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Genetic characterization of parvoviruses isolated from biological samples of Arctic foxes and Eurasian wolves

Master's thesis

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Declaration

"I hereby declare that I have done this thesis entitled "Genetic characterisation of parvoviruses isolated from biological samples of Arctic foxes and Eurasian wolves" independently; all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA."

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Abstract

This research was conducted in order to asses the possible risks that Canine parvovirus type 2 (CPV-2) poses for population of Eurasian wolf (*Canis lupus lupus*) living in Czech Republic, and to further deepen the knowledge of CPV-2 genetic evolution using any positive samples from Arctic foxes (*Vulpes lagopus*) located in Greenland and Iceland. Polymerase chain reaction (PCR) was used as a main diagnostic tool for detecting the presence of the virus in biological samples (DNA isolated from faeces). The NS1 (non-structural viral protein) specific primers designed by Hirasawa et al. in 1994 were used. Results of the PCR reaction were evaluated using gel electrophoresis with UV-light detectable colorant Ethidium Bromide (EtBr). Positive samples were afterwards sent for sequenation at sequencing laboratory at Charles University in Prague, and subsequently at State Veterinary Institute in Prague. All of the 124 samples from Eurasian wolves and 31 samples from Arctic foxes from Greenland were found negative. One sample from the 37 samples of Arctic fox from Iceland was found positive. However, after evaluating the sequence results, we have found 100 % homology with our positive control, therefore we have conducted that this sample was contaminated by our positive control, and was not genuinely positive. From these results we can conclude that Eurasian wolf population in Czech Republic has either not yet come into contact with the CPV-2 virus, or is immune against the infection this virus iduces. Therefore no immediate threat posed by CPV-2 to Czech wolf population is currently present. With the Arctic fox samples we have mainly gained the knowledge of health situation of wild fox populations in given countries. Since no such research was conducted before in Iceland or Greenland these primal results are useful as a confirmation of absence of highly pathogenic CPV-2 in wilderness of these countries. More immunological research would be needed to asses the reasons of absence of this virus in the studied populations.

Keywords: Canine parvovirus type 2, CPV, wildlife, Carnivore protoparvovirus 1, Parvoviridae, *Vulpes lagopus*, *Canis lupus lupus*, Czech Republic, Greenland, Iceland

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Abbreviations

AAVs - Adeno-associated viruses or

BFPV - Blue fox parvovirus

CPV-1 - Canine parvovirus type 1

CPV-2 - Canine parvovirus type 2

DNA - Deoxyribonucleic acid

ELISA - Enzyme linked immunosorbent assay

FPV - Feline panleukopenia

GIT - Gastrointestinal tract

HI - Hemagglutination inhibition

HSPG - Heparin sulphate proteoglycan

ICTV - Committee on Taxonomy of Viruses

IFA - Indirect immunofluorescent antibody

ITRs - Inverted terminal repeats

MDAs – Maternally derived antibodies

MEV - Mink enteritis virus

MLVs - Modified live vaccines

MODS - Multiple organ dysfunction syndrome

mRNA – Messenger ribonucleic acid

MTOC - Microtubule organizing centre

MVC - Minute virus of canines

MVM - Minute virus of mice

NS - Non-structural protein

ORF - Open reading frame

PCR - Polymerase chain reaction

PLA_2 - Calcium-dependent phospholipase A_2

PPV - Porcine parvovirus

RDPV - Raccoon dog parvovirus

rFeIFN-ω - Recombinant feline interferon-ω

RPV - Raccoon parvovirus

rt-PCR - Real-Time PCR

SIRS - Systematic inflammatory response syndrome

ssDNA - Single stranded DNA molecule

TfR - Transferrin type 1

UV – Ultra-violet

VP - Viral protein

1. Introduction

Parvoviruses are viruses belonging to family Parvoviridae. This family comprises of two subfamilies: Parvovirinae and Densovirinae. They can infect vertebrates and insects, respectively (Hoelzer & Parrish 2010; Decaro & Buonavoglia 2012).

The taxonomy of parvoviruses undergone major changes in year 2014, when according to the genetic research submitted into International Committee on Taxonomy of Viruses (ICTV) genera names were changed. The individual new genera had various novel species appointed to them. For example: Canine parvovirus type 2 (CPV-2), was placed in the species called Carnivore protoparvovirus 1 (genus *Protoparvovirus*), together with other carnivore parvoviruses like Feline panleukopenia virus (FPV), Raccoon parvovirus (RPV), Mink enteritis virus (MEV). According to this new taxonomy the subfamily Parvovirinae consists of eight genera: *Protoparvovirus*, *Amdoparvovirus*, *Aveparvovirus, Copiparvovirus, Tetraparvovirus*, *Bocaparvovirus*, *Dependoparvovirus* and *Erythroparvovirus* (Cotmore et al. 2014; Qiu et al. 2017) see Table 1 (data by Cotmore et al. 2014). All members of genus *Protoparvovirus* are very closely genetically related, with more than 98 % genome homology (Truyen 1999; Steinel et al. 2000). They do however, differ in their host ranges, antigenic variants or tissue tropism in hosts (Truyen 1999; Miranda & Thompson 2016).

The changes in taxonomy made were mainly fuelled by defining precise levels of sequence similarities that were needed for a specific virus to belong to the same taxonomic unit (genus, species). Phylogenetic analyses were based upon the specific amino acid sequence of the viral replication initiator protein-NS1, that has an AAA+ helicase activity (Cotmore et al. 2014). In this thesis we will be using the old taxonomy since it enables us to distinguish CPV-2 from the other carnivore parvoviruses and it is better suited for the needs of this research.

Table 1: Summary of changes between previous and current parvovirus taxonomy

Previous taxonomy			Current taxonomy		
Genus	# Species	# Strains	Genus	# Species	# Strains
Amdovirus	1	1	*Amdo **parvo** virus*	2	2
/	/	/	***Aveparvovirus***	1	2
Bocavirus	2	6	*Boca **parvo** virus*	12	22
/	/	/	***Copiparvovirus***	2	2
Dependovirus	12	13	*Dependo **parvo** virus*	7	23
Erythrovirus	4	9	*Erythro **parvo** virus*	6	12
Parvovirus	12	18	*Proto **parvo** virus*	5	25
/	/	/	***Tetraparvovirus***	6	10

CPV-2 is especially important virus causing enteric disease in wild carnivores (Miranda et al. 2017). This virus has very high mortality rates (up to 90 %) in cubs, is extremely contagious through faecal-oral (nasal) route, and also is very stable in the environment (indirect transmission is extremely frequent). Further, CPV-2 is an interesting example of virus that probably evolved by cross-species transmission and acummulation of mutations which make it able to cause pandemics (Hoelzer & Parrish 2010; Allison et al. 2014; Miranda & Thompson 2016).

Parvovirosis has emerged as a new pandemic viral disease of canines in 1978. At that time clinical similarities to already known FPV have been noted (Steinel et al. 2001; Allison et al. 2014). Through genetic and antigenic analyses it has been confirmed that there is a very close relationship between FPV and the newly named Canine parvovirus type 2. The name Canine parvovirus type 2 was used to distinguish the new virus from already known, though distantly related, Canine parvovirus type 1 (minute virus of canines, MVC or CPV-1) which is nowadays included in the genus *Bocaparvovirus* (Steinel et al. 2001; Decaro & Buonavoglia 2012; Cotmore et al. 2014).

The specific ancestral virus that gave rise to CPV-2 is not yet confirmed; however most probable ancestor according to several scientific studies is apparently FPV (Truyen et al. 1996, 1998; Hoelzer & Parrish 2010; Allison et al. 2013). A phylogenetic analysis of several viruses from FPV subgroup collected in the periods of 1960s, 1970s, and 1980s

revealed a FPV viral strain from a farmed arctic fox (*Vulpes lagopus*) in Finland as most closely related to CPV-2 (Veijalainen 1988; Truyen et al. 1996; Hoelzer & Parrish 2010). This is also supported by results of research made from 1991 to 1995 on CPV-2 in free-ranging European red foxes (*Vulpes vulpes*), where the virus showed several features of genomic similarities between FPV and CPV-2. It leads to the hypothesis of a possible link between CPV-2 and FPV (Truyen et al. 1998).

CPV-2 has high adaptation skills also proven by very quick emergence of new antigenic CPV-2 types called CPV-2a and CPV-2b between years 1979 and 1984 after introduction of vaccine against original CPV-2. Most recent strain CPV-2c has emerged in year 2000 (Steinel et al. 2000, 2001a; Decaro & Buonavoglia 2012; Allison et al. 2013; Miranda et al. 2017). CPV-2 has the ability to cross species barriers using only one or very few new mutations, and therefore to jump hosts, as is seen in studies concerning natural passage of CPV-2 in raccoons that may have facilitated its adaptation to dogs for example (Allison et al. 2012, 2014).

Summarising all this information has led to the theme of this diploma thesis. For fragmented wild populations of Eurasian wolves (*Canis lupus lupus*) of Czech Republic the emergence of this specific pathogen would be disastrous. The genetic evaluation of possibly positive samples from Arctic foxes (*Vulpes lagopus*) of Iceland and Greenland will also provide us with further information concerning the specific genetic characteristics of the involved CPV-2 strains, therefore enabling further study of phylogenetic relationships with already mapped genomes of other CPV-2 samples.

2. *Parvoviridae*

2.1. Parvovirus basics

Parvoviruses are small viruses. Their name comes from the Latin word "*parvus*" or small. In fact parvoviruses are the smallest among other animal DNA-based viruses. Their non-enveloped, and therefore very stable capsid, has 25 nm in diameter. The capsid contains one single stranded DNA molecule (ssDNA), which is 5,000-6,000 bases long (Lukashov & Goudsmit 2001; Cotmore & Tattersall 2013; Qiu et al. 2017).

It is important to mention specific genera of *Dependoviruses* containing so-called adeno-associated viruses or AAVs, which are unique in their nature. They specifically require co-infection with some kind of helper virus for successful infection of host organism (usually herpesvirus or adenovirus). AAVs are so-called cryptic viruses, meaning they do not induce (usually) any pathological response from the host organism. AAVs DNA usually integrates their DNA into a latent form and can be found in many different types of tissues (Siegl et al. 1985; Gao et al. 2004). AAVs are also useful in fields of human gene therapy. They are being developed to become vectors for gene therapy to treat diseases like haemophilia, cancer or cystic fibrosis (Xie et al. 2002).

