Molecular detection and characterization of canine parvovirus variants in Morogoro and Arusha regions of Tanzania

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SUMMARY

Canine parvovirus (CPV) is a significant pathogen that affects wild and domestic dogs globally, resulting in intricate pathological and epidemiological consequences, primarily observed in puppies, such as haemorrhagic enteritis and myocarditis. This virus is categorized into three strains: CPV-2a (which replaced the original strain CPV-2), CPV-2b, and CPV-2c. The objective of this study was to determine the status of CPV-2 and its circulating variants in domestic dogs residing in the Morogoro and Arusha regions of Tanzania. Rectal swabs were collected from a total of 143 domestic dogs with gastroenteritis between 2020 and 2021. Polymerase Chain Reaction (PCR) targeting the VP2 gene was used to detect canine parvovirus and its circulating variants. Out of 143 dogs, 10.48% tested positive for CPV-2. Five of the positive PCR products were sequenced using primers targeting the CPV-2b variant and showed nucleotide identities of 99.53% to 100% with other CPV-2 isolates deposited in GenBank from South Korea and China. This study documents the first detection of the CPV-2b variant in Tanzania using PCR and partial sequencing of the CPV-2 VP2 gene. The findings stand as significant milestone, shedding light on the global distribution of this variant of parvovirus. Further studies on the detection of circulating variants in other regions and characterization of the CPV-2 VP2 gene are recommended to aid strategic parvovirus infection preventive measures.

Keywords: *Dogs, Haemorhagic, Diarrhoea, Virology, Veterinary*

INTRODUCTION

Canine Parvoviral enteritis emerged as severe gastroenteritis in dogs in the late 1970s and has been responsible for a massive number of puppy deaths worldwide (Zienius et al., 2016). Central to the understanding of the disease is the antigenic variation of the virus, which has been noted since its emergence (Buonavoglia et al., 2001; Miranda and Thompson, 2016; Parrish et al., 1991). Recent developments in PCR-based typing have heightened the need for studying antigenic variants, and a range of assays have been developed and are used in diagnostic laboratories to detect CPV. These assays include real-time and conventional nested PCR for the detection of CPV-2 variants (Touihri et al., 2009). There is increasing concern about the antigenic variants and the vaccine response to existing field viruses, which calls for a greater understanding of the disease.

Vaccination remains the main method of preventing the disease, and so far, vaccines appear to be safe and 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, or when maternal antibodies interfere with their vaccination, they generally become naturally infected (Parrish, 1995). One of the most significant current discussions is the genetic/antigenic relationship between the viruses and the vaccine isolates. A study by Wang et al. (2019) demonstrated variation in antigenic response between rabies vaccine strains and street isolates. The evolution of the parvovirus raises questions about the

efficacy of vaccines used (Truyen, 2006; Hernández-Blanco and Catala-López, 2015) and calls for a strengthening of understanding of antigenic Parvovirus vaccines in Africa.

In Tanzania, most dogs are left to roam freely with minimal veterinary interference, such as vaccination and prophylactic measures. Mwalongo et al. (2014) and a subsequent study (Behdenna et al., 2019) reported Canine parvovirus infection in lions and domestic dogs kept in villages surrounding Serengeti National Park. Using conventional PCR, two antigenic variants (CPV-2a and CPV-2b) have been reported from domestic dogs around Serengeti National Park (Mwalongo et al., 2014). Subsequently, the disease has been reported in Morogoro based on clinical manifestations, specifically fetid bloody diarrhea (Raymond and Matondo, 2020). In most cases, the diagnosis of parvovirus infections in Tanzania depends solely on clinical manifestations, including enteritis and diarrhea, which are similar to other viral infections (Sykes, 2014). Moreover, there have been reports of vaccination failures, which might also be due to factors such as improper immunization scheduling, persistence of maternal immunity at the time of vaccination, or circulation of different antigenic variants of the virus (Decaro et al., 2020). Therefore, this study aimed to determine the status of CPV-2 and its circulating variants among dogs in selected areas of the Morogoro and Arusha regions of Tanzania.

MATERIALS AND METHODS

Study Area

The study was conducted in three districts of the Morogoro region (Morogoro Municipal, Morogoro Rural, and Mvomero) and one district of the Arusha region (Monduli District, Makuyuni division at Esilalei, and Mswakini ward) (Figure 1). Laboratory work was carried out 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 (SUA), Morogoro. Partial sequencing of the PCR products was performed on a commercial basis by Macrogen Inc in Seoul, Korea.

