Genetic variability among canine parvovirus strains currently circulating in Poland

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Summary

The aim of the study was to perform a molecular analysis of the CPV VP2 gene fragment isolated from faeces samples of 46 dogs (vaccinated, as well as non-vaccinated against parvovirus) with clinical parvovirus. In all animals, the infection was confirmed by PCR. The purified PCR products (fragment VP2 gene) were sequenced and compared to each other and to the sequences of CPV VP2 gene from NCBI GenBank. Readable sequences of the VP2 gene were obtained for 46 samples. In 23 out of 30 non-vaccinated dogs, the most frequently isolated CPV strain was 2b. In the remaining cases the dogs were infected with CPV-2a. The parvovirus strains isolated from faeces of vaccinated dogs were classified in most cases (14/16) as CPV-2b, one as CPV-2a and one as CPV-2c. Continuous monitoring of parvovirus in dogs in Poland plays an important role in updating vaccines against the disease and developing effective methods of its prevention.

Keywords: CPV, dogs, PCR

Parvovirus is an infectious viral disease caused by canine parvovirus (CPV-2), (family *Parvoviridae*, subfamily *Parvovirinae*, genus *Protoparvovirus*) (30). The genetic material of the virus is a single stranded DNA (ssDNA), which codes two non-structural proteins (NS1 and NS2) and three structural proteins (VP1, VP2 and VP3). The VP2 protein constitutes 90% of the viral capsid and acts as its main protein antigen (32).

Parvoviruses usually infect young animals (aged 6 weeks to 6 months) exposed to stress, parasitic invasions or kept in poor sanitary conditions (15). A lack of vaccinations is considered to be the most important risk factor of parvovirus (13). The main clinical symptoms of parvovirus are: diarrhoea, vomiting and fever. In intrauterine infected puppies or in the first two weeks of life, symptoms of congestive heart failure or inflammation may develop in the heart muscle with sudden death (1). Sometimes symptoms of cardiovascular failure they can appear even at 2 months of age (29). Some research also proves that parvoviral infection could be associated with myocarditis and myocardial fibrosis in dogs younger than 2 years (11). Haematological tests performed in sick animals may reveal leukopenia and anaemia associated with the loss of blood into the lumen of the gastrointestinal tract (5, 15).

The first cases of parvovirus in dogs, caused by the CPV-2 strain, were described in 1978 in the USA.

In the following years, the virus evolved and spread to other parts of the world. Three subsequent viral strains were described: CPV-2a (426Asn), CPV-2b (426Asp) and CPV-2c (426Glu) (3). These strains almost entirely superseded CPV-2 and occur almost everywhere in the world (20). Since the introduction of vaccinations against CPV, the prevalence of the disease in the dog population has been significantly reduced. Nevertheless, in Poland a growing incidence of the disease has been recently observed, also in adult animals regularly vaccinated against CPV-2.

The aim of the study was to perform a molecular characterisation of the CPV VP2 gene isolated from faeces samples of dogs with clinical parvovirus from the territory of eastern Poland.

Material and methods

Animals used in the study. The study was performed in the years 2017-2019 and involved 46 dogs from eastern Poland of various breeds and gender (females $n = 20$, males $n = 26$), aged 6 weeks to 8 years with confirmed parvovirus. Sixteen of the studied animals had a history of vaccination against parvovirus, while 30 animals were never vaccinated (Tab. 1). All dogs showed the clinical symptoms of parvovirus (apathy, fever, diarrhoea, vomiting). The disease was confirmed by the positive results of PCR (faeces samples from rectum served as material).

Tab. 1. Dogs used in the study

O.n.	Sample number	Vaccination type	Age	Gender	Breed
1.	2.	No vaccination	10 weeks	F	Mix
2.	3.	Nobivac 3x	6 months	M	Labrador retriever
3.	4.	No vaccination	8 months	M	German Shepherd
4.	5.	No vaccination	8 months	M	German Shepherd
5.	6.	No vaccination	8 months	M	German Shepherd
6.	7.	No vaccination	3 months	F	Mix
7.	8.	No vaccination	8 years	M	Mix
8.	9.	No vaccination	4 months	M	Mix
9.	10.	No vaccination	9 weeks	F	White Swiss Shepherd
10.	11.	Nobivac 3x	7 months	F	White Swiss Shepherd
11.	13.	Biocan 2x	9 weeks	M	German Shepherd
12.	14.	Biocan 2x	9 weeks	M	German Shepherd
13.	15.	Biocan 2x	9 weeks	M	German Shepherd
14.	17.	Vanguard, Canigen	7 months	M	Maltese
15.	18.	Biocan	3 months	M	Mix
16.	19.	Biocan	8 weeks	F	Mix
17.	20.	No vaccination	8 weeks	F	Mix
18.	21.	No vaccination	4 months	F	Mix
19.	22.	Nobivac	8 years	M	Mix
20.	23.	No vaccination	9 weeks	M	Mix
21.	24.	Nobivac	9 weeks	M	Mix
22.	25.	Nobivac	9 weeks	F	Mix
23.	26.	Nobivac	9 weeks	F	Mix
24.	27.	Nobivac	8 weeks	M	Maltese
25.	28.	No vaccination	9 months	F	Maltese
26.	29.	No vaccination	4 years	M	Czechoslovakian Wolfdog
27.	30.	No vaccination	10 weeks	F	Mix
28.	31.	Biocan	19 weeks	F	Mix
29.	33.	No vaccination	6 months	F	Rottweiler
30.	34.	Biocan	17 weeks	M	Welsh Terrier
31.	35.	No vaccination	8 months	M	Mix
32.	36.	No vaccination	1 year	M	Mix
33.	37.	No vaccination	2 years	F	Mix
34.	38.	No vaccination	4 months	F	Mix
35.	39.	Nobivac	4 months	M	Mix
36.	40.	No vaccination	6 weeks	M	German Shepherd
37.	41.	No vaccination	9 weeks	M	Mix
38.	42.	No vaccination	5 months	F	White Swiss Shepherd
39.	44.	No vaccination	4 months	M	Mix
40.	46.	No vaccination	4 months	F	Mix
41.	48.	No vaccination	4 months	F	Mix
42.	49.	No vaccination	4 months	F	Mix
43.	50.	No vaccination	2 years	M	Mix
44.	51.	No vaccination	5.5 years	F	Mix
45.	52.	No vaccination	4.5 months	M	Mix
46.	53.	No vaccination	7 weeks	M	Mix

