibodies were detected using indirect serological diagnostic technique; however, *S. Typhimurium* was rare and did not seem to be associated with diarrhea as major problem in the present study.

Based on the above conclusion, the following recommendations are forwarded:

- The strains of *E. coli* in calf diarrhea should be confirmed using modern diagnostic techniques
- Other *Salmonella* species prevalent in calf diarrhea should be isolated and serotyped
- Further studies are necessary to identify viral enteropathogens in the study area for complete understanding of pathogens of calf diarrhea
- Further molecular studies should be conducted to identify protozoal enteropathogens at a molecular level
- The general health status of the calves could be maintained by allowing the calves to obtain sufficient amount of colostrum soon after birth.
- After understanding the pathogens involved in calf diarrhea, appropriate control and preventive measures that could apply to the country should be designed

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8. APPENDICES

Appendix I: Questionnaire format

Farm identification

- Owners name------------------------------------
- Address: Kebele----------------------------------
- When it established------------------------------

1. Farm description

Owner/manager educations status

a. Illiterate b. Read and write c. Elementary school d. High school graduate
e. Professional

If, professional a. Related to animal production b. Unrelated to animal production

Herd size:

- Cows------------, Bulls------------, Heifers----------, Male calf-------, Female calf--------

Breed and age of animals kept in the farm

The farms as a source of income

a. Primary income b. Secondary income

Organization of farms

a. Family farm b. Partnership c. State

2. Management data

Calf care takers (attendants)

Ownership a. Owner (family member) b. Hired help

Sex a. Male b. Female

Experience of calf care takers

a. <5years b. > 5 years

Education of calf care taker

a. Elementary school b. High school c. College graduate d. Professional

Per parturient care

Calving facilities

a. Calving pen b. The same barn

Navel treatment a. Practiced b. Not practiced

Awareness of importance of colostrums feeding to neonates

a. Yes b. No

- if yes method of feeding a. Suckling b. Hand feeding
- Time of first feeding a. <6hours b. 6-24hours c. >24hours
- Duration of feeding a. For 24 hrs b. For 24 hrs to 4 days c. >24hrs
- If hand feeding – source of feeding

  a. From dam b. From another cow

Feeding

Types of feeding

a. Milk b. Milk replaces

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

Frequency of feeding

a. Once per day b. Twice/day c. Three times/day

Types of supplementary feed and quality per unit of body weight

a. Grazing b. Concentrates c. hay

Weaning age

a. 4-6 wks of age b. 6-8 wks of age c. 8-18 wks of age d. 12-16 wks of age

Housing

Housing a. Separate pen b. Together with cows in the same born c. Other

- If separate pen a. Individual b.Group pen

Bedding a. Present b. Absent

- If present what is the bedding material and how frequently it is changed
    a. > Once a week b. Once a week b. < One a week c. Daily

Types of floor

a. Concrete b. Soil c. Other

3. 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----------------------------

Appendix II: Isolation and identification of *E. coli*

Faecal samples were inoculated on Eosin Methylene Blue (EMB) agar and MacConkey agar medium for preliminary characterization and colonies showing characteristic metallic sheen on EMB agar were picked up and considered as presumptive *E. coli.* Colonies are medium size and bright pink to red with flat or elevated surface and complete white edges on MacConkey agar. The purified cultures of *E. coli* were stored in nutrient broth for further identification by biochemical tests. All the isolates were stained by Gram's Method to determine the purity of the isolates.

Gram's reagent

Crystal violet

Gram's iodine (mordant)

Ethanol 95%

Counter – stain (carbon fuchsine / safranin)

Gram stain 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.

E. coli isolates were preliminarily identified by biochemical tests viz. Indole, Methylred, Voges proskaeur and citrate utilization. The isolates were then be further characterized for their biochemical activity by the following tests viz. carbohydrate fermentation, urea hydrolysis, production of H2S on TSI as per Edwards and Ewing (1972).

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 37^0cfor 18 to 24hours. 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 370c 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% a-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.

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

Principle: urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea with the formation of ammonia (alkaline).

