UNIVERSITY OF PADUA

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Thesis

Molecular epidemiology of canine parvovirus in Namibia

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SUMMARY:

Dogs and cats have an increasing importance in the daily life of many families, and their care is a consolidated goal of Veterinary Medicine. It is in this context that canine parvovirus emerged as a very widespread disease. The causative agent is canine parvovirus (CPV-2), a virus that emerged recently, and is subject to a rapid and continuous evolution that has led it to mutate over the years, and to be able to acquire new host species .The three antigenic variants 2a, 2b, and 2c are widely distributed in the canine population globally, and more than one variant can exist in most cases.

CPV is a highly contagious viral infection that affects dogs, especially puppies, and can lead to severe illness or death if left untreated. It can be transmitted through direct contact with infected dogs or their feces, as well as through contaminated objects or environments thanks to its prolonged environmental persistence. Vaccination is an important preventive measure against CPV infection. Despite vaccination, CPV infections remain common in dogs, most often as a result of immunization failure or potentially because of mutations reducing cross-protection. The present work aims to increase the knowledge of CPV epidemiology and investigate the Namibian epidemiological situation.

To this purpose, newly generated sequences from Namibian domestic dogs and jackals were obtained and compared with already available ones. A dedicated phylogeographic analysis revealed that the introduction was more likely mediated by other African countries, highlighting the challenge of controlling illegal animal imports across the land borders. Similarly, the absence of any geographical clustering within Namibia testifies a substantially unconstrained viral circulation among districts. Although the limited wild animals' sample size prevents any definitive conclusion, the identity of the sequences from the jackal and the ones originating from the domestic dogs suggests a potential inter-species transmission.

ABSTRACT:

Canine parvovirus (CPV-2) has been shown to have originated as a host range variant from the feline panleukopenia virus (FPV), which primarily affects cats. Since it is a single-stranded DNA virus, it has a very high evolutionary rate and genetic variability compared to double-stranded DNA viruses. Through natural evolutionary processes, CPV-2 has adapted to the canine host. The canine parvovirus (CPV-2) is a relatively new virus that emerged in the late 1970s and has drawn a lot of interest globally and is responsible for severe systemic infection and high mortality if not treated. Soon after this discovery, CPV-2 underwent genetic evolution, and rise to two antigenic variants, CPV-2a and CPV-2b, which gradually took the place of the original type. A new antigenic variant, CPV-2c has been found in some countries since 2000 such as Italy and quickly spread around the world. The three antigenic variations CPV-2a, CPV-2b, and CPV-2c, which are distinguished by critical amino acid changes in the VP2 protein, have gradually supplanted the original CPV-2 type, which no longer exists in nature and is only found in a few commercial vaccines.

Namibia is a country in the southwest of Africa located between the Namib desert and the Atlantic Ocean. The country is home to diverse wildlife. Even though domestic dogs especially puppies, are in danger of this infection, wildlife also can be endangered. Molecular epidemiology data on CPV-2 in Africa, including in Namibia, are limited and outdated, showing the need for further research in these areas.

The present work aims to increase the knowledge of CPV epidemiology and investigate the Namibian epidemiological situation by analyzing Namibian dogs with clinical signs of acute gastroenteritis and jackal samples. These samples were tested by PCR and the complete VP2 of positive amplicons were sequenced. The VP2 of identified strains were characterized and evaluated to assess viral circulation and the linkage between host species, Namibian districts, and foreign nations.

Based on the obtained results, except one New-CPV-2a, all the detected strains belonged to the CPV-2c antigenic variant and were closely related to strains of Asian origin, which suggests the following conclusions:

1) The virus may have been introduced through the illegal movement of animals from regions where the CPV-2c variant is prevalent, mainly from Asia.

2) The absence of any geographical clustering within Namibia suggests a substantially unconstrained viral circulation among different districts of the country. This indicates that the virus has spread widely and is not restricted to specific regions within Namibia.

3) Absence or incomplete vaccination facilitated a huge unrestricted spread of the virus within districts. Vaccination and other control measures applications should be considered to protect wildlife.

4) Although a limited number of samples from jackals have been used in this study, the fact that the sequences from the jackal are closely related to those of domestic dogs indicates a possible sharing of the virus between these two species.

INDEX

1.INTRODUCTION:

To understand the problems that canine parvovirus (CPV-2) causes, it is important to trace the evolution of CPV to monitor the appearance of mutations that might affect vaccine effectiveness. Several diagnostic tests have been developed to detect parvoviral infections which are categorized into immunological tests (latex agglutination test, SIT-SAT and ELISA, etc.) and molecular-based tests (PCR, mPCR and RT-PCR etc.) (Figueiredo et al., 2017).

For CPV field diagnostics, the goal is to identify the virus, excluding the presence of other pathogens that can affect the gastrointestinal tract generating comparable clinical signs; for this purpose, commercial immunochromatography (IC) kits are used, or tested based on hemagglutination (HA) and viral isolation (VI) that can be performed in specialized laboratories. However, these methods have shown many weaknesses, such as being expensive in terms of time and costs and low sensitivity. Several factors might be involved, such as the excretion of a low viral load, the presence of antibodies in the intestinal lumen that masks the viral antigen, or the appearance of some strains that lost the ability to haemagglutinate (Flacke et al., 2013).

For this reason, biomolecular methods such as RFLP-PCR (Polymerase Chain Reaction Restriction Fragment Length Polymorphism) and RT-PCR (Real Time Polymerase Chain Reaction) are often chosen, since they are not affected by the immune response and are relatively robust to sample features. In particular, RT-PCR is very sensitive, it allows to detect and quantify the products generated during the reaction starting from a relatively simple preparation of the sample, and does not require post-PCR processing, thus reducing the time required compared to traditional PCR (Figueiredo et al., 2017).

The application of these techniques can also be used to further characterize the virus in addition to its detection. Initially, a combination of several methods was used to discriminate the variants of the CPV. Techniques like the inhibition of hemagglutination (HI) with monoclonal antibodies (Mabs), PCR-RFLP and finally sequencing, it was

possible to define whether the CPV found was a CPV-2a, CPV -2b or CPV-2c and even establish more precise epidemiological linkages among strains. However, this implies a great deal of time and money (Figueiredo et al., 2017; Maher & Wyatt, 2021).

With the validation of the Minor Groove Binder (MGB) probe assay, the differentiation between variants got much faster. Thanks to the use of MGB, having target regions characterized by SNPs (Single Nucleotide Polymorphism), it is possible to distinguish the variants by exploiting the single genetic differences at the nucleotide level. This technique is highly specific and sensitive and due to its affordability and practicality, is often used for epidemiological studies devoted to the identification of circulating genotypes (Dogonyaro et al., 2013a).

