CLINICAL CHARACTERIZATION, DETECTION OF CANINE PARVOVIRUS AND BACTERIAL CO-INFECTIONS, AND EVALUATION OF TREATMENT OUTCOMES IN CLINICALLY SUSPECTED PUPPIES AT AAU-CVMA VETERINARY TEACHING HOSPITAL, BISHOFTU, ETHIOPIA

MVS THESIS

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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 clinical medicine

By

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TITLE: CLINICAL CHARACTERIZATION, DETECTION OF CANINE PARVOVIRUS AND BACTERIAL CO-INFECTIONS, AND EVALUATION OF TREATMENT OUTCOMES IN CLINICALLY SUSPECTED PUPPIES AT AAU-CVMA VETERINARY TEACHING HOSPITAL, BISHOFTU, ETHIOPIA

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And recommend that it be accepted as fulfilling the thesis requirement for the degree of masters in veterinary clinical medicine

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LIST OF ABBREVATIONS

ALT	Alanine Transaminase
AST	Aspartate Transaminase
CBC	Complete Blood Count
CPV	*Canine parvovirus*
CRT	Capillary Refill Time
DNA	Deoxyribose Nucleic Acid
MLV	Modified Live Vaccines
PCV	Packed Cell Volume
SQ	Subcutaneous
SIRS	Systemic Inflammatory Response Syndrome
PCR	Polymerase Chain Reaction

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ABSTRACT

Canine parvovirus (CPV-2) remains a leading and potentially fatal viral disease in canine populations, especially in young puppies. The presence of co-infecting pathogens can exacerbate disease severity and complicate clinical outcomes. This study aimed to detect CPV, characterize clinical signs, identify major bacterial co-infections, and assess treatment outcomes in puppies clinically suspected of CPV infection. A case-series study was conducted over eight months (October 2024 to May 2025) at AAU-CVMA, Professor Fisseha Gebreab Memorial Veterinary Teaching Hospital, Bishoftu, Ethiopia. Thirty fecal samples were purposively collected from diarrheic puppies suspected of CPV-2 infection. Samples were analyzed using microbiological methods and conventional PCR. All the examined puppies exhibited diarrhea, vomiting, and anorexia; 60% (18/30) showed bloody diarrhea. CPV was detected in 28 samples. Ten puppies tested positive for CPV alone, while co-infections with *Salmonella* or *Escherichia coli* were found in 16 cases, and two cases had both bacterial pathogens alongside CPV. One sample showed co-infection with *Salmonella* and *E. coli* without CPV, and one sample was negative for all tested pathogens. Following antibiotic and supportive treatment, five puppies died, while 25 recovered. The results demonstrate a high rate of CPV and frequent bacterial co-infections in diarrheic puppies, underscoring the complication of canine parvovirus infection with bacterial pathogens. This study highlights the need for further research on the prevalence and molecular characterization of CPV in the study area to improve diagnostic, treatment and prevention strategies.

Keywords: *Canine parvovirus; Clinical Signs; Diarrhea; E. coli; Salmonella, Puppies*

1. INTRODUCTION

Canine parvovirus (CPV) is a highly contagious and often fatal disease in dogs, mainly affecting puppies, characterized by acute hemorrhagic enteritis and myocarditis. CPV is caused by three variants of canine parvovirus type 2 (CPV-2a, CPV-2b and CPV-2c) which is a leading cause of morbidity and mortality in dogs globally (Goddard and Leisewitz 2010). CPV-2 emerged as a cause of acute canine enteritis in mid-to-late 1970s, possibly from another carnivore parvovirus (cats or other hosts), spreading rapidly and triggering outbreaks worldwide (Greene, 2012). All three variants are thought to have similar pathogenicity leading to indistinguishable clinical disease (Decaro and Buonavoglia, 2012). CPV-2 is ubiquitous and can survive in the environment for more than a year, enabling exposure of susceptible dogs to infected feces, vomitus, or fomites. The incubation period following natural or experimental exposure ranges from 4 to 14 days, and virus shedding starts a few days prior to the occurrence of clinical signs, progressively declining 3–4 weeks post exposure (Decaro *et al.*, 2005).

Clinically, there are two forms of CPV disease, the enteric form characterized by acute fever, lethargy, anorexia, vomiting, and bloody diarrhea; whereas another type identified as a cardiac form that scarcely finds in neonates causing the failure of the respiratory and cardiovascular manifestations, with the infestation in utero (Shima *et al*., 2015). Neurologic signs in puppies with parvoviral enteritis may result from hypoxia secondary to myocarditis, hypoglycemia, or intracranial thrombosis or hemorrhage. (Schaudien *et al*., 2010). Uncommonly, neurologic signs such as tremors and seizures are observed. Puppies with myocarditis may be tachypneic and have increased lung sounds as a result of congestive heart failure (Woldemeskel *et al*., 2011).

Leukopenia due to neutropenia and/or lymphopenia is the prominent hematological abnormality in *Canine parvovirus* due to the destruction of bone marrow precursors, the depletion of lymphoid tissues, and the increased demands of the massively inflamed intestinal tract. Lack of significant leukopenia (≥4,500/μL) or lymphopenia (≥1,000/μL) at 24 hours post admission had a 100% positive predictive value for survival. The severity of the leukopenia and a failure of leukocytes to increase within the first 48hours of treatment are both negative

prognostic indicators during CPV infection (Goddard *et al*., 2008). Although nonspecific, serum biochemistry abnormalities consistently include hypoproteinemia, hypoalbuminemia, hypoglycemia reflecting an interplay among severe mal-nutrition, septicemia, and/or the stress-induced activation of catecholamine, hypocalcemia, and electrolyte abnormalities such as hypokalemia, hyponatremia, hypochloremia, and hypomagnesemia (Li and Humm, 2015). Death is usually due to the complications of the severe dehydration, circulatory shock, suppression of immune system and depression of bone marrow (Dogonyaro, 2010).

Co-occurrence of multiple pathogens in fecal samples of dogs remains unclear, but pathogens can also interact with one another in determining or aggravating disease. It is therefore important to screen for multiple pathogens in feces of diarrheic puppies to gain more insight into the role co-infections might play (Bagshaw *et al*., 2014). Parasitic, protozoal, bacterial and viral co-pathogens, all contribute to the development and severity (Savigny and Macintire, 2007). Other severe gastrointestinal infections such as salmonellosis can also cause diarrhea, so it is not specific for diagnosis. Therefore it is difficult to determine the cause of diarrhea without confirmatory diagnosis (Kalli *et al*., 2010). *E. coli* can also be associated with diarrhea in dogs, but fecal presence of these bacteria in dogs with diarrhea varies between studies, since the studies doesn't show the same number (Marks *et al*., 2011). *Giardia* spp., and *Cystoisospora* spp. are parasitic enteropathogens and are often present in puppies, their claimed role as a causative agent however varies per study (Grellet *et al.*, 2014).

The prognosis for infected dogs with timely and proper treatment is generally good. Success rates of 75–80% have been demonstrated with outpatient treatment (Sarpong *et al*., 2017). Appropriate fluid therapy and maintenance of adequate blood glucose concentrations are the most critical aspect of treatment (Sykes *et al*., 2014). The ideal mode of fluid therapy is intravenous to provide rapid fluid replacement (Goddard and Leisewitz 2010). The management of CPV also involves treatment of secondary bacterial complications with antimicrobial drugs. Whenever possible, the patient should be hospitalized in isolation and provided additional supportive care (Mylonakis *et al*., 2016).

Despite extensive global research on canine parvovirus (CPV), there is a lack of sufficient data from Ethiopia, particularly concerning the confirmation of the causative agent in clinical cases,

identification of characteristic clinical signs, and isolation of co-infecting pathogens. Moreover, treatment outcomes in puppies receiving available antibiotics for secondary complications and supportive therapy remain inadequately explored. These gaps justify the need for this study, which aims to detect CPV in fecal samples, identify typical clinical signs, isolate major bacterial co-pathogens, and assess treatment outcomes.

General Objective

- To detect canine parvovirus (CPV), characterize clinical signs, identify major bacterial co-infections, and assess treatment outcomes in clinically suspected puppies presented to AAU-CVMA Veterinary Teaching Hospital.

Specific objective

- To detect canine parvovirus (CPV) in fecal samples from clinically suspected puppies using conventional PCR
- To identify characteristic clinical signs associated with CPV infection
- To isolate major bacterial pathogens co-infecting puppies with CPV
- To evaluate the treatment outcomes in puppies received different antibiotics and supportive therapies

2. LITERATURE REVIEW

Canine parvovirus (CPV), caused by three variants of canine parvovirus type 2 (CPV-2; family Parvoviridae, Genus Parvovirus), is a leading cause of morbidity and mortality in dogs globally (Goddard and Leisewitz 2010). CPV-2 emerged as a cause of acute canine enteritis in mid-to-late 1970s, possibly from another carnivore parvovirus (cats or other hosts), spreading rapidly and triggering outbreaks worldwide (Greene, 2012). All three variants (CPV-2a, CPV-2b and CPV-2c) are thought to have similar pathogenicity leading to indistinguishable clinical disease (Decaro and Buonavoglia, 2012). CPV-2 is ubiquitous and can survive in the environment for more than a year, enabling exposure of susceptible dogs to infected feces, vomitus, or fomites. The incubation period following natural or experimental exposure ranges from 4 to 14 days, and virus shedding starts a few days prior to the occurrence of clinical signs, progressively declining 3–4 weeks post exposure (Decaro *et al.,* 2005).

