DETECTION AND MOLECULAR CHARACTERIZATION OF CANINE PARVOVIRUS INFECTION IN MOROGORO AND ARUSHA REGIONS OF TANZANIA

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DECLARATION

I, Caroline Eliona Uronu, hereby declare to the Senate of Sokoine University of Agriculture that this research paper is my own original work done within the period of registration and it has neither been submitted nor being concurrently submitted to any other institution.

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DEDICATION

This work is wholeheartedly dedicated to my late father and my mother. The Lord has blessed me with many forms of blessing but having them as my parents is the greatest blessing of them all. I would not be the woman I am today, if it weren't for their care, guidance, teachings, wisdom, prayers, time and financial investment.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percentage
°C	Degree Celsius
µL	Microliter
AFSCAN	African Small Companion Animal Network
bp	Base pair
CPV	Canine Parvovirus infection
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
MEGA	Molecular Evolutionary Genetics Analysis
ML	Milliliter
m-PCR	Multiplex-Polymerase Chain Reaction
NCBI	National Centre for Biotechnology Information
NS	Nonstructural protein
nt	Nucleotide
ORF	Open Reading Frame
PBS	Phosphate Buffer saline
PCR	Polymerase Chain Reaction
PVE	Parvovirus enteritis
RNA	Ribonucleic acid
rpm	Revolutions per minute
VP	Viral protein

Detection and molecular characterization of canine parvovirus infection in Morogoro and Arusha regions of Tanzania

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ABSTRACT

Canine parvovirus emerged as a viral pathogen of dogs in the late 1970s responsible for a severe global panzootic in dogs of all ages, characterized by haemorrhagic enteritis and myocarditis. The virus has evolved rapidly which has led to three antigenic variants: CPV-2a, CPV-2b and CPV-2c, replacing the original antigenic type (CPV-2). There is no information on the CPV -2 variants circulating in Tanzania, despite its worldwide distribution. Despite the canine parvovirus vaccine being one of the cores strategies of control, the virus is still widespread in the canine population. The aim of the present study was to detect canine parvovirus (CPV) from fecal samples of domestic dogs, in Morogoro and Arusha regions of Tanzania. This study was done in both vaccinated and unvaccinated dogs presented to SUA veterinary hospital with gastroenteritis. Rectal swab samples (n = 143) were collected from dogs in Morogoro and Arusha regions in 2020-2021. Polymerase Chain Reaction (PCR) targeting VP2 gene was used to detect canine parvovirus. Among the 143 samples, 15 were found positive by PCR. Five of the positive PCR products were sequenced. Sequence analysis comparison showed nucleotide identities of 99.53–100% among our CPV-2 isolates. The VP2 gene partial sequences revealed the presence of CPV-2b variant. Phylogenetic analysis of VP2 genes revealed that the CPV-2b variant clustered into two small evolutionary branch and shared the identical branch with two CPV-2b isolates from China and one isolate from South Korea. This study represents the first CPV molecular characterization conducted in Arusha and Morogoro regions of Tanzania.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Canine parvovirus emerged as a viral pathogen of dogs in the late 1970s responsible for a severe global panzootic in dogs of all ages, characterized by haemorrhagic enteritis and myocarditis (Zienius *et al.,* 2016). It is a non-enveloped, single stranded DNA virus that contains two large open reading frames (ORFs). The right end of the ORF encode for structural proteins (VP1 and VP2) which are a major component of viral capsid and the left end of the ORF encodes for non-structural (NS1and NS2) proteins which are important for controlling transcription and DNA replication (Giraldo-Ramirez *et al.,* 2020). CPV contains three major capsid proteins; VP1, VP2, VP3 and of all the three, VP2 is the most highly antigenic one and the determinant factor of host range and cell tropism (Decaro and Buonavugalia, 2012).

Over the past years, CPV-2 has developed new antigenic variants which have been described as CPV-2a, CPV-2b and CPV-2c and their variation is brought by residue 426 of the VP2 of the capsid protein. In 1980, CPV-2 original strain was replaced by the variant designated type 2a (CPV-2a), in 1984, CPV-2b was identified, and in 2000, CPV-2c was detected and reported in Italy (Faz *et al*., 2017). It is believed that CPV-2 has evolved from Feline Panleukopenia Virus (FPLV) due to mutation of the VP2 capsid protein gene that made it possible for a virus to change a host and infect canine while losing its ability to infect feline (Giraldo-Ramirez *et al*., 2020). This virus is extremely contagious, causing high morbidity with increased incidence in shelters, pet stores and breeding kennels which also compromises welfare due to unnecessary death and suffering (Greene and Levy, 2012). The disease is characterized by a rapid clinical course with

death that can often occur 2–3 days after onset of signs in non-protected hosts (Parrish, 1995).

It can affect dogs at any age, but severe infection is most common in puppies between 6 weeks and 6 months of age (Houston *et al.,* 1996). All breeds are susceptible to canine parvovirus infection even though mixed breeds have been reported to be at lesser risk for CPV enteritis than pure breeds (Glickman *et al*., 1985).

Due to the existence of multiple antigenic variants for CPV-2, the clinical signs can vary greatly. Several techniques have been developed in research laboratories, such as ELISA, hemagglutination assays, PCR, immunofluorescence, immunochromatography test and cell culture, however, the sensitivity and specificity of these procedures is not known (Faz *et al.,* 2017). A range of PCR based assays have been developed and been used in diagnostic laboratories for detecting CPV. These include real-time and conventional nested PCR for the detection of CPV-2 variants (Touihri *et al*., 2009).

The main method for controlling the disease in domestic animals is by vaccination. After the emergence of the disease, modified live virus vaccines were soon developed, the first CPV vaccine was made available in 1979 (Miranda and Thompson, 2016). The vaccines appear to be safe and to confer protective immunity allowing much of the disease to be controlled (Miranda and Thompson, 2016). However, the virus is still widely distributed in nature, and if pups are not vaccinated, and or when maternal antibodies interfere with their vaccination, they generally become naturally infected (Parrish, 1995). The evolution of the virus raises questions about the efficacy of some vaccines, so that an understanding of the variation is required (Truyen, 2006).

In Tanzania most dogs are left to roam freely with poor veterinary services such as vaccination and prophylactic measures hence increase the chances of dog bite injuries to people and the likelihood of spreading zoonotic diseases like rabies in the community (Knobel *et al*., 2008).

The aim of this study was to detect and to characterize the canine parvovirus variants circulating in dogs, in Tanzania. Phylogenetic analysis of this VP2 sequences has revealed research-based information of the correlations among geographical regions, types and circulating time, which lays a strong foundation for further research concerning the epidemiology, genetic variation, vaccination and molecular evolutionary relationships of the CPV-2 identified at different times and from different regions.

1.2 Problem Statement and Justification of the Study

Household's species are evidence of a close relationship between humans and animals. Dogs are the most popular pets worldwide hence people tend to create strong emotional attachments to dogs and develop very intimate relationships with them (Hao Yu Shih *et al.*, 2019).

Due to several significances of dogs such as hunting, guiding of blind people, detection of bombs and healer of both physical and emotional problems of humans, it is necessary to study various diseases that are responsible for high morbidity and case fatality rate. Viral diseases predominantly occurring in dogs include the Canine parvovirus infection, canine distemper, Corona virus infection, Canine parainfluenza and Rabies (Bargurjar *et al.*, 2011).

Despite worldwide distribution, and reports of its occurrence in dogs and other related species (Greene and Levy, 2012), Canine parvovirus infection has not been confirmed in

Tanzania. Diagnosis of the parvo cases had solely depended on clinical manifestations, some of which are similar to other common enteric diseases in the area such as ancylostomiasis.