The only weakness could be in relying on the slightest difference in SNPs to determine the CPV variant, since it means that even a single change in the probe-binding region can result in variant characterization error (Leisewitz et al., 2001).

The MGB probe assay can also be used as a technique to distinguish the field strains from the vaccine strains, defining whether the gastrointestinal symptoms, which appeared a few days after vaccination, are due to a reversion to virulence of the vaccine virus or an infection caused by a field strains. Depending on the characteristics of the MGB, it is possible to differentiate the variants, therefore a field strain CPV-2a, CPV-2b, CPV-2c, compared to the vaccine obtained on the CPV-2. With the introduction of the vaccine formulated on CPV-2b, the previous MGBs were not able to discriminate the new vaccine strain from the CPV-2b field variant and it was thus necessary to develop a MGB that was able to distinguish the two strains. This aspect outlines how the MGB probe assay is one technique that must be continually reworked and updated according to the evolution and viral epidemiology, as well as according to the objectives set (Dogonyaro et al., 2013a; Leisewitz et al., 2001).

Before describing the evolution and epidemiology of parvovirus, it is necessary to mention the taxonomy of the *Parvoviridae* family, which is constantly updated.

Protoparvovirus carnivoran1 species (Family: *Parvoviridae*, Order: *Piccovirales,* class: *Quintoviricetes*, Phylum: *Cossaviricota,* Kingdom: *Shotokuvirae,* class: *Monodnaviria*) is a small, non-enveloped, single-stranded linear DNA virus of approximately 5000 nucleotides, including two large open reading frames (ORFs): ORF1 encodes the two non-structural proteins NS1 and NS2, and ORF2 encodes the two structural proteins VP1 and

VP2. NS1, a nuclear phosphoprotein, plays a very important role in the replication of the virus and is responsible for cell apoptosis. The primary component of the capsid is VP2, which is engaged in (TfR) (transferrin receptor) binding and is the most essential protective antigen. As a result, the amino acid makeup of VP2 influences the majority of the virus's biological properties. The Capsid of CPV is small but complex (Maher & Wyatt, 2021).

VP1 contains the full length of VP2 plus an N-terminal domain. VP2 is the most present structural protein, representing 90% of the capsid and is the basis of the virus-host interaction. It is also cleaved into VP3 by the host proteases. The 60 protein subunits are formed by a β-sheet architecture, in which the β filaments are interconnected by amino acid loops that constitute the characteristic spikes of the capsid (Figueiredo et al., 2017).

As mentioned before, CPV is a single-stranded DNA virus and does not have its own polymerase to replicate its genome. For the virus, it is therefore necessary to enter the nucleus of an actively replicating host cell to benefit from its synthetic machinery. Furthermore, CPV does not possess the proteins to induce a cell entering the S-phase and is therefore forced to wait for this replicative phase to start autonomously. These important biological limits determine the pathogenesis of CPV, which is obliged to target cells that are most of the time in the S-phase, such as those of fetuses, new-borns, or hematopoietic cells and basal cells of the intestinal tract of young and adult animals (Tu et al., 2015).

Cell attachment and entry are mediated by the interaction between viral capsid and host cell transferrin receptor (TfR) (transferrin receptor).TfR is a cell surface glycoprotein expressed as a homodimer on most cells in the body and in particular on those with high levels of proliferation; its function is to bind transferrin, the proteins used to transport iron, and to bring them inside the cell through a mechanism of receptor-mediated endocytosis. Similarly, TfR binds the CPV virion, and rapidly transports it into the cell via the endosomal system (Fig.1). The binding with this receptor determines the ability to infect the cell (Darriba et al., 2012).

After infection, the virus replicates in the lymphoid tissue of the oropharynx, mesenteric lymph nodes and thymus, and spreads to the intestinal crypts of the small intestine in 1-5 days post-infection. The infection of the germinal epithelium of the crypts causes the destruction and collapse of the epithelium that should have constituted the lining of the intestinal villi with consequent alteration of cell turnover. The elimination of the virus in the feces begins around the third day after exposure and continues massively for 7-10 days.

During the infection, the virus also compromises the mitotically active precursors of circulating leukocytes and in particular lymphoid cells with consequent lymphopenia (decrease below 2000-3000 cells/ml of blood) often accompanied by neutropenia. Secondary bacterial infections, attributable to the alteration of the intestinal barrier, cause further complications such as bacteremia and endotoxemia. Although some dogs suffer from a subclinical or inapparent infection, severe enteritis can typically develop and fatal myocarditis can occur in puppies less than eight weeks of age. Symptoms of parvovirus enteritis are associated with vomiting, haemorrhagic diarrhea, anorexia and dehydration, hyperthermia and leukopenia. The development of serum antibodies occurs early 3-4 days after infection, while the development of local intestinal antibodies occurs towards the end of viral excretion. If a puppy heals from CPV enteritis, its antibodies remain at constant high levels for about a year, making it immune to possible reinfection (Decaro & Buonavoglia, 2012; Desario et al., 2005; Greene, 2011; Tu et al., 2015).

