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*Original article*

# Phylogenetic characterization of Canine Parvovirus VP2 partial sequences from symptomatic dogs samples

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## Abstract

The aim of the present study was to detect canine parvovirus (CPV) from faecal samples of clinically ill domestic dogs by polymerase chain reaction (PCR) followed by VP2 gene partial sequencing and molecular characterization of circulating strains in Lithuania.

Eleven clinically and antigen-tested positive dog faecal samples, collected during the period of 2014-2015, were investigated by using PCR. The phylogenetic investigations indicated that the Lithuanian CPV VP2 partial sequences (3025-3706 cds) were closely related and showed 99.0-99.9% identity. All Lithuanian sequences were associated with one phylogroup, but grouped in different clusters. Ten of investigated Lithuanian CPV VP2 sequences were closely associated with CPV 2a antigenic variant (99.4% nt identity). Five CPV VP2 sequences from Lithuania were related to CPV-2a, but were rather divergent (6.8 nt differences). Only one CPV VP2 sequence from Lithuania was associated (99.3% nt identity) with CPV-2b VP2 sequences from France, Italy, USA and Korea. The four of eleven investigated Lithuanian dogs with CPV infection symptoms were vaccinated with CPV-2 vaccine, but their VP2 sequences were phylogenetically distantly associated with CPV vaccine strains VP2 sequences (11.5-15.8 nt differences).

Ten Lithuanian CPV VP2 sequences had monophyletic relations among the close geographically associated samples, but five of them were rather divergent (1.0% less sequence similarity). The one Lithuanian CPV VP2 sequence was closely related with CPV-2b antigenic variant. All the Lithuanian CPV VP2 partial sequences were conservative and phylogenetically low associated with most commonly used CPV vaccine strains.

**Key words:** Canine parvovirus, dog, molecular epidemiology, Lithuania

## Introduction

Canine parvovirus (CPV) is the causative agent of acute haemorrhagic enteritis and myocarditis in dogs and it is one of the most important pathogenic viruses. CPV is a highly contagious virus and it causes often fatal diseases (Nandi and Kumar 2010). CPV, a member of the *Parvoviridae* family *Protoparvovirus* genus, contains a single strand DNA genome of about 5200 nucleotides that is packaged in an icosahedral capsid (Siegl et al. 1985, Cotmore et al. 2014). The genome is simple and contains two large open reading frames (ORF) as well some smaller or overlapping genes, mostly generated by alternative splicing. In the conventional orientation, the right-hand ORF encodes the capsid proteins and the left-hand one encodes the non-structural proteins (Hoelzer and Parrish 2010). CPV contains three capsid proteins: VP1, VP2 and VP3. VP2 is the highly antigenic major capsid protein, and it plays an important role in determining viral host range and tissue tropism (Hueffer et al. 2003). Amino acids substitutions in VP2 gene have been responsible for genetic and antigenic properties (Truyen 1999).

The virus emerged as dog pathogen in the late 1970's as host variant of feline panleukopenia virus (FPLV) (Truyen 2006). A few years after its emergence, the original virus type CPV-2 was replaced by two new antigenic variants, CPV-2a and CPV-2b (Parrish et al. 1988). During the last ten years, a novel CPV mutant, CPV-2c, has widely distributed and co-exists with other CPV types in Europe (Decaro et al. 2011), North (Hong et al. 2007) and South (Perez et al. 2007) America countries. The identification of the subtypes of CPV-2 that are currently circulating in the canine population is essential for the understanding of viral evolution and the development of measures to control its spread (Pinto et al. 2012). All the three antigenic variants differ from the original type CPV-2 for a few amino acids in the VP2 protein, whereas genetic differences among the variants are determined only by residue 426, with types 2a, 2b, and 2c displaying Asn, Asp, and Glu, respectively (Martella et al. 2006).

Polymerase chain reaction (PCR) is considered as the most reliable diagnostic technique having high degree of sensitivity and specificity in detecting CPV from faecal samples. PCR-based molecular typing of CPV also helps to gain new insights into pathogenesis of CPV-2 types (CPV-2a, CPV-2b and CPV-2c) and is extremely useful to understand antigenic differences between CPV types (Decaro et al. 2005a).