Excluding AAVs, the remaining parvoviruses are autonomous, even though they do need their host cell to undergo S phase (or G2 phase) in order for viral DNA replication to take place (Berns & Parrish 2013; Qiu et al. 2017). As Lukashov and Goudsmit (2001) say, there is a distinction between these two groups: autonomous parvoviruses contain primarily mRNA complementary DNA strands. On the other hand AAVs contain strands of DNA of either polarity.

Parvoviruses are widespread. They have most likely been spreading in animals for millions of years as can be concluded from the occurrence of integrated viral DNA in the

genomes of plenty different animals (vertebrates and invertebrates equally) (Belyi et al. 2010; Berns & Parrish 2013). Parvoviruses (including CPV-2) can be transmitted between hosts by several pathways: faecal-oral, urine, and respiratory tracts. They are very stable in the environment, and are easily transmitted by contaminated fomites, virus contaminated objects (Hoelzer & Parrish 2010; Berns & Parrish 2013; Miranda & Thompson 2016).

2.1.1 Viral capsid and general structure

Molecular weight of parvoviral capsid is ranging from 5.5 up to 6.2 × 10^6 daltons. Density measured in cesium chloride (CsCl) is usually between 1.39 up to 1.42 g/cm^3. Parvovirus virions are largely resistant to inactivation; they are therefore stable for up to 60 minutes in pH between 3 and 9 at 60°C. They can however, be inactivated by β-propionate, formalin, oxidizing agents and hydroxylamine (Berns & Parrish 2013; Cotmore et al. 2014).

Capsid of autonomous parvoviruses has icosahedral symmetry with triangulation number T =1 (Cotmore & Tattersall 2013; Qiu et al. 2017). It consists of 60 copies of structural proteins (in case of CPV-2 VP1, VP2, and VP3) see Figure 1 on page 7. The major structural protein in empty viral particles is VP2. VP2 is after filling of the particle by viral DNA proteolytically cleaved for VP3. VP1 protein is a product of alternatively spliced VP2 pre-mRNA. It bears calcium-dependent phospholipase A_2 (PLA_2) domain, which is normally buried deep in the viral capsid. It becomes exposed while the virus enters the host cell (with the exception of B19 where PLA_2 is exposed on the surface of the capsid suggesting different biology of this parvovirus). VP1 also bears the basic amino acids rich motif serving as a nuclear localization signal during cell entry and capsid assembly (Wu & Rossmann 1993; Simpson et al. 2000, 2002; Zádori et al. 2001; Xie et al. 2002; Hueffer & Parrish 2003; Kaufmann et al. 2004; Berns & Parrish 2013). There are many structural

similarities in the atomic structure of capsid between of autonomous parvoviruses and AAVs (Parris and Berns 2013) see Figure 1 and Figure 2 on pages 7 and 8.

The major structural protein is folded to the so-called “jelly roll” structure with antiparallel β-barrel motif by up to one third of its whole structure similarly as in Mengo virus or coxsackievirus B3 (Siegl et al. 1985; Agbandje et al. 1993; Rossmann 2012; Callaway et al. 2017). This conformation is also typical for many icosahedral viruses, like for example adenoviruses (Nandhagopal et al. 2014). Large loops that are connecting strands of this β-barrel create the capsid surface and have a major conformation role in interactions with host tissue where they fulfil the function of binding receptors. These loops also form the so-called conformation-dependent neutralizing epitopes that are recognized by specific antibodies in host organisms. These are important targets for the antibodies mainly on the surface of viruses like CPV-2, minute virus of mice (MVM), AAVs, and B19. Specifically they prefer to bind on raised regions around the threefold axes (Wu & Rossmann 1993; Simpson et al. 2000, 2002; Hueffer & Parrish 2003; Kaufmann et al. 2004) .

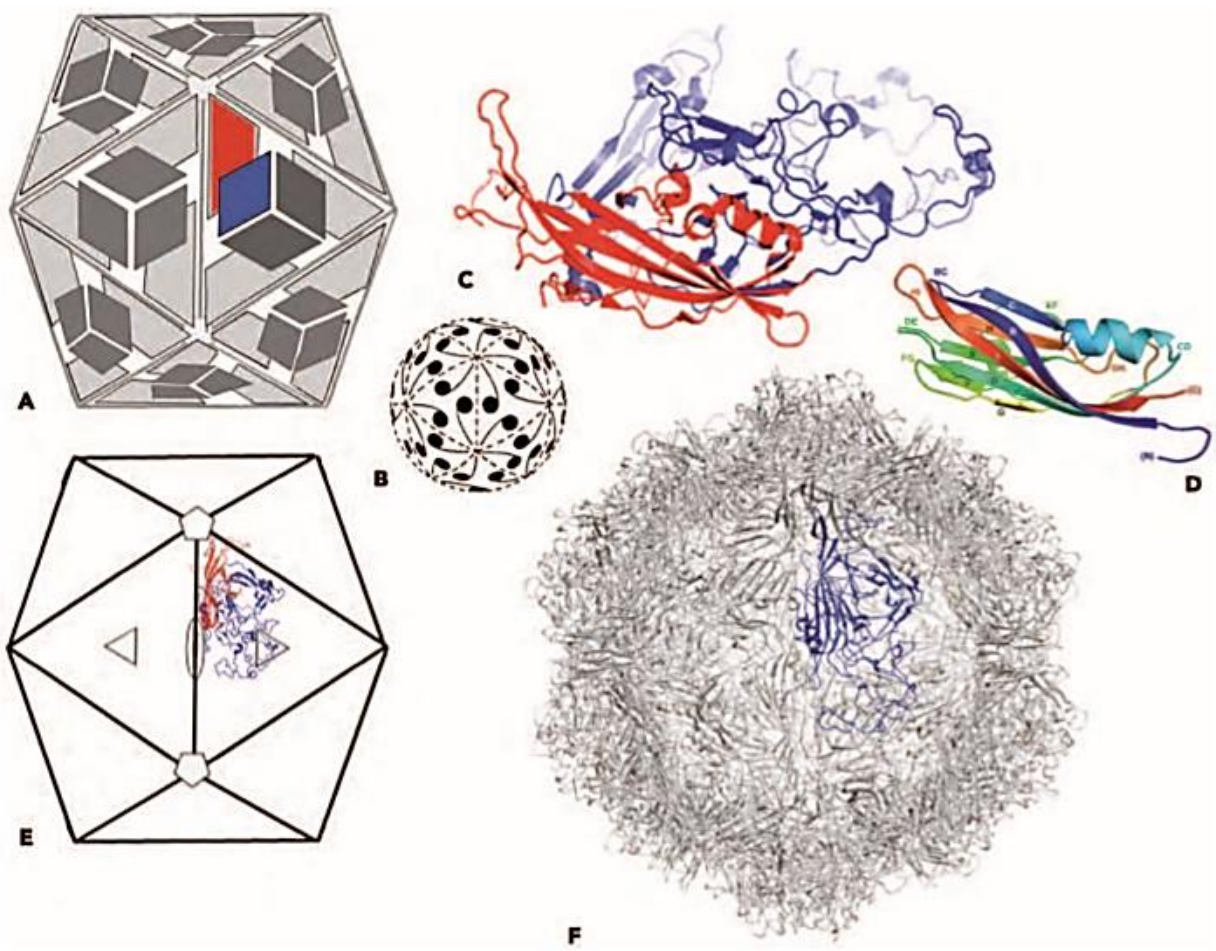

Figure 1: Canine parvoviru s (CPV) structure (from Harrison [2013] Fields virology) CPV-2 has a simple icosahedrally symmetric virion**. A:** Icosahedron, viewed along a twofold axis, with diagrammatic representations of a protein subunit with a core domain (colored red on one of the subunits) and a projecting region (blue). Compare the subunits with the representation of commas in **B**. **C:** Ribbon diagram of the CPV-2 protein subunit; the core domain (red) is a β-jelly-roll, from which emanate several loops that cluster to form a complex projecting region (blue). The simplified representation of the β-jelly-roll in **D** is in rainbow coloring, from blue at its N-terminus to red at its C-terminus. **E:** Icosahedron, as in **A**, but with a ribbon representation of one subunit; symbols for symmetry axes. **F**: Ribbon representation of all 60 subunits, with the subunit from **E** in blue and all others in gray (Harrison 2013).

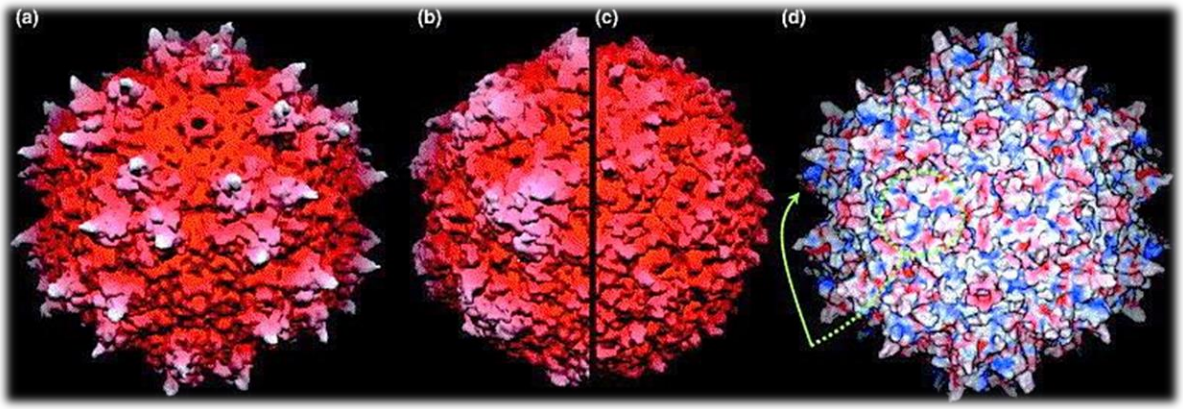

Figure 2: **Surface renderings of the capsids of AAV-2, CPV-2 and insect densovirus** The most structure elements are shared between AAV-2 (a) and CPV-2 (b), although the three-fold proximal peaks are more prominent in AAV-2 than the spikes in CPV-2. By contrast the surface of the insect densovirus (c) capsid appears smoother with no distinct spikes at the threefold axes of symmetry. The electrostatic surface potential of AAV with blue represents positive charge and red shows areas of negative electrostatic potential (d). The putative receptor binding sites are positively charged patches on the sides of the threefold proximal peaks and are shown head on (green circle) or edge on (green arrow) (Hueffer & Parrish 2003).

