THESIS

Jose Antonio Ayala Tola

2024

University of Veterinary Medicine Budapest
Department of Pathology

Examining the Typical Genotypes of Canine Parvovirus in Ecuador

By

Jose Antonio Ayala

Supervisor

Lilla Dénes

Budapest, Hungary

Table of Contents

Abstract

Canine parvovirus type 2 (CPV-2) is a highly contagious and widespread viral pathogen that primarily affects domestic dogs, causing severe gastroenteritis, particularly in puppies and unvaccinated dogs. Since its emergence in the late 1970s, CPV-2 has evolved into three major antigenic variants—CPV-2a, CPV-2b, and CPV-2c—each exhibiting distinct genetic and antigenic characteristic. In Ecuador, canine parvovirus continues to pose a significant threat to animal health, particularly in regions with inconsistent vaccination protocols. This study aimed to investigate the circulating genotypes of CPV-2 in the city of Quito, Ecuador, and assess the effectiveness of local vaccination protocols in controlling the spread of this virus.

Swab samples were collected from 18 dogs in various parts of Quito, all of which were suspected to be infected based on clinical signs such as vomiting, diarrhoea, lethargy, and dehydration. These samples were then transported to the Department of Pathology at the University of Veterinary Medicine, Hungary. After nucleic acid extraction, samples were kept on –80 ºC until further examination. We performed conventional PCR targeting the VP2 region of the CPV genome for viral detection. The samples were also screened by PCR for coronaviruses and canine circovirus (CanineCV), as both could cause similar symptoms as CPV. PCR results were visualized by agarose gel electrophoresis. We identified the whole VP2 gene of CPV in the PCR positive cases by Sanger-sequencing (Biomi Kft., Gödöllő). For PCR products obtained with sequencing primers, following agarose gel electrophoresis, amplicons of the appropriate lengths (~500 bp, ~600 bp, ~900 bp) were excised using a sterile scalpel. Sequence comparison was conducted through Maximum Likelihood analysis, and phylogenetic tree reconstruction using MEGA X software.

Of the 18 dogs sampled, only two were confirmed positive for CPV-2 infection and four others for coronavirus, none were positive for CanineCV. Genotyping of these positive cases revealed the presence of CPV-2c, providing crucial insights into the types of CPV-2 circulating in the region. Based on the phylogenetic analysis, the viral variants from Ecuador grouped together with sequences from India, Korea and Italy. The low detection rate might reflect the impact of local vaccination programs; however, the presence of

CPV-2 in these cases suggests potential gaps in the current vaccination protocols or vaccine-induced immunity. Further investigation into the vaccination history of the affected dogs revealed inconsistencies in adherence to recommended vaccination schedules, highlighting the need for stricter enforcement of vaccination guidelines.

Absztrakt

A kutya parvovírus (CPV-2) egy rendkívül fertőző és széles körben elterjedt kórokozó, amely súlyos gasztroenteritist okozhat elsősorban kutyáknál, különösképpen kölyökkutyákban és oltatlan állatokban. A CPV-2 1970-es évek végén való megjelenése óta három fő genotípusát különböztetjük meg – CPV-2a, CPV-2b és CPV-2c –, amelyek mindegyike eltérő genetikai és antigén jellemzőkkel bír. Ecuadorban a kutya parvovírus továbbra is komoly fenyegetést jelent az állategészségügyre, különösen azokon a területeken, ahol nem alkalmaznak egységes oltási protokollokat. Kutatásunk célja a Quitó városában keringő CPV-2 genotípusok azonosítása volt, valamint az oltási protokollok hatékonyságának értékelése a vírus terjedésének megfékezésében.

18 kutyától vettünk bélsár mintát Quitó különböző részein, amelyeknél a parvovírus fertőzés gyanúja a klinikai tünetek – például hányás, hasmenés, levertség és kiszáradás – alapján merült fel. A mintákat a magyarországi Állatorvostudományi Egyetem Patológiai Tanszékére szállítottuk. A nukleinsav kivonását követően a mintákat –80 °C-on tároltuk további vizsgálatokig. A CPV-2 genom VP2 régióját célzó hagyományos PCR-t alkalmaztuk a vírus kimutatására. A mintákat koronavírusok és kutya circovírus (CanineCV) jelenlétére is vizsgáltuk PCR-rel, mivel mindkettő a CPV-2-höz hasonló tüneteket okozhat. A PCR eredményeket agaróz gélelektroforézissel vizualizáltuk. A PCR-pozitív esetekben a teljes VP2 gént Sanger-szekvenálás alkalmazásával azonosítottuk. A szekvenciák filogenetikai összehasonlítását Maximum Likelihood analízissel végeztük el.

A 18 vizsgált kutyából mindössze kettőnél igazoltuk a CPV-2 fertőzést, négy másik esetben koronavírus jelenlétét mutattunk ki, circovírusra pedig mindegyik minta negatív volt. Az azonosított CPV-2 törzsek genotipizálása alapján az Ecuadorból származó minták a CPV-2c genotípusba tartoznak. A filogenetikai elemzés alapján az ecuadori vírusváltozatok Indiából, Koreából és Olaszországból származó szekvenciákkal alkottak egy szorosabb filogenetikai csoportot. Az alacsony kimutatási arány a helyi oltási programok hatását tükrözheti, azonban a CPV-2 jelenléte ezekben az esetekben azt sugallja, hogy a jelenlegi oltási protokollokban vagy a vakcinák által biztosított immunitásban hiányosságok lehetnek. További vizsgálataink az érintett kutyák oltási történetében következetlenségeket tártak fel az ajánlott oltási ütemterv betartásában, amely rávilágít az oltási irányelvek szigorúbb betartatásának szükségességére.

Abbreviations

CCoV: Canine Coronavirus

CanineCV: Canine Circovirus

CPV-2: Canine Parvovirus Type 2

DNA: Deoxyribonucleic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

FAT: Fluorescent Antibody Test

FPV: Feline Panleukopenia Virus

HA: Haemagglutination Assay

IC: Immunochromatography

LAMP: Loop-Mediated Isothermal Amplification

MLV: Modified Live Vaccine

PCR: Polymerase Chain Reaction

qPCR: Quantitative PCR

RVs: Rotaviruses

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcription Polymerase Chain Reaction

US: Ultrasound

VP1: Viral Protein 1

VP2: Viral Protein 2

VP3: Viral Protein 3

1. Introduction

Canine parvovirus 2 (CPV-2) is a highly contagious viral pathogen that emerged in the late 1970s and quickly spread globally due to its short incubation period and the absence of pre-existing immunity in dogs [1]. The virus primarily affects young, unvaccinated puppies, causing severe gastroenteritis, dehydration, and septicemia, which can be fatal if untreated [17]. In some cases, CPV-2 also targets the myocardium, leading to congestive heart failure [18]. By 1980, CPV-2 had become a worldwide concern, highlighting the need for effective diagnostics, vaccines, and control measures.

Research has shown that CPV-2 is antigenically similar to Feline Panleukopenia Virus (FPLV), differing by only minor DNA sequence changes (0.4% of the genome). This has led to speculation that CPV-2 may have evolved from FPLV, though definitive evidence is lacking [2].

Since its discovery, CPV-2 has undergone significant genetic evolution, resulting in three major antigenic variants: CPV-2a, CPV-2b, and CPV-2c. These variants differ in capsid protein sequences, which influence their antigenic properties and geographic distribution [7]. For example, CPV-2c, a particularly virulent variant, is predominantly found in South America, including Ecuador. Understanding this genetic diversity is essential for improving vaccine strategies and controlling outbreaks.

In Ecuador, inconsistent vaccination coverage exacerbates the impact of canine parvoviral infection, particularly in regions like Quito [63]. Limited epidemiological data on circulating CPV-2 genotypes hampers targeted prevention efforts. This thesis aims to characterize the genotypes of CPV-2 in Quito through molecular diagnostics and genotyping, providing insights into the effectiveness of current vaccination protocols and strategies to mitigate the virus's impact.

Additionally, the research will investigate co-infections with other viral pathogens, such as canine coronavirus (CCoV) and canine circovirus (CanineCV), to assess their role in worsening CPV-2 clinical outcomes. The findings will contribute to the global understanding of CPV-2 evolution, its impact on vaccine efficacy, and its control, especially in areas where emerging variants like CPV-2c are prevalent.

2. Literature Review

2.1 Viral taxonomy

The family *Parvoviridae* is currently comprised of three subfamilies: *Parvovirinae* and *Hamaparvovirinae* whose members infect vertebrates and *Densovirinae,* whose members infect invertebrates. [3]. They include 11, 11 and 5 genera, respectively.The subfamily *Parvovirinae* includes: *Dependoparvovirus, Erythroparvovirus, Protoparvovirus, Tetraparvovirus, Amdoparvovirus, Aveparvovirus, Copiparvovirus, Sandeparvovirus, Artiparvovirus, Bocaparvovirus,* and *Loriparvovirus [5]*. Since their discovery, viruses

belonging to the *Parvoviridae* family have been reported to cause relevant diseases in animals and humans, such as the aelutian mink disease virus (*Amdoparvovirus*), the chicken and turkey parvovirus (*Aveparvovirus*), the canine minute virus (*Bocaparvovirus*), the Human parvovirus *(Eryhtroparvovirus*), the feline and racoon parvovirus (*Protoparvovirus*) and many others [36].