There is concern that the antigenic differences may decrease the effectiveness of the vaccine based

on the original antigenic type CPV-2. Although the original vaccine has been shown to protect dogs against challenge from any of the current CPV types (Spibey et al. 2008), there are still many cases of clinical parvovirus infection in dogs. For example, in 2007, an outbreak of CPV-2c was reported in the dogs immunized with CPV-2-based vaccine in Italy (Decaro et al. 2008a).

In Lithuania, hemorrhagic enteritis in dogs caused by CPV-2 was first reported in 1995-1996 (Stankevicius and Salomskas 1998, Grigonis et al. 2002). The diagnostic criteria for CPV infection were based on clinical signs (enteritis, hemorrhagic gastroenteritis, gastroenteritis, vomiting and diarrhoea), the examination of blood and antibody titration: leukopenia (a white blood cell count – WBC of <5000 cells/ $\mu$ l) or a single CPV haemagglutination inhibition (HI) antibody titre  $\geq$ 1: 5120 (Glickman et al. 1985).

The aim of the present study was to detect CPV from faecal samples of clinically ill domestic dogs by polymerase chain reaction (PCR) followed by VP2 gene partial sequencing and molecular characterization of circulating strains in Lithuania. This is the first report of the CPV VP2 proteins partial nucleotide sequence analysis from clinical cases in Lithuania.

## Materials and Methods

### Sample collection

Faecal samples were collected from 25 dogs presented to different Veterinary Pet Clinics situated in Kaunas city (central part of Lithuania) during a period of 12 months from January 2014 to January 2015. At the animal hospital, the samples were emulsified (10%, wt/vol) in 1 ml sterile phosphate-buffered saline (PBS, pH 7.2) and stored at -20°C for 1-2 months prior to being sent for laboratory analysis. After thawing, the samples were centrifuged at 10,000 rpm for 10 min at 4°C. The cleared supernatants were frozen at -80°C and collected for PCR amplification.

### DNA extraction

DNA was extracted from samples of 10% faeces in PBS using the TRIzol method (Invitrogen, Life Technologies, MD, USA) following the manufacturer's recommendations. The supernatant containing the DNA was transferred to a new tube and stored at -20°C until it was used in PCR.

Table 1. Identification of Lithuanian CPV-positive dog faeces samples, used for PCR and sequence analysis.

| Sample ID | Breed                 | Age (months) | Sex    | CPV Vaccinated (+/-) | Sample type | CPV/CCV Ag Test Kits (+/-) | Sample DNA concentration ng/ $\mu$ l | Amplified PCR product (bp) |
|-----------|-----------------------|--------------|--------|----------------------|-------------|----------------------------|--------------------------------------|----------------------------|
| LTU2CPV2  | West Highland Terrier | 2.0          | Female | -                    | Faeces      | +                          | 34.6                                 | 681                        |
| LTU4CPV2  | German Shepherd       | 3.0          | Male   | -                    | Faeces      | +                          | 28.0                                 | 681                        |
| LTU5CPV2  | Mix                   | 3.0          | Male   | -                    | Faeces      | +                          | 19.7                                 | 681                        |
| LTU6CPV2  | Pekingese             | 1.5          | Male   | +                    | Faeces      | +                          | 31.7                                 | 681                        |
| LTU7CPV2  | Pekingese             | 2.5          | Female | +                    | Faeces      | +                          | 19.8                                 | 681                        |
| LTU8CPV2  | Yorkshire Terrier     | 2.0          | Female | -                    | Faeces      | +                          | 10.6                                 | 681                        |
| LTU9CPV2  | Beagle                | 1.5          | Female | -                    | Faeces      | +                          | 5.9                                  | 681                        |
| LTU10CPV2 | Mix                   | 5.5          | Male   | +                    | Faeces      | +                          | 8.5                                  | 681                        |
| LTU12CPV2 | Spice dog             | 2.5          | Female | -                    | Faeces      | +                          | 6.9                                  | 681                        |
| LTU13CPV2 | Mix                   | 2.5          | Female | -                    | Faeces      | +                          | 6.3                                  | 681                        |
| LTU31CPV2 | Chinese Crested       | 3.0          | Female | +                    | Faeces      | +                          | 8.9                                  | 681                        |