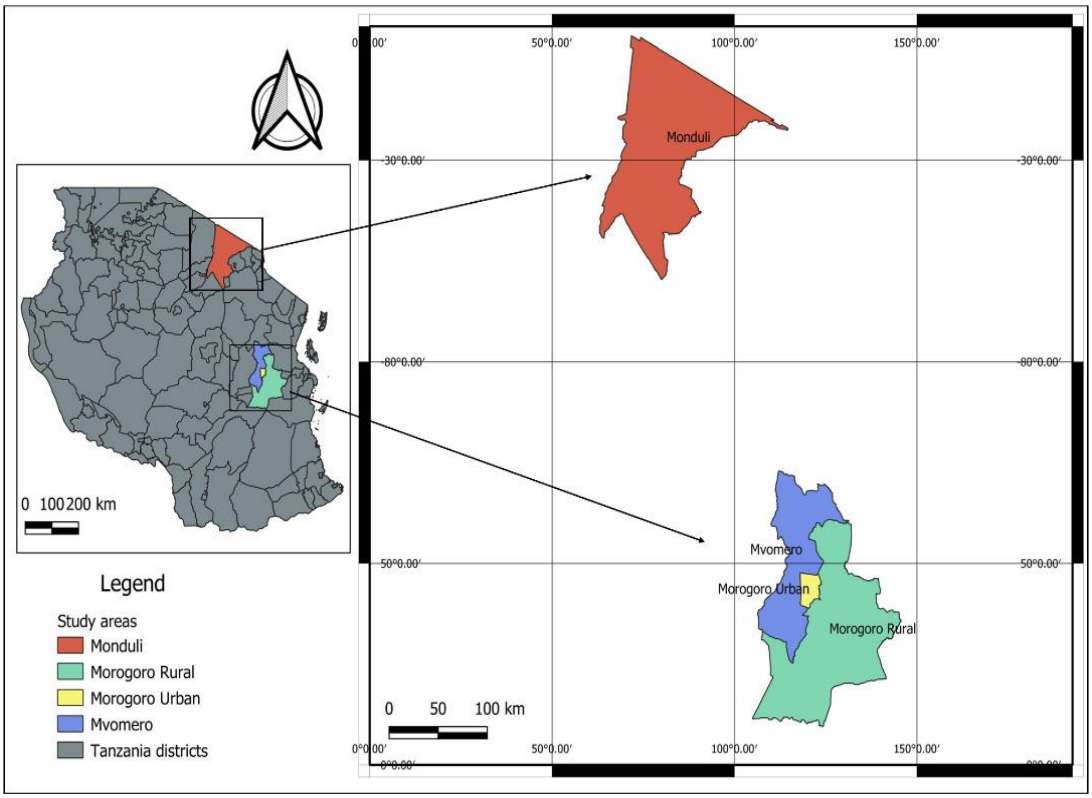

Figure 1: Map of Tanzania illustrating the study areas in Morogoro and Arusha regions. SOURCE: The map was constructed by the authors.

Study Design and sample size

The design of this research was a cross-sectional study, where dogs were purposively selected based on signs of diarrhea and sampled once without a prior history of their canine parvovirus vaccination status. The research strictly followed Sokoine University of Agriculture's Code of Conduct for Research Ethics (section 2.2) and obtained clearance from the Ethical Clearance Committee of the College of Veterinary Medicine and Biomedical Sciences, SUA, as well as from the District Executive Directors in each of the four districts studied, with prior consent obtained from dog owners. The sample size was calculated using a formula described by Naing et al. (2006): $N=Z^2*p\ (1-p)/d^2$, where *N* is the sample size, *Z* is the test statistic, *p* is the expected prevalence, and *d* is the precision. The values used in the formula were Z=1.96, d= 5% and p= 10.4% as prevalence of CPV in dogs (Mwalongo *et al.*, 2014). Therefore, the calculated sample size for the dogs was 143.