The etiologic agent of canine parvoviral enteritis is canine parvovirus (CPV), a non-enveloped, single-stranded DNA virus belonging to the family Parvoviridae, genus Parvovirus. Two types of CPV have been identified: CPV-1 and CPV-2. CPV-1 causes reproductive problems and mild diarrhea, whereas CPV-2 is responsible for hemorrhagic gastroenteritis and myocarditis in puppies that are six weeks to six months of age. CPV-2 is gradually being replaced in the canine population by new antigenic variants or biotypes, designated CPV-2a and CPV-2b (Decaro and Buonavoglia, 2012) and by a third biotype, CPV-2c, which has been increasing since its identification in 2001 (Hoelzer & Parrish 2010) Parvoviruses are small (diameter of 25 nm), none enveloped viruses infecting vertebrates and insects. The parvovirus virion consists of a spherical capsid, which is composed by three proteins and contains a linear, single strand DNA molecule (Decaro and Buonavoglia, 2012). Virus replication takes place in the cell nuclei and requires rapidly dividing cells of fetuses and newborns or of hematopoietic and intestinal tissues of young and adult animals. Their replication in vivo is rarely associated with the appearance of nuclear inclusion bodies, whereas the cytopathic effect induced in vitro is not always evident.

All parvoviruses are highly stable in the environment, as they are extremely resistant to pH and temperature changes and to treatment with lipid solvents, trypsin and most disinfectants. Virions can be inactivated by formalin, sodium hypochlorite, beta propiolactone, hydroxylamine, oxidizing agents and ultraviolet irradiation (Muzyczka and Berns, 2001). Canine parvovirus is the most widely recognized cause of transmissible viral diarrhea in dogs and one of the most common infectious diseases of dogs worldwide. It is a highly contagious disease that can transmit from the fecal waste of diseased individuals, whose severity can alter from slight to over 90% if the individuals do not get treatment or receive any faulty treatment (Hassan *et al*., 2017). Canine parvovirus (CPV) represents a common viral cause of acute enteritis in dogs (Han *et al*., 2014). It is an important cause of morbidity and mortality in puppies younger than 6 months (McCaw and Hoskins, 2006). As parvoviruses require cells with a high proliferation rate for replication they have a high affinity for the small intestine, bone marrow, and lymphatic tissues (Goddard *et al*., 2008). The virus is highly contagious and can be transmitted from fecal-oral route as well as inanimate objects. The clinical symptoms associated with CPV-2 are often non-specific and often signs of depression, lethargy and fever are observed (Sime *et al*., 2015).

Although severe clinical disease typically occurs in dogs younger than 6 months of age, adults with insufficient immunity may potentially be affected (Markovich *et al*., 2012). CPV-2 infected dogs gradually develop fever at the early stage and the progression of infection results in vomiting and diarrhea. The consistency of stool varies and it appears yellow or may contain blood. The clinical symptoms usually develop from 3 to 5 days of infection and lasts for 5–7 days (Tuteja *et al*., 2022). Puppies under one year of age are highly susceptible to gastrointestinal disorders resulting in diarrhea (Duijvestijn *et al*., 2016). Diarrhea is classified as acute (3-7 days), prolonged (8-13 days) and chronic (14-21 days) based on its duration. Chronic intermittent or persistent diarrhea is a common clinical sign in dogs with chronic enteropathy and might be a manifestation of gastrointestinal or extra gastrointestinal disorders (Volkmann *et al*., 2017). Most acute diarrhea cases, such as dietary diarrhea, are mild and self-limiting and likely associated with changes to the intestinal microbiota. However, a thorough and logical diagnostic plan must be followed to obtain an accurate diagnosis and guide appropriate therapy (Armstrong and Intervention, 2013). Despite its multifactorial etiology,

diarrhea may occur due to infectious diseases such as zoonotic and/or non-zoonotic endoparasites including Toxocara canis, protozoans such as Isospora spp., viruses including Canine Parvovirus (CPV) and Canine Coronavirus, bacteria, as well as dietary changes (Candellone *et al*., 2020).

Clinical symptoms such as anorexia, weight loss, abdominal pain, vomiting, watery to hemorrhagic diarrhea, mild fever, and prolonged or shortened CRT which may indicate hyperdynamic status as a result of pain, fever, and/or infection and dehydration have been reported in dogs (Hall, 2011). Diarrhea is a clinical sign characterized by a decrease of fecal consistency, leading to loose or liquid stools, by an increase in the frequency of evacuations in 24 h, with or without fever or vomiting (Guarino *et al*., 2018). There are four major pathophysiologic mechanisms that can result in diarrhea, although more than one mechanism can contribute to diarrhea simultaneously. Osmotic Diarrhea, Secretory Diarrhea, Increased Mucosal Permeability and Deranged Motility are the four types of pathophysiology of diarrhea (Marks, 2012).

Along with these non-specific clinical symptoms, findings related to systemic inflammatory response syndrome (SIRS), especially due to viral agents such as CPV, can be observed (Brady and Otto, 2001). Dog suffers from severe dehydration as a result of hemorrhagic diarrhea and vomiting that usually starts after 24 hrs of infection (Tuteja *et al*., 2022). The clinical manifestations of CPV-2 infection are non-specific or referable to enteritis, commonly including anorexia or lethargy, weakness, depression, foul-smelling diarrhea, which may range from mucoid to purely hemorrhagic, vomiting, dehydration, and fever (Kalli *et al*., 2010).

Diagnosis on the basis of clinical signs is not definitive, since several other pathogenic organisms can cause diarrhea in dogs. Various laboratory methods have been developed to detect CPV-2 in the feces of infected dogs, for example electron microscopy immunosorbent (EM), assay enzyme linked (ELISA), immunochromatographic tests (IC), viral isolation (VI), haemagglutination inhibition (HI) tests, conventional polymerase chain reaction (CPCR) and real-time polymerase chain reaction (RT-PCR) (Desario *et al.,* 2005). The most accurate method for diagnosis of canine parvovirus infection is the use of polymerase chain reaction on fecal samples which is highly recommended (Saasa *et al*., 2016). In many small animal clinics,

canine parvovirus is diagnosed based on the clinical signs and some can rely on the rapid CPV Test kits (Tinky *et al*., 2015). Confirmation of the diagnosis via necropsy can be made using histopathology to identify characteristic lesions or via immunofluorescence tissue testing (Greene and Decaro 2012).

Although treatment options are supportive, effective immunization is essential for the protection of the individual pet and the decrease of the population of susceptible animals in a region, thus promoting the "herd immunity" (Day *et al*., 2016). Modified live vaccines (MLVs) are currently used worldwide affording prolonged (7 years or longer) immunity that would confer protection against both disease and infection (Schultz *et al*., 2010).

2.1 Clinical Signs of Canine parvovirus

Clinically, there are two forms of CPV disease, the enteric form characterized by acute fever, lethargy, anorexia, vomiting, and bloody diarrhea; whereas another type identified as a cardiac form that scarcely finds in neonates causing the failure of the respiratory and cardiovascular manifestations, with the infestation in utero (Shima *et al*., 2015). Neurologic signs in puppies with parvoviral enteritis may result from hypoxia secondary to myocarditis, hypoglycemia, or intracranial thrombosis or hemorrhage. (Schaudien *et al*., 2010). Uncommonly, neurologic signs such as tremors and seizures are observed. Puppies with myocarditis may be tachypneic and have increased lung sounds as a result of congestive heart failure (Woldemeskel *et al*., 2011).

The clinical manifestations of CPV-2 infection are nonspecific or referable to enteritis commonly including anorexia or lethargy, weakness, depression, foul-smelling diarrhea, which may range from mucoid to purely hemorrhagic, vomiting, dehydration, and fever (Kalli *et al*., 2010). Leukopenia due to neutropenia and/or lymphopenia is the prominent hematological abnormality in *Canine parvovirus* due to the destruction of bone marrow precursors, the depletion of lymphoid tissues, and the increased demands of the massively inflamed intestinal tract. Leukopenia is a common finding in dogs with CPV infection, though is not present in all infected dogs. Lack of significant leukopenia (≥4,500/μL) or lymphopenia (≥1,000/μL) at 24 hours postadmission had a 100% positive predictive value for survival. The severity of the

leukopenia and a failure of leukocytes to increase within the first 48hours of treatment are both negative prognostic indicators during CPV infection (Goddard *et al*., 2008).