### Primers and PCR

The PCR was developed using primer set specific for pCPV-2ab. The pCPV-2ab primer set amplifies part of VP2 gene of both CPV-2a and CPV-2b variants (3025 to 3706 nucleotide position of CPV genomic DNA) to yield a product size of 681 bp. The PCR was carried out in principle as described earlier (Kumar et al. 2011) with some modifications. Five  $\mu$ l of DNA sample was mixed with 34.5  $\mu$ l water, nuclease free (Thermo Scientific, Lithuania); 10xPCR buffer (100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40) 5  $\mu$ l; 25 mM MgCl<sub>2</sub> 3  $\mu$ l (1,5 mmol/l); dNTP's (10 mM) 1  $\mu$ l; and Taq DNA polymerase (Thermo Scientific, Lithuania) (5 U/ $\mu$ l) 0,5,1 and primers pCPV-2ab (F) (5'-Gaa gag tgg ttg taa ata att -3', position 3025-3045) and pCPV-2ab (R) (5'-Cct ata taa cca aag tta gtac-3', position 3685-3706) 10 pmol each. The thermocycling format: denaturation at 95°C/5 min; 35 amplification cycles with denaturation at 95°C/30 s, annealing 55°C/60 s, extension at 72°C/60 s; and a final incubation at 72°C/7 min. A commercial CPV2a Virbagen Parvo-C vaccine (strain CPV 154, 107 TDCI50/1ml (tissue culture infective dose), Virbac Limited, UK) was used as a positive control and nuclease free water (Thermo scientific, Lithuania) – as a negative control.

### Agarose gel electrophoresis and PCR products purification

A 25  $\mu$ l aliquot of amplified PCR product from each sample were separated on 1.5% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), stained with ethidium bromide (10  $\mu$ l) and visualized under UV light. Consequently, PCR products were purified from agarose gel slice using GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania) following the manufacturer's recommendations. The GeneJET™ purification column was discarded and the purified DNA was stored at -20°C.

### DNA sequencing and phylogenetic analysis

Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) according to manufacturer's protocol. The DNA was sequenced with the same primer set that was used in the preceding RT-PCR steps. The following reaction mixtures were used: terminator ready reaction mix 1.0  $\mu$ l, template 10 ng, primer 3.2 pmol and deionised water up to 10  $\mu$ l. The following cycling condition was used: initial denaturation 96°C/1 min, followed for 25 cycles: 96°C/10 s, 50°C/5 s and 60°C/4 min. Ethanol/EDTA precipita-

Table 2. The CPV and FPLV reference strains used for Lithuania VP2 sequences phylogenetic analysis available from the GenBank database (<http://www.ncbi.nlm.nih.gov>).

