Szent István University

Postgraduate School of Veterinary Science

Polymerase chain reaction-based investigations of canine distemper and parvovirus strains from Hungary

Doctoral thesis

Dr. Zoltán Demeter

2009

SZENT ISTVÁN UNIVERSITY
POSTGRADUATE SCHOOL OF VETERINARY SCIENCE

Head:

Prof. Gyula Huszenicza, DSc

Supervisor:

…….
Prof. Miklós Rusvai, CSc
Szent István University, Faculty of Veterinary Science, Budapest
Department of Pathology and Forensic Veterinary Medicine

Members of the supervisory board:

Prof. Károly Vörös, DSc
Szent István University, Faculty of Veterinary Science, Budapest
Department and Clinic of Internal Medicine

Dr. Tamás Bakonyi, PhD
Szent István University, Faculty of Veterinary Science, Budapest
Department of Microbiology and Infectious Diseases

Copy … of eight

.....	.....
Prof. Gyula Huszenicza	Dr. Zoltán Demeter

Contents

Abbreviations

aa	amino acid
Asn	asparagine
Asp	aspartic acid
BFPV	blue fox parvovirus
BLAST	basic local alignment search tool
bp	base pair
CD	canine distemper
CDV	canine distemper virus
CNS	central nervous system
CPV2	type 2 canine parvovirus
CSF	cerebrospinal fluid
DIC	disseminated intravascular coagulopathy
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
F	fusion (gene/protein)
FP	feline panleukopenia
FPV	feline panleukopenia virus
Glu	glutamic acid
H	hemagglutinin (gene/protein)
h	hour
HE	haematoxylin and eosin
HI	hemagglutination inhibition
IFAT	indirect fluorescent antibody testing
IHC	immunohistochemistry
ISH	*in situ* hybridization
kb	kilobase
L	large polymerase (gene/protein)
M	matrix (gene/protein)
MEV	mink enteritis virus
$MgCl_2$	magnesium chloride

MHC	major histocompatibility complex
min	minute
MLV	modified live virus
mM	micromolar
mRNA	messenger RNA
N	nucleocapsid (gene/protein)
nm	nanometer
ORF	open reading frame
P	phospholipid (gene/protein)
p.i.	post infection
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RPV	raccoon parvovirus
RT-PCR	reverse transcription polymerase chain reaction
sec	second
SN	serum neutralization
SNP	single nucleotide polymorphism
UK	United Kingdom
USA	United States of America
UV	ultraviolet
VN	virus neuralization
VP	viral protein
µl	microlitre
µm	micrometer
°C	degrees Celsius

Summary

Samples taken from various species from 2004 to 2008 were analyzed for the presence of canine distemper virus (CDV), type 2 canine parvovirus (CPV2), or feline panleukopenia virus (FPV). The samples were collected from animals showing signs of clinical illness (clinical samples), or were collected from animals that succumbed following clinical signs suggestive of a viral infection, or when pathological changes indicative of such a disease were found during necropsy (necropsy samples). In case of clinical samples, the diagnosis was based on various molecular techniques applying polymerase chain reaction (PCR) based techniques, while in case of succumbed animals a wide spectrum of diagnostic methods was employed, such as macroscopic and routine light microscopic investigations, electron microscopy, and PCR-based techniques concluding with nucleic acid sequencing and subsequent phylogenetic analysis. The genetic analysis of Hungarian CDVs revealed the presence of strains belonging to several lineages: European, Arctic and European wildlife. The virus strains clustered in the Arctic group of CDVs were also demonstrated to be responsible for the endemic infection at the Dog Shelter of the City Council of Budapest. On the other hand, CDV infection was demonstrated in several other species from Hungary: fox *(Vulpes vulpes)*, raccoon *(Procyon lotor)* and ferret *(Mustela putorius furo)*.

A restriction fragment length polymorphism (RFLP) assay was also developed for the fast differentiation of vaccine and wild-type CDVs. The practical trials of the *Psi*I-based RFLP revealed that the virus strain present in one of the currently used vaccines reacted as a wild-type strain. Following the diagnostic PCR reactions, out of the 214 analyzed samples 58 (27.1 %) proved to be positive for CDV. Based on the subsequent nucleic acid sequencing and phylogenetic analysis, the incriminated strain was not clustered in the group of vaccine strains (America-1), as expected based on the product description provided by the manufacturer, but it was more closely related to pathogenic strains of different geographical origins. Vaccine batches of the same manufacturer were purchased in different countries (Israel, Malta, and USA), and batches dating back to 1992 and 1994 were also included in the analysis. The genetic analyses revealed that all these batches contained the exact same virus strains as the Hungarian vaccines purchased in 2006.

Parvovirus (FPV/CPV2) genetic material was successfully demonstrated by PCR in 72 (31.3 %) out of the 230 analyzed samples (in 17 cats, 1 lion, 1 Asian palm civet and 53 dogs). The initial genotyping attempts of 20 type 2 canine parvovirus (CPV2) strains from Hungary, using a previously described *Mbo*II-based RFLP test, revealed that

many of them were type 2c CPVs. Based on the subsequent nucleic acid sequencing, all Hungarian CPV2 strains turned out to be type 2a CPVs. The explanation of the initial misleading results was a point mutation that occurred at the other end of the amplicons used for the enzymatic digestion, emphasizing the need to constantly improve fast genotyping techniques and the fact that initial results can be misleading and they have to be double-checked using a more reliable technique.

The work also presents the first direct demonstration of a feline parvovirus (FPV) infection in an Asia palm civet *(Paradoxurus hermaphroditus)*. The pathological changes induced by the infection were similar to those described in other species, but the result emphasizes the fact that the host range of FPV is even wider than it was thought, and it includes even members of the *Viverridae* family. FPV infection was also demonstrated in a lion *(Panthera leo)* that belonged to a Hungarian lion tamer. The subsequent genetic analysis of the Hungarian FPV strains from the Asian palm civet, lion and two cats *(Felis catus)* revealed that there is relatively heterogenic group of FPV strains currently circulating in Hungary.

Összefoglaló (Summary in Hungarian)

Vizsgálataink során 2004 és 2008 között, különböző állatfajokból származó mintákban szopornyicavírus (canine distemper virus: CDV), 2-es típusú kutya parvovírus (canine parvovirus 2: CPV2) vagy macska panleucopaenia vírus (feline panleukopenia virus: FPV) kimutatását kíséreltük meg. A minták klinikai tüneteket mutató *(klinikai minták)*, illetve vírusos fertőzésre utaló klinikai megbetegedést követően elhullott állatok szerveiből *(szervminták)* származtak. A klinikai minták esetében a diagnózist polimeráz láncreakció (polymerase chain reaction: PCR) alapú molekuláris biológiai módszerek segítségével állítottuk fel, míg az elhullott állatok esetében széleskörű diagnosztikai vizsgálatokat alkalmaztunk, mint például makroszkópos vizsgálatot (boncolás), kórszövettant, elektronmikroszkópos vizsgálatot, PCR-alapú módszereket, illetve nukleinsav- és aminosav-szekvencia meghatározást és filogenetikai analízist. A PCR reakciók alapján a vizsgált 214 minta közül 58 (27,1 %) CDV pozitívnak bizonyult. A genetikai vizsgálatok során több CDV csoportba („európai", „északi-sarki" és „európai vadvilág") tartozó vírustörzset sikerült kimutatni Magyarországon. Az északi-sarki csoportba tartozó vírustörzsek a Fővárosi Ebrendészeti Telepen kimutatott endémiás fertőzésért bizonyultak felelősnek. Ugyanakkor szopornyicát több állatfajban is sikerült kimutatni: rókában *(Vulpes vulpes)*, mosómedvében *(Procyon lotor)* és vadászgörényben *(Mustela putorius furo)*.

A vakcina-eredetű és vad CDV törzsek gyors elkülönítése érdekében restrikciós fragmentumhossz polimorfizmus (restriction fragment length polymorphism: RFLP) alapú eljárást dolgoztunk ki. A *Psi*I alapú RFLP eljárás gyakorlati alkalmazása során az egyik jelenleg Magyarországon is alkalmazott CDV fertőzés elleni vakcinában található vírustörzs a vad vírustörzsekkel azonos reakciót eredményezett. A nuklein- és aminosav szekvenciák és a filogenetikai vizsgálat alapján a Vanguard (Pfizer Animal Health, USA) vakcinában található vírustörzs nem a vakcina-vírusok csoportjába, mint az a gyártó által közzétett leírás alapján elvárt volt, hanem sokkal nagyobb genetikai hasonlóságot mutatott különböző földrajzi régiókból származó vad vírustörzsekkel. Következő lépésként több országból (Izrael, Málta, USA) származó Vanguard vakcinát, valamint 1992-ből és 1994-ből származó vakcinákat vizsgáltunk meg. A genetikai vizsgálat alapján mindegyik vizsgált Vanguard vakcinában ugyanaz a vírustörzs volt jelen, mint a 2006-ban vizsgált magyarországi vakcinában, és semmiképp sem az, amit a gyártó a termékleírásban feltüntetett (Snyder Hill törzs).

Az FPV és CPV2 együttes kimutatására alkalmas klasszikus PCR-alapú eljárás segítségével a vizsgált 230 minta közül 72 (31,3 %) pozitívnak bizonyult (17 macska, 1 oroszlán, 1 pálmasodró és 53 kutya eredetű minta esetében). Egy korábban leközölt *Mbo*II alapú RFLP vizsgálat alkalmazását követően 20 magyarországi CPV2 törzsből 15 esetében CPV2c genotípusú törzseknek megfelelő eredményt kaptunk. A nuklein- és aminosav szekvenciák vizsgálata alapján az összes vizsgált magyarországi törzs tulajdonképpen 2a típusú CPV-nak bizonyult. A félrevezető eredmény magyarázata a PCR során keletkezett amplikonok másik végén létrejött pontmutációban rejlik. Ennek következtében a 20 magyarországi törzsből az RFLP alapú eljárást követően 15 esetében félrevezető genetipizálási eredményt kaptunk. Ez a tény a gyors genotipizálási technikák folyamatos felülvizsgálatának, illetve az eredmények megbízhatóbb eljárások segítségével történő alátámasztásának szükségességét emeli ki.

Vizsgálataink során elsőként sikerült direkt eljárásokkal kimutatni FPV fertőzést egy cibetmacskafélében *(Viverridae)*. A megvizsgált ázsiai pálmasodróban *(Paradoxurus hermaphroditus)* keletkezett kórbonctani és kórszövettani elváltozások megegyeztek az FPV által más állatfajokban előidézett elváltozásokkal. Ugyanakkor a fertőzés sikeres kimutatása fényt derített arra is, hogy a kórokozó gazdaspektruma szélesebb az eddig ismertnél, és hogy az FPV iránt a cibetmacskafélék is fogékonyak. FPV fertőzést egy Érd mellől származó oroszlánban *(Panthera leo)* is sikerült kimutatni. Az ázsiai pálmasodróban, az oroszlánban és két macskában kimutatott vírustörzsek genetikai vizsgálatának eredménye arra enged következtetni, hogy a jelenleg Magyarországon cirkuláló FPV törzsek viszonylag heterogén csoportot alkotnak.

1. Introduction

Based on modern scientific results an enormous variety of viral pathogens have been concurrently evolving with species belonging to all taxonomical classes, presumably ever since the early days of life on Earth. The life of all living creatures, starting with the simplest forms of life to the highest, most evolved forms of existence, *was*, *is* and most likely *will be* considerably influenced by these infinitesimal pathogens. Companion animals, such as the dog, cat and many other species are no exceptions. Virus infections, such as rabies, distemper, parvovirus infections, just to name a few of the most relevant ones, have shaped and decisively influenced receptive populations throughout the early, recent and present history. As "with great power comes great responsibility" the research, better understanding, treatment and most importantly the prevention of these diseases that threaten the well-being and survival of all receptive species fall under the responsibility of human beings, who *did* and *will* benefit of these species throughout the centuries.

Viral diseases such as distemper and parvovirus infection constantly threaten susceptible animal populations. The understanding of these diseases, including the molecular characteristics of the pathogens, represents the first line of defense and constitutes the basis of the better prevention of infections and protection of animals.

The hypothesis of the present study was that presently there is more than one genotype of CDV and CPV2 present in Hungary. The hypothesis was tested by detecting the pathogens in naturally infected animals and by analyzing relevant segments of the viral genomes. The infections were demonstrated using clinical and necropsy samples collected between 2004 and 2008 from several different species, such as dogs, foxes, ferrets, raccoons and an Asian palm civet. The genetic characterization of the pathogens was performed by determining the nucleic acid sequence of key segments of their genome and by using these data to determine the phylogenetic relationships between the Hungarian and strains previously isolated in other parts of the world.

2. Aims of the Study

The aims of the present study were to:

1. Design new primers for the diagnosis and phylogenetic analysis of Hungarian CDV, FPV and CPV2 strains.

2. Perform the phylogenetic analysis of Hungarian CDV strains in order to determine which variants are currently circulating in Hungary.

3. Design an RFLP-based test that would allow the differentiation of vaccine from wild-type strains of CDV.

4. Perform the genetic analysis of CDV strains present in vaccines currently used in Hungary.

5. Determine which CPV2 genotypes (2a, 2b or 2c) are currently present in Hungary.

3. Literature Survey

3.1 Canine distemper

3.1.1 General informations

Canine distemper (CD) is caused by the CDV which belongs to the *Morbillivirus* genus of the *Paramyxoviridae* virus family (Appel, 1987).

There are several hypothesizes regarding the origin of CDV. Some of the early scientists believed that CD was known "since time immemorial" in Europe, or at least France (Desmars, as cited in Panisset, 1938 and Blancou, 2004). Others, such as Heusinger (1853, as cited in Blancou, 2004), believed that the disease was "imported" from South America (Peru) shortly before 1760. He based his theory on a previous report by Ulloa from 1746, who in his opinion clearly described the some of the main clinical and epidemiological features of CD (Blancou, 2004). According to these theories, the disease was first described in Europe in 1760 in Madrid, Spain, where it caused severe losses in the canine population. Following its assumed arrival in Europe in 1760, the disease was soon reported in other countries such as England, Italy (1764), and Russia (1770). A severe CD outbreak occurred in Spain in 1763, and according to some reports approximately 900 dogs died of the disease in a single day in Madrid (Blancou, 2004).

Following numerous more or less scientific assumptions regarding the etiology of the disease, Henri Carré was the first to demonstrate that it was caused by an "ultravirus" (Blancou, 2004).

CD is a highly contagious viral infection of different carnivores that belong to numerous animal families, such as *Canidae, Mustelidae, Procyonidae, Felidae, Phocidae, Viverridae, Ursidae* and many others (Kovács et al., 1983; Appel, 1987; Blixenkrone-Møller et al., 1993; Gemma et al., 1996; Barret et al., 1999; Lan et al., 2006). Domestic canine populations and receptive wild species seem to act as reservoirs one for the other (Appel & Summers, 1995; Carpenter et al., 1998; Lednicky et al., 2004). CD-like diseases have been observed in large felids in the Tanzanian Serengeti National Park in 1994 (Roelke-Parker et al., 1996), in North American zoos in 1991 and 1992 (Appel et al., 1994), as well as in collared peccaries *(Tayassu tajacu)* and a non-human primate *(Macaca fuscata)* in Japan (Appel et al., 1994; Yoshikawa et al., 1989;

Greene & Appel, 2006). In some CDV outbreaks, including the mass mortalities among Baikal and Caspian seals and large felids in the Serengeti Park, terrestrial carnivores, including dogs and wolves, have been suspected as vectors for the infectious agent (Kennedy et al., 2000; Kreutzer et al., 2008; Beineke et al., 2009).

The virus is shed primarily by oro-nasal secretion, however, any discharge and secretion can carry the virus. CDV infects susceptible animals primarily by inhalation of airborne viruses or via infective aerosol droplets (Beineke et al., 2009).

CDV is susceptible to ultraviolet light, although protein or antioxidants help protect it from inactivation. Extremely susceptible to heat and drying, CDV is destroyed by temperatures greater than 50-60 °C for 30 minutes, but survival times are longer at colder temperatures. In warm climates, CDV does not persist in kennels after infected dogs have been removed. The virus remains viable between pH 4.5-9, and it is susceptible to ether, chloroform, dilute (<5 %) formalin solution, phenol (0.75 %) and quaternary ammonium disinfectant (0.3 %). Routine disinfection procedures are usually effective in destroying CDV in a kennel or hospital (Greene & Appel, 2006).

3.1.2 Pathogenesis

3.1.2.1 Systemic infection

The duration, severity and clinical manifestation of CD can present great variations, from virtually no clinical signs to severe disease with approximately 50 % mortality. Factors that influence these aspects as well as the incubation period are: virus strain and age and immune status of the host (Appel, 1970, 1987; Krakowa et al., 1980; Beineke et al., 2009).

The entering of pathogen in the host is followed by an incubation period that can last 1 to 4 weeks. Shortly after the infection, the virus starts to replicate in the lymphoid tissue of the respiratory tract. The first cell types that propagate the virus are represented by tissue macrophages and monocytes located on or in the respiratory epithelium and tonsils. The pathogen is then disseminated by lymphatics and blood to distant hematopoietic tissues during the first viremic phase (Appel, 1970; Beineke et al., 2009). This process coincides with the first phase of the biphasic fever, one very characteristic clinical finding in CD (Greene & Appel, 2006). Along with the transient fever, the onset of lymphopenia can be observed 3 to 6 days p.i. (Beineke et al., 2009). Within the first week of infection, the virus replicates in multiple lymphoid tissues such as thymus, spleen, lymph nodes, Kupffer cells of the liver, lamina propria of the intestine and

stomach, and mononuclear cells of the bone marrow (Pardo, 2006). Viremia occurs by spread of cell free virus as well as leukocyte and thrombocyte associated infectious pathogens (Fig. 1).

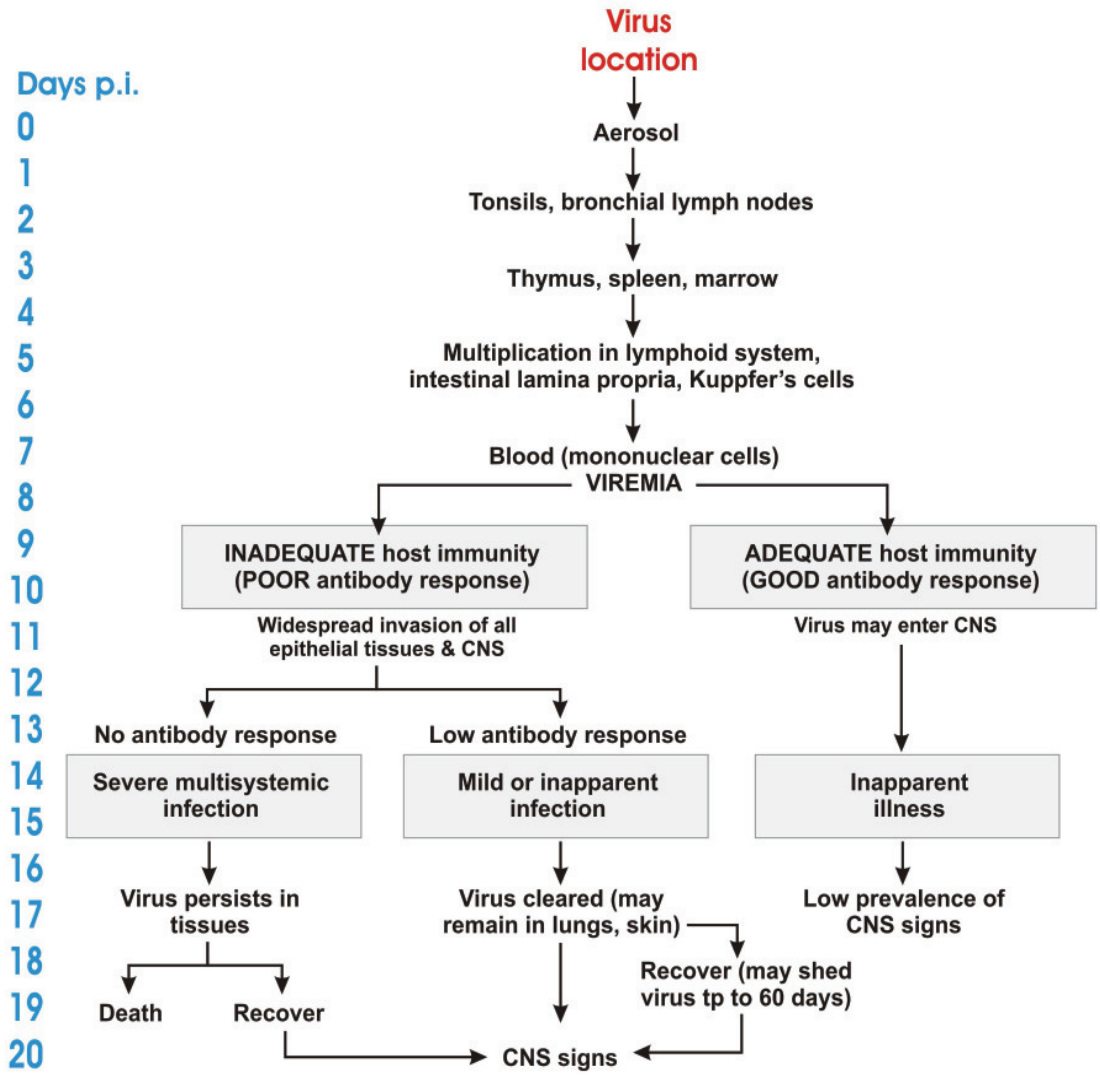

Fig. 1: Sequential pathogenesis of CD (Greene & Appel, 2006)

The second viremia follows several days later, frequently associated with high fever, and results in infection of parenchymal and tissue cells throughout the body (Appel, 1970; Beineke et al., 2009). Thus, CDV can be found in cells of the respiratory, gastrointestinal and urinary tract, endocrine system, lymphoid tissues, central nervous system and vasculature including keratinocytes, fibroblasts, thrombocytes and different lymphoid cell subsets, as well as bronchial, endothelial, epithelial and neuroectodermal cells (Baumgärtner et al., 1989; Koutinas et al., 2002, 2004; Beineke et al., 2009).

3.1.2.2 CNS infection

The spread of the virus to the CNS depends on the degree of systemic immune responses mounted by the host. Virus probably enters the nervous system of many viremic CDV-infected dogs whether neurologic signs are observed or not (Greene & Appel, 2006). Generally, a polio- and a leukoencephalitis, characterized by different distribution patterns of the lesions and pathogenesis, can be distinguished (Beineke et al., 2009).

Primary spread of CDV to the CNS is hematogenous (Greene & Appel, 2006). Studies tracking the route of CDV within the brain showed ependymal and subependymal white matter infection, indicating CNS spread along the CSF pathway (Vandevelde et al., 1985). A study using experimentally infected ferrets also suggests an olfactory nerve pathway for the neuroinvasion (Rudd et al., 2006). A direct spread from meningeal cells of the pia mater has been discussed (Baumgärtner et al., 1989).

Both a cell-free viremia during the first days following experimental infection and a cell-associated virus spread have been described (Summers & Appel, 1987). However, free infectious plasma virus cannot be observed after the appearance of virus-neutralizing antibodies. The leukocyte-associated viremia is believed to represent the major source of hematogenous infectivity. Viral antigen is first detected within CNS capillaries and venular endothelia at 5 and 6 days p.i. and/or in perivascular lymphocytes, astrocytic foot processes and pericytes at 8 days p.i. Furthermore, a productive CDV infection of the choroid plexus epithelium with release of progeny virus into the CSF followed by ependymal infection and spread of the virus to the subependymal white matter can be observed at 10 days p.i. (Beineke et al., 2009).

The type of lesion produced as well as the course and outcome of the infection within the CNS depend on numerous factors, such as the age and immunocompetence of the host at the time of exposure, the neurotropic and immunosuppressive properties of the virus, and the time at which lesions are examined (Greene & Appel, 2006).

- *Acute infection*

Acute CDV encephalitis, which occurs early in the course of infection in young or immunosuppressed animals, is characterized by direct viral replication and injury: virus antigen and messenger RNA (mRNA) are detected in lesions, whereas inflammatory cells and class II MHC antigen expression are absent or minimal. Virus causes multifocal lesions in the gray (neuronal infection and necrosis) and white matter

(noninflammatory demyelination), however neuronal infection can also occur with minimal signs of cytolysis (Greene & Appel, 2006).

The noninflammatory demyelination observed in acute CNS infections seems to be associated with viral infection of microglial and astroglial cells rather than oligodendroglial cells (Multinelli et al., 1989; Gaedke et al., 1999; Greene & Appel, 2006). Even though there is no evidence of active virus replication in oligodendroglial cells, the presence of the complete genetic material of CDV has been demonstrated to be present in these cells by ISH (Zurbriggen et al., 1993). The restricted infection likely leads to metabolic dysfunction and morphologic degeneration of oligodendroglial cells and results in demyelination via a down-regulation of myelin gene expression (Glaus et al., 1990; Zurbriggen et al., 1998; Greene & Appel, 2006) and myelin synthesis, due to the decrease in activity of a specific oligodendroglia myelin-producing enzyme: cerebroside sulfotransferase (Vandevelde & Zurbriggen, 2005).

- *Chronic infection*

Older or more immunocompetent dogs have a tendency to develop the chronic CDV lesions that are consistent with a leukoencephalomyelitis in the caudal brainstem and spinal cord (Pardo, 2006). In contrast to acute CDV encephalitis, subacute to chronic CDV encephalitis is characterized by reduced expression of CDV antigen and mRNA and a strong up-regulation of class II MHC expression. This results in perivascular mononuclear cell infiltrations (by mainly CD4+ and B cells) and a virus independent immunopathologic process. In chronic CNS infections the pathogenic mechanism for demyelination is not due to viral interference, but to the reaction of the immune system (Greene & Appel, 2006). The destruction of myelin is caused by the presence of antimyelin antibodies and the release of reactive oxygen radicals by the activated macrophages ("innocent bystander" theory; Greene & Appel, 2006).

If the animal survives the initial infection, CDV can be cleared from the inflammatory lesions, but it can persist in brain tissue in unaffected sites (Muller et al., 1995), presumably due to a noncytolytic infection (Vandevelde & Zurbriggen, 1995) or reduced expression of CDV proteins on the surface of inflammatory cells (Alldinger et al., 1993; Muller et al., 1995; Greene & Appel, 2006).

Two rarer forms of chronic CNS infection have also been described: old dog encephalitis (ODE) and inclusion body polioencephalitis (IBP). ODE occurs in infected animals that are immunocompetent and, following an acute CDV infection, have virus persisting strictly in the neurons in a replication-defective form (Axthelm & Krakowka,

1998; Greene & Appel, 2006). IBP can occur after vaccination or in dogs with a sudden onset of only neurologic manifestations of distemper (Nesseler et al., 1999), when multifocal gray matter necrosis, perivascular lymphocytic inflammation and cytoplasmic and intranuclear inclusion bodies are observed (Amude et al., 2007; Greene & Appel, 2006)

3.1.3 Clinical signs

CD is characterized by a wide variety of clinical manifestation that can be categorized as acute systemic form (catarrhal and/or nervous manifestations) and a chronic nervous manifestation. In addition, various other, more or less specific clinical signs (i.e. ODE, hard pad disease etc.) have been described and intensively studied (Greene & Appel, 2006; Beineke et al., 2009).