Moreover, there have been reports on vaccination failures, which might also be due to other factors such as improper immunization scheduling, persistence of the maternal immunity at the time of vaccination or circulation of different antigenic variants of the virus (Decaro *et al*., 2020). This study aims at detection and characterization of CPV-2 variants circulating in Tanzania.

1.2 Research Questions

i. Is parvovirus infection existing in Tanzania?

ii. What are the circulating strains of parvovirus in Tanzania?

1.3 Objectives

1.3.1 Overall objective

To understand the status of canine parvovirus infection in both vaccinated dogs and unvaccinated dogs with suggestive signs of parvovirus enteritis (PVE) in Morogoro and Arusha regions of Tanzania in order to provide baseline information for setting up control strategies.

1.3.2 Specific objectives

i. To determine the status of parvovirus infection via rapid test kit in Morogoro and Arusha regions.

ii. To confirm the occurrence of parvovirus infection and types of circulating canine parvovirus's strains using PCR in Morogoro and Arusha regions.

iii. To perform molecular characterization of canine parvovirus variants.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of the Disease and Its Etiology

Canine parvovirus type 2 is one of the main viral infectious agents that cause high morbidity and mortality in the canine population (Bargujar *et al*., 2011). It is a member of the *Parvoviridae* family, subfamily *Parvovirinae,* belonging to the *Protoparvovirus* genus and *Protoparvovirus* type 1 species (Giraldo-Ramirez *et al.,* 2020). According to the Baltimore classification; it is a single stranded, negative sense, non-enveloped DNA virus with approximately 5200 nucleotides with two open reading frames (Bajehson, 2010).

A few years after its emergence, in 1980 CPV-2 evolved and gave birth to the first antigenic variant, named CPV-2a, which differed from the original CPV-2 in the 5th and 6th amino acid positions of the major capsid (VP2) protein (Decaro *et al.,* 2020). In 1984, a second antigenic variant, named CPV-2b raised due to further mutation in the VP2 protein (mutation asparagine to aspartic acid at residue 426) (Decaro *et al.,* 2020). In 2000, a third antigenic variant, CPV-2c, was detected, which displayed amino acid change asparagine/aspartic acid to glutamic acid at residue 426 of the VP2 protein (Decaro *et al.,* 2020). A reason for this rapid antigenic diversity has been due to CPV-2 evolutionary rate which is close to about 10−4 substitutions per site per year (Parrish and Holmes, 2008).

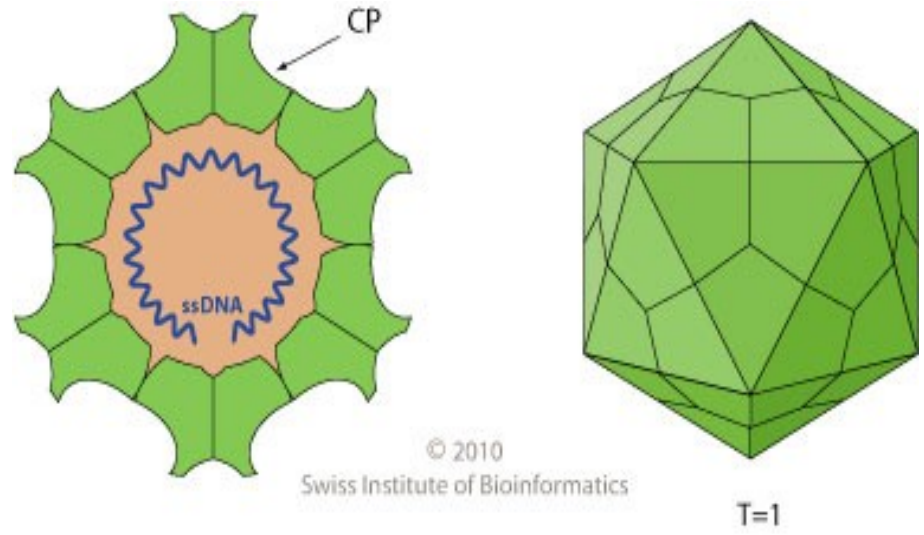
It is important to note that CPV-2 are contagious, highly stable, and capable of persisting in infected premises such as shelters, breeding kennels and pet stores for at least 1 year as they are extremely resistant to heat, cold, humidity, drought, pH and temperature change (Burrell *et al*., 2016).

2.2 Taxonomy and Classification of Parvovirus

Recent classification has shown that CPV belong to the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Protoparvovirus* along with mink enteritis virus, raccoon parvovirus and feline panleukopenia virus (Decaro *et al*., 2020).

Parvoviruses are small (diameter of 25 nm), single stranded, non-enveloped viruses infecting vertebrates and insects (Decaro and Buonavugalia, 2012).

The parvovirus capsid has been found to be formed by 60 copies of a combination of VP1, VP2 and VP3 (Callaway *et al.,* 2017). VP1 contains the full-length sequence plus an additional N-terminal domain. VP2, the most abundant structural protein, accounts for 90% of the viral capsid, representing the major determinant of host range and virus-host interactions, and is cleaved to VP3 by host proteases (Decaro and Buonavugalia, 2012). The parvovirus genome consists of a 5 000-nucleotide DNA molecule containing two large open reading frames (ORFs) and smaller or overlapping genes, encoding for two non-structural (NS1 and NS2) and two structural (VP1 and VP2) proteins through alternative splicing of the same mRNAs (Han *et al.,* 2015). Structural proteins are required for the assembly and packaging of the viral genome, and non-structural proteins aid in controlling DNA replication, assembly and regulation of genes expression (Sharma *et al.,* 2016). 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 (Zaher, 2018). Figure 1 describes the genome organization of canine parvovirus and its structure.