| GenBank Nr. | Strain/isolate ID          | Antigenic variants, country or vaccine producer | References                      |
|-------------|----------------------------|---|---------------------------------|
| FJ005252    | strain 96/02               | CPV2a, Italy                                    | Decaro et al. 2009              |
| GU362934    | strain 12/08-B             | CPV2a, Italy                                    | Decaro et al. 2010              |
| AF306447    | isolate 618                | CPV2a, Italy                                    | Battilani et al. 2001           |
| FJ005263    | strain 42/05-49            | CPV2b, Italy                                    | Decaro et al. 2009              |
| FJ005195    | strain 136/00              | CPV2c, Italy                                    | Decaro et al. 2009              |
| AY742935    | strain CPV-U6              | CPV2a, Germany                                  | Shackelton et al. 2005          |
| AY742953    | strain CPV-435             | CPV2a, Germany                                  | Shackelton et al. 2005          |
| FJ005260    | strain G82/97              | CPV2b, Germany                                  | Decaro et al. 2009              |
| FJ005198    | strain G133/97             | CPV2c, Germany                                  | Decaro et al. 2009              |
| DQ025986    | isolate 04S17              | CPV2a, France                                   | Unpublished (direct submission) |
| DQ025992    | isolate 04S23              | CPV2b, France                                   | Unpublished (direct submission) |
| KF500497    | isolate TR/CPV/K12-A       | CPV2a, Turkey                                   | Unpublished (direct submission) |
| KF500499    | isolate TR/CPV/K20-KM      | CPV2b, Turkey                                   | Unpublished (direct submission) |
| JF280912    | strain TR-SS               | FPLV, Turkey                                    | Muz et al. 2012                 |
| GQ865519    | strain GR09/09             | CPV2c, Greece                                   | Ntafis et al. 2010              |
| GQ865518    | strain GR51/08             | CPV2c, Greece                                   | Ntafis et al. 2010              |
| FJ005247    | strain 195/08              | CPV2c, Belgium                                  | Decaro et al. 2009              |
| FJ005214    | strain 67/06               | CPV2c, Spain                                    | Decaro et al. 2009              |
| Z46651      | strain 46                  | CPV2b, Poland                                   | Unpublished (direct submission) |
| EU145958    | isolate CPV/BJ069/O7       | CPV2a, China                                    | Unpublished (direct submission) |
| KC556950    | isolate CPV/Jiangsu/59     | CPV2a, China                                    | Unpublished (direct submission) |
| KC556956    | isolate CPV/Jiangsu/60     | CPV2b, China                                    | Unpublished (direct submission) |
| EF599097    | isolate DH326              | CPV2b, South Korea                              | Kang et al. 2008                |
| EU009206    | strain K031                | CPV2b, South Korea                              | Jeoung et al. 2008              |
| AJ698134    | isolate Indian             | CPV2a, India                                    | Unpublished (direct submission) |
| DQ182623    | isolate IIL P20            | CPV2b, India                                    | Chinchkar et al. 2006           |
| AB054220    | strain V217                | CPV2b, Japan                                    | Ikeda et al. 2000               |
| AB054225    | strain V142                | FPLV, Japan                                     | Ikeda et al. 2000               |
| M38245      | strain CPV-b               | CPV2b, USA                                      | Parrish 1991                    |
| M24003      | isolate CPV-15             | CPV2a, USA                                      | Parrish et al. 1988             |
| M74849      | isolate 39                 | CPV2b, USA                                      | Unpublished (direct submission) |
| AY742955    | strain CPV-436             | CPV2b, USA                                      | Shackelton et al. 2005          |
| FJ005235    | strain 67/07-11            | CPV2c, USA                                      | Decaro et al. 2009              |
| M38246      | isolate CU-4               | FPLV, USA                                       | Parrish 1991                    |
| JF796200    | strain PassoFundo/6        | CPV2a, Brazil                                   | Pinto et al. 2012               |
| JF796195    | strain Parana/9            | CPV2c, Brazil                                   | Pinto et al. 2012               |
| EU018144    | isolate ARG02              | FPLV, Argentina                                 | Unpublished (direct submission) |
| AJ007500    | isolate CPV-Africa 9       | CPV2a, Africa                                   | Unpublished (direct submission) |
| AJ007498    | isolate CPV-Africa 3       | CPV2b, Africa                                   | Unpublished (direct submission) |
| AY742933    | strain CPV-339             | CPV2a, New Zeland                               | Shackelton et al. 2005          |
| FJ011098    | strain Intervet/vaccine/06 | CPV2a, Intervet                                 | Unpublished (direct submission) |
| FJ011097    | strain Merial/vaccine/06   | CPV2a, Merial                                   | Unpublished (direct submission) |
| FJ197847    | strain CPVpf (vaccine)     | CPV2a, S. Korea, Pfizer                         | Yoon et al. 2009                |
| GU212792    | strain VAC_S quantum       | CPV2b, Thailand, Schering Quantum               | Phromnoi et al. 2010            |
| EU498681    | strain Felocell (vaccine)  | FPLV, Felocell                                  | Decaro et al. 2008b             |
| EU498680    | strain Purevax (valcine)   | FPLV, Purevax                                   | Decaro et al. 2008b             |

tion method used for the extension products purification. ABI Prism 310 Genetic Analyzer used for samples electrophoresis and data analysis. The sequencing results were prepared in the ABI format (chromatogram) and analyzed by Chromas (version 2.01, Technelysium, Australia), saved in the FASTA format and used for the next investigation.

Evolutionary analysis was conducted in MEGA6 (Tamura et al. 2013). Multiple alignments were carried out using ClustalX program (Thompson et al. 1997). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial tree for the heuristic search was obtained by applying the Neighbor-Joining method (Saitou and Nei 1987) to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach (Felsenstein 1981). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 nucleotide sequences. There were a total of 3651 positions in the final dataset, the percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test shown next to the branches.

