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MAJOR ENTEROPATHOGENES ASSOCIATED IN CALF DIARRHEA, WITH AN EMPHASIS ON *E. COLI* AND *SALMONELLA* SPECIES IN DAIRY FARMS OF MUKE TURI, DEBRE STIGE AND FITCHE TOWNS NORTH SHEWA, ETHIOPIA

MSc THESIS

BY

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BISHOFTU, ETHIOPIA

MAJOR ENTEROPATHOGENES ASSOCIATED IN CALF DIARRHEA, WITH AN EMPHASIS ON *E. COLI* AND *SALMONELLA* SPECIES IN DAIRY FARMS OF MUKE TURI, DEBRE STIGE AND FICHE TOWNS NORTH SHEWA, ETHIOPIA

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

By

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June, 2014

Bishoftu, Ethiopia

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Yimer Muktar** entitled: **Major enteropathogenes associated in calf diarrhea, with an emphasis on E. *coli* and *Salmonella* species in dairy farms of Muke Turi, Debre Tsige and Fitche towns North Shewa, Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of **Veterinary Microbiology.**

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DEDICATION

I dedicate this thesis manuscript to my late father Muktar Ali for nursing me with affection and love. I deeply wish that Allah would give him peaceful rest forever.

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (M.Sc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
BCoV	Bovine Corona virus
BVD	Bovine virus diarrhea
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CSA	Central Statistical Authority
CVMA	College of Veterinary Medicine and Agriculture
DAEC	Diffusely adherent E. coli
DNA	Deoxyribonucleic acid
DVM	Doctor of Veterinary Medicine
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhaghic *E. coli*
ELISA	Enzyme linked immunosorbent assay
EMB	Eosine methylene blue
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agriculture Organization
IMViC	Indole, Methylred, Vogaues prousker, citrate
ISO	International Organization for Standardization
LT	Labile toxin
MKTT	Muller-kauffmann tetrathionate
MOSH	Market oriented smallholder
MSc	Master of Science
PCR	Polymerase chain reaction
PVM	Phorous vacuolar membrane
RNA	Ribonucleic acid
SPSS	Statistical Package for the Social Sciences
ST	Stable toxin
ST	Stable toxin

STEC	Shiag-like toxin producing *E.coli*
TSI	Triple sugar iron
UK	United Kingdom
VT	Verotoxion
VTEC	Verotoxignic *E.coli*
XLD	Xylose-lysine-desoxycholate

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ABSTRACT

Cross sectional purposive type of study on entereopathognes associated in calf diarrhea in dairy farms of, Muke turi, Debre tisege and Fitche towns, North Shewa Zone, was conducted from November 2013 to April 2014 with the objective of identification of enteropathogens associated with calf diarrhea, investigating of potential risk factors related to the occurrence of enteropathognes causing calf diarrhea, determination of *E. coli* biotype and antimicrobial susceptibility patterns of bacterial isolates. Non-probability purposive sampling was used for the selection of farms and calf samples and 105 diarrheic calf samples were used in this study. Standard isolation techniques were used to identify the enteropathognes from diarrheic cases. The overall occurrence of *E. coli*, salmonella, cryptosporidium, eimeria and giardia found in this study were 69.5%, 25.7%, 27.6, 38.1% and 22.9% respectively. Single and mixed infection was common in this study. Among the potential risk factors age and age at first colotrum feeding were the factors that were found significantly associated with risk of occurrence of *E. coli* causing diarrhea (P= 0.01) using Chi square test and younger calves were more susceptible than older caves by odds of 14.74 times. Other risk factors (sex, breed) were not significantly associated with the occurrence of enteropathogns. Thirteen different biotypes of *E. coli* were found on the fermentation of nine sugars biotype XIII and XII were the dominant biotype found in calf diarrhea. Ciprofloxacin, trimethoprim-sulfamethoxazole and gentamycin were found to be susceptible for the bacterial isolates whereas erythromycin and tetracycline were developed resistance and most of the bacterial isolates showed multidrug resistance pattern. The occurrence rate of *E. coli*, salmonella, cryptosporidium and giardia causing diarrhea were serious problem in dairy caves. Great care should be given to the age of calves and time of first colostrum ingestion. Further epidemiological and microbiological studies on enteropathogns causing calf diarrhea to identify the serotype, virulent gene of the bacteria strongly recommended, in addition, the higher rate of antimicrobial resistance indicates the strategic use of the drugs in the future.

Key words: *Calf diarrhea, E. coli Eimeria, Cryptospordium, Giardia, North Shewa, Salmonella*

1. INTRODUCTION

Cattle production plays an important role in the economies and livelihoods of farmers and pastoralists. Dairy production and the livestock production in general have grown faster than crop production in most developing countries, and this trend is likely to continue with growth rates over the next twenty years estimated at 4.5 % per annum. The growing need for livestock production for their products and services makes the challenge more severe (FAO, 2006). Ethiopia, with 49.3 million heads of cattle, has the largest population in Africa (CSA, 2009).

Despite the large livestock population of Ethiopia, the economic benefits remain marginal due to prevailing diseases, poor nutrition, poor animal production systems, reproductive inefficiency, management constraints and general lack of veterinary care (Sissay *et al.*, 2008). Among the diseases, bovine calf scours or neonatal diarrhea of calves is a severe form of diarrhea that causes more financial loss to cow-calf producers than any other disease-related problem (Yamamoto and Nakazawa, 1997).

The future of any dairy production depends, among other things, on the successful raising of calves and heifers for replacement. Under modern dairy production in 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 must be replaced each year (Bath *et al.*, 1985). The impacts of calf diseases could be direct (causing calf deaths) and indirect through increased treatment expenses and decreased lifetime productivity and survivorship (Waltner-Toews *et al.*, 1986).

Enteric disease is a major health problem in calves, and diarrhea induces massive problems in the rearing of calves and is often caused by viral, bacterial and parasitic pathogens. Furthermore, diarrhea in young calves has been found to increase the risk of other diseases later in life (Van Donkersgoed *et al.*, 1993).

Calf diarrhea is any conditions characterized by passing of lose or watery feces with increased frequency, which could or could not be accompanied by other systemic signs like dehydration, decreased appetite or fever (Temesegen *et al.,* 2008). Calf diarrhea is a multifactorial disease entity that can have serious financial and animal welfare implications in both dairy and beef herds. It has been estimated that 75% of early calf mortality in dairy herds is caused by acute diarrhea in the pre-weaning period 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 and Bartels *et al.,* 2010). The incidence of diarrhea in calves under 30 days of age varies between 10 % and 20 % (Svensson *et al.,* 2003). In addition, calf diarrhea has an adverse effect on the calves' immediate health status, longevity in the herd and productivity performance and thus causes great economic losses. In order to increase the productivity per livestock unit without increasing livestock numbers, it is important to identify the etiological and predisposing factors involved in calf diarrhea in order to devise preventive measures and reduce losses during the initial months of the calf (Lorino *et al.,* 2005).

A number of infectious (bacteria, viruses, parasites) and non-infectious factors cause diarrhea in neonatal calves. Previous studies show that the most important infectious agents are *Rotavirus, Coronavirus*, enterotoxigenic *E. coli, Salmonella* species and Cryptosporidium species either singly or in combination (Steiner *et al.,* 1997; De la fuente *et al.,* 1998). However, other agents can also play a role in enteric diseases, as *Bovine Torovirus, Parvovirus, Pestivirus, Calicivirus, Astrovirus, Adenovirus*, eimeria species, Giardia, *Clostridium perfringens,* Campylobacter, Proteus and Klebsiella species (Achia *et al.,* 2004). Many of these enteropathogens cause severe intestinal lesions, alterations in enzyme activity and nutrient transport mechanism, or a combination of these effects. In some cases, diarrhea is self-limiting; others are associated with high morbidity and high mortality (Holland, 1990).

The most important group of bacteria causing calf diarrhea are *E. coli*, Salmonella and *Clostridium perfringens.* Pathogenic *E. coli* are one of the most important groups of

bacteria causing diarrhea and extra intestinal infections in humans and animals (Levine, 1987). Many of the bovine shiga toxins *E. coli* (STEC) have been isolated from cattle with diarrhea but they have also been recovered from healthy animals. Moreover, STEC have been associated with gastroenteric diseases, such as diarrhea and hemorrhagic colitis. Pathogenic *E. coli* strains cause significant loss of neonatal animals. Understanding the genetic basis and molecular mechanisms of the bacterial virulence of *E. coli* has shed light on the mechanisms of pathogenesis associated with this diverse group of bacteria (Kaper and O'Brien, 1998; Nataro and Kaper, 1998). In the debilitated or immunosupressed host or when gastrointestinal barriers are violated even normal nonpathogenic strains of *E. coli* can cause infection.

Salmonella infections in dairy calves have many impacts on animal and human health that are considered as a major worldwide problem. Substantial economic losses were manifested through mortality and poor growth of infected animals as well as the potential of zoonotic transmission (Smith *et al.,* 2004). Many outbreaks with high prevalence of clinical and subclinical Salmonella infections have been reported in cattle and calves worldwide (Berge *et al.,* 2008; Daly and Neiger, 2008). Cryptosporidium, eimeria and giardia species are the most important protozoan parasites causing gastrointestinal problems including diarrhea in calves. *Giardia* species is commonly found in cattle (Geurden *et al.,* 2010) and although this infection is often subclinical or even asymptomatic, it should be considered as a differential diagnosis in younger calves with acute or chronic diarrhea, reduced weight gain and ill thrift (Olson, 2004; Geurden *et al.,* 2010). Cryptosporidiosis one of the most important etiologies of acute diarrhea, especially in young calves (de Graaf *et al.,* 1999) and is caused by the intracellular protozoan parasite *cryptosporidium* species.

In developing parts of the world including Ethiopia there is a growing trend in the development of market oriented urban and peri-urban smallholder dairy farming, which is becoming an important supplier of milk and milk products to urban centers. However, 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 neonatal calf diarrhea indicated *Bovine Corona virus* as the major infectious case 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 (2007) in Debre Zeit and addis Ababa and Ynehewot (2008) Holeta and Debre zeit 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 (1998) in Debre Zeite, Tadesse (2004), Temsegen *et al.* (2008) in Debre Zeit included the bacterial and protozoal causes of calf diarrhea mainly focused in central Ethiopia. Recently, Dawit (2012) and Ashenafi (2013), have investigated the distribution of *E. coli* biotypes in calf diarrhea in dairy farms located at Debre Zeit and Addis Ababa and Kombolcha, respectively.

In Ethiopia, particularly in Debre stigae, Muke turi and Fitche towns, the isolation, identification and biotyping of enteropathogenes that cause diarrhea in calves has not been widely studied. In addition, considering the complex etiology of diarrhea and its economical importance in Ethiopian cattle industry the occurrence and etiology of calf diarrhea should be studied in these areas.

Therefore, the objectives of the study were:

- To identify the occurrence of the major enteropathogenes (*E. coli*, Salmonella, cryptosporidium, eimeria and giardia) involved in calf diarrhea.
- To investigate the association of the different enteropathognes with potential risk factors for infection.
- To determine the distribution of *E. coli* biotypes in calf diarrhea.
- To identify the antimicrobial susceptibility patterns of *E. coli* and *Salmonella* isolates.

2. LITERATURE REVIEW

2.1. Bovine calf diarrhea

Farm animals are born into environments with many potential enteropathogens and are initially exposed to resident micro flora in the vagina of the dam and subsequently to microbes harbored by herd mates. Some microorganisms are potentially harmful, while others are necessary for normal development and function of the gastrointestinal tract. However, once exposed, the intestinal tract is susceptible to infection with potential enteropathogens and in the absence of protective antibodies, various enteropathogens can become established and cause enteric disease. Infectious diarrhea of neonatal animals is a common disease (Holland, 1990). The disease not only causes a loss of the present value of the calf but also of the genetic potential for herd improvement (Bruning-Fann *et al.*, 1992).

Diarrhea in young pre-weaned calves is one of the most important causes of calf morbidity and mortality. The incidence of diarrhea in calves under 30 days of age varies between 10% and 20% (Bendali *et al.*, 1999a; Svensson *et al.*, 2003). Calf diarrhea has an adverse effect on the calves' immediate health status, longevity in the herd and productivity performance and thus causes great economic losses. In order to increase the productivity per livestock unit without increasing livestock numbers, it is important to identify the etiological and predisposing factors involved in calf diarrhea in order to devise preventive measures and reduce losses during the initial months of life (Lorino *et al.*, 2005).

In addition to the influence of varied environmental, managemental and nutritional factors, the infectious agents capable of causing diarrhea in calves are numerous. A number of infectious (bacteria, viruses, parasites) and non-infectious factors cause diarrhea in neonatal calves. Previous studies show that the most important infectious agents are Rotavirus, Coronavirus, enterotoxigenic *E. coli*., *Salmonella* species and

Cryptosporidium either singly or in combination (Snodgrass *et al.*,1986; Waltner-Toews *et al.*,1986; Reynolds *et al.*,1986; Steiner *et al.*,1997 and De la fuente *et al.*, 1998). However other agents can play a role in enteric diseases, such as *Bovine Torovirus, Parvovirus, Pestivirus, Calicivirus, Astrovirus, Adenovirus, Eimeria spp., Giardia, Clostridium petfringens, Campylobacter, Proteus and Klebsiella*. Calf diarrhea is the commonest disease in young calves and is the greatest single cause of death (Busato *et al*., 1997; Heinrichs and Radostits, 2001). It accounts for approximately 75% of the mortality of dairy calves less than three weeks of age (Haschek *et al.*, 2006). It is also a complex syndrome of great etiological complexity. In addition to the influence of varied environmental, managemental and nutritional factors, the infectious agents capable of causing diarrhea in calves are numerous.

Most cases of calf diarrhea are likely to be mixed infections, where more than one of the pathogenic agents is present. Many cases of scour proceed very rapidly, causing severe dehydration and metabolic imbalance within a few hours of the onset of disease. *E. coli* (K99 and F41) can cause very severe scour and dehydration in calves of less than one week old. In general, about 50% cases of neonatal diarrhea were ascribed to *E. coli* (Tripathi and Soni, 1984). Diarrhea caused by *E. coli* can occur as early as 24 hours after birth, but seldom occurs after three days of age unless it occurs as part of a mixed infection with rotavirus and *Cryptosporidium* species. *E. coli* can also invade the bloodstream and cause colisepticaemia (Quinn *et al.*, 1994). According to Blowey (2003), diarrhea in calves only a few days old is often caused by bacterial infections such as *Escherichia coli* or *Clostridium welchii*. Cryptosporidiosis and infections of rotavirus and corona virus commonly occur at an age of 10-14 days whereas Salmonella infection can occur at any age (Blowey *et al.*, 2003).

Failure of passive immunity transfer and overwhelming pathogen exposure are the main precipitating factors for calf diarrhea (Hunt, 1993). Four mechanisms have been demonstrated to be important in occurrence of diarrhea in calves. Hypersecretion of ions and water into the bowl, increased osmotic pressure from maldigestion and malabsorption

disease caused by damage to enterocytes, increased mucosal permeability due to inflammation, and the last and less important mechanism is alteration of intestinal motility (Hunt, 1993).

2.1.1. Major microbial causes of calf diarrhea

2.1.1.1. Pathogenic *Escherichia coli* strains

A Bavarian pediatrician, Theodor Escherichia, first described *Escherichia coli* in the late 19th century. In a series of pioneering studies of the intestinal flora of infants, he described a normal microbial inhabitant of healthy individuals (Kaper, 2005). It is gram negative rod-shaped motile or nonmotile, facultative anaerobic, non-spore forming member of the *Enterobacteriaceae* family found in the gastrointestinal tract of warm-blooded animals and humans (Frydendahl, 2002). *E. coli* is an important member of the normal intestinal micro flora of humans and other mammals; it has also been widely exploited as a cloning host in recombinant DNA technology. However, *E. coli* is not a laboratory workhorse or harmless intestinal inhabitant; it can also be a highly versatile and frequently deadly, pathogen (Kaper *et al.,* 2004). Moreover, *E. coli* is well known with the high genetic flexibility to acquire and/or transfer resistance or virulence genes from or to other strains of *E. coli* as well as other organisms (Hoyle *et al.,* 2005; Halawani, 2010). In addition, 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).

The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K (Bentley and Meganathan, 1982) and by preventing the establishment of pathogenic bacteria within the intestine (Hudault *et al.,* 2001; Reid *et al.,* 2001). New strains of *E. coli* evolve through the natural biological process of mutation and through horizontal gene transfer (Lawrence and Ochman, 1998). *E. coli* is a facultative habitant of the gastrointestinal tract and environment. However, the infection

is present due to break of the protection barrier, extreme pathogenic bacteria type or immunosuppression. Clinical disease due to *E. coli* in calves may be present as enteric or septicemic illness and is one of the most important causes of neonatal mortality in dairy calves (Lofstedt *et al.,* 1999). Nataro and Kaper (1998) have classified diarrhea causing *E. coli* into distinct groups based on virulence properties of *E. coli*, their difference in epidemiology and distinct O:H serotypes.viz: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC) or Verocytotoxigenic *E. coli* (VTEC), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). Each form of *E. coli* is associated with the production of specific enteric damage and physiological alterations by different toxins. In calves, the most common form is ETEC but STEC also play a role and are important for their impact on public health. *E. coli* strains for many years have been associated with intestinal disease in a variety of animal species. However, the role of STEC, EHEC or atypical EPEC as veterinary pathogens is unclear (Nataro and Kaper (1998).

Rahman (2002) studied 37 strains of *E. coli* isolated from cases of bovine calf diarrhea and gastrointestinal disorder. He reported prevalence of verotoxin genes among non-EHEC (non-O157) strains detected by PCR amplification technique. Of the 37strains of *E. coli* tested , five (13.5%) were found to be positive for VT genes: of which two strains harbored both VT1 and VT2 genes, while two strains only VT1 and one strain VT2gene only. Nishikawa *et al.* (2002) isolated 7.3% (67/924) diarrheic *E. coli* from sporadic cases of diarrheal illness in Japan using PCR technique. For detection of LT, ST and VT2 gene sequences of 132 bp, 171 bp and 228 bp respectively in *E. coli* isolates they used six sets of primers. These 67 strains were composed of 10 (15%) EHEC, and 4 (6%) ETEC along with other groups of diarrheic *E. coli*. Among the ETEC isolates, all the four strains carried ST gene and three carried LT gene.

Chattopadhyay *et al.* (2003) found a total of 20 STEC strains positive by PCR test out of 415 samples of different categories screened (fecal samples from 77 diarrheic calves, 78 healthy calves and 150 diarrheic children, 50 animal handlers, 60 food samples). The

isolation rate was highest in diarrheic calves where 17 (22.07%) were found positive for STEC out of 77 diarrheic calf fecal samples tested. On colony hybridization with *stx1* and *stx2* gene probes out of 29 samples that were positive by PCR technique, 10 contained both *stx1* and *stx2* genes while 3 contained only *stx2* gene. This study showed PCR to be more sensitive than hybridization technique.

Cobbold *et al.* (2004) studied STEC prevalence among dairy, feedlot and cow-calf herd in Washington and STEC strains were detected in 7.4% of fecal samples and farm environmental samples (of 1440 samples) using PCR technique. The prevalence for *stx* gene (6%) in samples from feedlots was significantly lower than those from dairy (20%) and range (*stx*: 21%) facilities.

Wani *et al.* (2003) reported the isolation and characterization of STEC serogroups associated with diarrhea in calves and lambs in India. They subjected 130 bovine and 15 ovine strains to multiplex PCR for detection of *stx1*, *stx2* genes along with other virulence factors. STEC strains belonging to different serogroups were detected in 9.73% of calves and 6% of the lambs studied. One of the most important serogroup O157 known to cause certain life-threatening infections in humans was isolated from both bovine and ovine fecal samples. Shaw *et al.* (2004) isolated 170 VTEC strains from 47% of dam samples (40 of 86 samples) and 20% of calf samples (130 of 664 samples). During their study, 14 calves showed symptoms of diarrhea. However, VTEC (serogroup O26) was detected in only one diarrheic calf.

Wani *et al.* (2004) serotyped *E. coli* isolates obtained from an outbreak of bloody diarrhea in 1-16 week old crossbred calves in an organized dairy farm in Kashmir. Seven out of 10 calves were affected. Serogroup O116 was recovered from 5 calves with diarrhea. The virulence gene profile revealed *stx1*, *eaeA* (attaching and effacing gene) and *hlyA* (α-hemolysin) genes.