Sample Collection and Processing

A total of 143 dogs were purposively selected from four clusters of districts, with an average of 36 dogs from each cluster. Fecal samples were collected from dogs with diarrhea using a rectal swab, which was introduced through the anus of the dogs. The swabs were immediately immersed in a 2 ml collection tube containing sterile phosphate buffered saline (10% w/v). After thorough mixing of the sample in the 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 a 0.45 µm sterile filter, and stored at −20°C. The samples were used within one month from the date of collection.

Screening of faecal samples using a rapid CPV Ag test kit

A commercially available rapid CPV Ag Test Kit (Synbiotic Corporation®), a chromatographic immunoassay, was used for the qualitative detection of Canine parvovirus antigen in canine feces. The test was performed following the manufacturer's protocol. Briefly, the swab was coated with a thin layer of feces, and then the feces-coated swab was immersed in the sample extraction buffer in the tube. The swab was swirled vigorously in the buffer, and by pressing the swab against the side of the tube, it was ensured that most of the fecal materials from the swab were removed. When removing the swab from the test tube, the swab was pressed against the side of the tube repeatedly until no more liquid came from the swab. Three drops of the sample were transferred to the sample well, drop by drop vertically. The results were read after ten minutes to determine the presence or absence of the pink/purple bands. Samples were considered positive if pink/purple bands were visible in both the sample and control windows. Samples were considered negative if there was only one pink/purple band in the control window and no band in the sample window.

Extraction of DNA and quantification

The DNA was extracted from the collected stool samples using a Quick-DNA fecal/soil microbe Miniprep Kit (Zymo Research Corp., USA, lot no. 206040) following the manufacturer's instructions. Briefly, 250 mg of fecal sample was added to a ZR BashingBead™ Lysis tube, and then 750 µl of Bashing Bead™ Buffer was added to the tube. A bead beater fitted with a 2 ml tube holder assembly was secured and centrifuged at maximum speed for 5 minutes. This was followed by centrifugation of the ZR Bashing Bead™ Lysis tube in a microcentrifuge at 8,000g for 1 minute. Up to 400 µl of the supernatant was transferred to a Zymo-Spin™ IIIF Filter in a collection tube and centrifuged again at 6,000g for 1 minute. Subsequently, 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the collection tube, mixed well, and 800 µl of the mixture from the previous step was transferred to a Zymo-Spin™ IICR Column in a collection tube. The column was centrifuged at 8,000g for 1 minute. The flow-through from the collection tube was discarded, and the last step was repeated.

Next, 200 µl of 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. Then, 500 µl of g-DNA Wash Buffer was added to the Zymo-Spin™ IICR column, and the mixture was centrifuged again at 8,000g for 1 minute. The Zymo-Spin™ IICR column was transferred to a clean 1.5 ml microcentrifuge tube, and 100 µl of DNA Elution Buffer was added directly to the column matrix. It was then centrifuged at 10,000g for 30 seconds to elute the DNA. The Zymo-Spin™ III-HRC Filter was prepared in a clean collection tube by adding 600 µl of Prep Solution, and it was centrifuged at 6,000g for 3 minutes. Finally, the eluted DNA was transferred to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuged at exactly 16,000g for 3 minutes.

The concentration of filtered DNA was measured in a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington) and stored at -80 °C for 15-30 days before the PCR analysis. The concentration of DNA was measured in ng/µL and the expected range of DNA concentration was from 50 to 250 ng/µL.
A freeze-dried multivalent vaccine (VANGUARD® PLUS 5 L4, Zoetis Inc., USA) containing a preparation of attenuated strains of canine distemper (CD) virus, canine adenovirus type 2 (CAV-2), canine parainfluenza (CPI) virus, canine parvovirus (CPV), and inactivated whole cultures of *L. canicola*, *L. grippotyphosa*, *L. icterohaemorrhagiae*, and *L. Pomona* was used as a positive control. This is the most currently used vaccine to control parvovirus infections in Tanzania.

Detection of CPV-2 by Conventional Polymerase Chain Reaction

The conventional PCR for the detection of CPV-2 utilized CPV-2 F and CPV-2 R primers (Table 1), as published in previous studies (Sharma et al., 2016), targeting the VP2 gene. These primers amplify a 681 bp fragment of the original CPV-2 strain. For each PCR reaction, 12.5 µL of One Taq® Quick-load® 2x Master Mix with Standard Buffer (New England Biolabs, catalog No. M0486S), 1 µL of each forward and reverse primer, 8.5 µL of nuclease-free water, and 2 µL of DNA template were used, making a total mixture of 25 µL for each reaction. The PCR amplification was optimized with the following conditions: Initial denaturation at 95 ºC for 10 minutes, followed by 35 cycles of denaturation at 95 ºC for 1 minute, annealing at 55 ºC for 1 minute, extension at 72 ºC for 1 minute, and final extension at 72 ºC for 10 min. Molecular-grade water was used as a negative control for amplifications, and a commercial vaccine (VANGUARD® PLUS 5 L4, Zoetis Inc., USA) served as a positive control.