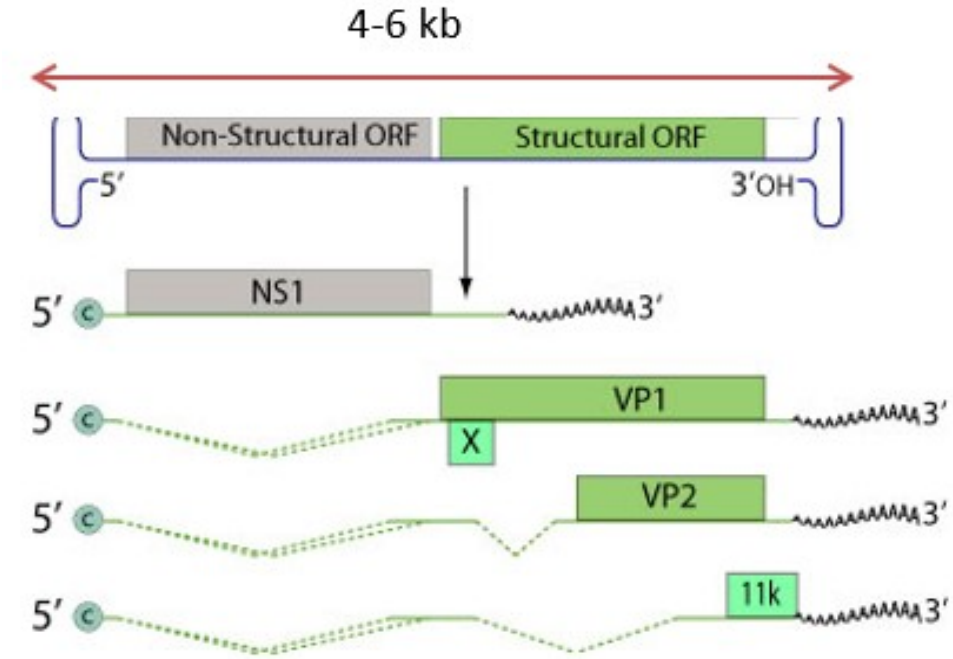


Figure 1: Genome organization of canine parvovirus and its structure

2.2 Epidemiology of Parvovirus Infection

Canine Parvovirus emerged as a dog pathogen in the late 1970s, as a host variant of Feline Parvovirus (Kwan *et al.,* 2021), likely through adaptation of an FPV-like parvovirus of wild carnivores. Although there is no definitive evidence, this hypothesis is supported by the active circulation of intermediate viruses between FPV and CPV in wild carnivores and by the inability of FPV to infect dogs (Decaro and Buonavugalia, 2012). In vitro, while FPV replicates efficiently only in feline cell lines, CPV can infect with the same efficiency cells of canine and feline origin (Nandi and Kumar, 2010). In vivo, FPV replicates in dogs in the thymus and bone marrow without being shed in the feces, and the original canine virus, CPV-2, does not replicate at all in cats (Nandi and Kumar, 2010). Conversely, both the type 2a and 2b variants have re-gained the ability to replicate in vivo in the feline host (Truyen, 2006). Studies on the interactions of FPV and CPV with their cellular receptor, the transferrin receptor type 1, have revealed that FPV specifically binds the feline TfR, whereas CPV-2 and its variants can bind both the feline and the canine TfRs. Interestingly, the antigenic variants of CPV-2 bind the canine and feline TfRs less efficiently than the original type-2 (Palermo *et al.,* 2006). Cases of feline panleukopenia caused by CPV-2a or 2b in wild and domestic felids have been reported in different parts of the world (Truyen, 2006).

Active virus circulation and initial vaccination programmes helped develop herd immunity in canine populations, which greatly reduced mortality and further spreading of the virus (Miranda and Thompson, 2016). However, host-immunity pressure may have also contributed to the progressive emergence of CPV-2 antigenic variants (Doley *et al.*, 2015). In the 1980s, two antigenic variants, distinguishable using monoclonal antibodies (MAbs), emerged within few years and were termed CPV types 2a (CPV-2a) and 2b (CPV-2b) (Zhou, 2017). Currently, the antigenic variants have completely replaced the

original type 2, which is still used in most commercial vaccines, and are variously distributed in the canine population worldwide (Battilani *et al.,* 2017). CPV-2a and CPV-2b differ from the original strain CPV-2 in five or six residues of the VP2 capsid protein. In contrast, only two residues differentiated CPV- 2a from CPV-2b, i.e., N426D and I555V (Truyen, 2006).

The distribution of parvovirus infection in a population is largely influenced by the susceptibility of host, environmental conditions such as housing, hygiene, population density, and pathogenicity of the infectious agent (Nandi *et al.,* 2008). Puppies between six weeks and four months old and dogs that have not been vaccinated against the virus are at increased risk for infection (Nandi *et al.,* 2013). Parvovirus transmission and spread from infected dogs to non-infected dogs occur through contact from contaminated environment such as body fluids, dog's feces and vomit (Decaro *et al.,* 2005). Dogs can also catch the virus by contact with contaminated bedding, food and water bowls, carpet, or a kennel that a dog with parvovirus has touched (Tuzio, 2021). Parvovirus can also be spread on contaminated shoes, clothing and human hands (Crawford, 2008). Figure 2, describes the global distribution of the CPV variants.

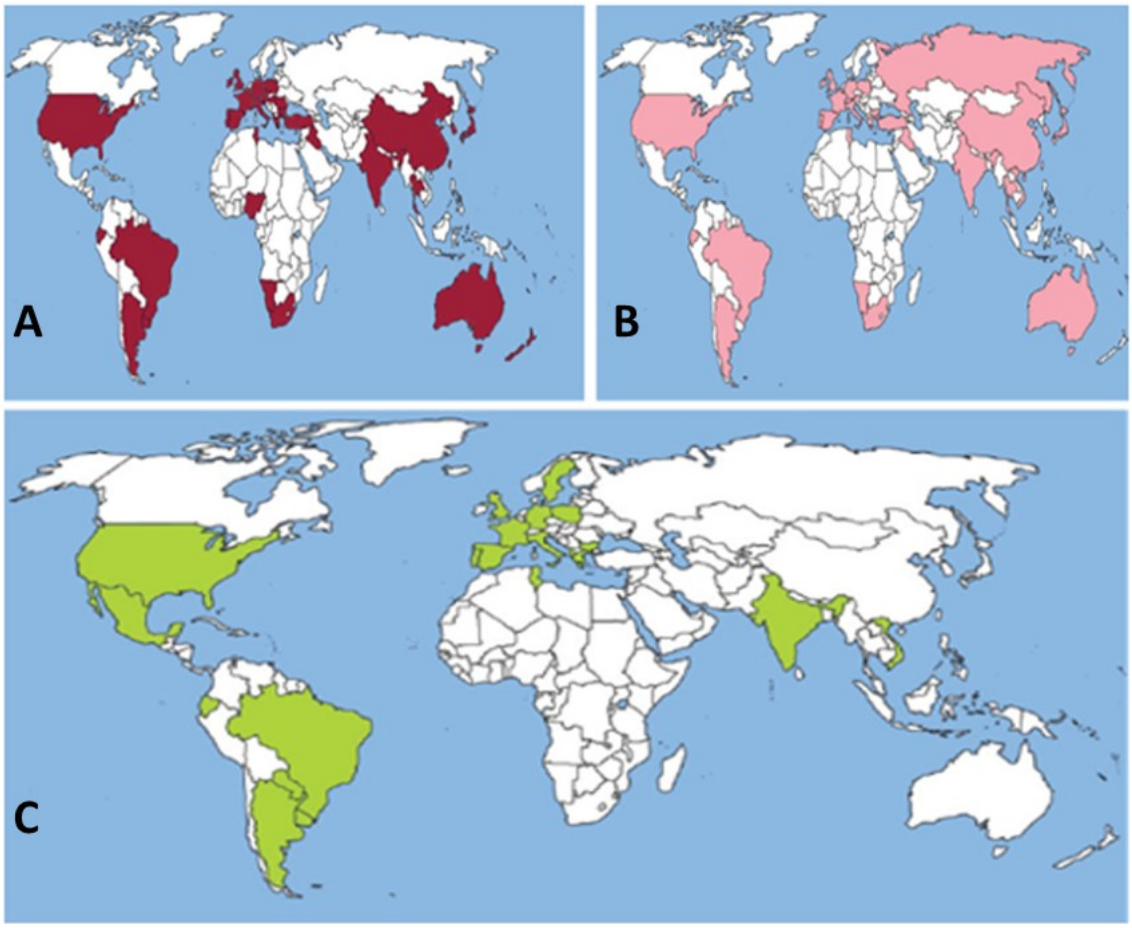


Figure 2: **Global distribution of CPV variant: Red, presence of CPV-2a variant (A); Pink, presence of CPV-2b variant (B) and Green, presence of CPV-2c variant (C) (Source: Miranda and Thompson, 2016)**

2.3 Pathogenesis of Parvovirus Infection

The infection is generally acquired by the fecal-oral route by direct contact from dog to dog or contact with contaminated feces, environment or people (Figueiredo *et al.*, 2016). The virus can also contaminate kennel surfaces, food and water bowls, hands and clothing of people who handle infected dogs (Appeal and Barr, 2009).

Infected dogs shed viruses in form of excretions such as saliva, urine, feces, and vomitus. Upon entering the body, CPV binds to its cellular receptor, the TfR; a transmembrane protein receptor that is expressed in many tissues (Harbison *et al.*, 2009). Virions enter cells by clathrin-mediated endocytosis (Parker and Parrish, 2000). Upon entering the cell,

the viral DNA is released from the capsid and since the virus does not possess its own DNA polymerase, then it hijacks that of the host for replication to occur (Parker and Parrish, 2000). The virus has its tropism for cells with high proliferation rate such as the lymphoid tissues, intestinal epithelium and bone marrow, as well as the heart in neonatal pups (kilian *et al.,* 2018). Following an incubation period of 3–7 days, the disease can be characterized by two clinical forms, the first one is enteric form, which is characterized by vomiting, haemorrhagic diarrhoea, depression, loss of appetite, fever and the second form is myocarditis in younger dogs (Zaher, 2018). Shedding of virus in feces may occur in the absence of clinical signs or before clinical signs of disease are detected.