Eriksson *et al.* (2005) performed prevalence study of VTEC O157 in 371 randomly selected Swedish dairy herds and analyzed by immunemagnetic separation and PCR methods. The isolation rate for VTEC O157 was 8.9%. Irino *et al.* (2005) reported a total of 202 individual shiga toxin producing *E. coli* (STEC) isolates among 1471 *E. coli* colonies screened for *stx*. One hundred and forty (69.3%) of them were typable. The great majority of the isolates carried *stx2* (40.6%) or *stx1, stx2* (56.4%) sequences. Only few isolates harbored *stx1* sequence alone (3%). In another study, Wani *et al.* (2005) demonstrated association of STEC O4 serotype with an outbreak of diarrhea in 4-7 week old calves. Six *E. coli* O4 strains carried *eaeA* and EHEC e*hlyA* genes and three possessed *Stx1* genes. Zweifel *et al.* (2005) reported a total of 42 STEC strains from slaughtered healthy cattle in Switzerland. The PCR analysis showed that 18 (43%) strains carried the *Stx1* gene, 20 strains (48%) had the *stx2* gene and four (9.5%) strains had both *stx1* and *stx2* genes.

Biotypes of *E. coli* isolates: Biochemical reactions have conventionally been used for identification of bacteria to the species level. Extensive studies of biochemical reactions of bacteria have been done to introduce biochemical-typing systems in epidemiological studies of bacteria (Barr and Hogg, 1979; Krishnan *et al.*, 1987). *E. coli* are able to ferment a variety of carbohydrate substrates, generally by converting them to glucose or to a substrate on the fermentative chain of the breakdown of glucose. The various fermentable carbohydrates include substances such as compounds of glucose with other sugars.

The ability to ferment a given sugar of the types described above by a strain of *E. coli* is dependent on the strain having the requisite enzymes to convert it to glucose or to a substance on the degradative chain from glucose. It has been found that different strains of *E. coli* differ in their ability to perform these conversions. Thus, while all strains are ferment glucose and over 90% ferment mannitol and lactose, with many of the other sugars mentioned above the fermentation reactions will vary. This is the basis of biotyping *E. coli*. These tests are also easy to perform, by determining, whether a strain

of *E. coli* will produce acid following growth in the presence of the carbohydrate (Crichton and Old, 1982).

Pandey *et al.* (1979) classified 29 isolates of *E. coli* obtained from 106 samples of milk, human urine, calf diarrhoea, poultry enteritis and infantile diarrhea into 19 biotypes by their fermentation reactions with dulcitol, starch, sucrose, salicin, raffinose and rhamnose. Hinton et *al*. (1982) examined 2973 *E. coli* isolated from six different groups of animals for their ability to ferment adonitol, dulcitol, raffinose, rhamnose and sorbose. Twenty fermentation patterns were recorded although 2443 (82%) of the *E. coli* belonged to seven of the 32 possible biotypes.

2.1.1.2. *Salmonella* species

The Gram-negative genus Salmonella is currently divided into two species, *Salmonella bongori* and *Salmonlla enterica.* A third species, *Salmonella. subterranea* was proposed in 2004 (Shelobolina *et al.* 2004), but this was later shown not to belong to the genus (Grimont and Weill, 2007). *Salmonella enterica* is further divided into six subspecies, enterica, salamae, arizonae, diarizonae, houtenae and indica, based upon DNA-DNA hybridization, 16S RNA analysis and multi-locus enzyme electrophoresis (Crosa *et al*. 1973; Reeves *et al.* 1989). The vast majority of *Salmonella enterica* serovars are found in subspecies enterica and account for greater than 99.9% of known human and animal infection (Selander *et al.* 1996).

Salmonella organisms are facultative anaerobic gram-negative rods within the family of *Enterobacteriacea*e (Yan *et al.,* 2003) and that can survive and multiply in the environment as result of fecal shedding. The Kauffmann-White scheme, first published in 1929, divides Salmonella into more than 2500 serotypes according to their antigenic formulae (Mortimer *et al.,* 2004). The majority of cattle isolates are the following four serotypes: *S. typhimurium, S. dublin, S. muenchen and S. copenhagen* (Davies, 2008). Infections are usually limited to the digestive tract, although the musculoskeletal and

nervous systems are occasionally affected too. In cattle Salmonella, infections comprise the second most economically important bacterial disease affecting the gastrointestinal system; following *E. coli* infections. Salmonella is a primary aetiologic agent of infectious diarrhea, and its high morbidity and mortality rates make it one of the most economically significant pathogens in livestock production (Fairbrother, 1999).

The majority of Salmonella strains found in cattle are not host-adapted (Rings, 1985). *Salmonella dublin*, host specific, and *S. typhimurium*, not host-specific, are quoted to be the most common servovars in bovine (Venter *et al.*, 1994), both affecting calves severely between six and twelve weeks of age and in the first three weeks of age, respectively (Bisping and Amtsberg, 1988). Salmonella organisms remain in the gastrointestinal tract as part of the host's communal flora and may be shed in feces. All food animals, except aquatic species in their usual habitats, are susceptible to natural infection with Salmonella species. An animal infected with *Salmonella* species may or may not develop salmonellosis (Ekperigin and Nagaraja, 1998). The frequencies of Salmonella serovars isolation vary from one location to the other due to different managmental and hygienic regimes as well as geographical, environmental and individual differences (Ritchie *et al.*, 2001 and Veling *et al.*, 2002).

Furthermore, salmonellosis on a farm is a potential zoonotic risk to farm workers and their families (Vanselow *et al.*, 2007). The organism spreads easily between operations, likely via manure contaminated clothing and footwear therefore, precautions and awareness programs should be adopted with focusing on animal contacts.

2.1.1.3. Cryptosporidium oocyst

The genus Cryptosporidium was established for *Cryptosporidium muris* by Tyzzer in 1907, 37 species name has been introduced. However, after redescription and confirmation, currently 21 names are associated with individual species (Robertson and Gjerde, 2001) and 16 species are actually regarded as valid based on different oocyst

morphology, site of infection, vertebrate class specificity, and genetic differences. Members of the genus cryptosporidia species are cyst-forming protozoan parasites, which have a monogamous life cycle and inhabit epithelial cells of the gastrointestinal or respiratory tracts and is a genus of protozoan parasites that infecting a wide range of hosts (Fayer, 2006). All groups of vertebrates are susceptible to Cryptosporidium infection worldwide. This parasite is the etiological agent of cryptosporidiosis, which is mainly characterized by diarrhea in humans and livestock.

Today three species are known to regularly infect cattle: *Cryptosporidium parvum*, *Cryptosporidium andersoni* and the recently described *Cryptosporidium bovis* (*Fayer et al.,* 2005; Langkjaer *et al.,* 2007; Thompson *et al.,* 2007). *C. andersoni* is found mainly in adult cattle but has also been found in young stock and *C. parvum* usually infects calves younger than one month (Olson *et al.,* 2004). *C. bovis* tends to infect older calves and young stock (Langkjaer *et al.,* 2007). Calves are primarily infected via the fecal-oral route and it takes less than 50 oocysts to infect a healthy calf (Fayer *et al.,* 2000).

Infection can rapidly spread from calf to calf when animals are communally housed and overcrowded or from cow to calf via the udders when they are contaminated with infected calf feces in the lying area of the dams (Nasir *et al.,* 2009). In cattle, *Cryptosporidium parvum* is considered to be the most common enteroparasite in calves during the first week of life, frequently as a coincidental infection with other viral, bacterial and parasitic pathogens (Fayer *et al.,* 1998; Degraaf *et al.,* 1999; O'handley *et al.,* 1999). Cryptosporidium infection can negatively influence growth rate, feed conversion and milk production (Esteban and Anderson, 1995) leading to a drop in the economic benefits derived from livestock production.

No drug therapy is yet available and the high resistance of oocysts to environmental conditions and chemical treatment make cryptosporidiosis difficult to control (Pozio, 2006). Cattle have been considered a primary reservoir for Cryptosporidium oocysts for zoonotic *C. parvum* (Chalmers and Giles, 2010). These animals could be a risk factor via

environmental contamination from their manure being spread on farmland or their grazing on watersheds (Monis and Thompson, 2003). On farms, transmission of *Cryptosporidium* species can result from ingestion of contaminated food or water, by direct transmission from host to host, or through insect vectors (Follet-Dumoulin *et al.*, 2001). Since vaccines have become commercially available against *Escherichia coli* K99, rotavirus, and corona virus, Cryptosporidium has emerged as the main neonatal diarrheic agent in calves (Moore and Zeman, 1991).

Infection occurs in immunocompetent as well as immunocompromised animals and humans, with diarrhea being the characteristic clinical sign. Enteric disease caused by *Cryptosporidium* species is usually self-limiting in immunocompetent individuals but debilitating and persistent in those immunocompromised (Ramirez *et al.*, 2004). There are no specific clinical signs to differentiate *C. parvum* infection from other kinds of diarrhea. Diarrhea is the most consistent clinical sign and is accompanied by increased frequency of defecation, tenesmus, anorexia, emaciation, central nervous system depression, dehydration and malnutrition. During the early stages of infection, blood-tinged feces and intestinal casts may be observed. Young calves do not show long-term shedding of oocysts. Excreted oocysts are very resistant in the environment and may remain infective for several months in either soil or water. Contamination of surface water and ultimately urban water supplies caused large outbreaks of water borne human cryptosporidiosis (Naciri *et al.*, 1999)

Cryptosporidium parvum is often the only pathogen found in diarrheic calves (Singh *et al.*, 2006). It has been reported from Norway that the prevalence of the parasite seems to increase as the number of calves in a herd increases, and that small farms tend to have fewer problems with cryptosporidiosis (Hamnes *et al.*, 2006). However, the importance of *C. parvum* infection as a cause of calf diarrhea is under debate since *C. parvum* is commonly found in healthy animals. In some studies, no association between infection with *C. parvum* and diarrhea or other clinical signs were found (de Rycke *et al.*, 1986 and Huetink *et al.*, 2001). However, results from Canada showed a three time higher risk for

calves shedding oocysts to be diarrheic than non-infected calves (Trotz-Williams *et al.*, 2005a). Another example is from India, where results show a 1.59 times greater risk for a calf to suffer from diarrhea if infected (Singh *et al.,* 2006). According to a study by Reynolds *et al.* (1986), there are more enteropathogenes, such as *C. parvum*, found in diarrheic calves than in clinically healthy calves.

2.1.1.4. *Eimeria* species

Members of two genera of intestinal coccidia, Eimeria and Isospora, are known to induce enteritis in livestock. The only pathogen of veterinary relevance in the latter genus is Isospora suis in piglets (Mundt *et al.*, 2005a), whereas a number of E*imeria* species are known to cause intestinal lesions and economic loss in farm animals, particularly poultry, rabbits and ruminants and young calves (Taylor and Catchpole, 1994 and Daugschies *et al.,* 2004). Eimeria is part of the genus of coccidian parasites, which belongs to the protozoan phylum Apicomplexa. At least 21 species of Eimeria are known to infect cattle. *Eimeria bovis* and *Eimeria zuernii* are considered the most pathogenic, but *Eimeria alabamensis*, *Eimeria aubumensis*, *Eimeria ellipsoidalis* and *Eimeria wyomingensis* can cause marked clinical signs too, if they are present in large numbers.

Bovine coccidiosis is an important protozoan disease that has been given to this disease of genus Eimeria affecting calves all over the world resulting in considerable economic losses each year beef and dairy industries (Daugschies and Najdrowski, 2005). The disease can be produced by several Eimeria species of which *E. bovis* and *E. zuernii* are the most pathogenic and the chief culprits' disease in cattle (Urquhart *et al.,* 1996; Radostits *et al.,* 2007). In many instances, coccidiosis is a silent thief robbing of performance but never visibly showing most of its symptoms, though the most common of which is bloody diarrhea (Urquhart *et al.,* 1996 and Radostits *et al.,* 2007).

The prevalence of Eimeria infection in cattle is generally high and can reach 100% in calves (Grafner *et al.,* 1982; Fox, 1985 and Cornelissen *et al.,* 1995). Calves at an age of

three weeks to six months are particularly susceptible to clinical coccidiosis (Taylor and Catchpole, 1994), which rather reflects lack of immunity than age resistance (Grafner and Graubmann, 1979), but high prevalence rates have also been documented in yearlings (Cornelissen *et al.,* 1995).

Adult cattle are generally resistant to clinical coccidiosis but they can carry subclinical infections, which may be a source of infection for calves (Svensson, 1993). Compared to clinical coccidiosis, subclinical coccidiosis is economically more important and may account for over 95% of all the losses associated with coccidiosis and can cost cattle ranchers more than US$400 million per annum. It can also delay growth of calves by as much as two months (Dedrickson, 2002).

Eimeriosis in cattle is particularly a problem of confined animals kept under intensive husbandry practices. The disease is more common in housed animals than in those on pastures. In associations with other enteropathogens, coccidia have been indicated as an important cause of diarrhea in calves (Ernst *et al.,* 1987). The occurrence of diarrhea depends upon the interaction of many factors, including the species of coccidia, the density of Eimeria oocysts in the environment, the related rate of exposure of naive calves to oocysts, environmental temperature, humidity and sunlight, which affect the development and survival of oocysts and stressors (Daugschies and Najdrowski, 2005).

2.1.1.5. *Giardia* cyst

Gastrointestinal protozoa of the genus Giardia are responsible for considerable economic losses in livestock associated with morbidity and mortality (Geurden *et al.,* 2010). Giardia is a parasite found in all parts of the world and in a large number of mammals, including humans, livestock, pets, wildlife, and aquatic animals (Lasek-Nesselquist *et al.,* 2010; Thompson and Monis, 2010 and Feng *et al.,* 2011) and also a frequent enteric parasite young animals including companion animals, livestock and wildlife (Thompson and Monis, 2012). According to Monis *et al.* (2009), there are eleven species within the

genus Giardia. Six of them, formally known as Assemblages A-G of the *Giardia duodenalis* morphological group, are genetically but not morphologically distinguishable. They can infect humans and mammals, with some being host specific and others having low host specificity. Giardia-infection in cattle is often subclinical or asymptomatic, but this infection can also cause symptoms including acute or chronic diarrhea, reduced weight gain and ill thrift in young calves (Geurden *et al.,* 2010). Although the prevalence of Giardia in cattle around the world varies considerably (Geurden *et al.,* 2010) longitudinal studies have shown cumulative infection rates in calves of 100% (O'Handley *et al.*, 1999; Ralston *et al.,* 2003). The two zoonotic species *G. duodenalis* (Assemblage A) and *G. enterica* (Assemblage B) and the livestock-specific species *G. bovis* (Assemblage E) are able to infect cattle with *G. bovis* being found most frequently followed by *G. duodenalis* (Becher *et al.,* 2004 and Dixon *et al.*, 2001).

Once infected, Giardia causes a generally self-limited clinical illness (i.e., giardiasis) characterized by diarrhea, abdominal cramps, bloating, weight loss, and malabsorption. However, asymptomatic giardiasis occurs frequently, especially in developing countries (Hellard *et al.,* 2000). Giardia is also a very common enteric parasite of domestic animals, including livestock, dogs, and cats and wildlife (Appelbee *et al.,*2005). One species within this genus, *Giardia duodenalis* (syn. *Giardia lamblia* and *Giardia intestinalis*) causes giardiasis in humans and most mammals. Thus, giardiasis is considered a zoonotic disease. The transmission of these protozoa occurs most often through ingestion of cysts in contaminated water and food (Robertson, 2009).

2.1.1.6. Rotaviruses

Rotavirus are the most frequently agents aassociated with diarrhea in children and domestic and animals. Rotaviruses are taxonomically grouped in the Reoviridae family (Derbyshire and Woode,. 1978) this family also includes the orthoreoviruses and orbiviruses, other genera known to infect animals and human beings (Derbyshire and Woode,. 1978). Rotaviruses are uniquely characterized by a double-stranded RNA

genome that consists of 11 gene segments enclosed in a double-shelled protein capsid. The 11 RNA gene segments encode viral proteins and have assigned functions (Matsu *et al.,* 1989).

Currently, group A and B rotaviruses are known to infect calves and lambs (Cheney *et al.*, 1984) but group A rotaviruses are most prevalent and clinically important. Four prominent group A calf diarrhea rotaviruses have been characterized: the neonatal calf diarrhea virus, the United Kingdom calf rotavirus, the B641 calf rotavirus, and the B223 calf rotavirus. Three of these strains (neonatal calf diarrhea virus, the United Kingdom strain, and B641 strain) have been assigned to rotavirus serotype six (Hoshino *et al.*, 1984) Although group B rotaviruses are infrequently detected in the feces of diarrheic calves, results of serologic or electron microscopic studies suggest that infection or at least exposure to group B rotavirus is common among young calves and lambs (Cheney et *al.*,1984).

Rotaviruses affect a variety of animal species, including laboratory rodents, wildlife, and humans. They are a common cause of diarrhea in lambs, calves, and pigs in which infection may be asymptomatic (Crouch, and Acres. 1984) varying in severity or fatal The importance of viruses as causes of neonatal calf diarrhea was first demonstrated by Mebus *et al*. (Holland,190).

Rotavirus an important aetiological in the neonatal calf diarrhea and mainly found in feces of diarrheic calves up to 3 rd weeks of life .these organisms are responsible for the vast majority (75 to 95%) of enteric infections in neonatal calves (Garcial *et al.*, 2000).

2.1.1.7. Bovine viral diarrhea virus (BVDV)

BVDV is a member of the genus Pestivirus in the family Flaviviridae Two antigenically distinct genotypes of BVDV exist, types 1 and 2, with further subdivisions discernable by genetic analysis (Donis, 1995). The two genotypes may be differentiated from each other,

and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the E2 major glycoprotein's, or by genetic analysis (Chasey and Davies. 1984).

2.1.1.8. Coronaviruses

Bovine Corona virus (BCoV) a member of the family Coronaviridae, order Nidovirales. The genome of *Corona virus* consists a single strand, caped and polyadenlated positive sense RNA molecule. A number of different viruses can be primary pathogens in the neonatal calf diarrhea complex. By far the most common viruses causing calf hood diarrhea found throughout the world are rotaviruses and corona viruses. Primary infection of newborn calves with either one of these viruses can cause severe intestinal alterations and diarrhea. Rotaviruses can produce high-morbidity outbreaks of diarrhea in calves less than ten days of age (Wengler, 1999).

BCoV isolates are generally recognized as being associated with enteric disease of newborn calves and winter dysentery or chronic diarrhea in adult cattle. Incidence of corona virus in neonatal diarrhea is slightly lower than Rotavirus (Mason and Caldow, 2005).

2.1.2. Pathogenesis of calf diarrhea

Enterotoxigenic *E. coli:* Enterotoxigenic *E. coli* (ETEC) produces profuse watery diarrhea that are mainly a problem in calves up to 4 days old, although they can occasionally produce diarrhea in older calves too (Naylor, 2002). ETEC strains produce plasmid-mediated enterotoxins, which bind to their specific receptors on the gut epithelium and by a complex interplay of biological mediators cause diarrhea. The osmotic diarrhea is due to secretory effect at the crypt cells and inhibition of absorption by villous tip cells. Several types of enterotoxins are identified and a single ETEC may be capable of producing one or more enterotoxins. Both heat-labile (LT I, LT) and heat

stable enterotoxins are found in ETEC. In calves, ETEC producing the low molecular weight STa cause the majority of neonatal diarrhea problems (Naylor, 2002).

There are two major subtypes of LT enterotoxins, LT-I and LT-II which do not cross-react immunologically. LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals. LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates but in neither animals nor humans has it been associated with disease. Thus, the term LT refers to the LT-I form (Nataro and Kaper, 1998).

Heat stable enterotoxin has two subtypes-STa and STb. STa toxins are produced by ETEC and several other gram-negative bacteria including *Yersinia enterocolitica* and *V. cholera*, while STb has been found only in ETEC (Levine, 1987). STa activates guanylate cyclase causing increased levels of cGMP in the entrecotes, thereby inhibiting Na^+, Cl^- co-transport. The clinical result is secretory diarrhea with loss of fluid, bicarbonate and other electrolytes (Nataro and Kaper, 1998). ETEC strains were first recognized as causes of diarrheal disease in piglets, where the disease continues to cause lethal infection in newborn animals (Alexander, 1994).

E. coli is capable of synthesizing various types of fimbriae that enable the organism to colonize the intestinal tracts of different animal species. Fimbriae F5 expressing ETEC strains are pathogenic in different animals such as calves, lambs and pigs, whereas *E. coli* F4 are only able to cause disease in pigs. However, ETEC possessing other fimbrial antigens including F41, F6, F17 and some types still not identified are capable of causing diarrhea in calves (Acres, 1985). Most ETEC isolated from cattle use the F5 pili, to bind to the enterocytes of the small intestine (Jay *et al.,* 2004).