Detection of CPV-2 antigenic variants (CPV-2a, CPV-2b and CPV-2c) by Conventional PCR

To detect the new antigenic variants of CPV-2, a 681 bp fragment encoding the capsid protein VP2 of both antigenic types CPV-2a and CPV-2b was amplified from the template DNA using forward (CPV-2ab F) and reverse (CPV-2ab R) primers (Table 1). The preparation of the reaction mixture, PCR amplification conditions, and visualization of PCR products were performed following the protocol used for the detection of the CPV-2 original strain above.
Since CPV-2ab F and CPV-2ab R primers can amplify both CPV-2a and CPV-2b antigenic types, another PCR was set using CPV-2b F and CPV-2b R primers (Table 1), which have the ability to recognize variant CPV-2b but not variant CPV-2a. Therefore, in this case, 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). The preparation of the reaction mixture, PCR amplification conditions, and visualization of PCR products were performed following the protocol used for the detection of the CPV-2 original strain above. PCR products were electrophoresed along with the GeneRuler™ 100 bp DNA plus ladder (Thermo Fisher Scientific®, Wilmington) in a 1% agarose gel stained with 4 µL of DNA Gel Red Stain (Biotium®, San Francisco Bay Area). The progress of mobility was monitored by the 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.

All 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). This

assay amplifies a 583 bp fragment of the gene encoding the capsid protein VP2 for the detection of antigenic variant CPV-2c. Again, the preparation of the reaction mixture, PCR amplification conditions, and visualization of PCR products were performed following the protocol used for the detection of the CPV-2 original strain above.

Partial sequencing of the VP2 structural protein gene

Approximately 45 µl of selected positive PCR products were sent to Macrogen Inc. (Seoul, Korea) for partial sequencing of the VP2 structural protein gene using forward and reverse primers on a commercial basis. The obtained sequences from the forward and reverse primers were edited and assembled using CLC Main Workbench 6.94 to obtain a consensus sequence. During editing process, quality analysis review of the sequences was performed manually by visual inspection of each sequence by comparing nucleotides to their corresponding chromatogram peaks. Nucleic acid sequences with poor chromatogram especially from both ends were trimmed before being assembled. The consensus sequences of CPV-2 obtained were searched in the NCBI GenBank database using the Basic Local Alignment Search Tool (BLASTN 2.12.0+) (Madden and Mancinelli 2008) to find regions of local similarity between CPV-2 sequences. Sequences to include in the multiple sequence alignment were chosen from the list generated during the BLAST search. Multiple sequence alignment was performed using Clustal W built into MEGA 11 (Tamura et al., 2021).

Phylogenetic analysis

A phylogenetic tree of the nucleotide sequences based on the partial length of the VP2-encoding gene was reconstructed using the Maximum Likelihood method and Tamura-3-parameter model (Tamura, 1992), as implemented in MEGA 11 (Tamura et al., 2021), with 1000 replicates. The substitution model with the lowest Bayesian Information Criterion (BIC) scores after computation using MEGA 11 was selected and used to reconstruct the phylogenetic tree. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All primers were obtained from the previous studies (Sharma *et al.*, 2016).

Table 1: List of primers used for detection of the original CPV-2 and 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	GGTGCTAGTTGATATGTAATAAACA		

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RESULTS

Canine parvovirus has been detected in domestic dogs by using a rapid CPV Ag test kit and PCR

A total of 143 fecal samples were collected from dogs with diarrhea in three districts of the Morogoro region (Morogoro Municipality, Mvomero District, and Morogoro Rural District) and one district of the Arusha region (Monduli District). A preliminary test was performed by screening the samples using a rapid CPV Ag test kit. Out of the 143 samples screened, 15 (10.48%) were found positive for CPV (Table 1). A total of 143 fecal samples were subjected to analysis through conventional PCR assay for the detection of CPV-2.