Myocarditis may be seen after infection of neonatal puppies, where the clinical signs are seen a number of weeks after infection (Sime *et al.,* 2015). Canine parvovirus infection is also characterized by a drop in the white blood cell counts as a result of the bone marrow infection and other lymphoid tissues (Prittie, 2004). Canine parvovirus (CPV) also leads to severe damage of the intestinal barrier (Kilian *et al.,* 2018). It has been speculated that dogs might develop chronic gastrointestinal disorders after surviving clinical manifestation of CPV infection (Kilian *et al.,* 2018).

2.4 Clinical Signs of Parvovirus Infection

Canine parvovirus is an acute illness, which means that symptoms develop suddenly, usually within 3–10 days of exposure (Decaro and Buonavoglia, 2012). Signs and symptoms of canine parvovirus include the following: bloody diarrhea (often severe), fever, lethargy (lack of energy), loss of appetite, malaise (discomfort associated with illness), rapid weight loss, vomiting, myocarditis and leukopenia (Zhuang *et al.*, 2018). Persistent vomiting and diarrhea usually cause rapid dehydration and damages to the intestines.

2.5 Diagnosis and Management of Parvovirus

In puppies and dogs that have not been vaccinated, CPV infection often is suspected when bloody diarrhoea, loss of appetite, and vomiting develop suddenly (Nandi and Kumar, 2010). Physical examination may reveal signs of dehydration (e.g. lethargy, sunken eyes, dry gums, rapid heart rate, and concentrated urine), fever, abdominal discomfort, swollen lymph nodes, and weight loss (Garigliany *et al*., 2016). Laboratory tests have also be used to diagnose the presence of the disease in dogs, where enzyme-linked Immunosorbent assay (ELISA) and Polymerase Chain Reaction (PCR), are among the tests that have been used to detects the presence of the virus in stool (Decaro *et al*., 2008).

Vaccination and good hygiene practices are the critical components of prevention of canine parvovirus infection (Mylonakis *et al.*, 2016). CPV vaccine is considered as a core vaccine by veterinary professionals, meaning that all dogs should be vaccinated, regardless of the geographical boundaries, since it protects animals from severe, life-threatening diseases that are globally distributed (Decaro *et al.*, 2020). However, despite the intensive vaccination programs that are adopted worldwide, CPV infection represents one of the most frequent infectious disease and cause of death in juvenile dogs even in developed countries (Decaro and Buonavoglia, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

The proposed study was conducted in three districts of Morogoro region (Morogoro Municipal, Morogoro Rural, and Mvomero) and one district of Arusha region (Monduli district, Makuyuni division at Esilalei and Mswakini ward).

Monduli is one of the seven districts of the Arusha region of Tanzania. It is located in the northeastern part of the country. It is bordered to the north by Longido district, to the east by Arusha Rural district, to the south by the Manyara region and to the west by Ngorongoro and Karatu districts. Its geographical coordinates are 3.3029° S and 36.4453° E. In Morogoro region the study was conducted in three districts, namely Mvomero, Morogoro Municipal and Morogoro Rural in Tanzania. Mvomero district is located at the north east of Morogoro region between the latitude 6.2555° S and longitude 37.5535° E. It is bordered to the north by the Tanga region, to the north east by the Pwani region, to the east and south east by Morogoro Rural district and Morogoro Municipality and to the west by Kilosa district.

Morogoro Municipality lies at the base of the Uluguru Mountain whose peak is about 500 to 600 meters above sea level. It is located between latitude 6.8278° S and longitude 37.6591° E. It is about 196 kilometers West of Dar es Salaam City and 260 kilometers East of Dodoma. Morogoro Rural District lies between latitude 7.2052° S and longitude 37.8937° E, covering 19056 square kilometers. It is bordered to the north and east by the Pwani Region, to the south by Kilombero District, to the southwest by the Kilosa District and to the west by the Mvomero District and the Morogoro Urban District. Figure 3 is a map of Tanzania, illustrating the sampling areas.

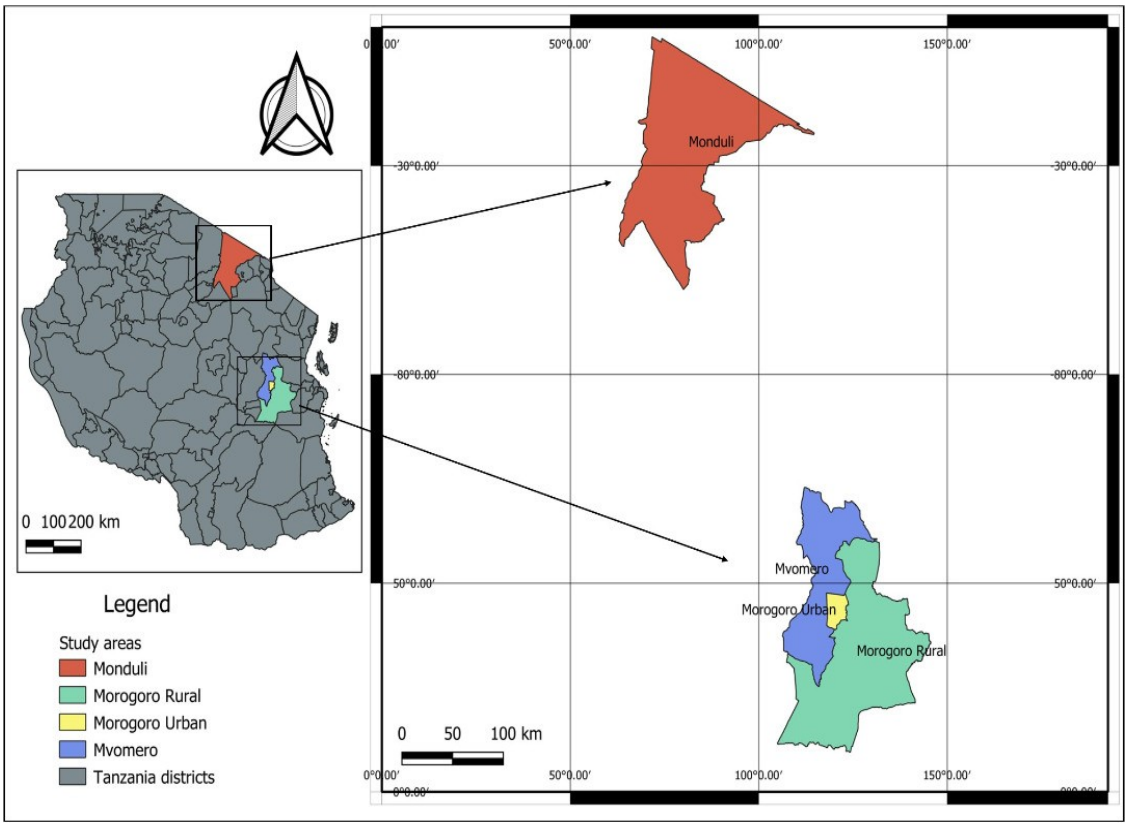


Figure 3: **Map of Tanzania illustrating the sampling areas.**

3.2 Study Population

The target study population for this study were both the vaccinated and unvaccinated dogs in the three districts of Morogoro region (Morogoro municipal, Morogoro rural, and Mvomero) and one district in Arusha region (Monduli District, Makuyuni division at Esilalei and Mswakini Ward). Another source of the study population was dogs with signs of enteritis (PVE) reported at SUA Animal hospital-Morogoro.