3.1.3.1 Acute systemic infection

Even though more than 50 % of CDV infections are probably subclinical, acute systemic infection is one of the most frequently encountered clinical manifestations. It can occur at any age, but it most commonly affects unvaccinated, exposed puppies 12 to 16 weeks of age that have lost their maternal immunity or younger puppies that have received inadequate concentration of maternal antibodies (Greene & Appel, 2006). Clinical signs are represented by serous to mucopurulent rhinitis and nasal discharge, conjunctivitis, interstitial pneumonia and necrotizing bronchiolitis, often complicated by a suppurative bronchopneumonia due to secondary bacterial infections (Beineke et al., 2009). The clinical evolution of the infection is characterized by a specific biphasic fever curve, and other clinical signs that do not respond to symptomatic antimicrobial therapy. Later on coughing, vomiting and diarrhea develop, which can lead to severe dehydration and emaciation (Greene & Appel, 2006). Nervous signs are diverse and progressive and include myoclonus, nystagmus, ataxia, postural reaction deficits and tetraparesis or tetraplegia (Vandevelde & Zurbriggen, 2005; Amude et al., 2007; Beineke et al., 2009). Animals can die suddenly from systemic illness, but adequate therapy can decrease the risk in many cases (Greene & Appel, 2006).

3.1.3.2 Chronic nervous manifestation

Neurologic signs frequently develop in the presence of nonexistent or very mild extraneural signs. Nevertheless, they are typically progressive. Neurological signs vary

according to the area of CNS involved, and can be represented by: myoclonus, seizures, cerebellar and vestibular signs, paraparesis or tetraparesis with sensory ataxia, hyperesthesia etc. (Greene & Appel, 2006).

3.1.3.3 Other manifestations

Skin lesions can be represented by vesicular and pustular dermatitis, and nasal and digital hyperkeratosis ("hard pad disease") (Greene & Appel, 2006).

Bone lesions: metaphyseal osteosclerosis of long bones or hypertrophic osteodystrophy has also been described in correlation with naturally occurring CDV infections and MLV vaccinations (Abeles et al., 1999; Greene & Appel, 2006).

Ocular signs: mild anterior uveitis is frequent in dogs with CDV encephalomyelitis (Greene & Appel, 2006). Other signs include blindness, optic neuritis, choroiditis, degeneration, necrosis and detachment of the retina (Pardo, 2006).

Transplacental infections: depending on the stage of gestation at which infection occurred, abortions, stillbirths, or the births of weak puppies may occur. Puppies infected transplacentally may develop neurologic signs during the first 4 to 6 weeks of life, or may suffer from permanent immunodeficiencies (Greene & Appel, 2006).

Neonatal infections: may result in dental impaction, partial eruption, oligodontia and enamel and dentin hypoplasia (Pardo, 2006). In a study using gnotobiotic puppies, following experimental CDV infection they have developed a virus-induced cardiomyopathy characterized by multifocal myocardial degeneration and necrosis (Higgins et al., 1981).

Rheumatoid arthritis: dogs with rheumatoid arthritis had high levels of antibodies to CDV in sera and synovial fluid, compared with dogs with inflammatory and degenerative arthritis (Bell et al., 1991).

3.1.4 Pathology

The pathologic findings depend on the type and severity of clinical symptoms. In many situations, especially when the examined animal died following an acute systemic infection, the changes can be minimal. The most frequently observed gross lesions are represented by dehydration, mucopurulent oculonasal discharge, serous/catarrhal/purulent pharingitis, tracheitis, pulmonary edema, interstitial pneumonia that can be aggravated by secondary bacterial pathogens to suppurative lobular bronchopneumonia, characterized by the consolidation of cranial and caudal

aspects of the lung lobes, catarrhal enteritis with depletion of the Peyer's patches, thymic atrophy, generalized lymphadenopathy, enamel hypoplasia, hyperkeratosis of the footpads and nose (Pardo, 2006; Greene & Appel, 2006; Beineke et al., 2009).

Microscopically, the presence of acidophilic cytoplasmic (in the epithelial cells) and nucleic (in the nervous tissue) viral inclusion bodies 1-5 μm in diameter is considered to be quite suggestive of a CDV infection, although similar structures were detected in healthy animals as well. Hence, the diagnosis of CD should not be based solely on histopathological examination. Formation of giant cells in different tissues (CNS, uvea, lymph nodes, lungs etc.) is also suggestive of a paramyxovirus infection (Greene & Appel, 2006).

3.1.5 Diagnosis, treatment and prevention

Clinical diagnosis of CD can be obtained by applying several techniques, such as blood work (the infection is characterized by a severe absolute lymphopenia due to lymphoid depletion, necrosis and apoptosis [Greene & Appel, 2006; Pardo, 2006]), the identification of inclusion bodies in lymphocytes, monocytes, neutrophils and erythrocytes obtained from peripheral blood and stained with Wright-Leishman stain (Greene & Appel, 1998), radiological identification of a viral pneumonia and various immunological assays (ELISA, direct and indirect immunofluorescent antibody tests [IFAT], SN, immunofluorescent techniques to detect viral antigen etc.), but most of these techniques have their diagnostic limitation and sometimes can lead to false positive results (Pardo, 2006). Other diagnostic techniques are represented by CSF analysis, IHC, serum antibody testing, virus isolation and techniques based on nucleic acid detection, such as the PCR-based methods (Greene & Appel, 2006).

Despite major advances in research of CD, currently there is no specific treatment for the disease. Therapy consists of supportive antimicrobial medicines administered for protection against secondary infections. Animals should be placed in a clean, stress-free environment, while fluid administration and anti-emetic therapy is necessary when digestive signs such as diarrhea and vomiting are present. The alleviation of neurological signs can be attempted using antiepileptic drugs and glucocorticoids (Greene & Appel, 2006). Hyperimmune serum administration can also be employed in the early stages of the infection or in heavily infected environments (Greene & Appel, 2006).

Edward Jenner was probably the first who tried to elucidate the nature of the disease and to prevent it by vaccination. He assumed that CD was a pox-like affection and performed vaccination experiments by which he concluded that out of the 43 vaccinated dogs none got infected, but due to the lack of control animals these results cannot be reliable interpreted (Blancou, 2004).

Since the beginning of the 20th century, significant progress has been made regarding the prevention, treatment and control of CD. Several types of vaccines are currently used on a routine basis to prevent the disease. The most frequently used types of vaccines are the ones containing modified live viruses (MLV) developed around the middle of the previous century. Some of the most commonly used strains are Onderstepoort (isolated from ranched foxes from North America in the 1930's; Haig, 1956) and Snyder Hill (isolated in Ithaca from the brain of a dog in the 1950's; Brown et al., 1972), but several others have been used throughout the years, such as Lederle, Rockborn etc. As recent genetic data show, all these strains belong to the so-called America-1 lineage, that have not been detected over the last 5 decades and it is not known whether they are still circulating in the field (Martella et al., 2007).

Recently other types of vaccines have been developed, besides the MLV ones, such as recombinant vaccines, DNA vaccines, subunit vaccines etc., but these are not as commercially available as the still more frequently used MLV based vaccines.

3.1.6 Genetic characteristics of the pathogen

The CDV genome is approximately 15 690 nucleotides in length and contains six genes that code for viral proteins (Fig. 2). The hemagglutinin or the H glycoprotein is important in the viral attachment to the host cell (Murphy et al., 1999). The H gene shows the greatest genetic variation, the reason could be that the protein is affecting and intimately interacts with the host's immune system (Bolt et al., 1997). This great genetic variability makes this gene suitable for phylogenetic analysis (Pardo et al., 2005). The F gene encodes for a glycoprotein that is essential for the fusion between the viral particle and the host cell. The F protein makes it also possible for the virus to move from one host cell to another (Murphy et al., 1999). The M protein provides the mechanism the virus needs to enter the host cell. It is also responsible for the assembly of new viral particles. The functional polymerase complex is crucial for the replication of viral RNA and is formed by the P and the L proteins. The P gene also encodes for two non

structural proteins: the C and V protein (Pardo, 2006). The N encapsulates the viral RNA (Simon-Martìnez et al., 2007).

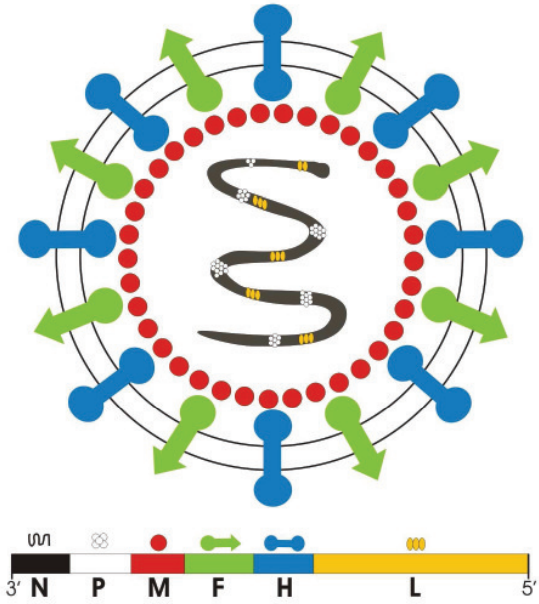

Fig. 2: Schematic representation of the ultrastructural morphology and genetic features of CDV (based on Greene & Appel, 2006)

3.1.7 Genetic diversity

Nucleic and amino acid sequence analysis of CDV has shown that the H gene of CDV is going through an antigenic drift depending on a geographic pattern (Martella et al., 2007). Furthermore, in a study made on the P gene and in lesser extent of the H gene showed that genetic differences of the isolates correlated with the geographic origin (Bolt el al., 1997). The H gene possesses the highest degree of variation and the F and P gene are genetically more stable (Bolt el al., 1997). This may be the consequence of the role the H protein plays in the host's immune reaction (Pardo et al., 2005). Considering this fact, the H gene has been the most studied gene to determine phylogenic relationships between different strains, although the F gene has also been used for this purpose. Another fact that makes it more advantageous to use the H gene is the higher number of complete H gene sequences than complete F gene sequences available in public databases for phylogenic comparison (Pardo et al., 2005). In addition to the H, F and P genes, studies have also been conducted on the N gene. In contrast to the H and the F gene, that shows high genetic variation, the N gene is more conserved (Simon-Martìnez et al., 2007). The mentioned study published results that showed 93-97 % similarity between their isolates and the Onderstepoort strain used as a reference. For

comparison, a study conducted by Bolt (1997) showed 7-10 % difference between the field isolates and the vaccine strains at the nucleotide level based on the H gene. The N gene is more stable, which is result of the fact that its product plays a smaller role in the host's immune reaction. However the changes in nucleic acid sequence of the N gene have proved to be coherent with the changes observed in the H gene segment of the viral genome (Simon-Martìnez et al., 2007).

Previous genetic studies demonstrated that CDV field strains can be grouped into six major genetic lineages: America 1 and 2, Asia 1 and 2, European and Arctic (Martella et al., 2007). The variation between different lineages of the CDV is more than 4 % of the aas. However the greatest genetic diversity is between the strains belonging to the America 1 group (vaccine strains) and the other CDV lineages (Martella et al., 2007). The Onderstepoort strain was as mentioned above isolated in America in the 1930s (Bolt et al., 1997). The temporal dynamics of a virus depends on transmission properties of the virus and the survival, immune response and the distribution of the host (Packer et al., 1999). Since the strain used for vaccination are kept in freeze dried form (Chappuis, 1995), it has consequently been protected from the above mentioned situations and therefore not been stimulated to evolve. The comparison between the wild isolates and the vaccine strains provides therefore a reference as to which extent the virus has evolved since then (Martella et al., 2007). Previous phylogenetic studies demonstrating differences between field strains and vaccine strains prove antigen drifting since the time of isolation of the strains currently still used for immunization.

3.2 Feline panleukopenia

3.2.1 General informations

FP is caused by FPV, a single stranded DNA virus belonging to the *Parvoviridae* virus family (Truyen et al., 1995). FPV is closely related to other parvoviruses, such as the mink enteritis virus (MEV), raccoon parvovirus (RPV), type 2 canine parvovirus (CPV2) and blue fox parvovirus (BFPV) (Parrish & Carmichael, 1983; Tijssen, 1999).

FPV-induced disease in domestic cats has been known since the beginning of the 20th century (Verge & Christoforoni, 1928; Steinel et al., 2000). Initially the disease was described as "feline infectious enteritis", "malignant panleukopenia", "feline distemper" or "spontaneous agranulocytosis" (Verge & Christoforoni, 1928; Steinel et

al., 2001). The first outbreaks of the disease in captive felids were reported in the 1930's and 1940's (Hindle & Findlay, 1932; Goss, 1942).

There are several reports of FPV infections that occurred in wild species affecting tigers *(Panthera tigris)*, leopards *(Panthera pardus)*, cheetahs *(Acinonyx jubatus)*, wild cats *(Felis sylvestris)*, lynx *(Lynx lynx),* servals *(Leptaillurus serval)*, tiger cats *(Felis tigrina; Felis aurata)*, ocelots *(Leopardus pardalis)*, lions *(Panthera leo)*, snow leopards *(Panthera unica)*, clouded leopards *(Neofelis nebulosa)*, mountain lions *(Felis concolor)*, African wild cat *(Felis lybica)*, leopard cats *(Felis bengalensis)* and maned wolves *(Chrysocyon brachyurus)* (Ikeda et al., 1999; Steinel et al., 2000; 2001). Based on the reported cases it is generally assumed that all members of the family *Felidae* are susceptible to FPV infection and disease (Steinel et al., 2001). Raccoons *(Procyon lotor)* and Arctic or blue foxes *(Alopex lagopus)* with clinical signs of a FPV disease were recognized in the 1940's (Waller, 1940; Phillips, 1943). The parvoviruses isolated from these species were described as FPV-like viruses and named after their respective hosts, RPV and BFPV (Appel & Parrish, 1982; Veijalainen & Smeds, 1988). A parvovirus infection of a South American coati *(Nasua nasua)* has also been reported (Johnson & Hallowel, 1968). Evidence of the susceptibility of members of the *Viverridae* animal family was represented only by the result of serological testings (Ikeda et al., 1999).

The epidemiology of FPV is characterized by an acute infection with shedding of high virus titers in the feces of diseased animals. Virus shedding usually lasts only 1 to 2 days, but virus can stay infectious in the environment for weeks or even months (Greene & Addie, 2006). Direct contact between carnivores is not required for efficient transmission (Steinel et al., 2001). FPV is maintained in a population by its environmental persistence rather than prolonged viral shedding; hence fomites play an important role in disease transmission because of prolonged survival of the virus on all sorts of contaminated surfaces, such as shoes, hands, food dishes, bedding etc. (Greene & Addie, 2006).

3.2.2 Pathogenesis

The pathogenesis of FPV infections is determined by the indispensable requirement of DNA replication of the virus for actively dividing cells. This necessity explains the differences in the outcome of infections in fetal, neonatal or older animals (Parrish, 1995).

3.2.2.1 Neonatal animals

Infection of neonates results in different disease from that seen in older animals, and is characterized by infection of the developing cerebellum. Enteritis, as one of the main pathological changes observed in older animals, is not even observed in very young animals (Parrish, 1995). One study applying PCR-based technique managed to demonstrate an association between myocarditis and FPV genome in the myocardium of cats with idiopathic hypertrophic, dilated and restrictive cardiomyopathy, hence suggesting that FPV plays an important role in the pathogenesis of these diseases (Meurs et al., 2000).

In utero or early neonatal infection frequently results in the replication of the virus in the cells of the external germinal epithelium of the cerebellum, resulting in changes such as cerebellar hypoplasia (Parrish, 1995), cerebellar agenesis, or hydranencephaly (Sharp et al., 1999). In surviving kittens, these pathological changes result in the so-called "feline ataxia syndrome" (Parrish, 1995).

3.2.2.2 Older animals

The virus enters the body of the receptive hosts most likely by infecting and replicating in the cells of the nasopharynx, the tonsils or other lymphoid tissues (Parrish, 1995). A plasma-phase viremia, occurring between 2 and 7 days p.i., disseminates the virus to all body tissues (Fig. 3), although pathologic lesions occur in tissues with the highest mitotic activity (Greene & Addie, 2006).

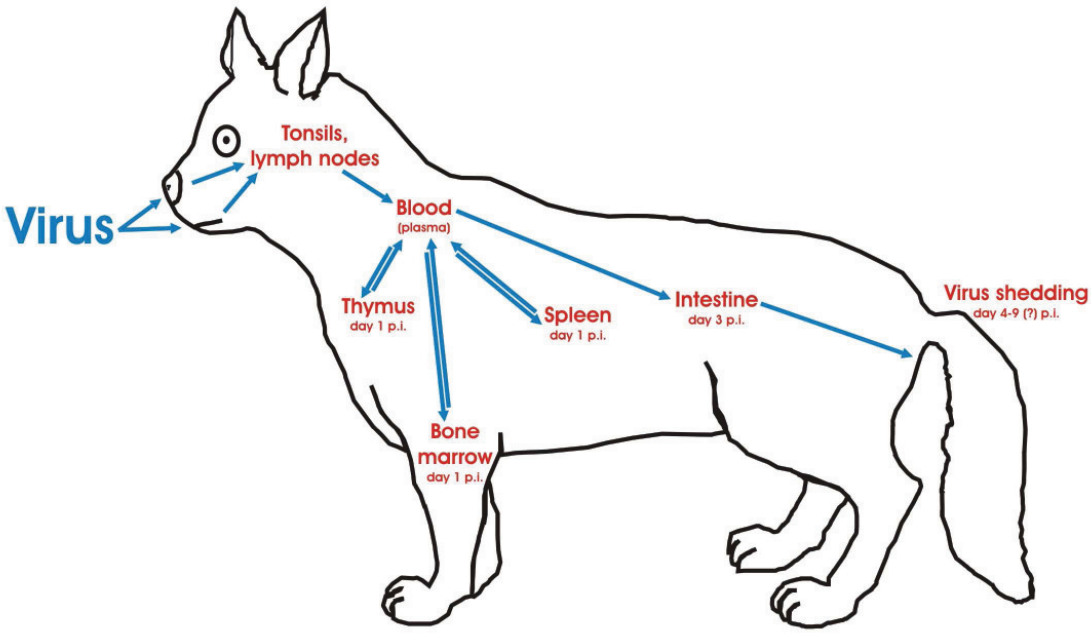

Fig. 3: Schematic representation of pathogenesis of FPV infection (based on Hoelzer et al., 2008a)

The incubation period before the onset of clinical signs is usually 4-5 days, and the clinical course can rapidly progress to death. Due to virus properties and needs, the primary pathologic site for viral replication is within the intestinal crypts, resulting in enteritis and diarrhea due to malabsorption and increased permeability (Lamm & Rezabek, 2008). The virus affects cells located deep in the intestinal crypts, while differentiated absorptive cells on the surface of the villi are non-dividing and are not affected. Thymic atrophy and extensive damage to all white blood cell populations and precursors, resulting in severe leukopenia are also common consequences of FPV infections (Greene & Addie, 2006).

Co-infections with other pathogens, such as *Clostridium piliforme* (Tyzzer's disease) and salmonellae have also been reported (Greene & Addie, 2006). Secondary infections caused by other bacteria are also considered to be common findings (Lamm & Rezabek, 2008).

3.2.3 Clinical signs

The number of animals that develop clinical signs is significantly lower than the number of infected animals. This fact is supported by the high prevalence of FPV antibodies in the cat population. Severe clinical illness is the rule in young, unvaccinated kittens, and the highest morbidity and mortality occurs between 3 to 5 months of age (Greene & Addie, 2006). FPV has also been demonstrated in young purebred kittens that died suddenly, without clinical signs suggestive of FP (Addie et al., 1998). In these *peracute cases* cats may die within 12 hours, as if poisoned. They may be found in terminal stages of septic shock, being profoundly dehydrated, hypothermic and comatose (Greene & Addie, 2006). In the more frequent *acute form* of the disease the clinical signs are represented by fever, depression, anorexia, vomiting, extreme dehydration, and sometimes in the later stages, diarrhea and hypothermia. Clinical signs suggestive of DIC, such as petechial and ecchymotic hemorrhages, are also common. Animals that survive infection for longer than 5 days without developing fatal complications usually recover, although recovery frequently takes several weeks (Greene & Addie, 2006).

FP causes infertility and abortion if the infection occurs during pregnancy. These females however never show clinical signs suggestive of FP. Kittens infected *in utero* may develop ataxia, incoordination, tremors, and normal mental status typical of cerebellar disease. Retinal degeneration is also a relatively common finding in affected kittens (Greene & Addie, 2006).

3.2.4 Pathology

Gross pathologic changes in naturally infected cats are usually minimal, and represented by dilated digestive tract, as well as hyperemia and firmness of the affected intestinal segments, with petechial and ecchymotic hemorrhages on the serosal surfaces (Greene & Addie, 2006). The histological changes within the small intestine include multifocal crypt necrosis, loss of crypt architecture with villus blunting, and signs of crypt regeneration (Lamm & Rezabek, 2008). The most severe lesion can be observed in the jejunum and ileum, while the duodenum and colon are less severely affected (Greene & Addie, 2006). Other pathological changes are the extreme lymphoid depletion which is most obvious in the follicles of lymph nodes, Peyer's patches, and spleen. Thymic atrophy is also a frequently observed change, especially in germ-free kittens, when it actually is the only observable pathological sign (Greene & Addie, 2006).

With *in utero* infections, FPV has a teratogenic effect that has a varied result depending on the stage of infection: in the latter stages of gestation the virus targets the brain and the eye because of the high degree of proliferative activity. This usually results in cerebellar hypoplasia, hydrocephalus, hydranencephaly and retinal dysplasia (Lamm & Rezabek, 2008).

Even though they are rare and transient, eosinophilic nuclear incusion bodies can also be found in FPV infection. Sometimes special fixating techniques, such as Bouin's or Zenker's fixatives must be used to reveal these formations (Greene & Addie, 2006).

3.2.5 Diagnosis, treatment and prevention

Diagnosis of FPV infections is based on clinical signs, clinical laboratory findings (mild to severe leukopenia, transient decrease in absolute reticulocyte count, thrombocytopenia), serologic testing, fecal enzyme-linked immunosorbent testing, immunochromatographic test, hemagglutination, virus isolation, and genetic detection (Greene & Addie, 2006).

Since currently there is no specific treatment for FP, infected cats should be treated symptomatically and nursed. Medication is usually represented by parenterally administered fluids, antiemetics, plasma or blood transfusion, broad-spectrum antibiotics and vitamins. Response to therapy can be followed by monitoring the total and differential leukocyte counts (Greene & Addie, 2006).

Protection against FPV infection is provided by maternal and passive immunity, and by active immunization. Vaccination of healthy cats is recommended, as active immunization has been the most important factor in reducing the incidence of the disease. There are currently several MLV and inactivated vaccines on the market that have been shown to have excellent efficacy, if administered appropriately (Greene & Addie, 2006; Lamm & Rezabek, 2008). A vaccination schedule should be created on an individual basis with consideration of age, environment, and the recommendation of the manufacturer. In general, a core vaccination of a MLV at 6 to 8 weeks, 9 to 11 weeks and 12 to 16 weeks of age is recommended (Lamm & Rezabek, 2008). After the kitten series, and a first booster 1 year later, triennial vaccination in conjunction with the rabies vaccine offers adequate protection (Greene & Addie, 2006). Special care should be taken when using inactivated vaccines and when vaccinating exotic felines or immuno-compromised individuals.

3.2.6 Genetic characteristics of the pathogen

Similarly to other parvoviruses, the genome of FPV contains two promoters which give rise to messages for either two non-structural genes (NS1 and NS2), or for the structural protein genes VP1 and VP2. The VP1 and VP2 proteins are translated from overlapping open reading frames (ORFs), and the complete sequence of VP2 is contained within the VP1 sequence (Reed et al., 1988; Parrish, 1995). The genetic structure of FPV displays greater than 98 % homology with the N strain of CPV2 in both nucleotide and amino acid sequence (Reed et al., 1988).

3.2.7 Genetic diversity

The results of a recent study by Decaro et al. (2008) presenting the genetic analysis of 39 Italian and British FPV strains reveal that strains detected in Italy and UK were highly related to each other, with a nucleotide identity of 99.1-100 and 99.4-99.8 % among Italian and British strains, respectively, whereas the similarities between all the sequences analyzed were 98.6-100 %. Based on the observed amino acid substitutions and the ratio between synonymous and non-synonymous substitutions on the VP2 gene segment (dS/dN = 0.10), the same authors conclude that the current evolution of FPV is driven by random genetic drift rather than by positive selection pressure, suggesting that FPV is in evolutionary stasis (Decaro et al., 2008).

3.3 Type 2 canine parvovirus infection

3.3.1 General informations

CPV2 infection is one of the most frequent causes of death in the young, susceptible canine populations worldwide. The causative agent is a member of the *Parvoviridae* virus family, closely related to the previously described FPV (Appel et al., 1979).

The disease was first reported in the late 1970's almost simultaneously in several parts of the world, such as in Europe (Appel et al., 1979; Burtonboy et al., 1979) and Australia (Kelly, 1978; Johnson & Spradbrow, 1979). Today there are several theories regarding the sudden emergence of CPV2, such as (1) the new pathogen arose as a host range mutant directly from FPV in the dog or cat populations; (2) another hypothesis was that CPV2 emerged from a FPV vaccine virus after propagation in tissue culture and that it was initially spread in vaccines; (3) CPV2 arose in a host different from the cat or dog, and that another, presumably wild carnivore may have harbored the immediate ancestor of CPV2 (Truyen, 1999). The result of the genetic investigations of wild-type and vaccine strains of FPV, early and recent CPV2 strains imply that the third hypothesis is the most probable one (Truyen et al., 1998; Horiuchi et al., 1998), but the exact origin of CPV2 still remains a mystery.

In 1979, shortly after the emergence and worldwide spread of CPV2, the virus suffered a few mutations that led the appearance of a new genotype, named CPV2a. This genotype differed from the original strains in only one amino acid (Parrish et al., 1988). Simultaneously to the emergence of CPV2a, the original genotype has completely disappeared from the receptive populations. In 1984 a new genotype named CPV2b, differing also by only one amino acid compared to CPV2a has emerged, and similarly to its "predecessors" has spread all around the world. Both CPV2a and CPV2b differ antigenically from CPV2, and in contrast to the original genotype, were able to infect cats (Parrish et al., 1988; Hoelzer et al., 2008b). In 2000 the emergence of a new genotype (CPV2c) has been reported in Italy (Buonavoglia et al., 2001) and subsequently in other European countries, such as Spain, Germany, the United Kingdom (Decaro et al., 2006b, 2007a), in Asia (Nakamura et al., 2004), as well as in South America (Pérez et al., 2007) and the United States of America (Hong et al., 2007). The genetic differences among the new genotypes are determined only by residue 426 of VP2, with types 2a, 2b and 2c displaying Asn, Asp and Glu, respectively (Decaro et al., 2007a).

Today a very diverse distribution of types 2a, 2b and 2c can be observed in most European countries (Decaro et al., 2007a), and there is evidence that the newest genotypes tend to replace previous variants (Martella et al., 2004; 2005; Decaro et al., 2007a). This replacement has been associated with increased ability to bind to canine transferrin receptors, although it might not rule out the possibility of mutations at residue 426 of the VP2 protein being selected also for their antigenic effects (Truyen, 2006; Decaro et al., 2007a).