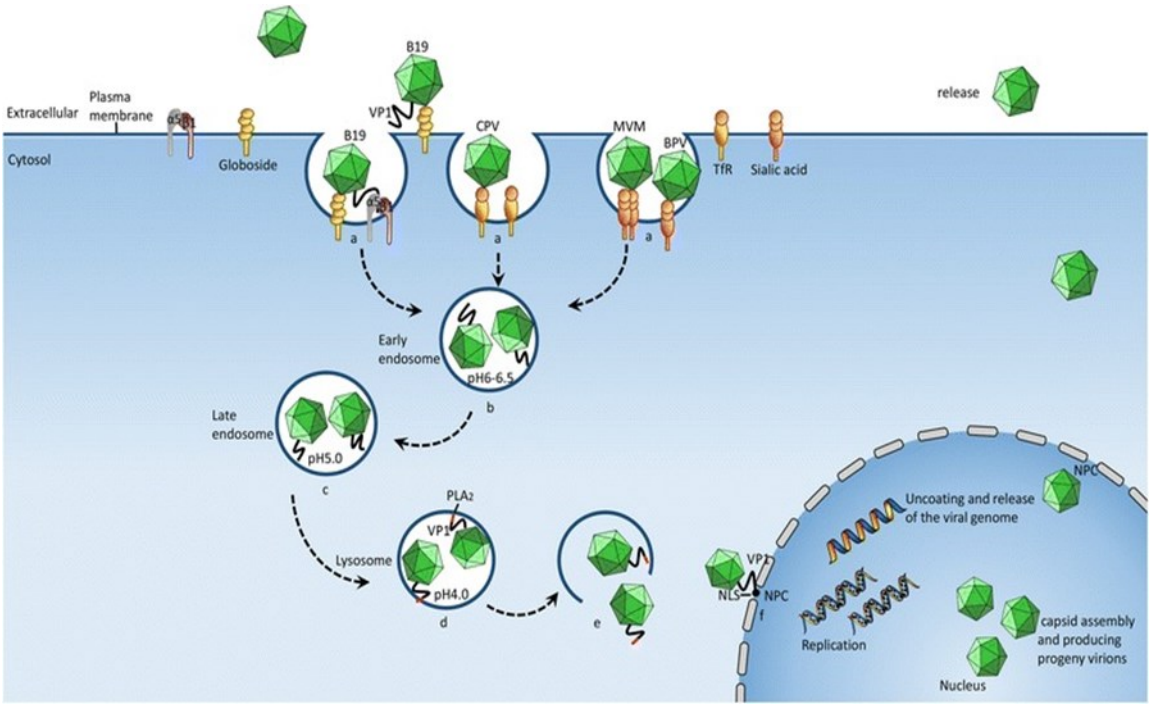

Fig. 1. A schematic of the parvovirus infection process, mediated by the Clathrin-dependent endocytic pathway. The internalization of the virus is mediated by the endocytosis pathway, primarily through the following steps. a Receptor binding-mediated internalization. The cellular receptor for parvovirus B19 is globoside, the cellular receptor for CPV is transferrin receptor (TfR), and the cellular receptor for MVM and BPV is sialic acid. b Form of the early endosome (pH 6.0-6.5). c Transformed into the late endosome at lower pH conditions (pH 5). d Transformed into the lysosome and the activation of phospholipase A2 (PLA2) (pH 4.0). e The activated PLA2 destroys the integrity of the lysosomal membrane. Thus, the viruses are released into the cytosol. f The viruses are transported towards the nucleus and target on the karyotheca with the help of the VP1 nuclear localization signal (NLS). The movement of microtubule and actin filaments are involved in the entire process of viral infection, from the early endosome to the peripheral nucleus. The viral genome begins to replicate and assemble after trafficking through the nuclear pore complex (NPC). The matured virions finally move through the NPC and are released extracellular. Adopted from (Tu et al., 2015).

The details of parvovirus infection of cells are still not fully understood, but the processes must involve small changes in the capsid structure that allow the endocytosed virus to escape from the endosome, pass through the cell cytoplasm, and deliver the single-stranded DNA (ssDNA) genome to the nucleus, where viral replication occurs (Fig1). Amino acid alterations on the capsid's outer surface control specific capsid structural changes and affect TfR binding, which is necessary for infection. However, the discovery of other amino acid changes in the VP2 protein, as well as the poor phylogenetic resolution supporting the three variants, are leading to a gradual shift away from the CPV-2a, CPV-2b, and CPV-2c terminology and toward the defining amino acid substitutions for each of these antigenic variants (Kumar et al., 2018b).

Some cases of co-infection of CPV-2 variants have been reported. In one of the cases, the dog was infected by CPV-2a and CPV-2c which strains distinguished by 29 nucleotides. In

other cases, the dog was infected by a recombinant strain, from recombination between CPV-2a and CPV-2c (Hu et al., 2020; Pérez et al., 2012).

Although CPV is an ssDNA virus and uses the host's cellular enzymes to replicate, thus benefiting from a certain degree of control and correction at the level of viral replication, it is not immune from possible errors, since it uses only some parts of this mechanism. The causes that can lead to frequent errors in the replication of ssDNA viruses are:

a) The characteristics of the viral DNA-template, which does not guarantee the fidelity of the DNA polymerase.

b) The reduced efficiency of the cellular repair and correction systems, which are not equally active in an infected cell.

c) The lack of the double helix (Mahy, 2010; Morley & Turner, 2017).

Although the high rate of mutations is certainly a key element of the great plasticity that characterizes CPV. Several factors, such as the size of the effective population, viral fitness, replication rate and natural selection also can play an important role too (Miranda & Thompson, 2016).

Many of the VP2 non-synonymous mutations occur in restricted sites, while those that occur outside these sites, but always on VP2, are synonymous substitutions, which do not modify the amino acid sequence and therefore protein functionality.

The canine parvovirus type 2 (CPV-2) first appeared in kennels and dog shelters in the late 1970s, causing severe epizootics. CPV-2 underwent genetic evolution shortly after it first appeared, giving rise successively to the two antigenic variants, CPV-2a and CPV-2b, which gradually replaced the original type. A brand-new antigenic variation called CPV-2c was discovered in Italy in 2000 and quickly spread to numerous other nations. Many studies have shown that Italy did not have CPV-2c before 2000 but then it quickly replaced CPV-2b, which had been reported less frequently in Italy in recent years. Sporadic isolation of CPV-2c has also been attained in Bulgaria, Greece, and the United Kingdom, where CPV-2a/2b detection was more common (Filipov et al., 2016). Since the earliest CPV-2c strain was recovered in 1996, there is proof that this variant was circulating in Germany for 4 years prior to its initial discovery in Italy in 2000. Epidemiological studies show that the most recent strain of CPV-2c is spreading to various geographical areas and is frequently linked to serious diseases in adult dogs. CPV-2c is currently predominant in Italy,

Germany, and Spain, and it is also extensively dispersed in Portugal, France, and Belgium, although CPV-2b or CPV-2a are more frequently detected (Decaro et al., 2011). Remarkably, it has been sometimes reported also in dogs who have finished the vaccination procedures (Kumar et al., 2018b).

CPV-2 was first detected in China in 1982 and then CPV-2 variants, namely CPV-2a/2b, and CPV-2c, circulating among dogs were reported. Many studies show the predominance in Southeast Asia such as China, Indonesia, Taiwan, Thailand and Myanmar of the CPV-2c. However phylogenetic analysis shows that Asian CPV-2c strains are in a separate cluster compared to CPV-2c from Europe and North America due to most of the CPV-2c stains had A5G, F267Y, Y324I, and Q370R mutations (Fig.2) (Dong et al., 2020; Mon et al., 2022; Wang et al., 2022).

Fig.2. Phylogenetic tree reporting the phylogenetic relationships between CPV-2 antigenic variants. Adopted from (Wang et al., 2022).