Genes coding for shiga toxins (stxl and stx_2), attaching and effacing mechanisms (eaeA) and haemolytic mechanisms (ehly) mainly mediate the pathogencity of shiga toxigenic *E. coli.* Although some stx_l- positive STEC isolates have previously been associated with severe disease, epidemiological studies have demonstrated that stx_2 is the most important

virulence factor associated with severe human disease (Beutin *et al.,* 2004). Shiga toxins have an A - B structure with the A subunit carrying the enzymatic activity and the B subunit binding to a host cell membrane receptor. The shiga toxin inhibits protein synthesis resulting in the death of cell by inhibition of globotriaosyl ceramide (Gb3) receptors on absorptive villus epithelial cells in the ileum. Originally, cattle are thought to be refractory to the effects of shiga toxin because of the absence of the Gb3 receptor. However, it has been shown these receptors are present in bovine intestines, localized in proliferating crypt cells. Intracellular trafficking in primary bovine intestinal cells excludes shiga toxin from endoplasmic reticulum and instead localizes it to lysosomes, a pathway that is likely to lead its inactivation. It has been postulated that shiga toxin plays a role in the ability of EHEC to cause persistent colonisation in cattle and this may be the reason that STEC are successful commensals of cattle (Naylor *et al.,* 2005).

Various studies attempted to demonstrate a link between the presence of STEC and intestinal diseases in ruminants have been developed (Leomil *et al.,* 2003 and Kang *et al.,* 2004). In some studies (Leomil *et al.,* 2003) the prevalence of STEC or fecal stx genes is higher in diarrheic than in healthy calves. Different virulence gene profiles of STEC have been seen in diarrheic and healthy calves. A higher proportion of STEC from samples of diarrheic calves contain *eae*A and stxl compared to STEC shed by healthy calves. Healthy animals were more likely to be *eae*A negative and stx2 positive.

It is possible that STEC promote colonization in younger animals even in the absence of disease. More direct evidence of STEC as animal pathogen comes from detailed examination of cases in which organisms are associated with intestinal Attaching and effacing lesions and relevant clinical signs. It appears that certain strains of *E. coli*, particularly serogroups 026, O111 and 0118 are virulent to cattle, particularly young calves (Wieler *et al.,* 1998). *E. coli* 026 has been isolated from clinical cases of hemorrhagic diarrhea in young and neonatal patients (Leomil *et al.,* 2005).

Salmonella: *Salmonella* species initiates the disease process by migrating to the lamina propria through the microvillus of mucosal cells and the tight junctions between those cells. The mucosal cells are damaged in the process and fibro necrotic plaques are formed. Damage to the mucosal cells disrupts the integrity of the mucosal lining of the gastrointestinal tract and causes accumulation of residue materials that would ordinarily have been confined to or excluded from the gut lumen, to leak out or in. Endotoxins and other materials seep out of the intestinal lumen into the body cavity while plasma proteins and other materials present in the exudates from the inflamed lamina propria leak into the intestinal lumen. The resulting acute inflammatory response is associated with an increase in vascular permeability resulting in mucosal edema. Furthermore, the influx of neutrophils is associated with necrosis of the upper-most ileal mucosa. The injury to the intestinal epithelium leads to leakage of extra vascular fluids and massive transmigration of neutrophils into the intestinal lumen, a process normally prevented by the epithelial permeability barrier. These data suggest that the severe fluid loss observed during Salmonella species enterocolitis is at least in part due to an inflammatory mechanism, which causes liquid to flow from the blood to the intestinal lumen (Zhang *et al.,* 2003).

Tremendous variation in clinical severity of disease exist based on the virulence and infection dose of Salmonella, the age, immune status and existence of concurrent disease in the calf. Clinical findings include a watery to fibrino-mucohaemorrhagic diarrhea with fetid odour, anorexia, marked depression, fever and shock and affected animals become recumbent within 12 to 48 h and die within 3 to 7 days (Tsolis *et al.,* 1999).

Cryptosporidium: The pathogenic mechanisms by which Cryptosporidium causes diarrhea, malabsorption and wasting are poorly understood. Whatever these mechanisms may be, the initial host–parasite interactions of attachment and invasion are critical primary events in pathogenesis. The ultra structural characteristics of attachment and invasion and various factors influencing attachment have been described. However, little is known about specific parasite and host molecules involved in these processes (Theodos, 1998). Knowledge of such molecules is crucial for understanding the

pathogenic mechanisms employed by this parasite**.** The parasite is intracellular but extracytoplasmatic (Holland, 1990). Infection is mostly concentrated to the lower parts of the small intestine (Bowman *et al.,* 2003).

The sporozoites can also invade the microvillus border of the gastric glands, bile duct or respiratory tract (Ballweber, 2001). The intracellular stages are formed within a parasitophoros vacuole, confined to the microvillus region of the host cell. The sporozoites become incorporated with the microvillus membrane and are internalized in a membrane sac of the host cell (Radostits *et al.,* 2000). The protozoa cause tissue reactions in the intestines, which may lead to diarrhea. Villous atrophy, a shortening of microvillus, villous fusion and cryptitis can be seen. The intestinal digestion and absorption of dietary nutrients is decreased due to the loss of microvillus and decreased activity in mucosal enzymes and villous function. It is mostly the lower part of the small intestine that is infected but cecum and colon can occasionally be affected (Radostits *et al.,* 2000). Sometimes the parasite causes bacterial overgrowth by decreasing disaccharides activity. Less disaccharidase reduces the breakdown of sugars and causes a good environment for bacterial growth (Ballweber, 2001).

Cryptosporidium, like other coccidia, sequesters itself inside the host cell during development. It is protected from the host immune response and the hostile environment of the gut, while accessing the nutritional and energy reservoirs of the host cell. Again, like other coccidia, it lies within a parasitophorous vacuole bounded by a parasite phorous vacuolar membrane (PVM), which, in other coccidians, is the portal through which nutrients from the host cytoplasm enter the parasite (Tzipori and Ward, 2002). Unlike any other coccidian, however, Cryptosporidium has, in addition, a unique structure known as the feeder organelle membrane, which directly separates the cell and parasite cytoplasm. It is assumed that the PVM in Cryptosporidium provides only a protective function, while the feeder organelle membrane is the site for nutrient and energy uptake from the host cell. It is conceivable, however, that the PVM is also selectively permeable to certain molecules from the gut lumen. This is because the PVM,

originally derived from the host cell membrane, may retain some of its absorptive and other functional activities (Tzipori and Ward, 2002).

Eimeria: In the pathogenesis of *Eimeri*a species, certain (unknown) target molecules allowing recognition, adhesion and subsequent penetration have been postulated. However, further development was exclusively observed in bovine cells and few gamonts and oocysts were obtained only in fetal gastrointestinal cells (Hermosilla *et al.*, 2002).

In the natural host sporozoites traverse mucosal cells without considerable alterations and finally invade endothelial cells of the central lymph capillaries of the ileal villi (Behrendt *et al.*, 2004). Invasion of the host cell is accompanied by the release of antigens from organelles located in the anterior region of the sporozoites (micronemes and rhoptries) that play a significant role in host cell recognition, penetration through the host cell membrane and formation of the parasitophorous vacuole (Heise *et al.*, 1999). The parasitized cell finally ruptures and releases the slender merozoites which are mobile and invade neighboring mucosal cells where they initiate the development of the much smaller second generation micromeronts in large intestine. Behrendt *et al. (*2000) explained, that in mouse model, that infected cells respond less to ATP in terms of intracellular Ca^{2+} and this could help the parasite to invade neighboring cells thus initiating the next step of the life cycle

Giardia: Giardia attaches intimately to the intestinal epithelium and to a variety of other inert substrates such as glass and plastic. The ventral disc is thought to be the primary organelle of attachment. Hydrodynamic forces generated beneath the disc by continuous activity of the ventral flagella have been proposed as a possible attachment mechanism (Holberton, 1974). It has also been suggested that flagella activity causes low pressure under the ventral disc due to fluid fluxes around the ventral and marginal grooves. However, attachment occurs with the morphologically distinct *G. psittaci,* in which the ventrolateral flange is incomplete; thus, it is unlikely that hydrodynamic forces can be the sole explanation for disc-mediated attachment (Meyer, 1990). The presence of contractile

proteins in the peripheral regions of the ventral disc suggests that they participate in attachment (Feely *et al.,* 1992). Inhibitors of microfilament function, such as cytachalasin-B and low calcium concentrations, inhibit attachment-again supporting a role for cytoskeletal proteins in attachment (Erlandsen and Feely, 1984; Feely *et al.,* 1992).

Giardia is usually found in close proximity to the apical surface of the enterocyte, often-penetrating deep into the intestinal crypts. Giardia is not an invasive organism, although there have been reports of trophozoites being found within the mucosa (Farthing,1989).The disease is now considered multifactorial, including apoptosis of enterocytes, loss of epithelial-barrier function, hyper secretion of chloride ions, inhibition of brush-boarder enzymes, and malabsorption of glucose, water, and sodium ions (Ankarklev *et al.,* 2010).

Several studies in animal models suggest that there are functional consequences of the structural abnormalities and the reduction in disaccharidase activity. In gerbils, basal transport of sodium and chloride ions was not different in non infected controls, but glucose-stimulated sodium absorption was significantly reduced in the jejunum but not in the ileum of infected gerbils in experiments using stripped small intestinal mucosa mounted in using chambers (Buret *et al*., 1992). Perfusion studies *in vivo* in animals have also shown impaired water, sodium, and chloride absorption in response to glucose, although basal transport was similar to that in controls (Buret *et al.,* 1992 and Cevallos *et al.,* 1995). In a neonatal rat model of infection, basal transport of water, sodium, and chloride ions was impaired with some animals actually in a net secretory state for sodium and chloride ions (Cevallos *et al.,* 1995). Perfusion of a lactose-containing solution enhanced these transport abnormalities. Studies with brush border membrane vesicles from infected mice provided further evidence that there is impairment of glucose and amino acid transport (Samra *et al.,* 1988).

Giardia trophozoites attach to the epithelium and have been shown by electron microscopy to disrupt and distort mcrovilli at the site where the ventral disc interfaces with the microvillus membrane (Samra *et al.,* 1988). It is possible therefore, that physical factors involved in this cell-cell interaction might account for microvillus damage at sites of adherence. There is some evidence to suggest that Giardia itself produces, and possibly release, cytopathic substances into the intestinal lumen (Samra *et al.,* 1988). Yet no parasite product has been identified to account for the morphological damage observed in the small intestine. Giardia does however, contain a number of proteinases, which might attack surface glycoproteins and disrupt microvillus membrane integrity (Parenti, 1989). In addition, the surface mannose-binding lectin of Giardia may contribute to epithelial injury (Inge *et al.,* 1988). Dietary plant lectins can directly damage intestinal epithelial cells and produce microvillus membrane abnormalities very similar to those seen in giardiasis (Lorenzsonn *et al.,* 1982; Dobbins *et al.,* 1986). It remains to be established whether any of these parasite products are injurious to the host enterocytes.

Rotaviruses: Rotaviruses cause disease that varies in severity. The viruses can be carried by an asymptomatic animal, can cause mild, self-limiting diarrhea, or can cause severe diarrhea with excessive fluid loss and severe electrolyte imbalances. Rotaviruses are transmitted by the fecal-oral route and selectively infect the mature villous absorptive epithelial cells (Theil *et al.,* 1978). Replication takes place within the cytoplasm of the mature enterocyte crypt cells are spared.

Infection results in the loss of mature epithelial cells, which are replaced by immature undifferentiated cells deficient in disaccharides and sodium-potassium ATPase activities (Argenzio, 1985). Absorptive capacity is much reduced, as indicated by D-xylose absorption studies, and morphologic changes are characterized by desquamation of infected cells followed by villous atrophy (Woode and Crouch. 1978). The atrophic villi become covered with immature squamous to cuboidal epithelial cells that have migrated from the crypts. The loss of digestive and absorptive functions leads to nutrient maldigestion and malabsorption.

Lactose, the primary carbohydrate of milk and the primary nutrient of young animals, passes through the small intestine undigested and unabsorbed. In the large intestine, lactose is fermented by colonic bacteria to produce short-chain organic acids and gases. The short-chain organic acids lower the colonic pH, causing a shift in the colonic bacterial flora. Bacteria that produce lactic acid and survive at the lower pH become established. Concurrent with the increase in luminal lactic acid, there is an increase in the intralumenal osmotic pressure, leading to further increased secretion. These two effects contribute to ongoing metabolic acidosis and systemic dehydration. (Woode and Crouch, 1978).

Corona virus: the virus has affinity for epithelial cells the villi of the small intestine. Replication of the virus in these cells is accompanied by loss of epithelial and blunting of the villi, which results in failure of digestion and absorption of nutrients. In the colon, surface epithelial cells are also attacked with loss of surface cells and cystic dilation and accumulation of cellular debris in underling crypts (Mason and caldow, 2005).

2.1.3. Diagnosis of calf diarrhea

An etiologic diagnosis is useful in selecting specific diagnostic and preventative regimens for bacterial infections. Establishing an etiologic diagnosis for bacterial infections may be more important now that there is some indication that effective vaccines are being developed. Diagnosis of salmonellosis and shiga toxigenic *E. coli* can have public health implications. Once an agent has been identified, one of the major problems is in interpretation whether it is responsible for diarrhea in the individual or herd, because most agents can also been found in healthy calves. Identification of one agent in pathologic material does not preclude the possibility that other agents are also contributing to the condition (Naylor, 2002). Culture techniques are designed to promote the growth and identify particular bacteria, while restricting the growth of the other bacteria in the sample. Often fecal specimens are cultured on selective media to identify organisms that cause diarrhea, while preventing growth of non-pathogenic bacteria. Once

a pathogenic organism has been isolated, it can be further characterized by its morphology, growth patterns such as (aerobic or anaerobic growth, patterns of hemolysis) and staining.

Many scours, regardless of cause, show similar clinical picture. However, the severity and character of the scour and the age of the affected calves can all help to make a professional judgment for the cause. Frequently, examination of faces samples from a group of calves can identify the organisms present in an outbreak. Occasionally however, routine tests fail to identify any specific organism and further examinations are required to make an accurate diagnosis, for example, history of predisposing causes, isolation of the agents , postmortem examination (Quinn *et al.*, 2002) and the use of PCR to test for enterotoxigenic genes (Ahmed *et al.*, 2007).

In diagnosing *E. coli* as a cause of diarrhea, demonstration of fimbrial antigens (K99 in case of or the enterotoxins is necessary (Quinn *et al.*, 1994). Fimbrial antigens can be detected by immunological tests like latex agglutination test or ELISA either directly from fecal samples or from culture of *E. coli* in special media that support expression of fimbrial antigens. Fluorescent antibody technique using conjugates prepared against colonizing antigens can be used on smears made from scraping from the ileum of fresh carcass. The most sensitive of the methods being developed for heat labile (LT) and heat stable (ST) toxins is Enzyme Linked Immune Sorbent Assay, which employs monoclonal antibodies. Cultural isolation of the organism from feces sample is the usual method of diagnosing of Salmonella from diarrheic calves. This involves inoculation of feces to selective enrichment broths. After overnight incubation in selective enrichment, it is transferred to selective plating media. Colonies showing characteristics of Salmonella are then being tested biochemically and seroptyped (Quinn *et al.*, 1994). Diagnosis of Cryptosporidium is usually done with acid-fast stain of fecal flotation smears. Commercially available ELISA tests have been also developed that have higher sensitivity and specificity than stained fecal floatation smears (Hendrix, 1998).

In the case of haemorrhagic feces containing tissue and fibrin strands, coccidiosis due to *E. bovis* and *E. zuernii* should always be considered. Moderate infection or infection with other Eimeria species may induce subclinical coccidiosis or transient non-haemorrhagic diarrhoea which is clinically rather unspecific and often attributed to other pathogens or totally ignored. Oocysts are easily found in the faces with light microscopy, preferably after concentration with conventional flotation techniques. The sensitivity of coproscopical methods is reduced in diarrheic faces due to dilution (Lentze *et al.,* 1999), and particularly in severe *E. bovis* or *E. zuernii* infections when large amounts of blood, tissue or mucus are shed. Examination of fecal samples from several animals to obtain a true estimate of the presence of coccidiosis within a group of calves is recommended (Joachim, 2002). If one or more oocyst shedding animals are identified all calves in the respective group are considered to be at increased risk.

Serological methods (ELISA and Western blot) have been developed for detection of *E. bovis* infection in calves but are subject to several drawbacks. In young calves fed colostrums, maternal antibodies that are not related to current infection may be detected in the serum (Fiege *et al.,* 1992; Faber *et al*., 2002). Although not suited for routine diagnosis serological methods are useful for epidemiological and experimental studies (Fiege *et al.,* 1992).

2.1.4. Predisposing factors for calf diarrhea

2.1.4.1. Calf factor

Factors related to birth: Several factors affect the health and vigor of calves immediately after birth. Calves born from dams with inadequate nutrition at late pregnancy or affected with prolonged anorexia, fever, or septicemia may be weak. Dystocia or prolonged parturition affects the calf's survival (Sivula *et al.,* 1996b). Research findings have indicated that metabolic, respiratory and mixed acidosis develop frequently at birth in calves, because of prolonged or difficult labor and dystocia. This

acidosis at birth may have detrimental effect on colostral immunoglobulin absorption (Besser *et al.,* 1990; Drewry *et al.,* 1999). Bendali *et al.* (1999) also found dystocia associated with risk of neonatal diarrhea.

Age of the calf: The age of the calf is the most important factor affecting morbidity and mortality. In a three-month study on Swedish dairy farms, 70% of the mortality occurred in the first month of the calves' age and the first week of age is with higher risk of dying than any other time there after (Waltner-Toews *et al.,* 1986; Olsson *et al.,* 1993). Similarly, Virtala *et al.* (1996b) in their 3 months study reported the peak occurrence of crude mortality and calf diarrhea at the second week of life. In a study on smallholder dairying in Debre Zeit 15% of the mortality rate was reported in the first month as compared to 8% mortality rate in 1 to 3 month of life (Gryseels and de Boodet, 1986). What all these studies showed was that young age is the critical age for calves and producers need special attention for young calves.

2.1.4.2. Environmental and managemental factors

Feed factors: Feed and feeding methods are important risk factors in morbidity and mortality of dairy calves. Feeding starts with colostrum soon after birth and involves feeding liquid feeds to preweaned calves and solid feeds to weaned calves (Bath *et al.,* 1985). Ingestion and absorption of enough quantity and quality of colostrum is a critical determinant for the health and survival of neonatal calves. Calves which do not received adequate colostrum are shown to have higher overall death rate are more likely to develop scouring and even at two to three months old (Blowey 1990). Wittum *et al.* (1994) found that calves with inadequate blood colostral immunoglobulin concentration in 24 hours of birth were at greater risk of neonatal calf diarrhea and mortality.

Time between birth and the first feeding is the prime factor for the failure of passive transfer of colostral immunity. Studies showed that calf mortality is significantly higher in calves that got colostrum late after birth than those that got colostrum soon after birth

(Bruning-Fann and Kaneene, 1992 and Wells *et al.,* 1996). In one study, it was found that each hour of delay within the range of 1 to 12 hours after birth increased the risk of illness by 10% (Olson *et al.,* 1993).

Pre-weaned calves in modern dairy herds are fed with milk or milk replacer. The quality of feed and the method of feeding require great care. The feeding method and time of feeding for pre-weaned calves that fail to insure the closure of esophageal groove, leads the feed to rumen and causes digestive upset (Blowey, 1990). Method of feeding was also associated with some contagious diseases (Wilson *et al.,* 1998). A transition from liquid pre-weaned feed to solid weaned calf feed is also a critical time in feeding calves. If this is not done carefully, the calf will get dietary stress and be susceptible for different diseases (Blowey, 1990; Cry *et al.,* 1998).

Housing: On most dairy farms, calves are taken from the maternity area soon after birth and placed in the calf-rearing barn. The rate of calf diarrhea is usually higher in calves housed indoors than outdoors. The increased illness and mortality in calves that are reared indoors is often attributed to a combination of inadequate control of thermal environment, poor air quality, undesirable relative humidity, inadequate exchange of air and poor sanitation (Blowey, 1990). Another study by Olsson *et al.* (1993) also indicated that keeping calves in single pen decreased incidence of enteritis as compared to group pens.

Herd size: Larger herd size was associated with an increased incidence of calf diarrhea. Cows in larger herds may be more densely housed, which could promote the spread of disease (Waltner-Toews *et al.,* 1986). A large number of cows in the maternity stall area would decrease the infectious disease control and can increase the incidence of diarrhea in neonatal calvesbecause of an increased number of enteropathogens in the environment (Frank and Kaneene, 1993).

A marked increase in population density commonly results in an increase in the incidence of infectious diseases. Different studies reported significantly lower calf mortality in dairies having small herds than large or medium herd size (Bruning-Fann and Kaneene, 1992).Garber *et al*. (1994) found higher prevalence of *Cryptosporidium* associated with larger herd size in dairy farms. Herd size by itself has not a biological effect on the calf health; rather, it may be a measurement of other factors like time available to observe and care for calves. Other possible reason for the apparent association between herd size and calf mortality could be that in case of small herd sizes enough time may elapse between successive births, which will reduce the concentration of infectious agents in the calf-rearing environment.