Other clinic-pathologic findings that can occur includes anemia, coagulopathies, electrolyte imbalances, and hypoalbuminemia (Greene and Decaro, 2012). The dehydration status that follows parvovirus infection results in increased liver enzymes activity, which is predispose to the occurrence of acute kidney injury. Early kidney damage was observed in canine parvovirus infection accompanied by elevation of blood urea nitrogen and creatinine (Van *et al*., 2018). There was an improvement in the results of the hemoglobin, erythrocytes, PCV, leucocytes, neutrophils and lymphocytes in blood samples after therapy compared to that before therapy. The virus's impact on the bone marrow, coupled with gastrointestinal bleeding, leads to decreased red blood cell production and increased destruction, contributing to the development of anemia (Decaro *et al.,* 2006). Anemia per se, is not a predictive biomarker for CPV but the degree of anemia can assist veterinarians to tailor supportive care, such as blood transfusions, accordingly (Decaro *et al.,* 2006). The serum albumin, globulin, serum ALT, AST, alkaline phosphatase and creatinine values were significantly returned to normal values as in clinically healthy dogs (Craven *et al*., 2011).

Although nonspecific, serum biochemistry abnormalities consistently include hypoproteinemia, hypoalbuminemia, hypoglycemia reflecting an interplay among severe mal-nutrition, septicemia, and/or the stress-induced activation of catecholamine, hypocalcemia, and electrolyte abnormalities such as hypokalemia, hyponatremia, hypochloremia, and hypomagnesemia (Li and Humm, 2015). Death is usually due to the complications of the severe dehydration, circulatory shock, suppression of immune system and depression of bone marrow (Dogonyaro, 2010). Necropsy can also help to diagnose infection as gross pathologic findings include thickened and discolored intestinal walls with rough serosal surfaces. These changes are typically worst in the distal duodenum for dogs in the early stage of disease while findings are more pronounced in the jejunum for more severely infected dogs (Greene and Decaro, 2012).

2.2 Major Pathogen Complications

Gastrointestinal signs are one of the most common clinical presentations of disease in shelter dogs. The disease can result from a multitude of causes and in many cases it is multifactorial. However, common etiologies for gastrointestinal disease in shelter dogs are infectious agents or enteropathogens. Canine Parvovirus-2 is one of the most common and significant enteropathogens in dogs (Doyle, 2021). Long term excretion of CPV is major source of infection among dogs. *E. coli* are considered as one of the major pathogens and found as natural commensals in gastrointestinal tract (Kumar *et al*,. 2014). Moreover, this virus is considered to be an important pathogen responsible for acute gastroenteritis and myocarditis in dogs (Mylonakis *et al*., 2016). Puppies are the most susceptible to canine parvovirus infection due to lack of protective immunity from maternally derived antibodies or from ineffective responses to vaccinations (Patterson, 2007). Escherichia coli, Serratia spp., Actinobacter anitratus, Klebsiella pneumoniae, Enterobacter spp., Staphylococcus intermedius and Streptococcus spp. can be found with CPV infection as the result of bacteria in gastrointestinal tract penetrated into the blood stream or nosocomial infection (Prittie, 2004).

2.3 Treatment Outcomes

Survival rate may be as low as 9% if no treatment is under-taken but may exceed 80% in tertiary care facilities (Mylonakis *et al*., 2016). Treatment for CPV is largely supportive and symptomatic. The principal components of treatment include 1) fluid therapy, 2) antibiotic treatment, 3) antiemetic treatment, and 4) nutritional support. An array of other treatment measures including, though not limited to, antiviral treatments and pain management have been assessed in the past or are currently under investigation regarding their potential utility in *Canine parvovirus* (Mylonakis *et al*., 2016).

Ultimately, the core components of a treatment plan include isolation, fluid support, enteral nutrition, anti-emetics, antibiotics, deworming, and analgesia. Close monitoring of infected dogs is vital to promptly adjust the treatment plan when needed. Euthanasia decisions are indicated for dogs that fail to respond to treatment or for shelters without the resources or transfer options to provide safe and humane treatment. (Doyle, 2021). Recovered dogs typically

develop life-long immunity to CPV but routine vaccination is still recommended to protect against other vaccine preventable diseases (Schultz *et al*., 2010).

2.3.1 Fluid therapy

Water is the most important nutrient for dogs with acute diarrhea, because of the potential for life-threatening dehydration due to excessive fluid and electrolyte losses. Oral fluid therapy, containing glucose, amino acids, and electrolytes, is typically reserved for non-vomiting patients with minor fluid deficits, or to supply maintenance fluid requirements in addition to water (Davenport *et al*., 2010). Maintenance of hydration and oncotic support as well as correction of acid–base and electrolyte disturbances are of utmost importance in *Canine parvovirus* treatment. Since subcutaneous fluid absorption is impaired in dehydrated animals, venous access is the cornerstone of fluid treatment (Lobetti *et al*., 2012).

Puppies admitted with severe hypovolemia need reestablishment of their circulating volume in 1–2 hours. As a rule, a balanced isotonic crystalloid solution (eg, Lactated Ringers) is the fluid of choice for initial restoration of intravascular volume and rehydration, with a rate titrated to improve perfusion parameters, including capillary refill time, mucosal color, pulse character, and mean arterial pressure or lactate concentrations (Anastasio *et al*., 2014). Crystalloid fluids are the standard fluid choice. However, colloid fluid support, such as with hydroxyethyl starch, may be indicated in cases of significant hypoalbuminemia or with signs associated with third space loss (Leisewitz, 2017). The physiologic basis for these solutions is the coupled transport of sodium and glucose, and other actively transported small organic molecules. However, such solutions are most beneficial in secretory diarrheas, which are quite uncommon in dogs (Mortier *et al*., 2015).

The volume of fluid administered should be calculated based on percent of dehydration at presentation and ongoing maintenance fluid needs. Dosage charts for SQ fluids are a helpful component of a written CPV treatment protocol, but flexibility in the protocol is necessary to adjust the treatment plan if fluids are not fully absorbed prior to the next treatment (Leisewitz, 2017). In dogs admitted without evidence of hypovolemic shock, hydration may be restored over12–24 hours. The daily fluid allowances should incorporate the maintenance

requirements, the current fluid deficits (body weight [kg] × % dehydration = volume [L] to correct), and the ongoing losses (might be subjectively estimated to 250 mL) (Davis *et al*., 2013).

2.3.2. Antibiotic treatment

Puppies with enteritis in combination with neutropenia are prone to become septic. Therefore, aggressive intravenous, broad-spectrum, bactericidal antibiotic treatment is part of the therapy (Doyle, 2021). Parenteral administration of wide-spectrum bactericidal antibiotics is warranted in dogs with severe *Canine parvovirus* due to the high risk of septicemia associated with the disruption of the mucosal barrier and the concurrent profound neutropenia (Prittie, 2004). Ampicillin and cefoxitin as single-agent treatments or in combination with enrofloxacin are rational empirical choices offering protection against Gram-positive, Gram-negative, and anaerobic organisms (Abrams, 2012). Broad spectrum antibiotic administration is indicated in CPV cases given the potential for bacterial translocation and reduced immune function. Cefovicin is another ideal antibiotic choice because of its broad spectrum efficacy and its ease of administration as a single-dose injection. Intravenous antibiotics such as ampicillin and amikacin are effective choices when intravenous access is present (Leisewitz 2017).

Oral antibiotics that are commonly used include amoxicillin and metronidazole. However, oral medication is often best postponed until vomiting has ceased to ensure adequate absorption. Though commonly utilized, metronidazole's efficacy against *E. coli* in an anaerobic environment may be limited (Pendland *et al*. 2002). As such, metronidazole would not be an appropriate single-agent antibiotic choice and may be better utilized when co-infection with flagellate parasites is diagnosed or suspected. Broad-spectrum treatment for intestinal helminths, if not previously administered, is an important adjunct treatment in all cases to reduce comorbidities (Doyle, 2021).

2.3.2 Nutritional support

Nutritional support is another cornerstone of treatment. Early enteral nutrition has been shown to reduce the duration of clinical signs and encourage weight gain during treatment (Mohr *et al*., 2003). Enteral feeding is associated with improved mucosal integrity, faster repair, and

as a result, reduced possibilities for bacterial translocation (Veir, 2014). This was accentuated in a relatively recent study, in which early enteral nutrition via naso-esophageal catheter starting 12 hours postadmission was associated with earlier clinical improvement, significant weight gain, and possibly improved gut barrier function compared to dogs subject to the traditional food withholding until cessation of vomiting for 12 hours (Mohr *et al*., 2013). Administration of nutritional support via nasoesophageal tube is ideal when possible, but treatment protocols using syringe feeding of a high caloric diet can also be successful and are more realistic in most shelter settings. The administration of enteral nutrition is best accompanied by anti-emetics to reduce nausea and vomiting. Maropitant is an ideal first-line choice for antiemetic therapy based on its efficacy, ability to be administered subcutaneously and once-daily dosing (Doyle, 2021).

2.3.3 Antiviral treatments

There have been anecdotal reports of passive immunization using convalescent serum from dogs that have recovered from CPV infection (Prittie, 2004). Time to hematologic recovery, viral load, severity of clinical findings, and length of hospitalization were among the parameters that were not improved in a recent study when a single 12 mL dose of CPV-immune plasma was administered as an adjuvant treatment for *Canine parvovirus* following the onset of clinical signs. (Bragg *et al*., 2012). However, a beneficial effect may still be possible to achieve if a larger volume of plasma is given prior to the occurrence of the clinical signs (Day *et al*., 2016).

2.3.4 Anti-pain drugs

Analgesia is also an important consideration during *Canine parvovirus* treatment as abdominal discomfort can be severe. Opioid therapy is the best choice for analgesia in CPV cases due to its safety profile and efficacy. Because opioids are a controlled substance (Bragg *et al*., 2012).

