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DETECTION OF MAJOR ENTEROPATHOGENS ASSOCIATED WITH CALF DIARRHEA IN DAIRY FARMS OF AFAR REGION, ETHIOPIA

MSc. Thesis

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LIST OF ACRONYMS

µg	Microgram
µm	Micrometre
Ag	Antigen
Ag-ELISA	Antigen Capturing Enzyme-Linked Immunosorbent Assay
Cm^2	Centimetre square
CPE	Cytopathic Effect
CSA	Central Statistical Agencies
CV	Corona Virus
DAEC	Diffusely Adherent *E. coli*
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxy Ribonucleic Acid
dsRNA	Double Stranded Ribonucleic Acid
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhaghic *E. coli*
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
ETEC	Enterotoxigenic *E. coli*
GRVA	Rotavirus group A
Kbp	kilobase pair
Km^2	Kilometre square
LT	Labile toxin
MB	Mega bit
Mbp	Mega base pair
MDBK	Madin Darby Bovine Kidney Cell
Ml	Millilitre
NAHDIC	National Animal Health Diagnostic and Investigation Center
NCD	Neonatal Calf Diarrhea
Nm	Nano meter
pH	Power of Hydrogen

rpm	revolution Per Minute
SPSS	Statistical Package for the Social Sciences
ST	Stable toxin
STEC	Shiaga-like toxin producing *E.coli*
TC	Tissue culture
VP	Virus Particle

ABSTRACT

Diarrhea is one of the most common causes of sickness and mortality among newborn calves. It has serious financial and animal welfare indications in both dairy and beef herds. Diarrhea in a newborn calf is one of the most common diseases in young animals, causing huge economic and productivity losses to livestock industry worldwide. Dairy farming is a growing cattle production system in Ethiopia. However, morbidity and mortality of calves are among the factors that have been hindering success of dairy industry. Multiple enteric pathogens such as virus, bacteria and parasite are the most common causes of calf diarrhea. However, various facets of diarrheal disease caused by rotavirus, coronavirus, *E. coli* K99 and *Cryptosporidium parvum* infections in calves in Afar region are inadequately understood. A Crossectional study was carried out from February 2020 to April 2021 with the aim of prevalence and associated risk factors of rotavirus, coronavirus, *E. coli* K99 and *Cryptosporidium parvum* infections in calves from selected districts of Afar national regional state, in north east Ethiopia. A total of 176 fecal samples of 0 up to 6 months old diarrheic calves were collected by purposive sampling. Samples were tested for those four pathogens using a commercially available enzyme-linked immunosorbent assay (ELISA). In addition, different factors were measured the level of association between variables. Out of the 176 samples tested 59 were positive for at least one pathogen by ELISA. Positivity rates for each pathogen were, coronavirus 45 (25.5%), *Cryptosporidium parvum* 29 (16.5%), rotavirus 18 (10.2%) and K99 enterotoxigenic *E.coli* 11(6.25%). From positive viral samples of ELISA test 38 were propagated in Madin Darby bovine kidney cells. After 3 following passage, progressive cytopathic effect (CPE) i.e. rounding, detachment as well as demolition of mono-layer cell of all samples was observed. The study sit, time of first colostrum feeding, and duration of colostrum feeding were significantly associated with the occurrence of rotavirus, coronavirus, *Cryptosporidium parvum* and *E. coli* and this are indicative of the need for strict prevention and control mechanisms such as practicing early colostrum feeding in newborn calves.

Key word: Afar, Calf diarrhea, *E. coli* and ELISA test.

1. INTRODUCTION

1.1. Background and Justification

Diarrhea is one of the most common causes of sickness and mortality among newborn calves. Neonatal calf diarrhea is defined as pathology having a complex multifactorial etiology, involving management, environmental, nutritional, physiological variations and variety of pathogens including bacteria, viruses and parasites are described as important agents causing diarrhea (Elham *et al*., 2012). In acute neonatal diarrhea, an important disease of calves, four micro-organisms in particular, are of widespread occurrence and have proven enteropathogenicity these are rotavirus (RV), coronavirus (CV), enterotoxigenic *Escherichia coli* K99 (ETEC) and *Cryptosporidium parvum* (Elham *et al*., 2012). Among these etiological agents rotavirus and coronavirus are known to be the most common and economically important viral agents cause neonatal calf diarrhea (Mayameei *et al.,* 2010*).*

Calf diarrhea have a common financial and animal welfare implications in both dairy and beef herds. Diarrhea in neonatal calves is one of the most important constraints in young animals. It causes huge economic and food animal production losses due to high morbidity and mortality rates in worldwide (xiaojuan *et al*., 2021).The future of any dairy production depends, among other things, on the successful raising of calves for substitution. In modern dairy production of the developed world, the average length of time a cow stays in a milking herd is about four years and, therefore, 25% of the milking herd necessarily replaced each year (Bath *et al*., 2012).

Dairy farming is a growing livestock production system in Ethiopia (Asefa Alemu *et al.*, 2016). Three major systems of dairy production recognized in Ethiopia based on climate, land holdings and integration with crop production, these are: urban, per-urban and rural dairy production (Yitaye Alemayehu 2008). Because of better availability of milk market in urban and peri-urban areas, dairy farms are concentrated in these areas of the country (Asefa Alemu *et al.,* 2016). A successful dairy farm operation requires that a high percentage of cows wean a live healthy calf every year. Raising healthy dairy calves to weaning period needs increasing the calf's level of immunity against disease while decreasing its exposure to the pathogen (Godden, 2008). But, out

of the agents that have been affecting achievement of dairy industry, calves morbidity and mortality is the one that causes main concern (Asefa Alemu *et al*., 2016).

Discontinuation course of treatment and continuous indiscriminate uses of antibiotic drugs against diarrheal infection of man and animal might have influenced to produce a new generation of virulent and resistant type of bacteria (Hemashenpagam *et al.,* 2009). In addition routine laboratory isolation, identification, characterization and drug sensitivity testing are expensive and impractical. Regular check of the pattern of the drug sensitivity of organisms is more significant.

1.2. Statement of the Problem

Calf diarrhea is the main problems for farmers in Ethiopia. These problems can lead to important economic losses since affected animals generally show reduced weight gain, productivity and sometimes resulting in death. In this country, 30% Pre-weaning calf mortality rate was reported in mixed crop-livestock production systems in Amhara Region (Yeshwas Ferede *et al*., 2014). and in market-oriented dairy farms, 18% mortality rate was obtained in Central Ethiopia (Wudu, Temesgen *et al*., 2008). To increase the productivity per livestock unit without increasing livestock numbers, quick identification of the infectious agents and predisposing factors is important in disease control, for implementation of preventive and treatment measures and also to reduce losses during the initial age of the calf.

Different research works reveal that viral, bacterial and parasites are the main causes of calf mortality. A study under taken by Umer Seid *et al*. (2020) in central part of Oromia was on viral causes of calf diarrhea. Abrham *et al.* (1992) and Demisse Demek (2007) in Debre Zeit and Addis Ababa and Ynehiwot Berhanu (2008) Holeta and Debre Zeit included the bacterial cause. Simachew Kebede (1998) and Tadesse Dessie (2004) in Debre Zeit also conducted studies on bacterial and protozoan causes of calf diarrhea. Ashenafi Germa (2013) studied *E.coli* biotypes from diarrheic calves in and around Addis Ababa. However, in the case of Afar region, detection of enteropathogens that cause diarrhea in calves has not been well studied.

In 2016 epidemiological study by Tsegaw Fentie and colleagues reported mortality rates of 15-25% in pri-urban, 9-14% in mixed crop-livestock and 26-29% in pastoralist calves. In 2019, a young stock mortality reduction package intervention baseline survey, conducted by Addis Ababa University's College of Veterinary Medicine and Agriculture and the Supporting Evidence Based Intervention project reported mortality rates of 21% in pre-urban, 10% in mixed crop-livestock's and 32% in pastoralist production system. In addition, based on the information obtained from local communities and district agricultural office there was high frequency occurrence of neonatal calf diarrhea. Therefore, this research was designed to study the presence of viral, protozoan and bacterial pathogens responsible for calf diarrhea from dairy farms in Afar region.

1.3. Objectives

1.3.1. General Objective

The general objective of this study is to detect the enteropathogenic virus, bacteria and protozoa associated with calf diarrhea.

1.3.2. Specific Objectives

- To detect and identify the major enteropathogens (*E. coli K99,* Coronavirus, rotavirus and *cryptosporidium parvum*) involved in calf diarrhea.
- To assess the associated risk factors for occurrence of calf diarrhea

2. LITERATURE REVIEW

2.1. Calf Scour

Calf scour is a clinical syndrome related to many diseases characterized by diarrhea. Despite of the cause, assimilation of fluids from the intestine is changed, directing to life-threatening electrolyte imbalances (Radostits *et al.,* 2007). The scouring calf loses fluids, rapidly dehydrates and suffers from electrolyte loss and acidosis. Infectious agents may cause initial damage to the intestine but death from scours usually results from dehydration, acidosis and loss of electrolytes (Elham *et al*., 2012).

Symptoms of calf diarrhea can easily know with high frequency and quantity of calf faces and having a higher than normal water content. Bright yellow or white faces, depressed calves who are reluctant to feed, calves with sunken eyes and a temperature skin remaining peaked or tented when lifted, indicating dehydration, weight loss and weakness, in severe cases, calves will collapse, become comatose and die (Smith, 2009).

For the newborn calf one of the most critical times is the first two weeks of life. Diarrhea is one of the major causes of mortality in newborn calves (Abu *et al.,* 2014). The incidence of diarrhea in calves less than one-month ranges between 15 to 20% in Switzerland with mortality rate between 15% to 8%, signifying that the greatest risk occurs during the first two weeks of life (Vandeputte *et al*., 2010).

The etiology of diarrhea is multifactorial and may include infective, environmental, nutritional and management factors but generally grouped into noninfectious and infectious causes. The noninfectious causes are often referred to as predisposing or contributing factors because there is a proven interaction between noninfectious causes and infection (Stoltenow and Vincent, 2003).Studies show that the most important pathogenic agents in calf diarrhea are bacteria (*Escherichia coli*, *Salmonella* specious., *Clostridium perfringens*, and other bacteria), Viruses (Coronavirus and Rotavirus) and Protozoa (*Cryptosporidium, Eimeria* specious and *Coccidia*) by attacking the lining of the calf intestine cause diarrhea. Rotavirus, coronavirus, *Enterotoxigenic E.coli* and *Cryptosporidium* cover from 75% to 90 % of infections in newborn calves (Radostits *et al*., 2007).

2.1.1. Viral Agents

Viruses are the smallest infectious agent that cannot reproduce by itself. The word “virus” is come from the Latin word for poison. The discoveries of viruses were after the formation of a porcelain Chamberland filters that could remove all bacteria greater than or equal to 0.2 µm which is visible in the microscope from any liquid sample passed through the device. In 1886, Adolph Meyer described tobacco mosaic disease. It could be transmitted from a diseased plant on to the leaves of a healthy plant via liquid plant extracts. The Russian scientist Dmitry lvanovsky in 1892 demonstrated that his disease could be passing on in this way even after the chamberland Pasteur filter had separate all viable bacteria from the extract. The “filterable” pathogen was proved and was new type of disease causing particle other than bacteria (Levine and Enquist, 2007).

Viruses are found in different shapes and sizes, and have different levels of complexity. A virus consists of two or three parts genes contain either DNA or RNA, long molecules that carry genetic information and protein coat that protects the genes; and also in some viruses, an envelope of fat. The nucleic acid either single or double stranded. Viruses vary in shape from the simple helical which consist of nucleic acid surrounded by a hollow protein cylinder or capsid and icosahedral to more complex structures. The size of the viruses can vary from 20 up to 300 nm (Zakariya, 2017).