After analysis, the obtained sequences from Lithuania (Table 1) were compared with those of other reference CPV and FPLV strains (Table 2) available from the GenBank database (<http://www.ncbi.nlm.nih.gov>).

## Results

All the 25 dogs, included in the CPV sample collection and investigation, showed the characteristic CPV infection symptoms of fever, diarrhoea or hemorrhagic diarrhoea and vomiting. All faeces samples of the suspected dogs were positive tested by practitioners using a rapid test for simultaneous detection of CPV and Canine coronavirus (CCoV) antigens (Anigen, Rapid CPV/CCV Ag Test Kits – chromatographic immunoassay for the qualitative detection of CPV and CCoV antigens in canine faeces, Bio Note, Korea).

The PCR investigations with the pCPV-2ab (F) and pCPV-2ab (R) primer set revealed positive results in all tested faeces samples. After DNA purification, the best 15 PCR positive CPV products (DNA concentration >5.0 ng/μl) were selected and identified as acceptable for sequencing step. Sequencing (forward and reverse) with the amplification primers yielded 681 bases of sequence on average, which each then trimmed to a consensus 675 nt for comparative analysis. The most specific eleven Lithuanian CPV2 sequences were selected for phylogenetic analysis

(Table 1). The sequence evolutionary relationships of the different Lithuanian CPV samples (n=11), CPV2 field (n=37) and vaccine (n=4) viruses as well as FPLV VP2 sequences (n=6) from various parts of Europe, Asia and America were identified (Fig. 1). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The sequences in Maximum Likelihood tree were subdivided into two main phylogenetic groups (I and II, bootstrap support of 62%) and eight main clusters in phylogroup I (bootstrap support of 48-55%). The FPLV field isolates from Turkey (JF280912FPLV), Japan (AB054225FPLV), Argentina (EU018144FPLV) and USA (M38246FPLV) were phylogenetically closely associated (99.4% nt identity) as the same as vaccine strains (99.6% nt identity) EU498681FPLV (Felocell) EU498680FPLV (Purevax) VP2 sequences clustered separately (Fig. 1, phylogenetic group II) as outgoing group. This group was distantly associated with all of phylogroup (I) CPV VP2 sequences (96.4% nt identity), including all the CPV VP2 sequences from Lithuania (96.7% nt identity).

Sequence comparisons showed nucleotide identities of 99.0-99.9% among the CPV strains VP2 sequences in Lithuanian samples. All the Lithuanian sequences were associated in phylogroup I (Fig. 1), but grouped in different clusters. The phylogenetic tree shows that the five Lithuanian CPV VP2 sequences isolated in this study predominantly clustered in a distinct clade (cluster 1) and were closely associated (99.6% nt identity), but the one sequence (LTU9CPV2) was rather divergent (99.0% nt identity). The all five Lithuanian CPV sequences from first cluster were associated (99.3% nt identity) with the CPV sequence from Italy (GU362934CPV2a). The cluster 2 was connected with the first cluster and contained two CPV sequences from Lithuania (LTU2CPV2 and LTU4CPV2) and the sequence FJ005252CPV2a from Italy (99.5% nt identity). The CPV sequence LTU7CPV2 was grouped together (99.3% nt identity, cluster 3) with CPV sequences from France (DQ025986CPV2a), Germany (AY742935CPV2a) and Italy (AF306447CPV2a). Two CPV2 sequences (cluster 4) from Lithuania (LTU6CPV2 and LTU13CPV2) were closely related (99.6% nt identity) to the CPV isolates VP2 sequences from Turkey (KF500499CPV2b; KF500497CPV2a).

