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

Brief version of the doctoral thesis

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INTRODUCTION

Canine distemper (CD) is still one of the most serious threats to the susceptible carnivore population in Central and Eastern Europe. CD is a highly contagious viral infection of different carnivores that belong to numerous animal families, such as *Canidae*, *Mustelidae*, *Procyonidae*, *Felidae* and many others. Several studies have demonstrated that domestic canine populations and other receptive carnivores seem to act as reciprocal reservoirs. CD is caused by the canine distemper virus (CDV) which belongs to the *Morbillivirus* genus of the *Paramyxoviridae* virus family. The clinical symptoms of the disease include severe respiratory, digestive and neurological signs with frequently fatal outcome. 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.

The CDV genome is approximately 15 690 nucleotides in length and contains six genes that code for viral proteins. The hemagglutinin (H) glycoprotein is important in the viral attachment to the host cell. 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. This great genetic variability makes this gene suitable for phylogenetic analysis. 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. The variation between different lineages of the CDV is more than 4 % of the amino acids. However the greatest genetic diversity is between the strains belonging to the America 1 group (vaccine strains) and the other CDV lineages. The temporal dynamics of a virus depends on transmission properties of the virus and the survival, immune response and the distribution of the host. Since the strain used for vaccination are kept in freeze dried form, 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. 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. 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) and Snyder Hill (isolated in Ithaca from the brain of a dog in the 1950's), but several others such as Lederle, Rockborn etc. have been

used throughout the years. As recent genetic data show, America-1 strains have not been detected over the last 5 decades, and it is not known whether they are still circulating in the field. This fact allows the development of genetic techniques for the differentiation of currently used vaccine strains from wild-type strains in case of recently vaccinated animals.

Feline panleukopenia virus (FPV) is a single stranded DNA virus belonging to the *Parvoviridae* virus family. FPV is closely related to other parvoviruses, such as the mink enteritis virus (MEV), raccoon parvovirus (RPV), canine parvovirus (CPV) and blue fox parvovirus (BFPV). FPV-induced disease in cats has been known since the beginning of the 20th century. The first outbreaks of the disease in captive felids were reported in the 1930's and 1940's. There are several reports of FPV infections that occurred in wild species. Based on the reported cases it is generally assumed that all *Felidae* are susceptible to FPV infection and disease. No direct report and diagnosis of an FPV infection of any member of the *Viverridae* has been reported so far, except indirect evidence represented by a serological survey in Formosan gem-faced civets (*Paguma larvata taiwana*). Based on observations of the outbreaks in the 1930's and 1940's, initially it was assumed that lions (*Panthera leo*) possess a natural resistance to the infection. However, later these conclusions were disproved by other observations. Previous observations on lion populations with demonstrated endemic FPV infection revealed no dramatic consequences of the disease outbreaks.

Canine parvovirus 2 (CPV2) suddenly emerged as a new pathogen in the late 1970's, but more recent studies on the rate of CPV molecular evolution have indicated that the virus must have emerged at least 10-15 years earlier. Soon after the emergence of the firstly isolated genotype (named CPV2), it was replaced by a different antigenic variant named 2a that could be distinguished by the means of monoclonal antibodies. In the mid 1980's the virus suffered another single mutation and the new variant (CPV2b) quickly spread around the world and decimated susceptible canine populations everywhere. Soon after the new antigenic variants (CPV2a and 2b) have completely replaced the original type 2 and are variously distributed and co-exist in canine populations worldwide. Since then a number of further mutations have been described, some of them associated with antigenic differences. Recent investigations have demonstrated that the recently reported CPV2c is progressively replacing previous variants in the Italian canine population. CPV2c has also been reported in several European countries, Asia, Americas and Tunisia. The knowledge of the exact current epidemiological situation is vital in the better understanding of CPV2 evolution, as history has already demonstrated that minor changes can cause great changes in the behavior of the virus.

MATERIALS AND METHODS

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).

CDV samples

Samples were collected from clinically ill dogs (*Canis familiaris*) and from the 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. A total number of 214 samples were analyzed by RT-PCR for the detection 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 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. *Necropsy samples* (n = 28) were represented by urinary bladder, spleen, kidney, lung, brain, and cerebellum.

Vaccine samples (n = 10) were obtained from commercially available vaccines 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.