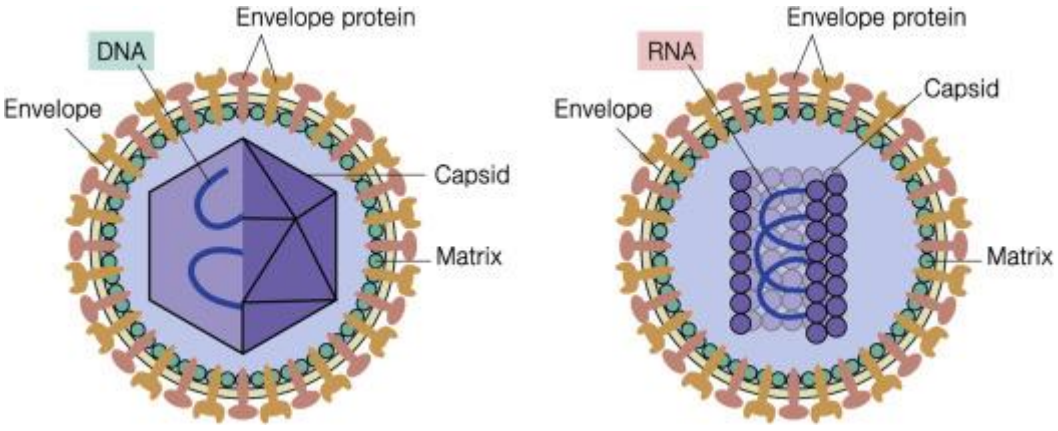

Figure 1: Diagrammatic representation of the virus particle. (Wang, 2017)

Viruses interrelate with the host at all phases of replication; cell entry, transcription, translation, genome synthesis and packaging and cell exit. The infection mechanisms of the virus are not only important for producing new virus progeny but also enable the host to know the presence of an infectious agent. As host species have inter in to mechanism of defend against pathogens, viruses have in turn evolved strategies to avoid the host immune response.

Viral infections alter cellular function and although the integrity of the epithelial cell layers initially maintain, within a very short period infected cells are desquamated in to the intestinal lumen. Functional alterations of the epithelial cells due to viral infection are thought to be responsible for abnormal absorption and secretion resulting in an imbalance with accumulation of fluid in the lumen of the intestine, which give to the diarrhea. In calves, group A rotavirus (GARV) and coronavirus are the most commonly associated viruses with neonatal diarrhea and it is not unusual that both viruses can concomitantly infect calves (Barry *et al*., 2009).

2.1.1.1. Coronavirus (CV)

The name coronaviruses are derived from Latin corona meaning "crown". Corona viruses are a group of related RNA viruses that causes diseases in human, other mammals and birds. The first corona virus was discovered by Arthur Schalk and M.C. Hawn in 1930 in chickens known as infectious bronchitis virus (Avian corona virus) and human corona virus were discovered in 1960s by E.C. Kendall, Malcolm Bynoe and david Tyrrell. In human and birds, the virus causes respiratory tract infections and in cows it cause diarrhea. It also associated with the occurrence of respiratory distress in calves and adults (Lathrop *et al*., 2000; Storz *et al*., 2000).

Corona viruses are belonging to the sub family orthcoronavirinae in the family *Coronaviridae*. They are enveloped viruses with the genome size ranging from 26 to 32 Kb in length, a positive sense single stranded total RNA which is group of related viruses that have positive-sense; single-stranded genomes made of ribonucleic acid and can act as messenger RNA. CVs are classified into three groups based on antigenic and genetic properties: α- CVs, β-CVs and γ-CVs. Bovine corona virus is including in group β-CVs (Enjuanes *et al*., 2000). The CV genome encodes four major structural protein that are spike, nuclocapsid, membrane and envelop (Wang *et al*., 2017). The most important protein of the virus is a nucleocapsid protein of helical symmetry. Coronaviruses replicate in the cytoplasm (Radostits *et al*., 2007; Fenner *et al*., 2011).

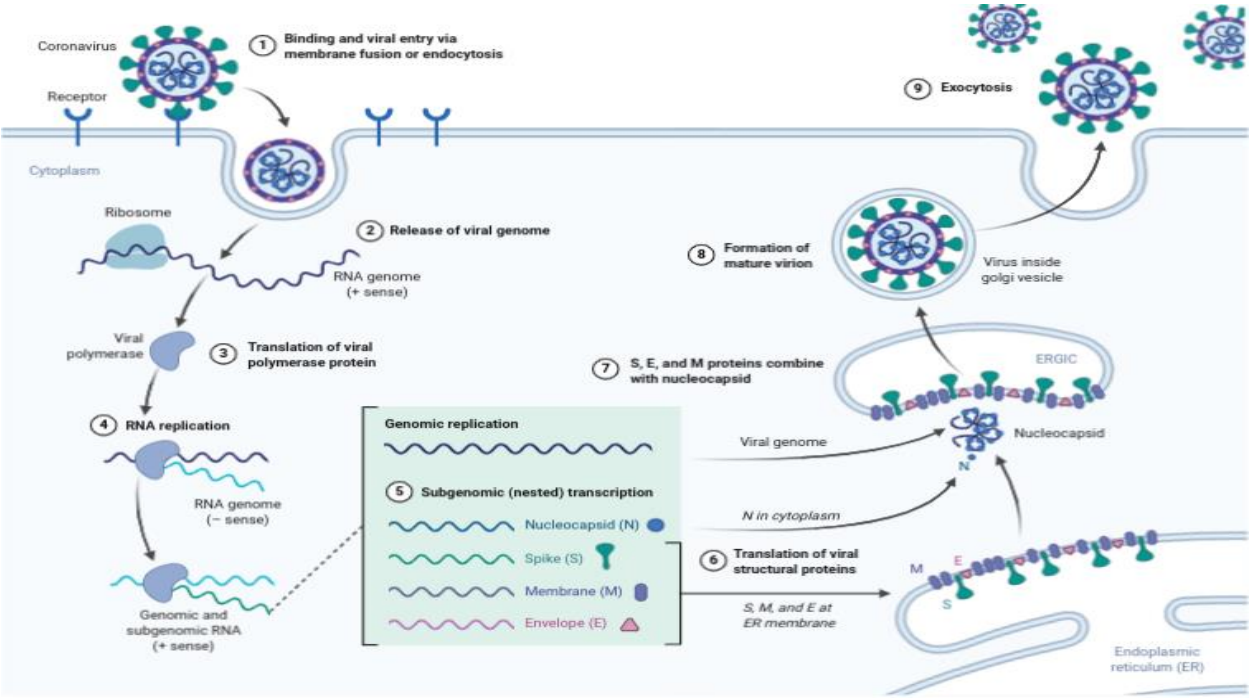

Figure 2: Diagrammatic representation of coronaviruses replication cycle.
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Corona viruses' infection generally caused as the result of orally use of food or water contaminated with infected faeces and respiratory route (Yavru *et al*., 2016). The virus has high affinity for epithelial cells in the villi of the small intestine. The infection of coronavirus is on epithelial cells in the respiratory tract and the intestine, trachea and lung, the nasal turbinates and also the villi and crypts of the small and large intestine, respectively. Replication leads to shedding of virus in nasal secretion and faeces. The main agents for the pathogenesis are still not fully investigate, such as how the virus infects enterocytes shortly after inter to an animal. This virus usually affects calves over one week of age. Clinical signs range from none to sever and include dehydration, depression, reduced weight gain and anorexia fever, respiratory signs and diarrhea with or without blood. Antigen capturing enzyme-linked immunosorbent assay (Ag-ELISA) and Electron microscopy (EM) are used for identification (Yimer Muktar 2014; Umer Seid *et al*., 2020).

2.1.1.2. Rotavirus (RV)

The discovery of rotavirus was in 1972 by an Australian research group led by Dr. Ruth Bishop (Bishop *et al*., 1973). It was recognize by direct electron microscopy visualization in the duodenal biopsies of a child with acute diarrhea and named duovirus. Rotavirus was one of the first recognized viral causes of diarrhea. The infection occur when calves ingesting the virus from fecal contamination of the environment (Foster and Smith, 2009).

Rotaviruses are double stranded RNA classified in the genus Rotavirus of the subfamily Sedoreovirinae in the family Reoviridae (Varani and Allain, 2002). This pathogen is characterized by segmented genomes comprising of 11 segments of dsRNA which a size range 16~21 kilo base pairs contained within a triple layered protein shell composed of a core, inner capsid and outer capsid (Estes and Cohen, 1989).

Rotaviruses are characterized by three important antigenic specificities: group, subgroup, and serotype (Umer Seid *et al*., 2020). From the six viral proteins (VP1, 2, 3, 4, 6 and 7); VP1, VP2 and VP3 are form the core of the rotavirus particle and the outermost layer is formed by two proteins, VP4 and VP7. Based on antigenic and genetic similarities of the intermediate capsid protein of VP6 rotaviruses can be divided into seven serogroups (A - G) (Fenner *et al*., 2011). Group A rotaviruses are the most frequently detected virus of rotaviral infection in domestic animals especially neonatal calf diarrhea (Steele *et al*., 2004).Most bovine rotavirus (95%) belong to group A, although group B and C are also seen in cattle (Helena, 2007).

The rotavirus genome consists of 18,555 nucleotides in total. Group A rotaviruses (RVA) classified into P or G types based on genetic and antigenic similarities of VP4 and VP7. VP4 (P protein for ‘protease-sensitive’ due to its trypsin mediated cleavage needed for virus adsorption into cells) determines the P serotypes. VP7 (G protein for ‘glycoprotein’ forming the matrix of the capsid) defines G serotypes (Laird *et al*., 2003).Sixteen G types and 27 P types have been reported in domestic animals (Desselberger *et al*., 2005). Bovine types of rotaviruses are G1, G6, G8, or G10. G6 and G10 are reported to be the most prevalent type in cattle (Martella *et al*., 2007).

Rotaviruses predominantly infect and replicate the mature non-dividing enterocytes by endocytosis in the middle and top part of the villi of the small intestine (Lundgren and Svensson, 2001). The virus genome is transcribed in the cell and causes degenerative changes that make the cell exfoliate. A massive loss of enterocytes leads to fusion of the villi (Dhama *et al*., 2009; Martella *et al*., 2010). Squamous or cuboidal epithelium takes the place of the columnar epithelium. The diarrhea is caused by the lack of cells that are able to process lactose and the reduced mucus area cause an increase of glucose and galactose in the lumen. In the large intestine the amount of lactose in the lumen leads to a reduced absorption of water because of osmotic pressure (Dhama *et al*., 2009). Mature absorbing cells in the small intestine are replaced by immature cells with secretory function. Functional balance may therefore change from absorption to secretion (Scott *et al*., 2004).

Rotaviruses are endemic and present in all cattle herds (Scott *et al.*, 2004). It is primarily transmitted by fecal-oral, although some studies have reported low titers of virus in respiratory tract secretions and other body fluids, indicating the possibilities for airborne and water-borne transmissions of rotavirus (Dennehy, 2000). The incubation period is 18-24 h. The definite drop of colostral antibodies at the age of 3 days match the peak of diarrhea at the age of 5-7 days (Radostits *et al*., 2000).