Other factors: Other environmental and managemental risk factors suspected to affect calf morbidity and mortality include: dam preventive practices by vaccination, the sanitation of calving area, perinatal care, grazing level, level of herd production, practice of prophylactic antibiotics, weaning age, separation or mixing of calves etc. (Lance *et al.,* 1992; Olson *et al.,* 19

2.1.5. Economic Importance of calf diarrhea

Calf diarrhea is the commonest disease in young calves and is the greatest single cause of death (Gitau *et al*., 1994; Sivula *et al*., 1996; Busato *et al*., 1997; Heinrichs and Radostits, 2001). The incidence of diarrhoea in calves under 30 days of age varies between 10 % and 20 % (Sivula *et al.,* 1996a; Bendali *et al.,* 1999a and Svensson *et al.,* 2003). It accounts for approximately 75% of the mortality of dairy calves under three weeks of age (Blowey, 1990).

In a survey of dairy calf morbidity and mortality in Holstein dairy herds, 20% of live-born calves were treated for diarrhea (Waltner- Toews *et al.,* 1986). In one study in Swedish dairy herds, a morbidity rate of 7% was found. In this study, enteritis occurred with frequency of 7.2% (Olson *et al.,* 1993). A major survey of beef producers in the

USA in 1997 found that 80% of calf morbidity from birth to 21 day is due to diarrhea (National Animal Health Monitoring System, 1997). Economic losses associated with an outbreak can be substantial and far-reaching. In addition to the costs associated with the additional labor, drug expenditures and calf death, there is the potential economic impact associated with the poor long-term performance of affected calves. Adjusted weaning weights have been shown to decrease by up to 15.9 kg per affected calf due to calf morbidity (Wittum, 1994). In Holleta dairy farm in Ethiopia, among the crosses and Boran breeds, a 6-month cumulative incidence of 38% was recorded for calf scour (Shiferaw *et al.*, 2002).

Calf diarrhea is of great economic importance to all dairy producers. Calfhood morbidity cause direct cost for treatment and nursing, affects days at first calving, affects dairy herd survivorship and future productivity (Waltner-Toews *et al.,* 1986 and Curtis *et al.,* 1989). In Waltner- Toews *et al.* (1986) study, heifers that have been treated for diarrhea during the first 3 months of life were 2.5 times more likely to die after 90 days of age than other heifers and heifers that had been treated for diarrhea were 2.9 times more likely to calve after 90 days of age than other heifers. Correa *et al.* (1988) on their study in Holstein heifer calves on several New York dairy farms observed that heifers without diarrheic illness during calf hood life were twice as likely to calve 6 months earlier compared with those that experienced diarrhea during calf hood.

Calf death also causes a loss of genetic material for herd improvement and decreases the number of dairy heifers available for herd replacement and expansion. Economic losses resulting from calf hood mortality and morbidity can be easily recognized, but the effect of morbidity on future health and performance, which may constitute a loss of even greater importance, is difficult to estimate. A dairy farm management system should employ a strategy that will reduce calf mortality and improve calf performance by controlling diseases. In a good management practices, annual mortality of calves under one months of age can be reduced to below 3-5% and first calving age at around 24 months (Heinrichs and Radostits, 2001).

2.1.6. Zoonotic importance of enteropathogns involved in calf diarrhea

Increasingly, food animals and their products are being identified as important sources of infectious pathogens for humans. Many of the organisms responsible for calf diarrhea are also important zoonotic pathogens. Numerous infectious pathogens capable of causing diarrhea among food animals have been associated with food borne disease and zoonoses in humans (enterohaemorrhagic *E. coli*, Rotavirus, *Cryptosporidium* species *Giardia* species, *Campylobacter* species and *Salmonella* species (Radostits, 1994 and Trevejo *et al.,* 2005). Producers and veterinarians managing an outbreak of calf diarrhea often overlook the potential for zoonotic transmission.

Many studies also showed that both healthy and diarrheic calves harbor STEC in their intestine (Roopnarine *et al.,* 2007) and shed the bacteria for several months and in great quantities (Widirasih *et al.,* 2004). Ruminants, especially cattle, are known to be the most important reservoir of STEC carrying them in their gastrointestinal tract (Osek *et al.,* 2000; Chinen *et al.,* 2003 and Irino *et al.,* 2005). Sporadic cases or large STEC outbreaks in humans are associated with the consumption of raw or undercooked meat of food animals and other foods contaminated by animal feces and by contact with STEC-positive animals or with their environment (Paton and Paton, 1998b). Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7 and they can carry it asymptomatically and shed it in their feces (Bach *et al.,* 2002). Food products associated with *E. coli* outbreaks include raw ground beef and raw seed sprouts or spinach (Sabin, 2006) raw milk, unpasteurized juice, unpasteurized cheese and foods contaminated by infected food workers via fecal-oral route.

2.1.7. Prevention and control of calf diarrhea

Multiple factors, both infectious and non-infectious, are involved in calf diarrhea outbreaks, which make disease control on farms difficult (Svensson *et al.,* 2003 and Trotz-Williams *et al.,* 2007). Different management and environmental factors have also

been associated with the disease. Therefore, timely prevention and control of calf diarrhea is important to reduce economic losses to producers and improve animal welfare (McGuirk, 2008). Dealing with such a large number of potential etiological agents as well as various management factors is an ongoing challenge for effective control of enteric disease in newborn calves (Booker *et al.,* 2008 and Uhde *et al.,* 2008). Therefore, a thorough investigation should include a study of dry cow management and calving practice as well as calf management (Bazely, 2003 and Andrews, 2004).

Studies of dairy herds have indicated that improved environmental management of calving pens and calf housing reduces calf diarrhea incidence and the extent of outbreaks. A system of "all in all out" calf housing, cleaning, steaming and disinfection of calf housing and calving pens, regular disinfection of utensils and adequate straw were identified as important management factors. In a study, calf diarrhea incidence was reduced from 36% to 11% within a year by the introduction of early colostrum feeding and improved housing hygiene (Lance *et al.*, 1992). Other factors mentioned are calf birth assistance due to dystocia, stress due to dystocia, many calves on a small surface and introduction of new cows to the herd (Bendali *et al.*, 1999b).

The passive immunity acquired from the colostrum and absorbed into the circulation from the gut is the calf's main defense mechanism against *E. coli* diarrhea. Inadequate amounts of immunoglobulins in the colostrum, inadequate intake of the colostrum and inadequate absorption of immunoglobulins from the gut render very young calves susceptible to infection (Groutides and Michell, 1990). Additionally, among calves aged 1-4 months old, carriage of VTEC *E. coli* O157 was reduced if the calf had suckled colostrum from the mother or if the calf had stayed more than 2 days with the mother after calving (Rugbjerg *et al.*, 2003).

In case of continuing problems with coccidiosis the management of the herd should be critically assessed, particularly with respect to hygiene, feeding, aeration, animal density, floor type, etc. Installation of slatted floors that allow less accumulation of feces in the

pens significantly reduces coccidian related health problems (Grafner *et al.*, 1985b). It has been doubted that improved barn hygiene is able to efficiently control coccidiosis at all (Fox, 1985). However, good hygiene is a valuable aid to reduce infection pressure (Hiepe *et al.*, 1978 and Joachim, 2002). Halofuginone is an anticoccidial that has been widely used in poultry production for control of Eimeria infections. In cattle, the only indication for use of halofuginone is cryptosporidiosis of suckling calves (Joachim *et al.*, 2003).

In case of diarrhea outbreak in a herd, it is important to attempt to diagnose the infective cause of the disease in order to target further control measures appropriately. Isolation of affected calves, effective treatment with rehydration solutions and provision of dry and warm conditions are vital in the treatment of calf diarrhea. The main aim of treatment is to restore the fluid balance in the animal by rehydration therapy. Oral therapy is the best way of providing rehydration fluids, but this may need to be replaced by intravenous therapy in severe cases where the calf is unable to drink. A return to whole milk feeding is recommended within two days of rehydration therapy to avoid a negative energy balance (Grove-White, 2004). Suckled calves should have limited access to the dam or suckler cow until full recovery has been achieved. During this period, they should receive regular rehydration therapy.

The use of antibiotics in diarrheic calves has been shown to be contraindicated in many studies, due to the further disruption of gut flora, the establishment of carrier states of salmonella infection and the development of antimicrobial resistance factors in the enteric flora. Although, very sick calves with Salmonellosis may benefit from antimicrobial therapy, most studies have also failed to show any beneficial effect of antimicrobial treatment (Rollin *et al.*, 1986). Some studies show-limited efficacy in reducing mortality and morbidity in an outbreak of diarrhea (Holck *et al.*, 1994).

Antibiotics should only be used for *E. coli* and Salmonella infection, after sensitivity test to choose the best drug, as inappropriate use of antibiotics can lead to serious antibiotic

resistance problems. Ciprofloxacin and probiotics such as *L. acidophilus* isolated from colostrum of goat and mares are highly effective in treatment of infection thus control of calf scour is based on feeding plenty of colostrum immediately after birth (Abd El-Moez *et al.,* 2010). Ciprofloxacin coated with gold nanoparticles showed high hindrance in vitro for the growth of *E. coli* and *S .typhimurium* (Zawrah and Abd El-Moez, 2011). Vaccination is very important in the control of calf scour, vaccines are to protect against *E. coli* and rotavirus. However, it is unlikely to be effective unless used in conjunction with good husbandry (Hirsh and Zee, 1999).

2.1.8. The status of calf diarrhea in Ethiopia

Limited works have been done on the incidence and causative agent of calf diarrhea in some parts of Ethiopia (Abraham *et al*, 1992; Simachew, 1998; Taddesse, 2004; Temsegen, 2008; Demisse 2004 and Yenhewoit, 2008). They reported variable incidence rate and causative agents of calf diarrhea in their respective study site and managemental conditions.

There are very few studies done to identify specific agents involved in disease syndromes such as the ones mentioned above. Abraham *et al.* (1992) tried to identify specific infectious agents associated with neonatal diarrhea in Ethiopian dairy calves. They found *bovine enteric coronavirus*, group A rotavirus and K99 Enterotoxogenic *E. coli* independently or in combination in diarrheic calves. Bovine enteric Corona virus was the most frequently detected pathogen followed by rotavirus. Salmonella was detected in diarrheic calves and was responsible for the death of calves in different parts of the country (Pergram *et al.*, 1981; Hussien, 1998 and Simachew, 1998) have also isolated *E. coli* from diarrheic calves, but this did tell little about the significance of the isolated bacteria to the causation of the disease. This is because most *E. coli* strains are normal flora of gastrointestinal tract of mammals and the strain causing with ability of causing disease should be identified before incriminating them as the causes.

Calf diarrhea was found to be the predominant calf health problem with incidence rate of 42.9% followed by pneumonia (4.9%). Diarrhea was also the leading cause of mortality in the study herds in Ethiopia (Hussein, 1998; Lemma *et al.*, 2001 and Temsegen *et al.*, 2008). According to Alemayehu *et al.,* (2009) the overall crude calf morbidity and crude mortality rates were 29.3% and 9.3%, respectively. The most frequent disease syndrome was diarrhea with incidence rates of 10% followed by septicemia (6.4%) and gastro-intestinal tract (GIT) disorder (5.4%) in smallholder dairy farms in Hawassa.

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted on dairy farms found in Mukaturi, Debre tsige and Fitche town in North Shewa Zone. Fitche**:** is located in North Shewa Zone of the Oromia National Regional State. North Shewa Zone is found in North-West direction of Addis Ababa. Fitche town, which is located at 147km away from Addis Ababa, is the capital of the zone. The zone has 13 rural districts with a total land area of 10,323 km^2. It is situated between $9^0$30'N and $38^0$40'E. The zone is bordered by Amhara Region in the North and the East, West Shewa Zone of Oromia Region in the West and Addis Ababa in the South. The altitude of the area ranges between 1300-2500 meters above sea level. It is divided into three agro-ecologies, namely, (15%) highland (>2500 meter above sea level), (40%) midland (1500-2500 meter above sea level) and (45%) lowland (500 -1500meter above sea level) and the area gets rainfall during both Belg (February to April) and Meher (June to September) seasons. The average annual rainfall of the area ranges from less than 840 mm to 1600 mm while the mean annual temperature varies between 15^0C and 19^0C (CSA, 2007). There have been 3.3 million cattle in North Shewa Zone; where cattle take the larger proportion (53%) followed by Shoat and Equines (25.56%) and (15.91) respectively. Livestock production also constitutes an important part of agricultural activities of the zone (RADD, 2010).

Mukie turi: is a town and the seat of the administration. Located at78 km North-West of Addis Ababa, and has three 3 agro climatic zones: temperate, subtropical and tropical each sharing 87%, 11%, and 2% of the total area. The altitude ranges from 1000- 3000 m.a.s.l. (NSDAD, 2010). It receives an annual mean rainfall of 1028 mm. the mean minimum and maximum temperatures of North Shewa Zone are 11.23^0C and 20.86^0C respectively. The climate of the area is favorable for crop and livestock production. The area is the main milk shed for Addis Ababa.

Debre tesige: is a town located in North Shewa Zone of Oromia, Ethiopia. In Debre-Libanos district is located 9^{o} 48'N and 38^{o} 44'E at about 89 km North of Addis Ababa. The altitude of the district is between 1500-2700 meter above sea level, its minimum and maximum temperature vary from 19^{o}C to 23^{o}C.It gets bimodal rain fall that ranges from 800-1200 mm and the predominant soil type are black soil (56%) and red soil (38%).Two agro ecologies are found in the area and mixed agricultural activities are performed. There are about 80,796 head of cattle 84,507, goats 23,723, sheep 10,899, equines 1,894, camels and 75,305 poultry in the districts. Mainly smallholder farmers under intensive, semi intensive and extensive production system rear all of these livestock species. The district is divided into 11 administrative Pas and 13000 liters of milk is collected from Debre-tsighe town only (Debre-Libanos district Agricultural and Rural development office, 2012).

3.2. Study Animals

Animals that were included in this study were exotic and cross breeds of dairy calves of both sex up to 4 months of age that were clinically affected with diarrhea and exhibiting signs of systemic disease (e.g. poor appetite, fever, sunken eye, dehydration, and reduced suckle reflex), and defecate pasty–watery feces. All diarrheic calves in dairy farms of Muke turi, Debre tisege and Fitche towns and its surroundings were incorporated in the study for a period of six months from November 2013 to end of April 2014. In the study area there were few relatively large dairy farms, with milking herd size of greater than 30, and a lot of market oriented smallholder (MOSH) dairy farms, with herd size of around two cows. During the study a total of 56 dairy farms, composed of three large and 53 smallholder dairy farms, were included. The majority of MOSH dairy farms were organized under dairy cooperatives in their respective localities. Both large and MOSH dairy farms kept exotic Holstein and crosses of Holstein breed of calves. Ages of diarrheic calves were categorized into three age groups: 1-4 weeks age, 5-8 weeks age and greater than 9 weeks, according to Lorino *et al.* (2005) and Lee *et al.* (2007).

3.3. Study design and sampling methodology

Cross sectional purposive type of study was conducted in dairy farms of, Mukaturi, Debre tisege and Fitche towns of North Shewa Zone. Selection of farms was done purposively based on availability of clinical case (diarrheic calves) in the farm and willingness of the owners. The health status of each calf was evaluated by clinical examination. Calves free from diarrhea were classified as healthy whereas sick calves shown abnormal fecal consistency and/or signs of dehydration, sunken eye, diarrhea and weakness. In addition, Farm management practices were evaluated, including age, sex, farm size, animal housing, cleaning and disinfection, feeding of the calves, colostrum feeding etc. Description of fecal amount, type, consistency, color, smell and mixtures (presence of blood or particles of undigested food, blood clots or pieces of intestinal tissue) were recorded. All relevant information about farm management conditions were collected during the time of sample collection. Questionnaire survey was also conducted on owners during the study period to assess the farm management system. (Appedix1)

3.3.1. Sample size determination

Non-probability purposive sampling was used for the selection of farms and calf samples. The sample size was determined based and availability of clinical case (diarrheic calves) and on willingness of the owners, in the farm. One hundred five diarrheic calf samples were used in this study.

3.3.2. Sample collection procedure

Fecal samples were collected directly from rectum of none treated diarrheic calves preferably soon after onset of diarrhea by using sterile wide mouth screw caped bottles. Fecal specimens were collected from all diarrheic calves aged from newborn to four month of age present on the farm at the time of the visit and on emergency calls from the farm owner. Enough amount of fecal samples were collected in sterile wide mouth screw

caped bottles samples were cooled on ice packs and transported to Microbiology Laboratory, Addis Ababa University, College of Veterinary Medicine and Agriculture in Bishoftu (AAU, CVMA). The samples were processed as soon as possible after collection. Collected samples were clearly labeled including the information on the date of sampling, the age, sex, breed and tag number of the calves (Appendix 2).

3.4. Identification of infectious agents associated with calf diarrhea

3.4.1. Isolation and identification of E. coli

Isolation of *E. coli* was conducted following standard procedures described in Quinn *et al.* (2002). Fecal samples were inoculated on to MacConkey agar medium, which selectively grows members of the *Enterobacteriaceae* and permit differentiation of enteric bacteria, and incubated at 37° C overnight. Colonies showing characteristic lactose fermenting (having pink colonies) were then picked up and sub cultured on to nutrient agar. The selected colonies were inoculated further on to Eosin methyl blue (EMB) agar to see the metallic sheen characteristics. From each MacConkey agar plate, all lactose-fermenting colonies were stored temporarily as nutrient broth cultures for further identification by biochemical tests. All the isolates were stained by Gram stain to determine the cell morphology, Gram reaction and purity of the isolates under the oil immersion objective (x100 magnification).Then, *E. coli* isolates were identified preliminarily by using indole, methyl red, and Voges-Proskauer and citrate utilization (IMViC) biochemical tests. *E. coli* isolates were presumptively identified (indole positive, methyl red positive, Voges-Proskauer negative and citrate negative (Quinn *et al.*, 2002) (Appendix 3).

3.4.2. Biotyping of E. coli isolates

The identified *E. coli* isolates were then further characterized for their sugar fermentation reactions on nine sugars viz., dulcitol, raffinose, rhamnose, salicin, sucrose, inositol,

lactose, maltose and xylose according to Edwards and Ewing (1972). The test isolates grown in phenol red broth were inoculated in to 1% of each sugar medium. Tubes were incubated at 37°C for three days and readings were then recorded after every 24 hours. Production of yellow color or acid production was considered as positive reaction and proper controls was kept for each of the sugar tests performed. Isolates showing similar fermentation reaction patterns on the nine sugars were considered as belonging to one biotype the result of each sugar were recorded (Appendix .6) and designation of biotype was done according to Pandey *et al.* (1979) and Ashenafi (2013).

3.4.3. Isolation and identification of salmonella species

Salmonella species were isolated and identified according to the techniques recommended by the International Organization for Standardization (ISO, 2002). (Microbiology of food and animal feeding stuffs horizontal method for the detection of Salmonella species) Accordingly, the detection of salmonella in fecal samples was followed four stages:

3.4.3.1. Non-Selective Pre-Enrichment

Buffered peptone water (BPW) was used at ambient temperature with the test portion of about 25ml of fecal sample which measured using a sterile measuring cylinder each sample was put into sterile universal bottle, and 225 ml of BPW (HiMedia, India) was added and mixed thoroughly using vortex mixer. In most cases, fecal samples smaller than 25ml were pre-enriched in BPW in a ratio of 1ml of the sample to 9 ml of BPW and incubated at 37°C for 16-18 hrs (ISO, 2002).

3.4.3.2. Selective Enrichment:

The pre-enrichment broth after incubation was mixed and 0.1 ml of the broth was transferred aseptically into a tube containing 10 ml of Rappaport-Vassiliadis medium

(RV broth). Another 1 ml of the pre-enrichment broth was transferred in to a tube containing 10 ml of Muller-Kauffmann tetrathionate broth (MKTT broth) (Oxoid, UK The inoculated RV broth was incubated at 41.5 °C ±1°C for 24 ± 3 hours and the inoculated MKTT broth was incubated at 37 °C±1 °C for 24 ± 3 hours (ISO, 2002).

3.4.3.3. Selective Plating out and Identification:

From the RV broth and MKTT broth Cultures, two selective solid media namely Xylose lysine desoxycholate (XLD) agar (Titan Biotech Ltd., Bhiwadi, India) and Brilliant green agar (Titan Biotech Ltd., Bhiwadi, India) plates were used for plating out and identification purpose. A loop-full of inoculums from the RV broth and MKTT was transferred aseptically and streaked separately onto the surface of xylose lysine deoxycholate agar (XLD agar) and Brilliant green agar separately. The plates were incubated at 37°C ± 1°C for 24 ± 3 hours. After proper incubation, the plates were examined for the presence of suspected Salmonella colonies, which appear pink with a darker pink center on XLD agar and red to pink color on Brilliant green agar.