In one Lithuanian dog CPV2 isolate VP2-sequence (cluster 6) was rather divergent: the sequence LTU31CPV2 isolate was associated (99.3% nt identity) with CPV isolates VP2 sequences from France (DQ025992CPV2b) and Italy (FJ005263CPV2b). Clusters 5 and 7 included more than two types of

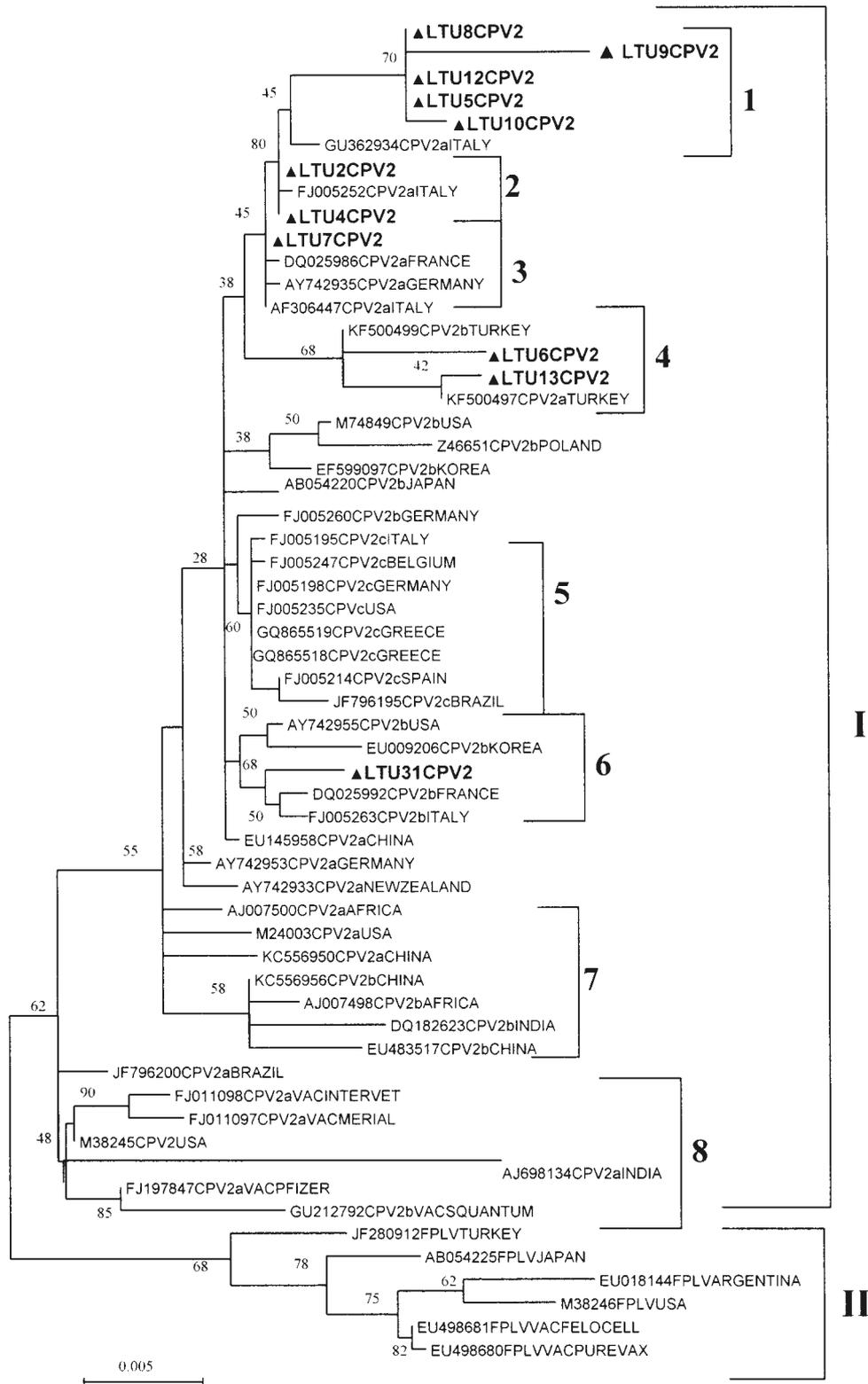


Fig. 1. The Maximum Likelihood phylogenetic tree of CPV VP2-coding (675 bp) region showing the relationships among 58 isolates of CPV. The tree with the highest log likelihood (-7330.6468) is shown. Lithuanian CPV isolates (n=11) VP2-sequences are presented in **▲Bold**. Evolutionary analysis was conducted in MEGA6 (Tamura et al. 2013).