The diagnosis of rotavirus was initially based on electromicroscopy, ELISA (Radostits *et al*., 2000), RNA electrophoresis, nucleic acid hybridization immunofluorescence, the conventional reverse transcription polymerase chain reaction (RT-PCR) and quantitative real time polymerase chain reaction (qRT-PCR) (Pang *et al*., 2014).

Rotavirus is the most frequent disease in less than one month old diarrheic calves (Radostits *et al*., 2007). It is present in most cattle herds and typically causes diarrhea in calves five to 14 days old. Rotavirus infections are self-limiting because once the epithelial cells are dead, the virus does not have anywhere to replicate. Carrier cows showing no symptoms are the main sources of rotavirus infection and shedding the virus around the time of calving. Rotavirus infections cause major economic losses in neonates of many domestic animals (Yimer Muktar 2014; Umer Seid 2020).

2.1.2. Bacterial Agents

Bacteria were discovered by Antonievan Leeuwenhoek in 1676.They are the primitive forms of life. Bacteria are microscopic organisms which are found everywhere. They are monerans and comprise a group of prokaryotic organisms which are characterized by Peptidoglycan call wall.

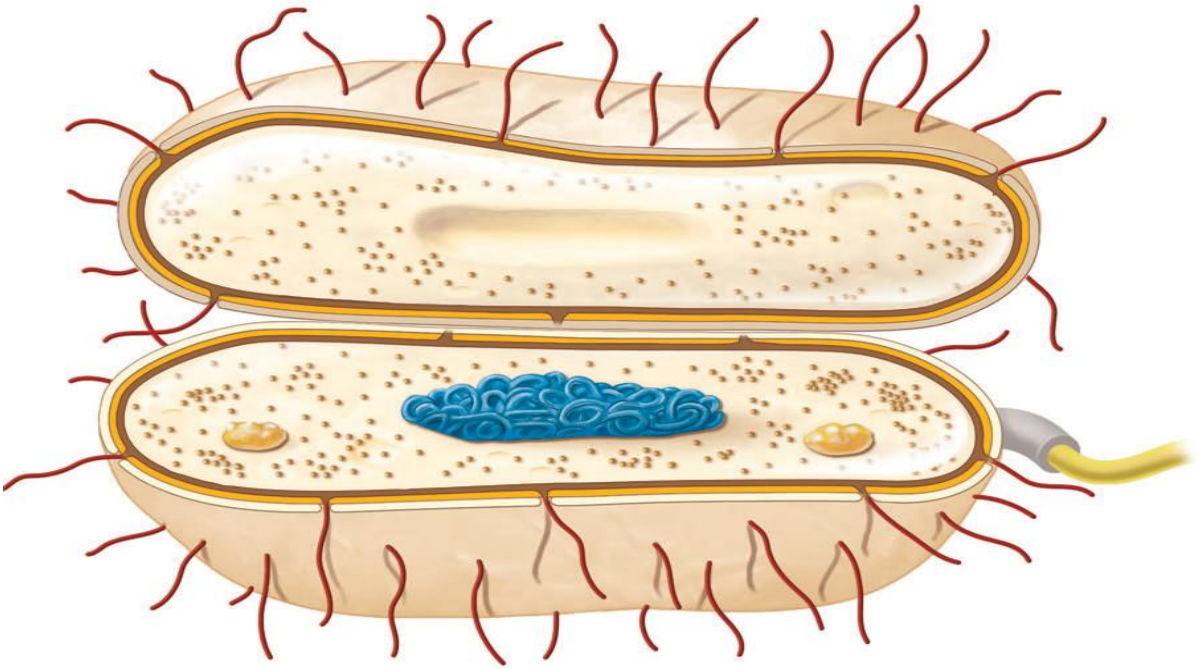

Figure 3: Structure of a bacterial cell. (Willy *et al*., 2017)

A bacterial genome irregular shape contain single circular DNA molecule, called the bacterial chromosome which sits in the cytoplasm, Reserve food material made up of glycogen and fats, Gas vacuoles may occur and all membranes bond cell organelles completely absent and 70S Ribosome occurs. The genomes size of bacteria can range from about 130kbp to over 14Mbp (0.6 to 8Mb). Most bacteria generally encode 600-6000 proteins. In addition bacteria may have one or smaller circular DNA molecules called plasmids that contain non-essential genes (Suyama and Bork, 2001).

Bacterium will be replicating its DNA whenever possible. When the genome is completely replicated, the two circular DNAs separate and the cell divides. Bacterial gene expressions (DNA replication, transcription and translation) work with astounding speed and fidelity in bacterial cells (Bustamante *et al*., 2011). Generally bacteria can show sexual means of reproduction by

binary fission, conidia, budding, cyst formation and reproduction through endospore formation are methods.

Bacteria can mediate diarrhea by producing enterotoxins that influence the crypt cells to hyper secrete, invading the intestinal mucosa and eliciting an inflammatory response that mediates hyper secretion through prostaglandins and other products of inflammation and by destroying villous absorptive epithelial cells and thus causing malabsorptive diarrhea (Torsein *et al*., 2011; Abdi, 2013; Sunday *et al.,* 2016).

2.1.2.1. K99 fimbrial adhesin-positive enterotoxigenic *Escherichia coli* (K99 ETEC)

Escherichia coli were first described by Theodor Escherich in 1885.In a series of pioneering studies of the intestinal flora of infants he described a normal microbial inhabitant of healthy individuals (Kaper, 2005).This microorganism commonly abbreviated as *E. coli*. It is a gram negative rod-shaped motile or non-motile, facultative anaerobic, non-spore forming member of the Enterobacteriaceae family found in the gastrointestinal tract of warm-blooded animals and humans (Frydendahl, 2002).

Novel strains of *E.coli* develop through the natural biological process of mutation and through horizontal gene transfer. The habitant of *E. coli* is a facultative in the gastrointestinal tract and also found in the environment. However, the infection is present due to break of the protection barrier, extreme pathogenic bacteria type or immunosuppression (Carlos, 2008).Clinical disease due to *E. coli* in calves may be present as enteric or septicemic illness, being one of the most important causes of neonatal mortality in dairy calves (Randhawa *et al*., 2012).

Calves diarrhea more recently is mainly caused by enterotoxigenic *E. coli* (ETEC) but others like attaching and effacing *E. coli* (AEEC) and Shiga toxin-producing *E. coli* (STEC) have also been identified as causes of diarrhea in calves (Mainil *et al*., 1993). Some strains of *E.coli* express virulence genes like stx1, stx2, α-hemolysin, ehly, cnf1, LT-II and STa that enhance organism ability to cause a variety of intestinal infections and diarrheal syndromes among animals and humans (Schmidt, 2010).

Diarrhea due to *E. coli* is one of the most frequent diseases occur in calves (Uhde, 2008), despite vaccination programs and management measures, necessitating treatment with antibiotics and

fluid therapy. Diarrhea caused by *E. coli* in neonatal calves is usually described by watery white or yellowish diarrhea, rapid onset and time course and high mortality. Diarrhea typically begins within 36–72 hours of birth in affected calves and they die within 2–3 days. Some calves die several hours after come into healthy and after free of diarrhea (Elham *et al*., 2012).

More virulent strains, such as *E. coli* O157: H7 cause serious illness or death in the elderly, the very young or the immune-compromised individuals (Hudault *et al*., 2001; Schmidt, 2010).Isolation and identification of certain virulence factors has served in defining mechanisms by which different strains result to various diarrheal syndromes, commensal *E. coli* strains sometimes hold virulence genes (Boerlin *et al*., 2005)

E.coli can be classified into six pathogroups based on their virulence scheme: enterotoxigenic *E.coli*(ETEC), shiga toxin-producing *E. coli*, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EAEC), enteroaggresive *E.coli* and enterohaemorrhagic *E.coli* (EHEC) (Kaper *et al*., 2004). From these pathogroups, the most frequent cause of neonatal calf diarrhea is ETEC stains that are producing the K99 (F5) adhesion antigen and also the heat-stable enterotoxin (Nataro and Kaper 1998). ETEC, EPEC and EHEC are the diarrheagenic types described as occurring in young farm animals. The significance of ETEC in the ethology of diarrhea among calves, lambs and pigs is well recognized and these organisms should not be confused with the rare EPEC and EHEC types that cause the less-common diarrheal syndromes (Randhawa *et al*., 2012).

New born calves are most harmed to enterotoxigenic *Escherichia coli* (ETEC) infection during first 4 days after birth and develop “watery” diarrhea if infected (Foster and Smith, 2009). After ingestion, ETEC infects the gut epithelium and multiplies in enterocytes in intestinal villi. The bottom part of small intestine is the best place of ETEC colonization due to the low pH (less than 6.5). For the attachment, bacteria express K99 antigen. As colonized on the gut epithelium, heat stable toxin is induced by ETEC and causes the secretory diarrhoea.

Many kinds of enterotoxins have been recognized and a single ETEC may be capable of producing one or more enterotoxins. Both heat-labile and heat stable Enterotoxins have been identified in ETEC. In calves, ETEC producing the low molecular weight heat-stable enterotoxins (STa) cause the majority of neonatal diarrhea problems (Gargan *et al*., 2013). Besides determination of the toxins and serotyping, i.e. determination of O serogroups related

with the cell wall lipopolysaccharides and H serogroups of the flagella has been applied for identification and characterization of ETEC (Boulianne *et al.,* 2011).

E. coli can synthesize various types of fimbriae that use for the organism to colonize the intestinal tracts of different animal species. Fimbriae *F5* expressing ETEC strains are pathogenic in many types of animals such as calves, lambs and pigs, whereas only able to cause disease in pigs are *E. coli F4*. However, ETEC own have other fimbrial antigens including *F41, F6, F17* and some types still not identified are capable of causing diarrhea in calves. ETEC which isolated from cattle use the *F5* pili, to bind to the enterocytes of the small intestine (Jay *et al.,* 2004).

A number of diagnostic tests are currently available for detecting ETEC, including Double-antibody enzyme-linked immunosorbent assay or latex agglutination test (Radostits *et al*., 2000), DNA gene probes specific for genes encoding toxins and adhesions of ETEC, multiplex polymerase chain reaction (PCR) for the rapid screening of ETEC toxins (Watterworth *et al*., 2005) and monoclonal antibody based coagglutination test.

2.1.3. Protozoan Parasitic Agent

The term protozoon implies ‘first animal’ or ‘little animal’. Protozoa are single-celled eukaryotic organisms that infect the cells lining the gut. Enteric infection with these parasitic agents is associated with villous atrophy, villous fusion, hypercellularity of the lamina propria and decreased activity of mucosal intracellular enzymes, causing nutrient malabsorption, diarrhea and debilitation (Wade *et al.,* 2000).

Protozoa do not have a cell wall and therefore they come in many different shapes and sizes. Protozoans have a relatively multiplex internal structure and carry out complex metabolic activities. Some protozoa have structures for propulsion or other kinds of movement. Vary from Amoeba which can convert its shape to Paramecium with its stable shape and structural complex.

Cryptosporidium is an important protozoan parasite that causes morbidity in neonatal calves. The genus was recognized for *Cryptosporidium muris* by Tyzzer in1907. This parasite is the etiological agent of cryptosporidiosis, which is commonly characterized by diarrhea in humans

and livestock. Cattle are commonly infected by *C. parvum, C. bovis, C. ryanae, and C. andersoni* (Ryan *et al.*, 2014). The most important *Cryptosporidium* species causing economic losses is *C. parvum (*Chalmers *et al*., 2011).