Procedure: the surface of the agar slant is streaked with the test organism and incubated at 37^0c for 18 to 24 hours.

Interpretation: organisms that hydrolyze urea rapidly may produce positive reaction with in 1 or 2 hours. Red (pink) color throughout medium indicates positive reaction.

Citrate utilization

Simmon citrate agar (Difco,USA) slope surface was streaked with the suspected bacterial colonies and incubated at 37^0c for 24 hours. Typical reaction for citrate utilization and possibility was declared by the medium from green to blue color (Quinn *et al.,* 1999)

Carbohydrate Fermentation Tests

Fermentation reactions of nine sugars viz., dulcitol, inositol, lactose, maltose, raffinose, rhamnose, salicin, and sucrose and xylose were studied. One per cent of each sugar in peptone water base with Andrade's indicator was used. For the test isolates grown in peptone water were inoculated into each sugar medium. Tubes were incubated at 37°C for seven days and readings were recorded after every 24 hours. Production of pink colour was considered as positive reaction. Proper controls were kept for each of the biochemical tests performed.

Appendix III**:** Sugar fermentation activity of *E. coli* isolates

Isolates	**Dulcitol**	**Inositol**	**Lactose**	**Maltose**	**Raffinose**	**Rhamnose**	**Salicin**	**Sucrose**	**Xylose**
1.	-	-	+	+	-	-	-	+	-
2.	-	-	-	-	-	-	-	-	-
3.	+	+	-	-	-	+	-	-	-
4.	-	-	-	-	-	-	-	-	-
5.	+	-	-	-	+	+	-	+	-
6.	+	+	-	-	-	+	-	-	-
7.	+	-	-	-	+	+	-	+	-
8.	-	-	-	-	-	-	-	-	-
9.	-	-	-	-	-	-	-	-	-
10.	+	-	-	-	+	-	-	-	+
11.	-	-	-	-	-	-	-	-	-
12.	-	-	-	-	-	-	-	-	-
13.	-	-	-	-	-	-	-	-	-
14.	+	+	-	-	-	+	-	-	-

15.	+	+	-	-	-	+	-	-	-
16.	+	-	-	-	-	+	+	-	-
17.	+	+	-	-	-	+	-	-	-
18.	+	+	-	-	-	-	-	-	-
19.	-	-	-	-	-	-	-	-	-
20.	+	-	-	-	-	+	-	-	+
21.	+	+	-	-	-	+	-	-	-
22.	+	+	-	-	-	+	-	-	-
23.	+	-	-	-	+	-	-	-	+
24.	+	-	-	-	-	+	-	-	+
25.	-	-	-	-	-	-	-	-	-
26.	+	-	-	-	+	+	-	+	-
27.	-	-	-	-	`	-	-	-	-
28.	-	-	-	-	-	-	-	-	-
29.	-	-	+	-	-	+	-	-	-
30.	-	-	-	-	-	-	-	-	-
31.	+	-	-	-	-	-	-	+	-
32.	+	+	-	-	-	+	-	-	-
33.	+	-	-	-	+	-	-	+	-
34.	-	+	-	-	-	-	-	-	-
35.	-	-	-	-	-	-	-	-	-
36.	-	-	-	-	-	-	-	-	-
37.	+	-	-	-	+	+	-	+	-
38.	+	+	-	-	-	-	-	-	-
39.	+	-	-	-	-	+	-	-	+
40.	+	+	-	-	-	+	-	-	-
41.	-	-	-	-	-	-	-	-	-
42.	+	-	-	-	+	-	-	+	-
43.	+	-	-	-	-	+	+	-	-