3. METHODOLOGY

3.1 Description of Study Area

The study was conducted in Bishoftu town of the East Shoa zone, Central Oromia, Ethiopia. Bishoftu town is situated at an elevation of 1,850 meters above sea level and is 47 kilometers southeast of Addis Ababa at latitude 9° N and longitude 40° E. With an average annual rainfall of 866 mm, the town experiences a bimodal rainfall pattern, with lengthy rains beginning in June and lasting until September, brief rains beginning in March and May, and dry seasons beginning in October and lasting until February. The average annual temperature is 18.7°C, with the mean minimum and maximum temperatures being 12.3°C and 27.7°C, respectively. 61.3 percent is the average relative humidity. (Anon, 2011).

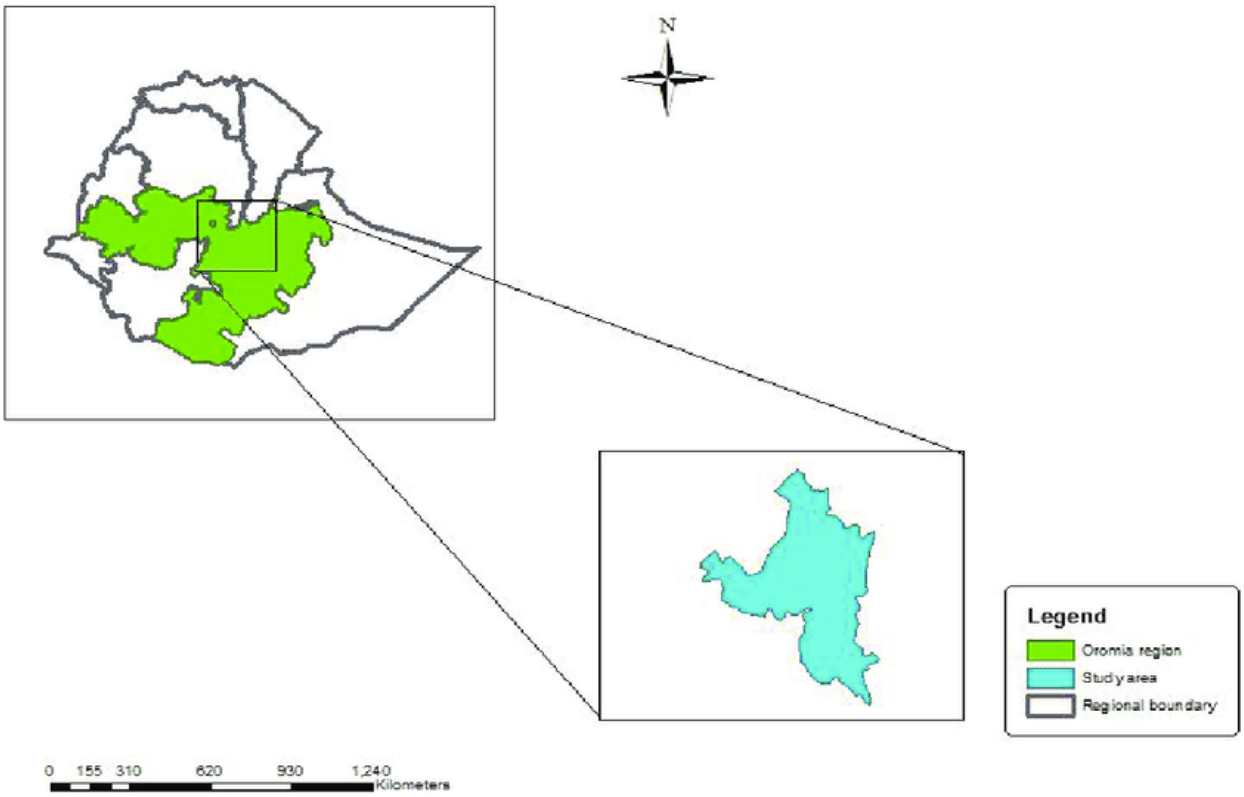


Figure 1 Map of study area Bishoftu, Ethiopia (Abunna *et al*., 2018)

3.2 Study animals

The study animals were puppies suspected of having CPV (canine parvovirus) and were admitted to Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA), Professor Fisseha Gebreab Memorial Veterinary Teaching Hospital. Puppies of different breeds and both sexes, less than six (6) months of age were included in the study.

3.3 Study Design and Sampling Method

A case-series study was conducted for a period of eight months, from October, 2024 to May, 2025 at AAU-CVMA, Professor Fisseha Gebreab Memorial Veterinary Teaching Hospital. Puppies under six months of age, with history of diarrhea or vomiting, showing clinical signs such as diarrhea (with or without blood), fever, lethargy, weakness, and dehydration were purposively included in this study. Vomiting or diarrhea may occur during the examination, or there may be evidence of diarrhea (Kalli *et al*., 2010).

3.4 Sample Collection Technique

During the study period fecal swab was collected from each puppy twice one for viral sample and one for bacterial sample. Using sterile swab the sample was collected directly from the animal and inserted into a test tube containing viral transport media for CPV detection and peptone water for bacterial isolation and identification.

3.5 Sample Size Determination

The sample size was determined purposively based on the number of puppies admitted to the AAU-CVMA Professor Fisseha Gebreab Memorial Veterinary Teaching Hospital during the eight-month study period. Accordingly, 30 puppies that met the inclusion criteria were included in the study.

3.6 Clinical sign observation

Clinical signs which are associated with *Canine parvovirus* suspected puppies was observed and recorded during the entire study period. Each and every clinical signs was observed and recorded by observation and history from the owner of the puppies. The clinical signs were bloody diarrhea, different consistency of diarrhea, vomiting, dehydration, anorexia and depression, they were measured based on their severity and their duration. Also vital parameters such as temperature, heart rate and respiratory rate were recorded.

3.7 Treatment protocol

Treatment was initiated based on history and clinical signs with antibiotics and supportive drugs. For this treatment protocol three different antibiotic drugs namely Sulfamethazine, Pen strep and Enrofloxacin were used in three groups of puppies. The puppies was systematically allocated to one of the three antibiotic treatments as the first puppy treated with Sulfamethazine, the second puppy with Pen strep and the third puppy with the third drug which is Enrofloxacin. The dose of the antibiotics were administered according to the prescription. In addition to antibiotic therapy, supportive treatments were administered based on clinical needs, including fluid therapy to address dehydration from diarrhea and vomiting, vitamin B complex to counteract anorexia, and 40% glucose to provide nutritional support for puppies that were inappetant or refused to feed. For treatment outcomes, puppies were closely monitored following therapy to assess clinical improvement. The duration of clinical signs, observable changes in symptoms, and overall recovery progress were recorded for subsequent analysis.

3.8 Laboratory Analysis

During this study period different laboratory works were performed depending on their importance. These laboratory procedures are viral and bacterial identification to see some major pathogen complications. During this study period each and every laboratory analysis findings was recorded and prepared for further analysis. Microbiology laboratory and molecular laboratory was used for bacteria and virus respectively.

3.8.1 Isolation and identification of bacteria

The two major bacterial species complicating with parvo virus on puppies, *Salmonella* and *E. coli* was isolated and identified using appropriate sample collection and identification procedure. Fecal samples was collected directly from the rectum of the puppies by using sterile swab and transferred to a prepared sample collecting material which is labeled to identify the puppies. They are inoculated into a non-selective broth medium, Buffered Peptone Water (BPW), transported to laboratory and incubated at 37°C for 24 hours (Karolenko *et al*., 2020).

To isolate and identify *E. coli* species the fecal samples was enriched in selective broths MacConkey broth to suppress non-lactose fermenting bacteria. Enriched cultures are then plated onto selective and differential media Eosin Methylene Blue (EMB) agar. *E. coli* tentatively identified based on its colony morphology on selective and differential media Eosin Methylene Blue (EMB) agar. The colonies typically appear as dark blue-black with a distinctive metallic green sheen due to their ability to ferment lactose and produce acid (Cheesbrough, 2006). After that biochemical test such as indole test, methyle red test, voges-proskauer test and citrate test were performed and only those sample showing positive for indole test and metyle red test and negative for voges-proskauer test were recorded as positive for *E. coli*.

In case of *Salmonella*, after a loopful of pre-enriched broth was transferred to a selective enrichment broths, Rappaport-Vassiliadis broth and incubated at 42°C for 18-24 hours. Finally, a loopful of enriched broth was streaked onto selective and differential agar plate XLD, *Salmonella* colonies appear as pink with black centers (Prasanna, 2024). After that biochemical test were performed and the samples were taken as *Salmonella* positive when the sample shows negative for indole test, positive for methyle red, negative for voges-proskauer and positive for citrate test.