3.4.3.4. Confirmation of Identity

Suspicious colonies were further sub cultured in nutrient media (Oxoid, England) for biochemical tests. Confirmation was done by using biochemical test according to ISO, 6579 (2002). Briefly, the suspected pure colonies from nutrient agar were inoculated on TSI slant (Oxoid, England), citrate slant (Oxoid, England), lysine iron agar (Oxod, England), urea broths (HiMedia, India), Voges proskauer and Indole tests. Isolates producing Red (alkaline) slant, yellow (acid) butt, H_2S positive/negative in TSI and lysine positive and unease negative, Voges proskauer positive and Indole negative results were identified as Salmonella (Appendix 4).

3.4.4. Detection of Cryptosporidium oocyst

Fecal samples were also examined for the presence of Cryptosporidium. This was done by modified Zeihl-Nelson staining technique as described by Kaufmann (1996). Thin smears were prepared directly from fecal samples, air-dried, and passed quickly over flame. Then the slides were fixed and stained with 0.5% of carbon fuchsine for two minutes. The slides were then washed with tap water and decolorized with acid alcohol (3ml HCl and 70% ethanol) for 1-3 minutes. Then, the slides were counter stained in methylene blue for another 1 minute, washed in tap water and allowed to dry on air. The slides were then observed under microscope for the presence of bright red granules (oocysts) on blue background. Identification of oocysts from smears was made by comparing with the slide photograph in Kaufmann (1996). Each slide was observed for 10 minutes to decide whether it is negative or positive and a smear was considered positive when one or more oocysts were observed. (Appendix 5)

3.4.5. Detection of Eimeria

Fecal samples were examined for the presence of *Eimeria* oocyst by flotation techniques according to Hendrix (1998). About three-gram portion of fecal samples were weighed out using a balance and put in a 50 ml beaker. Water (42 ml) was added, mixed thoroughly and poured in to a 100-ml glass beaker through a strainer. The 50-ml glass beaker was rinsed with 8 ml of water and the total fluid was poured in to four 15-ml conical tip centrifuge tubes. After centrifugation at 1,500 rpm for 5 min, the supernatant was decanted; then, concentrated sucrose solution (Sheather's sucrose solution) (specific gravity 1.27) was added to the sediment, until the tube is about half full. The content of each test tube was thoroughly mixed with a wooden applicator stick. With the aid of a conical flux, more sugar solution was added until a convex meniscus was formed on top of the tube. A glass cover slip was placed on top of each tube and was left for 30 min. Then, the glass cover slip was briskly lifted up and placed on a clean glass slide, not allowing formation of air bubbles. The entire area under each cover slip was examined under a binocular microscope at 40× magnification according to Hendrix (1998).

3.4.6. Detection of Giardia cyst

Fecal samples were also examined for the presence of Giardia. This was done by zinc sulfate centrifugal flotation technique as described by Dryden *et al.* (2006). About 3 g of faces were mixed with 15 ml of water. The mixture was then be stirred together with a tongue depressor and passed through a tea strainer or piece of. The solution was transferred into a 15-ml test tube and centrifuged for 10 min at 650 rpm and lugols iodine was in a 33% zinc sulfate solution (sp.gr 1.18).The supernatant was discarded and the pellet was resuspended in zinc sulfate (sp.g. 1.18) and the tubes were recentrifuged for 10 min. Then the test-tubes were filled with zinc sulfate until meniscus was formed, and a cover slip was placed on top making sure that it contacts the fluid in the tube. After waiting for 10 min the cover slip was placed onto a slide and the entire sample was scanned at a magnification of 40X. Cysts were detected in the specimen with the correct morphology by observing smooth oval wall, internal structure, size and shape under the microscope. A smear was considered positive when one or more cysts were observed.

3.5. Antimicrobial susceptibility testing for *E. coli* and Salmonella isolates

The antimicrobial susceptibility testing of *Salmonella* and *E. coli* isolates was conducted using disc diffusion method (Kirby-Bauer method) on Mueller-Hinton agar (oxoid, England) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009). All *E. coli* and Salmonella isolates were evaluated for antimicrobial susceptibility to eleven antimicrobial agents. A McFarland 0.5 (the turbidity of the test broth was adjusted with saline until the turbidity of the test suspension equated to that of the standard) standardized suspension of the bacteria in tryptone soya broth (Oxoid, England) was prepared; a bacterial suspension incubated for 6-8 hours was swabbed over the entire surface of Mueller Hinton agar (Oxoid, England) with a sterile cotton swab. The inoculated pates were allowed to stand for 3-5 minutes to observe any excess moisture from the medium before the antimicrobial disc were applied.

A ring of discs (Oxoid, England) containing single concentrations of each antimicrobial agent was then placed onto the inoculated surface using sterile forceps, gently pressed with the point of the forceps to ensuring complete contact with the agar surface. The discs were placed no greater than 24mm (center to center) and the plates were then inverted. After 16-18 hours of incubation at 35°C ± 2°C; aerobically, clear zones produced by antimicrobial inhibition of bacterial growth were measured in mm using a measuring caliper. For the susceptibility testing, the following eleven antimicrobial drugs and concentrations were used: ampicillin (10µg), chloramphenicol (30µg), gentamycin (30µg), ciprofloxacin (10µg), amoxicillin (25µg), tetracycline (30µg), Cefoxitin (30µg), Streptomycin (10µg), Erythromycin (15µg), trimethoprim-Sulfametoxazole (25µg), kanamycin (30µg) (Oxoid Ltd, England). Finally, the findings of antibiotic resistance testing were recorded as susceptible, intermediate and resistant according to Clinical and Laboratory Standards Institute break points (CLSI, 2012) (Appendix7).

3.6. Questionnaire survey

Pre-tested questionnaires were administered to dairy farm owners or farm managers 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 bovine calf diarrhea. These include age of calf, farm size, knowledge colostrum feeding, general health care, hygiene and sanitation of farms, occurrence of calf diarrhea, disease preventive and control measures practiced in the farms (Appendix 1).

3.7. Data collection, management and analysis

Data describing the diarrhegenic conditions suggestive of enterophatogeneic infection observed on calves along with age, farm size, colostrum intake, hygiene and sex were classified, filtered, coded and entered in to Microsoft Excel® 2007. The data were then be exported to SPSS windows version 20.0 (SPSS) for appropriate statistical analysis.

The occurrence of each enteropathogen from the total diarrhegenic calves were determined by using descriptive statistics. Chi square ($\chi 2$) (Fisher's exact test for data with a frequency of less than five in one cells) were used to measure the association between the different risk factors and occurrence of enteropathognes causing calf diarrhea. Effects were reported as statistically significant if p-value is less than 5 %.($P<0.05$) and multivariable logistic regression were used to see the odds of the associated risk factors.

4. RESULTS

4.1. Description of dairy farms based on questionnaire survey and observation

A total of 43 potential risk factors were considered for analysis for the occurrence of enterophatognes 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. However, some of the potential risk factors (age, breed, sex, time of first colostrum feeding) were different among the farms and were included in the analysis. In general, in all of the cases they kept exotic Holstein Friesian (HF) (69%) and cross breed (HF and local) (36%) calves. One of the farms in the study was intensively managed with an average number of 24 calves per herd and most of the smallholder farms were with an average number of two (2) calves per herd. In all dairy farms, male calves were sold soon after birth and females were kept for replacement stock.

Only one farm had calving facility (separate calving pen). Navel treatment during birth of calves was not practiced in all of the farms. Bedding was provided for calves in all large dairy farms and in none of the smallholder dairy farms. Calves were housed in separate pens with bedding of hay and the pens were cleaned twice a day and the calves were kept in-group pens during cleaning except in smallholder farms. All of the smallholder farms kept their calves' in-group pens separating from the cows. Almost 98 % of the dairy farmers had knowledge of the advantage of colostrum feeding but 82% were feed colostrums with in less than 6 hours birth of and 23% were feed greater than six hours of birth. All study farms fed whole milk for calves two times daily by bucket feeding with the exception of few smallholder farms that allowed calves to suckle their dams. No special starter feed was used in any of the farms; rather the same feed given to cows was used for calves. These include straw, hay and concentrates. Weaning age varied from farm to farm; most (76.2%) of farms weaned calves at three months of age whereas few smallholder farms (23.8%) weaned at 4 months of age. In general, the weaning age was lower for male calves, mostly under three months.

In all large dairy farms, there were vet personnel (veterinarian and/or animal health technician) either as fully employed or part time employed to deal with health aspects of the farms. Smallholder dairy farms call private veterinary practitioners whenever their animals face health problems. From farm managers or owners that mentioned calf health problems as a problem in dairy production and the majority of them complained calf diarrhea and pneumonia as major causes of calve death in the younger age.

4.2. Major Enterophatognes associated with calf diarrhea

Laboratory examination of 105 fecal samples from diarrheic calves that occurred during the study period was conducted to isolate and identify major bacterial and protozoal enterophatognes associated with calf diarrhea. Samples were examined for *E. coli*, Salmonella, Cryptosporidium, Eimeria and Garidia by standard bacteriological, differential staining by modified Ziehl-Neelsen (modified acid fast) and fecal flotation techniques

Of the 105 diarrheic fecal samples examined *E. coli*, Salmonella, Cryptosporidium, Eimeria and Giardia were detected in 73 (69.5%), 27 (25.7%), 29 (27.6%), 40 (38.1) and 24 (22.9) samples respectively as summarized in table 1.

Table 1. Bacterial and protozoa enteropathognes associated with calf diarrhea in dairy farms of Fitche, Muke turi and Debre Tsigie.

Enterophatognes	№.animals Examined	Frequencyof isolation	Proportion (%)
E. coli	105	73	69.5
Salmonella species	105	27	25.7
Cryptosporidium	105	29	27.6
Eimeria	105	40	38.1
Giardia	105	24	22.9

It was observed that mixed infections with more than one species of enteropathognes were found in diarrheic calves. As it can be observed in table 2, enteropathogenes were detected at higher rates as single isolates than in mixed infections. The frequency of occurrence of enteropathogens as single pathogen was 22 (21.0%) for *E. coil*, 10 (9.5%) for Eimeria; whereas 3 (2.9%) isolates, for each of Salmonella, Cryptosporidium and Giarida occurred in single infection. On the other hand, 7 (6.7%) of the cases were negative for any of the enetropathognes studied (Table 2).

Table 2. Occurrence of single and mixed infections with enteropathognes in calf diarrhea at the study areas

Enterophatognes	Frequency	Proportion (%)
E. coli	22	21
Salmonella Species	3	2.9
Cryptosporidium	3	2.9
Eimeria	10	9.5
Giarda	3	2.9
Cryptosporidium, Eimeria	2	1.9
Cryptosporidium, Eimeria, *E. coli*	3	2.9
Cryptosporidium, Eimeria, *E.coli, Salmonlla* spp	2	1.9
Cryptosporidium, Eimeria, Giardia	1	1.0
Cryptosporidium, Eimeria, Giardia, *E.coli*	3	2.9
Cryptosporidium, Eimeria, Giardia, *E.coli,Salmonella* spp	1	1.0
Cryptsoporidium, *E.coli*	5	4.5
Cryptsoporidium, *E.coli, Salmonella* spp	3	2.9
Cryptsoporidium, Giardia *E.coli*	3	2.9
Cryptosporidium, *Salmonella* spp	1	1.0
Emeria, *E.coli,*	8	7.6
Emeria, *E.coli, Salmonella* spp	3	2.9
Emeria,Giardia	1	1.0
Emeria, Giardia, *E.coli,*	5	4.5
Emeria, Giardia, *E.coli, Salmonella* spp	1	1.0
Emeria, Giardia, *Salmonella* spp	1	1.0
Giardia, Salmonella spp	4	3.8
E.coli, Salmonella spp	4	3.8
Giardia, *E.coli, Salmonella* spp	3	2.9
Giardia, *Salmonella* spp	3	2.9
No infection	7	6.7
Total	105	100.0

As shown in table 3 higher rate of detection of *E. coli* with (76.7%), Eimeria (36.7) and Cryptosporidium (30.0%) were observed in Fitche dairy farms whereas, higer detection rate of salmonella (29.4%) and Giardia (47.1%) were observed in Debre tesige dairy farms.

Table 3. Enteropathogens detected and isolated from diarrheic calves in different study sites.

	Enteropathognes identified				
Study site **(N)**	*E.coli* (%)	Salmonella (%)	Cryptosporidium (%)	Eimeria (%)	Giardia (%)
Muke turi (58)	37 (63.8)	15 (25.9)	16 (27.6)	21 (36.2)	16 (27.6)
Debretsige (17)	13 (76.5)	5 (29.4)	4 (23.5)	8 (47.1)	3 (17.6)
Fiche (30)	23 (76.7)	7 (23.3)	9 (30.0)	11 (36.7)	5 (16.7)
Total (105**)**	73 (69.5)	27 (25.7)	29 (27.6)	40 (38.1)	24 (22.9)

Key: N=Number of case

In the present study age of calves and time of colostrum feeding were strongly associated with the occurrence of diarrhea due to *E. coli* infection (P=0.01) whereas, other variables are not statically significant with any of the enteropathognes causing diarrhea (P>0.05) as summarized in table 4 and 5

Table 4. Association of bacterial enteropathogens with different factors (age, breed, Sex, time of first colostrums feeding) in diarrheic calves.

			Bacterial pathogens identified					
			E. coli			Salmonella		
Variables	Categories	N	№ +Ve (%)	χ2	P-value	№ +Ve (%)	χ2	P-value
Age	1-4	62	49(79)			17(27.4)		
(weeks)	5-6	36	22(61.1)	9.38	0.01*	8(36.3)	3.54	0.88
	>9	7	2 (28.5)			2(28.5)		
	Exotic	69	47(68.1)			18(26.1)		
Breed	Cross	36	26(72.2)	0.18	0.82	9(25.0)	0.01	1.00
	Female	76	52(68.4)			21(27.6)		
Sex	Male	29	21(72.4)	0.03	0.81	6(26.7)	0.07	0.62
TCF1st	<6 hrs	82	50 (60.9)			21(25.6)		0.9
	>6hrs	23	23(100)	12.9	0.01*	6 (26.1)	0.02	

*= stastically significant, χ2= Chi Square, TCF1st= Time of first colostrum feeding, No.+Ve = number of positive calves, N= number of cases.

Table 5. Association of protozoal enteropathogens with different factors (age, breed, Sex, time of first colostrums feeding) in diarrheic calves.

		Protozoal pathogens identified								
		Cryptosporidium			Eimeria			Giardia		
Variable	N	№ +Ve (%)	χ2	P-value	№ +Ve (%)	χ2	P-value	№ +Ve (%)	χ2	P-value
Age (week)										
1-4	62	22(35.4)			26(41.9)			19(30.6)		
5-8	36	7(19.4)	5.79	0.05	11(30.5)	1.32	0.54	5 (13.8)	5.85	0.05
>9	7	0(0.0)			3 (42.8)			0 (0.0)		
Breed										
Exotic	69	18(28.1)			23(33.3)	1.93		17(24.6)		
Cross	36	11(30.5)	0.24	0.65	17(47.2)		0.20	7 (19.4)	0.36	0.63
Sex										
Female	76	17(22.3)			30(39.5)			20(26.8)		
Male	29	12(46.1)	3.79	0.51	10(34.5)	0.22	0.63	4(13.8)	1.88	0.21
TCF1st										
<6 hrs	82	23(28.0)			29(35.3)			18(21.9)		
>6hrs	23	6 (26.0)	0.34	0.85	11(47.8)	1.18	0.27	6 (26.0)	0.17	0.67

*= statically significant, № =number of cases.

Multivariable logistic regression analysis of the occurrence of enteropathogens with the four potential risk factors (age, breed sex and time of first colostrum feeding) showed that only age of the calves was significantly associated with the occurrence of calf diarrhea due to *E. coli* (adjusted OR=14.74; CI: (2.18 - 9.80) indicating age group of calves in 1-4 weeks categories are more susceptible than calves with 5-8 weeks of age by OR of 14.74. All the other factors considered were not significantly associated for the occurrence of enterophatognes (Table 6 and7).

Table 6 Multivariable logistic regression analysis of bacterial pathogens with risk factors

Varibles	Bacterial pathogen	№of animals examined	№ postive (%)	COR (95% CI)	ADOR (95% CI)
Age					
>9 week		7	2 (28)	1	1
5-8week		36	22 (61.1)	3.93(0.67-23.09)	5.85(0.88-3.32)
1-4week		62	49 (79)	9.42(1.6454.23)	14.74 (2.18-9.80)*
Breed					
Exotic		69	47(68.1)	1	1
Cross	*E.coli*	36	26(72.2)	1.22(0.50-2.96)	2.04(0.67-6.24)
Sex					
Female		76	47(68.1)	1	1
Male		29	26(72.2)	1.21(0.473.120)	0.97(0.33-2.83)
TCF1st					
<six hour		82	50 (60.9)	1	1
>six hour		23	239 (100)	-	-
Age					
>9week		7	2 (28.5)	1	1
5-8week		36	8 (36.3)	0.71(0.12-4.40)	0.74 (0.12-4.94)
1-4 week		62	17(27.4)	0.94(0.17-5.34)	0.98 (0.16-6.13)
Breed					
Exotic		69	18(26.1)	1	1
Cross	Salmonella	36	9 (25)	0.98(0.37-2.38)	1.06 (0.37-3.07)
Sex					
Female		76	21(27.6)	1	1
Male		29	6(26.70)	0.68(0.24-1.91)	0.66 (0.23-2.010
TCF1st					
<sixhour		82	21(25.6)	1	1
>sixhour		23	6 (26.1)	0.98 (0.34 2.80)	0.66(0.23-2.010

TCF1st Time of first colostrums feeding, CI: confidence interval, OR: odds ratio, ADOR=Adjusted odds ratio, CODR= crude odds ratio, *= stastically significant

Table 7 Multivariable logistic regression analysis of Protozoal pathogens with risk factors.

Variables	Protozoal pathogen	animals examined	Positive (%)	COR(95%CI)	Adjusted odds ratio(95% CI)
Age					
>9 week		7	0(0.0)	1	1
5-8 week		36	7(19.4)	-	-
1-4 week		62	22(35.4)	-	-
Breed					
Exotic		69	18(28.1)	1	1
Cross	Cryptosporidim	36	11(30.5)	1.25 (0.51-3.03)	1.26 (0.44-3.60)
Sex					
Female		76	17(22.3)	1	1
Male		29	12(46.1)	2.45(0.98-6.11)	2.48(0.88-7.00)
TCF1st					
< six hour		82	23 (28.0)	1	1
>six hour		23	6 (26.0)	1.10(0.39-3.15)	1.27(0.41-3.98)
Age					
>9 week		7	3(42.8)	1	1
5-8wek		36	11(30.5)	0.59(0.11-3.07)	0.89(0.15-5.10)
1-4 week		62	26(41.9)	0.96(0.19-4.67)	1.55(0.28-8.50)
Breed					
Exotic		69	23(33.30	1	1
Cross	Eimeria	36	17(47.2)	1.79(0.78-4.07)	2.19(0.83-5.82)
Sex					
Female		79	30 (39.5)	1	1
Male		26	10 (34.5)	0.81(0.33-1.97)	0.55 (0.19-1.51)
TCF1st					
<six hour		82	29 (35.3)	1	1
>six hour		23	11(47.8)	0.59 (0.23-1.52)	0.63(0.24-1.67)
Age /weeks					
>9		7	0(0.0)	1	1
5-8		36	5(13.8)	-	-
1-4		62	19(30.6)	-	-
Sex					
Female		76	20(26.8)	1	1
Male	Giardia	29	4(13.8)	0.44(0.14-1.45)	0.37(0.10-1.38)
Breed					
Exotic		69	17(24.6)	1	1
Cross		36	7(19.4)	0.74(0.27-1.99)	1.26(0.40-3.96)
TCF1st					
>six hour		82	18(21.9)	1	1
<six hour		23	6(26.0)	0.79(0.27-2.31)	0.68(0.21-2.15)

TCF1st: Time of first colostrums feeding CI: confidence interval, COR: crude odds ratio

4.3. Biotypes of the *E. coli* isolates

In the present study, the fermentation reactions of carbohydrates were performed for all the 73 *E. coli* isolates and were found to be variable. Out of 73 *E. coli* isolates, all of the isolates fermented maltose (100%) while lactose, sucrose and rhamnose were fermented by 97.2% of *E. coli* strains. Xylose and sucrose were fermented by 93.2% strains. Dulcitol, inositol and salicin were fermented by 94.1%, 87.7% and 53.4% *E. coli* strains respectively as summarized in table 8

Table 8. Sugars fermented by strains of *Escherichia coli* isolated from diarrheic calves (N=73).