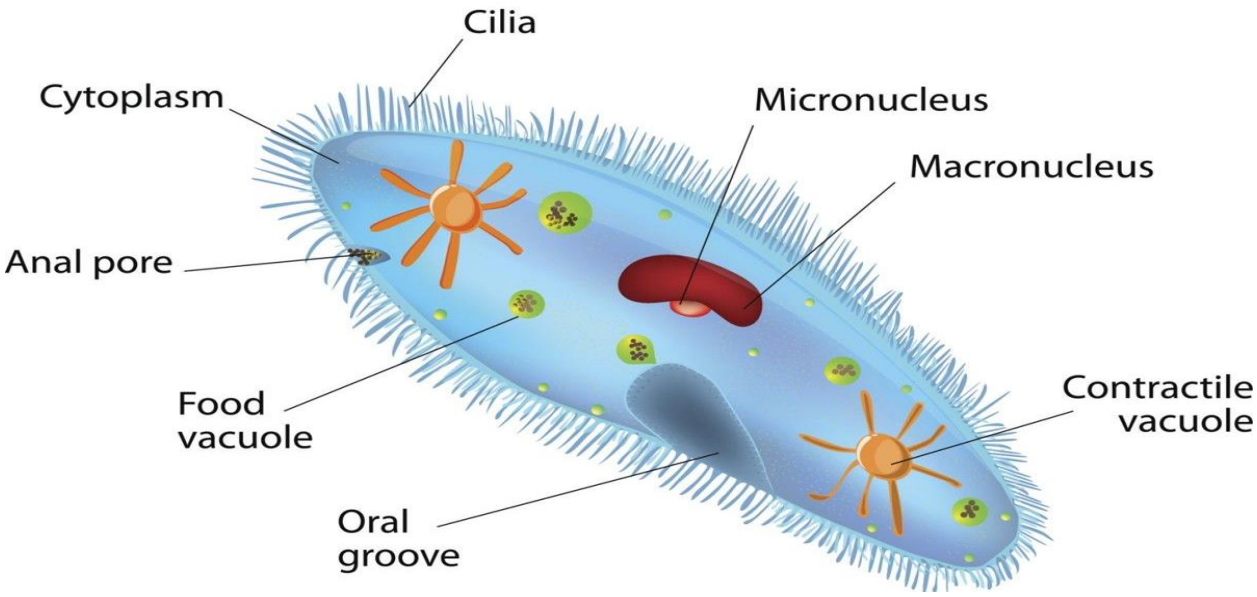

Figure 4: General protozoa structure.
(https://i.pinimg.com/originals/6b/41/db/6b41db5e7e314b369bf00b3abc107c55.jpg)

2.1.3.1. *Cryptosporidium parvum*

Cryptosporidium parvum is an intracellular protozoan parasite causing gastrointestinal disease and diarrhea in a variety of animal species including sheep, young farm animals and humans. C. parvum infection in calves is widespread (Al Mawly *et al*., 2015; Qi *et al*., 2015). *C. parvum* a protozoan that have sporozoites have been shown to express a shape change to a more spherical structure when the sporozoites age in vitro for a period of 12-24h (Robin and Franz, 2003).

The genome of *C. parvum* is of relatively small size and simple organization of 9.1mb, which is composed of eight chromosomes ranging from 1.04 to 1.5Mb. The genomes are very compact, and are one of the few organisms without transposable elements. Unlike other apicomplexans, *C.parvum* has no genes in its plastids or mitochondria.

Life cycle of *C. parvum* begins as sporulated oocysts, which enter the environment through the feces of the infected host. oocysts contaminate food and water. Infection happen when the oocysts are ingested by a suitable host. After Ingestion, the oocyst releases sporozoites which infect the brush border epithelium and undergo a complete life cycle. Primarily, the sporozoites undergo asexual reproduction. After that the sporozoites enter in to a sexual reproductive stage. Female macrogamonts and male microgamonts evolve and fertilization take place. The resulting zygote can develop in to a thick-walled oocyst that will exit the host or a thin-walled oocyst which will autoinfect the host (Borowskl *et al*., 2010).

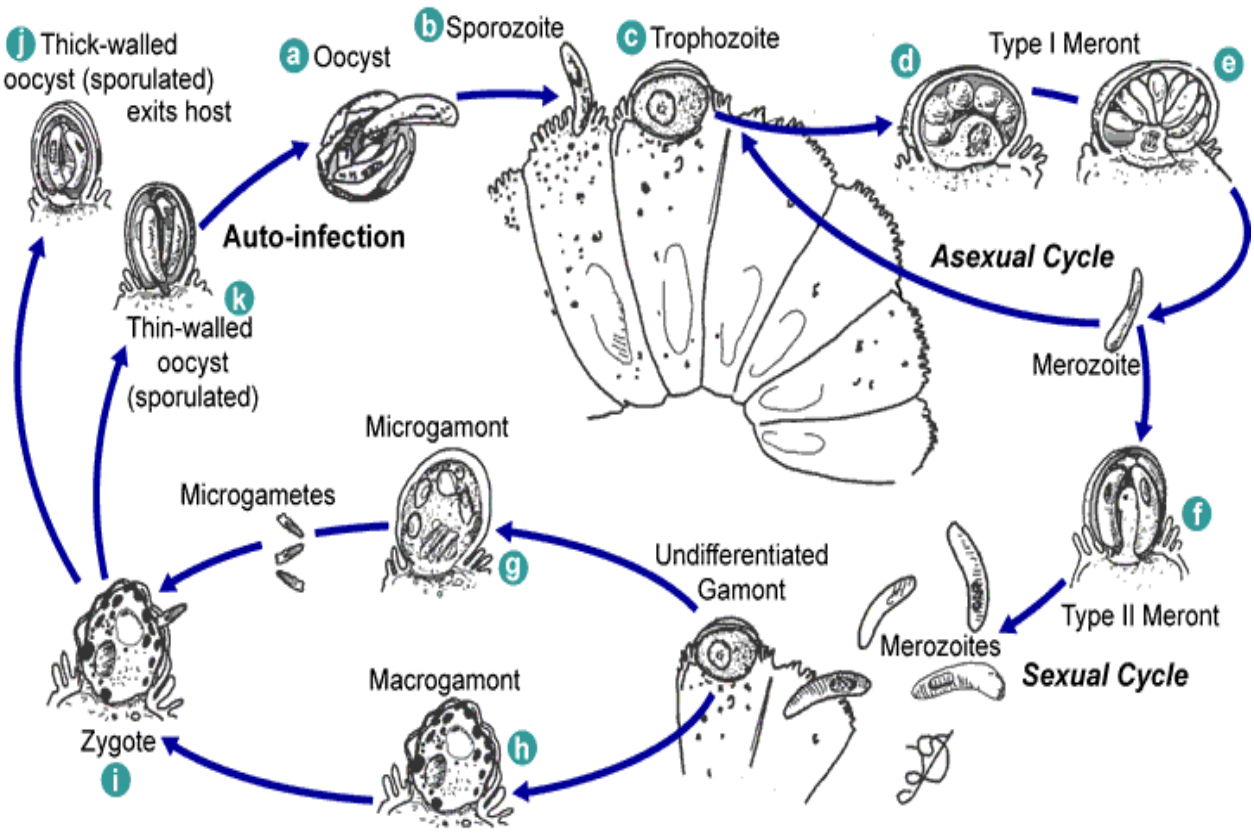

Figure 5: Life cycle of *C.parvum*.
(https://www.mcdinternational.org/trainings/malaria/english/DPDx5/images/ParasiteImages/A-F/Cryptosporidiosis/Crypto_lg.gif)

The invasion of *C. parvum* into enterocytes induces changes in intestinal cytoskeleton structures, such as loss of microvilli and shortening of columnar epithelial cells, leading to severe villous atrophy in infected animal (Wade *et al*., 2000). Damage to the intestinal epithelium causes prolonged malnutrition and reduced growth rates in affected calves due to malabsorption and fermentation of undigested milk in the intestinal lumen (Nydam and Mohammed, 2005).

Primary symptoms of *C. parvum* infection are severe, watery and non-bloody diarrhea. Most calves infected with *C. parvum* either appear no signs or develop mild diarrhea, sometimes express by fever, depression, in appetence, poor condition and dehydration for 2-3 days. It is most common causes of diarrhea in calves starting between 10 and 14 days of age, but can occur from about 7-30 days of age. These calves then develop immunity to *C. parvum* and are okay for life (Gurjar *et al*., 2008).

C. parvum parasite is transmitted by the fecal-oral route, but infection can result from consumption of contaminated groundwater or contaminated feed material as well as by contact with fomites and other animals shedding infective oocysts (Jennifer *et al*., 2013). Calves usually become infected with *C. parvum* between 1 up to 4 weeks of age. On most farms, environmental contamination is widespread and many potential sources of infection exist (Castro *et al*., 2002; Wegayehu Teklu *et al*., 2016).

2.2. Epidemiology of Calf Scour

2.2.1. Epidemiology of Calf Scour in the World

Diarrhea in calves is a leading cause of calf morbidity, mortality and economic losses in worldwide. It has been estimated that 75% of dairy herds is happen by acute diarrhea in the pre-weaning time (Svensson *et al.,* 2006) and also, a commonly reported disease in young animal and still a major cause of productivity and economic loss to cattle industry (Uhde *et al.,* 2008; Bartels *et al.,* 2010). The incidence of diarrhea in calves under 30 days of age differ between 10 % and 20% (Yimer Muktar 2014). The average incidence of diarrhea in newborn calves is around 20 % (Gilliss *et al*., 2013). Neonatal calf diarrhea causes a harmful effect on the calves' immediate health status, productivity performance and longevity in the herd and interrupts

production benefits with reduced weight gain and increased mortality and thus causes high economic losses.

2.2.2 Epidemiology of Calf Scour in Africa

African countries report a great range of calf mortality between 3% and 47% a period of the first year of life time, the majority of deaths occurring in the first 3 month of life (Zur *et al*., 2005).

Table 1: Calf mortality rates (0-12 months) compiled from different parts of Africa

Country	Management type	Mortality rate (%)	Study
Ethiopia	Smallholder and large	17.9	(Yeshwas Ferede 2015)
Ethiopia	Smallholder	9.3	(Bekele Megers *et al*., 2006)
Ethiopia	Smallholder	22	(Wudu T *et al*., 2008)
Kenya	Smallholder	15.8	(Muraguri *et al*., 2005)
Burkina Faso	Traditional/village	6	(Ganaba *et al*., 2002)
Zimbabwe	Smallholder	35	(French *et al.,* 2001)
Côte d' Ivoire	Smallholder	19	(Knopf *et al*., 2000)
Tanzania	Smallholder	12	(Swai *et al*., 2009)
Mali	Traditional/village	13	(Traoré and Wilson 1988)
Nigeria	Traditional/village	46	(Kudi *et al*., 1998)
Senegal	Traditional/village	12	(Fall *et al.,* 1999)
Gambia	Traditional/village	8-21	(Zinsstag *et al.*, 1997)

2.2.3. Epidemiology of Calf Scour in Ethiopia

Studies conducted on calf diarrhea in terms of pathogen identification and epidemiology in cattle industry of the country, in general, and market oriented dairy farms in particular, were very few. Previous study by Abraham *et al.* (1992) on new born calf diarrhea implies Bovine Corona virus (BCV) as the major infectious causes of neonatal calf diarrhea in some Ethiopian dairy herds, rotavirus and K99 *E. coli* also contributing to morbidity, either alone or as mixed infections.