FPV and CPV2 samples

A total number of 230 samples were analyzed by classical PCR for the joint 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, an Asian palm civet (*Paradoxurus hermaphroditus*), and a lion (*Panthera leo*). The 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. 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. A total of twenty-three parvovirus positive samples were selected for genetic analysis.

Methods

The examination methods applied in the current study were represented by (1) macroscopic examination (necropsy), (2) histopathology, (3) electron microscopy, and (4) genetic investigations. Genetic investigations were represented by (i) purification of the nucleic acid, (ii) designing of new primer pairs for diagnostic and phylogenetic purposes, (iii) amplifications (classical PCRs and reverse transcription PCRs), (iv) RFLP-based techniques (a newly designed *PsiI*-based test for the fast differentiation of vaccine and wild-type strains of CDV and a previously described *MboII*-based test for the quick identification of type 2c CPVs), and by (v) nucleic acid sequencing and phylogenetic analysis. The nucleotide sequences were identified using 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, 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.

RESULTS

CDV

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. In case of the dissected ferret and raccoon, macroscopic signs suggestive of a catarrhal enteritis, severe lung edema and state of shock were observed. The examined fox suffered of severe dermatitis caused by *Sarcoptes scabiei* infection. Most relevant histopathological 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. In the urine samples of two CDV positive dogs during the EM examination virus particles with a diameter of approximately 250 nm and other properties characteristic for paramyxoviruses were observed.

Following the diagnostic RT-PCR reactions, out of the 214 analyzed samples 58 (27.1 %) proved to be positive for CDV. Applying the newly designed *PsiI*-based RFLP 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, demonstrating that the RFLP test can be used for the differentiation of vaccine strains from currently circulating wild-type strains. When the newly designed enzymatic differentiation was tested in practice by using virus strains present in several CDV vaccines currently used in Hungary, 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 wild-type viruses and the Vanguard vaccine (Pfizer Animal Health, USA) remained undigested. In order to extend the investigations, different batches of Vanguard vaccines dating back to 1992 and 1994 were involved in the study. Furthermore, batches of the same brand were purchased in different countries as well, such as Israel, Malta and USA. The RFLP test unequivocally resulted in undigested amplicons in case of all batches from different time and geographical origin. The investigations performed on the full nucleic acid sequences of the H and F genes revealed that the Hungarian groups were differently clustered on the phylogenetic tree. 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.

In order to identify the strain present in the Vanguard vaccines, primers were designed to cover more than half of the viral 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. 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, but it showed a significantly higher similarity to a lesser panda isolate (AF178039: 99.45 %) and a wild-type strain isolated from a naturally infected dog (AY964114: 98.90 %) in the USA. 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, as well as the exact positioning of the virus strains present in the vaccines currently used in Hungary. As expected following the RFLP test, virus strains present in all vaccines but the Vanguard were positioned in the America-1 group. Snyder Hill strain has the complete H gene sequence determined and deposited in the GenBank (AF259552). As expected, this strain was clustered together with the other vaccine strains in the America-1 group. The phylogenetic trees constructed based on the nucleic acid sequences of the other investigated genes (H, N, M, P and F) only supported the conclusions of the H gene analysis. In conclusion, the phylogenetic trees constructed based on the nucleotide sequences of the investigated genes 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.

FPV

During necropsy pathological changes suggestive of 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. 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. Histopathologically, in all parvovirus positive 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. In case of the lioness, an extended necrotizing myositis in the left hind limb and the penetrating ulceration of the duodenum were also present. The EM examination of the diluted feces of 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. 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). All attempts to demonstrate the presence of an other pathogen (CDV, feline coronavirus, feline calicivirus, feline herpesvirus 1, *Chlamydophyla psittaci*) in the organ samples of the lion led to negative results.

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 (389/07) 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 %). 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 %). Strain 933/07 was positioned right next to strain 389/07 and the Chinese isolate JF-3, while strain 1335/07 seemed to be closely related to Argentinean FPVs. 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.