2.1.1 Protoparvovirus genus

The Protoparvovirus genus includes several relevant pathogens of domestic and wild vertebrates, the prefix "proto" meaning "first" is used to describe this genus as the first viruses identified in the *Parvoviridae* family. Regarding the virus replication of this genus, it takes place in the nucleus of cells, requiring rapidly dividing cells, therefore it has an affinity to foetuses' and newborns, as well as intestinal or hematopoietic tissues of both young and adult animals. As for the resistance of this genus, stability in the environment is high, they are resistant to changes of temperature and pH, as well as most disinfectants and lipid solvents [6]. Protoparvoviruses are responsible for several significant clinical diseases across various animal species, including CPV-2 and feline panleukopenia virus (FPV), mink enteritis virus (MEV), racoon parvovirus (RaPV), and the minute virus of mice (MVM) belonging to the *Protoparvovirus carnivoran1* species. Similarities in these species have been seen in cell culture, where during replication, characteristic cytopathic effects are observed when induced by these viruses. Many of the species could have the ability to hemagglutinate red blood cells of one or even more species, independently of them being their targeted host. Regarding the controversy around the origin of CPV-2, it is a widely believed theory that is derived from FPV, furthermore there have been three new antigenic variants of CPV-2 have been identified: type 2a, 2b and 2c. The genomes of these variants show around 98% similarity to each other. Further phylogenetic research on CPV-2 and FPV isolates shows that there are two defined clusters, the first one representing FPV type from cats, racoons and mink, and the second one representing CPV type from dogs and racoon dogs [7].

2.2 Genomic morphology of canine parvovirus

Canine parvovirus is a small, non-enveloped virus with an icosahedral structure, measuring between 18 and 26 nm in diameter. The CPV-2 genome is a linear, single-stranded DNA (ssDNA) of approximately 5.2 kilobases (kb) and contains only two primary open reading frames (ORFs). The left ORF encodes non-structural proteins NS1 and NS2, essential for replication and genome packaging, NS2 arises from differential

splicing, and it is further involved with the replication process. The right ORF encodes the viral capsid proteins VP1 (82 kDa) and VP2 (65 kDa), these proteins act as the main antigens at the time of inducing protective antibodies, with VP1 containing a unique N-terminal region of 143 amino acids not found in VP2. The third capsid protein, VP3 is produced during a proteolytic process of VP2. CPV-2 is characterized by a high mutation rate and includes three major antigenic variants forming the main CPV-2 types: CPV-2a, CPV-2b, and CPV-2c. These variants, which differ primarily in their VP2 gene sequences, exhibit distinct antigenic properties due to specific mutations, the characterization of the circulating a, b and c types is based on identifying the amino acid residue that is found in the 426th position of VP2 in the following order, asparganine N426, aspartic acid D426 and glutamic acid E426 respectively. Co-infection and recombination contribute to the virus's genetic diversity, leading to the emergence of recombinant strains [8,9]. This should look like:

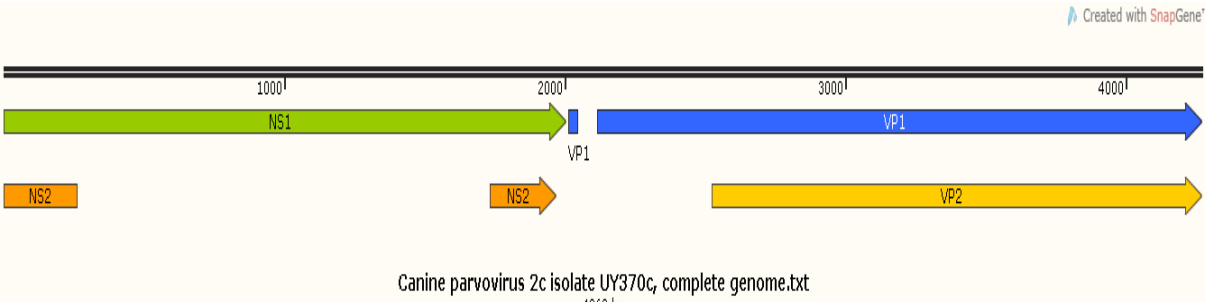

Figure 1: Canine parvovirus 2c complete genome. The figure was made using SnapGene [43]

2.3 Virus transmission

In the case of CPV-2, oronasal infection is the main route of infection in affected patients. The virus is spreading through direct contact as a primary route via infected faeces and vomit, but due to the high resistance of the virus in the environment, indirect contamination is a factor to be considered as well [10,11]. Although supposedly felines have lost susceptibility to CPV-2 and the main hosts of the virus are canids, in the last years, it has been shown that the virus can indeed spread efficiently in some cases in wild and domestic feline animals [12].

2.4 Geographical distribution

CPV-2 and its' 3 variants have been studied and demonstrated all around the world in a wide number of countries since the first discovery of the virus [13]. Studying and analyzing the evolution of the CPV-2 genome, especially its potential mutations in the amino acid sequence is important when aiming to improve global knowledge about the virus as well as vaccine effectiveness [14]. CPV-2a is predominantly found in Asia, with significant prevalence in countries such as China, Thailand, and India. Genetic analyses from outbreaks in these regions, particularly between 2008 and 2011, have highlighted its dominance. CPV-2b, on the other hand, remains the most frequently detected variant in North America, particularly in the United States, although it has also been reported in Asian nations like China and Japan, indicating a broader global distribution [15]. CPV-2c, the most geographically widespread of the three variants, is primarily associated with South America, where it has been extensively documented in Brazil, Argentina, and Uruguay during numerous outbreaks [44,16]. Despite being a DNA virus, CPV-2 exhibits a remarkably high mutation rate of approximately 10^{-4} substitutions per site per year, comparable to that of RNA viruses. This elevated mutation rate contributes to the rapid emergence of new variants, underscoring the need for continuous monitoring and genomic surveillance. Understanding the geographic distribution and genetic evolution of these variants is crucial for tailoring vaccination strategies and implementing effective control measures in regions where specific variants dominate [45].

2.5 Clinical Symptoms of CPV-2

Clinical signs of CPV-2 infection typically start appearing 3 to 5 days post-infection, lasting 5 to 7 days and ranging from mild digestive disturbances to severe, potentially fatal systemic disease. Outcomes vary depending on various factors, including the viral strain involved in the infection, immune status, age, and vaccination history of the animal. Symptoms can differ between adults and newborns; primary symptoms often can escalate to secondary complications or systemic infections. CPV-2 primarily affects the gastrointestinal tract, leading to haemorrhagic gastroenteritis, this condition is characterized by diarrhoea, vomiting, and bloody stool, which can result in dehydration, lethargy, and nutrient malabsorption, possibly advancing to hypovolemic shock if

untreated. In systemic infections, common signs include lethargy, anorexia, fever, and leukopenia, as the virus attacks bone marrow cells, weakening the immune response and therefore increasing vulnerability to secondary infections, worsening the prognosis. [18] Leukopenia is a common indicator of CPV-2 and aids in diagnosis. Systemic symptoms are particularly hazardous for puppies and unvaccinated dogs, whose immune systems are immature or weaker [17]. Additionally, studies show that CPV-2 infection in young animals can cause myocarditis, a serious condition that may occur without the typical gastrointestinal symptoms, it may be fatal and lead to chronic cardiac issues if the subjects survive [19,20,21]. Consistent vaccination has been shown to lower severe CPV-2 cases, particularly in areas where protocols may vary [62].

2.6 Pathogenesis

Following direct or indirect transmission, CPV-2 initially replicates in the lymphoid tissues of the oropharynx. From there, the virus disseminates through the bloodstream, targeting rapidly dividing cells and exhibiting a particular affinity for lymphopoietic tissues, bone marrow precursor cells, and enteric epithelial cells located within the crypts of the jejunum and ileum [29]. In some cases, particularly in puppies aged 2 to 3 weeks, CPV-2 invades myocardial cells, leading to sudden cardiac failure or the development of the intestinal form of the disease followed by myocarditis due to increased viral replication in the heart muscle [19].

A hallmark feature of CPV-2 infection is the profound immunosuppression caused by the virus's tropism for white blood cells, resulting in leukopenia and neutropenia. These conditions severely impair the immune response, rendering affected animals more susceptible to secondary infections. Viremia typically occurs within the first week post-infection, enabling further dissemination of the virus to various tissues. The primary replication site for CPV-2 is the intestinal crypts of Lieberkühn, where the virus targets germinal epithelial cells. This leads to the destruction of the crypts and migration of the virus to the intestinal villi, resulting in villous collapse and significant disruption of the intestinal architecture [49]. The collapse of the villi impairs nutrient absorption and exacerbates the severity of diarrhoea, contributing to dehydration and systemic imbalances. Furthermore, inflammation and necrosis associated with villous damage significantly alter the gut microbiota, increasing the likelihood of bacterial translocation across the compromised intestinal barrier. This can lead to severe bacterial complications,

including septicaemia, which further worsens the prognosis in severely affected animals [30].

2.7 Diagnostic Methods

2.7.1 Physical examination

Clinical signs of parvovirus infection in dogs are unfortunately nonspecific or general to a gastrointestinal disease, therefore complicated to diagnose exclusively by physical examination of the subject. Imaging methods such as ultrasound can provide additional information in the infection diagnosis, such as an abnormal motility in the intestine and fluid or gas filled intestinal loops, both secondary findings due to the damage caused by CPV-2 [33].