CPV, indicating weak variation within the two clusters (2a, 2b and 2c). However, the cluster 5 was monophyletic and joined all the CPV 2c new antigenic variants from Germany, Italy, Belgium, USA, Greece, Spain and Brazil (99.5% nt identity). The cluster 7 was more divergent and associated with CPV 2a and sequences from Africa, USA, China (98.8% nt identity), and CPV 2b from Africa, China and India (99.0% nt identity). Thus the majority of CPV sequences were clustered separately depending on their type. So the cluster 8 was based on CPV 2a antigenic variant and contained strongly connected (99.6% nt identity) CPV vaccine strains VP2 sequences FJ011098CPV2a (Intervet), FJ011097CPV2a (Merial) FJ197847CPV2a (Pfizer) GU212792CPV2b (Schering Quantum) and weak associated (98.3% nt identity) CPV isolates from USA (M38245CPV2) and India (AJ698134CPV2a).

The statistical comparative evaluation of nucleotides and amino acids (nt/aa) differences per sequence of all the identified CPV strains VP2 sequences in Lithuanian samples showed, that average of nt/aa differences within the Lithuanian isolates were 4.58/1.75 respectively. The identical investigation between Lithuanian and the others CPV isolates VP2 sequences included in statistical analysis indicated the 7.25/2.45 nt/aa differences. Although, ten of investigated Lithuanian CPV VP2 sequences were closely associated with CPV 2a antigenic variant (99.4 % nt identity), but one sample (LTU31CPV2) – with CPV 2b antigenic variant (99.3 % nt identity).

## Discussion

The analysis of canine parvovirus retrospective epidemiology showed, that CPV can be one of the most important agents of gastrointestinal diseases in Lithuania. As in the other countries (Filipov et al. 2011), in Lithuania, diagnosis of parvovirus in dogs was based only on clinical signs and rapid (enzyme-linked or chromatographic immunosorbent assay based) tests, which are often used in small animal practices without interpretation of possible false-negative results and without comparison with other methods. In contrast (Desario et al. 2005) with and similar (Filipov et al. 2011) to previous reports there was a good correlation between the antigen-detection test and PCR methods, all of PCR investigated Lithuanian dog faeces samples being recognized as positive. Most of the CPV PCR positive faeces samples (18 of 25 tested), were from dogs with signs of haemorrhagic gastroenteritis. The main source of the infection seems to be the faeces of infected dogs as more as  $10^9$  virus particles/g can be shed

during acute phase of the enteric form and virus survives at least 4 months in the faeces in the environment. So PCR is considered as the most reliable diagnostic technique having high degree of sensitivity and specificity in detecting CPV from faecal samples (Decaro et al. 2005b).

PCR based sequence analysis has revolutionized our knowledge of the spatial and temporal dynamics of CPV infection and also helps to gain new insights into pathogenesis and antigenic differences between CPV-2 types (Clegg et al. 2011). Phylogenetic analysis of the CPV2 variants circulating in the canine population of central Lithuania has shown that they are similar, but not identical to those found in other countries (including Europe). Except for a few nodes, the resulting Maximum Likelihood tree (Fig. 1) was not supported by high bootstrap values, showing that the partial sequences of the VP2 gene is not highly informative from phylogenetic point of view due to the low variability observed (2-42 nt). Phylogenetically the analysed canine parvoviruses fall into two distinct clades with the longest internal branch separating the FPLV-like viruses (Fig. 1, phylogroup II) from the viruses isolated from dogs (Fig. 1, phylogroup I). Phylogenetic analysis revealed that all CPV variants were descended from a single ancestor emerged during the mid-1970s and was closely related to the long-known FPLV which infects cats, minks, and raccoons but not dogs or cultured dog cells (Truyen 2006). There were more than 98% sequence homology and as few as six coding nucleotide differences in the VP2 gene at positions 3025, 3065, 3094, 3753, 4477 and 4498 (Truyen et al. 1995). Comparison of the entire genomes of the viruses showed that FPLV sequences were distinguished from CPV sequences by a total of 16 substitutions, of which 11 were located in the capsid protein genes, emphasizing the important role of the capsid in this emergence event (Hoelzer et al. 2008).