2.1.2 Genome of parvoviruses

Parvovirus genome consists of s single-stranded DNA molecule. The 5´and 3´ends of the genomic DNA contain inverted terminal repeats (ITRs), palindromic sequences forming specific hairpin structures and which are required for the DNA replication. 3´part of the genome bears an open reading frame (ORF) encoding for non-structural protein NS1, while 5´part bears ORF encoding for structural genes (VP1, VP2, and VP3). Other small but important proteins like the NS2 (in for example CPV-2), or the NP1 (nuclear phosphoprotein) protein specific for the bocaviruses, are produced by alternative splicing from the major transcripts.

Interactions between ITRs and structural proteins and mechanism of genome packaging are not fully understood in CPV-2. In B19 for example, this encodes a small specific protein responsible for interaction between viral genome and major structural proteins (Zádori et al. 2005; Sun et al. 2009; Berns & Parrish 2013; Tu et al. 2015).

2.2 Canine parvovirus type 2

2.2.1 Structure

CPV-2 has typical capsid characteristic for most parvoviruses (see chapter 2.1.1 and Figure 1). The structure of CPV-2, CPV-2a, MVM and FPV was already described by the use of X-Ray crystallography in three-dimensional near-atomic resolution (Tsao et al. 1991; Decaro & Buonavoglia 2012; Miranda & Thompson 2016; Callaway et al. 2017). This method allowed us to determine that capsid of these parvoviruses comprises characteristic 60 protein subunits VP1 (5-6 copies) and VP2 (54-55 copies), so approximately ninety percent of the protein in viral capsid is VP2, while only 10% is represented by the VP1 (Agbandje et al. 1993; Vihinen-Ranta et al. 2002; Parrish & Kawaoka 2005; Clegg et al. 2011; Miranda & Thompson 2016; Allison et al. 2016; Liu et al. 2017). Another CPV-2 capsid characteristics could then be the prolonged raised regions called spikes, positioned by the threefold axes, together with specific cylindrical structures at the fivefold axes enveloped by wide depression (or so-called canyon), and another depression, in this case called dimple, at the twofold axes (Tsao et al. 1991; Decaro & Buonavoglia 2012; Miranda & Thompson 2016; Allison et al. 2016).

2.2.2 Epidemiolgy and etiology

CPV-2 is one of the major causes of transmittable viral hemorrhagic gastroenteritis in dogs and also is one of the most common infectious diseases in dogs worldwide, highly infectious and dangerous for young animals up to 4 to 6 months depending on the age of weaning. Initially, when CPV-2 emerged in 1978, it was associated with two types of disease – enteritis, and myocarditis; however in most recent years the myocarditis had not been reported in domestic dogs, probably because the majority of domestic bitches are now immune and transfers this immunity via colostrum providing their maternally derived antibodies – MDAs. This protects the puppies in the most critical period of development (Decaro et al. 2005; Sykes 2014; Hernández-Blanco & Catala-López 2015; Luo et al. 2016). This immunity however, does not have to be present in wild carnivores since there are no vaccines applied; therefore this disease is still a concern for them. In 1970's no immunity was present in canids, and therefore the rapid spreading of CPV-2 was occurring all over the world in a pandemic scale. This pandemic was spreading at an alarming rate in a period of about 6 months, speed comparable to the spread of influenza virus in humans (Tatem et al. 2006; Hoelzer & Parrish 2010; Sykes 2014).

As mentioned before CPV-2 spreads via faecal-oral route (direct transmission), or through fomites contained in faeces of infected animals (indirect transmission) (Sykes 2014; Li & Humm 2015). Rodents and insects that came to contact with infected faeces can also serve as an indirect transmission route (the virus cannot replicate in these species, so they serve as just physical carriers) (Goddard & Leisewitz 2010; Sykes 2014).

CPV-2 replicates in the nucleus of actively dividing cells undergoing late S-phase or early G2-phase of the cell cycle (since CPV-2 cannot create its own DNA polymerase, it has to use host cell polymerase to reproduce) leading to the preferential infection of these cells, and this way affecting the bone marrow, thymus, lymph nodes, and the gastrointestinal tract severely (Goddard & Leisewitz 2010; Decaro & Buonavoglia 2012;

Sykes 2014; Li & Humm 2015). The virus can spread to all tissues including the brain (Elia et al. 2007; Decaro & Buonavoglia 2012).

CPV-2 starts its replication in oropharyngeal lymphoid tissue and later on it enters bloodstream where it infects leukocytes. Main target tissue of infection – the intestinal crypt epithelium is typically infected within 4 days of on-going infection (in experimental infections) or between 7 to 14 days (in the field). The infection of this tissue usually starts the onset of clinical signs – fever, lessened appetite, vomiting, rapid dehydration, and abdominal pain. The virus is disseminated by infected leukocytes to the germinal epithelium of intestinal crypts and destroys it, which causes the main symptom – diarrhoea. The infection of leukocytes causes acute lymphopenia.

In healthy animal, matured intestinal crypt epithelial cells migrate from the germinal epithelium of these crypts to the tips of the small intestine villi. There they acquire their absorptive capability and aid in digestion of nutrients. Since CPV-2 infects the germinal epithelium of these intestinal crypts it causes epithelial destruction and collapsing of the villi. Normal cell turnover is therefore disabled leading to pathologic lesion of atrophic and shortened villi (Humm & Hughes 2009; Goddard & Leisewitz 2010; Li & Humm 2015; Mylonakis et al. 2016). See Figure 3 to understand where the main targeted area of CPV-2 is.

Antibodies against CPV-2 usually appear after 5 days of infection and steadily increase until 10th day of infection (Li & Humm 2015). According to Sykes (2014) the virus is shed already before onset of clinical signs and it declines later, after usually 7 days. When female animal is infected during gestation, infertility, resorption or abortion can occur. Born offsprings that are infected in utero can develop myocarditis resulting in sudden death or congestive heart failure (Lenghaus et al. 1980; Sykes 2014). Coinfection by bacterial pathogens may lead to further worsening of the clinical signs adding for example respiratory distress or even multiple organ failure and death. Still the mortality

rate in adults is low, usually around 1 %. In offsprings however this rate can range between 70 % to 90 % (Decaro & Buonavoglia 2012; Mylonakis et al. 2016).

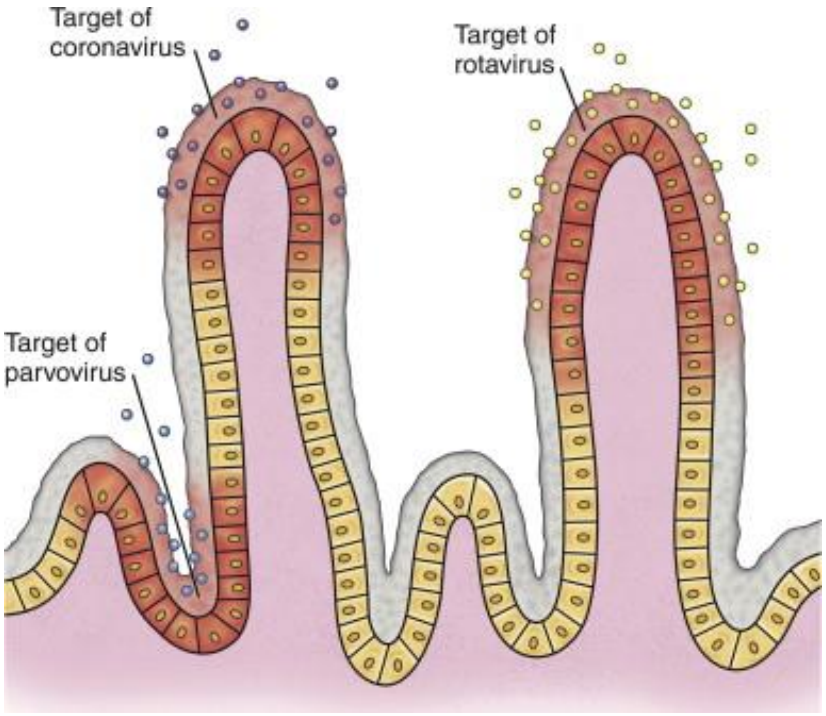

Figure 3: Target sites of replication of selected enteric viral pathogens (Sykes 2014).

To successfully develop in host cell, CPV-2 must go through complex infectious cycle that includes several steps: binding to cell receptors, endosomal and cytoplasmic trafficking, nuclear transport, DNA replication in host cell nucleus, new capsid assembly, another nuclear transport (out of the host cell), and finally the release from host cell – see Figure 4 (Parker & Parrish 2000; Callaway et al. 2017). For all these steps some very specific functions are required from capsid proteins. During receptor recognition the 3-fold spikes that are located centrally around VP2 residue 300 bind to transferrin type 1 (TfR), the main CPV-2 receptor. These spikes are also the most important antibody binding sites. They are usually divided by their position into two closely neighboring sites A and B. This binding to TfR on the host cell membrane then starts the whole process of infection and is quickly followed by clathrin-mediated endocytosis. Endocytosis pathway is finished in early endosome where the virus is exposed to significantly lower pH as the endosome

gradually acidifies, causing the release of capsid-bound calcium ions which cause the capsid loops to move thus exposing the VP1 unique region. This leads to the release of the virus to cytoplasm (Parker & Parrish 2000; Palermo et al. 2003; Harbison et al. 2008; Callaway et al. 2017). The transferrin receptor is expressed in biggest density on actively dividing cells, which helps to understand CPV-2 pathogenesis (Truyen 2006).

Recently it was found out that the virus capsid may also bind to sialic acid, more specifically the modified form of *N*-glycolylneuraminic acid (Neu5Gc) that is sometimes present in some of the hosts. Sialic acid belongs to a group of glycans. It is one of the cell surface carbohydrate components. It can serve as an attachment factor for many viruses including MVM or porcine parvovirus (PPV). Specifically in CPV-2 or FPV infections the sialic acid provides a binding site to erythrocytes; however the biological significance of this binding is not yet understood. This may be an important finding because even though this receptor is not the primary binding site, it can still potentially cause decrease of the infectivity of the virus (Harbison et al. 2008; Callaway et al. 2017).