2.7.2 Virus Isolation

Virus isolation is widely regarded as the gold standard for viral detection, including CPV-2, due to its high specificity and ability to confirm active infection. This method involves inoculating clinical specimens onto susceptible cell lines, allowing the virus to replicate and produce cytopathic effects (CPE) observable under a microscope. For CPV-2, both canine and feline cell lines are utilized, with canine kidney cells, such as Madin-Darby Canine Kidney (MDCK) cells, being particularly effective [30]. Additionally, Crandell-Rees Feline Kidney (CRFK) cells have demonstrated utility in propagating CPV-2. The process requires maintaining cell cultures in specific growth media to support viral replication and subsequent detection. While virus isolation offers high specificity, it is labour-intensive, time-consuming, and requires specialized laboratory facilities, which may limit its routine use in clinical settings [50].

2.7.3 Immunological methods

2.7.3.1 Haemagglutination Assay (HA)

The principle of the HA (hemagglutination) test is based on the virus binding to receptors located on the surface of red blood cells (RBCs) of a host, which leads to the agglutination of the virus. This test can be used to determine the antibody titer in a subject against the virus.[51]. It is shown that the CPV-2 virus can interact with and bind to sialic acid receptors, leading to the agglutination of red blood cells (RBCs). The HA (hemagglutination) test, used to observe this phenomenon, is sensitive to pH and temperature, with optimal conditions being a pH of 8 and a temperature of 4°C. While

the sensitivity of the HA test is generally good, it may be influenced by the handling and management of RBCs, as well as potential alterations in their sedimentation coefficient [30].

2.7.3.2 Fluorescent antibody test (FAT)

FAT detects specific viral antigens by using a fluorescent dye attached to monoclonal antibodies that react with the target antigens. It is characterized by high sensitivity and can provide clear quantitative results when detecting antibodies. However, factors such as the cost of the test and the expertise of the professional performing it should also be considered. [52].

2.7.3.3 ELISA test

Being one of the most cost-effective tests for detecting CPV-2, enzyme-linked immunosorbent assay (ELISA) rapid tests are widely used in clinics worldwide. The test is performed using fecal or rectal swab samples and provides results within 10–15 minutes, making it a widely used technique. The sensitivity of this test can reach up to 87%, while its specificity is typically above 90%. False negatives may occur, particularly due to irregular or decreased viral shedding during the early or late stages of infection. However, false positives are extremely rare and are often associated with a recent modified live vaccine (MLV) dose in the patient. [34].

2.7.3.4 Immunochromatography (IC)

The immunochromatographic strip test uses a detection component consisting of anti-CPV monoclonal antibodies to identify the virus, and a capture component made from polyclonal antibodies derived from rabbits, known as rabbit anti-CPV truncated VP2 (CPV-tVP2). Using polyclonal antibodies is beneficial as the assay can detect multiple epitopes, and their production is more cost-effective overall [30]. Commonly referred to as rapid tests, this technique provides results in just minutes by detecting CPV-2 antigens in fecal samples. However, compared to other diagnostic methods like PCR, it has lower sensitivity. Despite this limitation, immunochromatographic tests can still detect CPV-2a and CPV-2b with relatively high specificity and sensitivity, making them useful in environments where advanced diagnostic equipment is not available [35].

2.7.4 Molecular tests

2.7.4.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a widely used in vitro molecular platform for the detection and quantification of various pathogens across different sample types. PCR assays are highly specific as they rely on carefully designed primer pairs that target specific regions of the viral genome. This technique is particularly advantageous for amplifying unculturable pathogens, including CPV-2. Various PCR-based protocols have been developed to detect CPV-2 and its variants. For example, a differential PCR approach, which requires three sets of primers, has been optimized to identify CPV-2 antigenic types using a gradient of annealing temperatures. However, challenges such as polymerase enzyme inhibition can arise when processing homogenous materials like fecal matter. To overcome these limitations, touch-down PCR has been introduced, incorporating pretreatment steps such as sonication or boiling to improve enzyme activity and sensitivity during the amplification process [30]. Multiplex PCR (mPCR) is a molecular method which utilizes 2–3 sets of primers to carry out molecular typing in a single reaction tube. Several multiplex PCR have been developed for CPV-2 that can differentiate between three variants (CPV-2a, CPV-2b and CPV-2c). A mPCR is developed to differentiate between CPV-2a and CPV-2b by using 2 sets of primers [66]. Real-time PCR combines traditional PCR with a fluorescent probe while eliminating the need of gel electrophoresis analysis, providing a quick and accurate result in under an hour and it's commonly used when aiming for quantitative and qualitative analysis [52]. Another commonly used molecular test is Loop-mediated isothermal amplification (LAMP) test, which uses a specific DNA polymerase with high strand-displacing activity and special thermal conditions to generate an artificial so-called stem-loop structure of DNA and in this way detecting CPV-2 by targeting the VP2 gene. It is a highly sensitive and specific test and a rapid alternative to classic PCR or real-time PCR. It does not require sophisticated technology, the reaction can be maintained by a single temperature (~ 65 degrees). On the other hand, LAMP can generate a high number of false positives, and the application of several primers sets [50].

2.8 Vaccination Protocols

Vaccination is a cornerstone both in prevention and in eradication programs in veterinary medicine, including parvoviral infections [62]. It is important to follow strict protocols to

ensure the efficiency and correct use of this type of prophylactic medicine, however the vaccination schedules differ by country and are affected by external factors, such as public awareness, resource availability and veterinary infrastructure as well as government support in vaccination programs.

In Ecuador, the protocol seems to be influenced by two international organizations providing the guidelines of veterinary practices, the WSAVA (The World Small Animal Veterinary Association) and the AAHA (American Animal Hospital Association) [31]. According to the official recommended vaccination protocol from the WSAVA guidelines, CPV-2 vaccination in young animals can follow two distinct detailed plans. The first one consists of vaccinating the animal with the first dose of a modified live form of the virus (MLV) between 6-8 weeks of life, followed by 2 more doses in 2-4 weeks intervals, reaching the third dose when the animal is 16 weeks old, It is important to mention that in high risk situations such as kennels it is considered that vaccination can be given until 20 weeks of age. The second plan applies to puppies vaccinated between 4-6 weeks of life, it consists of a bivalent vaccine or a recombinant plus MLV forms protecting from CPV-2 or Canine Distemper Virus (CDV), respectively. After the first dose the plan suggests switching to the earlier mentioned essential vaccine by 8 weeks of age and continuing as stated before. In both vaccination plans, a fourth dose is administered between 6-12 months of age in each animal, or 12 months after the last dose given. In the case of adult animals, guidelines recommend revaccination with MLV vaccines at intervals no more frequent than every 3 years after completing the initial vaccination plan. However, for high-risk animals such as dogs living in kennels or environments with poor hygiene, annual revaccination may be recommended. However, for high-risk animals, such as dogs living in kennels or in environments with poor hygiene, annual revaccination may be recommended. [38].

When it comes to the standardized European vaccination protocol for CPV-2 data shows that, similar to Ecuador, they follow the guidelines of the WSAVA, in the case of core vaccination, using a MLV CPV-2 form of the vaccine from 6-8 weeks and two further doses until 16 weeks of age, followed by a revaccination at about 6 months rather than waiting until the dog is a year of age, in this way decreasing chances of infection for susceptible puppies that could not effectively create an active immune response, finally, adult dogs should be revaccinated every 3 years. In the case of using a CPV-2 recombinant vaccine alongside a MLV form of CDV vaccine, it is suggested to administer one dose at 4 weeks of age before switching to the routine primary plan of vaccination,

this form is aimed primarily for puppies rather than for the revaccination practice in older dogs [32].

2.9 Viral Pathogens Mimicking CPV-2 Symptoms

Although canine parvovirus infections are often diagnosed based on characteristic symptoms such as lethargy, vomiting and diarrhoea, these signs are not exclusive to the disease and are shared in other viral infections.

2.9.1 Canine Coronavirus

Coronaviruses are classified into antigenic groups, with group 1 further divided into subgroups 1a and 1b. Subgroup 1a includes closely related viruses such as porcine transmissible gastroenteritis virus (TGEV), its derivative porcine respiratory coronavirus (PRCoV), feline coronaviruses (FCoVs), and canine coronaviruses (CCoVs), highlighting their genetic interconnectedness [22]. To date, two CCoV genotypes are recognized: CCoV-I and CCoV-II. CCoV-II is further divided subtypes: CCoV-IIa and CCoV-IIb, based on recombination events. CCoV-IIa represents classical strains of CCoV, while CCoV-IIb includes TGEV-like CCoVs that likely originated from a double recombination event with TGEV [23]. The course and symptoms of CCoV are, in many cases, similar but milder than the ones of CPV-2, including diarrhea in adults, self-limiting enteritis and sometimes vomiting. However, these symptoms can become fatal in the case of a co-infection with CPV-2, resulting in severe and potentiated symptoms. There has also been research of a hypervirulent variant of CCoV, referred to as pantropic CCoV, which can result in a fatal disease of dogs, the pantropic variant has been shown not to be restricted to the gastrointestinal tract, but can reach other internal organs as well, with lymphopenia as the most prominent finding of the infection both in natural and experimental infection [24,25].