(Abrham *et al.*, 1992; Demisse Demek 2007) in DebreZeit and Addis Ababa and (Yenehiwot Beyene 2008) Holeta and Debrezeit also conducted studies concerning the cause of calf diarrhea, but all had emphasized mainly on bacterial and viral cause. Nevertheless, a study under taken by (Simachew Kebede 1998) in DebreZeite, (Tadesse Dessie 2004) in DebreZeit included the bacterial and protozoal causes of calf diarrhea mainly focused in central Ethiopia. (Dawit Meconen 2012) and (Ashenafi Germa 2013) have investigated the distribution of *E. coli* biotypes in calf diarrhea in dairy farms located at DebreZeit, Addis Ababa and Kombolcha respectively and (Yimer Muktar 2014) studied isolation, identification and biotyping of enteropathogenes that cause diarrhea in calves in Muketuri and Fitche towns. A study under taken by (Umer Seid *et al*., 2020) in central part of Oromia were on viral causes of calf diarrhea.

The rate of *E.coli, Cryptosporidium parvum,* rotavirus and coronavirus prevalence in diarrheic calves can be influenced by some risk factors such as different geographical regions, seasonal effects, and neonatal calf immune system status and farm management. The results of the several studies show the differences in the prevalence of *E.coli, Cryptosporidium parvum,* rotavirus and coronavirus in diarrheic calves in various regions.

The multifactorial character of calf diarrhea make this disease difficult to control successfully in modern cow-calf operations (Izzo *et al*., 2011). Calf diarrhea prevention and control should be based on a good understanding of the disease complexities such as multiple pathogens, co-infection, environmental factors, and feeding and management during the calving period before disease outbreaks (Bartels *et al*., 2010).

3. MATERIALS AND METHODS

3.1 Description of the Study Area

The present study was conducted in selected districts of Afar region (Dudub, Deho, Alledegi, Melkhawerer and Sheleko) from February 2020 to February 2021. The study area is located at a distance of 387 km from Addis Ababa. The region has a total population of 1,812,002 (CSA, 2017), a further 409,123 or 29.43% were pastoralists with an estimated area of 96,707 Km^2. The area is located at latitude and longitude of 11.75594°N and 40.95869°E respectively. According to National Meteorology Agency (NMA) (2021), it receives an average annual rainfall of 200mm and has all year temperature average from 10-30°C. The major economic activities in the region are livestock rearing (agriculture) and trade according to personal communication with Afar district Administration Office.

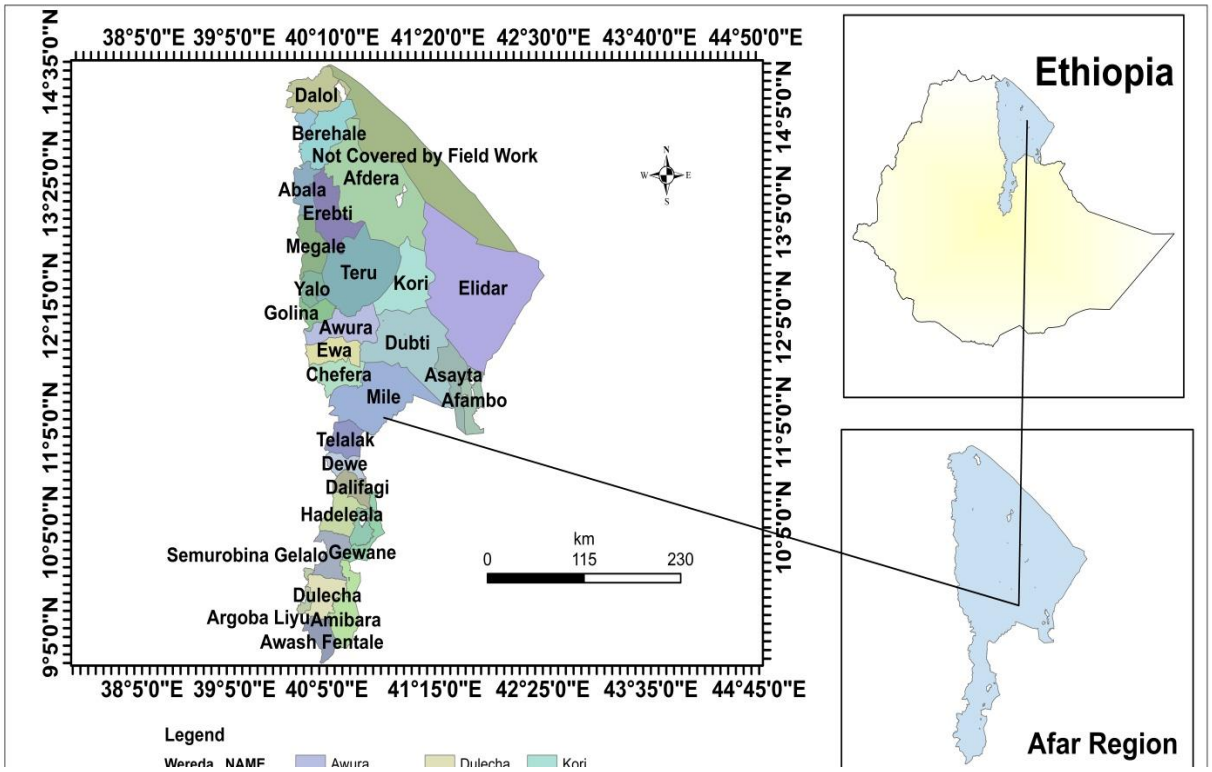

Figure 6: Map of the study area

3.2 Study Population

Animals that were included in this study were up to six months of age having clinical sign of diarrhea namely profuse watery diarrhea, systemic dehydration and depression during investigation. If the feces are semi-liquid to liquid, with or without other abnormal characteristics such as presence of blood or mucous was considered as diarrhea.

3.3 Study Design and Sampling Methodology

Cross sectional study design and purposive type sampling was employed where calves showing diarrhea in the study area during the study period were used as sample source. Information about the calves was gathered by interviewing the owners. The information collected was recorded on data collection sheet. During sampling date of sampling, consistency of feces, age, sex, the name of the farm and owner was recorded for each calf on proper recording format.

3.4. Sample Collection

Fecal samples were collected from selected diarrheic calves. About 10 ml of fecal sample was collected directly from the rectum of calves using sterile disposable latex glove into sterile plastic bottles after cleaning of the anal area with a paper towel and beats by rectal stimulation with the index finger using disposable sterile plastic gloves (Ammar *et al*., 2014).

The collected samples were clearly labeled including the information on the date of sampling, age and sex of the calves. Collected samples were placed in universal ice box containing ice packs and transported to the Parasitology laboratory at Aklilu Lemma Research Institute and were stored at -80 °C until test.

3.5. Detection of Bovine Rotavirus, Coronavirus, *E. coli* K99 and *C. parvum* Antigen Test (ELISA)

Enzyme linked immunosorbent assay (ELISA) is a method of target antigen (or antibody) capture in samples using a specific antibody (or antigen), and of target molecule detection using an enzyme reaction with its substrate. In ELISA various antigen-antibody combinations are used, always including an enzyme-labeled antigen or antibody. The enzyme activity is measured using a substrate that changes color or colorimetrically. Depend on the antigen-antibody combination, the assay is called a direct, indirect, sandwich and competitive ELISA type.

A commercial antigen-capture ELISA kit (Pathasure kit reference, Canada) is the type a sandwich ELISA capturing a mixture of monoclonal antibodies against bovine rotavirus, coronavirus, *E.coli K99* and *C. parvum* antigens in the fecal suspensions. The sandwich ELISA test was performed according to the manufacturer instruction (Kit reference). Each plate contains 96 well in the kit. Four wells were needed for each sample and control (1 well for each pathogen). Rows A1, A2, A3 and A4 were ready to use positive control wells and rows B1, B2, B3 and B4 wells were ready to use negative control. These control rows permit the differentiation between specific immunological reaction and non-specific bindings so as to eliminate false positives.

One gram (1ml) of feces was diluted in test tubes with dilution buffer provided in the kit based on the manufacturer instruction. A volume of 100µl of diluted samples was dispensed into wells following to both the positive and negative control wells per plate. The plate was incubated at 25°C for 30 minute and washed five times with 1x washing solution (diluted in the ratio 1:10 with distilled water) provided in the kit. Ready to use red conjugate (anti-rotavirus), ready to use blue conjugate (anti-coronavirus), ready to use green conjugate (anti-*E.coli K99*) and ready to use violet conjugate (anti-*Cryptosporidium*) were used as such and poured 100µl quantities into each well of column 1, each well of column 2, each well of column 3 and each well of column 4 per well respectively. The plates were incubated at 25°C for 30 minute and washed five times with the provided washing buffer. Then 100 µl of a chromogenes substrate solution added to each well on the plate. The plates were then incubated for 10 minutes at 25°C away from light.

Finally, the enzyme (horse radish peroxidase) reacts with the substrate (hydrogen peroxide). Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes color during reaction and a blue color developed. The strength of the color allows the determination of the type of sample tested. The test results were read by visual observation and for the development of blue coloration indicative of a positive result.

3.6. Viral Isolation Test

3.6.1. Sample Preparation

From viral ELISA positive fecal samples were taken forward for virus isolation. For each sample 1 gram (1ml for liquid sample) of feces were mixed with 9 ml sterile phosphate buffer saline with in screw-cupped bottle. Coarse glass beads were added and shacked vigorously. The fecal suspension samples were then poured into centrifuge tube and centrifuged at 3000 rpm for 10 min at 4°C in refrigerated centrifuge. The upper two-thirds of the supernatant fluids containing coronavirus and rotavirus positive were collected and filtered through 0.45 µm membrane syringe filter. Filtrates were treated with 20µg/inoculum trypsin for 2hrs at room temperature for enzymatic dissociation of cells and stored at - 20 °C until inoculation.

3.6.2. Cell Culture Preparation

Madine-Darby Bovine Kidney Epithelial cells (MDBK, passage 82) obtained from Athens Veterinary Diagnostic laboratory, University of Georgia, USA, were revived from liquid nitrogen and re-cultured in $25cm^2$ tissue culture flask. The confluent flask was then sub-cultured to multiple $25cm^2$ tissue culture flasks and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic solution at 37^oC in a humidified incubator at 5% CO_2.

3.6.3. Laboratory Investigation of Samples (Virus isolation)

The virus filtrates were mixed with an equal volume of Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal calf serum (FCS) and 10µg/ml crystalline trypsin (for dissociation of cells) and incubated at 37°C for 60 minutes. After incubation, 1ml of the mixture was

inoculated into the culture flasks with confluent monolayer of Madin-Darby bovine kidney (MDBK) cell lines (Arnold *et al*., 2009) and incubated for 1 hour at 37°C for adsorption of the virus. After the adsorption at 37°C for 1 hour, the cells were washed three times with a plain DMEM(Sigma-Aldrich) maintenance media and incubated at 37°C in a humidified incubator having 5% CO_2.

Monolayer cells were monitored every 24 h post-infection and inspected for development of cytopathic effects (CPEs), which is virus morphological change in culture and it is the characteristics of a particular group of viruses, using an inverted microscope. Viruses were subculture blindly every two days after being subjected to 3 cycles of freezing and thawing. CPE was observed after 48 hours and it was characterized by a destruction of the monolayer cell, cell rounding, and infected cells were disrupted and detached from the flask. Cells showing characteristic CPE were harvested by freezing and thawing thrice and centrifuged at 16,000rpm for 20 minutes at 4°C for the removal of cell debris. The supernatant containing the virus was collected and stored at −80°C for further passages.

If no CPE was observed, the sample was considered as “no virus detected” (NVD) and the culture was frozen at −80°C and then thawed and centrifuged at 3,000rpm for 10 minutes to collect the supernatant for second blind passage (P2).This was repeated for third passage (P3), and if no CPE was observed on the third passage after 48 hours inoculation, then the sample was considered as negative for coronavirus and rotavirus.