Fermented carbohydrates	№ of positive isolates	Proportion (%)
Dulcitol	69	94.5
Inositol	64	87.7
Lactose	71	97.2
Maltose	73	100.0
Raffinose	68	93.2
Sucrose	71	97.2
Xylose	68	93.2
Salicin	39	53.4
Rhamnose	71	97.2

Grouping in to various biotypes were performed using carbohaydrate fermentation reactions of nine different sugars viz. dulcitol, inositol, raffinose, rhamnose, salicin, lactose, maltose, sucrose and xylose. Grouping of biotype were done following the method described by Pandey *et al*. (1979) and (Ashenafi, 2013) and biotyping of the 73 *E. coli* isolates is summarized in table 9

Table 9. Biotypes of *E. coli* isolates based on fermentation reactions of nine sugars from diarrheic calves.

Positive sugars	№ Positive isolates	Proportion N (%)	Biotype assigned
Dul,Raf,Rah,Sal,Suc,Lac,Mal,Xyl	1	1.4	I
Dul,Sal,Suc,Ino,Lac,Mal	1	1.4	II
Dul,Rha,Ino,Mal	1	1.4	III
Dul,Rha,Ino,Mal	1	1.4	IV
Dul,Rha,Suc,Ino,Lac,Mal,Xyl	1	1.4	V
Dul,Raf,Sal,Suc,Ino,Lac,Mal	1	1.4	VI
Dul,Raf,Rha,Ino,Lac,Mal,Xyl	1	1.4	VII
Raf,Rha,Sal,suc,Lac,Mal	1	1.4	VIII
Dul,Raf,Rha,Suc,Ino,Lac.Mal,Xyl	22	30.1	IX
Rha,Sal,Suc,Lac, Mal,Xyl	1	1.4	X
Raf,Rha,Suc,Ino,Lac,Mal	7	9.6	XI
Dul,Raf,Rha,Suc,Ino,Lac.Mal,Xyl	2	2.7	XII
Dul,Raf,Rha,Sal,Suc,Ino,Lac,Mal,Xyl	33	45.2	XIII
Total	73	100	

Key: Dul=Dulcitol,Ino=Inositol,Lac=Lactose,Mal,Maltose,Raf=Raffinose,Suc=Sucrose,Xyl= Xylose,Sal=Salicin,Rha= Rhamnose.

In this study, the most commonly occurring biotypes were Biotype XIII (33 isolates, 45.2%), VIII (22 isolates, 30.1%), XI (7 isolates, 9.6%), I (2 isolates 2.7%) II and III, IV, V, VI, VII, IX, X, XII (1 isolates) for each biotype. As shown in table 9 the distribution of the dominant biotypes among the various calf age groups was observed in younger calves of the 1- 4 weeks age (45.2%) and 5-8 weeks (30.1%) of age were harboring the more dominant biotype than older calves aged above nine weeks. This finding is also

comparable with the result obtained from the analysis of age as a risk factors which indicated 1-4 weeks of calves were at a significantly high risk of being affected with *E. coli* causing diarrhea than the older age.(Tables 9 and 10).

Table 10. Distribution of *E. coli* biotypes among different age groups of diarrheic calves.

Age of calves	*E. coli* Biotypes													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	**N**
1-4 weeks	0	1	0	1	1	1	1	1	1	2	5	15	21	50
5-8 weeks	1	0	1	0	0	0	0	0	0	0	2	6	12	22
>9 weeks	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Total	1	1	1	1	1	1	1	1	1	2	7	22	33	73

N: Number

4.4. Antimicrobial susceptibility profiles of *E .coli* and S*almonella* isolates

Antimicrobial susceptibility testing was performed for all bacteria isolated from diarrheic calves. Eleven (11) antimicrobial impregnated discs (Oxoid, UK) were used. As shown on table 11, high resistance by *E. coli* isolates was seen against erythromycin (95.9%), tetracycline (74.0%), streptomycin (74.0%), and ampicillin (53.6%). On the other hand, 94.4%, 82.2, 82.2% and 61.6% of isolates were susceptible to gentamycin, ciprofloxacin, trimethoprim-sulfamethoxazole and chloramphenicol, respectively.

Table 11. Antimicrobial susceptibility pattern of *E. coli* isolated from diarrheic calves.

Antimicrobial agents	Susceptible N (%)	Intermediate N (%)	Resistant N (%)	Total
Gentamycin	66 (90.4)	0 (0.0)	7 (9.6)	73 (100%)
Tetracycline	19(26.0)	0 (0.0)	54 (74.0)	73 (100%)
Streptomycin	7 (9.6)	12 (16.4)	54 (74.0)	73 (100%)
Cefoxitine	34 (46.6)	7 (9.6)	32 (43.8)	73 (100%)
Ampicillin	8 (11.0)	26 (35.6)	39 (53.4)	73 (100%)
Chloramphenicol	45 (61.6)	9 (12.3)	19 (26.0)	73 (100%)
Erythromycin	2 (2.7)	1 (1.4)	70 (95.9)	73 (100%)
Trimethoprim-sulfamethoxazole	60 (82.2)	1 (1.4)	12 (16.4)	73 (100%)
Kanamycin	34 (46.6)	22 (30.3)	17 (23.3)	73 (100%)
Ciprofloxacin	60 (82.2)	11 (15.1)	2 (2.7)	73 (100%)
Amoxicillin	15 (20.	29 (39.7)	29 (39.7)	73 (100%)

Antimicrobial susceptibility testing was also performed for salmonella isolated from diarrheic calves. Eleven (11) antimicrobial impregnated discs (Oxoid, UK) were used. As shown on table 12, high resistance by salmonella isolates was seen against erythromycin (96.3%), tetracycline (53.3%), streptomycin (40%), and ampicillin (33.3%). On the other hand, 92.6%, 100, 88.9% and 77.8%% of isolates were susceptible to Gentamycin, Ciprofloxacin, Trimethoprim-sulfamethoxazole and Cefoxitine, respectively

Table.12. Antimicrobial susceptibility pattern of salmonella species isolated from diarrheic calves.

Antimicrobial agents	Susceptible N (%)	Intermediate N (%)	Resistant N (%)	Total
Gentamycin	25 (92.6)	0 (0.0)	2 (7.4)	27 (100%)
Tetracycline	3 (11.1)	8 (29.6)	16 (59.3)	27 (100%)
Streptomycin	16 (59.3)	0 (0.0)	11 (40)	27 (100%)
Cefoxitine	21 (77.8)	1 (3.7)	5 (18.5)	27 (100%)
Ampicillin	12 (44.4)	6 (22.2)	9 (33.3)	27 (100%)
Chloramphenicol	16 (59.3)	5 (18.5)	6 (22.2)	27 (100%)
Erythromycin	1(3.7)	0 (0.0)	26 (96.3)	27 (100%)
Trimethoprim-sulfamethoxazole	24(88.9)	2 (7.4)	1 (3.7)	27 (100%)
Kanamycin	13 (48.1)	8 (29.6)	6 (22.2)	27 (100%)
Ciprofloxacin	27 (100)	0 (0.0)	0(0.0)	27 (100%)
Amoxicillin	19 (70.4)	2 (7.4)	6 (22.2)	27 (100%)

N= number of isolates

For multidrug resistance profile, 91.78% of *E. coli* and 100% Salmonella isolates were develop multidrug resistance to different antibiotics. The most frequent multidrug resistance pattern consisting of four drugs is exhibited for erythromycin, streptomycin, kanamycin, tetracycline, and erythromycin, streptomycin, kanamycin, ampicilin with a resistance of 12 (17.91%) and 5% (18.51%) of *E. coli* and Salmonella isolates respectively as summarized in table 13.

Table 13. Antibiograms of the total *E. coli* and salmonella isolates form diarrheic calves

Antibiotics	**Resistance Strains**			
	E. coli		Salmonella	
	Frequency	%	Frequency	%
E, S	6	8.95	-	-
AMP, TE	2	2.98	-	-
E, K	1	1.49	1	3.70
E, AML	3	4.77	-	-
E, ET	1	1.49	4	14.81
E, C	-	-	1	3.70
E, AMP	-	-	2	7.40
E, FOX	-	-	1	3.70
E, S, AML	2	2.98	-	-
E, AMP, TE	2	2.98	-	-
E, S, TE	-	-	3	11.11
E, K, TE	-	-	1	3.70
CN, E, TE	-	-	1	3.70
E, S, K, TE	1	1.49	-	-
E, S, FOX, TE	12	17.91	-	-
E, K, AMP, TE	2	2.98	-	-
E, S, AMP, C	1	1.49	-	-
E, S, K, AMP	1	1.49	5	18.51
E, FOX, C, AML	-	-	1	3.70
E, S, C, AMP	-	-	1	3.70
E, S, C, AML	-	-	1	3.70
E, AMP, TE, AML	-	-	1	3.70
E, K, AMP, TE, AML	1	1.49	-	
CN, E, S, AMP	-	-	1	3.70
S, AMP, FOX, TE, AML	1	1.49	-	-
E, S, FOX, C, TE	1	1.49	-	-
CN, S, K, AMP, TE, SXT	3	4.77	-	-
CN, E, S, AMP, TE, SXT	2	2.98	-	-
E, S, K, AMP, FOX, TE, AML	1	1.49	-	-
E, S, AMP, FOX, C,ET, AML	2	2.98	-	-
E, S, K, AMP, C, TE, AML	2	-	-	-
E, S, AMP, FOX, TE, AML	3	4.77	-	-
E, S, AMP, FOX, C, TE	1	1.49	-	-
CN, E, S, K, AMP, SXT, AML	1	1.49	-	-
E, S, K, AMP, TE, SXT, CN	1	1.49	-	-
E, S, K, AML, C, TE, SXT, AML	2	2.984	-	-
E, S, K, AMP, C, TE, AML, CIP	1	1.49	-	-
E, S, K, AMP, FOX, C, TE, AML	-	-	2	7.40
E, S, K, AMP, C, TE, SXT, AML	-	-	1	3.70
E, S, K, AMP, FOX, C, TE, AML, CIP	1	1.49	-	-
Total	**67**	**91.78**	**27**	**100**

Key: E: Erythromycin, S: Streptomycin, K: Kanamycin, AMP: Ampicillin, TE: Tetracycline, SXT: Trimethoprim-sulfamethoxazole, AML: Amoxicillin, CIP: Ciprofloxacin, CN: Gentamycin, FOX: Cefoxitine, C: Chloramphenicol.

5. DISCUSSION

In the present study, an attempt has been made to isolate and detect major enterophatognes associated with calf diarrhea, as well as investigation of the potential risk factors, which could influence the occurrence of calf diarrhea. In addition, determination of the distribution of *E. coli* Biotypes and antimicrobial susceptibility of the bacterial isolates was conducted. Unlike previous studies on the subject in Ethiopia, which addressed mainly large scale dairy farms, the present study was also focused on both large and smallholder dairy farms. So the findings are important to contribute valuable information in understanding of pathogen diversity in calf diarrhea in the rapidly expanding smallholder dairy sector in the major urban centers of the country.

5.1. Risk factors

Many risk factors were tested for their association with the occurrence of enteropathognes causing calf diarrhea. Based on results, some factors were found to be significantly associated but others were not, although biologically they were hypothesized to be associated with the occurrence of enteropathogns causing diarrhea. Age and time of first colostrum feeding were the important calf (host) and managemental factors found to affect *E. coli* infection involved in calf diarrhea.

Analysis of risk factor in the association of enteropathogn occurrence has revealed that there was statistically significant association between age of calves, time of colostrum feeding and calf diarrhea due to *E. coli* infection. According to the result of the present study, young calves were at significantly high risk being affected by diarrhea due to *E. coli* infection. Young calves and calves that were feed colustrum greater than six hours of birth age were at a significantly high risk of being affected with diarrhea due to *E. coli* than older age and feed colostrums less than six hours of age. This finding in agreement with the finding of Trotz-williams *et al*. (2007) and Lorino *et al* (2005) who reported younger calves were at high-risk of calf diarrhea due to *E. coli* infection. (1993). The

finding that delayed colostrum intake (latter than 6 hours of age) associated with high risk of occurrence of *E. coli* agrees with other reports. Olson *et al*. (1993) 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%. Matte *et al.* (1982) found that 61% of colostral immunoglobulin containing 80mg/ml of IgG is absorbed in six hours and decreases sharply thereafter. This indicates that the first six hours are the period in which maximum absorption of coloistral immunoglobulin takes place (Bath *et al.,* 1985). In the present study, breed and sex of calve were not significantly associated with any of the enteropathgnes identified which was similar with the works of Yenehiwot (2008).

5.2. Enteropathognes associated with calf diarrhea

Identification of infectious agents causing calf diarrhea was one of the major tasks of the present investigation. Apart from the influence of varied environmental and managerial factors, the infectious agents involved in causing diarrhea are numerous. In the present study, *E. coli,* salmonella, cryptosporidium, eimeria and giardia were detected from diarrheic calves from smallholder and large dairy farms of North Shewa Zone (Muke turi, Debre tesige and Fitche. Similarly, these pathogens were also detected and isolated from calves by many other authors in different parts of the world as single and mixed infection (Reynolds *et al.,* 1986; Durham *et al.,* 1989; Ongerth and Stibbs, 1989; Brown *et al.,* 1990; Elschner, 1995; De la Fuente *et al.,* 1999; Deng *et al.,* 2003; Herrera-Luna *et al.* (2009); Yenehwot, 2008)

In this study, mixed infections were commonly detected in diarrheic calves. This was similar to the result obtained by Steiner *et al.,* (1997); de la Fuente *et al.,* (1999) Garcia *et al,* (2000), Joachim (2003); Herrera-Luna *et al.* (2009); Yenehiwot (2008). In similar way, mixed infections of different enteropathogens are common and often exaggerate the clinical outcome of the disease (Holland 1990).

It is suggested that the presence of more than one enterophatognes might be one of the factors determining an infection in a clinical or subclinical disease (de la Fuente *et al.*, 1998). On the other hand, mixed infection may be associated with more severe disease. In contrast, to Demissie (2007) who reported single infection, only by *Cryptosporidium parvum*, in diarrheic calves, the present study showed the occurrence of both single and mixed infections in study herds.

According to the present finding, *E coli* was the bacterial agent cultured with the highest frequency (69.6%) from diarrheic calves in this study, a finding that agrees with results of Bendali *et al.* (1999a) and Garcia *et al.* (2000). This finding is also comparable to that reported by China *et al.* (1999) with 55.6%, Khan and Khan (1997a) with 54%; Acha *et al*. (2004) with 76%, Ashanfi (2013) with 74% in kombolcha dairy farms, Dawit, (2012) with 64% in Addis Ababa and Debre Zeit dairy farms. But, the present result is much higher than that reported by Feunte *et al*. (1999), Bendali *et al.* (1999), Caple (1989), Brwon *et al.* (1990), Janke *et al.* (1990), Blanco *et al.* (1996), Herrera-Luna *et al.* (2009) and haschek *et al*. (2006) who reported isolation rates of 20.3%, 27.8%, 5%, 2.2%, 10.0%, 13.4%, 18.9% and 17.9%, respectively. In contrast to this study much higher detection of *E. coli* was reported by Adesiyun *et al.* (2001) 84.3% from diarrheic calves in Trinidad the variation might be due to the difference in the diagnostic technique used (the use of API 20 E system in the previous study might be increase the accuracy of detection rate).

The higher occurrence of calf diarrhea due to *E. coli* in the present study might be attributed to delay in first colostrum feeding in the study area because of poor farmers' awareness on the time of colostrums feeding. Olson *et al.* (1993) found that each hour of delay in colostrums ingestion in the first 12 hours of age increased the chance of a calves becoming ill by 10% Matte *et al.* (Matte *et al.*, 1982) found that 61% of colostral immunoglobulin containing 80mg/ml of 43 IgG is absorbed in six hours and decreases sharply thereafter and this idea was well explained by Blowey *et al.* (2003) diarrhea in calves only a few days old was often caused by bacterial infections such as *E coli*. Mixed

infection by protozoal parasite could also increase bacterial infection this idea was supported by (Ballweber, 2001) sometimes the protozoal parasite causes bacterial overgrowth by decreasing disaccharides activity. Less disaccharides reduces the breakdown of sugars and causes a good environment for bacterial growth

Moreover, the difference could be due to failures in proper management practices such as inadequate sanitation, improper hygienic management, which increase the opportunity for exposure to these organisms. In addition, the existence of disease predisposing stress factors such environmental stress (cold weather conditions) and feed shortage in the study might contribute for the high occurrence of *E. coli* infection. Although these pathogens can be isolated from healthy calves as well, the excretion rate is higher in diarrheic calves indicating their role as a cause of calf diarrhea (Reynolds *et al.*, 1986).

The overall detection percentage of *salmonella* species (25.7%) in the present study was higher than previously reported by Brown *et al.* (1990), Fuente *et al.* (1999), Khan and Khan (1997), Fuente *et al.* (1998), Achá *et al.* (2004), Haschek *et al.* (2006) and Herrera-Luna *et al.* (2009) who found 19.3%, 1.8 %, 13 %, 0.9%, 2%, 0 % and 0% of *salmonella* species were isolated from diarrheic calves in different countries respectively, In similar way, in Ethiopia a low prevalence of salmonella from diarrheic calves were reported by Demssie (2007) (16.7%) in and around Addis Ababa, Temesgen *et al.* (2008) (2.6%) in Debre Zeit and its environs. Moreover, Abraham *et al.* (1992) in their relatively comprehensive work on calf diarrhea in dairy farms of the central highlands of Ethiopia could not even detect salmonella in diarrheic calves. However, the results of the present study are comparable with the reports by Yenehiwot (2008) who found the organism at the rates of 24.11% in Ethiopia and Waltner-Toews *et al.* (1986) 22.0% in Canada.

The higher detection rate of salmonella in diarrheic calves' in the present study farms might be due to environmental contamination, ineffective cleaning and disinfecting of materials used as stated by different investigators (Davies, 2003). The housing system and herd size and the presence of calves of different ages on and in the farm (Mollenhorst

et al., 2005) could be important reasons for higher prevalence in this study as all were present around the farms examined. Moreover, *Salmonella* infection of a farm is maintained by transmission of the agent from faces of infected animals into susceptible animals, which is fecal oral route. The epidemiology of salmonellosis is primarily the epidemiology of fecal pollution (Gay, 1999). In the current study, it has been observed that most of the calves had a chance of being exposed to contaminated feed and water this might be one reason for the high detection of salmonella in the study area.

The result of the present study suggests that salmonella species contribute as a the common cause of calf diarrhea dairy farms of the study areas, also in agreement with other investigator (House, 1978) who suggests that salmonella is known to be the most common and economically important agents, among bacteria, causing calf diarrhea. Moreover, salmonella has been identified as a widespread diarrheal agent in dairy calves (Reynolds *et al*., 1986; Anou, 1997) in some European countries.

However, the present result was lower than the observations of Caple (1989), who found 36% of *salmonella* species from calves. In addition, Bulgin *et al*. (1982) reported salmonella as the most dominant isolate from diarrheic calves Pergram *et al*. (1981) also diagnosed Salmonella as an important cause of calf diarrhea in dairy farms of Ethiopia. These differences are most likely attributed to differences in the study country, environmental contamination, the nature of the production system, the specific control measures and husbandry practices of the study animals in different countries. The detection of Salmonella in diarrheic calves in the present study demonstrated its potential role in calf diarrhea at least in the study calves.

Moreover, the lower detection rate might be due to the fact that shedding of the agent did not coincide with the sampling occasion, failure to detect the causative agent, due to diagnostic method used, some cases of diarrhea might not be associated with infectious agents but, instead, due to management or to nutritional factors (Achia *et al.,* 2004). In similar way, the frequencies of Salmonella isolation vary from one location to the other

due to different managemental and hygienic regimes as well as geographical, environmental and individual differences (Ritchie *et al.,* 2001; Veling *et al.,* 2002).

In the present study, a significant number of calves were found harboring Cryptosporidium in the study farms. Cryptosporidium was detected at the rate of (27.6%) in diarrheic calves; this is in line with reports of 27.8%, by Alemayehu *et al*. (2013) in Eastern Ethiopia, 27.9% by Noordin *et al.* (2000) in England, 28.5% by Castro-Hermida *et al.* (2002) in Sirilanka, 25.6% by Herrera-Luna *et al*.(2009) in Austria in diarrheic calves. However, lower detection rates of 7.2% in Ethiopia (Temsegen *et al*., 2008), 19.2% in Zambia (Geurden *et al.,* 2006), 17.9% in France (Lefay *et al.,* 2000), 17.6% in central Ethiopia (Abeba *et al.,* 2008) and 11.9 in USA (Fayer *et al.,* 2008) were reported in diarrheic calves. This variation is most likely attributed to the differences in agro-ecology, management, and husbandry practices of the study animals in different countries (Radostits *et al.,* 2006).