Among these samples, 15 (10.48%) were identified as positive for the presence of the original strain of CPV-2 using the CPV-2 PCR assay, producing the expected 681 bp amplicon size (Figure 2-4, Table 1). It's worth noting that only the 15 samples that tested positive for rapid CPV Ag were detected by the PCR assay. All 15 samples that tested positive in the PCR analysis mentioned earlier underwent another round of PCR using CPV-2ab F and R primers designed for the detection of both variant CPV-2a and CPV-2b. The CPV-2ab PCR assay confirmed the presence of the VP2 gene in all 15 fecal samples, yielding the expected 681 bp amplicon size (Figure 3).

Table 1: A rapid CPV Ag and PCR test results of dog fecal collected in Morogoro and Arusha regions

Sampling location	Samples collected	Proportion of Ag positive (%)	Proportion of PCR positive (%)
Morogoro municipality	50	20	20
Mvomero district	30	10	10
Morogoro rural	20	10	10
Monduli district	43	0	0
Total positive	**143**	**10.48**	**10.48**

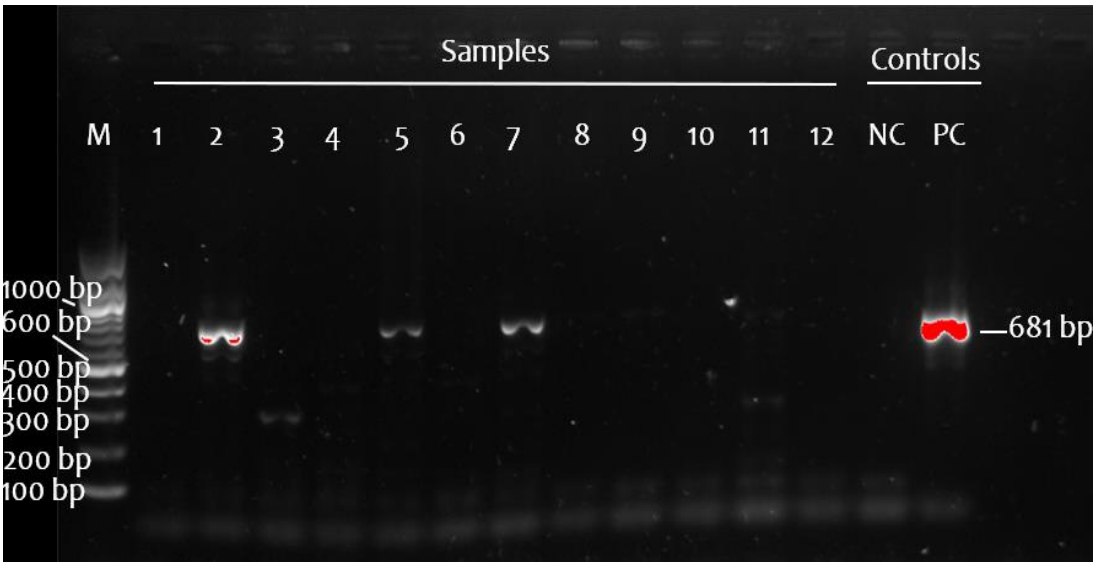

Figure 2: Gel electrophoresis of CPV-2 PCR products amplified using universal primers targeting 681 bp of VP2 gene. Lane M: DNA ladder, Lane NC: negative control (RNase free water), Lane PC: positive control (vaccine strain), and Lanes 1-11: test samples.