3.7. Questionnaire Survey

A total of 32 pre-tested number of questionnaires were administered to dairy farm owners to assess the general calf husbandry practices. Generally, the questionnaires included all practices in the farm, which could have impact on the proper rearing of calves and those risk factors responsible for calf diarrhea. These generally includes age of calf, knowledge colostrum feeding, general health care, calf separation, general health care, hygiene and sanitation of farms, occurrence of calf diarrhea, calf mortality, usage of vaccination and antibiotics to treat diarrhea, disease prevention and control measures practiced in the farms.

3.8. Data Collection, Management and Analysis

Structured questionnaire format was designed. It was used to collect information from responsible personnel and was interviewed after collected fecal samples from diarrheic calves. Data describing diarrheogenic conditions suggestive of rotavirus, coronavirus, *E.coli K99* and *C. parvum* infection observed on calves along with age, sex, colostrum feeding method, colostrum feeding duration, time of first feeding and other. The collected data were entered into Microsoft Excel and coded. The data was exported to SPSS windows version 25 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. An effect was reported as statistically significant if p-value was less than 0.05. In addition, all relevant information about farm management conditions was collected during the time of sample collection. Questionnaire survey was also conducted in Amharic language and translated in English on farmers during the study period to assess the farm management system.

3.9. Ethical Considerations

Ethical clearance was obtained from Addis Ababa University College of Natural and Computational Sciences Institutional Review Board and all procedures for this study was approved prior to conducting the experiment.

4. RESULTS

The prevalence of major viral, bacterial and protozoal enterophatogens associated with calf diarrhea from fecal samples of 176 calves was assessed. The highest proportions (25.6%) of the calves were infected with coronavirus and lowest proportion (6.2%) with *E. coli* K99 (Table 2).

Table 2: Viral, bacterial and protozoa enteropathogens associated with calf diarrhea

Enteropathogens	№.animals Examined	Frequency of isolation	Proportion (%)
C. parvum	176	29	16.4
E. coli K99	176	11	6.2
Coronavirus	176	45	25.6
Rotavirus	176	18	10.2

The positive and negative test results by antigen capture ELISA were indicated in (Figure 7) in selected farm of Afar, Ethiopia.

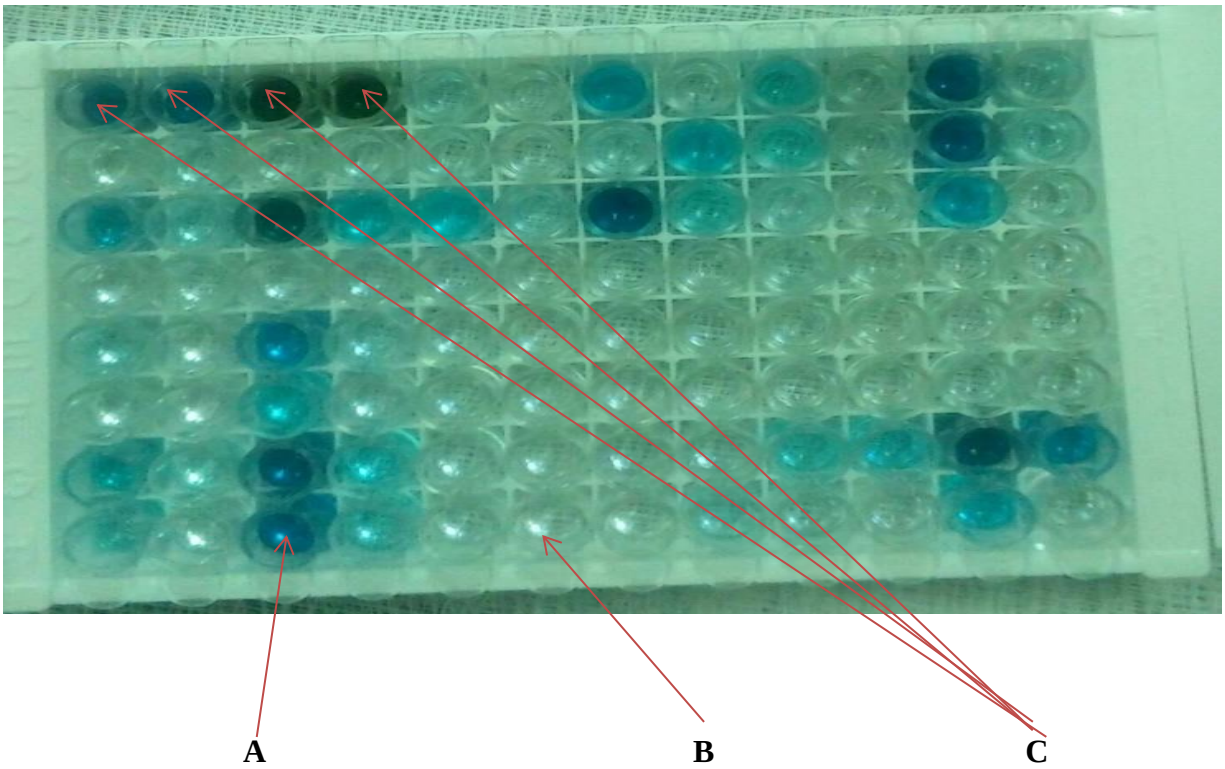

Figure 7: Results of ELISA in a Plate

Letter A represent the positive samples and arrow with letter B indicated the negative samples while Letter C all the arrow with blue color was positive control. The blue color indicated the positive sample and white color was negative sample.

Current research showed that except *C. parvum*, the highest frequency of enteropathogens was isolated from the fecal samples of male animals than female animals. The males were more susceptible to coronavirus, rotavirus and *E. coli* K99 infection as compared to female calves. However, statistically significant differences were not observed between the sex of the animals and the enteropathogens (Table 3).

Table 3: Proportion by sex of animal infected by the enteropathogens

Enteropathogens	Male (N=82)	Female (N=94)	X^2	P
Rotavirus	9 (10.9%)	9 (9.6%)	0.0936	0.76
Coronavirus	24 (29.3%)	21 (22.3%)	1.1045	0.293
E. coli K99	8 (9.7%)	3 (3.2%)	3.2210	0.073
C. parvum	13 (15.8%)	16 (17%)	0.0434	0.835

The general pattern of detection of the enteropathogens differs between different age categories of the animals with the highest detection during the one to three months of age (fig.8).

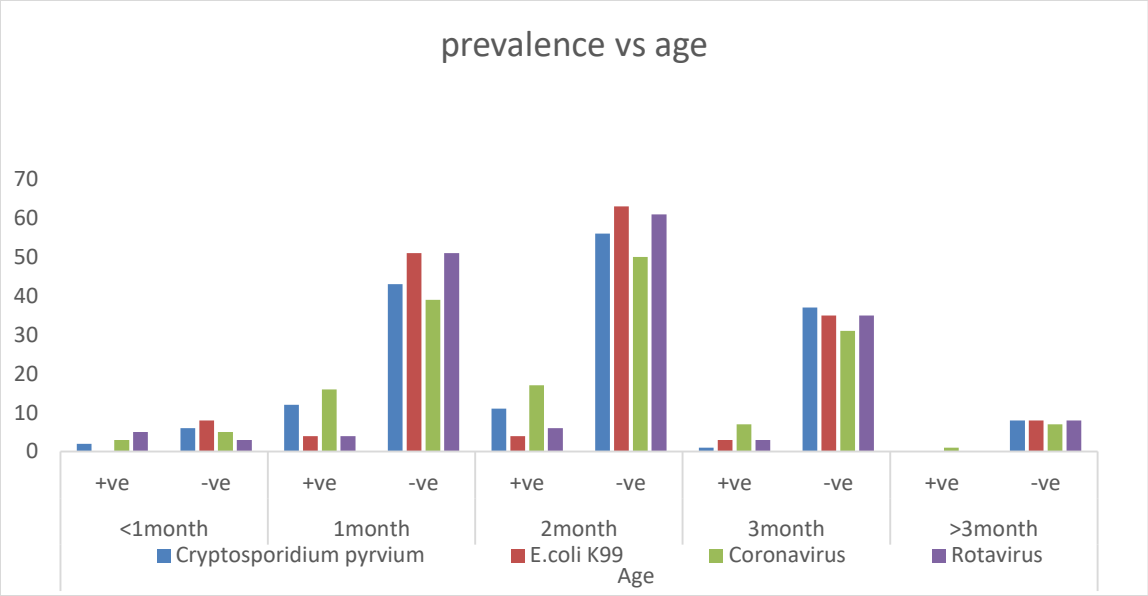

Figure 8: The prevalence of enteropathogens for different age categories of calves

Based on the findings, there was a significance association between occurrence of rotavirus and age of calf ($\chi 2$=37.29, p< 0.001).This age based difference in the prevalence of rotavirus is supported by the reason that younger calves are more susceptible to infection than older calves.

Different risk factors and their association with the prevalence of Enteropathogens were also assessed. A total of 32 potential risk factors were considered for analysis for the occurrence of enterophatogens causing calf diarrhea. Due to the similarity of farm management conditions in most of the farms no statistics was computed for most of the farm factors. The results indicate a prevalence of coronavirus, rotavirus and *C.parvum* for calves in less than one month of ages was 37.5%, 62.5% and 25% respectively. This shows that new born calves of 1-3 weeks of age were more susceptible to the above listed viral and protozoa infection. But the observed coronavirus, *E. coli* K99 and *C. parvum* prevalence in different calve ages were not significant (P> 0.05).

A higher prevalence (33.8%, 13.5%, 8.3% and 29%) of coronavirus, rotavirus, *E. coli* K99 and *C. parvum* antigen was observed among calves feed colostrum's from 30 minutes to 3 hours compared to calves given colostrum's within 30 minutes of birth (0%) and their prevalence were significant (P< 0.05) for between times of colostrums feeding. The current study indicated that the prevalence of coronavirus infection was higher in Melkhawerer (48.8%) as compared with other selected site and the prevalence of *E. coli* K99 and *C. parvum* were higher in Sheleko (24.2% and 54.5%). Rotavirus prevalence was higher in Dudub (26.7%). The coronavirus *E. coli* K99, *C. parvum* and Rotavirus prevalence was significant (P> 0.05) between the selected locations.

The result indicated that 22.2% of calves which separated immediately after birth were found positive, whereas coronavirus, *E. coli* K99 and *C. parvum* were detected in 25.7%, 2.4%, and 16.2% samples of calves never separated after birth respectively (Table 4 and 5). The rotavirus and *E. coli* K99 prevalence was significant (P= 0.02 and 0.04) between the separations of calves. See Table 4 and 5 for association and prevalence of other variables.