The African dog population is especially exposed to the disease because of low vaccination level, and their outdoor life exposes the country's wildlife resources to the risk of infection (Leisewitz et al., 2001). Except for 2009 research in Tunisia, no genetic characterization of CPV-2 has been published in Africa since 1998 (Table 1) (Dogonyaro et al., 2013b).

Limited data about the prevalence of CPV on the African continent, especially Namibia, is available so far. The current study aims to improve understanding of CPV epidemiology and investigate the Namibian epidemiological situation by analyzing 58 dog and 32 jackal samples from Namibia with clinical signs of acute gastroenteritis in order to assess viral circulation and the linkage between host species, Namibian districts, and foreign nations.

Distribution of the canine parvovirus variants in the world.

Continent/country	Number of strains detected		
	CPV-2a	CPV-2b	CPV-2c
Europe			
Italy	56	6	62
Portugal	0	16	15
Spain	3	1	9
France	0	9	7
UK	117	182	1
Belgium	17	0	9
Germany	13	18	21
Greece	81	1	2
Switzerland	1	0	0
Czech Republic	1	1	0
Romania	2	0	0
Hungary	27	0	0
Bulgaria	31	9	1
Slovenia	1	0	0
Africa			
Tunisia	15	21	14
North America			
USA	1	36	30
South America			
Uruguay	1	0	24
Argentina	9	4	14
Brazil	37	0	0
Asia			
India	37	4	0
India	b	b	3
Taiwan	2	34	0
Korea	119	7	0
Japan	4	21	0
China	27	5	0
Thailand	19	7	0
Oceania			
Australia	41	1	0

Table 1. Distribution of canine parvovirus variants in the world. Adopted from (Decaro & Buonavoglia, 2012).

2. MATERIALS AND METHODS

2.1. Sample collection and CPV screening

From September to November 2021 at the University Veterinary Academic Hospital of Windhoek, Namibia, rectal swabs (n = 58) were taken from dogs who had previously tested positive to a quick antigen test (WITNESS ParvoRapid Test - Zoetis, South Africa) for a possible CPV infection. All the collected dogs came from various Windhoek municipalities and showed symptoms consistent with canine parvovirus infection, such as lethargy, pyrexia, diarrhea, vomiting, and abdominal pain. When available from healthcare records, patient metadata, such as signalment and vaccination history, were included in the study.

Rectal swabs from 32 Black-backed Jackals (*Lupulella mesomelas*) killed between February and July 2021 during predator control culling operations on two farms each covering an area of around 10,000 hectares in the Windhoek district were also included in the study. All rectal swabs were stored at –20°C.

2.2. Sample preparation and real-time PCR

Total genomic DNA was extracted from the rectal swabs using a High Pure Viral Nucleic Acid Kit (Hoffman-La Roche, Switzerland) with an elution volume of 100 µl, according to the manufacturer guidelines. CPV-specific DNA was detected in both dogs and jackals using a real-time polymerase chain reaction (real-time PCR), as previously described by Decaro et al. (2005).

2.3.VP2 Amplification and sequencing analysis

The full VP2 gene was amplified as described in Tucciarone et al. To confirm the expected size, the presence, specificity, and concentration of the amplicon were tested in agarose gel electrophoresis and compared to molecular weight reference marker. The full VP2 sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands) using 4 different primers based on protocols, that generated overlapping sequences (Tucciarone et al., 2018).

Following the collection of the sequencing data, the chromatogram quality was determined using FinchTV software, which is available at http://www.geospiza.com and poor-quality regions and sequences corresponding to the primer areas were cut off or removed. The final consensus sequence for the complete VP2 region was generated with ChromasPro software (ChromasPro Version 2.0.0, Technelysium Pty Ltd)

Genomic regions flanking the VP2 were removed. The sequences were then aligned at the amino acid level to identify conserved areas and ensure the absence of frame shift mutations and premature stop codons. The MAFFT method, as employed by TranslatorX, was used for alignment.

Additional CPV VP2 sequences were downloaded from the GenBank library for studying the relationship of the Namibian strain in the international context.

1) Namibian-only complete VP2 sequences: This dataset contains complete VP2 sequences acquired from Namibian samples only. The dataset provides a detailed look at the genetic diversity and features of Namibian CPV strains.

2) Complete VP2 sequences from around the world. These sequences were gathered from several regions to enable a comparison of Namibian strains to those from other geographic regions.

3) A collection containing partial international VP2 sequences that represent a compromise between sequence length and coverage. It contains incomplete VP2 sequences from a variety of worldwide sources and was considered since it allowed the inclusion of sequences from other African countries.

Because of the large number of sequences, a representative sequence from those with 98% genetic identity was chosen for computational and graphical purposes. This selection was carried out using CD-HIT

2.4. Namibian sequence analysis

The Namibian strains of CPV were categorized into antigenic variants based on certain amino acid marker locations (Decaro & Buonavoglia, 2012). A maximum likelihood (ML) phylogenetic tree was created using the IQ-Tree software to better comprehend the evolutionary relationships among the strains. The best substitution model was selected based on the Bayesian Information Criterion (BIC). 10,000 ultrafast bootstrap replicates were performed to assess the robustness of the inferred clades in the ML tree. Bootstrap analysis provides statistical support for the tree's branching patterns and helps in determining the reliability of inferred linkages (Kumar et al., 2018a).

A neighbor-joining tree was also created using MEGA X software to compare the Namibian sequences to the international ones. Given the varying sequence lengths and partial overlap between sequences, the pair-wise deletion option was selected to optimize the available information. The neighbor-joining tree shows the genetic links between the Namibian strains and the international strains. It helps gain a better understanding of the Namibian strains' broader context and evolutionary linkages to other *Protoparvovirus carnivoran1* strains worldwide (Kumar et al., 2018a).

2.5. Phylodynamic analysis

A Bayesian serial coalescent approach was used to evaluate the population history, evolution, and migrating patterns of Namibian CPV (*Protoparvovirus carnivoran1*) strains and closely related foreign strains. BEAST 1.10.4, a popular software tool for Bayesian evolutionary research, was used for this analysis. Several population parameters have been

determined jointly using this method. These characteristics include the time to the most recent common ancestor (tMRCA), the evolutionary rate, and viral population dynamics.

The nucleotide substitution model was chosen using the Bayesian Information Criterion (BIC) obtained with the JModelTest program (Darriba et al., 2012). The BIC is a statistical metric that is used to evaluate and compare alternative models, and it helps in the selection of the best model for the dataset.

A relaxed log-normal model was adopted for the molecular clock to allow for differences in evolutionary rate among various lineages. This approach enables for rate across the phylogeny to be variable (Drummond et al., 2006).