3.3 Study Design

The design of this research is cross-sectional study which based on purposive sampling carried out to detect the presence of CPV in healthy unvaccinated dogs and dogs with signs of parvovirus enteritis where sample collection, screening and diagnosis of animals was done only once.

3.4 Sample Size

The sample size was calculated using a formula described by Naing *et al.* (2006). $N=Z^2*p(1-p)/d^2$, Where N= sample size; Z= test statistic; p= expected prevalence; d= precision. The values used in the formula are Z=1.96, d= 5% and p= 10.4% as CPV in dogs (Mwolongo *et al.,* 2014). Therefore, the calculated sample size for unvaccinated dogs was 143.

3.5 Sample Collection and Processing

Selection of individual dogs for the study was carried out using random sampling technique. A total of 143 dogs were randomly selected from four clusters of districts, 36 dogs from each cluster on average. Findings are not presented based on sex, age and breed. Fecal samples were collected from both suspected dogs with signs of PVE and non-suspected dogs by introducing a swab into the anus of dogs and homogenized (10% w/v) in a sterile phosphate buffer saline (PBS).

The samples were kept in 2ml collection tube with the swab. After thorough mixing of the sample in PBS, the swab was removed by squeezing on the inner surface of the collection tube. This emulsion was then centrifuged at 6 000 rpm for 15 min at 4°C. The supernatant was collected, filtered through 0.45 µm sterile filter and was stored at −20°C.

3.6 Laboratory Analysis

Laboratory work was conducted at the College of Veterinary Medicine and Biomedical Sciences (CVMBS) in the Molecular Biology and Biotechnology laboratory for viral research and training at Sokoine University of Agriculture, Morogoro. Positive PCR products were sent to Macrogen Inc (Seoul, Korea) for partial sequence analysis of the CPV-2 VP2gene.

3.6.1 Screening of fecal samples using a rapid CPV Ag test kit

For detection of CPV in healthy carrier's dogs, as well as in dogs with signs of PVE, rectal swabs were taken and tested with a commercially rapid CPV Ag test kit (Synbiotic Corporation®) which is based on Immuno Run assay technique as per manufacturer's protocol.

Fecal samples were collected by using a rectal swab. The procedures for screening fecal samples are described in Figure 4: 1-4. Briefly, the swab was coated with a thin layer of feces, then immersed the feces coated swab into the sample extraction buffer in the tube. The swab was swirled in the buffer vigorously and by pressing the swab against the side of the tube ensured that most of the fecal materials from the swab were removed. When removing the swab from the test tube, again the swab was pressed against the side of the tube repeatedly until no more liquid came from the swab. Disposable pippete was used to take the supernatant sample from the tube. Holding the pipette vertically, three drops of sample were transferred to the sample well, drop by drop vertically. The results were read after ten minutes for the presence or absence of the pink/purple bands. Sample results were read in window number 2 and the control band was read in window number 3 (Figure 4-4). Samples were considered positive if both pink/purple bands were visible in both windows number 2 and window number 3 (Figure 4-4). Samples were considered negative if there was no band in window number 2, with one pink/purple band in window number 3 (Figure 4-4).

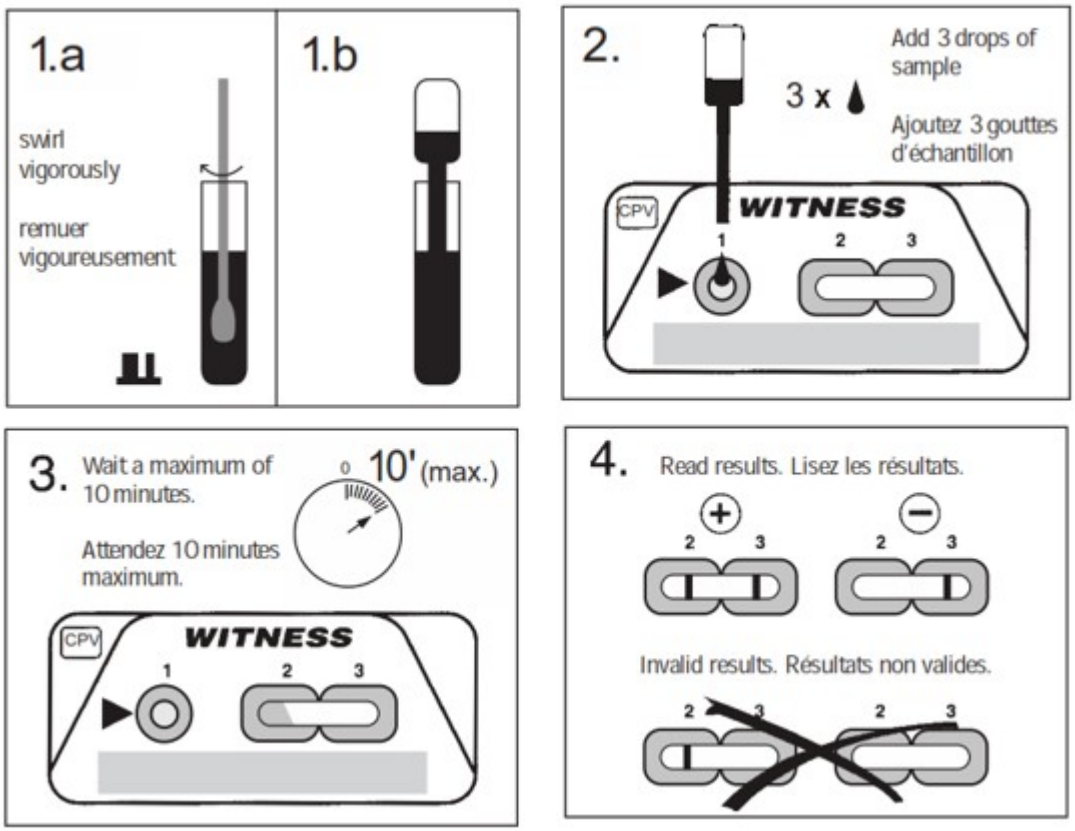


Figure 4: **A method for screening of fecal samples using a rapid CPV Ag test kit (Synbiotic Corporation®)**

3.6.2 Extraction of DNA and quantification

Viral DNA extraction from the collected stool samples was performed using Quick-DNA fecal/soil microbe Miniprep Kit (lot no. 206040) in accordance with the manufacturer's instructions. 250mg of fecal sample was added to ZR BashingBead™ Lysis tube and then 750µl BashingBead™ Buffer was added to the tube. Bead beater fitted with a 2ml tube holder assembly was secured and processed at maximum speed for 5 minutes. This was followed with centrifugation of the ZR BashingBead™ Lysis tube in a microcentrifuge at 8000g for 1 minute and transferred up to 400 µl of the supernatant to a Zymo-Spin™ IIIF Filter in a collection tube and centrifuged again at 6 000g for 1 minute. 1 200µl of the Genomic Lysis Buffer was added to the filtrate in the collection tube from the later step and mixed well and transferred 800µl of the mixture from the former step to a Zymo-Spin™ IICR Column in a collection tube and centrifuged at 8 000g for 1 minute. The

flow through from the collection tube was discarded and repeated the last step. 200µl of the DNA Pre-Wash Buffer was added to the Zymo -Spin™ IICR column in a new collection tube and centrifuged at 8 000g for 1 minute and then added 500µl g-DNA Wash Buffer to the Zymo-Spin™ IICR column and centrifuged again at 8 000g for 1 minute. Zymo-Spin™ IICR column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of DNA Elution Buffer was added directly to the column matrix, then centrifuged at 10 000g for 30 seconds to elute the DNA. Zymo-Spin™ III-HRC Filter was prepared in a clean collection tube and added 600 µl Prep Solution then centrifuged at 6 000g for 3minutes. Lastly, the eluted DNA was transferred to a prepared Zymo-Spin™ IIIHRC Filter in a clean 1.5ml microcentrifuge tube and centrifuged at exactly 160 000g for 3 minutes. The filtered DNA was then suitable for PCR analysis assay and other downstream applications. The DNA concentration was measured in a NanoDrop ND 1000 spectrophotometer.