3.6.2 Isolation and detection of Canine parvovirus

From each *Canine parvovirus* suspected puppies which are 30 in number, fecal swab was taken by using sterile swab and transported to laboratory by using virus transport media after labeling. For each sample every step of the Polymerase Chain Reaction (PCR) procedure was

followed to detect the virus in the feces. Those procedures include DNA extraction, amplification and detection. First the collected sample was extracted then amplified in the DNA amplifying room and finally the virus was detected by using gel electrophoresis in detection room. DNA extraction was performed with Spin Tissue Mini Kit for total DNA extraction. Amplification was performed using *Taq* DNA polymerase, employing the follow thermal profile: initial denaturation of 95ºC for 5 min, 94ºC for 30s secondary denaturation, 55ºC for 40s annealing, 72°C for 45s extension and final extension 72°C for 10 min with 35 number of cycle. Amplified products were purified with a purification kit and were sequenced with both forward and reverse primers with an automated sequencer according to the instructions. The primers used where 555_for 5'-CAGGAAGATATCCAGAAGGA-3' and 555'_rev 5'-GGTGCTAGTTGATATGTAATAAACA-3 that amplify a product of 583bp (Buonavoglia *et al*. 2001).

Table 1 Cycling conditions of the different primers during PCR

Gene	Primer denaturation	Secondary denaturation	Annealing	Extension	Final extension	No of cycle
VP2	95˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	35

Table 2 Oligonucleotide primers sequences (Metabion , Germany).

Virus	Gene	Primers	Primer/ probe sequence 5'-3'	Amplified Segment (bp)
Canine parvo	VP2	Screening primers	Hfor: CAGGTGATGAATTTGCTACA	630
			Hrev: CATTTGGATAAACTGGTGGT	
		Sequencing primers	Pbs: CTTTAACCTTCCTGTAACAG	427
			Pbas: CATAGTTAAATTGGTTATCTAC	

3.9 Data Analyze and management

The recorded data was stored on excel and then coded on SPSS for descriptive analysis. Using SPSS the descriptive analysis such as frequency and percentage was conducted to describe frequency of clinical signs observed, percentage of pathogens identified and treatment outcomes. The software also used to construct bar graphs to show the results frequency and percentage.

3.10 Ethical Clearance

After providing a detailed information about the research how to conduct clinical sign recording, how to collect sample in aseptic way and how to treat the puppies to the ethical clearance committee of Addis Ababa University and their comment, the ethical clearance approval sheet was provided as attached in the annex part.

4. RESULTS

4.1 Clinical Signs

As shown in Figure 2 below, all 30 puppies (100%) suspected of canine parvovirus presented with diarrhea, whith varying consistency from watery and mucoid to bloody.The frequency of diarrhea ranged from once to twice daily, with durations lasting from one to three days. Vomiting was the most commonly observed clinical sign, present in all puppies, and proved to be the most challenging symptom to manage despite treatment with anti-emetics. Vomiting lasted only one day in 16 puppies, persisted for two days in 14 puppies, and continued for three consecutive days in 4 puppies. Dehydration was another common clinical sign, observed at varying degrees from mild to severe based on clinical indicators. Although all puppies exhibited dehydration on the first day of admission, only 12 puppies (40%) remained dehydrated by the second day of treatment, and this number further decreased to 3 puppies (10%) by the third day.

All puppies (100%) exhibited anorexia, which persisted for at least three consecutive days, including the day of admission and the second day of treatment. By the third day of treatment, only 9 puppies (30%) had recovered from anorexia. Regarding bloody diarrhea, 18 puppies (60%) showed this symptom, with duration ranging from one to three days. Of these, only 5 puppies (27.7%) continued to have bloody diarrhea on the second day of treatment, and by the third day, only one puppy (5.5%) remained affected. These numbers exclude puppies with diarrhea that was merely tinged with blood. Vital signs, including body temperature and respiratory rate, varied from 37.4 degrees Celsius to 40.5 degrees Celsius and 24 to 36 breaths per minute, respectively. No significant changes were observed during the entire follow-up period, as vital signs generally returned to normal by the second or third day of treatment without notable fluctuations.

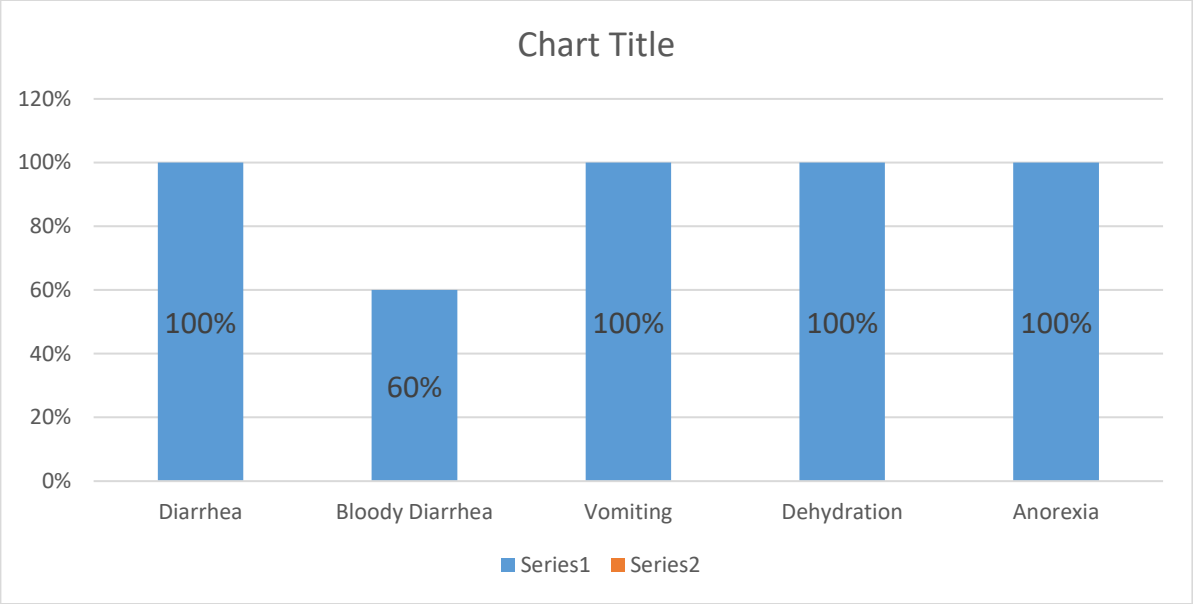


Figure 2 Frequency of clinical signs observed

4.2 Treatment Outcome

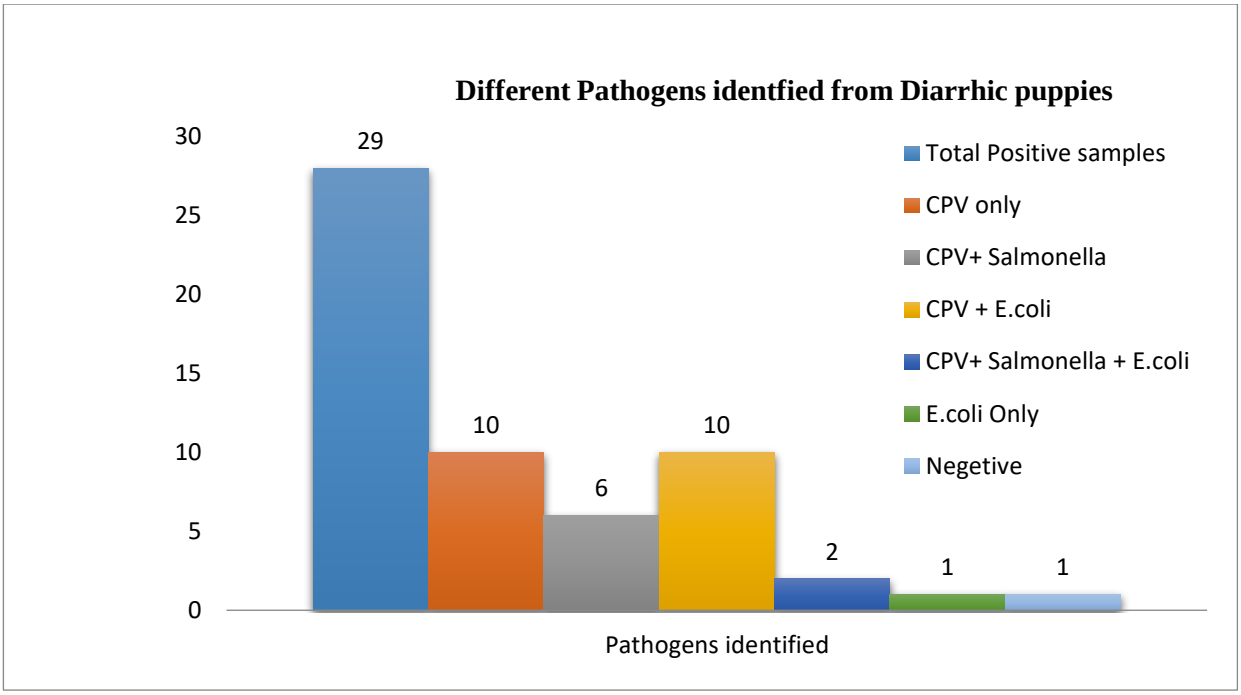
The prognosis for viral enteritis in puppies varies depending on the severity of the illness and the owner's ability to provide appropriate treatment. As presented in the table 3 below, out of 30 treated puppies, 25 (83.3%) recovered, while 5 (16.7%) died. The puppies were divided into three treatment groups based on the antibiotic administered. In the first group, treated with sulfamethazine, only one puppy died, resulting in a 90% recovery rate. Similarly, in the second group, treated with penicillin-streptomycin (pen strep), one puppy died, also yielding a 90% recovery rate. In contrast, the third group, treated with enrofloxacin, experienced a higher mortality rate, with three puppies (30%) dying and seven (70%) recovering. Comparatively, sulfamethazine and penicillin-streptomycin showed better outcomes with a 90% recovery rate and only 10% case fatality, while enrofloxacin had a lower recovery rate of 70% and a higher fatality rate of 30%.