CPV2 infections have been reported in several species, such as domestic dogs, bush dogs *(Speothos venaticus)*, coyotes *(Canis latrans)*, maned wolves *(Chrysocyon brachyurus)* and crab-eating foxes *(Cerdocyon thous)*, but it can be assumed that most if not all *Canidae* are susceptible (McCaw & Hoskins, 2006). The original CPV2 isolates infected only dogs, whereas the newer genotypes (2a, 2b) can also infect cats experimentally and naturally as well (Parrish et al., 1988; McCaw & Hoskins, 2006).

Not all infected animals develop clinical signs. Clinical illness however is most severe in young, rapidly growing puppies with concurrent parasitic infestation and/or bacterial, viral infections. In susceptible populations, the incidence of severe disease and death can be very high (McCaw & Hoskins, 2006).

CPV2 is highly contagious and most infections occur as a result of contact with contaminated feces in the environment or fomites. Previous observations have demonstrated that the infectious virus survives for at least one year in sandy or clay soils (Greene & Schultz, 2006). The incubation period can vary among genotypes from 7 to 14 days, but in field conditions in case of CPV2a and 2b it can be as brief as 4 to 6 days. Acute CPV2 infections can be seen in dogs of any breed, age or sex. Nevertheless, pups between 6 weeks and 6 months of age, and some large breeds seem to have an increased risk (McCaw & Hoskins, 2006).

3.3.2 Pathogenesis

Similarly to FPV, the pathogenesis of CPV2 is defined by the requirement for mitotic cells, dividing lymphoid and intestinal epithelial cells being the primary targets of the virus (Parrish, 1995).

The site of virus entry and initial natural infection occurs through the cells of the nasopharynx, the tonsils or other lymphoid tissues (Appel et al., 1979). Virus is isolated between 1 and 3 days after the infection from the tonsils, retropharyngeal lymph nodes, thymus and mesenteric lymph nodes, and after approximately 3 days virus is also

recovered from the intestinal-associated lymphoid tissues and Peyer's patches. Virus spreads systemically through a plasma viremia, resulting in widespread infection of the lymphoid tissues, including the thymus and all lymph nodes (Parrish, 1995). Marked plasma viremia is observed 1 to 5 days after infection. Following viremia, CPV2 localizes predominantly in the epithelium of the digestive tract and lymphoid tissues (McCaw & Hoskins, 2006). Infection of the bone marrow results in suggestive hematological features, such as leukopenia, lymphopenia and neutropenia, but differences in the severity of hematological values caused by the different genotypes have been demonstrated. Under experimental conditions, the severity of leukopenia coincided with the mortality of the CPV2 infected animals (Moon et al., 2008).

In the intestinal mucous membrane, CPV2 infects the germinal epithelium of the intestinal crypts, causing destruction, shortening and collapse of the villi. The most common complications of CPV2 infections are represented by secondary bacterial infections and DIC. Shedding of viral particles occurs 3-4 days after exposure, and usually lasts 7-10 days (McCaw & Hoskins, 2006).

3.3.3 Clinical signs

Severity of clinical signs is characterized by a marked variation, and can range from inapparent infection to acute fatal disease. Most dogs develop an inapparent or subclinical infection. The severity of CPV2 infection depends on the animal's age, stress level, breed, and immune status, but the most severe cases can usually be observed in puppies younger than 12 weeks, because they lack protective immunity and have an increased number of actively dividing cells (McCaw & Hoskins, 2006).

The most frequently encountered *digestive signs* are represented by vomiting, diarrhea, and mild to extreme dehydration. The feces appear yellow-gray and are streaked or darkened by blood. In severe cases fever and lekopenia can also occur. In case of secondary gram-negative sepsis or DIC, death can occur as early as 2 days following the onset of clinical signs (McCaw & Hoskins, 2006).

In utero infections or CPV2 infection of puppies younger than 8 weeks can result in *viral myocarditis*. When present, the illness affects all puppies of the same litter. These puppies die suddenly, without apparent previous illness, or following a short episode of crying, dyspnea and retching. Sometimes enteric signs are also present. Puppies that survive the initial infection can die weeks or months later as a result of congestive heart failure. The frequency of viral myocarditis decreased significantly and

became an occasional finding, compared to its occurrence during the widespread epizootic outbreaks of the late 1970's in CPV naive dogs (McCaw & Hoskins, 2006).

CPV2 infections have also been associated with *neurological diseases*, but these are more commonly secondary consequences of DIC, hypoglycemia, sepsis or acid-base electrolyte disturbance and hypoxia (Agungpriyono et al., 1999). *Cutaneous manifestations*, such as erythema multiforme have also been reported in case of a severe necrotizing CPV2 infection, suggesting that viral pathogens should also be considered in case of canine erythema multiforme in which a causative drug cannot be clearly established (Favrot et al., 2000).

Secondary clinical complications of CPV2 infections are represented by thrombosis due to hypercoagulability, asymptomatic bacterial urinary tract infections, and intravenous catheter infection (McCaw & Hoskins, 2006).

3.3.4 Pathology

The most relevant pathological changes can be found in the small intestine, myocardium, and lymphoid tissues. The most severely affected intestinal segment is the distal duodenum and jejunum. These intestinal loops appear thickened and segmentally discolored, with denudation of intestinal mucosa and the presence of dark, sometimes bloody, watery material within the stomach and intestinal lumen (McCaw & Hoskins, 2006). The enlargement of the mesenteric lymph nodes is also considered to be a very frequent finding. Histopathologically, the shortening and collapsus of the intestinal villi, and the presence of necrotic debris in the dilated crypts, as well as the presence of large, intranuclear inclusion bodies in the epithelial cells of the digestive tract are considered to be highly suggestive of a CPV2 infection. The pathologic changes may range from mild inflammation to diffuse, hemorrhagic enteritis (McCaw & Hoskins, 2006). Necrosis and depletion of lymphoid tissues, such as Peyer's patches, mesenteric lymph nodes, thymus and spleen, are also present.

Viral myocarditis is characterized macroscopically as pale streaks in the myocardium (McCaw & Hoskins, 2006). The lesions are usually represented by nonsuppurative myocarditis, mild fibrosis, myocyte degeneration, microcalcification, and the presence of intranuclear inclusion bodies (Agungpriyono et al., 1999). The presence of CPV2 particles can be demonstrated by the means of EM, IFAT, IHC and ISH (McCaw & Hoskins, 2006).

3.3.5 Diagnosis, treatment and prevention

Antemortem diagnosis of CPV2 infection is based on clinical signs, history, in-hospital fecal ELISA testing, virus isolation, EM scan of feces, HI, VN and PCR-based techniques. All of these techniques have their own limitations. Currently the PCR-based tests seem to be the most sensitive ones, and also allow the differentiation of CPV2 variants (Decaro et al., 2006a; McCaw & Hoskins, 2006; Lamm & Rezabek, 2008).

Postmortem diagnosis is confirmed by the presence of suggestive pathologic changes, histopathology, IHC, ISH, EM examination and PCR-based testing of tissue samples, as well as virus isolation from various organs (Lamm & Rezabek, 2008).

Treatment of parvovirus infection is supportive and symptom based, with the primary goals being the restoration of fluid and electrolyte balance and the prevention of secondary bacterial infections (McCaw & Hoskins, 2006; Lamm & Rezabek, 2008). With appropriate care, approximately 70 % of parvovirus cases should respond to medical therapy. Recovered animals maintain protective immunity against that strain for life (Lamm & Rezabek, 2008). In shelter and boarding facilities, such as kennels, due to the high resistance of the pathogen, very effective precautionary and disinfecting measures should be taken.

Just like in case of FP, protection against CPV2 infection is provided by maternal and passive immunity, and by active immunization (McCaw & Hoskins, 2006). Previous studies have irrefutably demonstrated that vaccination of dogs is critical and recommended (Decaro et al., 2007b; McCaw & Hoskins, 2006; Lamm & Rezabek, 2008). Currently the routinely used types of vaccines are represented by MLVs and inactivated vaccines.

The window of susceptibility for infection in pups with adequate levels of MDA actually begins 2 to 3 weeks before the waning of MDA at 8 to 12 weeks of age. To maximize the effectiveness of vaccination, a series of vaccinations over this window is recommended which should be developed on a case-by-case basis, with consideration of age, environment, and recommendation of the manufacturer (Lamm & Rezabek, 2008). Currently there are several effective brands of CPV2 MLV vaccines on the market, most of them still containing the initial type 2 strains, while the most recent products already contain newer type 2b strains.

Several studies have demonstrated that puppies can get infected even following vaccination, but they have also revealed that most cases of parvovirus-like disease occurring shortly after vaccination are related to infection with field strains of CPV2

(Decaro et al., 2007b). There is an ongoing dispute regarding the effectiveness of type 2 based vaccines against the new variants of the virus, but it is generally accepted that they induce a sufficient protective response (Truyen, 2006; Spibey et al., 2008).

3.3.6 Genetic characteristics of the pathogen

The genetic organization of CPV2 is very similar to the FPV genome. CPV2 contains a linear single stranded DNA of about 5200 kb. The genome has two ORFs: a left hand ORF encodes two non structural proteins (NS1 and NS2), and a right handed ORF encodes the two capsid proteins (VP1 and VP2), which are translated from alternatively spliced mRNA (Fig. 4; Reed et al., 1988; Horiuchi et al., 1998).

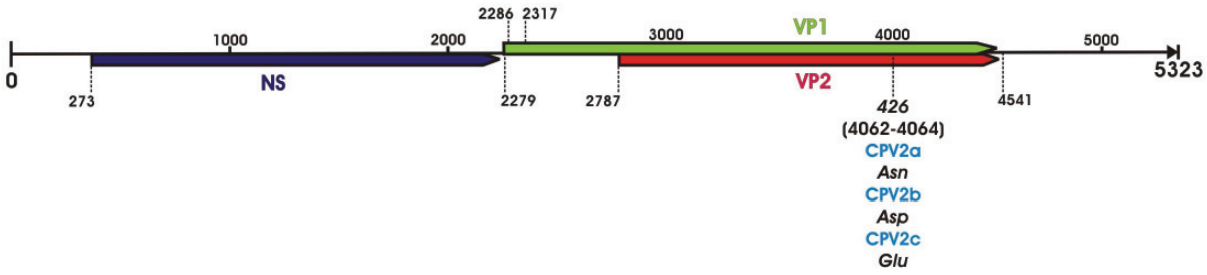

Fig. 4: Genetic organization of CPV2, with special emphasis on the aa present in postion 426 of VP2, the basis of CPV2 genotyping

Previous studies have demonstrated that the host range of parvoviruses is determined by the VP2 gene (Parrish, 1991; Chang et al., 1992). Characterization of CPV2/FPV chimeric viruses revealed that the host range of CPV2 is determined by aa93, 103 and 323 in VP2 (Chang et al., 1992; Horiuchi et al., 1998). Due to this fact the VP2 gene is most suitable for the genetic analysis of parvoviruses.

3.3.7 Genetic diversity

An extensive study by Decaro et al. (2007a) on the molecular epidemiology of CPV2 reveals that all genotypes of the virus are currently circulating in Europe, but there seems to be an evident diversity in the geographical distribution: while the new variant 2c is widespread in countries such as Italy, Portugal and Germany, and is sporadic in the UK, CPV2a is most frequent in Belgium, whereas in the UK, Germany and Italy it has

been overtaken by CPV2b and/or CPV2c. In Portugal on the other hand, 2a strains have not been detected at all, and type 2b and 2c strains seemed to be equally distributed.

A study performed by Pérez et al. (2007) reveals that type 2c strains are most frequent in Uruguay, and out of 30 analyzed samples only one turned out to be type 2a CPV. The situation in the USA seems to be somewhat different: out of 27 CPV2 positive samples only one turned out to be type 2a, 19 type 2b, and 7 type 2c CPV strains (Hong et al., 2007).

4. Materials and Methods

4.1 Examinations and investigations

All examinations and investigations were performed at the diagnostic laboratory of the Department of Pathology and Forensic Veterinary Medicine of the Faculty of Veterinary Science, Szent István University (Budapest, Hungary).

4.2 Samples

4.2.1 CDV samples

Samples were collected from clinically ill dogs *(Canis familiaris) (clinical samples)* and from organs of one fox *(Vulpes vulpes)*, one raccoon *(Procyon lotor)*, one ferret *(Mustela putorius)* and dogs that died following clinical signs suggestive of a viral infection, or when pathological changes indicative of such a disease were found during necropsy *(necropsy samples)*. The animals from which the samples were taken had various clinical backgrounds and mostly unknown immunization histories (Table 1).

A total number of 214 samples were analyzed by RT-PCR for the dectection of CDV. Most of these (n = 186) were represented by *clinical samples* such as blood, urine, nasal swab, and feces samples. As part of a monitoring survey, 99 samples were collected from animals living at the Dog Shelter of the City Council of Budapest. These samples were taken during the spring of 2005 and during the spring and summer of 2006. The interval of time between the collection of the first and the last samples lasted more than a month in both cases. The samples consisted of blood, urine and nasal swabs. Blood was collected in two separate tubes from each animal: one contained an anticoagulant (EDTA), while the other did not. The serum was obtained from the clotted blood following centrifugation (20 min at 1500 x g). In case of the anticoagulant-treated samples, the leukocytes were separated from the rest of the blood components using the hypo-osmotic shock technique. The rest of the clinical samples were collected at various small animal practices in Budapest and other parts of Hungary, and in the Department of Internal Medicine of our Faculty.

Table 1: Description of the analyzed CDV samples

Group	Sample code	Anamnesis*	Analyzed sample
I	H04Bp1F (N)	5 months old, female vizsla; normal temperature, only neurological signs; euthanized; histology: lymphoplasmocytic encephalitis; V	Urine and urinary bladder
IIa	H05Bp2S (C)	1 year old, mixed breed male; respiratory signs; UVH	Blood
	H05Bp3S (C)	10 months old mixed breed female; respiratory signs; UVH	Blood
	H05Bp4S (C)	3 years old, mixed breed male; respiratory signs; UVH	Blood
	H05Bp5F (C)	2 years old, mixed breed male; respiratory signs; UVH	Urine
	H05Bp6F (C)	4 months old, female labrador retriever; respiratory, digestive and neurological signs; UVH	Urine
	H05Bp7F (C)	1 year old, mixed breed female; respiratory signs; UVH	Urine
IIb	H06Bp8F (C)	4.5 years old, male rottweiler; respiratory signs; UVH	Urine
	H06Bp9S (N)	1 year old, mixed breed male; severe respiratory and digestive clinical signs; euthanized; pathological changes, characteristic to CD; UVH	Urinary bladder
	H06Bp10S (C)	1.5 years old mixed breed female; respiratory signs; UVH	Nasal swab
III	H06Ny11 (C)	2 years old, mixed breed male; respiratory & digestive signs; NV	Urine
	H06Ny12 (C)	1 year old mixed breed male; severe respiratory and digestive signs; UVH	Urine
	H06Ny13 (C)	2.5 years old mixed breed female; severe respiratory and digestive signs; UVH	Urine
X	712/07 (N)	1 year old female ferret; neurologic signs, enteritis; V	Lungs, brain
X	1210/2007 (N)	1.5-2 year old vixen; severe *Sarcoptes* infection, otherwise in advanced state of autolysis; NV	Urine and urinary bladder
X	1265/2007 (N)	1 year old male raccoon; sero-mucous nasal discharge, bronchitis and interstitial pneumonia, general lymphadenopathy; V	Urine, lungs

Group I: sample obtained from Budapest (winter of 2004)

Group IIa: samples obtained from animals from or retrieved from the Dog Shelter of the City Council of Budapest, during the first period of investigation (spring of 2005)

Group IIb: samples obtained from animals from or retrieved from the Dog Shelter of the City Council of Budapest, during the second period of investigation (spring and summer of 2006)

Group III: samples obtained from Eastern Hungary (winter of 2005)

*UVH: unknown vaccination history, V: vaccinated; NV: not vaccinated; X = not used for genetic analysis, C = clinical sample, N = necropsy sample

Necropsy samples (n = 28) were represented by urinary bladder, spleen, kidney, lung, brain, and cerebellum. Table 1 contains the description of the CDV positive animals, their provenience, clinical signs and immunological status, and type of sample(s) used to purify nucleic acid.

Vaccine samples were obtained from commercially available vaccines (Table 2), and were used to test a newly developed RFLP-based test. Nucleic acid sequences of virus strains present in these vaccines were also used in the subsequent phylogenetic analysis.

Table 2: Description of the CDV vaccines analyzed in the present study

	Vaccine brand	Manufacturer	Batch number*	Year of production	Country of origin
1	Vanguard	Norden Laboratories	NA	1992	Hungary
2	Vanguard	Smith Kline	NA	1994	Hungary
3	Vanguard Plus 5	Pfizer Animal Health	L53665	2006	Hungary
4	Vanguard Plus 5	Pfizer Animal Health	A602088C	2006	Israel
5	Vanguard Plus 7	Pfizer Animal Health	L60065	2006	Malta
6	Vanguard Plus 5	Pfizer Animal Health	A602620B	2006	USA
7	Canigen DH(A2)PPi	Virbac S.A.	12TU	2006	Hungary
8	Eurican DHP	Merial	L246779	2006	Hungary
9	Nobivac DHP	Intervet	A030C01	2006	Hungary
10	Canvac 8	Dyntec	040407	2007	Hungary

*NA: not available

4.2.2 FPV and CPV2 infections

A total number of 230 samples were analyzed by RT-PCR for the detection of FPV and CPV2. Samples were represented by fecal samples obtained from clinically ill patients and tissue samples acquired during necropsy from 33 cats *(Felis catus)*, 195 dogs, one Asian palm civet *(Paradoxurus hermaphroditus)*, and one lion *(Panthera leo)*. The analyzed samples were acquired by different departments of the Faculty of Veterinary Science, or they were sent in by veterinary practitioners from different parts of Hungary. Table 3 contains the description of the parvovirus (20 CPV2 and 3 FPV) positive samples selected for genetic analysis. The Asian palm civet was submitted by an exotic animal dealer from Budapest, while the lion carcass was sent in for diagnostic

purposes by a circus lion tamer from Érd, Hungary. Neither the palm civet nor the lion had received any vaccinations.

Table 3: Parvovirus positive samples analyzed in the present study

	Reference number	Year of collection	Description of the animals*
1	3657/04	2004	4.5 months old, ♀ Cane Corso (V)
2	1824/06	2006	3 months old, ♀ Chihuahua (V)
3	379/07	2007	3 weeks old, ♂ Yorkshire Terrier (NV)
4	590/07	„	3 months old, ♀ Rottweiler (V)
5	591/07	„	3 months old, ♀ Rottweiler (V)
6	653/07	„	1 month old, ♂ mixed breed (UVH)
7	663/07	„	2 months old, ♂ mixed breed (UVH)
8	889/07	„	2 months old, ♂ mixed breed (UVH)
9	1093/07	„	3 months old, ♂ Chihuahua (V)
10	1100/07	„	4 months old, ♂ Pug (V)
11	1309a/07	„	1.5 months old, ♂ English Bulldog (V)
12	1309b/07	„	1.5 months old, ♀ English Bulldog (V)
13	1309c/07	„	1.5 months old, ♂ English Bulldog (V)
14	1309d/07	„	1.5 months old, ♀ English Bulldog (V)
15	1353/07	„	2 months old, ♂ French Bulldog (V)
16	667/08	2008	3 months old, ♂ Cane corso (V)
17	772/08	„	7 months old, ♀ mixed breed (UVH)
18	773/08	„	2 months old, ♂Central Asian Shepherd (V)
19	789/08	„	1.5 months old, ♂Gordon setter (V)
20	794/08	„	1 month old, ♂ Havanese (NV)
21	389/07	2007	2 months old, ♂ Asian palm civet (UVH)
22	933/07	„	1 year old, ♂ domestic cat (UVH)
23	1335/07	„	7 weeks old, ♂ Persian cat (NV)
24	1259/08	2008	1.5 years old lioness (NV)

* V: vaccinated; NV: not vaccinated; UVH: unknown vaccination history

4.3 Histopathology

Tissue samples for the histopathological examination were fixed in 8 % buffered formaldehyde solution for 24 h, embedded in paraffin, then 4 µm thick sections were cut using a sledge type microtome (Reichert, Austria) and stained with HE according to the routine methods (Lynch et al., 1969), and then examined under light microscope.

4.4 Electron microscopic investigation

Urine and feces samples were suspended 1:3 in distilled water, cleared by low speed centrifugation, followed by 20 min at 9000 x g. The samples were prepared according to the single-droplet negative staining technique (Harris, 2007) and examined at a JEM 1011 transmission electron microscope (JEOL, Japan).

4.5 Genetic investigations

4.5.1 Purification of the nucleic acid

Tissue samples were homogenized in 10 ml phosphate buffered saline (PBS) and centrifuged at 1500 x g for 10 min. The nasal swabs were immersed in PBS solution with 10 ml/L antibiotic and antimycotic component (Sigma, USA) and then centrifuged, while the liquid samples (i.e. urine, serum) were only centrifuged. The viral nucleic acid was isolated from the supernatants using the QIAamp viral RNA Mini Kit (Qiagen, Germany) and High Pure Viral Nucleic Acid Kit (Roche, Switzerland), according to the manufacturers' instructions.

4.5.2 Primers

4.5.2.1 CDV

Primers were designed for diagnostic and for phylogenetic purposes as well. For diagnostic purposes, primer pair "CDV-A" specific to a 409 bases long segment of the conservative region of the L gene of the CDV genome was designed. Samples that tested positive with these primers underwent another RT-PCR assay using the "CDV-B" set of primers that partially encompassed the H gene of the CDV. The amplicons produced by primer pair "CDV-B" were subsequently used for an RFLP-based strain discriminating technique. The phylogenetic analysis of the Hungarian and other CDV strains was based on the full nucleotide sequence of the H and F genes, determined by using other sets of primers (Table 4). The sensitivity of the diagnostic PCRs using primer pair "A" was determined by using serial dilutions of a vaccine virus strain with a previously determined number of CDV RNA copies (10^5) (data not shown). Primers F to K were used only for vaccine 3 (Table 2).

Table 4: Primers used for the diagnosis and genetic analysis of CDV strains

	Sequence 5' to 3'	Target gene	Sense	Position	Annealing (°C)	Amplicon size (bp)
A	ATCCGCTCATCGATCAAGAC	L	+	12400-12420	52	409
	CAAGCCTCTTGCCAAGATTC		-	12788-12808		
B	AGGCCGTACATCACCAAGTC	H	+	7323-7343	52	1110
	TGGTAAGCCATCCGGAGTTC		-	8412-8432		
C	AACTTAGGGCTCAGGTAGTC	H	+	6994-7014	51	2023
	AGATGGACCTCAGGGTATAG		-	8996-9016		
D	AACTTAGGGTCCAGGACRTAGC	F	+	4845-4867	53	1091
	CGTAAACTCGGGCCAAAT		-	5917-5935		
E	GCATTGYTGGACTACCTGAG	F	+	5778-5797	49	1305
	CCTGAGCCCTAAGTTTTC		-	7062-7082		
F	YYACAAGGCTAGGGTTCAGAC	N	+	79-100	52	1739
	ACTCGGAGATGAGAAGGTGGAT		-	1797-1817		
G	GCCTGRTCCTCTGCCATTTA	N	+	676-698	53	1142
	ACTCGGAGATGAGAAGGTGGAT		-	1797-1817		
H	ACCCAGGTCCAACAAACC	P	+	1745-1763	50	1152
	GCCCCTCAATCGTGGAAA		-	2878-2896		
I	ACCCAGGTCCAACAAACC	P	+	1745-1763	53	3420
	GGACTTAGGCTCTTGTGTC		-	3401-3420		
J	AGGATACCGCACCTTCCAA	M	+	3164-3183	50	1025
	TCCCTCCTAGAGCAAACAC		-	4169-4188		
K	AGGATACCGCACCTTCCAA	M	+	3164-3183	52	1596
	GCAAGTGCGTGGRRGTATAA		-	4739-4759		

4.5.2.2 FPV and CPV2

The PCR-based diagnosis of FPV and CPV2 infections was performed using a newly designed primer pair that was able to demonstrate the presence of both pathogens in the analyzed samples. Hence, following nucleic acid isolation and a positive PCR-based test, it was not possible to differentiate FPV and CPV strains, nor distinguish type 2 CPV genotypes.

The phylogenetic analysis was performed using amplicons of several other sets of primer pairs, most of which were newly and specifically designed for the sequential amplification of VP2 of the viral genomes (Table 5).

Table 5: Primers used for the demonstration and genetic analysis of parvovirus strains

Primer pair	Sequence 5'-3'	Sense	Position*	Annealing (°C)	Amplicon size (bp)
CPV/FPV diag F	GACTTGTGCCTCCAGGTTAT	+	2388-2408	49	420
CPV/FPV diag F	GTTGAACTGCTCCATCACTC	-	2787-2807		
F2	ACCAACTTGGCGTTACTCAC	+	2154-2174	50	1014
R4	GTTGCCAATCTCCTGGAT	-	3149-3167		
CPV2-FPV-F	CACAGCAAACTCAAGCAGAC	+	2968-2988	48	898
CPV2-FPV-R	GTCCTGCTGCAATAGGTGTT	-	3846-3866		
VPM**	TGGAGGTAAAACAGGAATT	+	4094-4113	52	437
VPR**	TTTCTAGGTGCTAGTTGAG	-	4512-4531		

*positions are according to the nucleotide sequence of the CPV2 reference strain (accession number NC001539)

**Mochizuki et al., 1995

4.5.3 Amplifications

4.5.3.1 RT-PCR assays

Reverse transcription and amplifications were performed in a continuous RT-PCR method by using the Qiagen OneStep RT-PCR Kit (Qiagen, Germany). The 25 µl reaction mixtures contained 5 µl of 5 x buffer (final $MgCl_2$ concentration 1.5 mM), 0.4 mM of each deoxynucleozide triphosphate (dNTP), 10 U rRNasin™ RNase Inhibitor (Promega, USA), 0.8 µM of the appropriate forward and reverse primers, 1 µl of enzyme mix and 2.5 µl of template RNA.

Reverse transcription was carried out at 50 °C for 30 min. Following an initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to 35 cycles of heat denaturation at 94 °C for 45 sec, primer annealing for 45 sec at the corresponding temperature (Table 4) and DNA extension at 72 °C for 1 or 2 min, followed by a final extension of 10 min at 72 °C. The reactions were performed in a PCR Sprint Thermal Cycler SPRT001 (Hybaid, UK). Following RT-PCR, 7.5 µl of the amplicons were electrophoresed in a 1.2 % agarose gel (Merck, Germany) at 80 V for 80 min. The gel was stained with ethidium bromide and the bands were visualized at 312 nm using a TFX 35M UV transilluminator (Life Technologies, UK), and using the Kodak Digital Science 1D software program (Kodak, Japan). Product sizes were determined with reference to 100 bp and 1 kb molecular weight markers (Fermentas, Lithuania).

4.5.3.2 Classical PCR assays

The 50 µl reaction mixtures contained sterile deionized water, 10 x PCR buffer without $MgCl_2$, 2 mM dNTP mix with a final concentration of 0.2 mM of each dATP, dCTP, dGTP, dTTP, 25 mM $MgCl_2$ with a final concentration of 1-4 mM, 0.8 µM of the appropriate forward and reverse primers, 1 µl of 1.25 u/50 µl Taq DNA polymerase (Fermentas, Lithuania) and 2.5 µl of template DNA. Following an initial denaturation at 95 °C for 10 min, the reaction mixture was subjected to 35 cycles of heat denaturation at 94 °C for 45 sec, primer annealing at the corresponding temperatures (Table 5) for 45 sec, and DNA extension at 72 °C for 1 or 2 min, followed by a final extension at 72 °C for 10 min.