Table 4: Association of viral enteropathogens with different factors in diarrheic calves

			Coronavirus				Rotavirus			
Variables	Categories	N	№ +Ve	prevalence (%)	χ2	P-value	№ +Ve	prevalence (%)	χ2	P-value
Sex	male	82	24	29.3	1.105	0.293	82	10.9	0.094	0.76
	female	94	21	22.3			94	9.6		
Age (month)	<1	8	3	37.5	8.679	0.467	5	62.5	37.29	0.001 *
	1to3	160	41	25.6			13	8.1		
	>3	8	1	12.5			0	0		
location	Dudub	15	5	33.3	25.63	0.001 *	4	26.7	13.22	0.01*
	Deho	19	1	5.2			0	0		
	Alledeghi	66	7	10.6			2	3		
	Melkhawerer	43	21	48.8			6	13.9		
	Sheleko	33	11	33.3			6	18.2		
GCD1st	all of them	6	0	0	20.15	0.001 *	0	0	6.684	0.35
	most did	38	0	0			0	0		
	many didn't	132	45	34			18	13.6		
TCF1st	within 30min	43	0	0	19.55	0.001 *	0	0	6.483	0.01*
	from 30 min to 3 hrs	133	45	33.8			18	13.5		
DCF	for 15 min	133	45	33.8	19.55	0.001 *	18	13.5	6.483	0.01*
	for 40 min	43	0	0			0	0		
AMFD	<1 litre	134	45	1.3	18.95	0.001 *	17	12.7	3.705	0.157
	1-2 litres	41	0	0			1	2.4		
	>2 litres	1	0	0			0	0		
MR	almost always	2	0	0	16.16	0.001 *	0	0	3.882	0.144
	sometimes	41	1	2.2			1	2.4		
	always never	133	44	33.1			17	12.8		
SF	>2 month	31	10	32.2	0.885	0.347	5	16.1	1.427	0.232
	I don't use	145	35	24.1			13	8.9		
SCP	sometimes	9	2	22.2	0.056	0.813	3	66.7	5.516	0.02*
	never	167	43	25.7			15	8.9		
CE	almost always	3	0	0	4.031	0.133	1	33.3	2.657	0.265
	sometimes	8	0	0			0	0		
	always never	165	45	27.3			17	10.3		
VA	vaccine	32	8	25	3.192	0.203	6	18.75	4.361	0.113
	antibiotics	129	36	27.9			12	9.3		
	both	15	1	6.7			0	0		

Table 5: Association of bacterial and protozoan enteropathogens with different factors

Variable	Categories	N	*E. coli* K99				*C. parvum*			
			№ +Ve	prevalence (%)	$\chi 2$	P-value	№ +Ve	prevalence (%)	$\chi 2$	P-value
sex	male	82	8	9.7	3.2	0.07	13	15.8	0.04	0.83
	female	94	3	3.2			16	17		
Age (month)	<1	8	0	0	1.35	0.998	2	25	9.68	0.37
	1to3	160	11	6.87			26	16.2		
	>3	8	0	0			1	12.5		
location	Dududi	15	0	0	24.9	0.001*	2	13.3	28.7	0.001*
	Doho	19	0	0			0	0		
	Alhadega	66	0	0			2	3.03		
	Melkhawerer	43	3	6.98			16	37.2		
	Sheleko	33	8	24.2			9	54.5		
GCD	all of them	6	0	0	3.9	0.141	0	0	11.5	0.003*
	most did	38	0	0			0	0		
	many didn't	132	11	34.4			29	21.96		
TCF1st	within 30 min	43	0	0	3.79	0.05*	0	0	11.2	0.001*
	from 30 min to 3hrs	133	11	8.3			29	21.8		
DCF	for 15 min	133	11	8.3	3.79	0.05*	29	21.8	11.2	0.001*
	for 40 min	43	0	0			0	0		
AMFD	< 1 litre	134	11	0.82	3.68	0.159	28	20.9	7.97	0.019*
	1 to 2 litres	41	0	0			1	2.4		
	> 2 litres	1	0	0			0	0		
MR	almost always	2	0	0	3.79	0.15	0	0	8.29	0.016*
	sometimes	41	0	0			1	2.4		
	always never	133	11	8.3			28	21		
SF	>2 month	31	3	0.097	0.75	0.385	6	19.3	0.23	0.63
	I don't use	145	8	5.5			23	15.9		
SCP	sometimes	9	2	22.2	4.1	0.042*	2	22.2	0.23	0.63
	always never	167	4	2.4			27	16.2		
CE	almost always	3	1	33.3	4.29	0.117	0	0	2.31	0.31
	sometimes	8	0	0			0	0		
	always never	165	10	6			29	17.6		
VA	vaccine	32	1	3.12	2.0	0.362	3	9.4	5.4	0.067
	antibiotics	129	10	7.75			26	20.15		
	both	15	0	0			0	0		

NOTE: *= stastically significant, P value - Level of significance, χ2=Chi Square, −ve, negative; +ve, positive, TCF1st= Time of first colostrum feeding, No.+Ve = number of positive calves, N= number of cases, GCD1st= got colostrum in the first day , DCF= duration of colostrum feeding , AMFD= amount of milk feed daily, MR=milk replacers, SF= supplementary feed, SCP= separation of calves after parturition, CE= calves examined by health personnel, VA=vaccination and antibiotics.

In this study, MDBK cell line was used to isolate the virus from 38 virus samples of Ag-ELISA test positive samples. Out of 38 samples of both coronavirus and rotavirus cultured on MDBK cell line, CPE was observed in all samples. In the first pass, infected cells did not express any CPE. But from second passage onwards the infected cells started showing characteristic CPE. At 24 hours post infection the infected cells became round and clumped. At 48 hours post infection, the cells were thin and round shaped. At 72 hours post infection, the cells became small and majority of monolayer detached (Figure 7).

A B

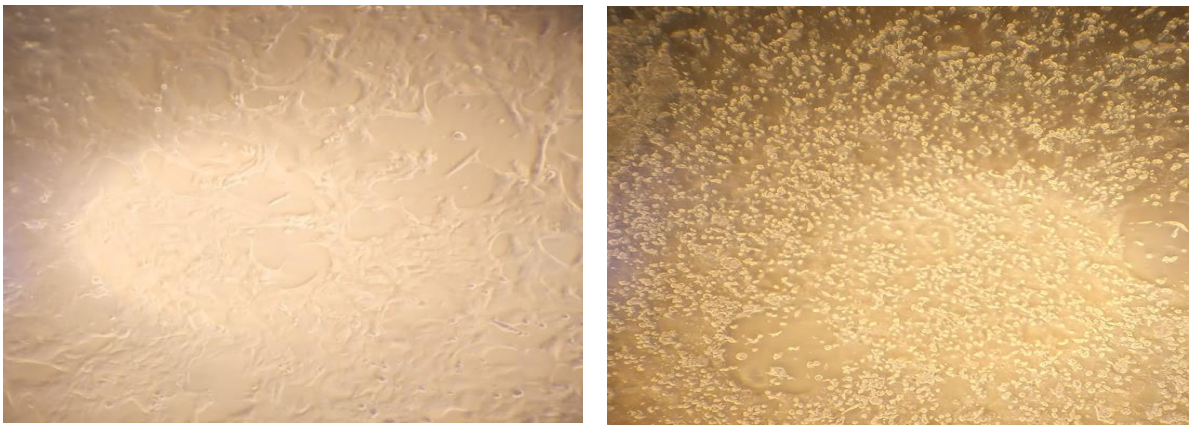

Figure 9: Characteristic CPE of rotavirus and coronavirus inoculated on Madin Darby bovine kidney cell line.

A. Shows cell swelling & obscure cell boundaries (20x objective)

B. Cells detachment & floating (10x objective).

5. DISCUSSION

Neonatal calf diarrhea is an enteric disease caused by many factors which includes the calf environment, nutrition, immunology and infectious agents. The most cause of diarrheal disease by the infectious agents. Viral, bacterial and protozoa has a great health problem in calves that interrupts production benefits with reduced weight gain and increased mortality and its potential for zoonotic spread.

The present study was undertaken to identify coronavirus, rotavirus, *C. parvum* and *E.coli K99* in diarrheic calves and to assess the association of different risk factors. The present findings show coronavirus was the predominant enteropathogen associated with calves' diarrhea. The prevalence of this agent is 25.6% (45/176). Our result is in agreement with those reported by Brandao *et al.* (2007) and Stipp *et al*. (2009) in Brazil, AKam *et al*. (2011) in Algeria and Izzo *et al.* (2011) in Australlia, in which the prevalence of coronavirus were 22.22%, 19%, 18.48% and 21.6% respectively, though much higher than the reports of Rai *et al*. (2011) (11.76%) and Dash *et al*. (2012) (4.76%) in India. However the result reached in our work remains lesser when comparing to that formerly cited by (Abraham *et al*., 1992) who has reported 38.9% in Ethiopia. This difference is due to the number of sample size, calf age, study site and sample collection season. Coronavirus is considered to be an important cause of diarrhea in calves.

In this study, the detection of rotavirus was found to be 10.8%. This result could also match with the previous findings of those reported by (Duman and Aycan, 2010) in Turkey (8.5%), (Kassem *et al*., 2017) 10% and (Umer Seid *et al*., 2020) in Ethiopia 7.2%.On the other hand, Rotavirus infections were detected at lower prevalence rate (%) than in previous studies compared to findings of (Smith, 2020) 47% in USA, (Abraham *et al*., 1992) 16.7% in Ethiopia and (Rai *et al*., 2011)15.68% in India and higher than the findings of (Beksisa Urge *et al*.,2020) who has reported 5% in Ethiopia.

The overall detection percentage of *C. parvum* (16.4%) in the present study was higher than previously reported by Wade *et al*. (2000) who found 2.4% of *C.parvum* was isolated from109 dairy herds in five counties of southeastern New York, USA. In the present study, *C. parvum* 16.4% (29/176) was, in agreement with previous reports in Argentina, the only species identified

in calves (Tomazic *et al*., 2013; Del *et al*., 2014). However, in contrast to previous studies done, a considerably higher overall prevalence was found (Trotz *et al.,* 2005) 40.6% in southwestern Ontario and (Nasir *et al*., 2009) 27.2% in Pakistan.

According to the present finding, *E coli k99* was the lowest prevalence (6.2%) from diarrheic calves in this study, a finding is in agreement with the result of Caple (1989) with 5%, but much less than the previous work reported by Yimer Mukta (2014), Zelalem Taressa and Soressa Bakale (2018), Dawit Meconen (2012) and Ashenafi Germa (2013) with 69.5%, 89.9%, 64% and 74% respectively in Ethiopia. The present result is higher than that reported by Brwon *et al.* (1990) in United Kingdom who reported isolation rates of 2.2%. The prevalence of the infectious agents varies among studies may be due to differences in sex, age, location, season, diagnostic techniques and other factors. Radostitis *et al*., (2007) in USA and Basera *et al*. (2010) in India.

Except *Cryptosporidium parvum,* the current result could suggest that more male calves were susceptible to coronavirus, rotavirus and *E.coli k99* infection though the difference is not statistically significant ($p > 0.05$). A higher prevalence of (29.3%) of coronavirus, (9.7%) of *E.coli k99* and 10.9% of rotavirus was observed among the males, while a prevalence of 22.3%, 3.2% and 9.6% was recorded in female calves, respectively. Other studies like (Umer Seid *et al*., 2020) and (Umer Seid 2019) also reported higher susceptibility of male calves in comparison to female calves.

In this study, young calves were at significantly high risk of being affected by diarrhea due to rotavirus infection. This finding was in agreement with the finding of (Trotz-williams *et al*., 2007) in southwestern Ontario and (Lorino *et al*., 2005) in France who reported younger calves were at high-risk of calf diarrhea. As compared to adult cattle, young calves are more likely susceptible to diarrhea. This is because of their liquid diet (milk), the higher water content in their bodies and their susceptibility to certain age related infectious diseases of the intestinal tract. In the present study, usage of vaccination and antibiotics to treat, calves examined by health personnel, supplementary feed to non-weaned calves and sex of calve were not

significantly associated with any of the enteropathgnes identified which was similar with the works of (Yenehiwot Beyene 2008).