Table 3 Treatment outcome results

Antibiotic	Treated	Recovered	Died	Recovery rate	Fatality rate
Sulfamethazine	10	9	1	90%	10%
Pen strep	10	9	1	90%	10%
Enrofloxacin	10	7	3	70%	30%
Total	30	25	5	83.3%	16.7%

4.3 Pathogen Isolation and identification

During the entire study period, 30 diarrhea samples were collected from the suspected puppies, and laboratory procedures were performed to isolate and detect *Canine parvovirus, Salmonella, and E. coli*. Out of 30 samples examined, CPV was detected in 28 cases. Among the 30 fecal samples tested for the three pathogens, ten puppies were positive for only CPV, six were positive for CPV with *Salmonella* co-infection, and ten samples were positive for CPV with *E. coli* co-infection. Two puppies were positive for all three pathogens, as indicated in Figure 4 below. One puppy was negative for all the tests conducted, and one was positive for both *E. coli* and *Salmonella*.



NB: *Negative indicates that none of the three pathogens: CPV, Salmonella, and E. coli, were isolated from the sample.*

Figure 3 Graph of pathogens identified

5. DISCUSSION

Clinical signs such as diarrhea (with or without blood), frequent vomiting, varying degrees of dehydration, anorexia, and depression were recorded. These findings are consistent with those reported by Soliman et al. (2018) and Zaghawa and Abualkhier (2019). The diarrhea observed in this study varied in consistency, ranging from watery to mucoid and blood-tinged, which is in agreement with the observations of Vasantha (2011). Bloody diarrhea was one of the most clearly observed signs in 60% of dogs which disagree with the report of 40% of puppies (Saho *et al.,* 2007) in dogs with CPV infection. The difference is maybe due the difference of our sample size as the study includes 180 dogs and the inclusion criteria was different as they used all ages of dogs. Such diarrhea may be due to destruction and collapse of the germinal epithelium of the intestinal crypts and the resulting villous atrophy (Bastan and Kurtdede, 2013). Zaghawa and Abualkhier (2019) reported bloody diarrhea as 77.5% from 40 puppies which slightly disagree with the current study.

Vomiting was one of the major clinical sign in infected dogs in the current study reported in all 30 (100%) which developed early within 24 to 48 post infection and remains for one day or two and sometimes three which agrees the report of Soliman *et al.,* (2018). Miranda *et al* (2015) also reported vomiting is not indicative suggesting the case as they arise from other health factors which disagree with current study. Another study was reported vomiting as 66.7% by Akanbi *et al* (2024) which is not in accordance with the current study of 100%. Dehydration is another characteristics clinical sign manifested by *Canine parvovirus* suspected puppies observed in all the puppies which is recorded from mild to severe which in accordance with Greene and Decaro (2012). Zaghawa and Abualkhier (2019) also reported dehydration was one of the most characteristic clinical complication in their study.

The study showed anorexia in 30 (100%) of the puppies in the current study even remaining positive for almost three consecutive days which disagree with a finding of Akanbi *et al*,. (2024). In a combination of clinical signs diarrhea, vomiting, anorexia and dehydration recorded in all 30 puppies (100%) which disagree with the finding of Akanbi *et al*,. (2024) of anorexia, and diarrhoea in 66.7% (20/30), and anorexia and vomiting in 60.0% (18/30). A combination of diarrhea, vomiting, anorexia, dehydration and bloody diarrhea was observed

only in 60% (18/30), which is nor inaccordance with Akanbi *et al*,. (2024) reported ony in 40.0% (12/30).

In the current study a death of 5 puppies from 30 (16.7%) was recorded after treatment with antibiotic, fluid therapy, and vitamin B complex which is in alignment with Frazão (2008) and the finding disagree with Mantione & Otto (2005) who reported a lower percentage of mortality (approximately 8%) and a mortality of 35% which is greater than the current study by Monteiro *et al* (2016). The difference with the two reports may be arrived due to a sample size difference, inclusion criteria and a treatment protocol used by the different authors. On the other hand differences in mortality can be explained by the period of delay by owners bringing their animals to the hospital for starting treatment. Out of the 30 puppies, to give different antibiotics they were categorized randomly in to three groups each group containing ten puppies. The first ten group were treated by Sulfamethazine alongside with fluid therapy, anti-emetics and vitamin B complex and 9 of them completely recovered were only one puppy died. As the same, group two which are treated with pen strep and other supportive therapy 9 of them recovered and only one death recorded. The last ten puppies were treated with enrofloxacin showed a mortality of 3 puppies which is higher than the first two group and only 7 puppies were recovered.

In the present study which includes thirty (30) *Canine parvovirus* suspected puppies which are tentatively diagnosed as *Canine parvovirus* 100% by seeing the clinical signs they were showing during the admission at the clinic and some evident history from the owners of the animal in accordance with Akanbi *et al.,* (2024) who has reported 100% as *Canine parvovirus* tentatively too. The current study which is conducted on 30 *Canine parvovirus* suspected puppies showed a positive result for *Canine parvovirus* (CPV) of 28 (93.3%) by PCR using the CPV-2 primer indicating that presumptive clinical diagnosis is reasonably reliable considering PCR assay highly sensitive to detect CPV-2 more than other diagnostic techniques (Desario *et al.*, 2005) which is slightly disagree with a report of Miranda *et al* (2015) of 77.5% from a total of 162 samples. This slight difference may arise from the difference of the sample size with the current study, the inclusion criteria difference and the study period covered by the authors which is more than two years.

Another report by Zaghawa and Abualkhier (2019) declared an 87.5% out of 40 puppies which disagree with the current study slightly. No significant change in prevalence of CPV-2 infection between male and female dogs, the prevalence of CPV-2 infection was higher in males when compared with females. The above observations are in accordance with other similar reports (Vivek *et al.,* 2013). Akanbi *et al.,* (2024) reported his finding as 28 out of 30 by clinical signs which also disagree with a current sudy reportin 30 out 30 (100%).

The current study observed 13 out of 30 (43.3%) puppies were positive for *E. coli* after examination in laboratory by observing on different selective and differential media then confirming by atleast four biochemical test such Indole test, methyl red test, vogus proskauer and citrate test. Of the 13 *E. coli* isolates, 12 were isolated from the fecal samples found positive for parvo viral infection indicating the positive correlation between the *E. coli* and canine parvo viral infection. Among those only one puppy showed positive for *E. coli* without complicating with others. This positive correlation might be due to the immunosuppression caused by *Canine parvovirus* in the infected dogs. Moreover *E. coli* are common in feces of animals and experimental studies on pathogenesis of *E. coli* in presence and absence of Parvo virus infection are required to be conducted before drawing any conclusion on their role. This finding is not in accordance with the finding of Kumar *et al,*. (2014) which is Only 6 (25 %) were identified as *E. coli*. The similar findings were observed by Ingle *et al*. (2009) who reported 20 per cent of samples to be positive for *E. coli*. The difference is may be due to the sample size difference, the laboratory procedure followed or the inclusion criteria set by the authors.

In case of *Salmonella* complicating with *Canine parvovirus* (CPV) suspected the study find 8 out of 30 (26.6%) of puppies positive for *Salmonella* and from those 7 (23.3%) of them were correlated with *Canine parvovirus* (CPV) positive puppies. These findings were reported after different laboratory procedures of selective and differential media as well as biochemical tests. The result is in accordance with the report of Marks *et al,*. (2018) with the report of 22% from 74 sample size. None of the puppies showed positive result without complicating with other pathogens.

6. CONCLUSION AND RECOMMENDATION

This study comprehensively investigated the clinical characteristics, detection of Canine Parvovirus (CPV) and bacterial co-infections, and treatment outcomes in clinically suspected puppies presented to the AAU-CVMA Veterinary Teaching Hospital in Bishoftu, Ethiopia. Diarrhea (with or without blood), vomiting, anorexia, and dehydration were frequently observed as characteristic clinical signs in puppies suspected of CPV infection. The study also identified *Salmonella* and *E. coli* as co-infecting bacteria in CPV-positive puppies, highlighting the complex interplay between viral and bacterial pathogens in exacerbating disease severity and complicating treatment. Timely treatment of CPV infection using antibiotics to manage bacterial co-infections, combined with supportive therapy to address dehydration, vomiting, and anorexia, resulted in favorable outcomes.

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

- Clinicians should follow the treatment approach that includes antibiotic drugs to reduce the effect of bacterial complications and supportive therapy to address underlying clinical signs like dehydration, vomiting and anorexia.
- Further studies should be conducted in the study area on molecular characterization and overall prevalence of Canine parvovirus to improve diagnostic, treatment, and prevention strategies.
- Investigations should be undertaken to isolate other pathogens complicating with Canine parvovirus.
- Relevant authorities should work on preventing the disease through improved vaccination coverage.