3.6.3 Polymerase chain reaction for detection of CPV-2

In order to identify CPV-2-positive samples, PCR amplification of VP2 was conducted using the conventional method. For each PCR reaction, 12.5 µL of Taq PCR Master Mix (2×) (Thermo Fisher Scientific®, New England biolabs, catalog No. M0486S) was used, with 1 µL of the forward primer and 1 µL of the reverse primer, 8.5 µL of a nuclease free water and 2µl of DNA template reaching a total mixture amount of 23.5 µL for each reaction. The primers used and locations that are being amplified by those primers are shown in Table 1.

Table 1: **List of all primers used for detection of the original CPV-2, CPV-2 antigenic variants (CPV-2a, CPV-2b and CPV-2c)**

CPV subtypes	Primer name	Primer sequence (5' to 3')	location	PCR Product
CPV-2	CPV-2 F	GAAGAGTGGTTGTAAATAATA	3025-3706	681bp
	CPV-2 R	CCTATATCACCAAAGTTAGTAG		
CPV-2ab	CPV-2ab F	GAAGAGTGGTTGTAAATAATT	3025-3706	681bp
	CPV-2ab R	CCTATATAACCAAAGTTAGTAC		
CPV-2b	CPV-2b F	CTTTAACCTTCCTGTAACAG	4043-4470	427 bp
	CPV-2b R	CATAGTTAAATTGGTTATCTAC		
CPV-2c	CPV 555 F	AGGAAGATATCCAGAAGGA	4002-4585	583bp
	CPV 555 R	GGTGCTAGTTGATATGTAATAAAC A		

NB: All primers were obtained from the previous studies (Sharma *et al.*, 2016)

The primer pair amplifies a 681 bp fragment of the original strain of CPV-2. Molecular grade water was used as a negative control for amplifications and commercial vaccine was used a positive control (VANGUARD® PLUS 5 L4, Zoetis Inc., USA). PCR amplification conditions used are in Table 2.

Table 2: PCR conditions for detection of the original CPV-2 and antigenic variants (CPV-2a, CPV-2b and CPV-2c)

Conditions	Temperature	Time	No. of cycles
Initial denaturation	95°C	10min	1
Denaturation	95°C	1min	
Annealing	55°C	1min	35
Extension	72°C	1min	
Final extension	72°C	10min	1

PCR amplification results were visualised using 1% agarose gel electrophoresis. Agarose gel was stained with the 4µL of DNA Gel red Stain (Biotium®, San Francisco Bay Area). In each well, 5µL of each sample obtained after amplification and 0.8 µL of the 6× DNA

loading buffer TAE were used, and the GeneRuler™ 100-bp DNA plus Ladder (Thermo Fisher Scientific®) was used as a molecular weight marker. Progress of the mobility was monitored by migration of dye.

The ultraviolet light Gel Doc™ EZ imager system (Bio-Rad, Molecular imager ®, USA) was used to visualize the PCR products in the gels and captured using Image Lab™ software.

3.6.4 Polymerase chain reaction for detection of CPV-2 antigenic variants (CPV-2a,CPV-2b and CPV-2c)

To detect the new antigenic variants of CPV, a 681 bp fragment encoding capsid protein VP2 of both antigenic types CPV-2a and CPV-2b were amplified from the template DNA using forward and reverse primers for CPV-2ab and CPV-2b (Table 1).

All the fecal samples that were found negative by PCR with CPV-2, CPV-2ab, and CPV-2b primer pairs were again subjected to another PCR assay using forward and reverse primers CPV-555 F and R (Table 1) that amplifies a 583 bp fragment of the gene encoding capsid protein VP2. For each PCR reaction, 12.5 µL of Taq PCR Master Mix (2×) (Thermo Fisher Scientific®) was used, with 1 µL of the forward primer, and 1 µL of the reverse primer, 8.5 µL of a nuclease free water and 2µl of DNA template making a total of 23.5 µL for each reaction mixture. List of primers used for the detection of CPV-2 antigenic variants are shown in Table 1. Molecular grade water was used as a negative control for amplifications and commercial vaccine (VANGUARD® PLUS 5 L4, Zoetis Inc., USA) was used a positive control. PCR conditions used are in Table 2.

The amplicons were visualised using 1% agarose gel electrophoresis. Agarose gel was stained with the 4µL of DNA Gel red Stain. In each well, 5µL of each sample obtained after amplification and 0.8 µL of the 6× DNA loading buffer TAE were used, and the GeneRuler™ 100-bp DNA plus Ladder (Thermo Fisher Scientific®) was used as a molecular weight marker. Progress of the mobility was monitored by migration of dye. The ultraviolet light Gel Doc™ EZ imager system (Bio-Rad, Molecular imager ®, USA) was used to visualize the PCR products in the gels and captured using Image Lab™ software.

3.6.5 Sequencing of VP2 structural protein gene

Approximately, 45 µl of positive PCR products after gel electrophoresis and visualization were sent to Macrogen Inc. (Seoul, Korea) for partial sequencing of VP2 structural protein gene using forward and reverse primers on commercial basis.

3.7 Data Analysis

3.7.1 Sequence editing, assembly and multiple sequence alignment

The obtained sequences from the forward and reverse primers were edited and assembled using CLC Main Workbench 6.94 to obtain consensus sequence. The consensus sequences of CPV-2 obtained were searched in the NCBI GenBank database using Basic Local Alignment Search Tool (BLASTN 2.12.0+) to find regions of local similarity between CPV-2 sequences. Sequences to include in the multiple sequence alignment were chosen from the list generated sequences during the BLAST search. Multiple sequence alignment was performed using Clustal W built in MEGA 11 (Tamura *et al*., 2021).

3.7.2 Phylogenetic analysis

A phylogenetic tree of the nucleotide sequences based on the partial length of VP2-encoding gene was reconstructed using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) as implemented in MEGA 11 (Tamura *et al*., 2021). The tree was drawn to scale, with branch lengths (0.03236613) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of Canine Parvovirus

A total of 143 dogs were tested from the three districts of Morogoro region namely, Morogoro Municipality, Mvomero district and Morogoro Rural district and one district of Arusha region namely, Monduli. Preliminary test was performed by screening the samples using rapid CPV Ag test kit whereby out 143 that were screened, 13(9%) samples were detected positive with an overall prevalence of 9% (n=143) from the four districts (Table 3).

Table 3: Prevalence of canine parvovirus in Morogoro and Arusha regions from dogs screened using a rapid CPV Ag test kit (n=143)

Location of origin	Total	Positive	Negative	Prevalence (%)
Morogoro municipality	50	8	42	16
Mvomero district	30	3	27	10
Morogoro rural	20	2	18	10
Monduli district	43	0	43	0
Total	**143**	**13**	**130**	**9.09**

4.2 Detection of Canine Parvovirus by PCR

This study based on molecular detection of CPV-2 by using PCR and characterization of the CPV-2 genome targeting the VP2 gene from dog fecal samples collected in Arusha and Morogoro regions of Tanzania. A total of 100 samples upon screening were subjected to conventional PCR using universal primers (CPV-2 F and R) targeting VP2 gene to detect the presence of CPV-2 original strain. Out of the 100 samples that were analyzed by CPV-2 PCR assay, fifteen (15%) were found to be positive for the presence of original strain of CPV-2, amplified a 681 pb (Figure 5).