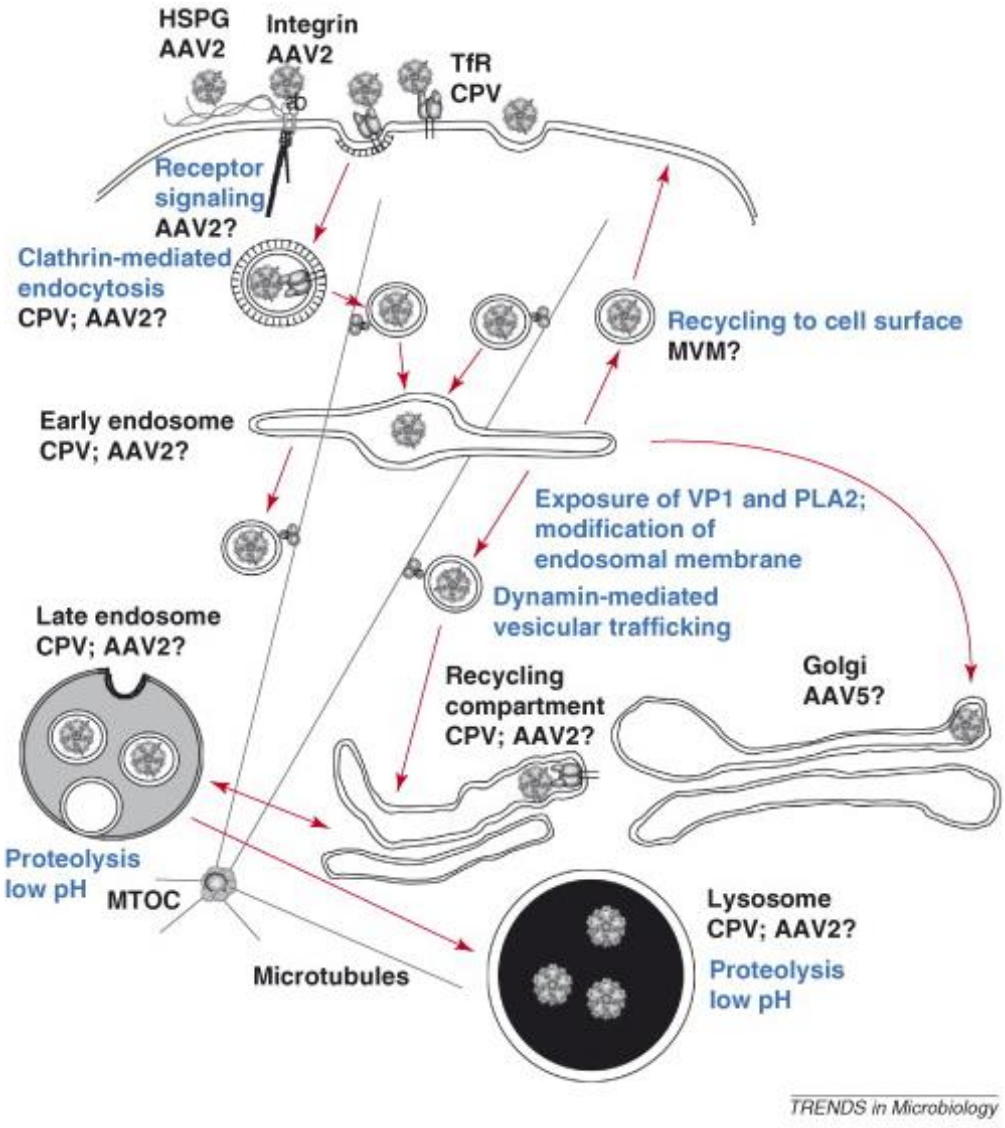

Figure 4: Cell uptake and endosomal trafficking of parvoviruses

Red arrows indicate internalization pathways that have been shown or suggested for various viruses. Abbreviations: HSPG, heparin sulphate proteoglycan; MTOC, microtubule organizing centre; TfR, transferrin receptor (Harbison et al. 2008).

2.2.3 Evolution and distribution

In 1967 parvovirus was first discovered as a cause of gastrointestinal and respiratory disease in dogs, and was called minute virus of canines (MVC) or later CPV-1. Later in 1978 new virus was discovered and named CPV-2. The suffix 2 was given the number to distinguish it from the unrelated CPV-1 (Carmichael et al. 1994; Truyen 1999; Hueffer et al. 2003; Goddard & Leisewitz 2010; Clegg et al. 2011).

As mentioned in introduction, CPV-2 most probably evolved from FPV. DNA sequencing of Arctic fox (*Vulpes lagopus*) samples revealed a blue fox parvovirus (BFPV) showing typical FPV viral sequence concerning all coding and most of the non-coding nucleotides but it also included three non-coding nucleotide changes that are typical for CPV-2 (Xiao-ying et al. 2011). This can be an intermediate stage between CPV-2 and FPV proposing the FPV origin of CPV-2. More recently a phylogenetic study of the VP2 protein were conducted by Miranda and Thompson (2016) mentioning that many of the raccoon parvovirus (RPV) sequences, and one bobcat parvovirus sequence, were located genetically in intermediate locations between the canine-associated CPV-2 and 2a. This can lead to a conclusion involving RPVs as a potential central role in transitioning of CPV-2 to CPV-2a (Allison et al. 2012). The specifics of CPV-2 evolution however are still an issue of discussion and no definitive proofs of specific origin have been found yet (Truyen 1999; Hueffer et al. 2003; Goddard & Leisewitz 2010; Clegg et al. 2011).

The mutations in VP2 region, allowed CPV-2 to specify to canine hosts (compared to for example FPV, where VP2 residue 93 changed from Lys to Asn causing host change). This specific mutation allowed the virus to bind to the TfR on canine cells, thus allowing the infection. Furthermore, new antigenic variants have emerged, thanks to mutations on VP2 of CPV-2 changing its amino acid sequence: CPV-2a – VP2 residues 300Gly, 87Met, 101Thr, and 305Tyr (discovered in 1979), CPV-2b - VP2 426Asp (discovered in 1984), and 2c - VP2 426Glu (discovered in 2000) (Cavalli et al. 2001; Hueffer et al. 2003; Desario et al.

2005; Decaro et al. 2007a, 2007c; Clegg et al. 2011; Decaro & Buonavoglia 2012; Sykes 2014; Lin et al. 2014; Allison et al. 2016; Wang et al. 2016) see Figure 5. Increased pathogenicity is typical sign of development in new strains of CPV-2. Antigenic variants 2a, 2b, and 2c show much higher shedding frequency then the original type, and they are invading the host more severely causing stronger infection (Carmichael 2005; Decaro & Buonavoglia 2012).

Original CPV-2 can infect feline cells *in vitro*, however it cannot cause infection *in vivo*; the more evolutionary developed strains 2a, 2b, and 2c have re-gained this ability to be able to replicate *in vivo* in felines (Truyen 2006; Decaro et al. 2010a; Allison et al. 2012, 2013). Later it was also found out why even though so closely related to CPV-2, FPV cannot infect canine hosts. It is due to unique glycosylation site found only on TfR of dogs and closely related canids like wolves (*Canis lupus*) or coyotes (*Canis latrans*) that blocks the binding of FPV-like viruses in these species (Decaro & Buonavoglia 2012; Allison et al. 2013; Franzo et al. 2017).

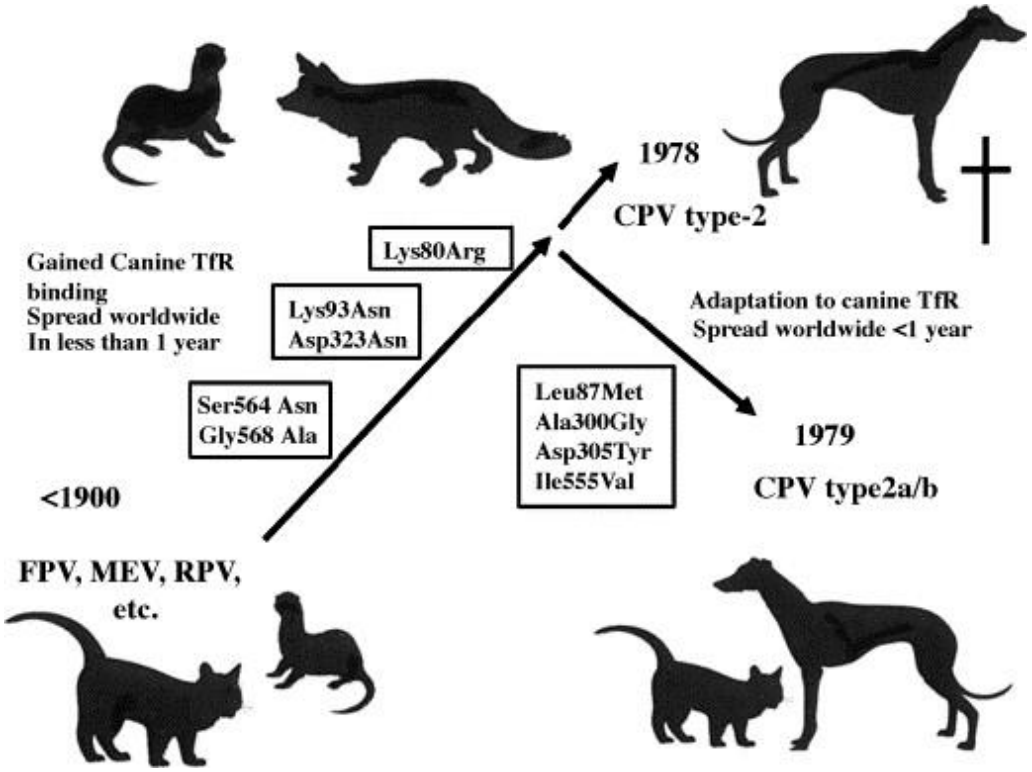

Figure 5: Host evolution of CPV-2 by amino acid changes on VP2 by Truyen (2006)

The dynamics of spreading and evolution of CPV-2 had undergone some changes since after 1978. The strains of CPV-2 that followed the initial outbreak (meaning CPV-2a, CPV-2b, and CPV-2c) have been going through continued evolution on population level and became increasingly more and more geographically subdivided. Now it appears they are circulating mainly locally (Hoelzer et al. 2008b; Hoelzer & Parrish 2010).

Decaro and Buonavoglia (2012) found out, the frequency of CPV-2 variants in Italy specifically fluctuated during years 1995 to 2005 when CPV-2c rapidly overtook CPV-2b. Furthermore this new strain of CPV-2 was detected in several other countries: for example in 2006 it emerged in Spain (Decaro et al. 2006a). It is now predominant in Italy, Germany, and also is present in countries like France, Belgium or Portugal where still older strains are predominant. All three CPV-2 variants are present in Tunisia. In North America types 2b and 2c predominate (Hong et al. 2007; Kapil et al. 2007), while in South America CPV-2c is most common (Pérez et al. 2007). The exception in South America being Brazil where CPV-2a is main source of infection (Decaro & Buonavoglia 2012). In Asia and Australia CPV-2a is the most common strain, even though Nakamura et al. (2004) found CPV-2c strain in Vietnam. In India some CPV-2c were also detected (Decaro & Buonavoglia 2012). For world map with occurrence of genetic variants of CPV-2 in domestic dogs see the Appendices chapter.