To estimate viral population dynamics, the Bayesian Skygrid model was used. The Bayesian Skygrid is an effective tool for estimating population size changes over time. It models population size changes as a piecewise-linear function using a non-parametric Bayesian technique.

These models were selected based on Bayesian Factor (BF) calculation utilizing the marginal likelihood estimation via path sampling and stepping-stone approaches (Hill & Baele, 2019)

A discrete state phylogeographic analysis was performed in addition to the previous studies to evaluate the spreading process and migration patterns of CPV strains. The asymmetric migration model was used in the investigation, in addition to Bayesian Stochastic Search Variable Selection (BSSVS). This method determines the statistical importance of inferred migration paths between nation pairs by identifying the most parsimonious description of the spreading process and calculating a BF (Lemey et al., 2009).

For this research, the Markov Chain Monte Carlo (MCMC) run was set to 500 million generations, with population parameters and trees sampled every fifty thousand generations. Tracer 1.6 was used to examine the resulting log files in order to determine convergence, mixing, and estimate the effective sample size (ESS) of the parameters. If the ESS was larger than 200 and the convergence and mixing were regarded satisfactory, log files were considered acceptable.

The mean and 95% higher posterior density (HPD) were used to summarize parameter estimation. Tree annotator from the BEAST program was used to build and annotate maximum clade credibility (MCC) trees.

SPREAD3 software was used to reconstruct the migration of CPV strains throughout time. It allows the visualization of migration patterns as well as the estimation of migration rates between countries. SPREAD3 was also used to generate BF values to estimate the significance of migration rates between nation pairs. A BF larger than 10 was viewed as proof of non-zero, or significant, migration rates (Bielejec et al., 2016).

A similar approach was used to reconstruct the dynamics of the main Namibian CPV clade after its introduction. This analysis contributes to a better understanding of the temporal dynamics and dissemination of the specific CPV clade inside Namibia. The study gives insights into the migration patterns, dynamics, and statistical significance of CPV strain migration rates both internationally and within Namibia.

3. RESULTS

3.1. Namibian Dataset

The population of domestic dogs included 29 males and 29 females. The majority are categorized as mixed-breeds. The average age is about 4 months, ranging from 1 month to 2.5 years of age. Information on vaccination status was available for 37 dogs: 23/37 were unvaccinated. 12/37 were just partially immunized and 2/37 completed the immunization schedule. Nobivac Canine 1-DAPPvL2, Eurican DA2PPI2, Canigen DHPPi, and Vanguard Puppy 5 vaccines are routinely used to prevent CPV in Namibian dogs.

All 58 domestic dogs previously tested positive for CPV by immunochromatography rapid test. Real-time PCR revealed CPV DNA in all samples with high virus loads (Ct values ranging from 9.09 to 21.2) (Table 2).

In the Jackals population, 10 males and 22 females were considered. Except for five adolescent jackals, all of the jackals were adults. Real-time PCR identified the CPV genome in one of 32 jackals (Table 2).

ID	date	name	sample	qPCR (Ct)
P1	07/09/2021	Bubbles	rectal swab	17
P2	06/09/2021	Blacky	rectal swab	21.50
P3	07/09/2021	Dora	rectal swab	15.97
P4	07/09/2021	Spotty	rectal swab	20.48
P5	08/09/2021	Duks	rectal swab	15.09
P6	13/09/2021	Captain	rectal swab	23.20
P7	13/09/2021	Cookie	rectal swab	17.30
P8	13/09/2021	Chungus	rectal swab	20.10
P9	15/09/2021	Bula	rectal swab	13.80
P10	16/09/2021	Rose	rectal swab	12.57
P11	16/09/2021	Winter	rectal swab	13.86
P12	17/09/2021	Luna	rectal swab	16.32
P13	17/09/2021	Rowly	rectal swab	17.75
P14	20/09/2021	DK	rectal swab	21.20
P15	21/09/2021	Lulu	rectal swab	17.41
P16	21/09/2021	Rambo	rectal swab	18.84
P17	21/09/2021	Spencer	rectal swab	12.11
P18	22/09/2021	Gringer	rectal swab	12.97
P19	22/09/2021	Gorgeowb	rectal swab	14.92
P20	23/09/2021	Chop	rectal swab	17.81
P21	23/09/2021	Rax	rectal swab	18.90
P22	24/09/2021	Mienie	rectal swab	18.74
P23	27/09/2021	Mercy	rectal swab	16.72
P24	28/09/2021	Bondjic	rectal swab	16.14
P25	28/09/2021	Bully	rectal swab	13.99
P26	29/09/2021	Taylor	rectal swab	18.41
P27	29/09/2021	Brown	rectal swab	14.61
P28	29/09/2021	Khenny	rectal swab	20.28
P29	30/09/2021	Pabulo	rectal swab	16.02
P30	04/10/2021	Brown	rectal swab	20.64
P31	05/10/2021	Tiny	rectal swab	10.12
P32	05/10/2021	Grace	rectal swab	12.99
P33	06/10/2021	Pluto	rectal swab	14.66
P34	07/10/2021	None	rectal swab	17.71
P35	09/10/2021	Lion	rectal swab	11.19
P36	09/10/2021	Kundi	rectal swab	14.35
P37	09/10/2021	Rambo	rectal swab	14.50
P38	11/10/2021	Puppy 1	rectal swab	18.65
P39	11/10/2021	Puppy 2	rectal swab	12.01
P40	11/10/2021	Puppy 3	rectal swab	9.02
P41	28/10/2021	Skylar	rectal swab	12.92
P42	13/10/2021	Billie	rectal swab	13.27
P57	05/11/2021	Ouma	rectal swab	15.25
P58	05/11/2021	Smerfi	rectal swab	13.05
J16	20/02/2021	jackal	intestine	26.45

Table 2. List of CPV-positive samples, their features, and RT-PCR values.

The positive results of the fast test and the presence of CPV DNA in all tested samples show that CPV is common in the domestic dog population. The viral load was high in the domestic dogs, suggesting the current infection. CPV was found in only one jackal, with a reduced virus burden.

Based on the entire VP2 gene sequence obtained from the positive samples, it was found that all strains, except for sample 33 (CPV-2a), belonged to the CPV-2c antigenic variant.

The amino acid profile of the CPV-2c strains was featured by glycine (Gly) at position 5, tyrosine (Tyr) at position 267, isoleucine (Ile) at position 324, and arginine (Arg) at position 370. In relevant papers and articles, this profile was referred to as the Asian CPV-2c profile.