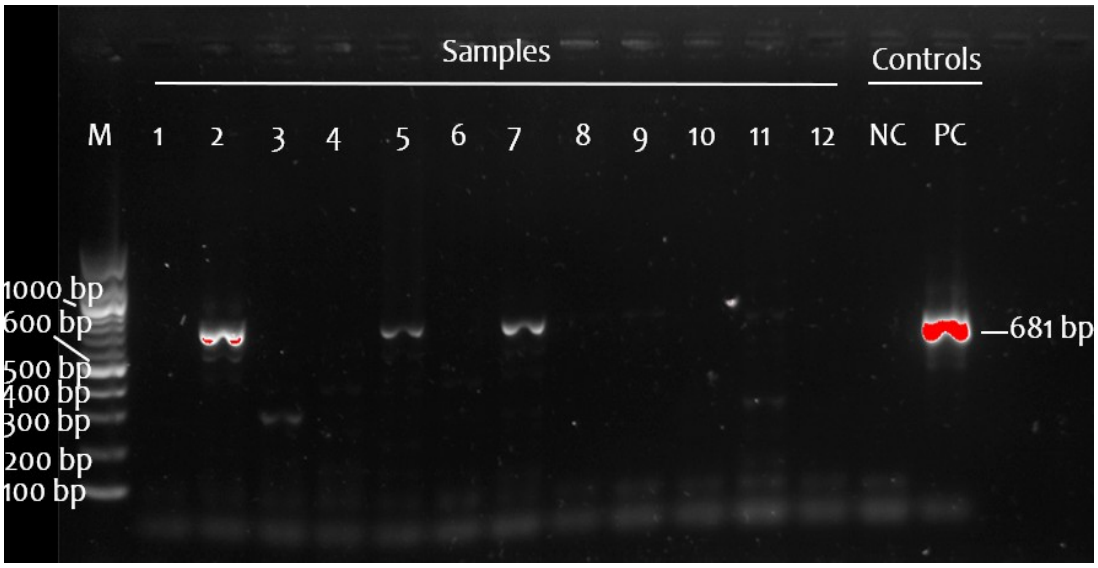


Figure 5: Gel electrophoresis of CPV-2 PCR products amplified using universal primers targeting 681 bp of VP2 gene. Lane M: DNA ladder, Lane NC: negative control (RNase free water), Lane PC: positive control (vaccine strain), and Lanes 1-11: test samples.

All the 15 PCR positive samples above were subjected to another round of PCR using a CPV-2ab F and R primers designed for detection of both variant CPV-2a and CPV-2b. CPV-2ab PCR assay also detected 15 out of 100 fecal samples tested by PCR to be positive VP2 gene of the CPV-2 (Figure 6). The amplicons detected, as expected were of 681 bp.

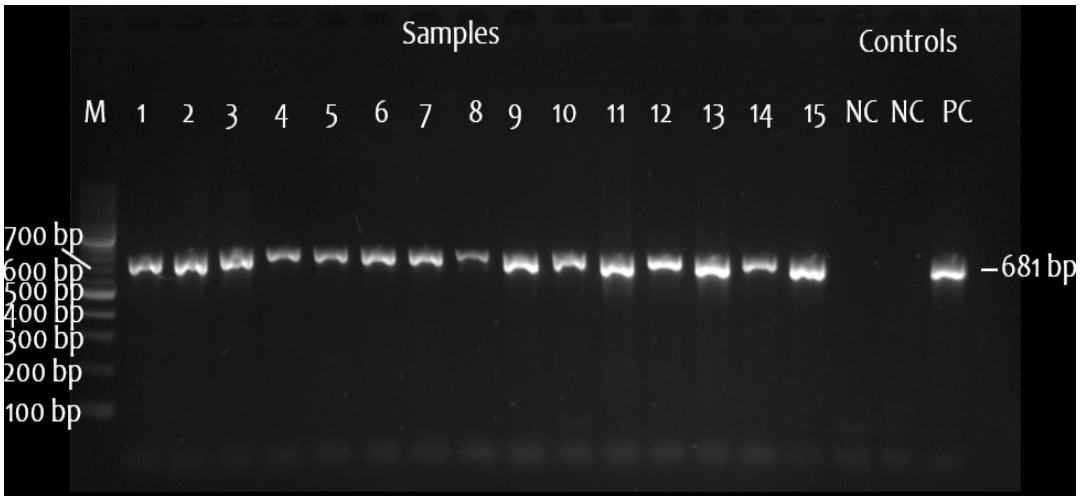


Figure 6: Gel electrophoresis of CPV2 PCR products amplified using CPV-2ab primers. Lane M: DNA ladder, Lane NC: negative control (RNase free water), Lane PC: positive control (vaccine strain), and Lanes 1-15: test samples.

To confirm whether the detected amplicons are of variant CPV-2a or CPV-2b, the samples were again subjected to another PCR assay by using primers CPV-2b F and R. All the 15 samples (15%) were detected by the primers (Figure 7) implying that the samples contained CPV-2b variant. The primers amplified a 427 bp fragment of the gene encoding the capsid protein VP2 as expected.

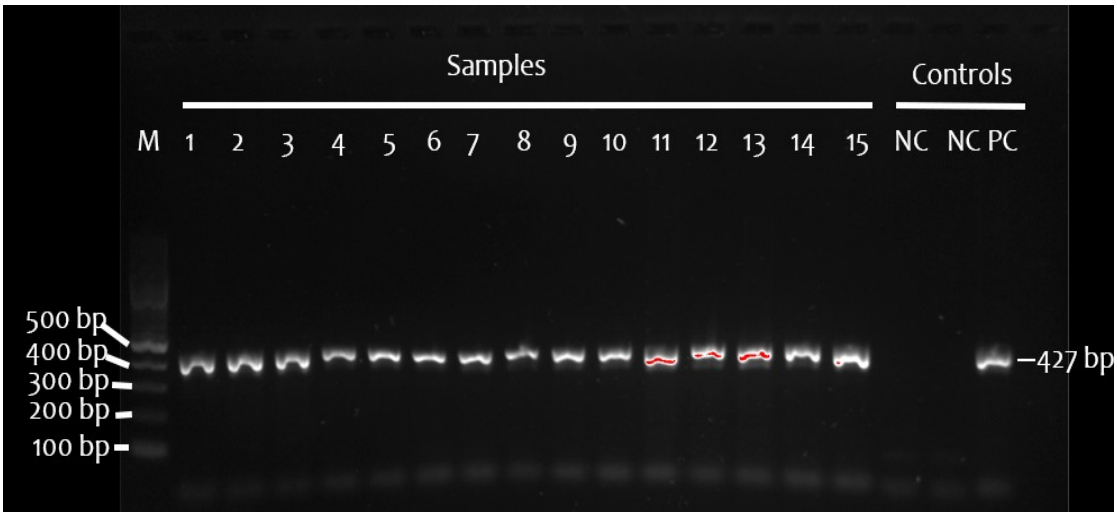


Figure 7: Gel electrophoresis of CPV2 PCR products amplified using CPV-2b primers. Lane M: DNA ladder, Lane NC: negative control (RNase free water), Lane PC: positive control (vaccine strain), and Lanes 1-15: test samples.

In addition, no sample could be amplified when PCR assay was performed by using 555 forward and reverse primers (data not shown) targeting 583 bp fragment, which meant that CPV-2c variant was absent in the samples.

4.3 Nucleotide Sequence and Phylogenetic Analysis

The amplified PCR products were sent for sequencing on commercial basis to Macrogen Inc. (Seoul, Korea). The obtained sequences were edited, assembled and the consensus sequences were used for to find the local regions of similarity in the NCBI GenBank database using BLAST. Upon blast search, the highest identity ranged from 99.53% to

100% with other CPV-2 deposited sequences from China and South Korea isolates respectively.