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8. ANNEX

Annex 1.

Annex 1 Laboratory procedure for isolation and identification of *E. coli* (Gupta,. *et al* 2017)

Materials:

- Homogenised 10 grams of eggshell powder in 90 ml of sterile peptone water that incubated at 37°C for 24 hours.
- MacConkey Agar and EMB (eosin methylene blue)
- Bunsen burner
- Sterile Petri dishes
- Vortex mixer
- Balance
- Incubator
- Sterile inoculation loop
- Autoclave

Preparation of MacConkey Agar

- Required amount of suspension was prepared by the ratio of 50 grams of MacConkey Agar powder in 1000 ml distilled water.
- Suspension was boiled to dissolve the medium completely.
- Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes

Preparation of Eosin Methylene blue Agar

- Required amount of suspension was prepared by the ratio of 36 grams of Eosin Methylene blue Agar powder in 1000 ml distilled water.
- Suspension was boiled to dissolve the medium completely.
- Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Preparation of Nutrient agar

- Required amount of suspension was prepared by the ratio of 28 grams of Nutrient agar powder in 1000 ml distilled water.
- Suspension was boiled to dissolve the medium completely.
- Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes

Inoculation

- Approximately 15–20 mL of melted Agar of each media (cooled to 45 °C) was poured into the sterile Petri dishes.
- The plates were allowed to solidify, inverted and incubated at 37°C for 24 hours to check any contaminations.
- A loopful of the incubated homogenized eggshell powder suspension sterile peptone water was streaked onto MacConkey Agar plates, EMB (eosin methylene blue) and Nutrient agar plates with no contaminations.

Incubation

- Inoculated plates were inverted to prevent condensation from dripping onto the agar and incubated at 37°C for 24hours.

Identification

- On MacConkey Agar, *E. coli* produces pink colonies due to lactose fermentation.
- Pink colonies on MacConkey Agar and dark blue-black colony with a distinctive green metallic sheen on EMB were subcultured again on MacConkey Agar, EMB and nuitrent agar for clear identification and confirmed by further biochemical tests (e.g., IMViC test).

Annex 2 Laboratory procedure for Isolation and identification of *Salmonella* (Heymans,. *et al* 2018).

Materials:

- Xylose Lysine Deoxycholate (XLD) Agar
- Rappaport-Vassiliadis (RV) Enrichment Broth (for enrichment)
- Bunsen burne
- Sterile Petri dishes
- Vortex mixer
- Balance
- Incubator
- Sterile inoculation loop
- Autoclave

Procedure:

Preparation of Rappaport-Vassiliadis (RV) Enrichment Broth

- Required amount of suspension was prepared by the ratio of 30 grams of Rappaport-Vassiliadis (RV) Enrichment Broth powder in 1000 ml distilled water.
- Suspension was boiled to dissolve the medium completely.
- Ten ml of the suspension was dispensed into screw-capped tubes
- Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Inoculation into Pre-enrichment

- 1ml of the incubated homogenized eggshell powder suspension in peptone water was transferred into in 9ml of Rappaport-Vassiliadis broth to promote the growth of *Salmonella spp*. .
- Then homogenized eggshell powder suspension were incubated at 41 °C for 24 hours.

Preparation of Xylose Lysine Deoxycholate (XLD) Agar

- Required amount of suspension was prepared by the ratio of 55 grams of Xylose Lysine Deoxycholate (XLD) Agar powder in 1000 ml distilled water.
- Suspension was boiled to dissolve the medium completely.
- Autoclaving was not performed, as boiling is sufficient for sterilization of XLD agar.
- Approximately 15–20 mL of melted Agar (cooled to 45 °C) was poured into the sterile Petri dishes.

The plates were allowed to solidify, inverted and incubated at 37°C for 24 hours to check any contaminations.

Selective Plating

- A loopful of the incubated enriched broth was streaked onto XLD plates with no contaminations.

Incubation

- Inoculated plates were placed upside down to avoid excessive moisture in the surface of the medium and incubated at 37°C for 24 hours.

Identification

- Red colonies with black centers on XLD were confirmed by further biochemical tests

A. Biochemical Tests

Indole Test

The indole test was performed to determine the ability of the organism to split the tryptophan molecule into indole. Indole was one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possessed the enzyme tryptophanase were capable of hydrolyzing and deaminating tryptophan with the production of indole, pyruvic acid, and ammonia.

Preparations of tryptophan broth

- Required amount of suspension was prepared by the ratio of 16.0 g of tryptophan broth powder suspended in 1 litre of distilled or deionized water**.**
- The mixture was heated to boiling and shaken until the powder was completely dissolved.
- The medium was then sterilized at 121°C for 15 minutes using an autoclave.
- After sterilization, 4 ml of broth was dispensed into final sterile test tubes under aseptic conditions.

Materials and Reagents

- Test tubes
- Inoculating loop or needle
- Pure bacterial colony
- Incubator
- Labeling materials
- Bunsen burner

- Rack for test tubes
- Protective gloves and lab coat
- Kovac's reagent

Procedure

- Sterilized test tubes containing tryptophan broth were prepared.
- Each tube was inoculated aseptically using pure bacterial colony sub cultured in nutrient agar.
- The inoculated tubes were then incubated at 37°C for 28 hours.
- After incubation, 5 drops of Kovac's reagent was added to each broth culture.
- The tubes were then observed for the presence or absence of a red ring at the interface, which indicated a positive indole reaction.

Interpretation of Results

- Development of a bright red color at the interface of the reagent and broth within seconds after adding the reagent indicated the presence of indole and was recorded as a positive test.

i. Methyl Red (MR) Test

Preparation of MR-VP Broth

- Required amount of suspension was prepared by the ratio of 17.0 grams MR-VP broth to 1000 mL distilled water. The mixture was heated to boiling and shaken until the powder was completely dissolved.
- After the broth was completely dissolved, 10 mL of the solution was dispensed into clean test tubes.
- Each tube was then loosely covered with a screw cap to allow for steam penetration during sterilization.
- The test tubes containing the MR-VP broth were sterilized in an autoclave at 121°C and 15 lbs (psi) pressure for 15 minutes.
- After autoclaving, the tubes were allowed to cool down before being used for inoculation.
-

Materials and Reagents

- Test tubes
- Inoculating loop
- Labeling materials
- Rack for test tubes
- Protective gloves and lab coat
- MR-VP broth
- Pure bacterial culture
- Bunsen burner
- Incubator
- Methyl red indicator

Principle

The test was used to determine the ability of the organism to produce and maintain stable acid end products from glucose fermentation and to overcome the buffering capacity of the medium.

Procedure

- MRVP broth was inoculated with a pure culture of the organism.
- The broth was incubated at 37°C for a 48 hours.
- Five drops of methyl red reagent were added per 5 mL of broth.
- The broth medium was observed for a color change.

Interpretation of Results

- A positive result was indicated by a red color (pH below 6).
- A negative result was indicated by a yellow color (no acid production).

ii. Voges-Proskauer (VP) Test

Principle

- The VP test was performed to determine the ability of the organism to produce the neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation.

Materials and Reagents

- Test tubes
- Inoculating loop
- Labeling materials
- Rack for test tubes
- Protective gloves and lab coat
- MR-VP broth
- Pure bacterial culture
- Bunsen burner
- Incubator
- α-naphthol and 40% potassium hydroxide

Procedure

- A pure culture of the test organism was inoculated into VP broth and incubated at 37°C for 24 hours.
- Then, 1 ml of the broth was transferred to a sterile test tube. 0.6 ml of VP reagent I (5% α-naphthol in ethanol) was added, followed by 0.2 ml of VP reagent II (40% potassium hydroxide).
- The tube was gently shaken to expose the medium to atmospheric oxygen and left undisturbed for 10 to 15 minutes.

Interpretation of Results

- A positive result was indicated by a pinkish-red color at the surface of the medium.
- A negative result was indicated by a yellow color.

iii. Citrate Utilization Test

Principle of Citrate Utilization Test

Citrate agar is used to test the ability of an organism to utilize citrate as a source of energy. The agar medium contains citrate as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen. The growth of the organism is indicative of the utilization of citrate as it is an intermediate metabolite in the Krebs cycle. The enzyme citrase breaks down citrate into

oxaloacetate and acetate, where oxaloacetate is further broken down to form pyruvate and carbon dioxide. The release of carbon dioxide induces the metabolism of ammonium salts, causing the formation of ammonia or sodium carbonate, both of which increase the alkalinity of the medium. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6

Materials

- Test tubes
- Simmons Citrate Agar
- Inoculating loop
- Labeling materials
- Rack for test tubes
- Protective gloves and lab coat
- Pure bacterial culture
- Bunsen burner
- Incubator

Preparation of the media

- Required amount of suspension was prepared by the ratio of 24.28 grams of Simmons Citrate Agar powder to 1000 mL distilled water.
- The solution is then heated to bring it to a boil in order to dissolve the medium completely.
- The dissolved medium is then dispensed into tubes and sterilized in an autoclave at 15 lbs pressure (121°C) for 15 minutes.
- After autoclaving, the tubes were taken out and cooled at a slanted position to a temperature of about 445°C. T
- The position was be maintained in order to obtain butts of 1.5 – 2.0 cm depth.