On the other hand, there were relatively higher rates of detection reports of cryptosporidium in different countries. Detection prevalence of, 35.5% in USA by Nguyen *et al.* (2007), 33.5% in Vietnam by Brook *et al.* (2008) and 47.9 in Spain by Santin *et al.* (2004) were reported in diarrheic calves. Exceptionally, some of the reports revealed the highest prevalence of detection 52.6% in central Spain de la Fuente *et al.* 1999 50% in Netherlands (Huetink *et al.,* 2001) and 70% in USA (Fayer *et al.,* 2000), 64% in Debre Zeit and its environs Temesgene (2008), 51.79% in Holeta, Debre Zeit, and Muke Turi, Ethiopia (Yenehiwot, 2008) 63.9%, Demisse (2007) in and around Addis Ababa, Ethiopia. These differences in the prevalence among countries may be attributed to discrepancy between the sensitivity of the diagnostic techniques employed (use of antigenic ELISA kit), the difference in the stocking rate and husbandry system of livestock production system of the countries which may increase opportunity for exposure to these organisms. Besides, these variations could also be due to the difference in the susceptibility of the target population that related to age differences. (Geurden *et al.,* 2006). Moreover, higher detection rate of *Cryptosporidium* might be due to several

factors like early contamination soon after birth by contact with their dams, contaminated calf house, asymptomatic carriers and contaminated environment (Castro-Hermida *et al.*, 2002).

The present study showed a higher rate of Eimeria (38.1%) which was higher than previous reports by Kassa *et al*. (1987) in Bahirdar (24.9%), by Kebadu (1998) in Debre Zeit (20%), by Ferid *et al.* (2012) in Eastern Ethiopia (22.7%), Nagwa *et al.* (2011) in Egypt (25.4%) and Herrera-Luna *et al*. (2009) in Austria (10.4%). This higher rate of detection of oocyst of eimeria in the study area might be due to the cold stress and changing weather which might predisposes to the opportunistic, pound-robbing coccidian protozoa; hence, severe outbreaks of coccidiosis are common shortly after very cold weather (Ward *et al.*, 2008). This high prevalence detection of eimeria oocyst in infected calves and the greater proportion of subclinical infections could negatively influence animal productivity and cause economic losses from poor feed efficiency, slow weight gain, weight loss, failure of the calves to grow to their full potential, and increased susceptibility to other diseases (Cornelissen *et al*., 1995; Chibuanda *et al.*, 1997). Moreover, continuous oocyst sheding from subclinically infected calves contaminate the environment of calves or the hair coats and cause severe coccidiosis in highly susceptible new calves that are kept in these areas (Cornelissen *et al.*, 1995; Kennedy *et al.*, 2001).

During the study period, almost all the calves were housed in overcrowded condition, with poor management and have easy contact with adult animals. This might have given more chance for the animals to lick each other and ingest large number of oocysts, which is in agreement with previous reports (Rodriguez-Vivas *et al.*, 1996; Kennedy, 2001; Radostits *et al*., 2006; Abebe *et al.*, 2008). Coccidiosis occurs most commonly in young animals with a seasonal incidence when young calves are brought together for weaning or moved into feedlots or fed in small areas for the winter months. The prevalence of infection and the incidence of clinical disease are also age related (Radostits *et al*., 2007). The detection rate of Gairdia (22.9%) in this study was in agreement with previous finding reported by Geurden *et al*. (2010) with a detection rate of 22.4% by Bjorkman *et al.* (2003) 29% in diarrheic calves and 23% in health calves and a report by (Ljungström

et al., 2001), 25% in diarrheic calves and 20% health calves in Sweden. However, much lower than Olson *et al.* (1997) who reported 73% in diarrheic calves.

On the other hand the report of the present study was higher than a report by Wade *et al* (2000) and Herrera-Luna *et al*. (2009) who reported detection rate of 10% and 6.1% respectively. According to Olson *et al.* (1997), the detection of *Giardia* species in calves worldwide varies between 10 and 73%. This variation might be due to differences related to a difference between studies, especially regarding animal age and type of diagnostic techniques employed. For instance, in a study at the Canary Island, Ruiz *et al.* (2008) found positivity of 42.2% in 315 young animals through the association of three diagnostic techniques (microscopy, immunofluorescence and PCR). However, if only the microscopy results are considered, the positivity goes down to 26%, emphasizing the need to combine techniques to achieve a more reliable diagnosis.

Thus, in this study the use of only one diagnostic technique (microscopy), coupled with the use of only one fecal sample from each animal, could have underestimated the real detection rate of this pathogen in the studied farms. Additionally, studies demonstrate that because of its easy transmission and high viability of cysts in the environment, when a longitudinal research of animals was made the detection rate could reach 100% (Koudela and Vitovec, 1998; Becher *et al.*, 2001). Besides, microscopy of Giardia cysts and trophozoites is more straightforward, and there is little risk of confusion with other parasites. Moreover, only ghost cysts with an empty appearance were sometimes not recognized as giardia parasites (Collins *et al.*, 1978) which was the method used in this study might also be under estimate the detection rate of the parasite. In addition to the differences inherent to the research methodology employed, several authors highlight that the differences in management and hygienic-sanitary conditions between the farms could affect the infection rate (Bomfim *et al.*, 2005; Geurden *et al.*, 2010).

5.3. Biotypes of the *E. coli* isolates

Biochemical reactions have conventionally been used for identification of bacteria to the species level. Extensive studies of sugar fermentation reactions of bacteria have been conducted to introduce biochemical-typing systems in epidemiological studies of bacteria (Barr and Hogg, 1979; Krishnan *et al.*, 1987)

In the present study, the fermentation reactions of carbohydrates by all the *E. coli* isolates were found to be variable. All of the *E. coli* isolates, obtained from diarrheic calves were able to utilize one or more sugars tested. The isolates could be grouped into various biotypes considering the fermentation reactions of nine different sugars. All the *E. coli* isolates were distributed into thirteen different biotypes. This might reflect underlying variations in strain patterns from place to place in different countries (Kasper *et al.*, 2005). This is in accordance with findings of (Dawit, 2012) and Pandey *et al.* (1979) who used the same nine sugars for biotyping of *E. coli* isolates. Such variability in biotypes amongst *E. coli* isolates have also been reported by Hinton *et al*. (1982), Camguilhem and Milon (1989) and Blanco *et al*. (1996) in different countries.
Such variability in fermentation reactions biotypes and distribution amongst *E. coli* isolates have also been reported by Hinton *et al.* (1982), Camguilhem and Milon (1989) and Blanco *et al.* (1996) in different countries.

In the present study, all the strains have been found to ferment maltose (100%). This is in agreement with Tewari and Aggarwal (1982), who found fermentation of maltose by all (100%) of the *E.coli* isolates. In the current study, fermentation of xylose (93.2%) and lactose (97.3%) is comparable with the finding of Singh *et al.*(2013), Tewari, and Aggarwal (1982), Forbes *et al.* (2002), who reported (98.54%) and 94.5 %. respectively. In the present study, 94.1% (73) strains have been found to ferment dulcitol; this was higher than Kaur *et al.* (1991) and Forbes *et al.* (2002) who reported 71.1% and 60% of their strains to ferment dulcitol respectively. This might be attributable to specific characteristics of these organisms isolated from different locality. Higher formation of dulcitol indicates that dulcitol fermenting *E. coli* strains are significantly more virulent

than the others since a correlation between the virulence of *E. coli* and dulcitol fermentation has been reported (Kalmansen *et al.,* 1975).

In this study all the 73 isolates fermented dulicitol togethere with the other sugar the result is agrees with Dawit (2012) reported 15 biotypes based on 6 sugars (dulcitol, raffinose, rhamnose, salicin, starch and sucrose) on his work in the central part of Ethiopia. His data showed that the biotype that fermented duclcitol was the most dominant one (20%), followed by the biotypes that ferment dulicitol, raffinose and rhamnose (13.3%).

In the present study, 93.2% *E. coli* isolates had raffinose fermentation activity which is much higher than other reports (61.96%) (*Singh et al.,* 2013). However, Braaten and Myers (1977) have reported even higher percent of *E. coli* isolates showing fermentation activity of this sugar. The variable raffinose fermentation activity might be due to its genetic locus, which is reported to be plasmid coded (Smith and Huggins, 1978). Fermentation of sucrose in this study (97.2%) was higher than finding of Singh *et al.* (2013) who reported 49.96%.

The present study also investigated the identity of different types of *E. coli* biotypes in calf diarrhea. This, biotyping could be a useful method of identification as supported by Gargan *et al.* (2013). The most dominant (45.2%%) biotype was biotype XIII In the presnt study 13 differnte *E.coli* biotypes were found based on the fermentation of nine sugars; this was in accordance with Ashenafi (2013) and Gargan *et al.* (2013) and Similarlly Chattopadhyay *et al.* (2003) found that thirteen strains out of 20 *E. coli* samples from diarrheic calves found positive for verotoxin gene by PCR test could be isolated and characterized for biotyping on the basis of sugar fermentation reactions in three sugars.

5.4. Antimicrobial susceptibility profiles of *E. coli* isolates

The emergence and dissemination of antimicrobial resistance is an important issue in public health, animal health, and food safety. Thus, antimicrobial susceptibility test was performed to all of the bacterial isolates. In this study, almost most bacterial isolates were susceptible to gentamycin, ciprofloxacin and trimethopprim-sulfamethaxzole. High susceptibility of *E. coli* to gentamycin in this study, were comparable with Holland *et al.* (1999) who reported all *E. coli* strains were highly sensitive to gentamycin. These results are similar to those of Wereckenthin *et al.,* (2002) and Aksoy *et al.* (2007), Ynehiwot (2008), who reported high susceptible rate of *E. coli* and Salmonella to the aformtioned antimicrobial agents. *E. coli* isolates were resistant to erythromycin. The finding of the erythromycin with resistance was comparable with (Nazir, 2004) who reported 100% Erythromycin resistant *E. coli* isolates. The resistance acquired possibly due to cross-resistance with lincosamides (Von Recklinghausen *et al.,* 1989).

On the other hand, most of the *E. coli* isolates were resistant to tetracycline, Streptomycin, ampicilin and cefoxitine. While the resistance to tetracycline, Erythromycin and streptomycin is consistent with the Ynehewot (2008), Werckenthin *et al.* (2002) and Aksoy *et al*. (2007) who reported high resistance rate of *E. coli* to many antimicrobial agents. Similarly Chattopadhyay *et al*. (2001) studied the antibiotic sensitivity pattern of STEC strains from animal, human and reported that STEC strains were uniformly sensitive to common antibiotics except tetracycline, and streptomycin, erythromycin.

Resistance to tetracycline in *E. coli* and related species is principally plasmid mediated and an inducible trait. Mechanisms of resistance include decreased accumulation of tetracycline due to either acquisition of an energy-dependant efflux pathway or to decreased influx, or to decreased access of tetracycline to the ribosome (site of action) due to acquisition of ribosome protected proteins and enzyme inactivation (Speer *et al.,* 1992). Microorganisms that have been resistant to one tetracycline frequently exhibit

resistance to the others. Tetracycline was found initially to be highly effective against ETEC, but resistance has been emerging in the recent past and becoming a constraint in the treatment (Kapusnilk-uner *et al.,* 1996). The susceptibility pattern of *E. coli* observed in the present study indicates that the resistance patterns were more evident to antibiotics frequently used like tetracycline and Ampicilin in accordance with Hariharan *et al*. (2004).

Ampicillin and amoxacillin was found to be resistance in this study, which is in agreement with Bradford *et al.* (1999), and (Nasir, 2004). The high resistance of these drugs in gram-negative bacteria might be due to the transfer of resistance genes form gram-positive bacteria of β-lactamase genes. Al-Assil *et al*., 2013 explained that among the 25 isolates, the most prevalent *β-lactamase* gene was *βlaCTX-M,* which was detected in all of the isolates; the *β laTEM* gene was found in eight isolates of *E. coli.*

In this study, the high resistance pattern against tetracycline, erythromycin and streptomycin might be most likely due to selective pressure resulting from uncontrolled and inappropriate use of these agents in veterinary clinic and in study farms as well as in the country as a whole. This is promoted by the lack of an antibiotic use policy and the availability of antibiotics distributed in the country since *E. coli* is part of the normal fecal flora and hence used as a potential indicator for resistance trend in human and animals (Werckenthin *et al.,* 2002). Antibiotics use leads to resistance in pathogenic bactriea as well as the development of resistance strains in flora bacteria. Historically, much of the interest has focused on pathogenic bacteria; more recently, the role of commensal organisms as a reservoir or vehicle to transfer resistance genes to more harmful, pathogenic bacteria has been postulated (Bartoloni *et al.,* 2006; Dyer *et al.,* 2006). One such mechanism is the transfer of antimicrobial resistant plasmids between *Escherichia coli* and *Salmonella* spp. (De Francesco *et al.,* 2004).

Moreover, the high level of antibiotic resistant among *E. coli* isolates might be due to self-prescription policy, comparatively cheaper antibiotics intake, lack of dependency on

laboratory guidance and in adequate doses of antibiotics intake, and indiscriminate use of antibiotics in animal husbandry practices. In many areas of Ethiopia, antibiotics can be easily available over the counter without prescription of registered veterinary medical practitioner. This is the main cause of misuse of antibiotics. The resistance that develops in flora bacteria may be transferred to another bacteria and infect humans through direct or indirect routes .in particular, resistance strains from association with the antibiotics used in veterinary medicine (tetracycline, Erythromycin, streptomycin (Aksoy *et al.*, 2007). Information about the prevalence of antibiotics resistance in communal enteric bacteria and represent reservoirs of resistance genes in potentially pathogenic bacteria (O'connor *et al.,* 2002).

The antimicrobial sensitivity patterns of the Salmonella isolates in this study was in agreement with Van Duijkeren *et al.,* 2003), Murugkar *et al.,* (2005) and Ynehewot (2008) who reported resistance to different antimicrobial agents like erythromycin, tetracycline. The indiscriminate use of different kinds of antibiotics creates a potential health risk to animals and humans in terms of drug residues and the development of resistant bacterial strains.

Recently published studies described an increased incidence of resistance for different antimicrobials (Orden *et al.,* 2000). No resistance was observed in ciprofloxacin. The high rate of sensitivity to ciprofloxacin in this study in *salmonella* species might be attributed to the fact that ciprofloxacin has not been used in veterinary medicine in most of the farms to date. Least resistance by these isolates was observed in gentamycine, trimethoprim-sulfamethoxazole. The finding compares well with the finding of other researchers (Murugkar *et al.,* 2005 and Sibhat 2006). Excessive or inappropriate use of antibiotics in the rearing of farm animals represents a major factor in the emergence, persistence and spread of resistance salmonella even in the humans who are the participant of the food chain. Hence, it is imperative that judicious use of antibiotics in the treatment and prophylaxis, after *in vitro* testing, be practiced to sustain the usefulness of the antibiotics in controlling salmonellosis on long-term basis (Murugkar *et al.,* 2005).

In the present study multidrug, resistance was common in both *E. coli* and Salmonella isolates. The finding is in agreement with Sibhat (2006) and Ynehiwot (2008) in Ethiopia Saravanbava *et al.* (1990) in India, Nazir (2004) in Bangladesh, who reported multidrug resistance pattern. This might be due to indiscriminate use of antibiotics, lack of proper knowledge and negligence it was well explained by Jawetz *et.al.* (1984) indiscriminate use of antibiotics eventually supersedes the drug sensitive microorganisms from antibiotic saturated environment. The drug resistant bacteria can spread in the environment where man and animal acquire infection with bacteria carrying drug resistant plasmids (Joseph *et al.* 1979) leading to multidrug resistance. Moreover, multidrug resistance of *E. coli* and salmonella isolates in this study could be largely due to acquired antimicrobial resistance phenotypes most often develop via conjugative transfer of plasmids this was well explained by Di Conza *et al.* (2002) Plasmids may carry class I integrons, which are mobile DNA elements that are important in the proliferation of bacterial multidrug resistance (MDR), especially among the gram-negative enteric species.

6. CONCLUSION AND RECOMMENDATIONS

Diarrhea represented an increase and recurrent problem in young calves. It remains an important cause of morbidity and mortality in dairy calves. In the present study it has been found that both bacterial and protozoal enteropathogens had contributed for the occurrence of calf diarrhea in Mukaturi, Debretsige and Fitche areas five enteropathognes *E. coli*, Salmonella, Cryptosporidium, Eimeria and Gairdia involved in causing calf diarrhea isolated and characterized with the available laboratory facility. *E. coli* was detected in higher rate than others followed by eimeria. In most of the cases mixed infection were common

In the investigation of potential risk factors for the occurrence of enteropathognes causing calf diarrhea age of calves and time of first colostrum feeding were found to be significantly associated with the occurrence of *E. coli* that causing calf diarrhea. Young calves were found to be more susceptible to infection by *E. coli.* However, sex of calves and breed were not significantly associated with the occurrence of the enterophatognes-causing calf diarrhea. The *E. coli* biotyping test result indicted that the most commonly occurring biotypes were Biotype XIII fermented all the sugars tested. The distribution of the isolates in to different biotypes indicates a wide variation in the presence of enzymes that ferments a given sugar that further implies the diverse nature of the bacteria.

The Antimicrobial susceptibility test result indicated that an increased antimicrobial resistance to the different antimicrobial agents was tested. However, from all the drugs ciprofloxacilln, gentamycin and trimethoprim-sulfamethoxazole were effective to both of *E. coli* and *salmonella* species isolated. Majority of both isolates were resistance to erythromycin and tetracycline. Multidrug resistance profile was developed by both *E. coli* and salmonella isolates to different antibiotics. Erythromycin, streptomycin, kanamycin, tetracycline and ampicilin were the most frequent drugs that showed multidrug resistance pattern in both bacterial isolates.

Therefore, based on the above conclusion the following recommendations are forwarded:

- Calf diarrhea is a syndrome of great etiological complexity and hence a more comprehensive study should be conducted to identify the major infectious causes involved in calf diarrhea and to design cost effective, appropriate prevention and control strategies.
- Since age of the calves and the time of first colostrums feeding are the most important potential risk factors creation of awareness to the farm owner through agriculture extension service should be given.
- Education of the farm owners and attendants on the risk of zoonotic infection should be carried out since some of the pathogen (*E. coli, Salmonella spp and Cryptosporidium Spp)* can result infection in human especially in immunocompromised individuals (young, older and HIV patients).
- The bacterial strains identified revealed high resistance rates to the antibiotics, including a presence of multidrug resistance, which indicate the need for proper and strict usage of antibiotics should be practiced in the future.
- The distribution of the *E. coli* isolates in to different biotypes indicates the diverse nature of the organism. Therefore, further detailed study should be carried out to understand the role of *E. coli* and identify the virulent strains involved in calf diarrhea.
- As this work was a cross sectional type of study, further longitudinal study with repeated samplings is required to obtain a more accurate data on the detection prevalence of these enteropahtognes.

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APPENDICES

Appendix 1: Questionnaire format for management and description of calves

1. FARM IDENTIFICATION

Animal code: _____ Farm name: _____

Owners name _____

Address: Kebele _____

When it established _____

Sex: Male ☐ Female ☐

Age: Day 0-1 Weeks ☐ 2-4 Weeks ☐ 5-6 Weeks ☐ 6-8 Weeks ☐

Breed: Local ☐ Cross ☐ Exotic ☐

2. FARM DESCRIPTION

2.1. Owner/manager educations status

Illiterate ☐ Read and write ☐ Elementary school ☐ Highschool ☐ graduate ☐ professiona☐

If professional: Related to animal production ☐ Unrelated to animal production ☐

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

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

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

3. MANAGEMENT DATA

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

3.1.1. Sex of attendants': Male ☐ Female ☐

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

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

College graduate ☐ Professional ☐

3.2. Perparturient care:

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

3.2.2. Navel treatment: Practiced ☐ Not practiced ☐

4. AWARENESS OF ATTENDANTS

4.1. About importance of colostrum feeding to neonates: Yes ☐ No ☐

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

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

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

4.5. If hand feeding – source of feeding: From dam ☐ From another cow ☐

4.6. Types of feeding: Milk ☐ Milk replace ☐

5. AMOUNT OF MILK/MILK REPLACES GIVEN DAILY PER UNIT OF BODY WEIGHT

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

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

Grazing ☐ Concentrates ☐ hay ☐

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

12-16 wks of age ☐

Appendix 2**:** Data Recording Format

N.	Calf ID	Age (week)	Sex	Breed	Diarrhea type	*E.coli*	Salmonella	Cryptosporidium	Emiria	Giardia
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										
14										
15										
161										
17										
18										
19										
20										
21										
22										
23										
24										
25										
26										
27										
28										
29										
30										

Appendix 3: Laboratory record format for *E. coli*

No	Farm/ site	Calf ID	Age	Sex	Mac (pink/Pal)	EMB (MS/NMS)	IMVCi Tests			
1							I	MR	Vp	Ci
2										
3										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										
14										
15										
16										
17										
18										
19										
20										

KEYS: Mac=MaCconkey agar, EMB=Eosine methylen blue agar, I= Indole, Ur= Urea agar, MR=methyl Red, Vp=Vagouse prouskure, Ci= citrate, TSIA=Triple Sugar Iron Agar, B=Butt, S=Slant, Gp=Gas production, H_2S=Hydrogen Sulphide Production.