The phylogenetic tree (Figure 8) was reconstructed using different nucleotide sequences of CPV subtypes (CPV-2, CPV-2a, CPV-2b and CPV-2c) circulating worldwide retrieved from the blast search in the NCBI Genbank. Phylogenetic analysis revealed that all five isolates identified clustered in the CPV-2b subtype in the phylogeny together with the two Chinese isolates (Accession Number JQ268284 and JQ743891) and one isolate from South Korea (Accession Number MN053893).

In these serotype, one group consist of TZ8 CPV2b/Tanzania/2021, TZ9 CPV2b/Tanzania/2021 and TZ10 CPV2b/Tanzania/2021 are closely related to two Chinese viruses of the year 2010 and 2011 (100% nucleotide identity) and one South Korea virus (100% nucleotide identity) of 2017.

The second group, consisting of TZ6 CPV2b/Tanzania/2021, TZ7 CPV2b/Tanzania/2021 were also closely related to two Chinese viruses of the year 2010 and 2011 (99.53% nucleotide identity) and one South Korea virus (99.53% nucleotide identity) of 2017.

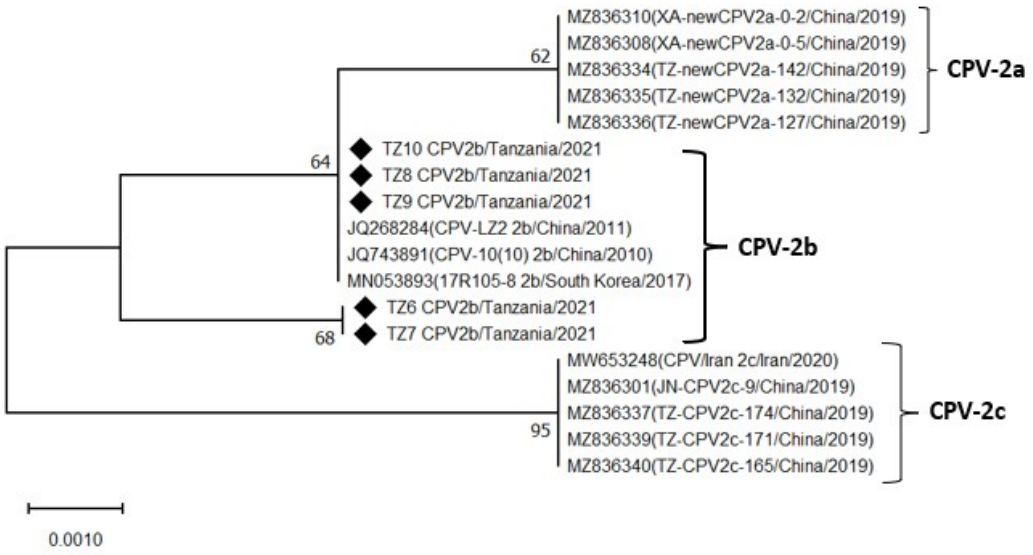


Figure 8: Evolutionary analysis by maximum likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-590.06) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

CHAPTER FIVE

5.0 DISCUSSION

The desire to keep pets in Africa is increasing greatly, hence it is becoming difficult to ignore the diseases of dogs and cats (Muhairwa *et al.,* 2008). Due to several significances of dogs, it is necessary to study various diseases that are responsible for high morbidity and case fatality rate, among those diseases are viral diseases including the Canine parvovirus infection, Canine distemper, Corona virus infection, Canine parainfluenza and Rabies (Bargurjar *et al.,* 2011). Despite worldwide distribution, and reports of its occurrence in dogs and other related species (Greene and levy, 2012), the disease has not been confirmed in Tanzania.

This study aimed at detection of Canine parvovirus and its circulating variants in Morogoro and Arusha regions using PCR and sequencing of the PCR products. The finding from this study indicated that CPV-2 were detected in the fecal samples collected in the two regions. The primers used in this study were able to discriminate the known three CPV variants (CPV-2a, CPV-2b and CPV-2c). Variant CPV-2a is recognized by primers CPV-2ab only, while variant CPV-2b is recognized by both primers CPV-2ab and CPV-2b (Sharma *et al.,* 2016). Since all the 15 samples were amplified by both CPV-2ab and CPV-2b primers producing specific bands of the expected sizes, it means variant CPV-2a was not present in the samples. There was no amplification observed when primers targeting variant CPV 2c was used implying that the variant was not detected in the samples. Therefore, only one variant (CPV-2b) was detected in the samples. As all the fifteen samples were positive for CPV-2 original strain, it can be assumed that the original strain of CPV that is normally incorporated into CPV vaccines, still circulates in the local canine population in Morogoro region.

No information is available if the vaccinated animals are adequately protected against newer antigenic types (CPV-2b) using the available CPV vaccines that only contains the original CPV-2 and CPV-2c strains. Nevertheless, there have been complaints that vaccinated dogs against parvovirus infection have also been succumbing to the disease in Tanzania. There are several factors for vaccine failure including improper cold chain and the use of vaccine which do not match with the circulating virus strains.

The VP2 partial sequence analysis showed that, the five samples from Morogoro region were closely related with CPV-2b antigenic variant and showed 99.0-99.9% identity. All sequences from Tanzania were associated with one phylogroup, but grouped in different clusters. Five of the investigated Tanzania CPV-VP2 sequences were closely associated with CPV-2b antigenic variant (99.53%-100% nucleotide identity) from China and South Korea. This implies that the virus could have been introduced in/from those countries during exportation or importation of dogs.

Canine parvovirus infection is one of the most important enteric viral pathogens of dogs (Jia-yu *et al.*, 2018). Since its emergency the virus has evolved rapidly, leading to the three antigenic variants, CPV-2a, CPV-2b and CPV-2c, which are now circulating in the world and have completely replaced the original strain (Jia-yu *et al.*, 2018). The occurrence of canine parvovirus infection has received considerable attention in other countries of Africa (Bajehson, 2010). Little information has been documented in Africa about the genetic characterization of CPV-2 (Figueiredo *et al.*, 2016). Despite the intensive vaccination, this virus still represents one of the main causes of acute gastroenteritis and death in the canine population (Decaro *et al.*, 2020).

Most of the studies have only been carried out in a small number of dogs submitted to the clinics and the information obtained does not provide details on the epidemiology and

variants of the virus involved in the disease. Diagnosis of parvovirus in dogs usually has based only on clinical signs and rapid (enzyme-linked or chromatographic immunosorbent assay based) tests, which are often used in small animal practices without interpretation of possible false-negative results and without comparison with other methods (Zienius *et al.,* 2016). In view of that, the results from this study will provide additional knowledge to evolutionary relationships between the worldwide circulating variants versus the circulating variants in Morogoro. This is the first study to demonstrate the CPV-2 original strain and CPV-2b variant in Morogoro and Arusha by PCR and sequencing.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The following conclusions were made basing on the study findings:

i. The overall prevalence of Canine Parvovirus (CPV) was 9% when tested the fecal samples using rapid CPV Ag test kit (n=143). Only dogs of Morogoro region were found to be infected.

ii. Out of the 100 samples that were analyzed by CPV-2 PCR assay, fifteen were found to be positive for the presence of an original strain of CPV-2 and CPV-2b variant. Also, the PCR positive samples were from Morogoro region only.

iii. The VP2 partial sequence analysis showed that, the five samples from Morogoro region were closely related with CPV-2b antigenic variant from China and South Korea, showing 99.53-100% identity.

6.2 Recommendations

i. Appropriate control measures should be implemented to control canine parvovirus infection in the society and of the core strategic control of this disease is through vaccination using the circulating variant in Tanzania. This can be done by incorporating variant CPV-2b in the existing vaccine which contain the original CPV-2 strain and CPV-2c variant.

ii. Further studies on detection and characterization of the virus should be conducted in other regions of Tanzania to capture all the possible variants circulating in the country.

iii. Finally, whole genome sequencing is recommended to fully understand the antigenic characteristics of CPV viruses circulating in Tanzania.

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