2.2.4 Diagnosis

Since CPV-2 poses such a significant influence on pet dog populations, many clinical diagnostic tools were developed to pinpoint the infection as soon as possible. However, CPV-2 has many clinical signs comparable to other diseases that cause acute gastrointestinal enteritis or inflammation (Pollock & Coyne 1993; Sykes 2014; Mylonakis et al. 2016). For example these can be: canine distemper infection, pancreatitis, inflammation of bowels, salmonellosis, intoxication or even presence of foreign objects

inside the animal intestines. It is therefore necessary to very specifically prove or disprove the presence of CPV-2 in the host organism. It is usually done by detecting the viral antigen (ELISA - enzyme linked immunosorbent assay, hemagglutination inhibition - HI), or by polymerase chain reaction (PCR) amplification of the specific viral DNA from biological samples. Comparison of clinical and clinicopathologic signs may also be helpful, however these can vary from animal to animal, or from one virus strain to another (see Table 2) (Prittie 2004; Kalli et al. 2010; Miranda et al. 2015).

Clinical signs of CPV-2 infection are nonspecific, and connectable to infection of the intestines (see Table 2). They usually consist of lethargy, depression, malaise, diarrhea, weakness, vomiting, depression, dehydration and fever. Most affected cubs begin vomiting and experience diarrhea within 24 to 48 hours of initial clinical signs (Pollock & Coyne 1993; Macintire & Smith-Carr 1997; Prittie 2004; Li & Humm 2015; Mylonakis et al. 2016). There can also be more severe or even fatal complications like intussusception (folding of small intestine part into the section of intestine next to it thus blocking the passage of feed-nutritions), heart failure, esophagitis (caused by vomiting), and neurological problems. All these are usually uncommon. In very serious cases (usually in young animals) systematic inflammatory response syndrome (SIRS), sepsis, septic shock, and multiple organ dysfunction syndrome (MODS) can appear (Sykes 2014). Animals that have shown three of the four SIRS criteria (tachycardia, tachypnea, fever, and leukopenia) usually have mortality rate up to 55% according to Kalli et al. (2010).

Common trait in connection with CPV-2 is the presence of subclinical infection, usually present in adult animals that have not been vaccinated. These animals can shed the virus and spread the disease. Nevertheless, presence of anti-CPV-2 antibodies in these animals can cause false-positive results in serological tests. These false-positive results can mislead veterinarians into believing that an animal has already been vaccinated (due to higher number of antibodies), thus leading to conclusion no shedding or further spreading of the disease is possible. This of course can lead to undetected spreading of the virus and

cause misinformation in veterinary databases (Decaro & Buonavoglia 2012; Mittal et al. 2014; Altman et al. 2017).

Table 2: Findings of clinical signs on 94 puppies with spontaneous parvoviral enteritis Used from Mylonakis et al. (2016)

Clinical sign	Number of dogs (%)
Depression/lethargy	67 (71.3)
Anorexia	67 (71.3)
Diarrhea	65 (69)
Hemorrhagic	48 (51)
Nonhemorrhagic	17 (18)
Vomiting	62 (66)
Dehydration	60 (64)
Mucosal pallor	32 (34)
Prolonged capillary refill time	31 (33)
Fever	31 (33)
Abdominal pain	18 (19)
Hypothermia	4 (4)

Hematological abnormalities are another way to assess the CPV-2 infection. Leukopenia or in other words decrease in the count of white blood cells (usually connected with neutropenia – neutrocytes decrease and lymphopenia – lypmhocytes decrease) ensues in the host animal. This is due to the inability of infected bone marrow to produce the needed precursors, inflamed GIT (gastrointestinal tract) which has higher demand of leukocytes, and the exhaustion of multiple lymphoid tissues. Leukopenia is therefore one of the most prominent hematological signs occurring in sick animals with CPV-2 infection. Other, not so common signs may appear including thrombocytosis, pancytopenia, monocytosis or neutrophilic leukocytosis. Malnutrition, even though non-specific, is usually prevalent in biochemistry results including hypoproteinemia, hypoglycemia, and hypoalbuminemia. This is often connected to loss of some electrolytes like Cl or Mg (Decaro & Buonavoglia 2012; Sykes 2014; Li & Humm 2015; Miranda et al. 2015; Mylonakis et al. 2016).

In common veterinary praxis several diagnostic tools are used to describe the infection. It can be diagnostic imaging, serology, or detection of antigens. Detection of viral DNA in animal faeces can also be used. Abdominal radiography or ultrasonography can be useful to detect signs on the intestines like: fluid or gas present in them, loops, decreased intestinal motility, and sometimes even thinning of mucosa-layers on the walls of the intestines (Sykes 2014; Mylonakis et al. 2016).

Another way to make a diagnosis is to quantify the antibodies present in the host animal blood serum. It is done so by using hemagglutination inhibition, or as already mentioned, the method ELISA. With serological methods there are several problems with the interpretation of the results. Substantial number of animals can be seropositive in response to previous, usually subclinical infection. Also the MDAs or vaccination-originated antibodies can be present and change the serological results of the tests; therefore serology by itself cannot be considered as a reliable and valid diagnostic tool for an active CPV-2 infection (Sykes 2014; Li & Humm 2015; Hernández-Blanco & Catala-López 2015; Mylonakis et al. 2016). Methods like ELISA and other similarly functioning tests like immunomigration assay and immunochromatography assay when used with faeces or rectal swab samples are the most cost efficient; however there is some discussion about their sensitivity. It can range from 16 to 80% depending on the comparing “gold standard” method like PCR or immune-electron microscopy that is chosen. It was a topic of discussion in previous years whether sensitivity of ELISA test can be influenced by the viral strain, however has been proven recently that the test should not be affected by the type of genetic variant (Decaro et al. 2010b; Markovich et al. 2012; Decaro et al. 2013; Mylonakis et al. 2016).

Diagnostic veterinary laboratories often use PCR (real-time or conventional) in order to detect different CPV-2 antigenic strains. Usually when antigen-negative faeces sample is found, it signals the need to use PCR as a detection method to conclude the presence of the virus (Sykes 2014; Mylonakis et al. 2016). Positive PCR results however can be misleading and positive findings can be seen also in animals without signs typical

for CPV-2, or animals with prevailing diarrhea leading to uncertainty when it comes to reliability of this test in clinical laboratories (Schmitz et al. 2009). Due to very high sensitivity of PCR test even animals vaccinated with modified live viral strains can be detected as positive (even though it is not that common) when testing biological samples after vaccination. It is not known how long after vaccination this may occur (Decaro et al. 2014; Mylonakis et al. 2016). There are currently specialized PCRs which apply the "minor groove binder probe" methods which can specify between vaccine and the real non-artificial virus. These methods can be also used to identify if these two types coexist in one animal (Decaro et al. 2006b, 2013).

2.2.5 Treatment

The treatment of CPV-2 is very important since the survival rate in non-treated animals can be as low as 9%, while in contrast when treatment is used it can be as high as 80%. The treatment alone is usually targeted to support of the animals own immune system and to suppress the pathogenic side effects like vomiting, secondary bacterial infections or fever. There are four main components to the treatment: a) fluid therapy, b) nutritional support, c) antibiotics, and d) antiemetic treatment (against vomiting) (Sykes 2014; Li & Humm 2015; Mylonakis et al. 2016).

The antibiotic treatment is usually based on wide-spectral antibiotics like ampicillin and cefoxitin, sometimes in combination with enrofloxacin granting protection against G+ and G- bacteria, and anaerobic bacteria present in the intestine. The antibiotics have to be provided intravenously or by injection. The antibiotic treatment is crucial because of the substantially increased risk of sepsis and concurrent bacterial infection in the host animal (Prittie 2004; Sykes 2014; Li & Humm 2015; Mylonakis et al.2016).

The antiemetic treatment is given throughout the healing process to stop vomiting, using drugs like metoclopramide a dopaminergic antagonist for animals experiencing continual and problematic vomiting; next it can be ondasetron with dolasetron (which both function as serotonin receptor blockers) for constant vomiting. There is also the novel maropitant (antagonistic to neurokinin1 receptors) that has yet to be fully tested in CPV-2 infection usage (Sedlacek et al. 2008; Mylonakis et al. 2016).

The nutritional support is usually provided via GIT, not intravenously. This type of support is connected with repaired intestine mucosal structure and overall faster recovery and healing of the intestine itself; thus resulting in reduction of the danger of spreading bacterial infection from intestines to other organs. Since the disease has such a quick onset and course, intravenous feeding is usually not necessary (Prittie 2004).

As with any diarrheic infection, fluid therapy is of utmost importance. The animal must stay as hydrated as possible with careful monitoring of oncotic pressure. The control of acids, bases and electrolytes is needed for preservation of homeostasis in the organism. This treatment is usually provided intravenously since severely dehydrated animals have only limited access to subcutaneously absorbed water (Lobetti et al. 2002; Prittie 2004).

The usage of antivirotics is currently in development. There have been some trials using serum from immune dogs that have undergone, and recovered from, CPV-2 infection as a way to achieve passive immunity. In one study 12 ml of plasma with CPV-2 active antibodies was given to animals displaying clinical signs of CPV-2. This treatment had no effect and it did not improve any of the clinical and serological symptoms of the disease (like the amount or virus in organism, duration of time spent in hospital, recovery from leukopenia etc.) (Bragg et al. 2012; Mylonakis et al. 2016). Recombinant feline interferon-ω (rFeIFN-ω) is being studied and has shown some very positive and hopeful results (Martin et al. 2002). However the scarce availability and high cost of this substance have stopped it from being used standardly as a clinical drug. There are some other drugs

currently studied like for example oseltamivir (Savigny & Macintire 2010; Mylonakis et al. 2016).

Pain inhibiting analgetics like butorphanol or buprenorphine can be given to reduce pain caused by the severe enteritis (Sykes 2014; Mylonakis et al. 2016). Also maropitant previously mentioned as antiemetic treatment has some potential uses even as a pain management drug reducing internal body pain (Boscan et al. 2011).

2.2.6 Prevention in domestic animals

Due to the history of parvoviral infections, prevention is one of the most important topics concerning the CPV-2. Even more so, since the virus is able to maintain its virulence for up to 6 months (or longer) at standard room temperature (Prittie 2004). In closed environment like kennels, very thorough and proper hygienic cleansing must be present. Sodium hypochloride is one of the common chemicals successfully killing viruses and therefore is widely used. The dilution should be 1:30 with water and at least 10 minute period must pass before it is removed from the targeted surface (Mylonakis et al. 2016).