2.9.2 Canine Circovirus

CanineCV is a non-enveloped, circular, single-stranded DNA virus of the *Circoviridae* family and *Circovirus* genus, together with porcine and canary disease-causing viruses as well. Although the main enteric pathogens of canine species are the CPV-2 and CCoV, CanineCV has been increasingly detected in dogs presenting haemorrhagic diarrhoea [26]. Besides bloody diarrhoea and enteritis, CanineCV has also been known to be able to cause necrotizing lymphadenitis and vasculitis in canine subjects, possibly relating to the epithelial damage that Circoviruses in general can cause as it is seen in other species,

like skin diseases in swine and beak and feather disease in birds [27]. However, there have been only a few to no cases that report CanineCV as a primary agent in enteritis, in most cases, it has been found accompanying other viral pathogens, such as CPV-2 and CCoV. This leads us to emphasize the importance of correct diagnostic methods when it comes to co-infections, especially in canine enteritis, which presents very similar symptoms independently of the pathogen responsible for the infection [28].

2.9.3 Canine Rotavirus

Rotaviruses (RVs) are non-enveloped RNA viruses belonging to the *Sedoreoviridae* family [64]. That typically cause gastroenteritis in puppies under three months of age, as well as in various mammalian and avian species [46] .The R*Vs* consists of 9 species referred to as A-D and F- J, distinguished by the antigenic properties of VP6 [64]. The RVs genome consists of 11 double-stranded RNA (dsRNA) segments enclosed within a triple-layered particle (TLP). It encodes six structural proteins (VP1–VP4, VP6, and VP7) and five or six nonstructural proteins (NSP1–NSP6) [65]. RVA is the most common RVs species infecting dogs and other mammals. Transmission occurs via the faecal-oral route, with the apical part of the intestinal villi being targeted during its pathogenesis [47]. This results in villous necrosis and therefore having as a direct consequence diarrhoea, characterized by its increased mucoid content and watery consistency, lasting usually around 10 days. Histopathological examination aids in the differential diagnosis of RVs infections by identifying intestinal villi fusion and epithelial necrosis. Currently, there is no specific treatment for RVs infection in dogs. Disease management primarily focuses on preventing and treating dehydration, with the use of antiviral and anti-emetic drugs recommended in certain cases [37].

2.9.4 Canine Distemper Virus

CDV shares similarities with CPV-2, particularly in the way of transmission, which primarily occurs through nasal and oral exposure. Upon infection, CDV initially

replicates in the lymphoid tissues, leading to significant immunosuppression, with T lymphocytes being more severely affected than B lymphocytes. The incubation period for CDV can extend up to four weeks or more, during which clinical signs such as anorexia, depression, tonsillitis, and nasal or ocular discharge may develop. Fever typically peaks within 3 to 6 days of post-infection, after which viremia disseminates the virus to epithelial cells in various organs [37]. This epithelial involvement can result in hyperkeratosis of the skin, enamel hypoplasia, and respiratory and gastrointestinal symptoms, such as purulent discharge, dyspnoea, diarrhoea, and vomiting, often complicated by secondary bacterial infections. Neurological symptoms may emerge approximately 20 days of post-infection or later, as the virus reaches the central nervous system. These symptoms include paralysis, seizures, and behavioural abnormalities such as head tilt and circling. The multisystemic nature of CDV and its ability to cause severe complications highlight the importance of early diagnosis and supportive care [48].

3. Materials and Methods

3.1 Sampling and nucleic acid extraction

In 2024, we gathered rectal swabs from 17 dogs treated in 11 veterinary clinics in Quito, Ecuador. We sampled 6 females, 11 males aged 1-17 years-old with various vaccination status, data shown on Table 1. To keep nucleic acid integrity, swabs were immersed in 1ml DNA/RNA shield (Zymo Research Corporation, USA.) and kept on 4℃, until transported to the University of Veterinary Medicine, Budapest. Samples were vortexed for 15 seconds and the homogenized solutions were transferred to a centrifuge tube. After centrifugation for 3 minutes on 3000g using the Pico™ 17 microcentrifuge (ThermoFisher Scientific, USA), nucleic acid extraction was performed with 200 µl of the supernatant using the 203 MagCore Viral Nucleic Acid Extraction Kit (RBC Bioscience, USA) and the MagCore II Plus extraction machine (RBC Bioscience, USA). Samples were eluted in 60 µl elution buffer and kept on -80℃ until further examination.

Table 1:Sample collection data

Samples	Age	Gender	Vaccination Status	Clinic location
S1	5 years	Male	Complete	B
S2	1 week	Female	No vaccination	C
S3	2 years	Male	No registered	C
S4	1 week	Male	No vaccination	C
S5	1 year	Male	Completed	D
S6	2 years	Female	2017 last	C
S7	1 year	Male	Complete	D
S8	7 years	Male	Complete	D
S9	3 years	Male	No registered	A
S10	5 years	Male	No vaccination	C
S11	11 months	Female	No registered	A
S12	5 years	Male	Complete	C
S13	2 years	Male	No registered	C
S14	3 years	Female	2022 Last	F
S15	3 months	Female	No vaccination	E
S16	1 week	Male	No vaccination	C
S17	1 year	Female	Complete	E

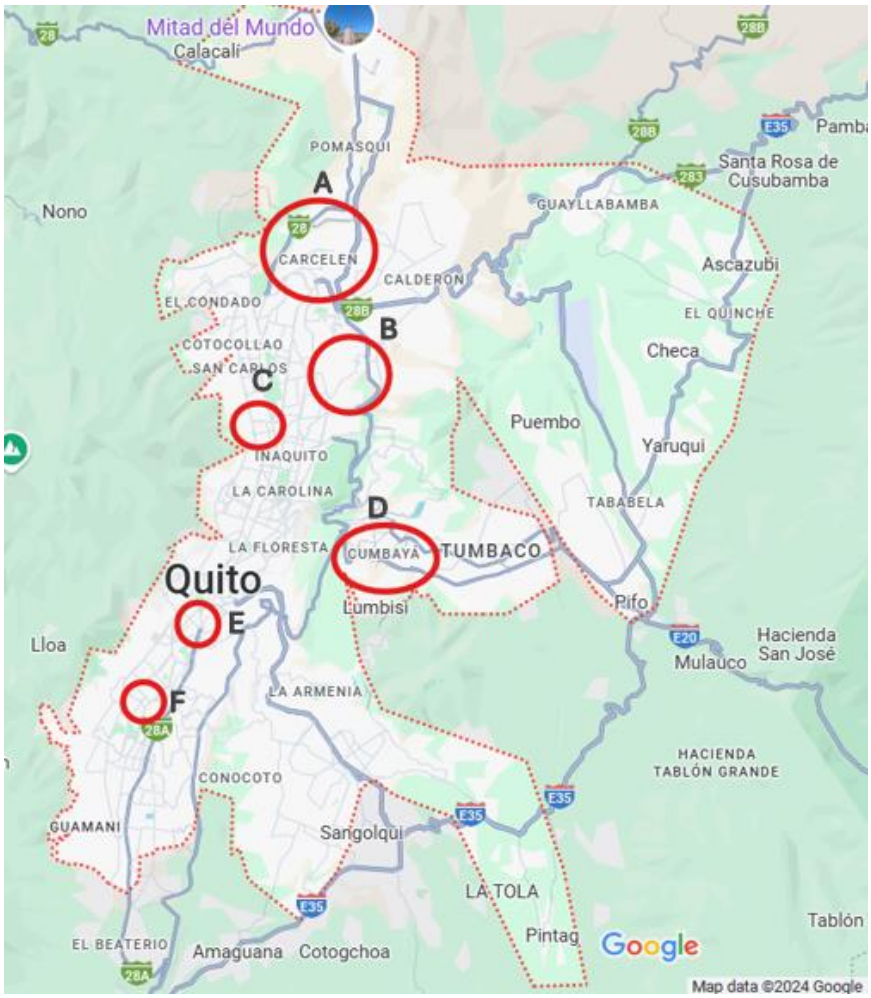

Figure 2: The geographical locations of the veterinary clinics from which the samples were collected are marked with a red circle (A-F, Google maps, accessed: 20.11.2024).

3.2 Viral detection

We investigated the presence of CPV-2 by conventional polymerase chain-reaction (PCR), canine coronavirus (CaCoV) by reverse-transcriptase PCR (RT-PCR) and canine circovirus (CanineCV) by real-time PCR (qPCR) with systems routinely used in the Molecular Laboratory of the Department of Pathology. PCR and RT-PCR were run in a QIAamplifier 96 machine (QIAGEN, Germany), the real-time PCR reactions in the Q qPCR machine (Quantabio, USA).

CPV2 detection was performed using the AllTaq PCR master Mix kit (QIAGEN, Germany) with the following protocol: 95 °C for 2 min, 35 cycles at 95 °C for 1 min, 53°C for 40 sec and 72 °C for 1 min, then 72 °C for 10 min. The end volume of the PCR mix was 20 µl and contained 5 µl 4X AllTaq MM, 0.1 µ MM Tracer, 11 µl RNase free water, 0.25µM end concentration of the forward and reverse primers (CPV/FPV diag F: 5'-GACTTGTGCCTCCAGGTTAT-3', CPV/FPV diag R: 5'-

GTTGAACTGCTCCATCACTC-3') designed for the detection of the VP2 segment of the CPV genome (Demeter et al., 2009) and 2.5 µl template [40].