The average genetic distance across all Namibian CPV strains was determined to be 0.0017, with a range of 0.000 to 0.0103. The average genetic distance across CPV-2c strains was calculated to be 0.0014, with a range of 0.000 to 0.0057. There was no grouping based on administrative divisions in the geographical distribution of the strains within Windhoek city (Fig.4). This shows that geographical variables have little influence on the distribution of CPV strains in Windhoek. Furthermore, the genetic sequence obtained from the CPV strain taken from the jackal was found to be 100% identical to some canine strains from Namibia's major cluster. This indicates that the CPV strain from the jackal and the canine strains have a tight genetic link, indicating a potential cross-species transmission or shared source of infection (Fig .3).

Fig.3. Maximum likelihood phylogenetic tree reconstructed using the complete VP2 sequences of the Namibian CPV strains obtained in the present study. The host species is represented by different shapes (i.e dog = full circle; jackal=full triangle) nearby the sequence label, while the collection districts are color-coded. Adopted from (Franzo et al., 2022).

3.2. International Dataset

The phylogenetic analysis based on the complete VP2 alignment, besides confirming the variant classification, demonstrated the presence of a main Namibian cluster comprising 34 strains, plus a series of minor clades, interspersed among other foreign strains in the phylogenetic tree. In all instances, Namibian strains were identical or closely related to strains of Asian origin (Fig.4). A fully comparable pattern was observed using the partial VP2 dataset. However, when the pairwise deletion method was used, the research revealed a link to other African strains, particularly those from Egypt, Nigeria, and Zambia (Fig.5).

A phylogeographic analysis was done using representative partial VP2 sequences closely related to the Namibian strains to further study the phylogeography of the CPV strains. The investigation included sequences from Asia and Africa, providing full coverage of the selected region. The strains' time to the most recent common ancestor (tMRCA) was determined to be in 2009, with a high posterior density (95% HPD) ranging from 1993.28 to 2010.91. The estimated evolutionary rate was 9.70×10^{-4} with a 95% confidence interval ranging from 2.58×10^{-4} to 1.98×10^{-3}. The study demonstrated that the CPV strains originated in Asia before spreading to several African countries. The introduction of CPV strains into Namibia was attributed to other African countries, with Egypt having a larger posterior probability (PP>85%) but Nigeria and Ethiopia also having considerable probability (Fig .5).

The calculation of the BF indicated statistically supported migration rates between countries. Migrations from China to Egypt, Egypt to Namibia, Nigeria to Zambia, China to Nigeria, Namibia to Ethiopia, and South Korea to India, for example (Fig.6). The serial coalescent phylodynamic study was carried out only on the largest Namibian cluster. The most likely origin of this cluster was determined to be around February 2020.

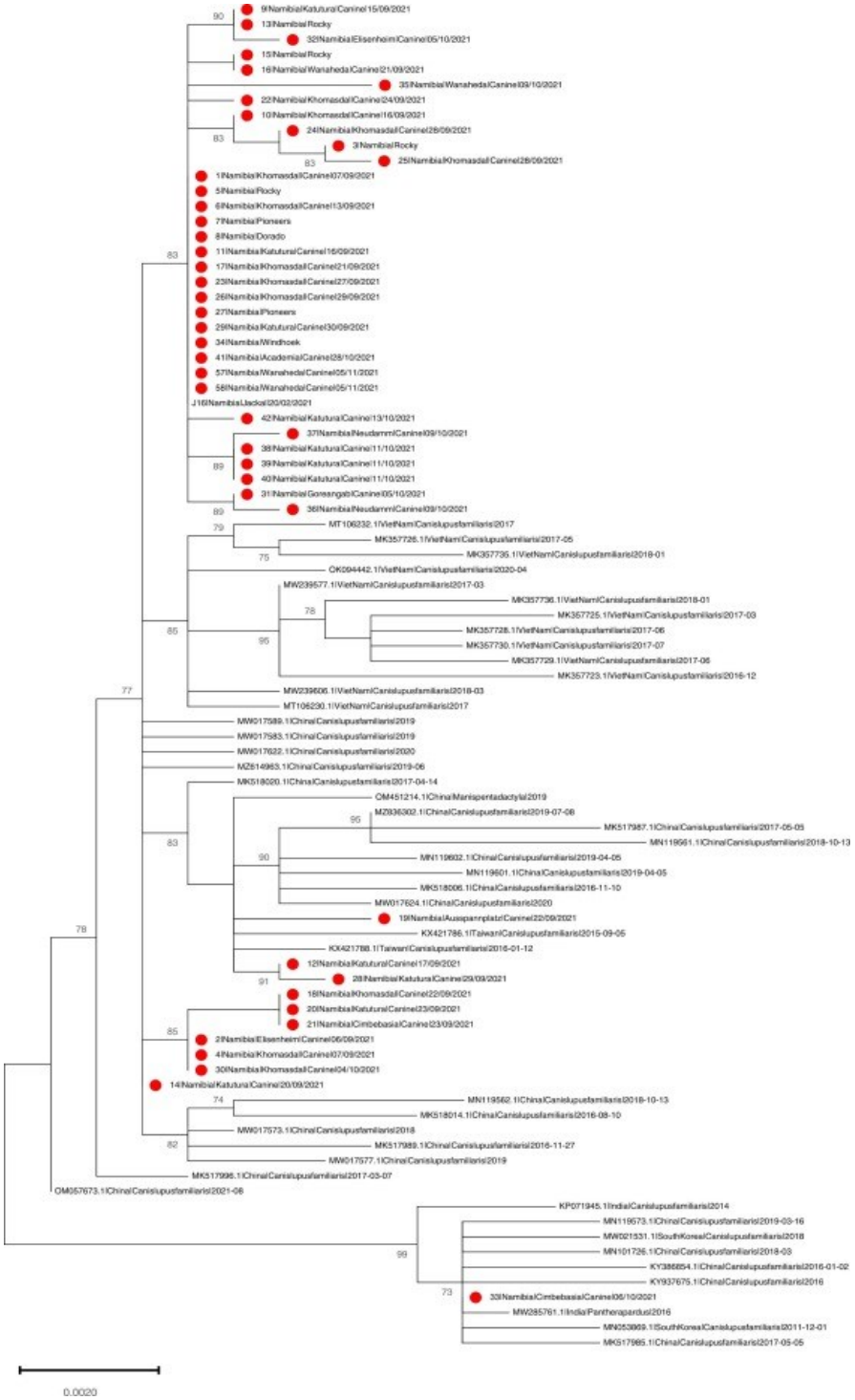

Fig. 4. Maximum likelihood phylogenetic tree reconstructed using the complete VP2 sequences of the Namibian CPV strains obtained in the present study plus a set of international ones. For graphical reasons, only one international sequence representative of all those sharing 98% genetic identity was selected. The host species is represented by different shapes (i.e., dog = full circle; jackal=full triangle). Adopted from (Franzo et al., 2022).