Excluding disinfection and perfect hygienic conditions, the importance of vaccination is paramount. It helps to lower the numbers of vulnerable animals in general population and like so it induces the so-called herd immunity (Day et al. 2016). Serum antibody titer is usually used as a standard measurement for the need of vaccination, since it is strictly connected to immunity. Seronegative animals are susceptible; animals with low titers usually do not become clinically sick, they can however shed the virus and spread it to environment. Animals with high titers will not develop any active infection, and they do not shed or spread the virus since they are immune to any form of the disease (Pollock & Coyne 1993; Prittie 2004).

Modified live vaccines (MLVs), with high-titer low passage CPV-2 strain, are the most commonly used all around the world. Low passage is characterized by the time spent in various tissue cultures in order to decrease the virulence of targeted virus; high-titer refers to the amount of viral units in the vaccine itself (Prittie 2004). They can provide immunity for up to 7 years (possibly even longer) including immunity against infection and disease as well (Abdelmagid et al. 2004; Schultz et al. 2010). Immunization with some MLVs can result in faecal viral shedding for several days after vaccination which can cause some misinterpretation of faecal antigen results (Smith-Carr et al. 1997; Mylonakis et al. 2016). According to the vaccine manufacturers, the incidence of the disease after vaccination is less than 1% (Prittie 2004).

Inactivated CPV-2 vaccines can be used for immunization as well; however they have been proven inferior to the MLVs. They do not prevent viral shedding, thus allowing the virus to spread, plus they are less likely to effectively cause proper immunization during MDAs (maternally derived antibodies) interference. They can however be useful in some situations like vaccination of pregnant female or an immunosuppressed animal (Smith-Carr et al. 1997; Reddy et al. 2000; Prittie 2004; Decaro et al. 2005).

The interference of MDAs is one of the leading causes of immunization failure; thereafter the effect of vaccination depends on MDAs titer, and on the type of vaccine that is used. There are currently several laboratory methods that are used to determine the CPV-2 antibody titers in pregnant females and their offsprings. These tests include ELISAs, IFA (indirect immunofluorescent antibody), and HI tests (Waner et al. 1996; Decaro et al. 2005). The HI measurements of titers for CPV-2 antibodies are considered as so-called “golden standard” for quantification of the present antibodies in the host animal. Also HI MDAs titers are used to indicate how many MDAs are present in the cub; thus providing the information needed to assess the affectivity of the vaccine. For example cubs with antibody HI titers ≤ 1:80 are considered to be susceptible to parvovirus infection, and are in need of further immunization. Furthermore, when titer of MDAs is ≥ 1:80, or even in some cases with just 1:10 or 1:40 is likely to interfere with the vaccine

(Pollock & Coyne 1993; Waner et al. 1996; Decaro et al. 2005). In comparison, dogs that have recovered from actual infection of CPV-2 have HI titers ranging from 1:640 to 1:2560 even after two years of undergoing the infection, which may possibly suggest that immunization after in recovered animal is lifelong, or at least long-lived (Buonavoglia et al. 1992).

Normally the vaccination should be provided from 6 to 8 weeks of age, further each 2 to 4 weeks until 16 weeks or possibly even more (Day et al. 2016; Mylonakis et al. 2016). After the age of 16 weeks, usually only two (or even just one) doses of MLVs separated by only 2 to 4 weeks should provide sufficient protection from the CPV-2 virus (Schultz et al. 2010).

3. Wildlife and carnivore parvoviruses

Carnivore parvoviruses, present in wildlife and in domestic animals, include several genetically close viruses like: FPV, CPV-2, MEV, BFPV, RPV, and RDPV (raccoon dog parvovirus). They are now present in many domestic and feral carnivore species and can affect many kinds of host species (Steinel et al. 2001; Hueffer & Parrish 2003; Hoelzer & Parrish 2010). In our current knowledge, most Canidae (wolf, dog, coyote,…), Hyaenidae, Felidae (cheetah, mountain lion, leopard, lynx…), Mustelidae (mink...), Procyonidae (raccoon, Asiatic raccoon dog), and Ailuridae (red panda) include some animals that are natural hosts for FPV and/or CPV-2a, CPV-2b, and CPV-2c (Gese et al. 1991; Steinel et al. 2000, 2001a; Biek et al. 2002; Qin et al. 2007; Kapil et al. 2010). Their geographical range, host range and prevalence have been increasing steadily since the end of 20th century; therefore carnivore parvoviruses can still be classified as emerging epidemic pathogens (Dobson & Foufopoulos 2001; Shackelton et al. 2005).

Emerging epidemics can be divided into three categories. First is characterized by novel invasion of wildlife population by a virus. High host susceptibility due to lack of immunity is therefore present in the population, and is often followed by rapid spread through host population; therefore it is called a “virgin ground epidemic”. To this category we can easily fit the explosive spreading of CPV-1 and CPV-2 in their original forms. Second type of emerging epidemic is characterized by viruses that are endemic to a specific host and geographical region, where they are spreading as a result of new (changed) external factors that influence the restart of spreading. Final category of epidemics is a combination of the ones mentioned previously - recently emerged viruses invading non-immune host populations that are in addition negatively influenced by current environmental conditions. The uniqueness of CPV-2 epidemics is embedded in the rate with which the virus can achieve nucleotide substitution. This characteristic places CPV-2 in a special subgroup of pathogens emerging via mutations, more or less placing it in the first subgroup of emerging epidemics (Daszak et al. 2000; Dobson & Foufopoulos 2001; Cunningham et al. 2017).

With wildlife, where close contact between animals is scarce, there can still be high risk of infection due to the stability of parvovirus in environment and its spreading via fomites and faeces. This also adds cross-contamination through a domestic animal hosts like dogs or cats as one of the possible routes of transmission. There are also so-called anthropogenic factors influencing the spreading of carnivore parvoviruses in wildlife populations. From these, the most important factor would be environmental degradation including habitat fragmentation or atmospheric pollution; habitat fragmentation then being one of the most influential. Habitat fragmentation is closely connected to disease outbreaks since it increases the contact between wild living and domestic animals (Mech et al. 2008; Park 2012). As Hoelzer and Parrish (2010) indicate, there are no immunological, epidemiological and pathological known interactions between CPV-2 or AMDV and CPV, FPV or MEV respectively in their hosts, suggesting that the individual viruses cannot coexist in one host. However more CPV-2 strains can coexist together in

one animal boosting its virulence, genetic potential and evolution rate (Battilani et al. 2007, 2011).

Parvoviruses can spread rapidly and in populations, that had not undergone the disease yet (therefore they lack immunity), there can be high associated mortality that can lead to substantial decrease in population size. On the other hand populations in which these infections occur endemically have new cases appearing mainly in young animals that are developing infection after MDAs protection fades. When such population belongs to the group of seasonal breeders, then the infections can occur cyclically, and affect the population in major and very harmful way, since the mortality in cubs is quite high (Steinel et al. 2001; Hoelzer & Parrish 2010).

Perhaps the most striking distinctive attribute of CPV-2 compared to other carnivore parvoviruses is its ability to fairly easily cross between species, thus posing especially big threat on various wild animal populations. This evolutionary process is not yet very well understood on epidemiological level (since genetics of the mutations have been more or less specifically studied) (Steinel et al. 2001). We do know however, that usually with cross-species viral transfers, the final result is a “dead-end” infection in the newly acquired host, disabling any further transmission. Rather than separate adaptation to a specific new host, the evolution and mutations of the virus are occurring in randomly susceptible animal host, not specifically selected before (Shackelton et al. 2005; Hoelzer et al. 2008a). Compared to its FPV predecessor, CPV-2 evolves significantly faster at a population level (Hoelzer et al. 2008b). It has a nucleotide substitution rate (Li et al.[2017] state the frequency of approximately 10^{-4} substitutions per site, per year) comparable to rapidly evolving RNA viruses like HIV-1 and human influenza A (Shackelton et al. 2005; Hoelzer et al. 2008b). These unique attributes of CPV-2 can be explained by two possible ways: either CPV-2 mutagenic rate is simply high enough to fulfill these extreme abilities, or persistent positive selection pressure has influenced the virus to elevate the rate of mutations in order to replicate. As Shackelton et al. (2005) and Raj et al. (2010) point out in their research, the current data point to both of these influences correlating together.

Both of their teams have found strong evidence pointing towards positive selection at a several sites of the VP2, and an evolutionary patterns suggesting directional selection on a branch leading from FPV to CPV-2.

Vaccination plays a major factor in controlling the diseases in domestic animals and in captive populations of wild carnivores. Vaccines for captive wild carnivores appear to be safe; however these vaccines have not yet been tested in wildlife species, so the license for usage in nature was not yet granted. Therefore some of the most important questions like dosage, frequency of usage, level of protection conferred, and appropriate type of vaccines still remain unanswered (Hoelzer & Parrish 2010). In one interesting field test provided by Belsare and Gompper (2015) on Indian wild dog populations, some surprising results have been shown. In their study they have given vaccines against several canine-based viral diseases (including CPV-2) to several feral and domestic dog populations. Afterwards they compared the increase of immunization with control groups that have not been vaccinated. In the case of CPV-2 (but also with other vaccines) the vaccination was practically non-effective, not providing the dogs with any bonus protection, since most of the dogs had already undergone the disease and had strong natural immunity present; thus rendering the vaccines useless. This also proves that CPV-2 and other viruses are enzootic and are actively circulating in feral dog populations in India, suggesting similar situations may be occurring throughout the world's feral dog populations. To further complicate the matter of vaccination, some animals can remain without sufficient antibody response even after vaccination, and like so, they can also remain unknowingly unprotected (Decaro et al. 2007b; Larson & Schultz 2008). The question of wild animal vaccination is therefore still an open topic waiting for more research to be conducted.

4. Aims

The goals of this Msc. thesis were to evaluate the possible risks posed by CPV-2 to Czech population of Eurasian wolf (*Canis lupus lupus*) and then to further examine the samples of Arctic fox (*Vulpes lagopus*) providing us with information needed about the impact and genealogy of this virus in populations of Greenland and Island.

Since the populations of wolves in central Europe (Czech Republic included) are fragmented, the threat CPV-2 poses to them is significant. The populations of Arctic foxes are not so fragmented and their survival is not threatened by CPV-2, therefore their samples were tested mainly to evaluate the phylogenetic development of given strains of CPV-2.