We used the OneStep RT-PCR Kit (QIAGEN, Germany) for the identification of CaCoV with the following protocol: 50°C for 30 min, 95 °C for 15 min, 40 cycles at 94 °C for 1 min, 50°C for 1 min and 72 °C for 1 min, then final elongation at 72 °C for 10 min. The mix contained 2 µl 5X buffer, 0.4 µl dNTP (10 mM), 0.4 µl Enzyme Mix, 0.1 µl RNase inhibitor (ThermoFisher Scientific, USA), 5.7 µl RNase free water, 0.75 µM end concentration of both primers (cor11_F: 5'-TGATGATGSNGTTGTNTGYTAYAA-3', cor13_R: 5'-GCATWGTRTGYTGNGARCARAATTC-3') designed for the ORF 1b gene (Escutenaire et al., 2006) [41].

Real-time PCR was carried out with PerfeCTa SYBR Green FastMix (Quantabio, USA) in 20 µl reaction volume: 10 µl 2X SYBR Green MM, 3,8 µl RNase free water, 0.3 µM end concentration of both primers (CaCV_qPCR_F: 5'-CTGAAAGATAAAGGCCTCTCGCT-3', CaCV_qPCR_R: 5'-AGGGGGGTGAACAGGTAAACG-3') amplifying a 132 bp segment in the intergenic region between the ends of the two major open reading frames (de Arcangeli et al. 2020) and 5 µl template [42]. The reaction was run on the following program: 95 °C for 30 s, 40 cycles at 95 °C for 5 s, 60°C for 20 s, then the melt curve: 60 °C to 95 °C at a rate of 0.3 °C/s. The fluorescent signal was registered on the FAM channel.

PCR and RT-PCR products were separated on 1.6% agarose gel (TopVision Agarose, Thermo Fisher Scientific, USA). We suspended 1.6g powdered agarose in 100 ml 1X TAE buffer, heated in a microwave until clear, and after cooling to ~55°C, homogenized with 5 µl SYBR Safe Gel stain (Thermo Fisher Scientific, USA) then poured into the bed supplemented with a comb. After solidification, the comb was removed, and the gel was immersed in 1X TAE buffer in a gel electrophoresis machine (Major Science MINI-300, Taiwan). 5 µl of the PCR products were mixed with 1 µl Loading Dye (Thermo Fisher Scientific, USA), pipetted into the wells and run on 120V for 40 min next to a DNA Ladder (GeneRuler 50 bp DNA Ladder, Thermo Fisher Scientific, USA). The bands were visualized in blue light using a UV plate machine (DUO View, Cleaver Scientific Ltd, UK). For sequencing, the appropriate bands were cut out of the gel and the PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany), eluated in 35 µl Elution Buffer and kept on -20 °C until further examinations.

3.3 Sequence analysis

In samples we detected CPV2 were then processed further for sequencing. The VP2 gene of CPV2 was amplified by PCR with three primer sets (Fig 3). PCR products were sent to a commercial provider (Eurofins Biomi Kft., Gödöllő) for Sanger-sequencing. Raw electropherograms were visualized using Chromas software version 2.6.6 (Technelysium Pty Ltd., Australia). The reliability of the bidirectional sequencing results was compared with one another and with a reference strain downloaded from GenBank (JX660690), with any errors and discrepancies corrected. The obtained sequences were identified using the BLAST (NCBI) online software, and representative VP2 sequences were obtained from the GenBank. Sequence alignment was carried out with the mafft 7 online tool, using the E-INS-i method (Katoh and Toh, 2008). Maximum Likelihood (ML) analysis and phylogenetic tree reconstruction were performed with MEGA X software (Kumar et al., 2018), using the MODELS setting to select the best-fitting model (Nei and Kumar, 2000), and ML bootstrap values were calculated based on 1,000 repetitions. The phylogenetic tree was visualized and edited in MEGA X.

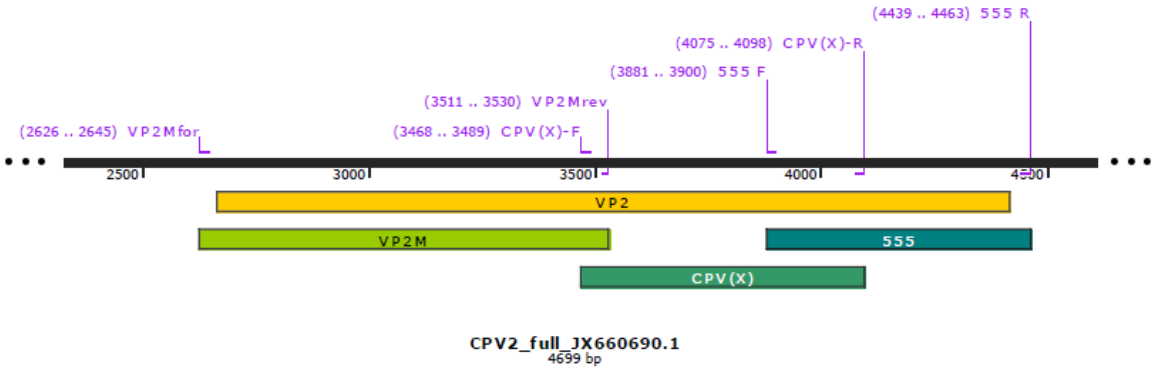

Figure 3: Schematic representation of the VP2 segment of the CPV-2 genome. The 3 sequencing primer pairs and their positions are noted in purple. The figure was made using SnapGene [43].

4. Results

4.1 PCR and Viral Detection Results

The molecular analysis of the 17 canine fecal samples collected from Quito, Ecuador, revealed the presence of CPV-2 in two samples through conventional polymerase chain reaction (PCR). The detected viral sequences corresponded to the VP2 region, a highly

conserved segment of the CPV-2 genome, critical for distinguishing its genetic variants. Four samples tested positive for canine coronavirus (CaCoV), while none of the samples demonstrated the presence of canine circovirus (CanineCV), as presented on Table 3. These findings confirm CPV-2 and CaCoV as the predominant enteric pathogens among the tested cases.

Table 2: Results of the diagnostic PCR tests. The positive results are marked "+," the negative tests "- ".

SAMPLES	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
CPV-2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
CCoV	-	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-
CCV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.2 Phylogenetic analysis

The full sequences of the CPV-2 VP2 protein-coding regions were determined from fecal samples of two dogs. Based on the analysis of the 426th amino acid (CPV-2a: N426, CPV-2b: D426, CPV-2c: E426) of the VP2 protein, the identified viral strains from Ecuador belong to CPV-2c (Figure 4).

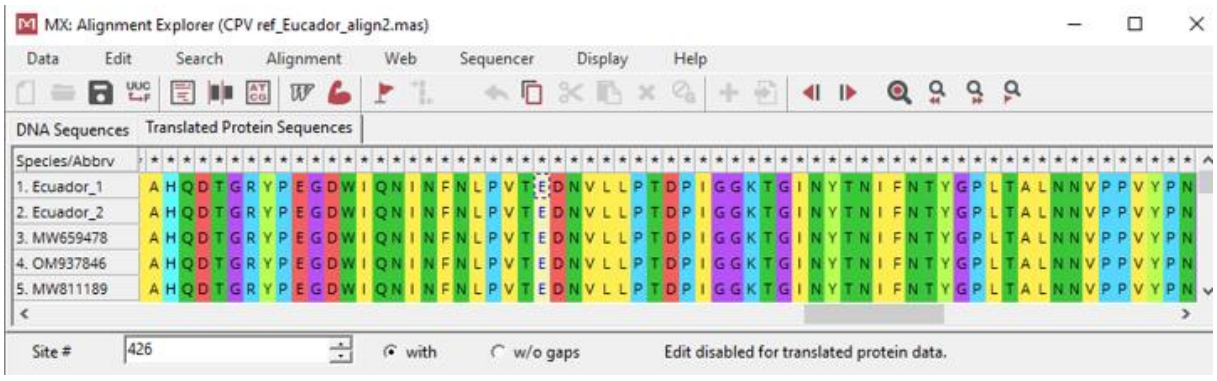

Figure 4 Identification of the CPV-2 genotype of the samples from Ecuador. The assumed protein sequences are shown, the 426th position is highlighted. N=asparagine, D= aspartic acid, E= glutamic acid. The analysis was conducted using the MEGA X software.

Phylogenetic analysis showed that the Ecuadorian sequences form a distinct group with most of the CPV-2c sequences from Asia (Figure 5). The CPV-2b strains from Thailand, Vietnam and China are grouped closely and show 0.4-0.52% genetic distance among them. A unique CPV-2a strain from China (KR002805) and Singapore (KX618915) are closely related to CPV-2c variants from Myanmar, with a genetic distance of 0.29%. We identified a smaller monophyletic group of CPV-2c that includes sequences from the USA, Italy, France, Uruguay and Brazil. The genetic distance among this group was 0.46%-0.63%. The vaccine strains formed a monophyletic group with the two FPV sequences, which were chosen as an outgroup in our analysis. The highest genetic distance between the analyzed CPV-2 sequences was 2.3% and the highest observed distance was between an FPV reference sequence (EU659111) and a sequence from Ecuador (Ecuador_1). The average genetic distance of the vaccine strains to the analyzed CPV-2 strains was 1.56%.