The visualization of the amplicons was performed according to the previously described protocol.

4.5.4 RFLP-based techniques

4.5.4.1 Differentiation of vaccine and wild-type strains of CDV

Discrimination of vaccine and the 13 Hungarian wild-type strains of CDV was performed using the amplicons produced by primer pair "CDV-B" as follows: 5 µl of the RT-PCR amplicons was mixed with 3.5 µl ddH_2O, 1 µl of *Psi*I enzyme (SibEnzyme, Russia) and 1 µl of B9006S buffer solution provided by the manufacturer. The mixture was then heated to 36 °C for 60 min and mixed at 100 x g, at intervals of 30 sec in a Thermomixer Comfort device (Eppendorf, Germany). The amplicons were then detected by electrophoresis in a 2 % Tris acetate-EDTA-agarose gel at 80 V for 80 min and fragment sizes were determined using the same procedure.

4.5.4.2 Identification of type 2c CPVs

Twenty CPV2 positive samples (Table 2) collected from 2004 to 2008 were analyzed. The identification of type 2c CPVs based on *Mbo*II digestion was performed according to a protocol previously described by Bounavoglia et al. (2001), using primers *555for* and *555rev* that amplified a segment of approximately 582 bp of the VP2 gene of CPV2 strains, encompassing nucleotide positions 4003-4585. In short, 10 µl of the PCR products was mixed with 15 µl double distilled water, 1 µl of *Mbo*II (Fermentas, Lithuania) and 1 µl of puffer B supplied by the manufacturer. The mixture was then incubated overnight at 56 ºC. The subsequent electrophoresis was performed as previously described.

4.5.5 Nucleic acid sequencing and phylogenetic analysis

Following electrophoresis in a 2 % Low Melting Agarose Gel (Bio-Rad Laboratories, USA), the amplicons were cut out from the gel, and DNA was extracted with the QiaQuick Gel Extraction Kit (Qiagen, Germany). Fluorescence-based direct sequencing was performed in both directions on the amplicons at Biomi Kft. (Gödöllő, Hungary) and Biogon Kft. (Budapest, Hungary), using ABI 3100 genetic analyzers (Applied Biosystems, USA). The nucleotide sequences were identified using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The two direction sequences were compiled and aligned using the Align Plus 4 software (Scientific and Educational Software, USA) and Clustal X. Phylogenetic trees were constructed using the nucleic acid sequences of the Hungarian CDV, FPV and CPV2 strains, as well as sequences of corresponding lengths acquired from the GenBank (http://www.ncbi.nlm.nih.gov) by neighbor-joining with two parameters distance matrix (Kimura, 1980), using the Phylip program. The robustness of the groupings in the neighbor-joining analysis was assessed with 1000 bootstrap resamplings in case of the CDV strains and 100 in case of the parvovirus strains.

5. Results

5.1 Macroscopic findings

5.1.1 CDV infections

The macroscopic examination of the carcasses of all infected animals, regardless of species, revealed dehydration of a various degree, the presence of a mucopurulent discharge in the airways, conjunctivitis, enlargement of the mesenteric lymph nodes, interstitial pneumonia and/or catarrhal or suppurative bronchopneumonia (Fig. 5).

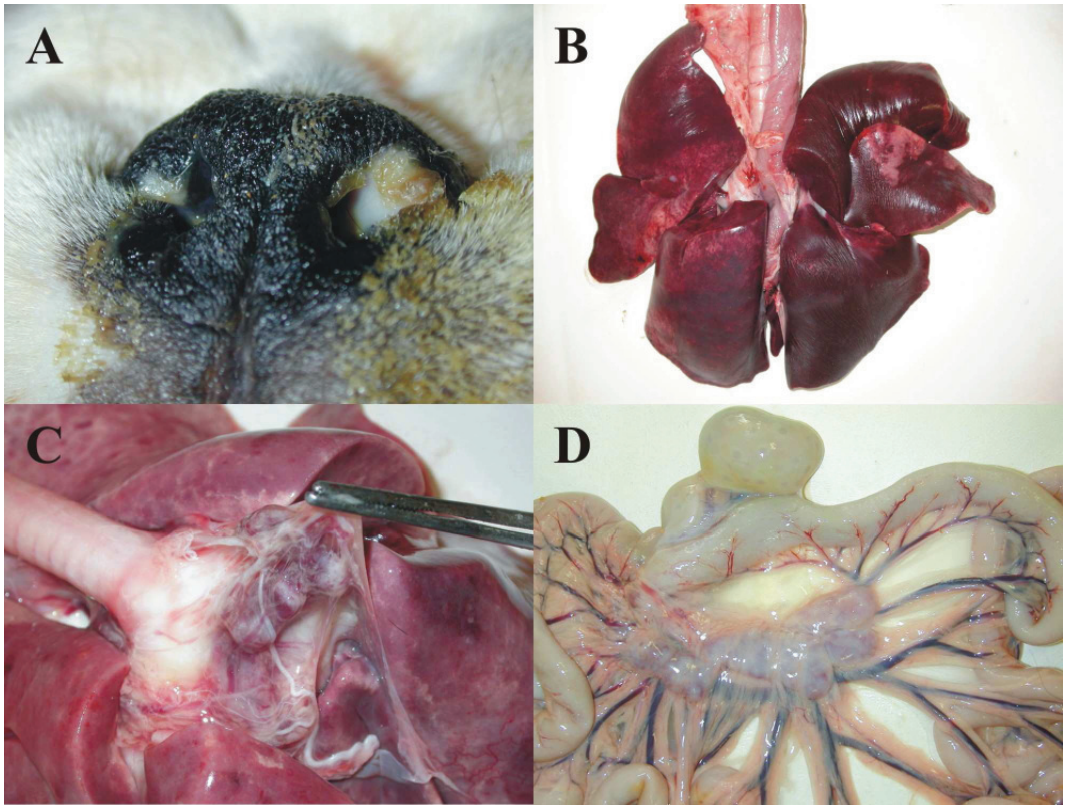

Fig. 5: Macroscopic findings of a dog succumbed following CDV infection. A: mucopurulent nasal discharge; B: interstitial and suppurative bronchopneumonia; C: severe enlargement of the peribronchial and D: mesenteric lymph nodes

In case of the dissected ferret and raccoon, macroscopic signs suggestive of a catarrhal enteritis, severe lung edema and state of shock were observed (Fig. 6). The examined fox suffered of severe dermatitis caused by *Sarcoptes scabiei* infection (Fig. 7).

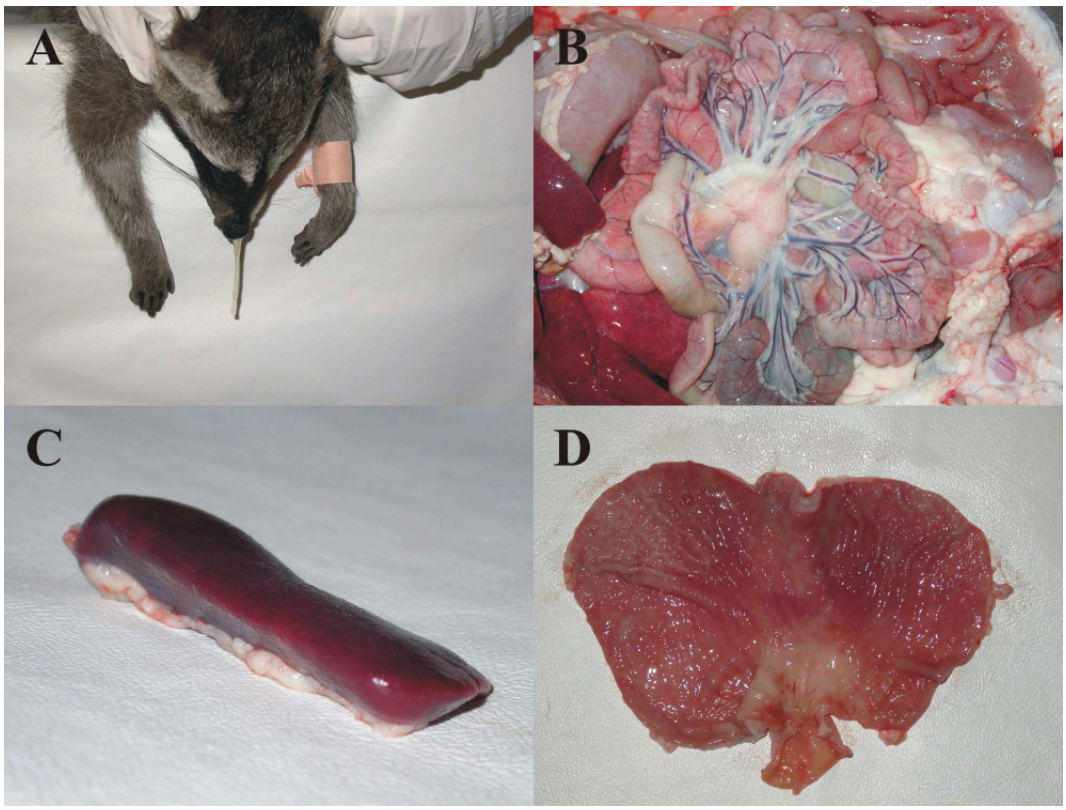

Fig. 6: Macroscopic findings of a CDV positive raccoon. A: mucous nasal discharge; B: catarrhal enteritis and severe enlargement of the mesenteric lymph node; C: enlargement of the spleen (rounded edges); D: catarrhal gastritis

Fig. 7: Signs of *Sarcoptes scabiei* infection on the ears of the examined fox

5.1.2 FPV infections

During necropsy pathological changes suggestive of a parvovirus infection, such as: dehydration, anemia, enteritis and enlargement of the abdominal lymph nodes and of the spleen were observed. In case of the lioness, an extensive bacterial necrotizing myositis, following a traumatic injury that penetrated the skin, could be observed on the left hind limb.

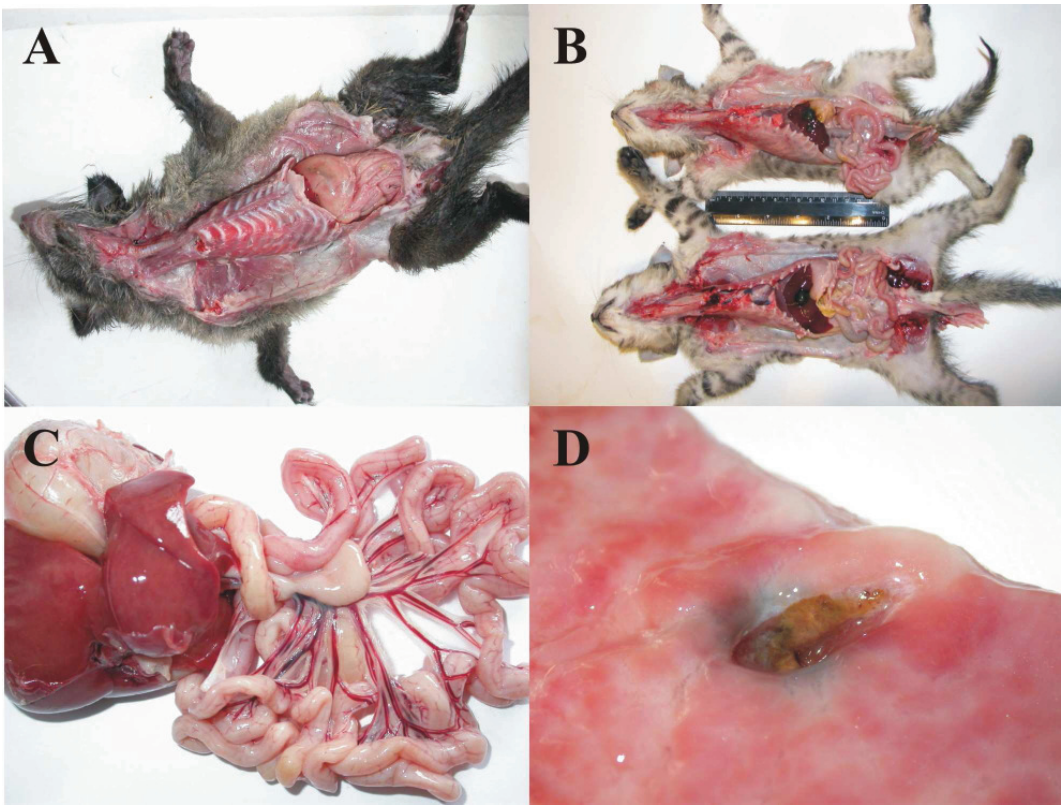

Fig. 8: Macroscopic findings of FPV positive animals analyzed in the present study. A: extreme dehydration and anemia in an Asian palm civet; B: carcass of two dehydrated kittens with firm, extremely dilated intestinal loops; C: enlarged mesenteric lymph node and dilated intestinal loops with characteristic, rigid walls (Asian palm civet); D: ulcerative enteritis in an African lion *(Panthera leo)*

Other findings were represented by a penetrating ulcer in the cranial segment of the duodenum and a subsequent serous peritonitis. In case of the Asian palm civet, the most relevant pathological signs were the severe dehydration, firmness of the affected intestinal loops, enlargement of the mesenteric lymph node and of the spleen (Fig. 8).

5.1.3 CPV2 infection

Pathological changes observed during the dissection of animals succumbed following a CPV2 infection were represented by severe dehydration, anemia, evident signs of diarrhea, severe inflammation of the anterior segment of the small intestine (duodenum and jejunum), enlargement of the gallbladder, lymphadenopathy (especially in the mesenteric lymph nodes) and the presence of a hemorrhagic or yellowish-brown mucous content in the intestinal lumen (Fig. 9).

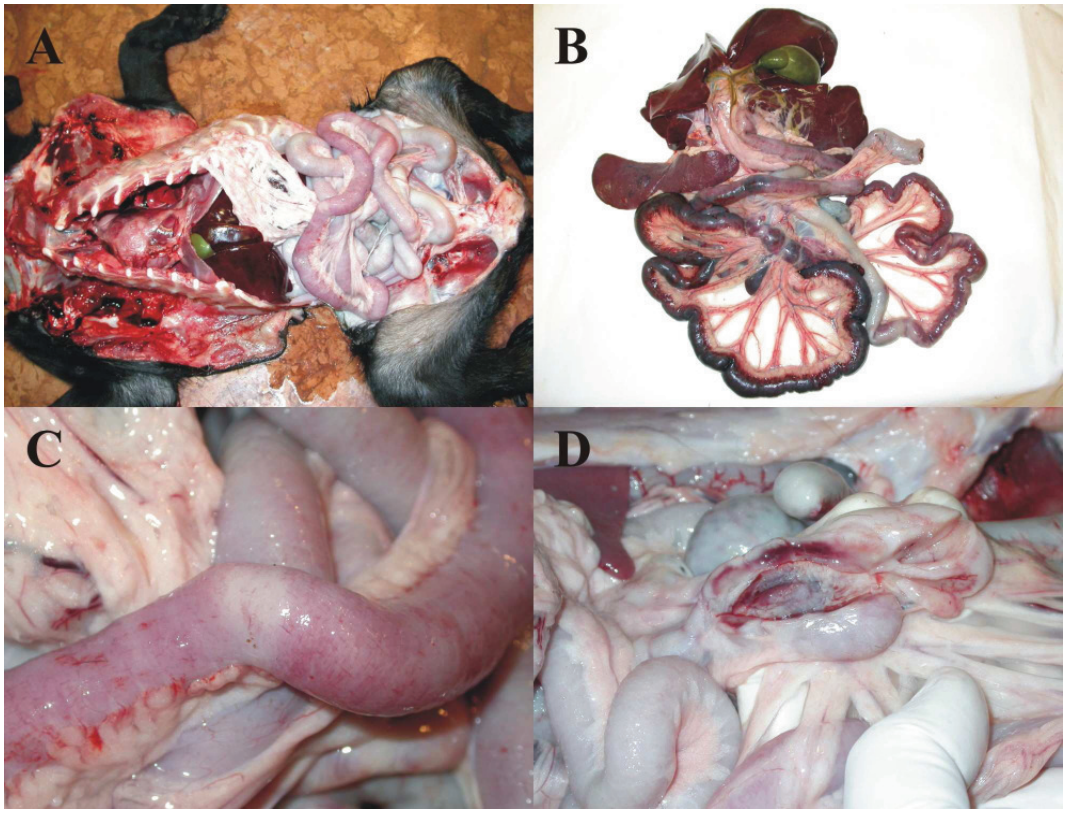

Fig. 9: Macroscopic changes induced by CPV2. A: anemia and evident enteritis affecting the jejunum; B: abdominal organs of a dog showing signs of hemorrhagic enteritis, lymphadenopathy and dilatation of the gall bladder; C: lymphoid depletion as seen in a Peyer's patch; D: cut section of an enlarged mesenteric lymph node with hemorrhagic infiltration

5.2 Histopathology

Routine histopathological examination has been performed in all cases when complete carcasses were available for examination. All findings were in accordance with the changes described in the literature.

5.2.1 CDV infection

Most relevant changes were represented by interstitial and catarrhal or suppurative bronchopneumonia, lymphoid depletion and catarrhal gastroenteritis, perivascular mononuclear cell infiltration in the brain and cerebellum (in case of dog H04Bp1F) and the presence of eosinophilic cytoplasmic inclusion bodies in the digestive and urinary epithelia (Fig. 10).

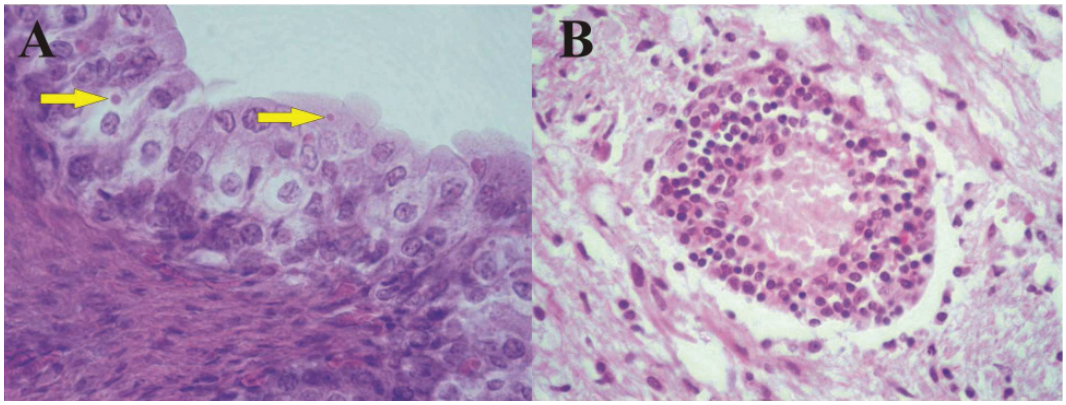

Fig. 10: Histopathology of the urinary bladder and brain of a CDV positive dog. A: eosinophilic cytoplasmic inclusion bodies in the transitional epithelial cells (yellow arrows); B: perivascular mononuclear cell infiltration in the brain (HE staining)

5.2.2 FPV infection

In all FPV infection cases, regardless of species, multifocal crypt necrosis, moderate mononuclear cell infiltration in the propria, shortening of intestinal villi, signs of regeneration tendencies, lymphoid depletion and the presence of nuclear inclusion bodies could be observed (Fig. 11). In case of the lioness, an extended necrotizing myositis in the left hind limb and the penetrating ulceration of the duodenum were also present.

5.2.3 CPV2 infection

In case of the CPV2 positive dogs the most important findings were represented by focal mucous membrane necrosis in the small intestine, shortening of the intestinal villi, marked infiltration of mononuclear inflammatory cells (Fig. 12) and the presence of well differentiated, basophilic nuclear inclusion bodies in the epithelial cells of the

duodenum and jejunum. Signs of severe depletion of the lymphoid elements in the spleen and other lymphoid organs were also observed.

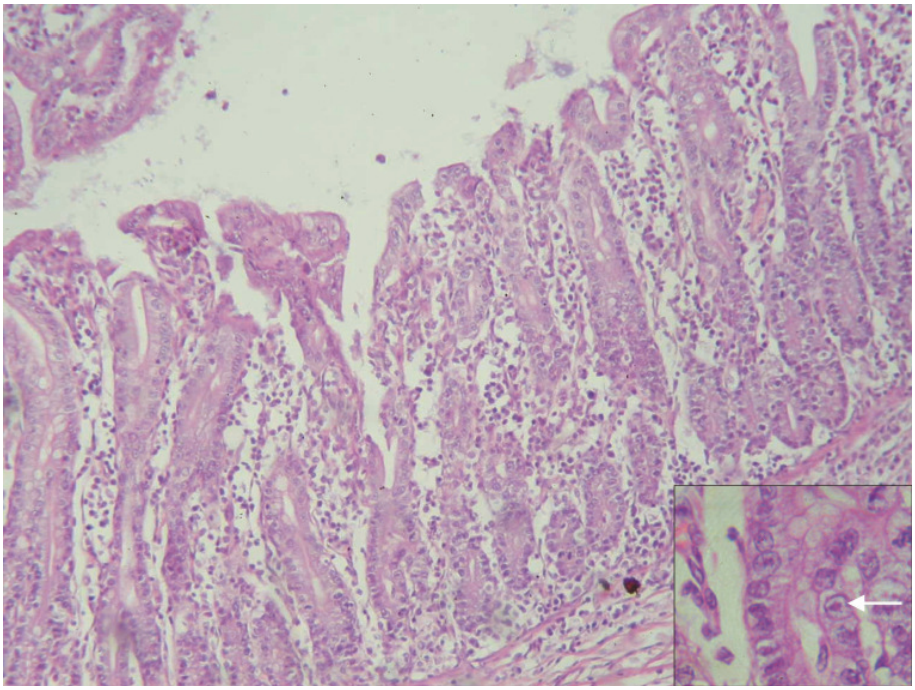

Fig. 11: Intestinal histopathology of the FPV positive Asian palm civet – insert: higher magnification with nuclear inclusion body (white arrow) in an epithelial cell (HE staining)

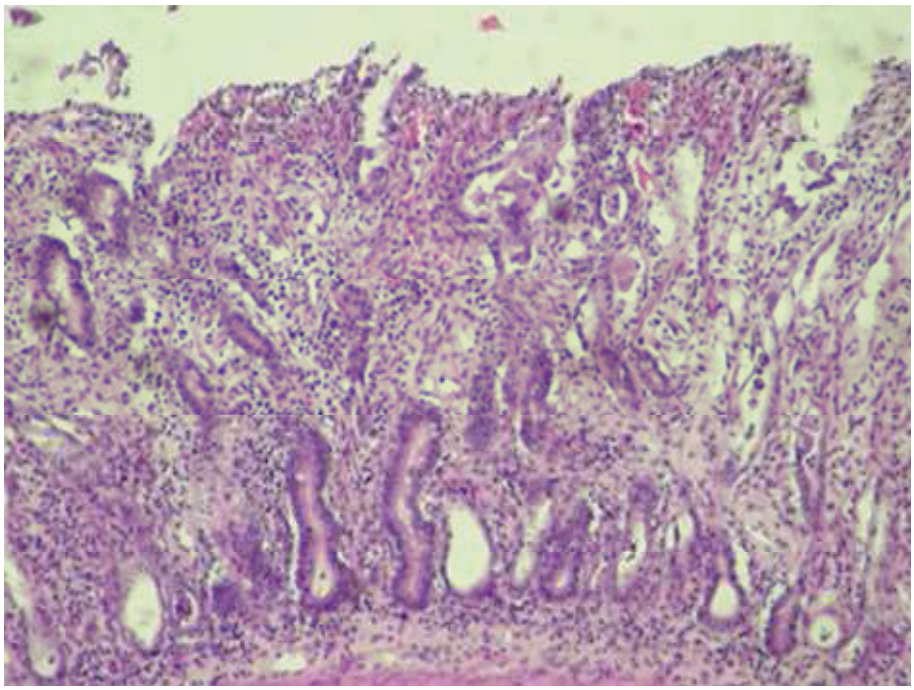

Fig. 12: Histopathological picture suggestive of CPV2 infection: shortening and fusion of the intestinal villi, cryptal regeneration and inflammatory cell infiltration (HE staining)

5.3 Electron microscopy

In the urine samples of CDV positive dogs (samples H04Bp1F and H06Bp9S), virus particles with a diameter of approximately 250 nm and other properties characteristic for paramyxo-viruses were observed (Fig. 9: A). The EM examination of the diluted feces of CPV2 positive dogs and the Asian palm civet revealed the presence of numerous rounded viral particles, measuring about 18 to 21 nm in diameter. Based on their ultrastructural morphology, the viral particles were identified as members of the *Parvoviridae* family (Fig. 13: B).

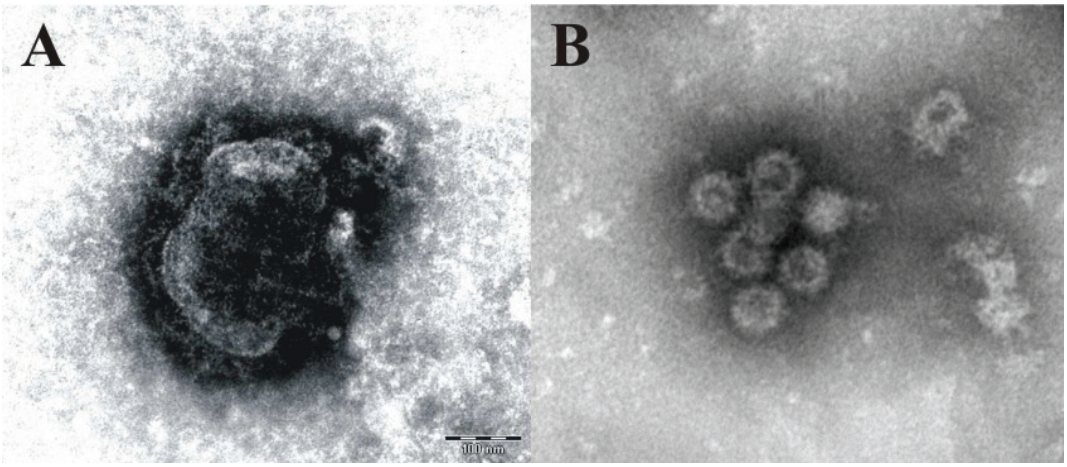

Fig. 13: Negative staining electron microscopic pictures of the viral pathogens. A: viral particle showing characteristics specific for paramyxoviruses; B: viral particles presenting morphological characteristics of the *Parvoviridae* family (negative contrast, 150 000 X)

5.4 Genetic investigations

5.4.1 Amplifications

All amplifications (RT-PCRs and PCRs alike) performed according to conditions described earlier resulted in amplicons of the expected sizes in case of each primer pairs, without the presence of nonspecific bands. Out of the 214 analyzed samples, 58 (27.1 %) proved to be positive for CDV. Parvovirus (FPV/CPV2) genetic material was successfully demonstrated in 72 (31.3 %) out of the 230 analyzed samples (in 17 cats, 1 lion, 1 Asian palm civet and 53 dogs).