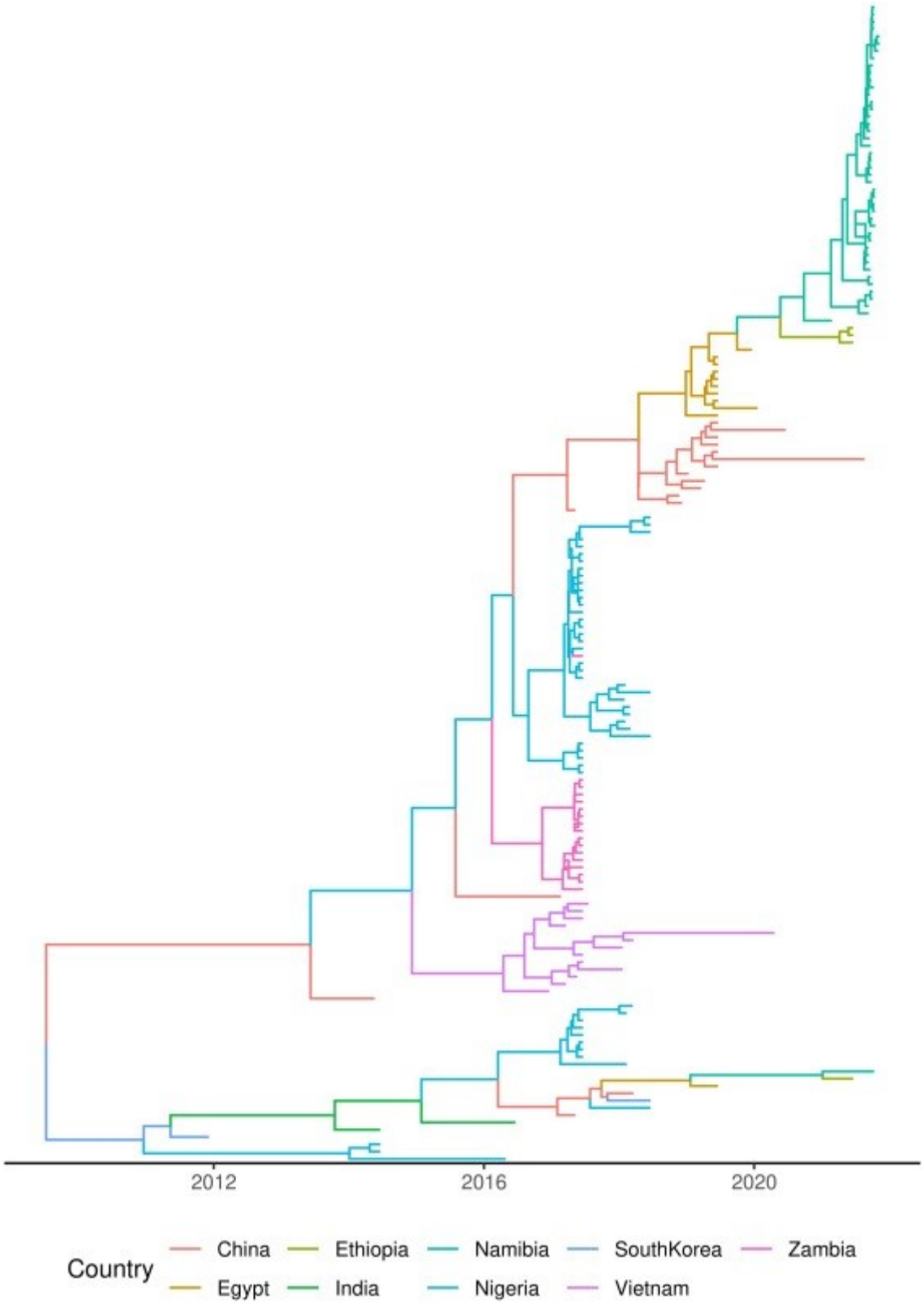

Fig. 5. Time calibrated maximum clade credibility phylogenetic trees based on Namibian and closely related strains. The tree branches have been color-coded according to the location predicted with the highest posterior probability. Adapted from (Franzo et al., 2022).

4. Discussion

Canine Parvovirus (CPV) is a serious issue for domestic and wild canine populations globally. CPV remains a challenge, despite the development and use of effective vaccinations.

CPV, like all parvoviruses, is extremely stable and can persist for more than five months on objects, such as cages, floors, and clothes, and it is therefore possible that humans play a key role in viral transmission. Infected dogs can shed the virus in their feces, contaminating the environment and making it challenging to eradicate the virus completely. Even in places with a relatively high vaccination rate, this persistence raises the risk of infection for susceptible animals. In fact, some dogs and cats may have weaker immune systems or inadequate immune responses to the vaccine, making them more susceptible to CPV infection. Puppies and kittens are especially vulnerable to the virus until they finish their immunization (vaccination) series and achieve full protection.

CPV can also harm wild animals populations like foxes and wolves, which can act as virus carriers. Even though CPV vaccines are generally effective, no vaccine gives complete protection. It is important to promote appropriate pet ownership, which includes routine vaccines, good hygiene, and rapid veterinary care, to solve ongoing CPV issues.

The current study focuses on updating the molecular epidemiology of canine parvovirus (CPV) in Namibia. Most probably, the high frequency of CPV-related disease in dogs can be attributed to incomplete vaccines rather than vaccine failure. To solve this issue, it is important to focus on improving the timely administration and correct application of vaccines in puppies. In addition, education of dog owners about the importance of vaccination and other control measures can be another important factor.

The molecular characterization of the CPV strains identified in the study shows the presence of different antigenic variants, specifically CPV-2a and CPV-2c, with a significant predominance of the CPV-2c variant. Understanding the distribution of CPV variants is important for vaccine development and ensuring that vaccination protocols effectively protect against the prevalent strains. Deeper research will help in adapting control strategies and improving vaccine efficacy in regions affected by CPV infections. The absence of any within-Namibia geographical clustering supports an unconstrained viral circulation.

Therefore, the application of more effective control measures and restrictions could contribute to limit infection relevance in the country.

The Namibian strains were compared to strains from other regions in the worldwide context, demonstrating that the closest genetically linked strains were of Asian origin Accordingly, all the detected CPV-2c strains were featured by the 5Gly, 267Tyr, 324Ile and 370Arg which are commonly reported in Asian strains (Abascal et al., 2010).