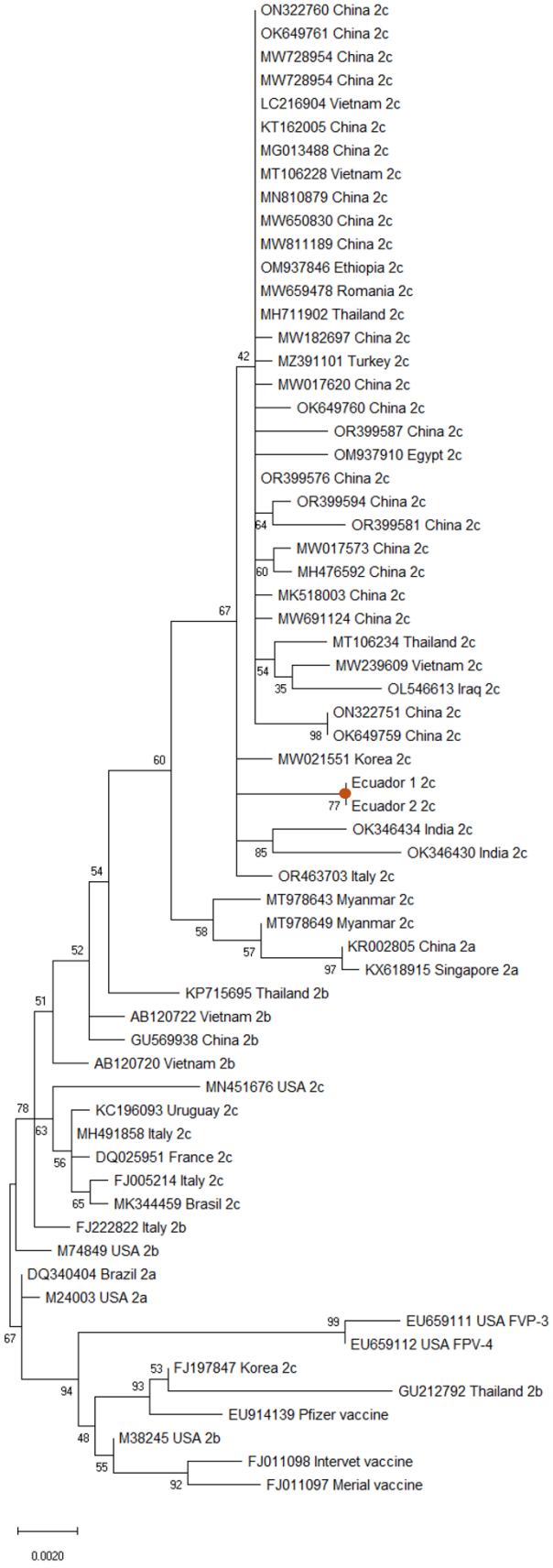

Figure 5: Phylogenetic tree based on the comparison of reference and our VP2 coding sequences of CPV-2, downloaded from GenBank.The strains identified in Ecuador are marked by an orange dot.

The comparison was conducted using the Maximum Likelihood method, and the resulting bootstrap values are displayed above the branches as percentages. The scale indicates 0.002 expected changes at specific locations and branches. The coding of strains appearing on the phylogenetic tree is as follows.

5. Discussion

Our study is one of the few describing CPV-2 infection of dogs in Quito, Ecuador and provides an insight into the epidemiology of this virus in the region. The identification of CPV-2c, a variant known for its high virulence and adaptability, highlights the need for enhanced surveillance and effective vaccination protocols in Ecuador. This finding is consistent with prior studies in South America, particularly in Brazil and Chile, where CPV-2c has emerged as the predominant variant [53]. The prevalence of CPV-2c in these areas underscores the importance of monitoring the genetic evolution of the virus to adapt vaccination strategies accordingly.

Phylogenetic analysis showed close genetic similarity between the Ecuadorian CPV-2c samples and strains reported in Italy, India, and Korea, suggesting potential intercontinental transmission pathways facilitated by global animal trade or movement. The Ecuadorian sequences grouped within the CPV-2c clade, indicating a shared evolutionary origin. This finding aligns with studies conducted in Sicily [54] and Colombia [55], where similar evolutionary patterns were observed. The Ecuadorian isolates displayed unique mutations, possibly driven by local evolutionary pressures, as also suggested in studies from India and Chile [56,53]. These genetic variations could reflect the influence of inconsistent vaccination coverage, regional population dynamics, or environmental factors specific to Ecuador. Franzo et al. emphasized the adaptability and global dispersal of CPV-2c, underscoring the evolutionary dynamics of this lineage [57]. The grouping of Ecuadorian isolates with OR463703 from Franzo's work highlights the interconnectedness of CPV-2c across diverse ecological settings and reinforces the importance of genomic surveillance for understanding viral adaptation and informing control strategies.

The detection of CPV-2c raises significant concerns about the efficacy of current vaccination strategies in Ecuador. While vaccines based on CPV-2a and CPV-2b are widely used, their cross-protective efficacy against CPV-2c remains uncertain. This issue has been documented in regions such as Brazil [10] and Ethiopia [58], where inconsistent vaccination schedules have facilitated the circulation of CPV-2c. The low detection rate of CPV-2 (2/17 samples) in this study may be due to the low number of investigated sample but could also reflect the partial success of vaccination programs. However, the presence of CPV-2c indicates potential gaps in herd immunity. Comparative analyses

with vaccination strategies in other regions highlight the importance of rigorous immunization protocols. Studies from North-East India [56] and Chile [53] demonstrate how strict vaccination schedules reduce the prevalence of CPV-2c. These findings underscore the need for stricter adherence to vaccination guidelines in Ecuador and public education to enhance compliance. Recent evidence also suggests that vaccine-induced immunity may not fully prevent the spread of CPV-2c variants, necessitating the development of next-generation vaccines targeting emerging strains [59].

In terms of diagnostics, this study highlights the importance of molecular methods such as PCR for identifying and characterizing CPV-2 variants. PCR's high sensitivity and specificity allowed for the detection of CPV-2, while sequencing and phylogenetic analysis provided deeper insights into the virus's genetic diversity. Rapid diagnostic methods, such as immunochromatographic tests, while practical in resource-limited settings, are less sensitive for variant identification [7]. Studies from Turkey [60] and Colombia [55] emphasize the need to integrate molecular diagnostics with routine surveillance to ensure early detection of emerging variants and support targeted control efforts. By combining robust diagnostic techniques with genomic analyses, researchers and clinicians can gain a more comprehensive understanding of the viral landscape and mitigate its impact.

Globally, the findings of this study contribute to a growing understanding of the evolution and dissemination CPV-2. The identification of CPV-2c in Ecuador aligns with international trends, where this variant is increasingly dominant due to its adaptability and potential for immune escape. Findings from Ethiopia [58], Sicily [54], and Colombia [55] emphasize the role of genetic recombination and mutation in shaping the evolutionary trajectory of CPV-2. Global collaboration in monitoring and controlling CPV-2 is imperative. Sharing genomic data and harmonizing vaccination strategies across regions can enhance efforts to control the virus. Studies from Chile and North-East India [53,56] demonstrate how coordinated action can prevent the spread of highly transmissible variants like CPV-2c. These efforts should focus on bridging gaps in vaccination coverage and improving diagnostic infrastructure in regions with limited resources.

Despite the insights provided by this study, several limitations must be acknowledged. The small sample size restricts the generalizability of the findings, and additional studies with larger datasets are needed to better characterize CPV-2 variants in Ecuador. Investigating the vaccination history and immune status of sampled dogs would clarify

the factors influencing CPV-2c persistence. Future research should focus on evaluating the cross-protective efficacy of current vaccines against CPV-2c and developing next-generation vaccines to address emerging variants. Longitudinal studies tracking the evolutionary dynamics of CPV-2 in Ecuador and neighbouring regions will be critical for designing effective control strategies [61].

Bibliography

(1): Parrish CR (1990) Emergence, natural history, and variation of canine, mink, and feline parvoviruses. In: Advances in virus research. pp 403–450

(2): Pollock R DVM, Coyne M VMD (1993) Canine parvovirus. Veterinary Clinics of North America: Small Animal Practice 23(3). https://doi.org/10.1016/S0195-5616(93)50305-4

(3): Pénzes JJ, Söderlund-Venermo M, Canuti M, Eis-Hübinger AM, Hughes J, Cotmore SF, Harrach B (2020) Reorganizing the family Parvoviridae: a revised taxonomy independent of the canonical approach based on host association. Archives of Virology 165(9):2133–2146. https://doi.org/10.1007/s00705-020-04632-4

(4): Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (2005) Virus taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses.