5.4.2 RFLP-based investigations

5.4.2.1 Differentiation of wild-type CDVs from vaccine strains

Following the previously described protocol, in case of the CDV strain present in a currently used vaccine (Canigen, Virbac S.A., France) two clearly differentiable bands at the predicted sizes of 294 bp and 816 bp were obtained, while the amplicons of all tested wild-type viruses remained undigested. Therefore, in these cases only one band was visible at the UV examination of the agarose gels in which the electrophoresis was performed (Fig. 14).

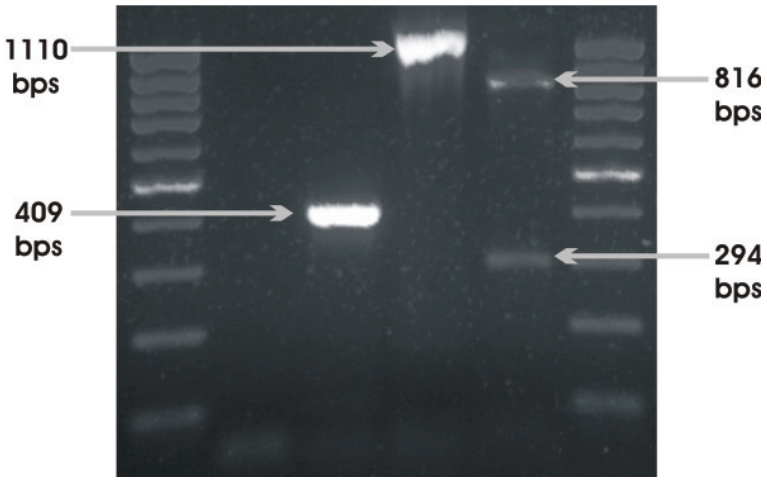

Fig. 14: Results of the PCRs with two of the primer pairs used in the present study, and result of the RFLP analysis based on *Psi*I restriction enzyme recognition site of the CDV H gene amplicons produced by primer pair "B". Lane 1 and 6: 100 bp molecular weight marker (Fermentas, Lithuania); Lane 2: negative control; Lane 3: positive control for primer pair "A"; Lane 4: positive control for primer pair "B"; Lane 5: vaccine strain (Canigen, Virbac S.A., France) digested by the *Psi*I enzyme

The alignment of the sequences with the cleavage site of the *Psi*I enzyme is shown in Fig. 15. It demonstrates that according to the nucleotide sequences deposited in the GenBank, the vaccine strains (Onderstepoort, Snyder Hill, Convac, Lederle) contain at the position of interest a thymine (T), while the wild-type strains contain a cytosine (C). This nucleotide change is present in all of the examined nucleotide sequences of wild-type strains deposited in the GenBank.

When the newly designed enzymatic differentiation was tested in practice by using several virus strains present in CDV vaccines currently used in Hungary (Table 2), the RFLP resulted two clearly differentiable bands in case of most strains present in currently used vaccine at the predicted heights of 294 bp and 816 bp, while the

amplicons obtained from field viruses and a Vanguard vaccine (Table 2, vaccine 3; Pfizer Animal Health, USA) remained undigested (1110 bp) (Fig. 16).

```
ONDERSTEPOORT   1039 ATAACAAACCACCGTGGTTTTATAAAGATTCAATTGCAAC
SNYDER HILL     1039 ......................................
CONVAC          1039 .....T................................
VACCINE JAPAN   1039 ......................................
LEDERLE         1039 ......................................
AB212730        1039 ..C.....T..........C.............G.A.....
AY386315        1039 ........T........G..C..............A.....
AY964110        1039 ........T........G..C..............A.....
AF164967        1039 ........T........G..C..............A.....
AY438597        1039 ........T........G..C..C...............
Z47764          1039 ........T........G..C..............A.....
X84999          1039 ........T........G..C..............A.....
DQ191765        1039 ........T........G..C..............A.T...
AY964114        1039 ........T..........C.............G.A.....
AY964108        1039 ........T..........C.............G.A.....
H04BP1F         1039 ........T........G..C..............A.....
H05BP2S         1039 ........T..........C.............G.A.....
H06NY13         1039 ........T........G..C..............A.....
H06BP10F        1039 ........T..........C....G.......G.A.....
```

Fig. 15: The *Psi*I enzyme cleavage site (shaded) and the exact location of the genetic marker (red) that allows the differentiation of wild-type viruses from the vaccine strains

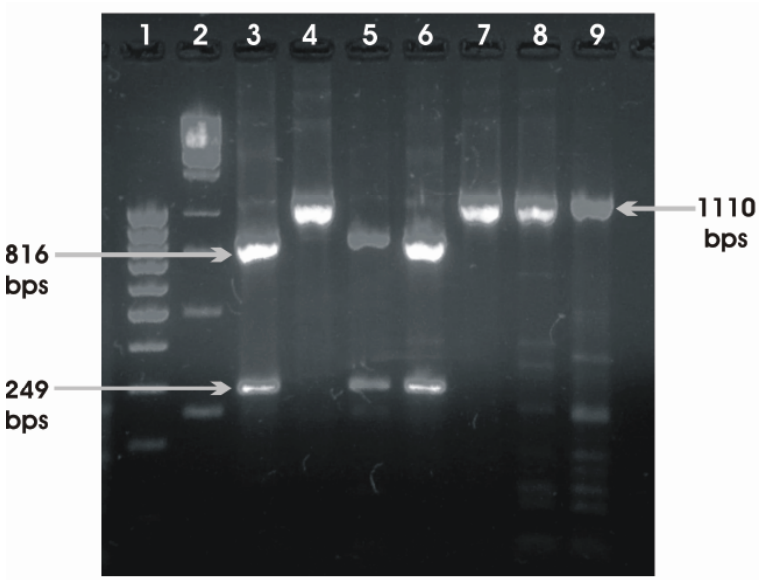

Fig. 16: Results of RFLP after cleavage by restriction enzyme *Psi*I at the recognition site within the CDV H gene. Lane 1: 100 bp molecular weight marker (Fermentas, Lithuania); Lane 2: 1 kb molecular marker (Fermentas, Lithuania); Lane 3: "Nobivac" vaccine strain; Lane 4: "Vanguard" vaccine strain; Lane 5: "Eurican" vaccine strain; Lane 6: "Canigen" vaccine strain; Lanes 7-9: wild-type strains

The same result was obtained from the repeated RFLP test. In order to extend the investigations, different batches of Vanguard vaccines (Pfizer Animal Health, USA) dating back to 1992 and 1994 were involved in the study (Table 2, vaccines 1-2). Furthermore, batches of the same brand were purchased in different countries as well, such as Israel, Malta and USA (Table 2, vaccines 4-6). The RFLP test unequivocally resulted in undigested amplicons in case of all batches from different time and geographical origin.

5.4.2.2 Identification of type 2c CPVs

The attempt to identify type 2c CPVs was performed on twenty PCR-positive samples acquired from dogs. Following the *Mbo*II-based enzymatic digestion one clearly visible band (at approximately 490 bp) was obtained (the "shorter" amplicons of approximately 90 bp were barely visible) in case of samples 1 and 4-17, while the amplicons of samples 2, 3, 18-20 and of the control type 2b vaccine strain (Quantum, Schering-Plough, USA) remained undigested (Fig. 17).

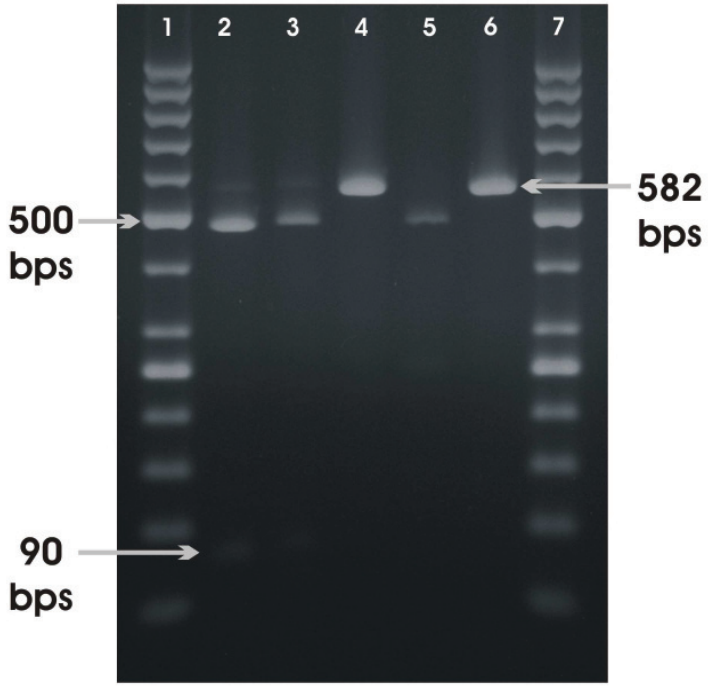

Fig. 17: Result of the agarose gel electrophoresis with the digestion of the amplicons of some of the Hungarian CPV2 strains. Lanes 1 and 7: molecular weight markers (50 bp Gene Ruler Ladder, Fermentas, Lithuania); lanes 2-5: Hungarian CPV2 strains; lane 6: control type 2b CPV vaccine strain (Quantum, Schering-Plough, USA)

5.4.3 Sequence analysis and phylogeny

5.4.3.1 CDV strains

- Wild-type strains

The results of the sequence analysis demonstrated that the Hungarian CDV strains are similar to other typical CDVs reported in different parts of the world. Following BLAST searches, the Hungarian sequences showed different levels of identity with nucleotide sequences deposited in the GenBank.

H gene

The initial investigations performed on the full nucleic acid sequences of the H gene revealed that the Hungarian groups were differently clustered on the phylogenetic tree (Fig. 18). The virus strains obtained from the Dog Shelter of the City Council of Budapest were positioned in the group of Arctic strains; sample H04Bp1F was grouped in the cluster of European isolates, while samples H06Ny11, H06Ny12 and H06Ny13 showed the highest level of identity with strains belonging to the so-called "European wildlife" group (Fig. 18).

The Hungarian strain dating back to 2004 (H04Bp1F) showed the highest level of identity with other European strains, such as isolates from Germany (AY386315: 98.2 %), and Denmark (AF478544: 97.9 %), and only 92.0 % to the Onderstepoort reference strain (AF378705). Hungarian CDV strains obtained from the shelter (Groups IIa and IIb) clustered to the Arctic group were closely related to virus strains from countries geographically distant from Hungary, such as the United States of America (98.95-99.01 %), Italy (99.23-99.28 %), China (97.86-97.91 %) and Greenland (97.31-97.36 %), as well as to phocine distemper virus 2 (PDV-2, X84998, 96.71-96.76 %). The strains clustered in this group showed a level of identity of only 92.2 % to the Onderstepoort strain.

The three Hungarian strains demonstrated from dogs from Eastern Hungary (Group III) showed the highest level of identity with viruses isolated from European wildlife, such as a mink from Denmark (Z47759: 96.8 %-96.9) and a ferret from Germany (X84999: 96.3-96.4 %), and only 92.4-92.5 % compared to the Onderstepoort strain.

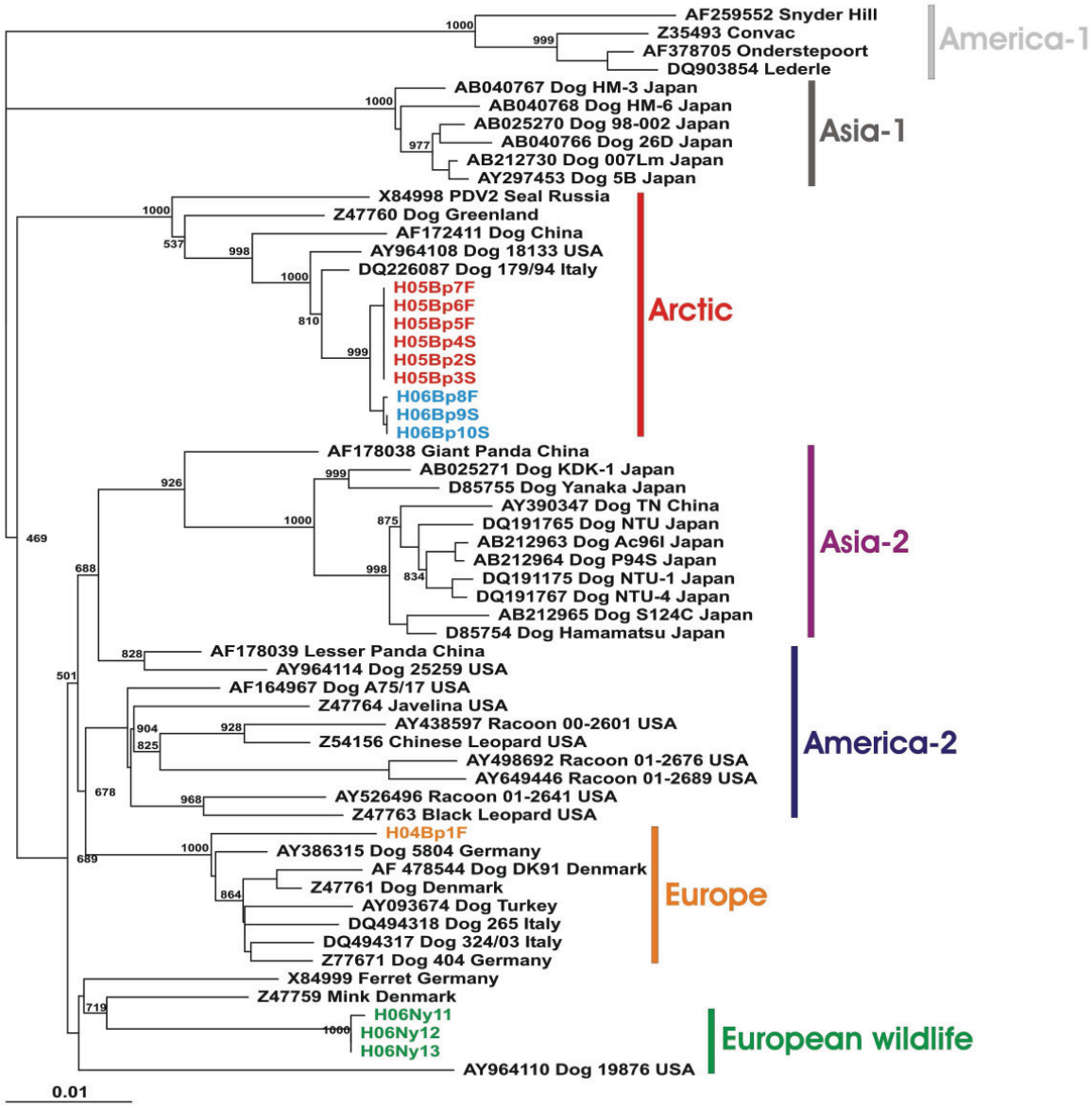

Fig. 18: Phylogenetic tree constructed upon the complete nucleotide sequences of the H genes of representative CDVs and the analyzed Hungarian strains. Accession numbers, the species and country of origin are shown for each sample

F gene

The genetic analysis performed on the full sequence of the F gene revealed findings similar to those observed on the H gene. Most clusters could be easily identified, only the two Asian groups were more difficult to distinguish. The grouping of the Hungarian strains of CDV followed the same pattern as in case of the H gene (Fig. 19).

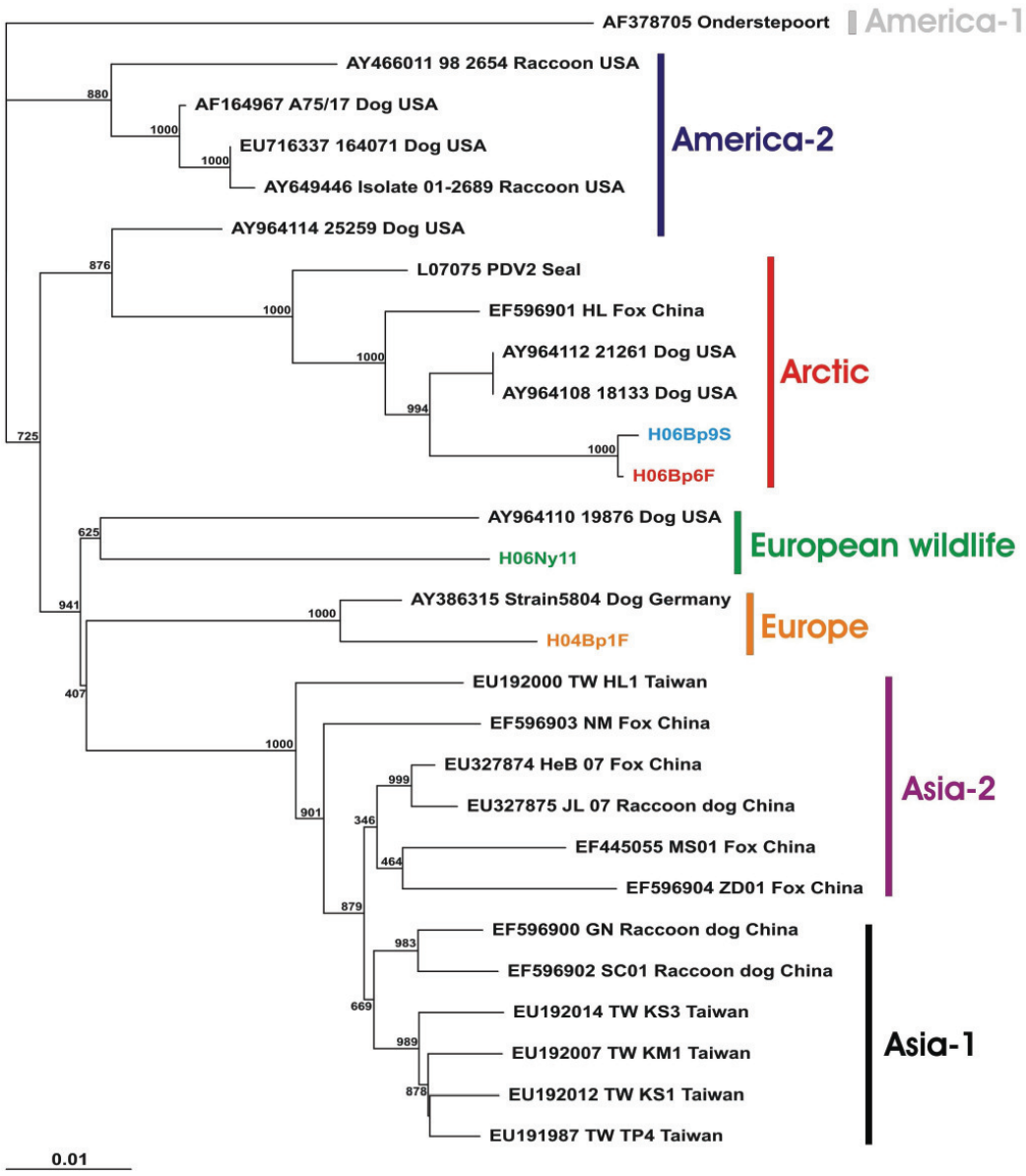

Fig. 19: Phylogenetic tree constructed upon the complete nucleotide sequences of the F genes of representative CDVs and the analyzed Hungarian strains. Accession numbers, the species and country of origin are shown for each sample

- Vaccine strains

To identify the strain present in the Vanguard vaccines, primers were designed to cover most of the genome. The nucleotide sequences of all CDV genes, except the highly conserved L gene were determined. The findings on all genes of the Vanguard vaccines have revealed that the virus strain present in all of them is more closely related to different wild-type strains, than to the one stated by the manufacturer (Snyder Hill strain) or any of the viruses from the group of vaccine strains (Table 6). As a next step the full nucleotide sequences of the highly variable H gene of all vaccine brands currently used in Hungary were included in the study (Table 2). Since the obtained

nucleotide sequences of vaccines 1 to 6 turned out to be 100 % similar in case of all batches (data not shown), the subsequent investigations performed on other domains of the virus genome were carried out using only vaccine number 6 (Table 2).

Table 6: Level of identity between the nucleotide and aa sequence of the CDV genes and proteins of the strain present in the Vanguard vaccines and those of the viruses from the group of vaccine strains (shaded) and of the wild-type strains, which showed the highest level of identity. The values in italics represent the level of identity at the aa level.

H gene

	Vanguard	Panda	Dog	Snyder Hill	Convac	Canigen	Onder.	Eurican	Japan
Vanguard	-	***98.84***	***98.35***	***91.77***	*90.95*	*90.95*	*91.44*	*90.13*	*91.44*
Panda	**99.45**	-	*97.86*	*91.61*	*91.44*	*90.78*	*91.28*	*89.96*	*91.28*
Dog	**98.90**	98.57	-	*90.78*	*89.96*	*89.96*	*90.46*	*89.14*	*90.46*
Snyder Hill	**92.92**	92.70	92.32	-	95.39	95.23	95.72	93.91	95.72
Convac	93.09	93.31	92.48	97.09	-	*96.87*	*97.53*	*95.55*	*97.36*
Canigen	93.03	92.81	92.43	96.87	98.46	-	99.01	*98.19*	*99.17*
Onder.	93.25	93.03	92.65	97.09	98.68	99.39	-	*97.69*	*99.50*
Eurican	92.59	92.37	91.99	96.32	97.91	99.23	98.84	-	*97.69*
Japan	93.25	93.03	92.65	97.09	98.68	99.45	99.61	98.90	-

Vanguard: EF095750; Snyder Hill: AF259552; Convac: Z35493; Canigen: DQ903854; Onder: AF378705, Eurican: AF014953; Japan: AB212966; Panda (Lesser Panda): AF178039; Dog (25259): AY964114

N gene

	Vanguard	Raccoon	A75/17	Onder1	Onder2	Lederle	China	Onder3
Vanguard	-	***99.61***	***99.42***	*97.51*	*97.70*	*97.70*	*97.13*	*97.70*
Raccoon	**99.10**	-	*99.80*	*97.90*	*98.09*	*98.09*	*97.51*	*98.09*
A75/17	**99.04**	99.80	-	*97.70*	*97.90*	*97.90*	*97.32*	*97.90*
Onder1	94.52	94.78	94.59	-	*98.66*	*97.70*	*97.13*	*99.80*
Onder2	94.59	94.84	94.65	99.23	-	*97.90*	*97.32*	*98.85*
Lederle	94.21	94.33	94.14	97.39	97.64	-	*99.42*	*97.90*
China	94.02	94.14	93.95	97.20	97.45	99.55	-	*97.32*
Onder3	94.59	94.84	94.65	99.93	99.30	97.45	97.26	-

Vanguard: EU072200; Onder1: NC001921; Onder2: AF378705; Onder3: AF305419; China: EF375619; Lederle: EF418783; Raccoon (01-2689): AY649446; A75/17: AF164967

P gene

	Vanguard	A75/17	Ferret	Onder1	Onder2	Rockborn	Onder3
Vanguard	-	***98.62***	***98.03***	*94.29*	*94.29*	*94.09*	*92.12*
A75/17	**98.75**	-	*97.83*	*93.70*	*93.70*	*93.50*	*91.53*
Ferret	**98.49**	98.03	-	*94.09*	*94.09*	*93.89*	*91.92*
Onder1	95.80	95.47	95.40	-	*99.21*	*99.80*	*97.44*
Onder2	95.86	95.53	95.47	99.40	-	*99.01*	*96.65*
Rockborn	95.66	95.34	95.27	99.86	99.27	-	*97.63*
Onder3	95.66	95.34	95.27	99.86	99.27	99.86	-

Vanguard: EU072201; A75/17: AF164967; Ferret (Germany): AF259550; Onder1: AF305419; Onder2: AF378705; Rockborn: AF181446; Onder3: M32418

M gene

	Vanguard	A75/17	Raccoon	Onder1	Onder2	Onder3	Onder4
Vanguard	-	***98.80***	***98.51***	*95.83*	*97.02*	*94.94*	*95.53*
A75/17	**98.71**	-	*99.70*	*97.02*	*98.21*	*96.13*	*96.72*
Raccoon	**98.41**	99.10	-	96.72	97.91	*95.83*	*96.42*
Onder1	94.04	94.64	94.54	-	*98.21*	*99.10*	*99.70*
Onder2	94.94	95.53	95.54	98.90	-	*97.32*	*97.91*
Onder3	93.55	94.14	95.43	99.50	98.41	-	*98.80*
Onder4	93.94	94.54	94.44	99.90	98.80	99.40	-

Vanguard: EU072199; A75/17: AF164967; Raccoon (00-2601): AY443350; Onder1: AF305419; Onder2: AF378705; Onder3: M12669; Onder4: NC001921

F gene

	Vanguard	Dog	A75/17	Onder1	Onder2	Onder3	Onder4
Vanguard	-	***97.58***	***96.07***	93.06	93.31	90.19	93.16
Dog	**98.13**	-	*96.07*	*92.00*	*93.06*	*91.40*	*92.15*
A75/17	**97.33**	94.26	-	*91.25*	*92.00*	*90.64*	*91.40*
Onder1	90.79	92.00	92.91	-	*97.88*	*99.39*	*99.69*
Onder2	91.85	93.06	92.86	98.39	-	*97.28*	*98.19*
Onder3	92.86	92.81	92.70	99.69	*98.19*	-	*99.09*
Onder4	90.95	92.15	93.01	99.89	*98.49*	99.59	-

Vanguard: EU072198; Dog (25259): AY964114; A75/17: AF164967; Onder1: AF305419; Onder2: AF378705; Onder3 (pCDV AS3): M21849; Onder4: NC001921

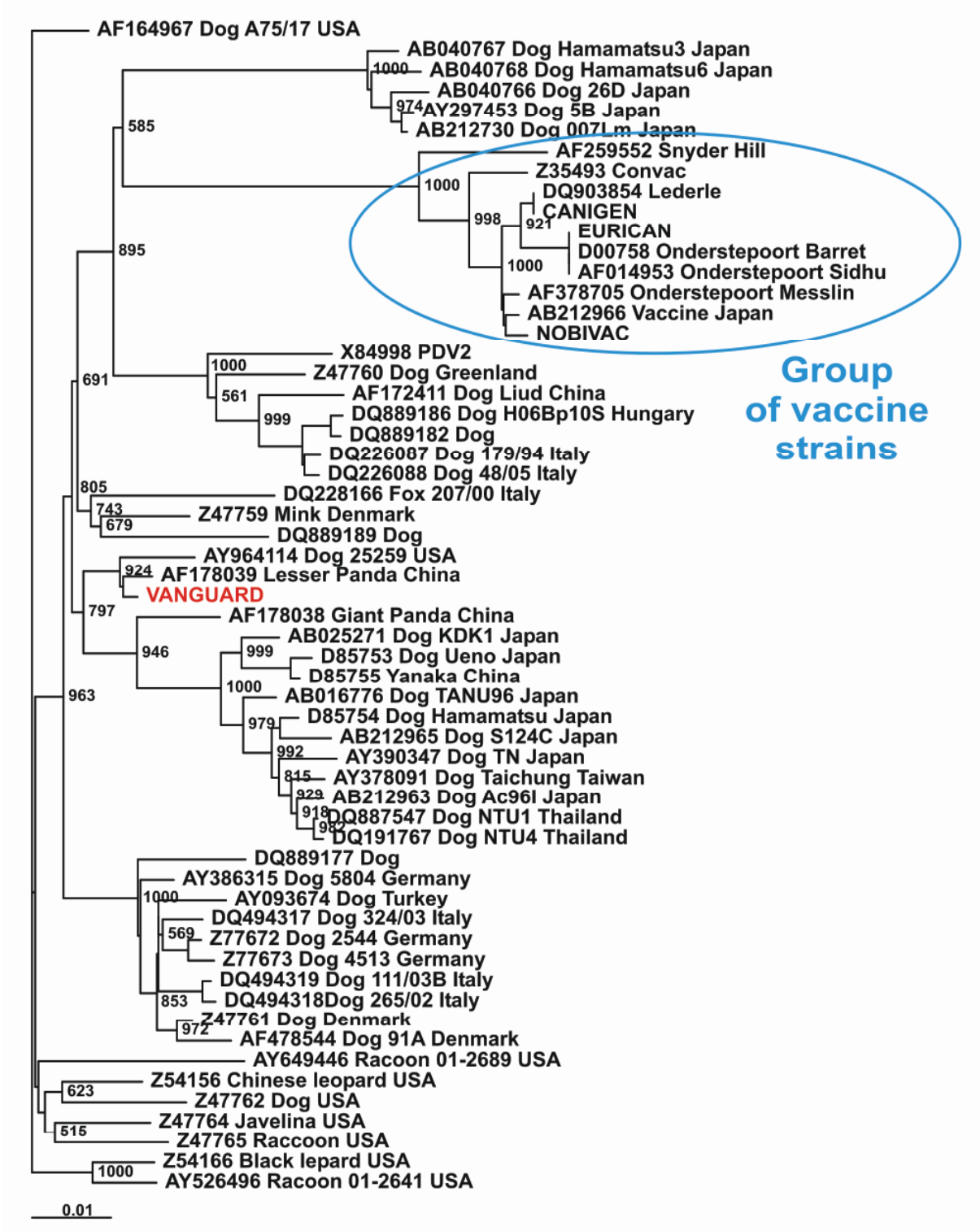

Fig. 20: Phylogenetic tree constructed based on the complete nucleotide sequences (1824 bases) of the H gene

According to the investigation of the complete sequence of the H gene, the investigated virus strain was not positioned anywhere close to the Snyder Hill strain (that has a known nucleotide sequence for the complete H gene), but it showed a significantly higher similarity to a lesser panda isolate (99.45 %) and a wild-type strain

(98.90 %) isolated from a naturally infected dog (strain 25259, accession number: AY964114) in North America. As expected following the initial RFLP the virus strains present in vaccines 7-10 were positioned in the group of vaccine strains (America-1).