Appendix 4. Laboratory record format for Salmonella

S.No	Farm	Calf ID	Sex	XLD	BGR	Ur	Vp	I	LIA			TSIA			
									B	S	H_2S	B	S	H_2S	GP
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															
17															
18															
19															
20															

KEYS: XLD=Xylose Lyisen Dexoxchalte BGR=Brilliant Green Agar, Indole, Ur= Urea agar, Vp=Vagouse prouskure, TSIA=Triple Sugar Iron Agar, LIA=Lyisen Iron Agar, B=Butt, S=Slant, Gp=Gas production, H_2S=Hydrogen Sulphide Production.

Appendix 5.Laboratory record format for protozoa parasites

No.	Farm	Calf ID	Age	Sex	ZNS	Fecal Flotation		Remarks
					Crypto (P/N)	Eimeria (P/N)	Giardia (P/N)	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								

KEYS: ZN= Zilele Nielsen Stain, Crypto. =Cryptosporidium.

Appendix 6. Laboratory record format for sugar fermntion *E. coli* biotyping).

S.No	Farm	Calf ID	Sex	Age	Dul	Raf	Rha	Sal	Suc	Ino	Lac	Mal	Xyl
1													
2													
3													
4													
5													
6													
7													
8													
9													
10													
11													
12													
13													
14													
15													
16													
17													
18													
19													
20													
21													
22													
23													

KEYS: Dul=Duclitol, Raf=Raffinose, Rha=Rhamnose, Sal=salicin, Suc=Sucrose, Ino=Inositol, Lac=Lactose, Mal=maltose, Xyl =Xylose.

Appendix 7.Zone diameter interpretive standard chart for Enterobacteriacea

Antimicrobial agents and symbols	Disc contents(µg)	Zone diameter, nearest whole mm		
		Resistance	Intermediate	Susceptible
Ampicilline(AMP)	10	≤ 13	14-16	≥ 17
Erytheromycin(E)	5	≤ 13	14-22	≥ 18
Gentamycin(CN)	10	≤ 12	13-14	≥ 15
Kanamycin(K)	30	≤ 13	14-17	≥ 18
Streptomycin(S)	10	≤ 11	12-14	≥ 15
Trimethoprim-Sulfamethoxazole(SXT)	1.25/23.75	≤ 10	11-15	≥16
Amoxacillin(AML)	20	≤ 13	14-16	≥ 17
Tetracyclin(TE)	30	≤ 11	12-14	≥ 15
Chloramphenicol(C)	30	≤12	13-17	≥ 18
Cefoxitine (FOX)	30	≤ 14	15-17	≥ 18
Ciprofloxacin(CIP)	5	≤ 14	15-17	≥ 21

Source: CLIS, 2012.

Appendix 8. List of media and preparation used for isolation of *E. coli* and salmonella.

1. Buffered peptone water (Oxoid, England)

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

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

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

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

Preparation: Suspended 36.00 gm of dehydrated EMB in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121oC for 20 minutes. The molten medium was cooled to about 50oC temperature and poured into sterile petri plates.

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

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

Preparation: Suspended 55.07 gm of dehydrated MCA in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121^{o}C for 20 minutes. The molten medium was cooled to about 50^{o}C temperature and poured into sterile Petri plates.

4. Mueller Kauffman Tetrathionate broth (Oxoid, England):

Ingredients (g/l): Meat extract 0.9 g, Peptone from meat 4.5 g, Yeast extract 1.8 g Sodium chloride 4.5 g, Calcium carbonate 25.0 g, Sodium thiosulfate 40.7 g, Ox bile, dried 4.75 g, Sterile water 1000 ml Brilliant green solution 1:1000 10 ml Iodine-Potassium iodine solution 20 ml.

Preparation: Dissolve the tetrathionate in sterile water in a flask by shaking. Aseptically add brilliant green solution and then iodine-potassium iodine solution. Adjust pH to 7.4 - 7.8 at 25^{o}C. Store at about 4^{o}C.

5. Rappaport Vassiliadis broth (HiMedia, India)

Ingredients g/l: Soya peptone 4.500, Sodium chloride 8.000, Dipotassium phosphate 0.400, Potassium dihydrogen phosphate 0.600, Magnesium chloride hexahydrate, 29.000, Malachite green, 0.036 pH, after sterilization (at 25°C)5.2±0.2

Preparation Suspend 27.11 grams of dehydrated medium (the equivalent weight of dehydrated medium per liter) in 1000 ml purified/ distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired into tubes and sterilize by autoclaving at 115°C as per validated cycle.

6. Brilliant Green Agar (BGA) (Titan Biotech Ltd., Bhiwadi, India)

Ingredients (g/l): Proteose peptone 10.000, Yeast extract, 3.000, Lactose, 10.000 Sucrose 10.000, Sodium chloride, 5.000, Phenol red, 0.080, Brilliant green, 0.0125, Agar 20.000, Final pH (at 25°C) 6.9±0.2.

Preparation: Suspend 29.045 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. AVOID OVERHEATING. For more selectivity, aseptically add rehydrated contents of 1 vial of Sulpha Supplement (FD068). Mix well before pouring into sterile Petri plates.

7. Xylose-Lysine Deoxycholate Agar (XLD Agar) (Titan Biotech Ltd., Bhiwadi, India)

Ingredients (g/l) Yeast extract**,** 3.000 L-Lysine**,** 5.000**,** Lactose 7.500**,** Sucrose**,**7.500**,** Xylose**,** 3.500**,** Sodium chloride**,** 5.000**,** Sodium deoxycholate**,** 2.500**,** Sodium thiosulphate**,** 6.800**,** Ferric ammonium citrate**,** 0.800**,** Phenol red**,** 0.080**,** Agar**,** 15.000**,** Final pH (at 25°C) 7.4±0.2.

Preparation: Suspend 56.68 grams in 1000 ml distilled water. Heat with frequent agitation until the medium boils. Do not autoclave or overheat. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates. It is advisable not to prepare large volumes that will require prolonged heating, thereby producing precipitate

8. Nutrient Agar (Oxiod, England)

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

Preparation: Suspend 28.0 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

9. Triple Sugar Iron Agar (TSIA) (HiMedia, India)

Ingredients (g/l): Beef extract 3.000 Peptones (Casein and Beef) 20.000, Yeast extract 3.000, Lactose monohydrate 10.000 Sucrose, 10.000 Glucose monohydrate 1.000, Ferric ammonium citrate 0.300, Sodium chloride, 5.000, Sodium thiosulphate, 0.300 Phenol red, 0.025, Agar, 12.000 pH after sterilization (at 25°C) 7.4±0.2

Preparation: Suspend 64.0 3 grams (the equivalent weight of dehydrated medium per liter) in 1000 ml purified /distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes or as per validated cycle allow the medium to set in sloped form with a butt about

10. Lysine Iron Agar (HiMedia, India)

Ingredients (g/l). Peptic digest of animal tissue5.000, Yeast extract 3.000, Dextrose1.000, L-Lysine, 10.000, Ferric ammonium citrate, 0.500, Sodium thiosulphate, 0.040, Bromocresol purple, 0.020, Agar 15.000 Final pH (at 25°C) 6.7±0.2

Preparation: Suspend 34.56 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs

pressure (121°C) for 15 minutes. Cool the tubes in slanted position to form slants with deep butts

11. Urea broth medium (HiMedia, India)

Ingredients (g/l) Monopotassium phosphate 9.100, Yeast extract 0.100, Dipotassium phosphate 9.500, Urea 20.000, Phenol red, 0.010, Final pH (at 25°C), 6.8±0.2

Preparation: Suspend 38.7 grams in 1000 ml distilled water. Mix well and sterilize by filtration. Do not autoclave or heat the medium. Dispense in sterile tubes.

12. Phenol Red Agar (Difco, France)

Ingredients (g/l) Proteose peptone 10.000, Beef extract 1.000, Sodium chloride 5.000, Phenol red 0.018 Final pH (at 25°C) 7.4±0.2.

Preparation: Suspend 16 .02 grams in 1000 ml purified/ distilled water,mix well. Heat if necessary to ensure complete solution. Distribute in fermentation tubes (tubes containing inverted Durham's tubes). Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Aseptically add filter sterilized or autoclave sterilized carbohydrate solution to sterile basal medium.

13. MR-VP Medium (HiMedia, India)

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

Preparation: Suspend 17 grams in 1000 ml of distilled water. Heat if necessary to dissolve the medium completely. Distribute in test tubes in 10 ml amounts and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

14. Tryptone Soya Broth (Oxoid, England)

Ingredients (g/l) Pancreatic digest of casein 17.000Papaic digest of soyabean meal 3.000 Sodium chloride 5.000 Dextrose (Glucose) 2.500 Dipotassium hydrogen phosphate 2.500 Final pH (at 25°C) 7.3±0.2.

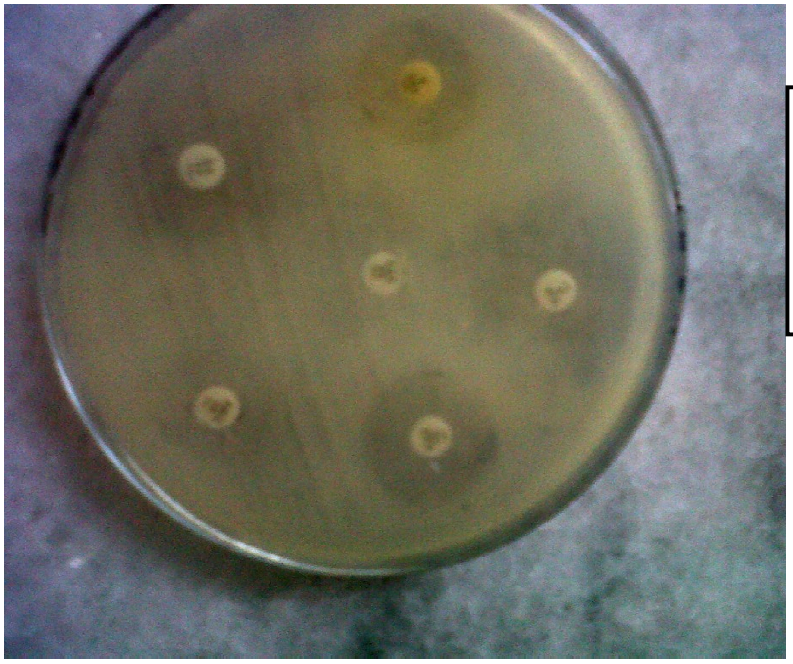
Preparation: Suspend 30 grams in 1000 ml purified/ distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense as desired.

15. Mueller Hinton agar (Oxoid, England)

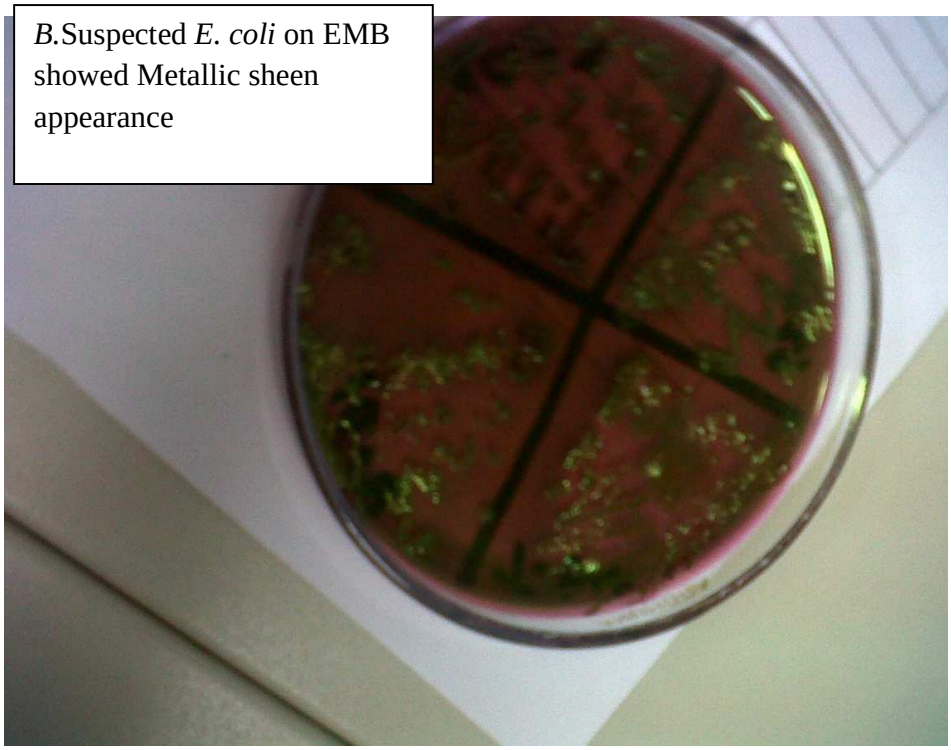
Ingredients g/l: Casein acid hydrolysate 17.50, Beef heart infusion 2.00, Starch, soluble 1.5, Agar 17.00 gm, Final pH (at 25°C) 7.3 +0.2.

Preparation: Suspended 38 gm in 1000 ml distilled water. Sterilized by autoclaving at 15 psi pressure, 121oC for 20 minutes. The molten medium was cooled to about 50oC temperature and poured into sterile Petri plates.

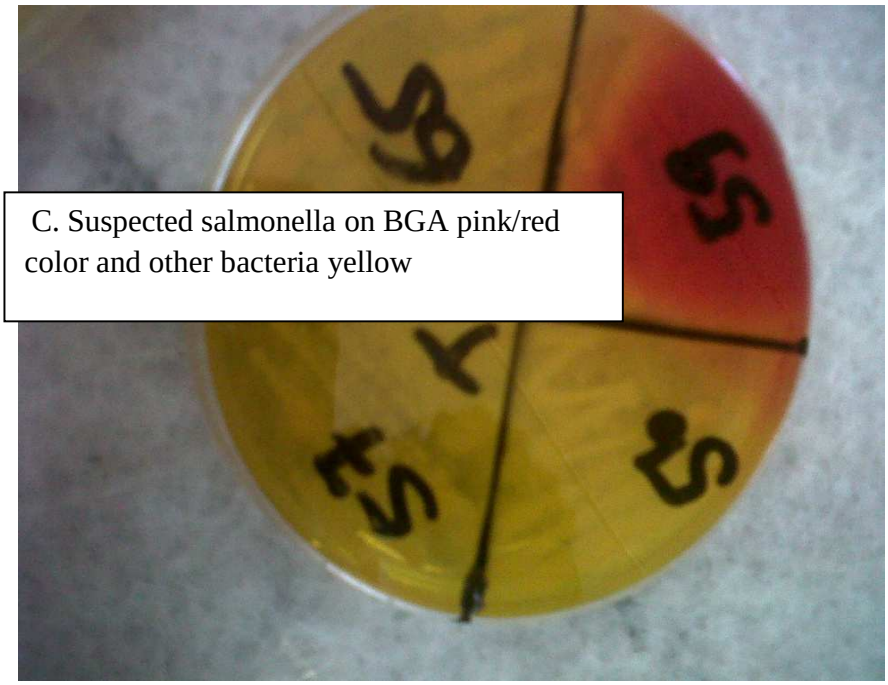
Appendix 9 list of some photos captured during laboratory works



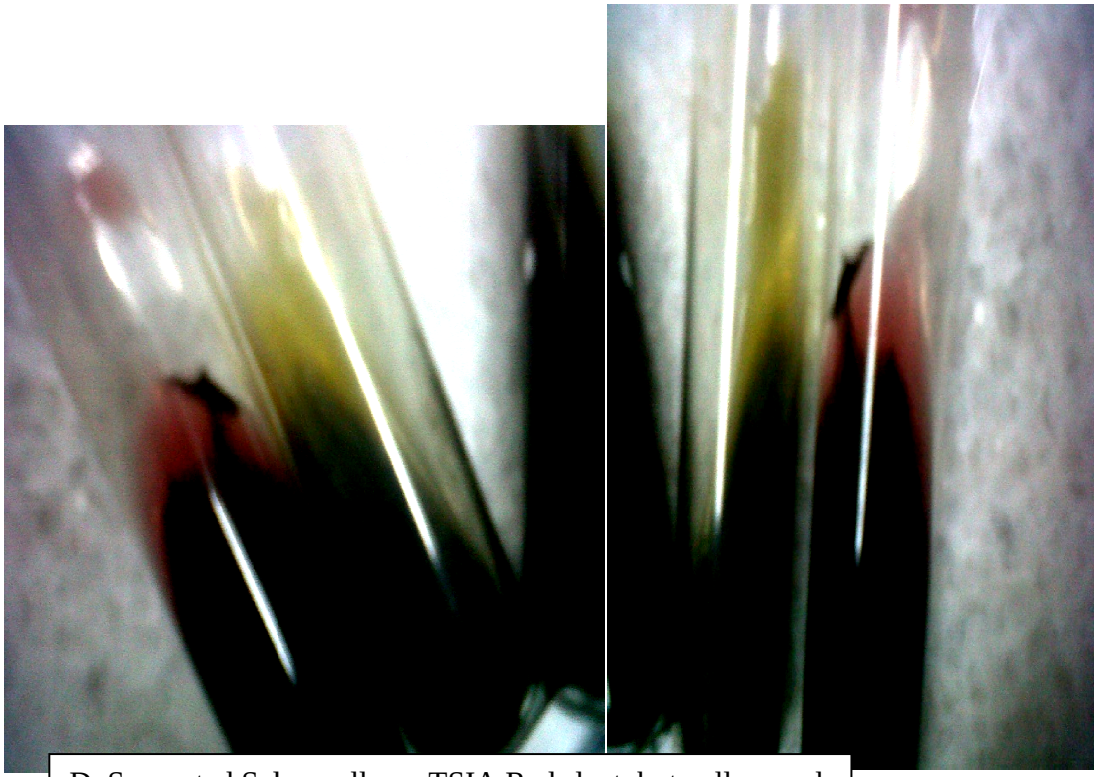
A Photo of drug sensitivity test bacterial isolates



*B.*Suspected *E. coli* on EMB showed Metallic sheen appearance

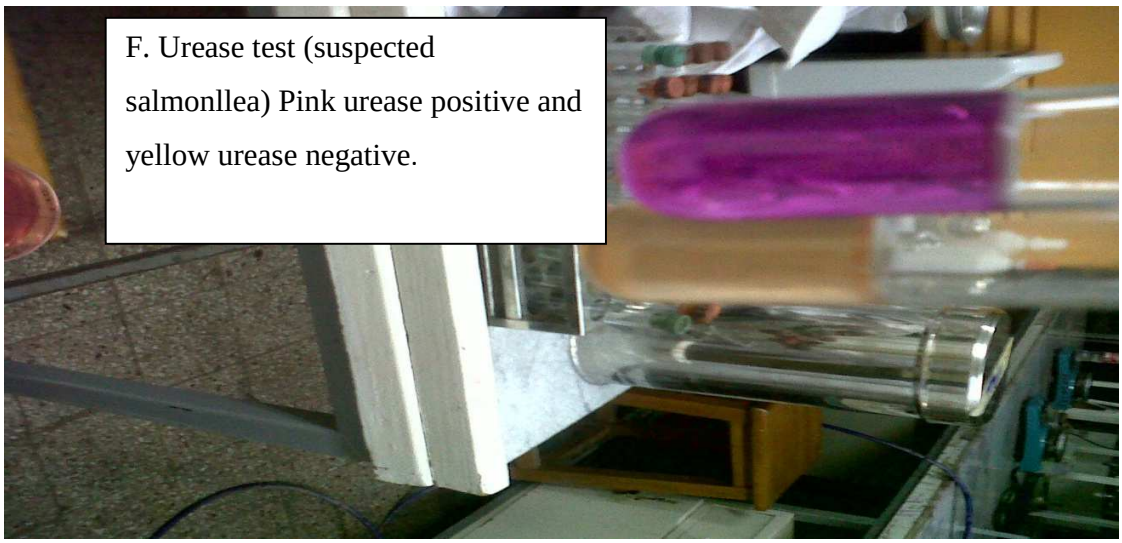


C. Suspected salmonella on BGA pink/red color and other bacteria yellow



D. Suspected Salmonella on TSIA Red slant, but yellow and H_2S

E.Suspected Salmonella on XLD medium



F. Urease test (suspected salmonllea) Pink urease positive and yellow urease negative.

G. Photo of Sugar testes Red positive and yellow positive and negative for the tested sugar

H. photo of Citreat utilization test blue positive and green negative

F. Photo of some of the study calves