(5): Cotmore SF, Agbandje-McKenna M, Canuti M, Chiorini JA, Eis-Hübinger AM, Hughes J, Mietzsch M, Modha S, Ogliastro M, Pénzes JJ, Pintel DJ, Qiu J, Söderlund-Venermo M, Tattersall P, Tijssen P (2019) ICTV Virus Taxonomy Profile: Parvoviridae. Journal of General Virology 100(3):367–368. https://doi.org/10.1099/jgv.0.001212

(6): Decaro N, Buonavoglia C (2012b) Canine parvovirus—A review of epidemiological and diagnostic aspects, with emphasis on type 2c. Veterinary Microbiology 155(1):1–12. https://doi.org/10.1016/j.vetmic.2011.09.007

(7): Chung H-C, Kim S-J, Nguyen VG, Shin S, Kim JY, Lim S-K, Park YH, Park B (2020) New genotype classification and molecular characterization of canine and feline parvoviruses. Journal of Veterinary Science 21(3). https://doi.org/10.4142/jvs.2020.21.e43

(8): Pérez R, Calleros L, Marandino A, Sarute N, Iraola G, Grecco S, Blanc H, Vignuzzi M, Isakov O, Shomron N, Carrau L, Hernández M, Francia L, Sosa K, Tomás G, Panzera Y (2014) Phylogenetic and Genome-Wide Deep-Sequencing Analyses of Canine Parvovirus Reveal Co-Infection with Field Variants and Emergence of a Recent

Recombinant Strain. PloS One 9(11):e111779. https://doi.org/10.1371/journal.pone.0111779

(9): Touihri L, Bouzid I, Daoud R, Desario C, Goulli AFE, Decaro N, Ghorbel A, Buonavoglia C, Bahloul C (2008) Molecular characterization of canine parvovirus-2 variants circulating in Tunisia. Virus Genes 38(2):249–258. https://doi.org/10.1007/s11262-008-0314-1

(10): Mazzaferro EM (2020) Update on canine parvoviral enteritis. ˜the œVeterinary Clinics of North America Small Animal Practice/Veterinary Clinics of North America Small Animal Practice 50(6):1307–1325. https://doi.org/10.1016/j.cvsm.2020.07.008

(11): Behdenna A, Lembo T, Calatayud O, Cleaveland S, Halliday JEB, Packer C, Lankester F, Hampson K, Craft ME, Czupryna A, Dobson AP, Dubovi EJ, Ernest E, Fyumagwa R, Hopcraft JGC, Mentzel C, Mzimbiri I, Sutton D, Willett B, Haydon DT, Viana M (2019) Transmission ecology of canine parvovirus in a multi-host, multi-pathogen system. Proceedings - Royal Society Biological Sciences/Proceedings - Royal Society Biological Sciences 286(1899). https://doi.org/10.1098/rspb.2018.2772

(12): Ikeda Y, Mochizuki M, Naito R, Nakamura K, Miyazawa T, Mikami T, Takahashi E (2000) Predominance of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. Virology 278(1):13–19. https://doi.org/10.1006/viro.2000.0653

(13): Khatri R, Singh P, Mohan H, Minakshi, Cs P (2017) Epidemiology, Pathogenesis, Diagnosis and Treatment of canine parvovirus disease in dogs: A mini review. Journal of Veterinary Science & Medical Diagnosis 06(03). https://doi.org/10.4172/2325-9590.1000233

(14): Sarabandi S, Pourtaghi H (2023) Whole genome sequence analysis of CPV-2 isolates from 1998 to 2020. Virology Journal 20(1). https://doi.org/10.1186/s12985-023-02102-2

(15): Li G, Ji S, Zhai X, Zhang Y, Liu J, Zhu M, Zhou J, Su S (2017) Evolutionary and genetic analysis of the VP2 gene of canine parvovirus. BMC Genomics 18(1). https://doi.org/10.1186/s12864-017-3935-8

(16): Amrani N, Desario C, Kadiri A, Cavalli A, Berrada J, Zro K, Sebbar G, Colaianni ML, Parisi A, Elia G, Buonavoglia C, Malik J, Decaro N (2016) Molecular epidemiology

of canine parvovirus in Morocco. Infection, Genetics and Evolution 41:201–206. https://doi.org/10.1016/j.meegid.2016.04.005

(17): Umar S, Gao D, Kim S, Cheng Y, Fang Z, Zhongqi Q, Yu W, Anderson BD (2024) Molecular characterization of canine parvovirus type 2 (CPV2) reveals a high prevalence of the CPV2c genotype among dogs suffering from diarrhea. Animal Diseases 4(1). https://doi.org/10.1186/s44149-023-00107-6

(18): Zaher KS, El-Dabae WH, El-Sebelgy MM, Aly NI, Salama ZT (2020) Genotyping and phylogenetic analysis of canine parvovirus circulating in Egypt. Veterinary World 13(2):326–333. https://doi.org/10.14202/vetworld.2020.326-333

(19): Lenghaus C, Studdert MJ, Finnie JW (1980) ACUTE AND CHRONIC CANINE PARVOVIRUS MYOCARDITIS FOLLOWING INTRAUTERINE INOCULATION. Australian Veterinary Journal 56(10):465–468. https://doi.org/10.1111/j.1751-0813.1980.tb02555.x

(20): Sime TA, Powell LL, Schildt JC, Olson EJ (2015) Parvoviral myocarditis in a 5-week-old Dachshund. Journal of Veterinary Emergency and Critical Care 25(6):765–769. https://doi.org/10.1111/vec.12347

(21): Ford J, McEndaffer L, Renshaw R, Molesan A, Kelly K (2017) Parvovirus infection is associated with myocarditis and myocardial fibrosis in young dogs. Veterinary Pathology 54(6):964–971. https://doi.org/10.1177/0300985817725387

(22): Cavalli A, Desario C, Kusi I, Mari V, Lorusso E, Cirone F, Kumbe I, Colaianni ML, Buonavoglia D, Decaro N (2014) Detection and genetic characterization of Canine parvovirus and Canine coronavirus strains circulating in district of Tirana in Albania. Journal of Veterinary Diagnostic Investigation 26(4):563–566. https://doi.org/10.1177/1040638714538965

(23): Decaro N, Mari V, Elia G, Addie DD, Camero M, Lucente MS, Martella V, Buonavoglia C (2009) Recombinant canine coronaviruses in dogs, Europe. Emerging Infectious Diseases 16(1):41–47. https://doi.org/10.3201/eid1601.090726

(24): Decaro N, Mari V, Von Reitzenstein M, Lucente MS, Cirone F, Elia G, Martella V, King VL, Di Bello A, Varello K, Zhang S, Caramelli M, Buonavoglia C (2012) A pantropic canine coronavirus genetically related to the prototype isolate CB/05.

Veterinary Microbiology 159(1–2):239–244. https://doi.org/10.1016/j.vetmic.2012.03.039

(25): Chen C, Ji X, Zhang T, Ge L, Sun M, Yang M, Li C A systematic review and meta-analysis of canine enteric coronavirus prevalence in dogs of mainland China. Virology Journal 21(1). https://doi.org/10.1186/s12985-024-02425-8

(26): Decaro N, Martella V, Desario C, Lanave G, Circella E, Cavalli A, Elia G, Camero M, Buonavoglia C (2014) Genomic Characterization of a Circovirus Associated with Fatal Hemorrhagic Enteritis in Dog, Italy. PLoS ONE 9(8):e105909. https://doi.org/10.1371/journal.pone.0105909

(27): Hsu H-S, Lin T-H, Wu H-Y, Lin L-S, Chung C-S, Chiou M-T, Lin C-N (2016) High detection rate of dog circovirus in diarrheal dogs. BMC Veterinary Research 12(1). https://doi.org/10.1186/s12917-016-0722-8

(28): Kotsias F, Bucafusco D, Nuñez DA, Borisovsky LAL, Rodriguez M, Bratanich AC (2019) Genomic characterization of canine circovirus associated with fatal disease in dogs in South America. PLoS ONE 14(6):e0218735. https://doi.org/10.1371/journal.pone.0218735

(29): (2024) Importance of biomarkers and cytokines in the prognosis of canine parvovirus infection. Pakistan Veterinary Journal. https://doi.org/10.29261/pakvetj/2024.214

(30): Tuteja D, Banu K, Mondal B (2024) Canine Parvovirology – A brief updated review on structural biology, occurrence, pathogenesis, clinical diagnosis, treatment and prevention. Comparative Immunology Microbiology and Infectious Diseases 82:101765. https://doi.org/10.1016/j.cimid.2022.101765

(31): Murillo sánchez se (2020) revisión de literatura sobre las dos principales enfermedades virales que afectan a caninos en ecuador: actualización de tratamientos. facultad de ciencias agropecuarias carrera de medicina veterinaria y zootecnia, :37–40

(32): Squires RA, Crawford C, Marcondes M, Whitley N (2024) 2024 Guidelines for the vaccination of dogs and cats – compiled by the Vaccination Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA). Journal of Small Animal Practice 65(5):277–316. https://doi.org/10.1111/jsap.13718

(33): Mylonakis ME, Kalli I, Rallis TS (2016) Canine parvoviral enteritis: an update on the clinical diagnosis, treatment, and prevention. Veterinary Medicine 7:91–100

(34): Kaur G, Chandra M, Kaur H (2024) The Gastroenteritis Virus of Dogs: Canine Parvovirus and Its Diagnosis over the Years. Asian Journal of Biology 20(9):15–29. https://doi.org/10.9734/ajob/2024/v20i9435

(35): C N (2020) Detection of canine parvovirus in dogs by means polymerase chain reaction. American Journal of Biomedical Science & Research 7(6):540–547. https://doi.org/10.34297/ajbsr.2020.07.001219

(36): Cotmore SF, Agbandje-McKenna M, Chiorini JA, Mukha DV, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen P, Gatherer D, Davison AJ (2013a) The family Parvoviridae. Archives of Virology 159(5):1239–1247. https://doi.org/10.1007/s00705-013-1914-1

(37): Decaro N (2020) Enteric viruses of dogs. Advances in Small Animal Care 1:143–160. https://doi.org/10.1016/j.yasa.2020.07.012