Procedure

- A well-isolated colony **was taken** from fresh culture using a sterile inoculating needle.
- The citrate agar tubes **were inoculated** by streaking the surface of the slant.

- The slant **was streaked** back and forth with the inoculating loop.
- The caps of the test tubes **were left loosened** to ensure adequate aeration.
- The tubes **were incubated** aerobically at 37°C for up to 4 days.
- The test tubes **were examined** daily for 4 days before discarding the result as negative.
- Any change in color, if present, **was observed**.

Interpretation of Results

- Positive Reaction: Growth with color change from green to intense blue along the slant.

Negative Reaction: No growth and No color change; Slant remains green.

Annex 3 Laboratory procedures of PCR (Landry and Leland, 2016).

Principle

The principle of PCR for canine parvovirus (CPV) detection is the selective amplification of a specific DNA sequence unique to CPV, enabling its detection even from a very small amount of viral genetic material in a sample.

Materials, Chemicals and reagents used

1. DNA template: Extracted DNA from the canine sample (e.g., feces) that may contain the CPV DNA.
2. CPV-specific primers: Short DNA sequences designed to flank the target CPV DNA region.
3. DNA polymerase: A thermostable enzyme (e.g., Taq polymerase) that synthesizes new DNA strands.
4. Deoxynucleotide triphosphates (dNTPs): The building blocks (A, T, C, G) for the new DNA strands.
5. Buffer solution: To provide the optimal chemical environment for the polymerase.
6. Magnesium chloride ($MgCl_2$): A cofactor for the DNA polymerase.
7. Nuclease-free water: To bring the reaction to the final volume.

For detection of the amplified product, additional materials are often used, such as:

- Agarose gel and electrophoresis buffer: For separating DNA fragments by size.
- DNA stain (e.g., ethidium bromide or SYBR Safe): To visualize the DNA bands in the gel.
- DNA ladder/marker: To determine the size of the amplified DNA fragment.

Procedures

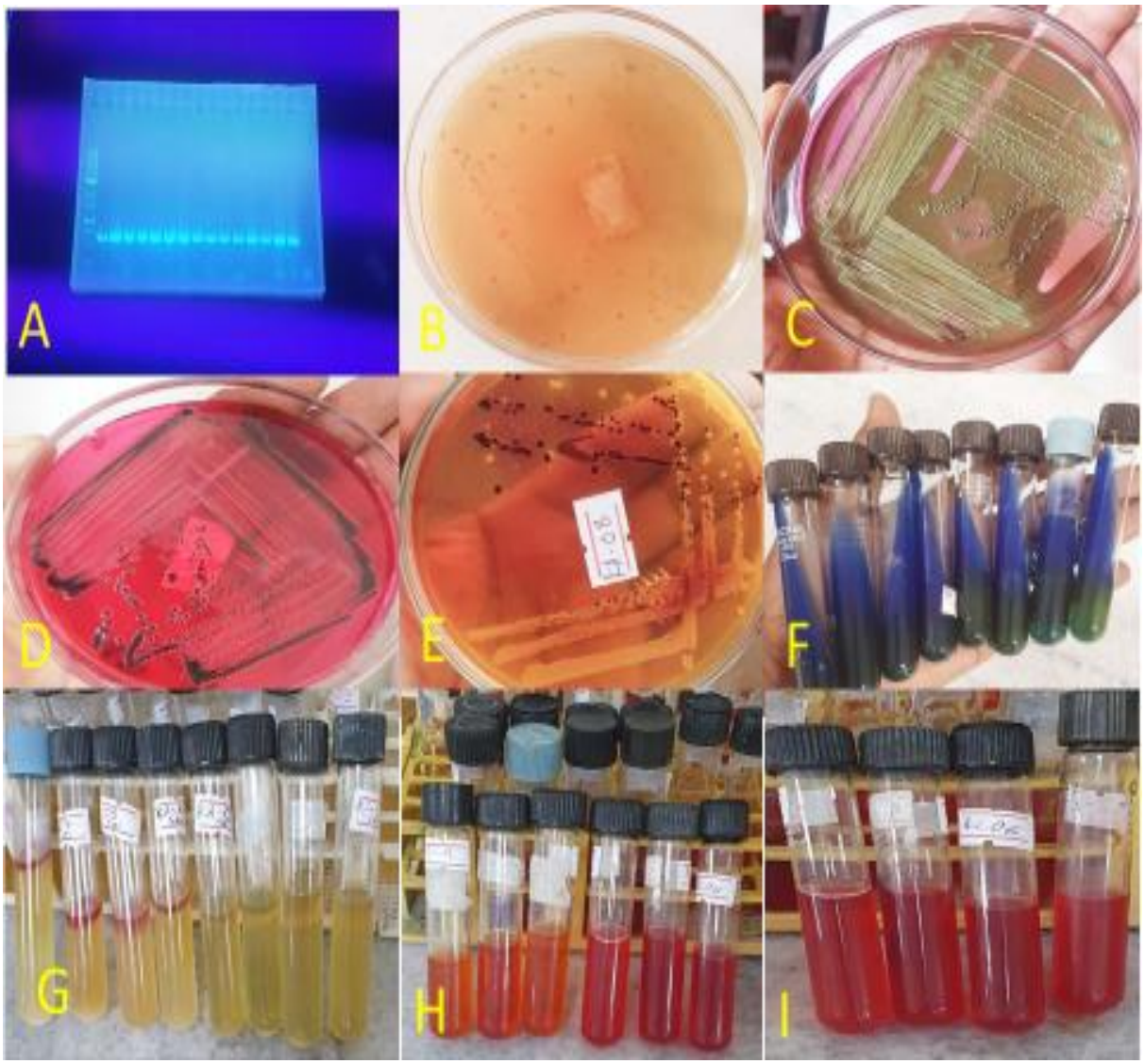
- Sample Collection
    - Fecal sample was collected using sterile swab and placed in a labeled test tube containin 10ml of virus transporting media taken to laboratory.
- DNA Extraction
- Lysis: Breaking open the cells and viral particles to release the DNA.
- Purification: Separating the DNA from other cellular debris, proteins, and inhibitors that might interfere with the PCR process.
- PCR Amplification, This is the core of the PCR procedure, where specific segments of the CPV DNA are amplified
- DNA template: The extracted DNA sample.
- Primers: Short, synthetic DNA sequences that are complementary to regions flanking the target CPV DNA sequence. There will be a forward and a reverse primer.
- DNA polymerase: A heat-stable enzyme (e.g., Taq polymerase) that synthesizes new DNA strands.
- Deoxynucleotide triphosphates (dNTPs): The building blocks (A, T, C, G) for the new DNA strands.
- Buffer solution: To provide the optimal chemical environment for the polymerase.
- Denaturation: Heating the reaction to a high temperature (e.g., 94−95∘C) to separate the double-stranded DNA into single strands.
- Annealing: Cooling the reaction to a lower temperature (e.g., 50−65∘C) to allow the primers to bind (anneal) to their complementary sequences on the single-stranded DNA.
- Extension: Raising the temperature to an optimal temperature for the DNA polymerase (e.g., 72∘C), allowing it to extend the primers and synthesize new DNA strands complementary to the template.

- The number of cycle for this process was 35 cycles
- After the PCR amplification, the presence of the amplified CPV DNA fragment is confirmed, usually by Gel electrophoresis
- Gel electrophoresis: The PCR products are loaded onto an agarose gel, and an electric current is applied. DNA fragments are negatively charged and migrate through the gel based on their size. A DNA ladder (a mixture of DNA fragments of known sizes) is usually run alongside to determine the size of the PCR product.
- Staining: The gel is stained with a dye (e.g., ethidium bromide or SYBR Safe) that binds to DNA and fluoresces under UV light, allowing visualization of the DNA bands.The presence of a band of the expected size for the CPV target sequence indicates a positive result.

Annex 4 Pictures of Clinical signs observed *(A. Bloody diarrhea, B. Depression, C and D. indicators of dehydration (sunken eyeball)).*

Annex 5 Pictures of chemicals and reagents used *(A. DNA stain, B. Buffer AE elusion buffer, C. Nuclease free water, D. Buffer AW2).*

Annex 6 Pictures of Some laboratory works



Annex 7 Pictures of Some Laboratory Results *(A. PCR result positive for CPV, B. E.coli colony on mackonkey agar, C. E.coli colony on EMB agar, D. Salmonella colony on XLD, F. Citrate utilization tes positive resul, G. Indole test positive (left) and negative (right) results, H. Methyl red test positive (left) and negative (right) results, I. Vogues proskeour negative result).*

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/80/17/2025

Name of Applicant: **Kemal Ahmed** (BVSc, MSc student)

Address: Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Clinical characterization, detection of canine Parvo virus and bacterial co-infections and evaluation of treatment outcomes in clinically suspected puppies at AAU-CVMA veterinary teaching hospital, Bishoftu, Ethiopia*

Date of application:	**December, 2024**
Nature of the project:	**clinical investigation**
Target animal species:	**dogs**
Number of animals involved:	**30**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/04/17/025, 25/02/2025**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Kemal Ahmed**.

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Annex 8 Ethical clearance certificate