All currently known CPV-2- and CPV-2a-derived viruses are monophyletic, indicating that a single cross-species transmission event gave rise to all currently known CPV strains (Shackelton et al. 2005, Hoelzer et al. 2008). The same monophyletic clustering was observed in the Maximum Likelihood tree (Fig. 1) where the CPV sequences from Lithuania were closely related (more than 99% nt identity) to CPV-2a VP2 sequences from Italy (cluster 2), France, Germany (cluster 3) or Turkey (cluster 4). However, five CPV VP2 sequences from Lithuania (cluster 1) were related to CPV-2a, but were rather divergent (6.8 nt differences) from others tree clusters. Only one CPV VP2 sequence from Lithuania (LTU31CPV2) was associated with CPV-2b VP2 sequences from France, Italy, USA and Korea (cluster

6). As it was expected and reported previously (Decaro et al. 2009) all the CPV-2c VP2 sequences formed a monophyletic cluster (cluster 7). Despite the strong phylogenetic association with CPV-2a ancestor, the Lithuanian CPV VP2 sequences show more or less geographically defined evolution pattern (especially five CPV samples in the first cluster), as were identified in other regions and studies (Battilani et al. 2002, Wang et al. 2005, Kang et al. 2008). Phylogenetic analysis showed some evidence for geographical clustering at an international level, suggesting that currently there are limited opportunities for global transmission, as has previously been suggested by others (Hoelzer et al. 2008). Despite this observation, sequences from individual countries, as exemplified by the United Kingdom, were generally not monophyletic, implying that national diversity is produced by a combination of local evolution occasionally supplemented by importation of new sequence types. This geographical restriction of certain virus types highlights the importance of rigorous, epidemiologically representative sampling strategies for the study of viral molecular epidemiology (Clegg et al. 2011).

Whether the CPV epidemiology in other Eastern European countries (Filipov et al. 2011) is related to different canine vaccination protocols or trade practices should be evaluated carefully in the future. The four of eleven investigated Lithuanian dogs (Table 1) with CPV infection symptoms were vaccinated with CPV-2 vaccine (one time), but their VP2 sequences were phylogenetically distantly associated with CPV vaccine strains VP2 sequences (11.5-15.8 nt differences). All the CPV-2a vaccine VP2 sequences were strongly determined and closely related (bootstrap support 90 and 85) in cluster 8 (Fig. 1). As previously found (Decaro et al. 2007), the occurrence of CPV-induced gastroenteritis in regularly vaccinated dogs poses intriguing questions about the real efficacy of currently available (type 2 based) vaccines against the infection and diseases caused by the antigenic variants. In this case, this was indeed the main reason that the virus was sent for further identification following the initial confirmation of the presence of CPV in the faeces using an in-clinic test kit. Quick test kits can detect the presence of parvovirus, but will not be able to distinguish the type involved. In order to confirm whether the isolate is a field strain or a vaccine one it is often sufficient to type the virus since the original 'type 2' virus is no longer present in the field (Parrish et al. 1985). In such cases, the identification of type 2a, 2b or 2c would be sufficient to confirm a field infection. The manufacturers were initially able to use specific PCR probes to differentiate between the unique genetic fingerprint of the vaccine strain

and all other CPV strains, thus confirming that the isolate was not a vaccine one (Sutton et al. 2013). Moreover, nowadays there is a particular interest in the CPV once the virus has involved to a new viral variant that has been detected in several parts of the world. This becomes significant as most of the currently available vaccines are manufactured with CPV-2, and the crossimmunity between these different genotypes is not totally understood. Some authors have suggested an update of the virus strains in current vaccines, taking into account the existing partial protection (Truyen 2006). Because of this, the isolation of new CPV circulating variants is important in order to be used in vaccine manufactures more effective from an immunogenic point of view (Puentes et al. 2012).

## Conclusion

Phylogenetic investigations of the Lithuanian CPV VP2 partial sequences identified monophyletic relations among the close geographically associated CPV samples. Ten of the eleven VP2 sequences from Lithuania were related to CPV-2a antigenic variant, but 5 of them were rather divergent and demonstrated 1.0% less sequence similarity to CPV-2a isolates from France, Germany or Turkey. The one Lithuanian CPV VP2 sequence was closely related with CPV-2b antigenic variants from France, Italy, USA and Korea. All of the Lithuanian CPV VP2 partial sequences were conservative and phylogenetically low associated with most commonly used CPV vaccine strains.

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