The phylogenetic tree constructed based on the complete nucleic acid sequence of the H gene reveals the previously described geographic distribution pattern of CDV strains (Martella et al., 2007), as well as the exact positioning of the virus strains present in the vaccines currently used in Hungary. The virus strains present in all vaccines but the Vanguard were positioned, as expected based on the product description provided by the different manufacturers, in the group of vaccine strains (America-1). The vaccine strain present in the Vanguard vaccine was positioned among the wild-type CDVs, on the same branch of the phylogenetic tree with a virus strain demonstrated in a lesser panda from China (AF178039) and a North American dog (dog 25259: AY964114). Another very important finding is the positioning of the Snyder Hill strain that has the complete H gene sequence determined and deposited in the GenBank (accession number AF259552). As expected, this strain was clustered together with the other vaccine strains in the America-1 group (Fig. 20).

The fact that the vaccine strain present in the Vanguard vaccines is different from the Snyder Hill strain is also emphasized by the differences highlighted by the amino acid sequence alignment of the H protein of these CDV strains. Figure 21 demonstrates the differences observed following the alignment, as well as the numerous similarities observed between the amino acid sequence of the Vanguard strain and the one of the CDV strain isolated from a lesser panda in China (AF178039).

The phylogenetic trees constructed based on the nucleic acid sequences of the other investigated genes only supported the conclusions of the H gene analysis (Figs. 22 to 25). In conclusion, the phylogenetic trees constructed based on the nucleotide sequences of the investigated genes (H, N, M, P and F) revealed that the virus strain present in the Vanguard vaccines was not positioned in the group of vaccine strains (America-1), as expected based on the product description, but it showed a considerably higher level of identity with wild-type virus strains.

```
Snyder Hill       1 mlsyqdkvgafykdnaranpsklslvteehggrrppyllfvllillvgil
Vanguard          1 ...................s.........q...................m
Lesser Panda      1 ...................s.........q...................m

Snyder Hill     151 allsitgvrfhkvstsnmefsrllkedmekseavhhqvidvltplfkiig
Vanguard        151 ...a.......q.....................................
Lesser Panda    151 ...a.......q.............n.......................

Snyder Hill     301 deiglrlpqklneikqfilqktnffnpnrefdfrdlhwcinppskvkvnf
Vanguard        301 ..............................................i...l
Lesser Panda    301 ....s.........................................i...l

Snyder Hill     451 tnycetigirksiasaanpillsalsggrsdifppyrcsgattsvgkvfp
Vanguard        451 ....d...l.................r..g................rf..
Lesser Panda    451 ....d...l.................r..g................rf..

Snyder Hill     601 lsvslsmslisrtsviinmltaisdgvygktyllvpddierefdtqeirv
Vanguard        601 ..............e.....................y..g.....k...
Lesser Panda    601 ..............e.....................y..g.....k...

Snyder Hill     751 feigfikrwlndmpllqttnymvlpenskakvctiavgeltlaslcvees
Vanguard        751 ................................................d..
Lesser Panda    751 ..............s.................................d..

Snyder Hill     901 tvllyhdsrgsqdgilvvtlgifgatsmdhieevipvahpsmekihitnh
Vanguard        901 ........n.................p..qv..........v........
Lesser Panda    901 ........n.................p..qv..........v........

Snyder Hill    1051 rgfikdsiatwmvpalasekqeeqkgclesacqrktypmcnqtswepfgg
Vanguard       1051 ................v........n.........s..............
Lesser Panda   1051 ................v........n.........s..............

Snyder Hill    1201 gqlpsygrltlpldasvdlqlnisftygpvilngdgmvyyespllnsgwl
Vanguard       1201 ..............p.i.....................d...g...d....
Lesser Panda   1201 ..............p.i.....................d.......d....

Snyder Hill    1351 tippkngtilglinkasrgdqftviphvltfapresggncylpiqtsqii
Vanguard       1351 ........v............................s.........f..m
Lesser Panda   1351 ........v............................s.........f..m

Snyder Hill    1501 drdvliesnlvvlptqsfryviatydisrndhaivyyvydpfrtifytyp
Vanguard       1501 .k...t..........n......k.....d...........i.k.sf...
Lesser Panda   1501 .k...t..........n............d...........i...sf...

Snyder Hill    1651 frlttkgrpdflriecfvwddnlwchqfyryeaniansttsvenlvrirf
Vanguard       1651 ......................d........f..d.t.f............
Lesser Panda   1651 .q....................d........f..d.t.f............

Snyder Hill    1801 scnrsnp*
Vanguard       1801 .....k.*
Lesser Panda   1801 .....k.*
```

Fig. 21: Amino acid sequence alignment of the following strains: Snyder Hill (AF259552), Vanguard vaccine (EF095750) and the virus isolated from a lesser panda (AF178039). Similar residues are represented by dots

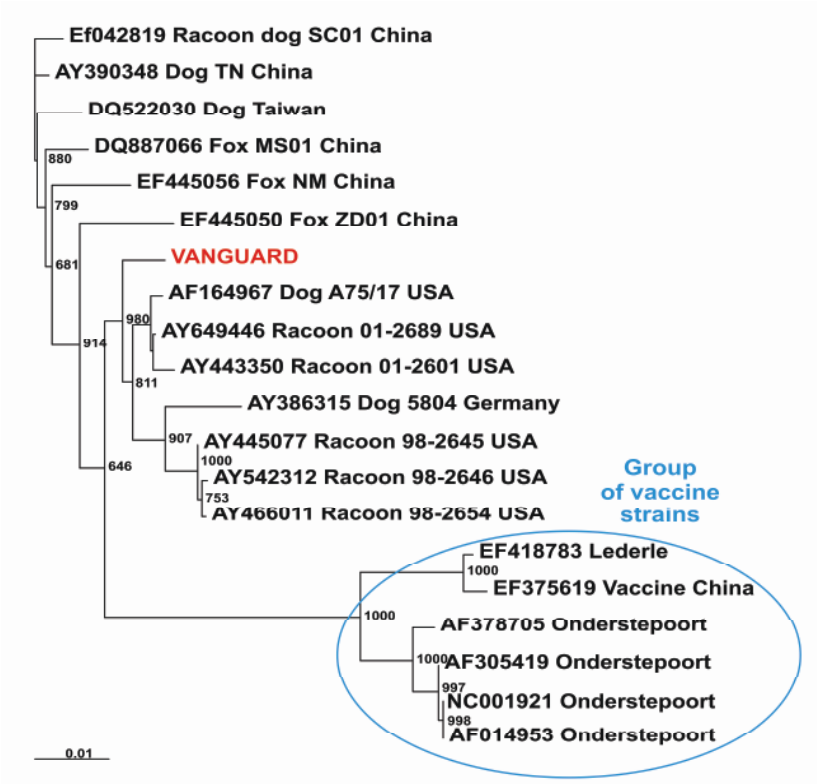

Fig. 22: Phylogenetic tree constructed based on the complete nucleotide sequences (1572 bases) of the N gene

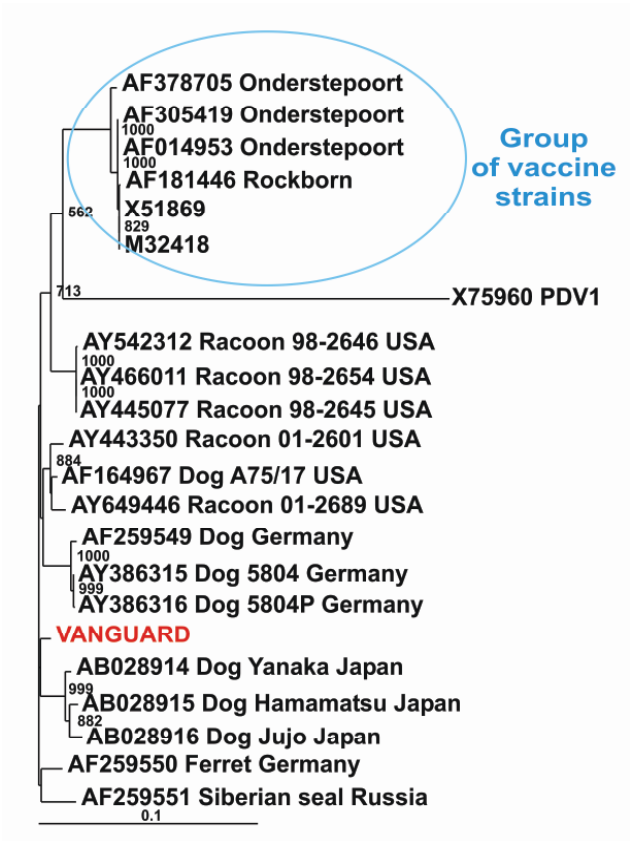

Fig. 23: Phylogenetic tree constructed based on the complete nucleotide sequences (1524 bases) of the P gene

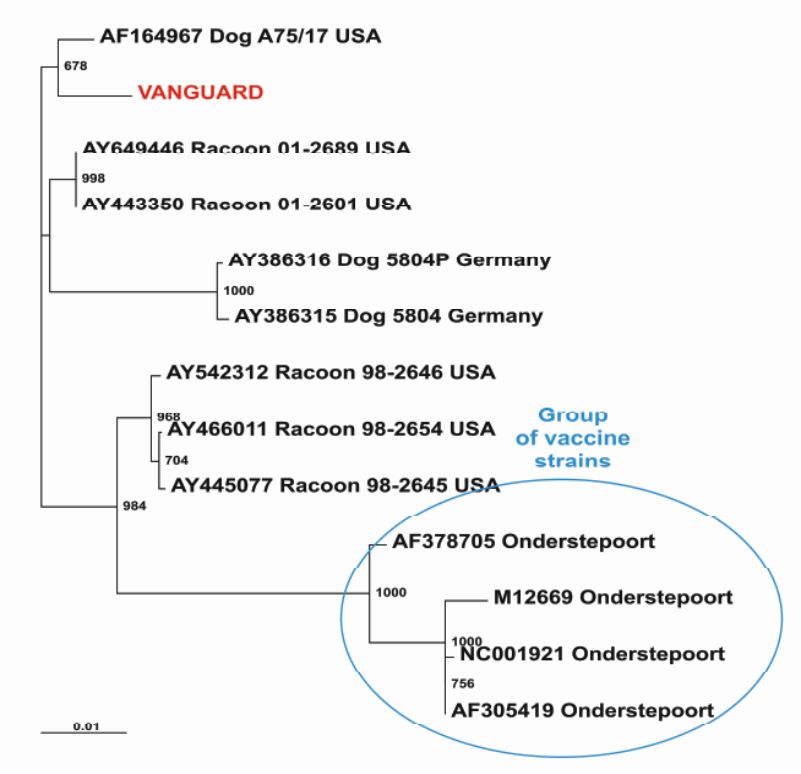

Fig. 24: Phylogenetic tree constructed based on the complete nucleotide sequences (1008 bases) of the M gene

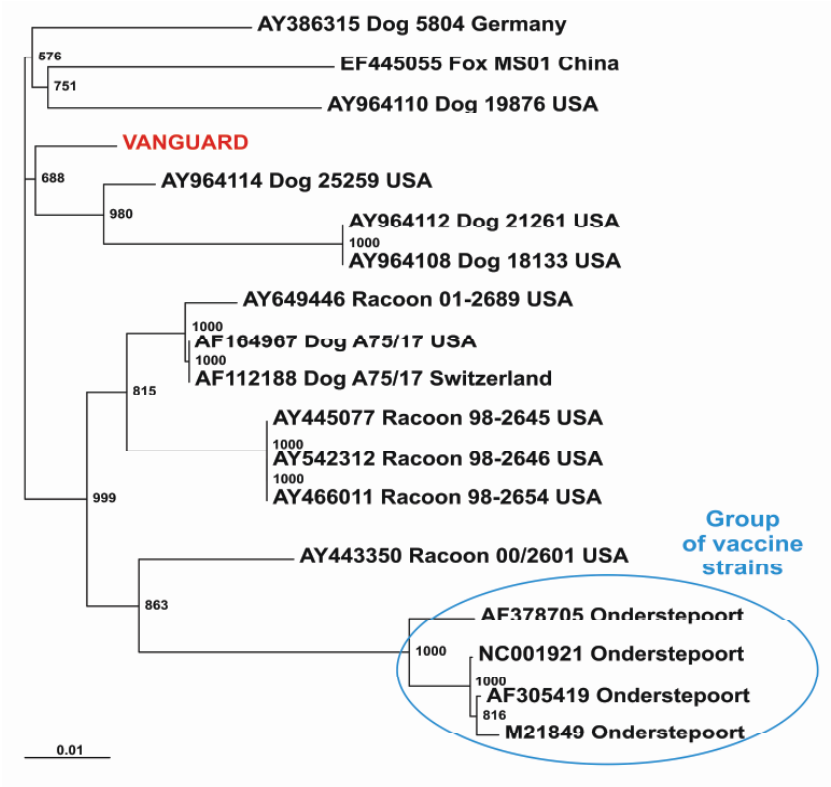

Fig. 25: Phylogenetic tree constructed based on the complete nucleotide sequences (1989 bases) of the F gene

5.4.3.2 FPV strains

PCR positive samples from two cats, one lion and one Asian palm civet were selected for phylogenetic analysis. Based on these analyses, all strains turned out to be FPVs. The analysis of the Hungarian FPV strains demonstrated that the virus strain that infected the Asian palm civet is most closely related to other FPV strains such as JF-3 (DQ099431) isolated in China (99.54 %), Gercules Biocentr (AY665655) from Russia (99.54 %) and XJ-1 (EF988660) reported in China (99.48 %), and to an RPV strain (M24005) as well (99.48 %).

Fig. 26: Phylogenetic tree constructed based on the complete nucleotide sequence of the VP2 gene

In the attempt to clarify the time and place of infection, two Hungarian FPV strains demonstrated in cats (933/07: EU360958 and 1335/07: EU360959) were also included in the genetic analysis. Both strains showed the similar level of identity with strain 389/07 at the nucleic acid level (99.43 %), but they were positioned on different branches of the phylogenetic tree. Strain 933/07 was positioned right next to strain 389/07 and the Chinese isolate JF-3, while strain 1335/07 seemed to be more closely related to Argentinean FPVs (Fig. 26).

The FPV strain demonstrated in the dissected lion showed the highest level of identity with a virus strain isolated in Russia (AY665655: 99.83 %), while on the phylogenetic tree was placed on the same branch with strain 377 (U22188), isolated in Germany.

5.4.3.3. CPV2 strains

Sequence analysis was performed on the twenty PCR-positive samples used for the *Mbo*II-based RFLP. The sequence analysis of the amplicons (582 bp) amplified by primers *555for* and *555rev* (Buonavoglia et al., 2001) revealed that all analyzed viruses are CPV2 strains. The results have also demonstrated that these Hungarian type 2 CPVs have Asn at position 426, characteristic for type 2a strains. The investigation also revealed the explanation for the misleading result of the RFLP-based genotyping attempts: none of these "false" type 2c CPV strains presented the *Mbo*II cleavage site at position that is characteristic for "true" type 2c strains (position 4064), therefore at position 426 not aa suggestive of type 2c strains (Glu) was present, but an Asn, typical for type 2a CPVs. However all of these strains presented an *Mbo*II cleavage site, but at the other end of the amplicons obtained following the PCR (Fig. 27). The observed SNP did not alter the deduced aa sequence.

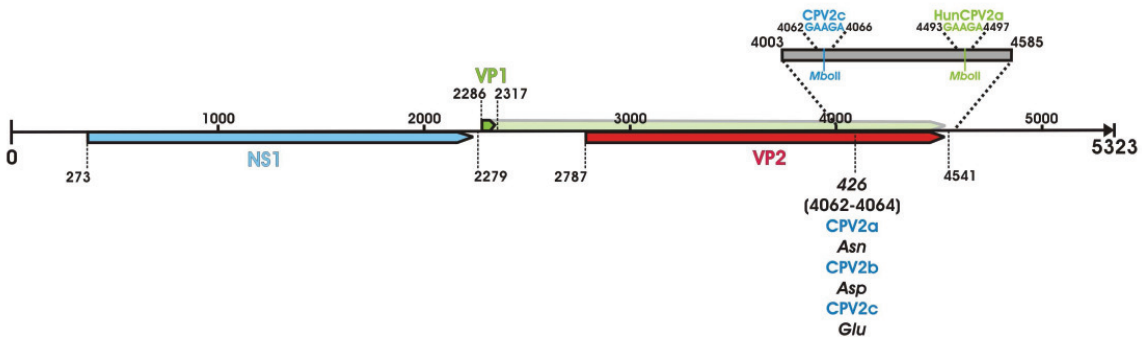

Fig. 27: Position (green) of the point mutation that led to the appearance of the misleading *Mbo*II cleavage site on the analyzed segment (gray)

The alignment of the Hungarian CPV2 strains revealed only a few other, apparently randomly scattered SNPs, but none of these produced any change in the deducted aa sequences. None of the observed SNPs seemed to be characteristic only for Hungarian strains of CPV2. Due to the relatively short length of the amplified genome segment phylogenetic analysis was not performed.

The nucleic acid sequences of the Hungarian CPV2 strains analyzed in the present study were deposited in the GenBank and were assigned with the following accession numbers: EU815830-EU815849.

6. Discussion

Despite the vaccination procedures applied in Hungary, CDV is still a serious threat to the susceptible animal population. For a fast and reliable diagnosis of CD a pair of "diagnostic" primers that targeted the L conserved domain of the viral genome was designed. Due to the conservative character of the amplified segment, this primer pair could most likely be used to detect all CDV strains currently present in Hungary.

In many situations, the cause of the respiratory and/or digestive clinical signs is not the CDV infection, but infections caused by other viral (parainfluenza virus, canine adenovirus 2, canine respiratory coronavirus, etc.) and/or bacterial (*Bordetella sp., Pasteurella sp.*, etc.) agents (Greene & Appel, 2006). Because the initial clinical signs following infection can be indicative not only of CD, the signs can be misleading and the regular use of the specific diagnostic tests, such as those based on PCR, are indicated for the proper diagnosis of CD. Based on the nucleotide sequences deposited in the GenBank, and the conserved character of the L gene, primer pair "A" (Table 4) theoretically attaches to the genome of all currently circulating wild-type viruses. Since the sensitivity test demonstrated that it can detect CDV RNA concentrations as low as 10^2, it can be concluded that primer pair "A" can be used as a specific diagnostic test for CDV infections. Using these newly designed diagnostic primer pairs, CD has been demonstrated in Hungary in other species as well: a free-roaming fox, a raccoon, and a ferret. Out of the 214 analyzed samples, 58 (27.1 %) proved to be positive for CDV.

In order to clarify the relationship of the Hungarian strains with other CDV strains reported in other parts of the world, 13 Hungarian wild-type virus strains collected between 2004 and 2006 were selected, and a phylogenetic analysis on the full segment of nucleotide sequence of the H gene was performed. The analysis has shown that the diversity of the Hungarian strains is high, and that they are placed on different branches of the phylogenetic tree, in three groups. Molecular analyses performed in other countries demonstrate similar findings: in a given geographical region more than one genotype of CDV can be present at the same time (Haas et al., 1999; Lednicky et al., 2004; Martella et al., 2007). The studies indicate that genotypes from far away countries appear sooner or later in the dog population, most likely due to the intense, uncontrolled trade and travel of dogs. This observation is also validated by the fact that Hungarian strains are closely related to other CDVs currently present in other parts of the world, including Europe and other distant regions, such as China and North

America. The similarity of the Hungarian strains to the Onderstepoort reference strain varied between 90.86 and 91.91 %.

The Hungarian strain H04Bp1F from Group I (Table 1) obtained from Budapest in the winter of 2004 is closely related to virus strains reported in other European countries, such as Denmark, Italy, Germany and Turkey (European cluster; Fig. 18).

Although the diversity of the CDV in the total canine population is obvious, one large, relatively homogenous cluster (Group II) is formed based on the sequences obtained from one population, that of the Dog Shelter of the City Council of Budapest (Fig. 18). The investigations demonstrated that there is a high incidence of CD at the above mentioned establishment: from the 99 clinically ill dogs examined during the 16 months period, 25 turned out to be positive for CD. Several samples from different periods of time were analyzed. We determined the complete sequence of the H gene of six samples from the spring of 2005 and three samples from the spring and summer of 2006, as well as the partial H gene sequence of many other samples collected from the same shelter. The relatively long period of time between the collection of the first and last samples, and the level of similarity among the obtained nucleotide sequences demonstrate that there is an endemic infection at the above mentioned establishment. Based on this conclusion, we have considered that further investigation of other samples obtained at the same establishment was not necessary, since they would have only confirmed the endemic infection. In the present study we have included only the sequences that had the full sequence of the H gene determined. The analyses also demonstrate that the investigated virus strains from the second period of examination had suffered a homologous amino acid change when compared to the strains from the first period of the investigation, specifically, the change of lysine to arginine at position 278 of the H protein.

The investigations performed at the above mentioned establishment have also revealed that in the case of large, open populations, such as in dog shelters, nasal swab samples are not always reliable in the diagnosis of CD. Out of the 99 clinically ill dogs that were examined in the shelter during a one year period, there were 16 cases when both blood samples and nasal swab samples were analyzed. For six dogs, both the blood (serum and separated leukocytes) and nasal swab samples proved to be positive, and hence the results were regarded as proof of viremia caused by the CDV infection. Animals that had a positive PCR test using their serum were also tested positive on the separated leukocytes. We could not identify animals with only one of their blood tests (serum or leukocytes) positive. On the other hand, for ten dogs, only the nasal swab

samples turned out to be positive, while the repeated examination of both blood samples gave only negative results. This means that the virus remained at the entrance site, on the nasal mucosa and did not actually cause viremia, or it could mean that viremia had been overcome. Therefore, in 10 out of 16 of these situations (62.5 %), the positive results obtained using only the nasal swab samples gave misleading, diagnostically false positive results. These results, as well as the decreasing number of clinical cases at the shelter also demonstrate the efficiency of the CD preventive program started during the fall of 2005.

Interestingly, the Hungarian CDV strains obtained from the above mentioned establishment belong to the so called Arctic group (Martella et al., 2006), closely related to virus strains from countries geographically distant from Hungary, such as the United States of America (98.95-99.01 %), Italy (99.23-99.28 %), China (97.86-97.91 %) and Greenland (97.31-97.36 %), as well as to the PDV-2 (X84998), isolated from seals from Lake Baikal (96.71-96.76 %). These findings seem to emphasize the unfortunate consequences of uncontrolled animal movement and trade.

The rest of the Hungarian strains are located in other parts of the phylogenetic tree (Fig. 18). Three viruses (Group III) from samples collected in Eastern Hungary (H06Ny11, H06Ny12 and H06Ny13) seem to join a different cluster, which include other strains isolated from a Danish mink (96.82-96.92 %), from a ferret in Germany (96.27-96.38 %) and from a dog in North America (94.62-94.73 %). These findings are in accordance with the conclusions of other authors who concluded that there are strong interconnections among the susceptible wild and domestic animals, and that they can act as reservoirs one for the other (Haas et al, 1997; Lednicky et al., 2004). The H proteins of these three strains have eight potential glycosylation sites, same as all virus strains belonging to the Arctic group.

The phylogenetic analysis of the deduced aa sequences shows a similar clustering: the strains from the same establishment are positioned very close to each other (99.83 % identity between the strains from the two examination periods), while the positions of the other aa sequences are quite similar to that of the nucleotide sequences. The identity between the aa sequence of the H protein of the Hungarian Arctic strains and PDV-2 varies from 95.71 to 96.04 %. Bootstrap resampling analyses support most of the clustering, both on the nucleotide and on the aa based phylogenetic trees. In conclusion, the study demonstrates that more than one genotype of CDV is present currently in Hungary. Furthermore, point mutations resulting in the emergence of new variants can be observed in populations where endemic infection is present.

Only one strain from each subgroup of Group II was selected for the F gene analysis. These results only emphasized our previous findings regarding the endemic infection at the above mentioned establishment, as well as the evidence of viral mutation. These results are most probably the evidence of viral evolution. Nevertheless, one can not ignore the possibility that a new strain was introduced and replaced the earlier one. The overall results of the genetic analysis of the F gene supported the findings obtained from the H gene analysis: the clustering of CDV strains presented a similar geographical pattern (Fig. 19), and the Hungarian strains were grouped as in case of the H gene (Fig. 18).

The observed genetic heterogeneity of the CDV strains is intriguing, since Hungary is a relatively small country. The presence of more than one virus genotype in such a small territory can be explained by the epizootiological openness of the canine population, maintained by the lack of geographic barriers, as well as the high number of foreign citizens who own dogs and are living in Hungary or visit the country. The import of exotic canine breeds and other receptive species, as well as the uncontrolled movement of the receptive wild species also contribute to the heterogeneity of the Hungarian CDV strains.