44.	-	-	-	-	-		-	-	-
45.	+	-	-	-	-	+	+	-	-
46.	-	-	-	-	-	-	-	-	-
47.	-	-	-	-	-	-	-	-	-
48.	-	-	-	-	-	-	-	-	-
49.	-	-	-	-	-	-	-	-	-
50.	-	-	-	-	-	-	-	-	-
51.	-	-	-	-	-	-	-	-	-
52.	-	-	-	-	-	+	-	-	-
53.	-	-	-	-	-	-	-	-	-
54.	+	+	-	-	-	+	-	-	-
55.	-	-	-	-	-	-	-	-	-
56.	-	+	-	-	-	-	-	-	-
57.	-	-	-	-	-	-	-	-	-
58.	-	-	-	-	-	-	-	-	-
59.	-	-	-	-	-	-	-	-	-
60.	-	-	-	-	-	-	-	-	-
61.	+	+	-	-	-	+	-	-	-
62.	-	-	+	+	-	-	-	+	-
63.	-	-	-	-	-	-	-	-	-
64.	+	-	-	-	-	+	+	-	-
65.	-	-	-	-	-	-	-	-	-
66.	-	-	-	-	-	-	-	-	-
67.	+	+	-	-	-	+	-	-	-
68.	-	-	-	-	-	-	-	-	-
69.	-	-	-	-	-	-	-	-	-
70.	-	-	-	-	-	-	-	-	-
71.	-	-	-	-	-	-	-	-	-
72.	-	-	-	-	-	-	-	-	-

73.	-	-	-	-	-	-	-	-	-
74.	-	-	-	-	-	-	-	-	-
75.	-	-	+	+	-	-	-	+	-
76.	-	-	-	-	-	-	-	-	-
77.	+	+	-	-	-	+	-	-	-
78.	-	-	-	-	-	-	-	-	-
79.	-	-	-	-	-	-	-	-	-
80.	-	-	-	-	-	-	-	-	-
81.	-	-	-	-	-	-	-	-	-
82.	-	+	-	-	-	-	-	-	-
83.	+	+	-	-	-	-	-	-	-
84.	-	-	-	-	-	-	-	-	-
85.	-	-	-	-	-	-	-	-	-
86.	+	+	-	-	-	+	-	-	-
87.	-	-	-	-	-	-	-	-	-
88.	-	-	-	-	-	-	-	-	-
89.	+	-	-	-	+	+	-	+	-
90.	+	-	-	-	-	+	+	-	-
91.	+	-	-	-	-	+	-	-	+
92.	-	-	-	-	-	-	-	-	-
93.	+	-	-	-	-	-	-	+	-
94.	+	+	-	-	-	+	-	-	-
95.	+	-	-	-	-	+	+	-	-
96.		-	-	-	-	-	-	-	-
97.	+	-	-	-	-	+	+	-	-
98.	-	-	-	-	-	-	-	-	-
99.	+	+	-	-	-	+	-	-	-
100.	-	-	-	-	-	-	-	-	-
101.	-	-	-	-	-	-	-	-	-

102.	-	-	-	-	-	-	-	-	-
103.	-	-	-	-	-	-	-	-	-
104.	+	-	-	-	+	+	-	+	-
105.	+	-	-	-	-	-	-	+	-
106.	+	+	-	-	-	+	-		-
107.	+	-	-	-	-	-	-	+	-
108.	-	-	-	-	-	-	-	-	-

Appendix IV: laboratory preparation and procedures for protozoan

Preparation of Sheather's Flotation Solution and Procedure of Flotation

Preparation

1. 454 gm of table sugar is dissolved in 355 ml of very hot water.
2. Stir until dissolved and allow cooling. Then add 6ml of liquid phenol.

Procedure

1. 5 ml of feces was measured and placed in the beaker.
2. 42ml of water was added, mixed thoroughly and poured into another glass beaker through a strainer.
3. Then the mixture was poured into 12ml centrifuge tube and centrifuged in1500rpm for 3-5minutes and the supernatant was decanted and a sugar solution (specific gravity 1.27) was added to the sediment, thoroughly mixed with a wooden applicator stick and the tube was filled until 1inch from the top of the tube and
4. The mixture was again centrifuged in 1500 rpm for 3minutes.

5. After centrifugation the tube was filled to the top until a convex meniscus was formed and a glass cover slip was placed on top of the tube and left for 10 minutes.
6. Then, the glass cover slip was briskly lifted up and placed on a clean glass slide, not allowing formation of air bubbles.
7. The slide was examined under microscope with ×40 magnifications.