The relationships between Namibia and Asian countries, particularly China, might suggest that pet travel could be a potential pathway for viral introduction. However, pet vaccination is mandatory for pet introduction in Namibia, therefore other hypotheses such as illegal animal introduction must be considered.

The analysis of the partial VP2 dataset reveals genetic similarities with strains from African countries such as Egypt, Ethiopia, Nigeria, and Zambia. It has been suggested that the CPV-2 strains seen in these African countries are likewise of Asian origin (Maher & Wyatt, 2021). So neighboring African countries may also play a role, as animal movement control across borders is often difficult. It is important not to overstate Egypt's unique role in the introduction of CPV strains into Namibia, as intermediate steps in the transmission pathway may be hidden due to data scarcity.

Based on all this information it can be suggested that many nations, including Namibia, encounter issues from illegal trade and animal introduction, and these activities may contribute to the spread of CPV.

In Africa, there is a significant presence of wildlife species that hold high ecological relevance. The close contact of domestic and wild animals raises the possibility of pathogen transmission, including CPV. Domestic animals that have not been vaccinated may come in contact with wildlife, potentially favoring the infection spreading between different populations. Stray animals frequently lack access to veterinary treatment and are more likely to be unvaccinated, rendering them more vulnerable to illnesses such as CPV. These animals can operate as virus carriers and help spread the virus in both domestic and wild populations. Wild animals, especially jackals, can become infected with CPV-2 through a variety of mechanisms, including feeding on infected carcasses or coming into contact with contaminated settings where the virus persists (Flacke et al., 2013). The possibility of early puppy mortality is especially concerning since it can have a negative impact on wild animal population.

It’s a true fact that the loss of young population can have an impact on population growth and sustainability, potentially leading to population decreases or even local extinctions. Understanding the transmission dynamics of CPV-2 in wild populations is important for wildlife conservation efforts. It highlights the importance of monitoring and applying proper disease control measures, such as vaccination programs and promoting hygiene practices, not only to protect domestic animals but also wild populations that are at risk of CPV-2 infection.

Several factors, including the likelihood of development of a carrier status, could be involved in the infection dynamics of CPV-2 in wild populations. No research that specifically characterizes viral infection dynamics and the link with the onset of clinical symptoms in wild species is currently available. Unluckily given the biological and ecological value of this topic, further dedicated studies should be performed to find out the true incidence of CPV-2 in wild populations. Such studies would also aid in determining the possible function of wild populations as virus hosts and the directionality of viral flux, including infection between domestic and wild populations.

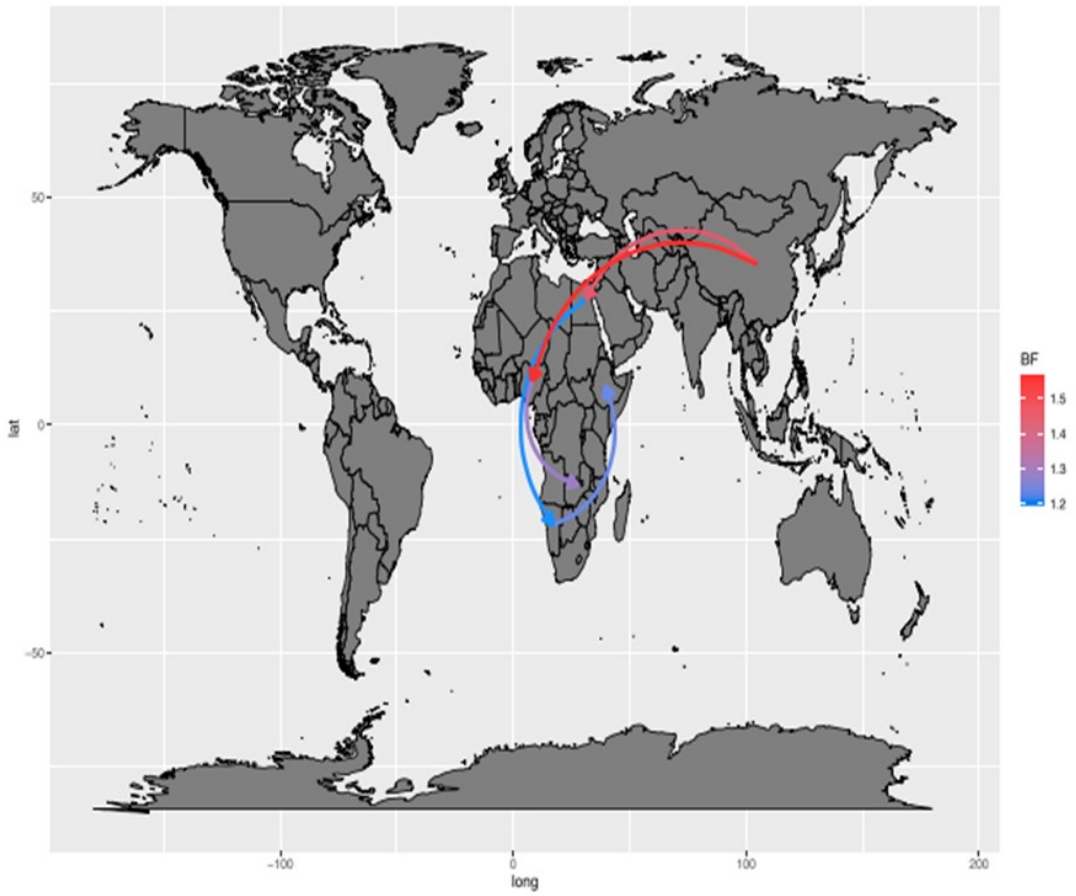

Fig. 6. Well-supported migration paths (i.e. BF>10) among countries are depicted as edges whose colour is proportional to the BF. The location of each country has been matched with its centroid. Adapted from (Franzo et al., 2022).

5.Conclusion

The current investigation showed the prevalence and importance of canine parvovirus (CPV) as a cause of gastrointestinal disease in Namibia. The most typical strains observed were the CPV-2c antigenic variant, which had probably an Asian origin. The CPV-2c variant is widely prevalent throughout Africa, based on previous studies and the spreading of the virus is likely through cross-border illegal animal trade between these countries, even though specific patterns and causes of viral import into Namibia are still unclear.

The absence of geographical clustering within Namibian regions shows the need for further investigation into the epidemiological spreading pattern and circulation of viral pathogens within the country that could be useful to improve biosecurity and control measures. Incomplete or absence of vaccination reported by the dog's owner also shows a lack of information and knowledge which need to be improved.

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