(38): Squires, Crawford, Marcondes, Whitley (2024a) Guidelines for the vaccination of dogs and cats – compiled by the Vaccination Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA). In: WSAVA Org. https://wsava.org/wp-content/uploads/2024/04/WSAVA-Vaccination-guidelines-2024.pdf. Accessed 12 Nov 2024

(39): Llc GB SnapGene | Software for everyday molecular biology. In: SnapGene. https://www.snapgene.com/

(40): Demeter Z, Gál J, Palade EA, Rusvai M (2009) Feline parvovirus infection in an Asian palm civet (Paradoxurus hermaphroditus). Veterinary Record 164(7):213–215. https://doi.org/10.1136/vr.164.7.213

(41): Escutenaire S, Mohamed N, Isaksson M, Thorén P, Klingeborn B, Belák S, Berg M, Blomberg J (2006) SYBR Green real-time reverse transcription-polymerase chain reaction assay for the generic detection of coronaviruses. Archives of Virology 152(1):41–58. https://doi.org/10.1007/s00705-006-0840-x

(42): De Arcangeli S, Balboni A, Kaehler E, Urbani L, Verin R, Battilani M (2019) Genomic Characterization of Canine Circovirus Detected in Red Foxes (Vulpes vulpes)

from Italy using a New Real-Time PCR Assay. Journal of Wildlife Diseases 56(1):239. https://doi.org/10.7589/2018-11-270

[43]: Tello, M., Oporto, B., Lavín, J. L., Ocejo, M., & Hurtado, A. (2022). Characterization of a carbapenem-resistant Escherichia coli from dairy cattle harbouring blaNDM-1 in an IncC plasmid. Journal of Antimicrobial Chemotherapy, 77(3), 843–845. https://doi.org/10.1093/jac/dkab455

[44]: Maya L, Calleros L, Francia L, Hernández M, Iraola G, Panzera Y, Sosa K, Pérez R (2013) Phylodynamics analysis of canine parvovirus in Uruguay: evidence of two successive invasions by different variants. Archives of Virology 158(6):1133–1141. https://doi.org/10.1007/s00705-012-1591-5

[45]: Shackelton LA, Parrish CR, Truyen U, Holmes EC (2004) High rate of viral evolution associated with the emergence of carnivore parvovirus. Proceedings of the National Academy of Sciences 102(2):379–384. https://doi.org/10.1073/pnas.0406765102

[46]: Estes, M.K., G. Kang, C.Q. -Y. Zeng, S.E. Crawford, and M. Ciarlet, 2001: Pathogenesis of Rotavirus Gastroenteritis, pp. 82–100.

[47]: Chamsai E, Charoenkul K, Udom K, Jairak W, Chaiyawong S, Amonsin A (2024) Genetic characterization and evidence for multiple reassortments of rotavirus A G3P[3] in dogs and cats in Thailand. Frontiers in Veterinary Science 11. https://doi.org/10.3389/fvets.2024.1415771

[48]: Creevy KE, Evans JB (2022) Canine distemper. In: MSD Veterinary Manual. https://www.msdvetmanual.com/generalized-conditions/canine-distemper/canine-distemper

[49]: Canine parvovirus - WSAVA 2014 Congress - VIN. https://www.vin.com/apputil/content/defaultadv1.aspx?id=7054783&pid=12886

[50]: Cassedy A, Parle-McDermott A, O'Kennedy R (2021) Virus Detection: A review of the current and emerging molecular and immunological methods. Frontiers in Molecular Biosciences 8. https://doi.org/10.3389/fmolb.2021.637559

[51]: Uma M, Nivetha K, Pradeepika N, Mohanakrishnan H (2022) Validation of haemagglutination, haemagglutination inhibition and antibody neutralization assays used

to determine the titre of CPV -2 virus and anti CVP – 2 egg yolk antibodies. International Journal of Health Sciences :2820–2831. https://doi.org/10.53730/ijhs.v6ns8.12686

[52]: P M, Brar B, K S, Thomas JV, J SJ, Na I, Lambe U, Guray M, Bansal N, Kumar P, Joshi VG, Khatri R, Mohan H, Pundir CS, Khurana SK, Prasad G (2016) Canine parvovirus- an insight into diagnostic aspect. Journal of Experimental Biology and Agricultural Sciences 4(3S):279–290. https://doi.org/10.18006/2016.4(3s).279.290

[53]: Alexis V-A, Sonia V, Daniela S, Miguel G, Timothy H, Valentina F, Lisette L, Leonardo S (2021) Molecular analysis of Full-Length VP2 of canine parvovirus reveals antigenic drift in CPV-2B and CPV-2C variants in central Chile. Animals 11(8):2387. https://doi.org/10.3390/ani11082387

[54]: Mira F, Schirò G, Franzo G, Canuti M, Purpari G, Giudice E, Decaro N, Vicari D, Antoci F, Castronovo C, Guercio A (2024) Molecular epidemiology of canine parvovirus type 2 in Sicily, southern Italy: A geographical island, an epidemiological continuum. Heliyon 10(4):e26561. https://doi.org/10.1016/j.heliyon.2024.e26561

[55]: Giraldo-Ramirez S, Rendon-Marin S, Ruiz-Saenz J (2020) Phylogenetic, evolutionary and structural analysis of canine parvovirus (CPV-2) antigenic variants circulating in Colombia. Viruses 12(5):500. https://doi.org/10.3390/v12050500

[56]: Jayappa K, Rajkhowa TK, Gaikwad SS (2024) Canine parvovirus in North-East India: a phylogenetic and evolutionary analysis. Veterinary Quarterly 44(1):1–13. https://doi.org/10.1080/01652176.2024.2408742

[57]: Franzo G, Mira F, Schirò G, Canuti M (2023) Not Asian anymore: Reconstruction of the history, evolution, and dispersal of the “Asian” lineage of CPV-2C. Viruses 15(9):1962. https://doi.org/10.3390/v15091962

[58]: Tegegne D, Tsegaye G, Faustini G, Franzo G (2022) First genetic detection and characterization of canine parvovirus type 2 (Carnivore protoparvovirus 1) in southwestern Ethiopia. Veterinary Research Communications 47(2):975–980. https://doi.org/10.1007/s11259-022-10027-4

[59]: Hao X, Li Y, Xiao X, Chen B, Zhou P, Li S (2022) The Changes in Canine Parvovirus Variants over the Years. International Journal of Molecular Sciences 23(19):11540. https://doi.org/10.3390/ijms231911540

[60]: Karapinar Z, Karaman M, Kisadere İ, Usta M, TiMurkan MÖ (2023) Virological and pathological investigation of Canine parvovirus-2 (CPV-2) with the assessment of the genetic variability of field strains. TURKISH JOURNAL OF VETERINARY AND ANIMAL SCIENCES 47(6):544–555. https://doi.org/10.55730/1300-0128.4324

[61]: Bahoussi AN, Wang P-H, Ma Z-H, Rani N, Wu C, Xing L (2022) Identification of novel recombinants and proposed standard reference genomes for phylogenetic classification of canine parvovirus-2 (CPV-2): Comprehensive analysis revealing global evolutionary trait. Frontiers in Veterinary Science 9. https://doi.org/10.3389/fvets.2022.1030522

[62]: Decaro N, Buonavoglia C, Barrs VR (2020) Canine parvovirus vaccination and immunisation failures: Are we far from disease eradication? Veterinary Microbiology 247:108760. https://doi.org/10.1016/j.vetmic.2020.108760

[63]: Basurto-Sacón MG, Zambrano-Yépez JD, Vallejo-Romero S (2024) Factores de riesgo asociados con la parvovirosis canina en diferentes regiones climáticas de Ecuador. journalgestar.org. https://doi.org/10.46296/gt.v7i14.0168

[64]: Matthijnssens J, Attoui H, Bányai K, Brussaard CPD, Danthi P, Del Vas M, Dermody TS, Duncan R, Fāng Q, Johne R, Mertens PPC, Jaafar FM, Patton JT, Sasaya T, Suzuki N, Wei T (2022) ICTV Virus Taxonomy Profile: Sedoreoviridae 2022. Journal of General Virology 103(10). https://doi.org/10.1099/jgv.0.001782

[65]: Desselberger U (2014) Rotaviruses. Virus Research 190:75–96. https://doi.org/10.1016/j.virusres.2014.06.016

[66]: Matthijnssens J, Attoui H, Bányai K, Brussaard CPD, Danthi P, Del Vas M, Dermody TS, Duncan R, Fāng Q, Johne R, Mertens PPC, Jaafar FM, Patton JT, Sasaya T, Suzuki N, Wei T (2022) ICTV Virus Taxonomy Profile: Sedoreoviridae 2022. Journal of General Virology 103(10). https://doi.org/10.1099/jgv.0.001782

Acknowledgments

First, too my family, I extend my heartfelt thanks. To my mom and my dad, your unconditional love and trust in me have been the cornerstone of all my achievements. To my grandparents, my role models in every way, your upbringing has been a source of wisdom and strength for me throughout all these years. To my brother, thank you because I know you are always there for me even with the distance. To you, GM, I could not have done this without your support.

I would also like to express my gratefulness to my supervisor, **Lilla Dénes**, for her support and understanding throughout this project. For giving me the chance to learn from her and being very patient in the process of guiding me in this process. Her dedication to mentorship has not only enriched my knowledge but also encouraged me to strive for excellence and learned a few things about discipline.