Using the diagnostic test employing the newly designed primers, CDV infection has been demonstrated in other species as well: a fox, a raccoon and a ferret. The fox was a free-roaming animal found dead in a forested area of Western Hungary. Due to the advanced autolysis, only a macroscopic examination revealing a severe *Sarcoptes scabiei* infection and sample acquisition could be performed. It can only be assumed that the parasitic infection was most likely the result of the immunosuppression caused by the CDV infection. The macroscopic examination of the CDV positive raccoon and ferret revealed changes suggestive of the infection: severe dehydration and anemia, generalized lymphadenopathy, moderate enteritis and interstitial pneumonia. The demonstration of CDV infection in these animals only seems to emphasize the wide range of receptive species of the virus, the necessity of implementation of the already existing vaccination protocols and prophylactic measures for receptive wild species, as well as the importance of limitation of contact between susceptible species.

Like in other cases when MLV vaccines are used for the prevention of certain infectious diseases, the immunization of dogs with MLV against CDV infection sometimes leads to misleading results in the PCR-based test due to the presence of the vaccine strain in the clinical samples. Since the introduction of vaccinations for the

prevention of CD, several virus strains (such as the Onderstepoort, Rockborn, Snyder Hill, Lederle or the Convac strains) have been used. Due to the fact that primer pair "A" also attaches to the genome of vaccine strains, and that recent immunization of animals with an attenuated live vaccine might cause difficulties in the evaluation of the PCR test (Greene & Appel, 2006), we developed an RFLP test to accurately differentiate the vaccine strains from the wild-type strains. This test is based on the presence of a constant enzyme cleavage site on the region amplified by primer pair "B" (Table 4). Since the nucleotide sequence of the Lederle strain is not present in the GenBank, to make sure that the result of our RFLP test is reliable, prior to designing the test, we sequenced the amplicons obtained using primer pair "C" on one of the vaccines (Canigen DH(A2)PPi/L, Virbac S.A., France) that according to the manufacturer contains the above mentioned strain. Based on the obtained nucleotide sequence (DQ903854), and on the sequences of other vaccine strains already available in the GenBank, the newly designed RFLP test seemed to work on all vaccine strains currently applied to prevent CD in Hungary. According to these sequences, the cleavage site on which the newly designed RFLP test is based is present on all investigated vaccine strains, and it is absent from all of the analyzed wild-type strains retrieved from the GenBank. More precisely, it is based on the change of the nucleotide at the position 8139 of the viral genome from thymine (T) in the vaccine strains to cytosine (C) in the wild-type strains. This point mutation can be found in the nucleotide sequences of all wild-type CDVs deposited in the GenBank. The presence of the mutation does not cause any change in the amino acid sequence of the wild-type strains at the given position. The nucleotide sequence that suffered the described mutation is also the recognition sequence for the *Psi*I endonuclease. Due to this fact, the *Psi*I enzyme will cut only the amplicons of the vaccine strains and will leave the amplicons of all wild-type strains undigested; therefore, at least in theory, it can be used for the fast and reliable differentiation of the wild-type CDV strains from the currently used vaccine strains. When testing the RFLP test in practice by using a vaccine strain (Canigen DH(A2)PPi/L, Virbac S.A., France) and several wild-type strains, the positive result of the enzymatic digestion was clearly visible in case of the virus strain from the vaccine as a double band (294 bp and 816 bp) by transillumination of the gels in UV light. Meanwhile, the amplicons of the field samples remained uncut and formed a single characteristic band (1110 bp) (Fig. 14). As an attempt to test the newly designed assay further, the virus strains present in most of the CDV vaccines currently used in Hungary were also included in the trials. These tests revealed that the CDV strain present in the

Vanguard (Pfizer Animal Health, USA) vaccine reacted as a wild-type strain (Fig. 15). According to the statement provided in the product description by the manufacturer, the European Vanguard products used to immunize against CD contain the Snyder Hill strain. The Snyder Hill strain was isolated in Ithaca, N.Y., USA in the 1950's from the brain of a dog and passaged *in vivo* in dogs before being adapted to cell growth in NL-DKC cells (Brown et al., 1972; Martella et al., 2007). Based on the nucleotide sequence available in the GenBank (AF259552), the amplicons of primer pair "B" (Table 4) should have been digested by the *Psi*I enzyme, but those of the virus strain present in the Vanguard vaccine remained undigested, hence reacted as a wild-type virus. Further three repetitions of RFLP test gave the same result.

These partial results raised the first questions regarding the identity of the virus strain present in the Vanguard vaccines hence an extensive genetic and phylogenetic analysis of the incriminated virus strain was conducted. Primer pairs that enabled the amplification of complete genes of the CDV genome were designed and the nucleotide sequences of all CDV genes (H, N, M, P and F), except the highly conserved L gene were determined. For phylogenetic analysis initially the H gene was chosen, because this gene is considered as one of the most variable one in the CDV genome (Haas et al., 1997; Harder & Osterhaus, 1997; Mochizuki et al., 1999; Martella et al., 2006) therefore it is profoundly suitable for phylogenetic analysis. H gene analysis further emphasized the previous findings: the investigated virus strain was not positioned anywhere close to the Snyder Hill strain (that has a known nucleotide sequence for the complete H gene: AF259552), but turned out to be most closely related to a wild-type virus strain isolated from a lesser panda (AF178039) (Fig. 20). Similar observations were reported by another research group (Pardo et al., 2005), but the investigated segment was too small to obtain relevant results (979 bases). Based on the findings of this research group the virus strain present in the Vanguard vaccine was most closely related to the A75/17 wild-type strain isolated from a dog, but the investigation of the complete sequence of the H gene has revealed that it is more closely related to the lesser panda isolate (99.45 %) and a wild-type strain (98.90 %) isolated in North America from a naturally infected dog (strain 25259, accession number: AY964114) by the same research group (Pardo et al., 2005). The analysis of other genes of CDV have also revealed that the virus strain present in the Vanguard vaccine is more closely related to different wild-type strains, than to the one stated by the manufacturer or any of the viruses from the group of vaccine strains (Table 6). The phylogenetic trees constructed based on the nucleotide sequences of the investigated genes further emphasized these

findings: in each tree the incriminated virus strain was not positioned in the group of vaccine strains (America-1), as expected based on the product description, but it showed a considerably higher level of identity with wild-type virus strains (Figs. 20 and 22-25).

The results irrefutably demonstrated that the virus strain present in the Vanguard vaccine purchased in 2006 in Hungary does not contain the Snyder Hill strain, as claimed by the manufacturer. In order to extend our investigations in time and geographical respect, vials of vaccine produced in different times (1992 and 1994) and different geographical origin (Malta, Israel and USA) were purchased. The same RFLP test was applied to determine whether these vaccines contain the same virus strain as the vaccine purchased in Hungary in 2006. The result of the RFLP test and the complete sequencing of the H gene of the virus present in all these Vanguard vaccine batches revealed that all of them contain exactly the same virus strain, showing not even one nucleotide difference on this relatively large and highly variable gene (data not shown). Due to these findings, the vaccine batches dated back to 1992 and 1994 and the ones purchased in other countries were not investigated on other segments of the CDV genome, since those results most probably would have only confirmed the previous observations. On the other hand, these findings directed us to the conclusion that if a contamination or any other event caused the replacement of the originally claimed seed virus, it must have happened before 1992.

There could be several possible explanations for the findings of the present study, such as (1) the incorrect labeling of the Snyder Hill strain deposited in the GenBank (AF259552) – this suggestion is contradicted by its positioning among the group of vaccine strains in all phylogenetic trees; (2) a possible recombination of the seed virus with a wild-type strain – this theory is eliminated by the similar results obtained during the parallel investigation of the different genes; (3) due to a taxonomical and technical error the strain was incorrectly labeled when the vaccine was registered; (4) due to unknown reasons the vaccine contains a different virus strain and finally (5) a contamination of the seed virus stock used in the vaccine production, also suggested by Pardo (2006). At this point the last three explanations cannot be demonstrated neither eliminated. It has to be emphasized that no claims regarding the safety or efficacy of any of the analyzed vaccines (including the Vanguard vaccines) were reported. The significance of these findings is inconclusive without further studies. At this point it can only be declared that the vaccine does not contain the virus strain stated by the manufacturer in its product description and has not been containing it since at least as 1992. Additional research, such as experimental infections should be

performed in order to reveal whether the strain is virulent as its phylogeny suggests, or a new avirulent strain having higher identity to the wild-type strains. If this theory turned out to be true, it is possible that this recently and supposedly successfully attenuated wild-type strain would be a breakthrough and could provide a better protection against the wild-type strains that are currently and increasingly infecting even the vaccinated canine populations. The genetic investigations were not so developed and widespread at the time of the registration of the vaccine. Conventional (serological) tests and experimental infections are not suitable for identification and discrimination of CDV strains.

FPV-induced disease in cats has been known since the beginning of the 20th century (Verge & Christoforoni, 1928; Steinel et al., 2000). There are several wild species from different animal families that are known to be receptive for FPV (Horiuchi et al., 1998; Steinel et al., 2001). Up to this point no reports were submitted regarding the direct demonstration of an FPV infection in any member of the *Viverridae* animal family, but one publication reported FPV seropositivity survey in Formosan gem-faced civets (Ikeda et al., 1999). The PCRs produced in each FPV infection case amplicons of the expected size, demonstrating the presence of the parvovirus genome. The subsequent genetic analyses demonstrated that the virus strains demonstrated in Hungarian cats and Asian palm civet are closely related and are positioned on the same cluster of the phylogenetic tree (Fig. 26). The virus strain demonstrated in the Asian palm civet was most closely related to other FPV strains such as JF-3 (DQ099431) isolated in China (99.54 %), Gercules Biocentr (AY665655) from Russia (99.54 %) and XJ-1 (EF988660) reported in China (99.48 %), but to an RPV strain (M24005) as well (99.48 %). In the attempt to clarify the time and place of infection, two Hungarian FPV strains demonstrated in cats (933/07: EU360958 and 1335/07: EU360959) were also included in the genetic analysis. Both strains showed similar level of identity with strain 389/07 at the nucleic acid level (99.43 %). Due to the positioning of the Hungarian strains and an Asian virus strain in the same cluster, the genetic analysis of these samples unfortunately did not help to clearly elucidate the time and place of infection. The only indirect evidence is the moment of the onset of clinical signs and eventual death of the animal (approximately 50 hours following its arrival to Hungary). Even though FPV has a very short incubation period (Greene & Addie, 2006), based on the severity of the macro- and microscopical findings and on the genetic data available, at

this point it can be only assumed that the infection occurred in Malaysia, prior to the animal's arrival to Hungary.

The overall result irrefutably demonstrates the parvovirus infection in the examined animal. The nucleotide sequence of the amplicons resulted following the PCRs have revealed that the causative agent is an FPV strain. On the other hand these investigations revealed that more wild species are susceptible to FPV infections than known before, hence the causative agent has a host range even wider than it has been suspected. It may also mean that more rigorous measures need to be taken such as quarantine and other prophylactic measures when importing wild and exotic species with unknown immunological history in order to prevent infections and the spreading of pathogens. The examinations represented by macroscopical findings, histopathology, EM and especially those based on a PCR technique described, are the first direct diagnosis of an FPV infection in an Asian palm civet and in any member of the *Viverridae* animal family.

Pathological changes observed during the dissection of the analyzed lion were suggestive of a viral infection. The histopathological examination of tissue specimens only emphasized the initial observations. The presence of FPV was demonstrated by the means of diagnostic PCR. All attempts to demonstrate the presence of other pathogens (CDV, feline coronavirus, feline calicivirus, feline herpesvirus 1, *Chlamydophyla psittaci*) gave negative results. The necrotic myositis present in the hind limb, cerebral edema, ulcerations of the tongue were most likely consequences of the severe immunosuppresion caused by FPV. The phylogenetic analysis of the FPV strain that infected the lion revealed that it is most closely related to a German isolate (U22188), and it is positioned on a different branch of the phylogenetic tree than the other Hungarian strains (Fig. 26). These findings seem to emphasize the genetic diversity of Hungarian FPVs, somewhat similarly to the CDV situation. The infection of wild and exotic species is unfortunately another evidence for the susceptibility of these species, and the necessity for the implementation of more severe quarantine and prophylactic measures in order to avoid contact of these animals with potential carriers of FPV.

Following its sudden emergence at the end of 1970's, type 2 CPV infection became one of the most frequent causes of death among the young, susceptible canine populations worldwide. Despite the rapidly developed and still existing effective vaccination procedures the disease is still causing serious losses, especially in young, unvaccinated canine populations. Even though CPV2 is a small DNA virus, it possesses

a relatively quick evolutionary pattern compared to other closely related viruses, such as FPV. Following its emergence and rapid spread throughout the world (Kelly, 1978; Appel et al., 1979; Burtonboy et al., 1979; Johnson & Spreadbrow, 1979) the original type 2 CPV was soon replaced by a different antigenic variant (named CPV2a) that differed from the original type by only 5 aas in the VP2 coat protein (Parrish, 1994; Truyen, 2006; Pérez et al., 2007), whereas the type 2b CPV genotype that emerged a few years following this event differs from the 2a type by only one aa (Asn426Asp) (Parrish, 1994; Pérez et al., 2007). More recently a new antigenic variant (named CPV2c) was reported in several countries around the world, including Italy (Buonavoglia et al., 2001), Spain (Decaro et al., 2006b), Germany (Shackelton et al., 2005), the United Kingdom (Decaro et al., 2007a) and Uruguay (Pérez et al., 2007). The genetic analysis and genotyping of CPV2 strains is of enormous interest in the better understanding of virus evolution and as an irreplaceable help in the designing of even better vaccines and vaccination protocols than the ones currently used.

Presently in Europe a very diverse distribution of these genetic variants can be observed (de Ybanez et al., 1995; Greenwood et al., 1996; Buonavoglia et al., 2001; Martella et al., 2005; Decaro et al., 2006a,b). The aim of the present study was to have an insight on the current distribution of CPV2 variants in Hungary and to demonstrate or disprove the presence of CPV2c in Hungary. Following the collection of the analyzed samples and the certain positive diagnosis of CPV2 infection, we have conducted a previously described RFLP-based rapid identification technique of CPV2c variants. Surprisingly, based on this identification method, 15 (75 %) out of the 20 analyzed samples were identified as type2c CPVs. The result was unexpected, because based on previous reports CPV2c did not seem to have such a high incidence in Europe, but only in South America so far (Pérez et al., 2007). In order to verify the results of the *Mbo*II-based RFLP test we have determined the nucleic acid sequence of the incriminated amplicons used in the enzymatic digestion. Since these amplicons also contained the nucleic acid sequence responsible for coding the aa at position 426 of VP2, the obtained genetic information was able to indicate the genotype of the analyzed sample as well: Asn in case of CPV2a, Asp in case of CPV2b and Glu in case of CPV2c (Fig. 27). The analysis of the nucleic acid and deduced aa sequences revealed a most surprising outcome: based on the aa at position 426 of VP2, all of the analyzed samples were type 2a CPVs. The analysis has also revealed the explanation for the results of the RFLP: the *Mbo*II cleavage site was present not at the site characteristic for CPV2c, but it appeared following a SNP at the other end of the amplicons (Fig. 27). Hence the rapid enzymatic

identification of the incriminated Hungarian type 2a CPVs gave a misleading genotyping result and acted as "true" CPV2c strains (Fig. 17). Another interesting finding was the high number of vaccinated yet infected animals (13 [65 %] out of 20). Obviously many things can interfere with a successful vaccination, such as transportation and storage conditions of the vaccine vials, the moment of vaccination and the clinical condition of the animals etc., but the high incidence of demonstrated infection in these vaccinated animals only fuels the ongoing dispute (Truyen, 2006) regarding the vaccination protocols of puppies and the type of strains that should be used. The findings of the present work also emphasize the devastating effect CPV2 infections can have in smaller, confined canine populations, such as the ones living in breeding facilities: samples 11-14 (Table 3) were obtained from puppies living in the same breeding establishment. Even though the breeders had an extensive hands-on experience in canine breeding and the animals received their vaccinations, a total number of 18 (75 %) animals (out of 24) died in the incriminated breeding season, all showing clinical signs suggestive of an enteric infection. The pathological and virological examinations performed on 6 of the puppies have identified the causative agent as CPV2a. Even though the relatively short length of analyzed segment (577 bp of the VP2 gene) did not allow a thorough and reliable phylogenetic analysis, the phylogenetic tree constructed based on this region of the analyzed Hungarian strains and several other strains retrieved from the GenBank revealed that these strains were closely related to each other and other type 2a CPVs from other distant parts of the world, such as USA, Asia, Africa and New Zeeland (data not shown).

In conclusion, based on the results of the present work it can be stated that currently in Hungary only type 2a CPVs seem to be present. On the other hand, the genetic analysis provided substantial evidence to demonstrate that due to a seemingly constant point mutation present in most of the Hungarian CPV2a strains, the previously described *Mbo*II-based rapid identification of CPV2c strains unfortunately cannot be reliably used any more.

7. New Scientific Results

Most important scientific results of the present study:

1. Design of new, PCR-based assays for the reliable diagnosis of canine distemper and feline and canine parvovirus infections and the genetic analysis of the causative agents.
2. Design of a *Psi*I based restriction fragment length polymorphism assay for the fast and reliable differentiation of vaccine and wild-type strains of CDV.
3. Demonstration of the presence of a different strain than the "Snyder Hill" (or any other strain belonging to the America-1 cluster) in Vanguard (Pfizer Animal Health, USA) vaccines, including batches from different countries and batches dating back to 1992 and 1994.
4. Demonstration of the presence of three different genotypes (European, Arctic and European wildlife) of CDV in Hungary, diagnosis of CDV infection in various species from Hungary (dogs, a fox, a raccoon and a ferret), and demonstration of an endemic infection at the Dog Shelter of the City Council of Budapest.
5. First direct demonstration of an FPV infection in a member of the *Viverridae* animal family (in an Asian palm civet – *Paradoxurus hermaphroditus*), and demonstration of FPV infection in a lion *(Panthera leo)* from Hungary.
6. Demonstration of only type 2a CPVs in Hungary and of type 2a Hungarian CPV variants that react as type 2c CPVs following a previously described *Mbo*II-based 2c genotyping assay, hence leading to diagnostically misleading results.

8. References

ABELES V., HARRUS S., ANGLES J.M., SHALEV G., AIZENBERG I., PERES Y., AROCH I.: Hypertrophic osteodystrophy in six Weimaraner puppies associated with systemic signs. In: *Veterinary Record*, 1999, 145, 130-134.

ADDIE D.D., TOTH S., THOMPSON H., GREENWOOD N., JARRET J.O.: Detection of feline parvovirus in dying pedigree kittens. In: *Veterinar Record*, 1998, 142, 353-356.

AGUNGPRIYONO D.R., UCHIDA K., TABARU H., YAMAGUCHI R., TATEYAMA S.: Subacute massive necrotizing myocarditis by canine parvovirus type 2 infection with diffuse leukoencephalomalacia in a puppy. In: *Veterinary Pathology*, 1999, 36, 77-80.

ALLDINGER S., BAUMGÄRTNER W., ORVELL C.: Restricted expression of viral surface proteins in canine distemper encephalitis. In: *Acta Neuropathologica*, 1993, 85, 635-645.

AMUDE A.M., ALFIERI A.A., ALFIERI A.F.: Clinicopathological findings in dogs with distemper encephalomyelitis presented without characteristic signs of the disease. In: *Research in Veterinary Science*, 2007, 82, 416-422.

APPEL M.J.G.: Distemper pathogenesis in dogs. In: *Journal of the American Veterinary Medical Association*, 1970, 156, 1681-1684.

APPEL M.J.G., SCOTT F.W., CARMICHAEL L.E.: Isolation and immunisation studies of a canine parvo-like virus from dogs with haemorrhagic enteritis. In: *Veterinary Record*, 1979, 105, 156-159.

APPEL M.J.G., PARRISH C.R.: Raccoons are not susceptible to canine parvovirus. In: *Journal of the American Veterinary Medical Association*, 1982, 181, 489.

APPEL, M.J.G.: Canine distemper virus. In: *Virus infections of vertebrates, Vol. 1.* Editor: Horzinek, M.C.M. Elsevier Science Publishers B.V., Amsterdam, 1987, pp. 133-159

APPEL M.J.G., YATES R.A., FOLEY G.L., BERNSTEIN J.J., SANTINELLI S., SPELMAN L.H., MILLER L.D., ARP L.H., ANDERSON M., BARR M., PEARCE-KELLING S., SUMMERS B.A.: Canine distemper epizootic in lions, tigers and leopards in North America. In: *Journal of Veterinary Diagnostic Investigation*, 1994, 6, 277-278.

APPEL M.J.G., SUMMERS B.A.: Pathogenicity of morbilliviruses for terrestrial carnivores. In: *Veterinary Microbiology*, 1995, 44, 187-191

AXTHELM M.K., KRAKOWKA S.: Experimental old dog encephalitis (ODE) in a gnotobiotic dog. In: *Veterinary Pathology*, 1998, 35, 527-534.

BARRETT T.: Morbillivirus infections, with special emphasis on morbilliviruses of carnivores. In: *Veterinary Microbiology*, 1999, 69, 3-13.

BAUMGÄRTNER W., ÖRVELL C., REINACHER M.: Naturally occurring canine distemper virus encephalitis: distribution and expression of viral polypeptides in nervous tissues. In: *Acta Neuropathologica*, 1989, 78, 504-512.

BEINEKE A., PUFF C., SEEHUSEN F., BAUMGARTNER W.: Pathogenesis and immunopathology of systemic and nervous canine distemper. In: *Veterinary Immunology and Immunopathology*, 2009, 127, 1-18.

BELL S.C., CARTER S.D., BENNETT D.: Canine distemper viral antigens and antibodies in dogs with rheumatoid arthritis. In: *Research in Veterinary Science*, 1991, 50, 64-68.

BLANCOU J.: Dog distemper: imported into Europe from South America? In: *Historia Medicinae Veterinariae*, 2004, 29, 35-41.

BLIXENKRONE-MØLLER M., SVANSSON V., HAVE P., ORVELL C., APPEL M., PEDERSEN I.R., DIETZ H.H., HENRIKSEN P.: Studies on manifestations of canine distemper virus infection in an urban dog population. In: *Veterinary Microbiology*, 1993, 37, 163-173.

BOLT G., JENSEN T.D., GOTTSCHALCK E., ARCTANDER P., APPEL M.J.G., BUCKLAND R., BLIXENKRONE-MØLLER M.: Genetic diversity of the attachment (H) protein gene of current field isolates of canine distemper virus. In: *Journal of General Virology*, 1997, 78, 367-372.

BROWN A.L., VITAMVAS J.A., MERRY D.L.JR., BECKENHAUER W.H.: Immune response of pups to modified live-virus canine distemper-measles vaccine. In: *American Journal of Veterinary Research*, 1972, 33, 1447-1456.

BUONAVOGLIA C., MARTELLA V., PRATELLI A., TEMPESTA M., CAVALLI A., BUONAVOGLIA D., BOZZO G., ELIA G., DECARO N., CARMICHAEL L.: Evidence for evolution of canine parvovirus type 2 in Italy. In: *Journal of General Virology*, 2001, 82, 3021-3025.

BURTONBOY G., COIGNOUL F., DELFERRIERE N., PASTORET P.P.: Canine hemorrhagic enteritis: detection of viral particles by electron microscopy. In: *Archives of Virology*, 1979, 61, 1-11.

CARPENTER M.A., APPEL M.J.G., ROELKE-PARKER M.E., MUNSON L., HOFER H., EAST M., O'BRIEN S.J.: Genetic characterization of canine distemper virus in Serengeti carnivores. In: *Veterinary Immunology and Immunopathology*, 1998, 65, 259-266.

CHANG S.F., SGRO J.Y., PARRISH C.R.: Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. In: *Journal of Virology*, 1992, 66, 6858-6867.

CHAPPUIS G.: Control of canine distemper. In: *Veterinary Microbiology*, 1995, 44, 351-358.

DE YBANEZ R.R., VELA C., CORTES E., SIMARRO I., CASAL J. I.: Identification of types of canine parvovirus circulating in Spain. In: *Veterinary Record*, 1995, 136, 174-175.

DECARO N., ELIA G., MARTELLA V., CAMPOLO M., DESARIO C., CAMERO M., CIRONE F., LORUSSO E., LUCENTE M.S., NARCISI D., SCALIA P., BUONAVOGLIA C.: Characterization of the canine parvovirus type 2 variants using minor groove binder probe technology. In: *Journal of Virological Methods*, 2006a, 133, 92-99.

DECARO N., MARTELLA V., DESARIO C., BELLACICCO A.L., CAMERO M., MANNA L., D'ALOJA D., BUONAVOGLIA C.: First detection of canine parvovirus type 2c in pups with haemorrhagic enteritis in Spain. In: *Journal of Veterinary Medicine B Infectious Diseases Veterinary Public Health*, 2006b, 53, 468-472.

DECARO N., DESARIO C., ADDIE D.D., MARTELLA V., VIEIRA M.J., ELIA G., ZICOLA A., DAVIS C., THOMPSON G., THIRY E., TRUYEN U., BUONAVOGLIA C.: The study of molecular epidemiology of canine parvovirus. In: *Emerging Infectious Diseases*, 2007a, 13, 1222-1224.

DECARO N., DESARIO C., ELIA G., CAMPOLO M., LORUSSO A., MARI V., MARTELLA V., BUONAVOGLIA C.: Occurrence of severe gastroenteritis in pups after canine parvovirus vaccine administration: A clinical and laboratory diagnostic dilemma. In: *Vaccine*, 2007b, 25, 1161-1166.

DECARO N., DESARIO C., MICCOLUPO A., CAMPOLO M., PARISI A., MARTELLA V., AMORISCO F., LUCENTE M.S., LAVAZZA A., BUONAVOGLIA C.: Genetic analysis of feline panleukopenia viruses from cats with gastroenteritis. In: *Journal of General Virology*, 2008, 89, 2290-2298.

FAVROT C., OLIVRY T., DUNSTON S.M., DEGORCE-RUBIALES F., GUY J.S.: Parvovirus infection of keratinocytes as a cause of canine erythema multiforme. In: *Veterinary Pathology*, 2000, 37, 647-649.

GAEDKE K., ZURBRIGGEN A., BAUMGÄRTNER W.: Lack of correlation between virus nucleoprotein and mRNA expression and the inflammatory response in demyelinating distemper encephalitis indicates a biphasic disease process. In: *European Journal of Veterinary Pathology*, 1999, 5, 9-20.

GEMMA T., WATARI T., AKIYAMA K., MIYASHITA M., SHIN Y.S., IWATSUKI K., KAI C., MIKAMI T.: Epidemiological observations on recent outbreaks of canine distemper in Tokyo area. In: *Journal of Veterinary Medical Science*, 1996, 58, 547-550.

GLAUS T., GRIOT C., RICHARD A., ALTHAUS U., HERSCHKOWITZ N., VANDEVELDE M.: Ultrastructural and biochemical findings in brain cell cultures infected with canine distemper virus. In: *Acta Neuropathologica,* 1990, 80, 59-67.

GOSS L.J.: Diagnosis and treatment of diseases of wild animals in captivity. In: *Cornell Veterinarian*, 1942, 32, 155-161.

GREENE C.E., APPEL M.J.G.: Canine distemper virus. In: *Infectious diseases of the dog and cat, 2nd edition*. Editor: Greene C.E. Saunders Philadelphia, PA, USA, 1998, pp. 1-22.

GREENE C.E., ADDIE D.D.: Feline parvovirus infections. In: *Infectious diseases of the dog and cat, 3rd edition*. Editor: Greene C.E. Saunders Elsevier, St Louis, USA, 2006, pp. 78-88.