These aims were established to provide veterinarians and wildlife specialists in Czech Republic with crucial information about the Czech population of wolves by providing them with more data about the health status of this population. The results will show what steps (if any) are needed to take concerning CPV-2 virus infection. The samples of Arctic foxes that would be used for genetic study may possibly reveal the missing links of FPV to CPV-2 evolution. This genetic evaluation is crucial for phylogenetic studies concerning CPV-2 in order to understand the development and mutation tendencies of this virus. To further support our aims we have designed the following research questions:

1. Is CPV-2 present in the tested samples of Eurasian wolves (*Canis lupus lupus*) in Czech Republic and Arctic foxes (*Vulpes lagopus*) in Island and Greenland?

2. If CPV-2 is present in the samples, then what are the impacts for the selected populations and how it is it phylogenetically characterized?

3. If CPV-2 is not present in the samples, what does it mean for selected populations?

5. Methods

5.1 Sample collection

We have tested 37 samples of frozen Arctic fox intestines from Iceland, and 31 faecal samples from Greenland. The samples from Iceland and Greenland were collected by Eva Fuglei (the Norwegian Polar Institute). For wolves we have used 124 faecal samples collected by volunteers from Friends of the Earth Czech Republic.

5.2. DNA isolation

All used samples contained DNA that has been isolated by Milena Smetanová (Faculty of Tropical AgriSciences, Czech University of Life Sciences) and Eva Myšková (Biology Centre, Czech Academy of Sciences) using QIAamp DNA Stool Mini Kit (QIAGEN). Precise steps are provided by QIAGEN together with the kit. For more information the manual is provided with this work in a pdf. format copied on provided CD.

5.3 PCR

PCR method based on amplifying of certain segment of DNA of the virus genome using CPV-2 specific primers was implemented in this diploma thesis.

5.3.1 Primers

Forward: TGAGCTGCATTTAGTTAGTTTTGA; **Reverse:** TGTTTGCCATGTATGTGTTAGCT. Binding to NS1 protein gene on CPV-2 genome (Designed by Hirasawa et al. 1994)**.**

5.3.2 Mastermix

2x PPP mastermix (TopBio), which already includes Taq polymerase (from bacteria *Thermus aquaticus*) and necessary reaction buffers, was used in our testing. The samples were divided into test tubes precisely in volume 1 µl of each test DNA (10-50ng whole genomic DNA) mixed with 5 µl PPP mastermix 1 µl forward and 1 µl reverse primers (10 mM each) and filled to 10 µl by distilled water.

5.3.3 Thermal cycle

PCR reaction was performed in T100 Thermal Cycler (BioRad) under this program: 96°C for 10min for initial denaturation, followed by 40 cycles of DNA amplification (96°C - 30s for denaturation; 55°C - 30s for primer annealing; and 72°C - 1min for DNA amplification).

5.4 Gel Electrophoresis

Gel electrophoresis was used to visualize and evaluate the PCR results. Electrophoresis is based on separation and analysis of macromolecules, like DNA, based on their size and charge. We used 2% agarose gel (100ml) colored by 1 µl of Ethidium bromide (EtBr). TBE (Tris/Borate/EDTA) buffer was used as a solution with voltage of 120 V on the PowerCac (BioRad) power source and HU13 (Scie-Plads) electrophoresis pool, which makes 5 V/cm of voltage between our electrodes.

The electrophoresis was run for 60 minutes. After electrophoresis the separated DNA molecules were evaluated (photographed) under UV, ultraviolet light reflected from fluorescent EtBr (transluminator Schoeller).

5.5 Sequenation

Positive samples were sequenced in the laboratory of DNA sequenation (Charles University). For further validation of results samples were also sequenced in State Veterinary Institute using different set of PCR primers. Sequence of the positive sample was compared with other CPV-2 sequences stored in the database GenBank.

6. Resuls

From the 124 faecal samples of Eurasian wolf from Czech Republic, none were found positive.

The 31 samples of Arctic fox faeces from Greenland were also all negative.

The 37 samples of Arctic fox frozen intestines from Iceland had one positive result. This sample was then sequenced twice, first at the Charles University, then at the State Veterinary Institute in Prague with different set of primers resulting in this sequence: CAGATGATGTTCAATTTTATACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATG AATTTGCTACAGGAACATTTTTTTTTGATTGTAAACCATGTAGACTAACACATACATGGCAAACAAA TAGAGCATTGGGCTT.

Since the sample had shown very weak positive feedback on Real-Time PCR, rt-PCR, and since the blasted sequence shown 100% homology with our positive control (which was isolated dog CPV-2 virus), we concluded that this sample was contaminated by our positive control.

7. Discussion

Concerning Arctic foxes, the results that were found provide us with some initial data about the health of their populations in Greenland and Iceland. They do not however provide any further development of knowledge about genetic evolution of CPV-2 since all samples were, in the end, proven negative. Since we cannot make any conclusions about the phylogenetics of CPV-2 strains in these regions, we can at least fill in the gaps of natural conservation questions for this species. Arctic foxes have been studied for numerous reasons, mainly for genetic development of the species (Dalén et al. 2006; Norén et al. 2016) and for ecological impacts on the populations in main regions of origin (Norén et al. 2010). The health of these populations in connection with different types of viral diseases had not been properly monitored in recent years, with some exceptions - for example rabies disease is under continued surveillance (Raundrup et al. 2015). The health-connected research conducted, focuses mainly on chemical contaminants in fox's habitats which include both sea shores and inland areas; therefore these animals are susceptible to contaminants from both of these sources since they come into contact with them as apex predators (Alonso Aguirre et al. 2000; Letcher et al. 2010). Another frequently studied field concerning health of wild Arctic fox populations is parasitology (Jenkins et al. 2011).

With our thesis results, we have proven that CPV-2 virus infection is probably not present in the populations of these animals in Greenland and Island. This finding is important mainly because Arctic foxes tend to migrate with the changes of availability of food resources during the year, lemming for inland foxes vs. sea birds and eggs for seashore foxes (Levy et al. 2016), and thus the spreading of such highly transmittable and lethal animal virus would be dangerous. Gradually more and more animals would come into contact with the pathogen since the virus could easily spread via faeces (fomites) to even other species of animals as well (for example wolves in Greenland or domestic dogs in Iceland) given the nature of CPV-2 host variability. This would of course have dramatic

impact on domestic dogs and cats which could serve as an intermediate in spreading the virus to wild animals. These results therefore can serve as an ecological and health survey about these animals, and can be used as a milestone for assessing the needed health-protection measures in their wild populations.

For Eurasian wolves, the negative results of tested samples indicate that no active infection of CPV-2 virus is currently circulating in this Czech population that comprises mix of two European wolf subpopulations - Carpathian and Central European lowland (Hulva et al. 2017). Since CPV-2 is highly contagious, and the ranges of wolf territories are usually covering more than one country (Hindrikson et al. 2017), the virus would easily spread even among neighboring packs and countries. With social arrangement of wolves the whole pack would usually end up infected. This might cause serious setbacks in the conservation efforts. Therefore our results can be used by wildlife conservation specialists as an insurance of healthy (from CPV-2 virus) population, providing them with the possibility to focus the health awareness research about these animals towards some other dangerous pathogens like helminths or various bacterial infections. The research in the field of virology however is somewhat lacking for these protected animals. Again the populations are mainly monitored for their genetic characterization, their feeding behavior and their ecological impact (Trouwborst et al. 2017; Sidorovich et al. 2017; Hindrikson et al. 2017). The importance of proper monitoring of health of these animals is paramount, and given the possible dangers of infection that Eurasian wolves encounter (domestic animals, rodents, insects…) the monitoring and prevention of most dangerous viral diseases should be increased if possible - given the difficulties with obtaining suitable samples or detecting some viral diseases like for example canine distemper.

In Czech Republic so-called wolf watches and lynx watches are established mainly for prevention of poaching which is still currently the highest threat for these big predators (Kutal & Blaha 2008). These watches collect faeces and track the animals' movement. Similar conservation group efforts could be used all over Europe as a way to obtain samples for testing; thus providing scientist with the needed material. Our research

can serve as a step stone for other virological studies of wolves in Europe, and as an appeal towards further investigation of wild European wolves' health status.

Given the epidemic and biological nature of CPV-2 virus, our results can further indicate several possibilities when concerning CPV-2 epidemiology. With Arctic foxes from Greenland and Iceland, which were never (to our knowledge) before tested positive for CPV-2 presence, we cannot make any specific conclusions about developed immunity or whether the infection was never present there in wild animals in the first place. The only two confirmed parvoviral infection in wild Arctic foxes were found in Finland in 1988 (Veijalainen, 1988) and in China in 2011 (Xiao-ying et al. 2011). Both of these countries are isolated from Greenland and Iceland by sea, so animals from these places were not able to meet. We could therefore indicate that only CPV-2 infections that could be present in wildlife of Greenland and Iceland are of domestic animal origin. Due to transportation of pets from different countries negligence in veterinary inspection could be the cause of CPV-2 possible spreading. However mainly the domestic pets would be the endangered point in such a case, and the wildlife should not be in severe danger given the development of wildlife protection levels in these countries. Endemic strains of parvoviruses can however be present in the populations, and given the limited number of samples we had, the infection could have been missed. Further testing would be necessary to prove or disprove this hypothesis.

In European wolves however the infection was found and proven to be circulating in the wild packs of Italy in 1997 (Martinello et al., 1997) and then in 2001 (Battilani et al. 2002). In domestic dogs this disease was found even more recently in Italy, Germany, United Kingdom, Portugal, Spain, Czech Republic, Switzerland, and Belgium in 2007 (Decaro et al. 2007a) see Figure 6 on the next page.

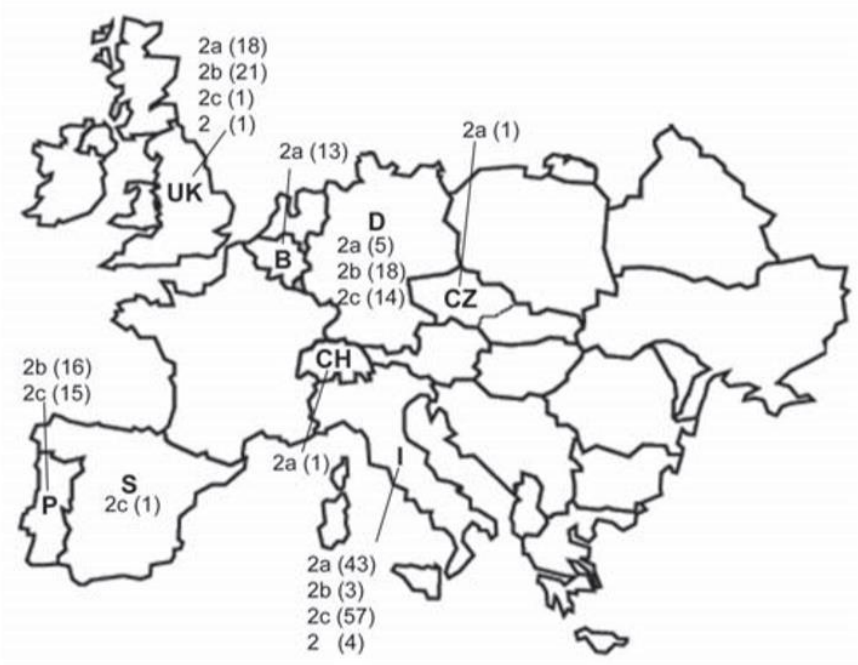

Figure 6: Geographic distribution of CPV-2 in domestic dogs of Europe in 2007

Antigenic variants are indicated. In brackets the number of samples tested is given (Decaro et al. 2007a).