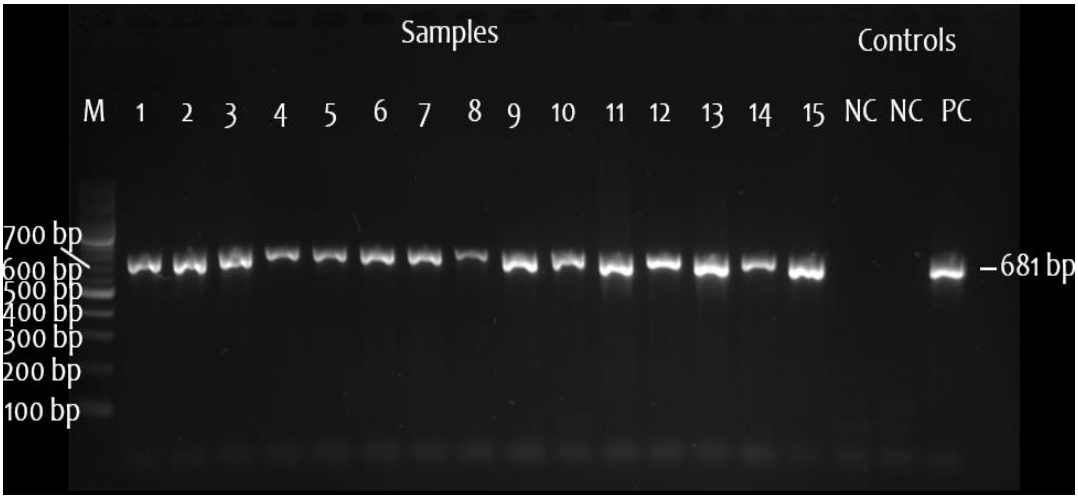

Figure 3: Gel electrophoresis of CPV2 PCR products amplified using CPV-2ab primers. Lane M: DNA ladder, Lane NC: negative control (RNase free water), Lane PC: positive control (vaccine strain), and Lanes 1-15: test samples.

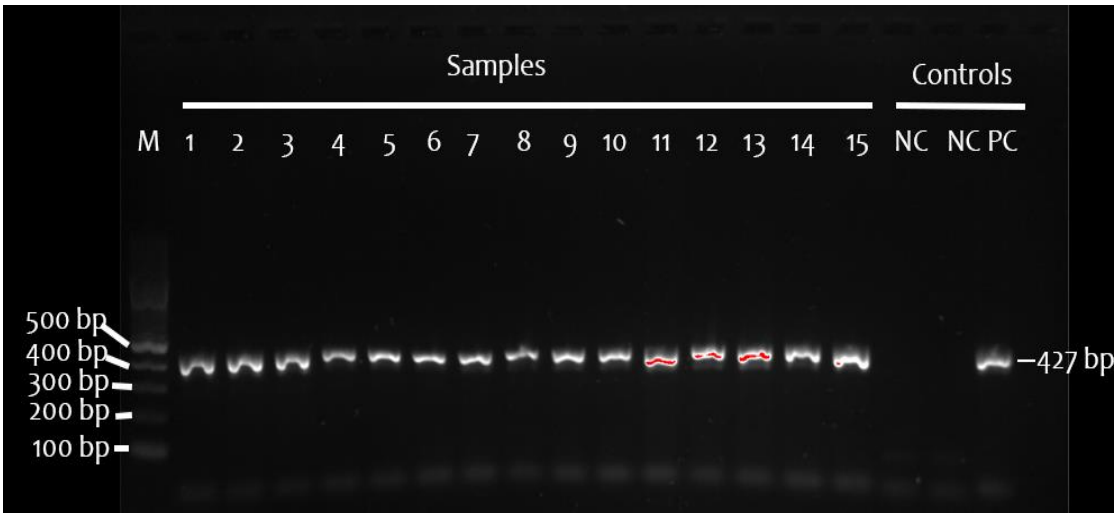

Figure 4: Gel electrophoresis of CPV2 PCR products amplified using CPV-2b primers. Lane M: DNA ladder, Lane NC: negative control (RNase free water), Lane PC: positive control (vaccine strain), and Lanes 1-15: test samples.