GREENE C.E., APPEL M.J.G.: Canine distemper. In: *Infectious diseases of the dog and cat, 3rd edition*. Editor: Greene C.E. Saunders Elsevier, St Louis, USA, 2006, pp. 25-41.

GREENE C.E., SCHULTZ R.D.: Immunoprophylaxis. In: *Infectious diseases of the dog and cat, 3rd edition*. Editor: Greene C.E. Saunders Elsevier, St Louis, USA, 2006, pp. 1101-1118.

GREENWOOD N. M., CHALMERS W.S.K., BAXENDALE W., THOMPSON H.: Comparison of isolates of canine parvovirus by monoclonal antibody and restriction enzyme analysis. In: *Veterinary Record*, 1996, 138, 495-496.

HAAS L., MARTENS W., GREISER-WILKE I., MAMAEV L., BUTINA T., MAACK D., BARRETT T.: Analysis of the haemagglutinin gene of current wild-type canine distemper virus isolates from Germany. In: *Virus Research*, 1997, 48, 165-171.

HAAS L., LIERMANN H., HARDER T.C., BARRETT T., LÖCHELT M., VON MESSLING V., BAUMGÄRTNER W., GREISER-WILKE I.: Analysis of the H gene, the central untranslated region and the proximal coding part of the F gene of wild-type and vaccine canine distemper viruses. In: *Veterinary Microbiology*, 1999, 69, 15-18.

HAIG D.A.: Canine distemper: immunization with avianized virus. In: *Onderstepoort Journal of Veterinary Research*, 1956, 17, 19-53.

HARDER T.C., OSTERHAUS A.D.M.E.: Canine distemper virus – A morbillivirus in search of new hosts? In: *Trends in Microbiology*, 1997, 5, 120-124.

HARRIS R.J.: Negative staining of thinly spread biological samples. In: *Electron microscopy methods and protocols, 2nd edition*. Editor: Kuo J. Humana Press Inc., Totowa, New Jersey, USA, 2007, pp 116-119.

HEUSINGER C.F.: Recherches de pathologie comparée. Cassel chez H. Hotop, Vol. 1, 1853, p. 674.

HIGGINS R., KRAKOWKA S., METZLER A., KOESTNER A.: Canine distemper virus-associated cardiac necrosis in the dog. In: *Veterinary Pathology*, 1981, 18, 472-486.

HINDLE E., FINDLAY G.M.: Studies on feline distemper. In: *Journal of Comparative Pathology and Therapy*, 1932, 45, 11-26.

HOELZER K., SHACKELTON L.A., HOLMES E.C., PARRISH C.R.: Within-host genetic diversity of endemic and emerging parvoviruses of dogs and cats. In: *Journal of Virology*, 2008a, 82, 11096-11105.

HOELZER K., SHACKELTON L.A., PARRISH C.R., HOLMES E.C.: Phylogenetic analysis reveals the emergence, evolution and dispersal of canine parvoviruses. In: *Journal of General Virology*, 2008b, 89, 2280-2289.

HONG C., DECARO N., DESARIO C., TANNER P., PARDO M.C., SANCHEZ S., BUONAVOGLIA C., SALIKI J.T.: Occurence of parvovirus type 2c in the United States. In: *Journal of Veterinary Diagnostic Investigations*, 2007, 19, 535-539.

HORIUCHI M., YAMAGUCHI Y., GOJOBORI T., MOCHIZUKI M., NAGASAWA H., TOYODA Y., ISHIGURO N., SHINAGAWA M.: Differences in the evolutionary pattern of feline panleukopenia virus and canine parvovirus. In: *Virology*, 1998, 249, 440-452.

IKEDA Y., MIYAZAWA T., NAKAMURA K., NAITO R., INOSHIMA Y., TUNG K.C., LEE W.M., CHEN M.C., KUO T.F., LIN J.A., MIKAMI T.: Serosurvey for selected virus infections of wild carnivores in Taiwan and Vietnam. In: *Journal of Wildlife Diseases*, 1999, 35, 578-581.

JOHNSON R.H., HALLOWELL R.E.W.: Natural susceptibility to feline panleucopaenia of the coati-mundi. In: *Veterinary Record*, 1968, 82, 582.

JOHNSON R.H., SPRADBROW P.B.: Isolation from dogs with severe enteritis of a parvovirus related to feline panleucopaenia virus. In: *Australian Veterinary Journal*, 1979, 55, 151.

KELLY W.R.: An enteric disease of dogs resembling feline panleucopaenia. In: *Australian Veterinary Journal*, 1978, 54, 593.

KENNEDY S., KUIKEN T., JEPSON P.D., DEAVILLE R., FORSYTH M., BARRETT T., VAN DE BILDT M.W.G., OSTERHAUS A.D.M.E., EYBATOV T., DUCK C., KYDYRMANOV A., MITROFANOV I., WILSON S.: Mass die-off of Caspian seals caused by canine distemper virus. In: *Emerging Infectious Diseases*, 2000, 6, 637-639.

KIMURA, M.: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. In: *Journal of Molecular Evolution*, 1980, 16, 111-120.

KOUTINAS A.F., POLIZOPOULOU Z.S., BAUMGÄRTNER W., LEKKAS S., KONTOS V.: Relation of clinical signs to pathological changes in 19 cases of canine distemper encephalomyelitis. In: *Journal of Comparative Pathology*, 2002, 126, 47-56.

KOUTINAS A.F., BAUMGÄRTNER W., TONTIS D., POLIZOPOULOU Z., SARIDOMICHELAKIS M.N., LEKKAS S.: Histopathology and immunohistochemistry of canine distemper virus-induced footpad hyperkeratosis (hard pad disease) in dogs with natural canine distemper. In: *Veterinary Pathology*, 2004, 41, 2-9.

KOVÁCS G., MOCSÁRI E., SZTOJKOV V., VETÉSI F., KISS J.H.: Canine distemper epizooty on a large scale mink ranch. Epizootiological and diagnostic experiences. In: *Magyar Állatorvosok Lapja*, 1983, 38, 305-308. [in Hungarian]

KRAKOWKA S., HIGGINS R.J., KOESTNER A.: Canine distemper virus: review of structural and functional modulations in lymphoid tissues. In: *American Journal of Veterinary Research*, 1980, 41, 284-292.

KREUTZER M., KREUTZER R., SIEBERT U., MÜLLER G., REIJNDERS P., BRASSEUR S., HÄRKÖNEN T., DIETZ R., SONNE C., BORN E.W., BAUMGÄRTNER W.: In search for virus carriers of the 1988 and 2002 phocine distemper virus outbreaks in European harbour seals. In: *Archives of Virology*, 2008, 153, 187-192.

LAMM C.G., REZABEK G.B.: Parvovirus infections in domestic companion animals. In: *Veterinary Clinics of North America Small Animal Practice*, 2008, 38, 837-851.

LAN N.T., YAMAGUCHI R., INOMATA A., FURUYA Y., UCHIDA K., SUGANO S., TATEYAMA S.: Comparative analyses of canine distemper viral isolates from clinical cases of canine distemper in vaccinated dogs. In: *Veterinary Microbiology*, 2006, 115, 32-42.

LEDNICKY J.A., DUBACH J., KINSEL M.J., MEEHAN T.P., BOCCHETTA M., HUNGERFORD L.L., SARICH N.A., WITECKI K.E., BRAID M.D., PEDRAK C., HOUDE C.M.: Genetically distant American canine distemper virus lineages have recently caused epizootics with somewhat different characteristics in raccoons living around a large suburban zoo in the USA. In: *Virology Journal*, 2004, 1, 2.

LYNCH M.J., RAPHAEL S.S., MELLOR L.D., SPARE P.D., INWOOD M.J.: Medical laboratory technology and clinical pathology, 2nd edition. WB Saunders Co, Philadelphia, 1969, pp. 25-29.

MARTELLA V., CAVALLI A., PRATELLI A., BOZZO G., CAMERO M., BUONAVOGLIA D., NARCISI D., TEMPESTA M., BUONAVOGLIA C.: A canine parvovirus mutant is spreading in Italy. In: *Journal of Clinical Microbiology*, 2004, 42, 1333-1336.

MARTELLA V., DECARO N., ELIA G., BUONAVOGLIA C.: Surveillance activity for canine parvovirus in Italy. In: *Journal of Veterinary Medicine B Infectious Diseases Veterinary Public Health*, 2005, 52, 312-315.

MARTELLA V., CIRONE F., ELIA G., LORUSSO E., DECARO N., CAMPOLO M., DESARIO C., LUCENTE M.S., BELLACICCO A.L., BLIXENKRONE-MOLLER M., CARMICHAEL L.E., BUONAVOGLIA C.: Heterogeneity within the hemagglutinin genes of canine distemper virus (CDV) strains detected in Italy. In: *Veterinary Microbiology*, 2006, 116, 301-309.

MARTELLA V., ELIA G., LUCENTE M.S., DECARO N., LORUSSO E., BANYAI K., BLIXENKRONE-MOLLER M., LAN N.T., YAMAGUCHI R., CIRONE F., CARMICHAEL L.E., BUONAVOGLIA C.: Genotyping canine distemper virus (CDV) by a hemi-nested multiplex PCR provides a rapid approach for investigation of CDV outbreaks. In: *Veterinary Microbiology*, 2007, 122, 32-42.

MCCAW D.L., HOSKINS J.D.: Canine viral enteritis. In: *Infectious diseases of the dog and cat, 3rd edition*. Editor: Greene C.E. Saunders Elsevier, St Louis, USA, 2006, pp. 63-70

MEURS K.M., FOX P.R., MAGNON A.M., LIU S.K., TOWBIN J.A.: Molecular screening by polymerase chain reaction detects panleukopenia virus DNA in formalin-fixed hearts from cats with idiopathic cardiomyopathy and myocarditis. In: *Cardiovascular Pathology*, 2000, 9, 119-126.

MOCHIZUKI M., SAN GABRIEL M.C., NAKATANI H., YOSHIDA M.: Comparison of polymerase chain reaction and haemagglutination assays for the detection of canine parvoviruses in faecal specimens. In: *Research in Veterinary Science*, 1995, 55, 60-63.

MOCHIZUKI M., HASHIMOTO M., HAGIWARA S., YOSHIDA Y., ISHIGURO S.: Genotypes of canine distemper virus determined by analysis of the hemagglutinin genes of recent isolates from dogs in Japan. In: *Journal of Clinical Microbiology*, 1999, 37, 2936-2942.

MOON H.S., LEE S.A., LEE S.G., CHOI R., JEOUNG S.Y., KIM D., HYUN C.: Comparison of the pathogenicity in three different Korean canine parvovirus (CPV-2) isolates. In: *Veterinary Microbiology*, 2008, 131, 47-56.

MULLER C.F., FATZER R.S., BECK K., VANDEVELDE M., ZURBRIGGEN A.: Studies on canine distemper virus persistence in the central nervous system. In: *Acta Neuropathologica*, 1995, 89, 438-445.

MULTINELLI F., VANDEVELDE M., GRIOT C., RICHARD A.: Astrocytic infection in canine distemper virus-induced demyelination. In: *Acta Neuropathologica,* 1989, 77, 333-335.

MURPHY F., GIBBS J., HORZINEK M., STUDDERT J.: *Veterinary Virology, 3rd edition.* Academic Press, San Diego, USA, 1999, 411-428.

NAKAMURA M., TOHYA Y., MIYAZAWA T., MOCHIZUKI M., PHUNG H.T., NGUYEN N.H., HUYNH L.M., NGUYEN L.T., NGUYEN P.N., NGUYEN P.V., NGUYEN N.P., AKASHI H.: A novel antigenic variant of canine parvovirus from a Vietnamese dog. In: *Archives of Virology*, 2004, 2261-2269.

NESSELER A., BAUMGÄRTNER W., ZURBRIGGEN A., ORVELL C.: Restricted virus protein translation in canine distemper virus inclusion body polioencephalitis. In: *Veterinary Microbiology*, 1999, 69, 23-28.

PACKER C., ALTIZER S., APPEL M., BROWN E., MARTENSON J., O'BRIEN S., ROELKE-PARKER M., HOFMANN-LEHMANN R., LUTZ H.: Viruses of the Serengeti: patterns of infection and mortality in African lions. In: *Journal of Animal Ecology*, 1999, 68, 1161-1178.

PANISSET L.: Traité des maladies infectieuses des animaux domestiques. Vigot Freres, Paris, 1938, p. 562.

PARDO I.D.R., JOHNSON G.C., KLEIBOEKER S.B.: Phylogenetic characterization of canine distemper viruses detected in naturally infected dogs in North America. In: Journal of Clinical Microbiology, 2005, 43, 5009-5017.

PARDO I.D.R.: Phylogenetic characterization of canine distemper viruses detected in naturally infected North American dogs. Doctoral Thesis. Graduate School of the University of Missouri-Columbia, Missouri, USA, 2006.

PARRISH C.R., CARMICHAEL L.E.: Antigenic structure and variation of canine parvovirus type-2, feline panleukopenia virus, and mink enteritis virus. In: *Virology*, 1983, 129, 401-414.

PARRISH C.R., HAVE P., FOREYT W.J., EVERMAN J.F., SENDA M., CARMICHAEL L.E.: The global spread of replacement of canine parvovirus strains. In: *Journal of General Virology*, 1988, 69, 1111-1116.

PARRISH, C.R.: Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. In. *Virology*, 1991, 183, 195-205.

PARRISH, C.R.: The emergence and evolution of canine parvovirus – an example of recent host range mutation. In: *Seminars in Virology*, 1994, 5, 121-132.

PARRISH C.R.: Pathogenesis of feline panleukopenia virus and canine parvovirus. In: *Baillière' s Clinical Haematology*, 1995, 57, 57-71.

PÉREZ R. FRANCIA L., ROMERO V., MAYA L., LÓPEZ I., HERNÁNDEZ M.: First detection of canine parvovirus type 2c in South America. In: *Veterinary Microbiology*, 2007, 124, 147-152.

PHILLIPS C.E.: Haemorrhagic enteritis in the arctic blue fox caused by the virus of feline enteritis. In: *Canadian Journal of Comparative Medicine*, 1943, 7, 33-35.

REED A.P., JONES E.V., MILLER T.J.: Nucleotide sequence and genome organization of canine parvovirus. In: *Journal of Virology*, 1988, 62, 266-276.

ROELKE-PARKER M.E., MUNSON L., PACKER C., KOCK R., CLEAVELAND S., CARPENTER M., O`BRIEN S.J., POSPISCHIL A., HOFMANN-LEHMANN R., LUTZ H.: A canine distemper virus epidemic in Serengeti lions *(Panthera leo)*. In: *Nature*, 1996, 379, 441-445.

RUDD P.A., CATTANEO R., VON MESSLING V.: Canine distemper virus uses both the anterograde and the hematogenous pathway for neuroinvasion. In: *Journal of Virology*, 2006, 80, 9361-9370.

SHACKELTON L.A., PARRISH C.R., TRUYEN U., HOLMES E.C.: High rate of viral evolution associated with the emergence of carnivore parvovirus. In: *Proceedings of the National Academy of Sciences*, *USA*, 2005, 11, 379-384.

SHARP N.J.H., DAVIS B.J., GUY J.S., CULLEN J.M., STEINGOLD S.F., KORNEGAY J.N.: Hydranencephaly and cerebellar hypoplasia in two kittens attributed to intrauterine parvovirus infection. In: *Journal of Comparative Pathology*, 1999, 121, 39-53.

SIMON-MARTINEZ J., ULLOA-ARVIZU R., SORIANO V.E., FAJARDO R.: Identification of a genetic variant of canine distemper virus from clinical cases in two vaccinated dogs from Mexico. In: *Veterinary Journal*, 2007, 175, 423-426.

SPIBEY N., GREENWOOD N.M., SUTTON D., CHALMERS W.S.K., TARPEY I.: Canine parvovirus type 2 vaccine protects against virulent challenge with type 2c virus. In: *Veterinary Microbiology*, 2008, 128, 48-55.

STEINEL A., MUNSON L., VAN VUUREN M., TRUYEN U.: Genetic characterisation of feline parvovirus sequences from various carnivores. In: *Journal of General Virology*, 2000, 81, 345-350.

STEINEL A., PARRISH C.R., BLOOM M.E., TRUYEN U.: Parvovirus infections in wild carnivores. In: *Journal of Wildlife Diseases*, 2001, 37, 594-607.

SUMMERS B.A., APPEL M.J.G: Demyelination in canine distemper encephalomyelitis: an ultrastructural analysis. In: *Journal of Neurocytology*, 1987, 16, 871-881.

TIJSSEN P.: Molecular and structural basis of the evolution of parvovirus tropism. In: *Acta Veterinaria Hungarica*, 1999, 47, 379-394.

TRUYEN U., GRUENBERG A., CHANG S.F., OBERMAIER B., VEIJALAINEN P., PARRISH C.R.: Evolution of the feline-subgroup parvoviruses and the control of canine host range in vivo. In: *Journal of Virology*, 1995, 69, 4702-4710.

TRUYEN U., GEISSLER K., PARRISH C.R., HERMANNS W., SIEGL G.: No evidence for a role of modified live virus vaccines in the emergence of canine parvovirus. In: *Journal of General Virology*, 1998, 79, 1153–1158.

TRUYEN, U.: Emergence and recent evolution of canine parvovirus. In: *Veterinary Microbiology*, 1999, 69, 47-50.

TRUYEN U.: Evolution of canine parvovirus – a need for new vaccines? In: *Veterinary Microbiology*, 2006, 117, 9-13.

VANDEVELDE M., ZURBRIGGEN A., HIGGINS R.J., PALMER D.: Spread and distribution of viral antigen in nervous canine distemper. In: *Acta Neuropathologica*, 1985, 67, 211-218.

VANDEVELDE M., ZURBRIGGEN A.: The neurobiology of canine distemper virus infection. In: *Veterinary Microbiology*, 1995, 44, 271-280.

VANDEVELDE M., ZURBRIGGEN A.: Demyelination in canine distemper virus infection: a review. In: *Acta Neuropathologica*, 2005, 109, 56-68.

VEIJALAINEN P., SMEDS E.: Pathogenesis of blue fox parvovirus on blue fox kits and pregnant vixens. In: *American Journal of Veterinary Research*, 1988, 49, 1941-1944.

VERGE J., CHRISTOFORONI N.: La gastroenterite infectieuse des chats; est-elle due à un virus filtrable? In: *Comptes Rendus des Sciences del Société de Biologie et de ses filiales* 1928, 99, 312.

WALLER E.F.: Infectious gastroenteritis in raccoons *(Procyon lotor)*. In: *Journal of the American Veterinary Medical Association*, 1940, 96, 266-268.

YOSHIKAWA Y, OCHIKUBO F, MATSUBARA Y., TSUROUKA H., ISHII M., SHIROTA K., NOMURA Y., SUGIYAMA M., YAMANOUCHI K.: Natural infection with canine distemper virus in a Japanese monkey *(Macaca fuscata)*. In: *Veterinary Microbiology*, 1989, 20, 193-205.

ZURBRIGGEN A., YAMAWAKI M., VANDEVELDE M.: Restricted canine distemper virus infection oligodendrocytes. In: *Laboratory Investigations,* 1993, 68, 277-284.

ZURBRIGGEN A., SCHMID I., GRABER H.U., VANDEVELDE M.: Oligodendroglial pathology in canine distemper. In: *Acta Neuropathologica,* 1998, 95, 71-77.

9. Scientific Publications of the Thesis

Demeter Z., Palade E.A., Lakatos B.,. Kozma T., Rusvai M.: Experiences on the Control of Endemic Distemper at the Flaying-House of Budapest. In: *Magyar Állatorvosok Lapja*, 2006; 128, 665-673. [in Hungarian, with English abstract]

Demeter Z., Lakatos B., Palade E.A., Kozma T., Forgách P., Rusvai M.: Genetic diversity of Hungarian canine distemper virus strains. In: *Veterinary Microbiology*, 2007, 122, 258-269.

Demeter Z., Gál J., Palade E.A., Rusvai M.: Feline parvovirus infection in an Asian palm civet *(Paradoxurus hermaphroditus)*. In: *Veterinary Record*, 2009, 164, 213-215.

Demeter Z., Palade E.A., Hornyák Á., Rusvai M.: Controversial results of the genetic analysis of a canine distemper vaccine strains. In: *Vaccine* – submitted for publication

10. Other Publications in Peer Reviewed Journals

Jakab Cs., Bánky Á., Kincses K., Balka Gy., **Demeter Z.**: Histopathology and frequency of canine skin tumors. In: *Magyar Állatorvosok Lapja*, 2006, 128, 140-149. [in Hungarian, with English abstract]

Gál J., Pásztor I., **Demeter Z.**, Palade E.A., Ursu K., Bálint Á., Pap T., Farkas Sz.: Viral serofibrinous tracheitis and resulting suffocation in an Amur ratsnake (*Elaphe schrenki*). In: *Magyar Állatorvosok Lapja*, 2008, 130, 421-424. [in Hungarian, with English abstract]

Palade E.A., Biró N., Dobos-Kovács M., **Demeter Z.**, Mándoki M., Rusvai M.: Poxvirus infection in Hungarian great tits *(Parus major)*. In: *Acta Veterinaria Hungarica*, 2008, 56, 539-546.

Palade E.A., **Demeter Z.**, Dobos-Kovács M., Rusvai M., Mándoki M.: Demonstration of infectious bursal disease virus, chicken nephritis virus and infectious bronchitis virus by multiplex RT-PCR diagnostic technique. In: *Magyar Állatorvosok Lapja*, 2008, 130, 559-564. [in Hungarian, with English abstract]

Gál J., Landauer K., Palade E.A., Ivaskevics K., Rusvai M., **Demeter Z.**: Squamous cell carcinoma and consequent otitis in a Long-eared Hedgehog *(Hemiechinus auritus)*. In: *Acta Veterinaria Hungarica*, 2009, 57, 69-74.

Gál J., **Demeter Z.**, Palade E.A., Rusvai M., Géczy Cs.: Harderian gland adenocarcinoma in a Florida red-bellied turtle *(Pseudemys nelsoni)*. In: *Acta Veterinaria Hungarica*, 2009 – accepted for publication

Palade E.A., Bajnok L., Dobos-Kovács M., **Demeter Z.**, Rusvai M.: Genetic characterization of Hungarian chicken anaemia virus strains. In: *Magyar Állatorvosok Lapja*, 2009 – accepted for publication [in Hungarian, with English abstract]

Jakab Cs., Schaff Zs., Kulka J., Szász A.M., **Demeter Z.**, Gálfi P., Rusvai M.: Angiogenesis – literature review. In: *Magyar Állatorvosok Lapja*, 2008 – accepted for publication [in Hungarian, with English abstract]

11. Congress Abstracts

Demeter Z., Forgách P., Tapaszti Zs., Rusvai M.: Detection of canine distemper virus strains by polymerase chain reaction technique and their phylogenetic analysis. In: *Acta Microbiologica et Immunologica Hungarica*, 2005, 52, 28-29.

Demeter Z., Palade E.A., Lakatos B., Kozma T., Rusvai M.: Endemic canine distemper infection at a dog shelter: epizootology and viral variability. In: *Acta Microbiologica et Immunologica Hungarica*, 2006, 53, 28-29.

Palade E.A., Dobos-Kovács M., **Demeter Z.**: Different diagnostic methods for turkey rhinotracheitis virus infections. In: *Acta Microbiologica et Immunologica Hungarica*, 2006, 53, 326.

Demeter Z., Lakatos B., Magyar T., Kozma T., Rusvai M.: Partial results of the epidemiological survey performed at the Dog Shelter of the City Council of Budapest. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, 24th of January, 2006. [in Hungarian]

Demeter Z., Palade E.A., Rusvai M.: Development of a multiplex RT-PCR test for the simultaneous detection of some of the major respiratory viral pathogens of the dog. In: *Acta Microbiologica et Immunologica Hungarica*, 2007, 54, 23-23.

Demeter Z., Palade E.A., Farsang A., Soós T., Balka Gy., Rusvai M.: Controversial results of the genetic analysis of a canine distemper vaccine strain. Vaccine Congress, 9-11 December 2007, Amsterdam, The Netherlands. *Abstract Book. Poster abstract P151*.

Palade E.A., Biró N., Dobos-Kovács M., **Demeter Z.**, Benyeda Zs., Mándoki M., Rusvai M.: Poxvirus infection in great tit *(Parus major)* in Hungary. In: *Acta Microbiologica et Immunologica Hungarica*, 2007, 54, 97-98.

Demeter Z., Palade E.A., Rusvai M.: Differentiation of vaccine and wild-type strains of CDV by restriction fragment length polymorphism. Congress of the Hungarian

Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, 23rd of January, 2007. [in Hungarian]

Palade E.A., Mándoki M., **Demeter Z.**, Dobos-Kovács M., Benyeda Zs., Rusvai M.: Development of a multiplex PCR assay for the simultaneous detection of infectious bronchitis virus and avian nephritis virus. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, 23rd of January, 2007.

Demeter Z., Palade E.A., Gál J., Rusvai M.: Demonstration of distemper and parvovirus infection in different species. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, 22nd of January, 2008. [in Hungarian]

Palade E.A., Mándoki M., Dobos-Kovács M., **Demeter Z.**, Rusvai M.: Diagnosis of infectius bronchitis, avian nephritis and infectious bursal disease by multiplex RT-PCR, and the phylogenetical analysis of infectious bursal disease virus strains circulating in Hungary. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, 22nd of January, 2008.

Palade E.A., Bajnok L., Dobos-Kovács M., **Demeter Z.**, Rusvai M.: Genetic characterization of Hungarian chicken anaemia virus strains. Congress of the Hungarian Academy of Sciences and the Doctoral (PhD) School of Veterinary Medicine, 27th of January, 2009.

12. Acknowledgements

First of all I would like to express my gratitude to my thesis supervisor, **Professor Miklós Rusvai**, for his unlimited support and confidence. I am grateful to all my colleagues from the Department of Pathology and Forensic Veterinary Medicine, especially **Mihály Dobos-Kovács, Csaba Jakab, Ferenc Vetési, Ferenc Baska, János Gál, Míra Mándoki, Gyula Balka**, and **Zsófia Benyeda** for all their valuable help and guidance. My thanks are also for all staff of the same department, especially **Ilona Herczeg, Renáta Pop** and **Judit Szilágyi.** I am grateful to all staff members of the Department of Microbiology and Infectious Diseases, especially **Tamás Bakonyi, Ákos Hornyák, Petra Forgách, Csaba Kővágó** and **Zsuzsanna Tapaszti** for guiding my first steps in the fascinating world of molecular biology.

I would also like to thank all veterinarians who kindly submitted the clinical samples, especially **Tamás Kozma** and **Béla Lakatos.** I am grateful to **Tibor Soós and Attila Farsang** for their help and suggestions regarding the CDV vaccines. I deeply appreciate the support and assistance of Pfizer Animal Health division of Pfizer Inc. showed during the investigations regarding the Vanguard vaccines.

I would like to express my gratitude towards the Hungarian Ministry of Education and the Agora Foundation for the scholarship that made my PhD studies possible.

None of my achievements would have been possible without the help and support of my family, especially my parents, brothers, uncle and aunt: none of this would have been possible without your love and support.

Finally I would like to express my gratitude to **Elena Alina Palade**, my colleague and true love for her unlimited help, understanding and patience.