The present results can be associated with many risk factors. Risk factors supposed with the occurrence of diarrhea associated with coronavirus, rotavirus, *C. parvum* and *E. coli* were location, amount of milk feeding, colostrum feeding, duration of first colostrum feeding and time of first colostrum feeding. The finding that time of first colostrum feeding (calves feed colostrum's from 30 minutes to 3 hours) associated with high risk of being affected with diarrhea than within 30 minutes and this result was in agreement with other reports which found that each hour of delay in colostrum ingestion in the first 12 hours of age increased the chance of a calf becoming ill by 10% (Wudu Temesgen *et al*., 2008).

The resistance of the calf to disease depends predominately on the quality and amount of colostrum it receives from the cow during the first hours of life after birth, as there is no resistance transfer from cow to calf before birth. Colostrum feeding was significantly associated with the occurrence of diarrhea coronavirus, (x^2= 20.1510, p = 0.001) and *C. parvum* (x^2= 11.5, p= 0.003).

6. CONCLUSION AND RECOMMENDATION

The present study was done to investigate the prevalence of coronavirus, rotavirus, *C. parvum* and *E. coli* in calves' up to 6 months of age at different selected farms of Afar region. The effect of associated risk factors on the prevalence of diarrhea was also assessed. Based on the present findings, calf diarrhea caused by coronavirus, rotavirus, *C. parvum* and *E. coli* were found from diarrheic calves using ELISA kit and 59 samples were identified as positive for at least one pathogen. When the 38 samples of Ag-ELISA positive samples of both coronavirus and rotavirus were cultured on MDBK cell line, CPE was observed in all samples. Questionnaire survey also indicated that awareness of the advantage of colostrum feeding and times of colostrum administration to neonate calves are crucial for the final development of immune status against infection.

This result indicated that calf diarrhea was found to be high in the study area and could affect dairy production system. For the higher detection of coronavirus from diarrheic calves in the study area location, amount of milk feeding daily, colostrum feeding, time of first colostrum feeding and duration of first colostrum feeding, frequency of provide water for non-weaned calves, usage of milk replacer to newborn calves were found to be incredibly important risk factors.

The present findings show that viral, bacterial and protozoan infection occurs in calves. In this study it can be concluded that improving producer knowledge and practices regarding colostrum feeding and other key risk factors such as farm hygiene and improved farm hygiene management could be especially effective for reducing coronavirus, *E.coli, C.parvum* and rotavirus exposure on calves.

Based on recent findings, we are highly recommending improved calf management practice and early colostrum feeding. In order to provide high resistance to disease calves need to be protected by practicing early colostrum feeding

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ANNEXS

Annex I: Record Format for Calf Diarrhea

No.	Date	Owners name	Sample ID	Location	Sex	Age	Sample type	Remark
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								

Annex II: Questionnaire format for management and description of calves

1. Respondent's data

1.1. Owners name:

1.2. District:

1.3. Village:

1.4. Kebele:

1.5. Address:

1.6. Education:

2. Pastoral: Calves

2.1. Number of adult cows above 2 years of age in the household_____cows.

2.2. Number of calves born (both dead and alive) during the last 12 months?_____calves.

2.3. Number of calves born alive during the last 12 months that died_____calves.

2.4. Number of calves born during the last 12 months which develop navel infection___calves.

2.5. Number of calves born during the last 12 months that were treated for navel infection__calves.

2.6. Number of calves born dead (stilibirth) during the last 12 months.

2.7. Number of calves born during the last 12 months that died within 1 week of birth.___calves.

2.8. did the calves born during the last 12 months got colostrium in the first day of life?

1. all of them 2. Most did 3. Many didn't

2.9. Number of calves born during the last 12 months that died due to malnurtrition/ feed shortage____calves?

2.10. What is the amount of milk fed daily to newborn calves?

1. <1 litre 2. 1-2 litres 3. >2 litres

2.11. Do you provide milk replacers to new born calves?

1. almost always 2. Sometimes 3. Always never

2.12. Do you provide supplementary feed to non-weaned calves?

1. <1 month 2. 1-2 month 3. >2 month 4.i don't use supplementary feed

2.13. When do you introduce feed different from milk/milk replacer to calves?

1. twice per day 2. Oncer per day 3. Every other day 4. Do not provide water

2.14. How often do you provide water to non weand calves?____cows

2.15. How many cows had been pregnant in the previous 12 months?___cows

2.16. how many cows in your herd abort in the last 12 months?___cows

2.17. Of the cows that gave birth in the last months, how many had mastitis?

1. almost always 2. Sometimes 3. Always never

2.18. Do you keep pregnant cows separated during parturition?

1. almost always 2. Sometimes 3. Always never

2.20 do you provide supplements to cows approaching parturition

1. almost always 2. Sometimes 3. Always never

2.21. Are sick calves examined for disease by health personnel?_____calves with diarrhea

2.22. how many calves develop diarrhea during the last 12 months?____calves with respiratory problems.

2.23. number of calves with respiratory problem in the last 12 months?

1. yes 2. No

Annex III: Principle of the ELISA Test

In this test, the entire microtitration plate is sensitized with a mixture of antibodies that are specific for the pathogen. These antibodies capture the corresponding pathogens in the fecal samples. The fecal material is diluted in dilution buffer and incubated on the microplate for half hour at 21°C +/ - 3°C. Positive and negative controls are also deposited on the plate. The plate is incubated and washed and then ready-to-use conjugates are added to the wells. Following a second incubation for half hour at 21°C +/- 3°C, the plate is washed again and the chromogen tetramethylbenzidine (TMB) is added. This chromogen has the two advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If the pathogens being sought is present in the faeces, the corresponding conjugate or conjugates remain bound to the corresponding micro wells and the enzyme catalyses the transformation of the colourless chromogen into a blue compound. The intensity of the resulting colour is proportionate to the titre of the pathogen in the sample.

Annex IV: Composition of the Kit

An ELISA assay is typically performed in a multi-well plate (96 wells). The entire plate is sensitised by antibodies specific for the pathogens for which the test is designed. Washing solution: One 100 ml bottle of 20 X concentrated washing solution. The solution crystallises

spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until all crystals have disappeared. Mix the solution well and take up the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water. Dilution buffer: One 50 ml bottle of 5x colored and concentrated buffer for diluting samples. Dilute this concentrated dilution buffer 1:5 with distilled or demineralised water. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper. Conjugate: 4 X 12 ml vials of coloured conjugate. The specificity of each conjugate is indicated on the bottle. The reagents are ready to use. Positive control: 4 vial containing 3 ml of the positive control. The reagent is ready to use. Negative control: 4 vial containing 3 ml of the negative control. The reagent is ready to use. Single component TMB: One bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C.protected from light. This solution is ready to use. Stop solution: One bottle of 1 M phosphoric acid stop solution. This reagent is ready to use.

Annex V: Procedure of ELISA Test

1- Bring all the reagents at 21°C +/- 3°C before use.
2- Dilute faecal samples volume per volume into dilution buffer. This is a qualitative dilution only, which must allow the pipetting of faecal suspensions. Discard any gruds by natural decantation for about 10 minutes. Do not centrifuge the suspensions.
3- Remove the microplate from its wrapper.
4- Pipette the diluted samples into the wells at the rate of 100 µl. Take care to change pipettes between two different samples. The arrangement of samples on the plate must be set by the user according to the number of faecal samples to test and the valences selected for each sample. Distribute the positive and negative controls over the plate as well (one well per valence tested). The control solutions are ready to use. If the distribution scheme for the samples and conjugates is complicated, fill out the layout forms.
5- Cover with a lid and incubate the plate at 21°± 3°C for 1/2 hour.
6- Rinse the plate with the washing solution prepared as instructed in the section "Composition of the Kit". To do this, dispose of the microplate's contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the

containment vessel. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times proceed to the next step.

7- Add the ready to use conjugates into the wells at the rate of 100 µl per well.
8- Cover with a lid and incubate the plate at 21°± 3°C for 1/2 hour.
9- Wash the plate as instructed in Step 6.
10- Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated.
11- Incubate 10 minutes at 21°C +/- 3°C without covering and away from light
12- Interpret the results visually by checking for a blue colour, unless you want to record the signals using a plate reader. In the latter case, skip to Step 13 and stop the reaction with the stop solution (read in the yellow range).
13- Add 50 µl of the stop solution to each well directly from the bottle. The blue colour will change into a yellow colour.
14- Record the optical densities using a plate reader and a 450 nm filter. The readings must be made as soon as possible after applying the stop solution, for in the event of a strong signal the chromogen can crystallise and lead to incorrect measurements.

Annex VI: Interpreting the Results

If spectrophotometer readings are made, calculate the net optical density of each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control. Precede in the same way for the positive control antigens. The test is validated only if the positive control antigens yield difference in the optical densities at 10 minutes that are greater than the values: Rotavirus, coronavirus, *E.coli, C.parvum* > 1000 Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage. Using the following table, determine each sample's status (positive, negative). Rotavirus, coronavirus, *E.coli, C.parvum* > = 600 % Any sample that yields a difference in optical density that is greater or equal the percentages above is considered positive for the valence in question. Conversely, any sample that yields a difference in the

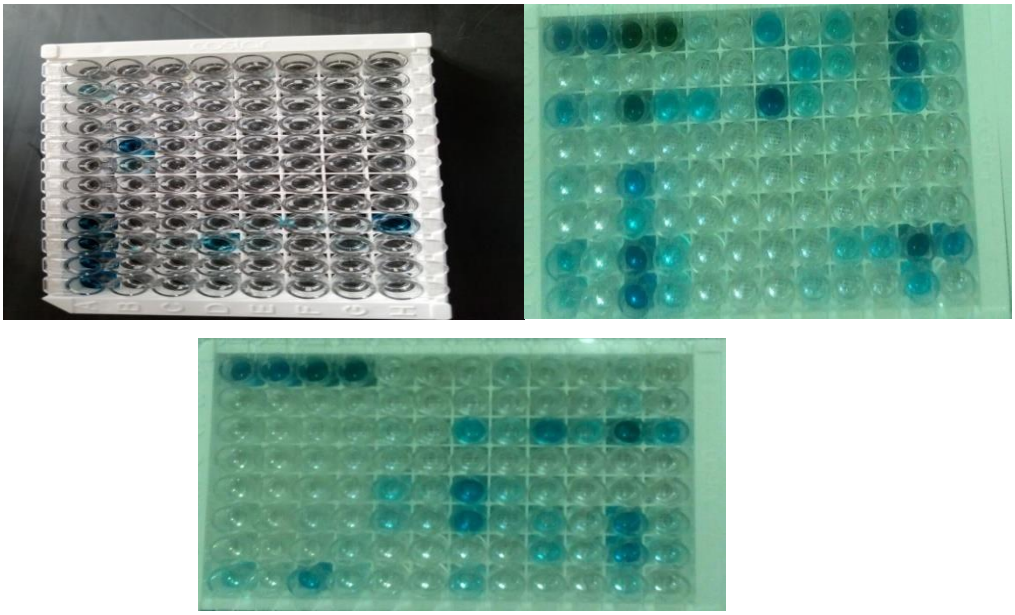
optical density that is less than the percentages above is considered negative for the valence in question.

If the results are interpreted visually (reading of the blue colour), the samples that produce a more intense blue colour than the colour in the corresponding negative control wells are considered to be positive.

Annex VII: Laboratory Data Recording Format

Sample number	Age	sex	*E.coli* K99		*C.parvum*		Coronavirus		Rotavirus	
			+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										

Annex VIII: List of some photos captured during laboratory works and Sample Collection



A Photo of ELISA test

Photo taken at the study area and photo of uninfected monolayer of MDBK cells (10x objective)