CPV2

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. In case of the CPV2 positive dogs the most important histopathological findings were represented by focal mucous membrane necrosis in the small intestine, shortening of the intestinal villi, marked infiltration of mononuclear inflammatory cells 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. As mentioned earlier, 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). 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 bps) was obtained (the “shorter” amplicons of approximately 90 bps were barely visible) in case of 15 out of the 20 selected samples, while in case of the other five samples and of the control type 2b vaccine strain (Quantum, Schering-Plough, USA) the amplicons remained undigested. Sequence analysis was performed on the 20 PCR-positive samples used for the *Mbo*II-based RFLP. The sequence analysis of the amplicons (582 bp) amplified by primers *555for* and *555rev* 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 amino acid 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. The observed mutation did not alter the deduced amino acid sequence. The alignment of the Hungarian CPV2 strains revealed only a few other, apparently randomly scattered mutations, but none of these produced any change in the deduced amino acid sequences. None of the observed mutations 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.

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. Using these newly designed diagnostic primer pairs, CD has been demonstrated in Hungarian dogs and in other species as well: a free-roaming fox, a raccoon, and a ferret. In order to determine the CDV genotypes present in Hungary, a total number of 13 CDV positive samples collected between 2004 and 2006 were analyzed. These samples were taken from dogs that presented clinical symptoms suggesting CD, and from carcasses of dogs that succumbed following CD clinical signs, or that presented pathological changes that could have been attributed to CDV infection. The phylogenetic analysis was performed on the complete nucleic acid sequences of the H and F genes. The sequences of the Hungarian samples were compared to other complete sequences deposited in the GenBank. The phylogenetic analysis performed on the nucleic and amino acid sequences of the H and F genes and proteins respectively, revealed that the Hungarian groups were differently clustered on the phylogenetic tree: 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. 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. 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 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.

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. One of the several possibilities to differentiate vaccine strains from wild-type viruses is the use of RFLP-based tests. Based on the obtained nucleotide sequence (accession number: DQ903854), and on the sequences of other vaccine strains already available in the GenBank, our *PsiI*-based 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. Due to this fact, the *PsiI* 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. 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, even though 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. Based on the nucleotide sequence available in the GenBank (AF259552), the amplicons used in the RFLP test should have been digested by the *PsiI* enzyme, but those of the virus strain present in the Vanguard vaccine remained undigested, hence reacted as a wild-type virus. 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. H gene analysis further emphasized the previous findings: the investigated virus strain was not positioned anywhere close to the Snyder Hill strain, but turned out to be most closely related to a wild-type virus strain isolated from a lesser panda (AF178039). 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. 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. 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. 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 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.

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. There are several wild species from different animal families that are known to be receptive for FPV. 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. 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. 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, 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. 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. These findings seem to emphasize the genetic diversity of Hungarian FPVs, quite 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 the original type 2 CPV was soon replaced by a different antigenic variant (CPV2a) that differed from the original type by only 5 amino acids in the VP2 coat protein, whereas the type 2b CPV genotype that emerged a few years following this event differs from the 2a type by only one amino acid (Asn426Asp). More recently a new antigenic variant (named CPV2c) was reported in several countries around the world, including Italy, Spain, Germany, the UK, USA, Tunisia and Uruguay. 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. 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. 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 amino acid 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. The analysis of the nucleic acid and deduced amino acid sequences revealed a most surprising outcome: based on the amino acid 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. Hence the rapid enzymatic identification of the incriminated Hungarian type 2a CPVs gave a misleading genotyping result and acted as “true” CPV2c strains. 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 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: three of the analyzed samples 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 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 Zealand.

In conclusion, we managed to demonstrate the presence of only CPV2a in Hungary so far. On the other hand, the genetic analysis provided evidence to demonstrate that due to a seemingly constant mutation present in most of the Hungarian CPV2a strains, the previously described and routinely used *Mbo*II-based identification of CPV2c cannot be reliably used any more.

NEW SCIENTIFIC RESULTS

- Design of new primers for the diagnosis of CD and feline and canine parvovirus infections and for the genetic analysis of the causative agents.
- Design of a *PsiI* based RFLP assay for the fast and reliable differentiation of vaccine and wild-type strains of CDV.
- Demonstration of the presence of a different strain than “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.
- 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.
- 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 an FPV infection in a lion (*Panthera leo*) from Hungary.
- Demonstration of only type 2a CPVs in Hungary, and identification of type 2a Hungarian CPV variants that react as type 2c CPVs following a previously described *MboII*-based 2c genotyping assay, hence leading to diagnostically misleading results.

LIST OF PUBLICATIONS

Scientific publications of the thesis

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