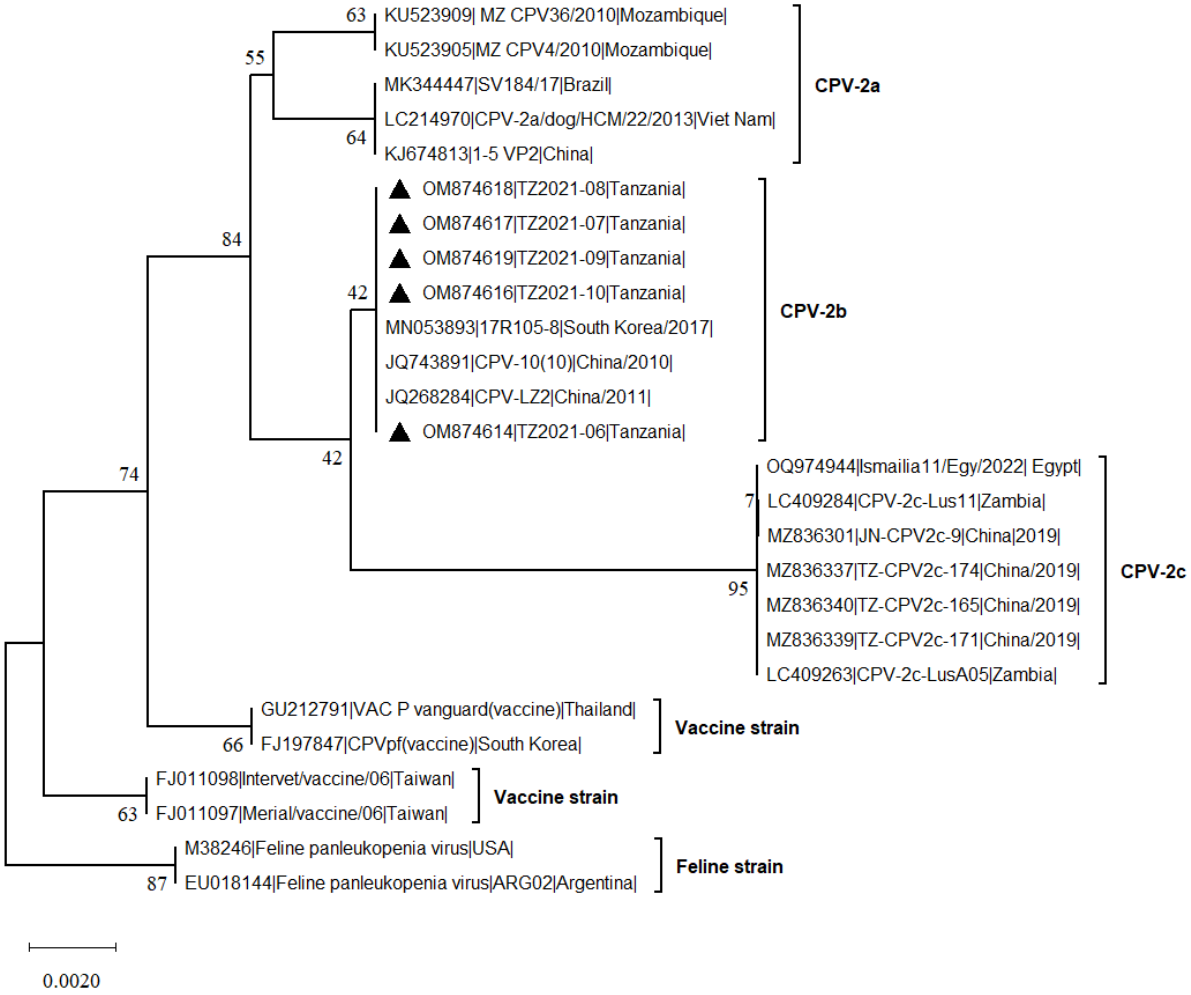

Figure 5: Evolutionary analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-652.33) is shown. The percentage of trees in which the associated taxa clustered 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 3 parameter 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).

To confirm whether the detected amplicons correspond to variant CPV-2a or CPV-2b, the samples underwent another round of PCR using CPV-2b F and R primers. All 15 samples were successfully detected by these primers, yielding an expected 427 bp amplicon size (Figure 4). This indicates that the samples contained the CPV-2b variant and not CPV-2a. It is noteworthy that variant CPV-2a is recognized exclusively by CPV-2ab primers, whereas variant CPV-2b is identified by both CPV-2ab and CPV-2b primers (Sharma et al., 2016).

Canine Parvovirus and its circulating variant CPV-2b have been confirmed in domestic dogs through sequencing. The PCR products were amplified and sent for sequencing on a commercial basis to Macrogen Inc. (Seoul, Korea). The obtained sequences were edited, assembled, and the consensus sequences were used to identify local regions of similarity in the NCBI GenBank database using the BLAST Search Tool. Upon the blast search (Megablast), the highest identity ranged from 99.53% to 100% with other CPV-2 deposited sequences from South Korea and China. The consensus nucleotide sequences were submitted to NCBI GenBank and accession numbers were asigned (OM874614-OM874619).