Preparation of Modified Ziehl Neelsen Staining Solution

1. 1.0g of basic fuchsin is dissolved in 10ml of 95% ethanol.
2. 5.0 gm of phenol is dissolved in 100.0 ml of distilled water.
3. The two solutions then mixed together and allowed to stand for a few hours and then filtered in to a clean container.
4. 10ml of the above mixture is taken and mixed well with 90ml of distilled water.

Procedure of Modified Ziehl Neelsen Staining Technique

1. The samples are concentrated by Sheather's centrifugal flotation. Drops are taken by pipettes from the top of Sheather's centrifugal floated sample and smears are made on the slide and allowed to air dry.
2. The smears are fixed in 95% methanol for 5 minutes.
3. The smears are stained with Modified Ziehl Neelsen staining solution for 10-15 minutes.
4. Then wash in tap water thoroughly.
5. The smears are decolourised in acid alcohol (1% HCl in 97% ethanol) for 15-20 seconds.
6. Rinse thoroughly in tap water.
7. Counter stain with 0.4% malachite green for 30-60 seconds.
8. Rinse thoroughly and air dry.
9. Examine using x100 magnification with oil immersion. Cryptosporidium oocysts appear spherical bright red in a green back ground.

Materials Used

- Plain microscope slides,
- Beaker
- Distilled water
- Microscope
- Cover slips,
- Cheesecloth or a strainer,
- Stirring rod (a pencil works well),
- Fecal floatation solution (sugar)
- Test tube holding rack.
- Disposable plastic glove
- Ice box
- Flask
- Plastic container
- Universal bottle
- Modified Ziehl Neelson staining solution
- Centrifuge
- Phenol
- Malachite green
- Tap water
- Basic carbol fuchsin
- Centrifuge tube
- Potassium dichromate
- Applicator stick

Appendix V**:** Test procedure for anti-*Salmonella* antiserum

Material required

Glass or white test cards

Disposable stirrer

Test tubes (12x100 mm)

Automatic pipettes

Saline solution (NaCl 0.9%)

Mechanical rotator, adjustable at 100 r.p.m

Theromostatic bath (30-20°C)

Procedure:

I. qualitative test

1. Bring the test reagents and samples to room temperature
2. Resuspend the antigen vial gently, aspirate dropper several times to obtain a thorough mixture
3. Place 50 microliter of the serum under test into a row of circles on the card. Dispense 1 drop of Negative Control serum into two additional circles.
4. Add 1 drop of the appropriate well shaken suspension to each circles next to the samples to be tested
5. Mix the contents of each circle with a disposable stirrer and spread over the entire area enclosed by the ring .use separate applicator for each mixture

6. Rock the sling gently by hand or by means of a mechanical rotator (100 r.p.m) for a period of 1 minute.
7. Observe immediately under a suitable light source for any degree of agglutination.

Reading

Non-reactive: smooth suspension with no visible agglutination, as shown by negative control

Reactive: any degree of agglutination visible macroscopically

II. Semi-quantitative Test

1. For each specimen to be tested place 80, 40, 20, 10, and 5 microliter of serum into each of the circles of a card.
2. Test each dilution as described in steps 4-7 for the qualitative test

Reading: Same as in Qualitative Test. The titer of the specimen is reported as the highest dilution that shows reactivity.

III. Tube Agglutination Test

1. Using saline solution as a diluent, prepare for each antigen to be tested a row of doubling dilutions of the specimen.
2. Add 1 drop of the appropriate well-shaken suspension to each tube of a given row. Mix Final serum dilution will be: 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640.
3. Incubate at 37°C for 24
4. Examine macroscopically for agglutination

Reading

Read the results of all control tubes first. After the examining the patterns of the sediment shake the tube gently

Non-reactive: In a negative reaction and in the Suspension Control tube there is no clumping visible. Suspension shows a typical swirl when the tube is flicked

Reactive: Partial or complete agglutination with variable degree of clearing of the supernatant fluid.

The titer is reported as the highest dilution that shows agglutination. The next higher dilution should be negative.