This quite substantial prevalence of CPV-2 in domestic dogs can potentially have impacts on wildlife as we explained in chapter 3. of our literature review. As Figure 7 indicates, the possibly active wolf infection from Italy could spread to Croatia and Romania, and from there to Austria, Slovakia, Poland, and even Czech Republic according to the gene flow patterns that correlate with movement of the animal packs (Simonis Juniper L. et al. 2018). These findings can warn us about the possibility of active wildlife infection with CPV-2 in central Europe even though it had not been confirmed in most recent years. Since our results are negative, we can have several hypotheses about the wolves of Czech Republic: the wolves could have developed long-term immunity against CPV-2, or the wolves had not come to contact with the virus yet. These would have to be further examined by another research; preferentially ELISA tests from blood samples of wolves. These are however difficult to obtain, so careful research design would have to be

implemented. Since the population in Czech Republic is very small and it is basically only a fragment of Carpathian and Lowland wolf populations, the results we have found are helpful, yet they cannot be conclusive for the whole central European wolf population. Given the high mutagenic and epidemic potential of CPV-2 it is crucial to keep monitoring European wildlife for signs of any parvoviral infection. New antigenic variants could unexpectedly arise and cause serious damage to wildlife populations all over Europe.

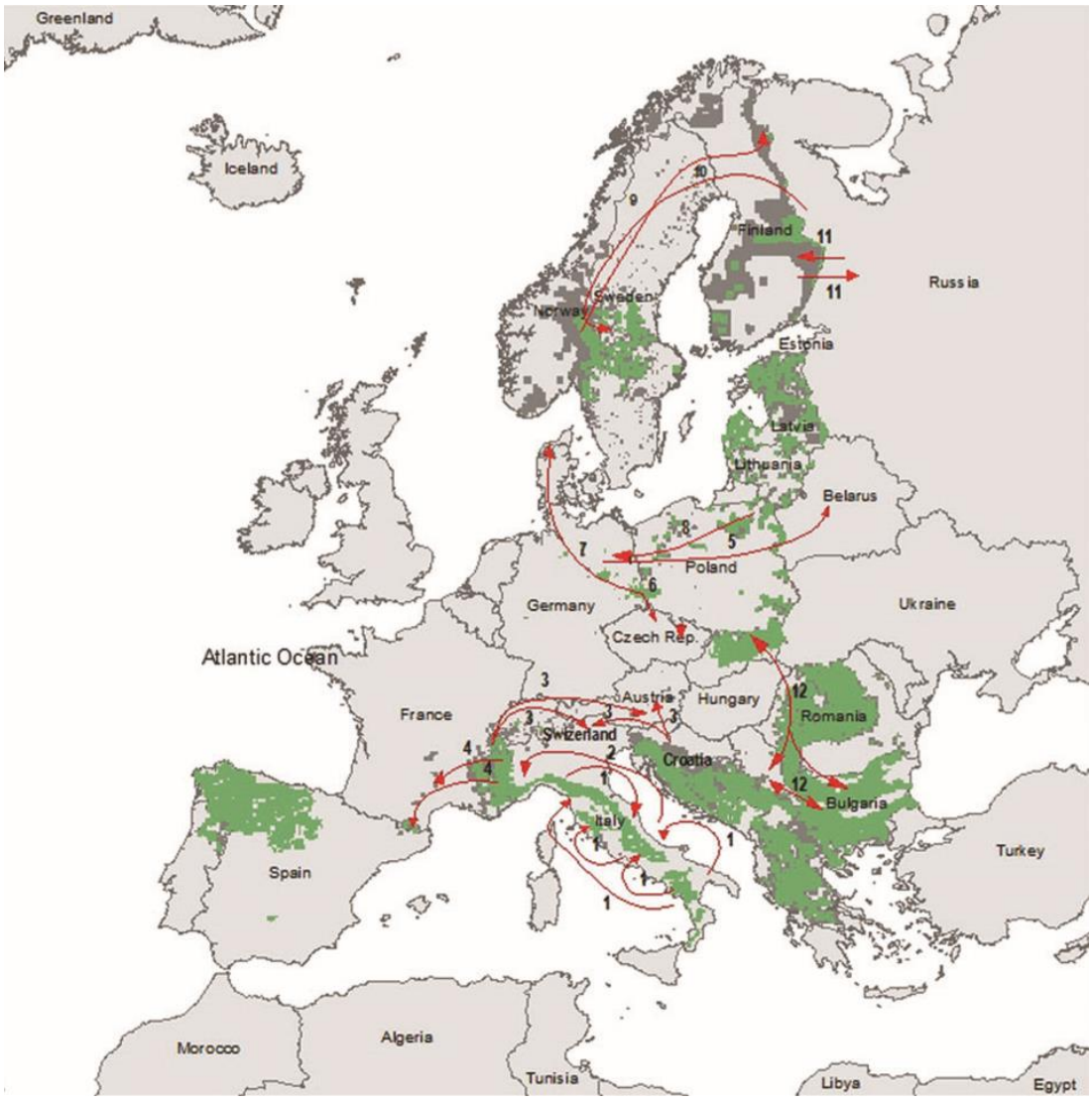

Figure 7: Wolf distribution and directions of gene flow in Europe (Hindrikson et al. 2017) Green indicates wolf permanent occurrence, and dark grey sporadic occurrence. Wolf occurrence in Russia, Ukraine and Belarus is not marked on the map. Confirmed dispersal between and within wolf populations is indicated by red arrows.

8. Conclusions

The absence of CPV-2 infection in Eurasian wolves in Czech Republic suggests the so-called Carpathian and European lowland wolf populations are either immune to the CPV-2 infection due to undetected subclinical infections, or they didn't come to contact with actively circulating CPV-2 in nature yet. Very low concentrations of the virus may have also been the reason why we did not detect any positive results

For the research of CPV-2 in Arctic foxes the negative results provide us mainly with completely novel study of local populations concerning CPV-2 occurrence. Since CPV-2 was never before studied or confirmed in these countries it provides vital reassurance for local veterinarians and scientists that active infection is indeed absent in the wilderness.

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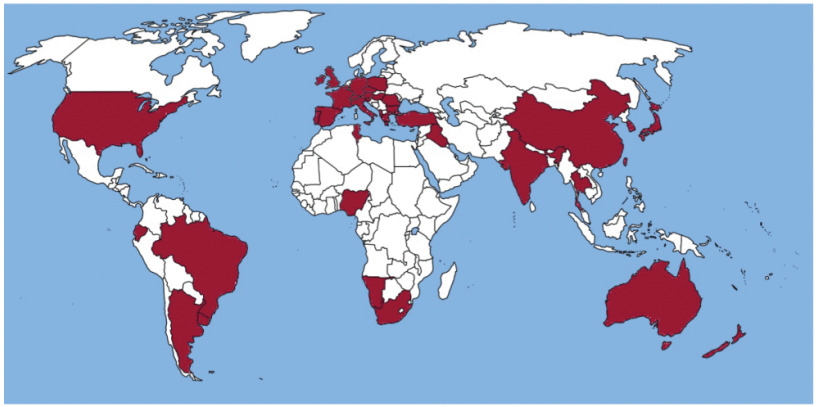
10. List of Appendices

(a)



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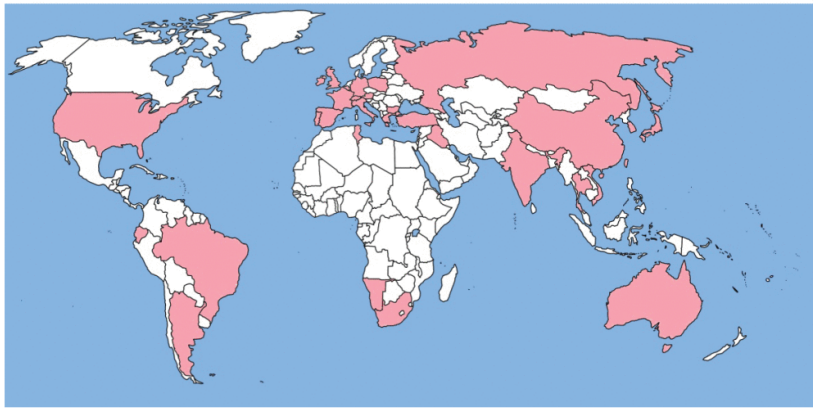
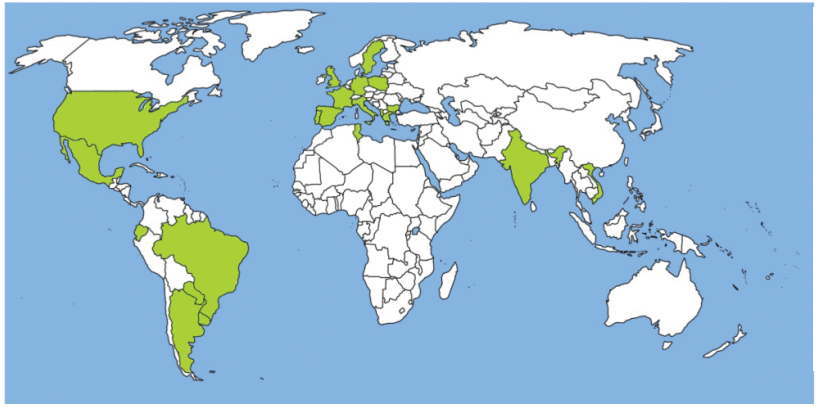


(c)



Worldwide distribution of CPV-2 variants in domestic dogs (Miranda & Thompson 2016)

Red, presence of CPV-2a variant (a)

Pink, presence of CPV-2b variant (b)

Green, presence of CPV-2c variant