The phylogenetic tree (Figure 5) was reconstructed, incorporating other 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. Two isolates of Feline Panleukopenia Virus (FPLV) were also included to root the tree, as it is believed that CPV-2 evolved from FPLV. The CPV-2 vaccine strains used to control CPV-2 infections in dogs were also included in the reconstructed phylogeny. Phylogenetic analysis revealed that sequences from this study (marked with ▲) clustered within the CPV-2b subtype in the phylogeny, alongside the Chinese and South Korean isolates (Figure 5).

DISCUSSION

In Tanzania, the diagnosis of the virus relies on clinical signs and, to some extent, rapid diagnostic tests. Molecular detection and partial sequencing of the virus have only been documented in Serengeti National Park in Northern Tanzania from apparently healthy domestic dogs (Mwalongo et al., 2014). This was confirmed by this study where CPV-2 was detected in the fecal samples collected from healthy dogs. 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 as previously reported by Sharma et al., (2016). Since all 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. Furthermore, there was no amplification of CPV-2c when primers targeting variant CPV-2c were used, implying that the variant was not present in the samples. The nucleotide sequence analysis of the CPV in the present study showed that the sequences were 99.53 to 100% identical to CPV-2b antigenic variants isolated from China (isolate 17R105-8 with GenBank accession number MN053893.1) and South Korea (strain CPV-10(10) with GenBank accession number JQ743891.1). Moreover, the phylogenetic analysis of the CPV-2b VP2 nucleotide sequence also showed that all CPV-2b variants detected formed a monophyletic cluster with the CPV-2b variants from China and South Korea.

This study confirms the presence of CPV-2b in Tanzania, which is consistent with Mwalongo et al. (2014), who found the variant in healthy domestic dogs. All three CPV-2 variants have been reported to circulate in Tunisia (Touihri et al., 2009) and Morocco (Amrani et al., 2016), whereas

CPV-2b variant has been reported only in South Africa (Dogonyaro et al., 2013; Oosthuizen et al., 2019), Mozambique (Figueiredo et al., 2017). This study, however, failed to demonstrate the presence of CPV-2a, as shown by Mwalongo et al. (2014). All fifteen samples were positive for CPV-2 original strain, suggesting that the original strain of CPV, normally incorporated into CPV vaccines, still circulates in the local canine population in the Morogoro region. No information is available if the vaccinated animals are protected against newer antigenic types (CPV-2b) using the available CPV vaccines that only contain the original CPV-2 and CPV-2c strains.

Based on the phylogenetic analysis performed in this study (Figure 5), there is a possibility that the currently used vaccines, especially from isolate VAC_P vanguard (GU212791.1/Thailand) and isolate CPVpf (FJ197847.1/South Korea), can still protect the dogs against parvovirus infection. The isolate VAC_P vanguard is currently used in Tanzania to vaccinate dogs against parvovirus infection. Nevertheless, there have been complaints that vaccinated dogs against parvovirus infection in Tanzania have also been dying with clinical signs suggestive of parvovirus infection. Findings from this study indicate that about 15% of the samples collected from dogs with diarrhea in the Morogoro region were positive for parvovirus. A 12-year retrospective study on the pattern and relative frequency of preventable canine diseases in Morogoro based on clinical signs indicated that parvoviral diarrhea is among the top five most frequently admitted cases at Sokoine University of Agriculture (SUA) Animal hospital (Raymond and Matondo, 2020). The trend for parvoviral diarrhea cases at the hospital was found to be increasing with time, calling for a reassessment of the strategies used to control parvoviral infection in dogs. Vaccination of dogs against parvovirus infection is the main control strategy worldwide. However, there are several factors for vaccine failure, including improper cold chain and the use of vaccines that do not match the circulating virus strains (Decaro et al., 2020).

The results from this study provide additional knowledge by indicating that the vaccines currently in use for controlling parvoviral infection may partially protect against the parvovirus variant circulating in the Morogoro region subject to confirmation through experimental and field studies in Tanzania. This is the first study to demonstrate the CPV-2 original strain and CPV-2b variant in the Morogoro region by PCR and partial sequencing of the VP2 protein. Sequence comparison showed that the circulating CPV-2b variant was identical to isolates from China and South Korea. It is not currently known how the strain was imported to Tanzania. There are possibilities that the strain was imported from China and Korea; however, this possibility remains to be ascertained through research.

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CONFLICT OF INTEREST

Authors do not have any conflict of interest.

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