Molecular analysis. DNA for analysis was extracted from stool samples with GeneAll Exgene Stool DNA mini kit. Polymerase chain reaction (PCR) was performed using a programmable thermal cycler (Biometra). The PCR reaction for CPV VP2 gene was carried out according to a method described by Buonavoglia (3) using the following primers: 555for (positions 4003-4022) 5' → 3' CAGGAAGATATCCAGAAGGA and 555rev (positions 4585-4561) 5' → 3' GGTGC-TAGTTGATATGTAATAACA. This limited the DNA section to a length of 583 bp of the VP2 gene. The positive control was the DNA of CPV-2b (strain CPV-2b-Bio 12/B), isolated from a commercially available vaccine (Versican Plus DHPPI, Zoetis Belgium SA), and the negative control was DNA from the stool of a healthy dog. All samples were examined only once. Each reaction was composed of 30 cycles with the denaturation stage at 94°C for 60 s, annealing at 58°C for 60 s and elongation at 72°C for 90 s. The reaction mixture (50 µL) contained 100 µM of each dNTP, 1.6 mM of MgCl₂, 0.25 µM of each primer, 2.5 U of Taq DNA polymerase, and 5 µL of DNA template. The PCR products were analysed with the electrophoresis method in a 1% agarose gel and Tris-borate-EDTA (TBE) buffer at a voltage of 10 V/cm for 50 mins. The PCR reaction products purified with the QIAquick PCR Purification Kit (Qiagen) were sequenced by the DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics at Polish Academy of Science in Warsaw. The sequencing results were received via email and developed using the computer software Lasergene DNA Star MegAlign software (Madison USA). The same software was used to analyse the sequence of CPV isolates and compare them with sequences available in the National Center for Biotechnology Information (NCBI) Genbank from: Germany (AY742953, AY742935), Brazil (DQ340434), South Africa (AJ007500, AJ007498), New Zealand (AY742933), China (EU145958), Turkey (EU145958, KF500499), Italy (GU362934, FJ005252, AF306447, FJ005263, FJ005195), USA (M24000, M38245, AY742955, M74849, FJ005235), France (DQ025986, DQ025992), India (AJ698134, DQ182623), South Korea (EU009206, EF599097), Japan (AB054220), Poland (Z46651), Spain (FJ005214), Belgium (FJ005247) and Greece (GQ865518, GQ865519) and with sequences VP2 of CPV strains used in commercial vaccines. Double phylogenetic analysis was performed. The phylogenetic trees were created separately

for CPV VP2 amplicons obtained from vaccinated dogs and non-vaccinated dogs (Fig. 1 and Fig. 2).

Results and discussion

All 46 samples gave the amplicon size of 583 bp in agarose gel electrophoresis. Vaccinated dogs ranged in age from 9 weeks to 8-years-old and 13/16 (81%) of them were up to 6-months-old. Within unvaccinated dogs were animals at age from 6-weeks- to 8-years-old, and 19/30 (63%) dogs were up to 6-months-old. Generally, fourteen out of 46 animals (30%) were over six months of age and 32/46 (70%) were 6-weeks- to 6-months-old.

Within vaccinated dogs 8/16 (50%) were mixed breed, 3/16 (19%) German Shepherd, 2/16 Maltese (13%) and there were single representants, 1/16 (6%), of such breeds as Labrador Retriever, White Swiss Shepherd and Welsh Terrier. Within non-vaccinated dogs 21/30 were mixed breed (70%), 4/30 (14%) were German Shepherds, 2/30 (7%) were White Swiss Shepherds and there was one case (3%) of such breeds as Maltese, Rottweiler and Czechoslovakian Wolfdog.

Generally, twenty nine out of fifty-two dogs (63%) with parvovirus confirmed by molecular test were mixed breed, 7/46 (15%) were German Shepherds, 3/46 (7%) Maltese Dogs, 3/46 (7%) White Swiss Shepherds and there were single cases, while 1/46 (2%) of such breeds as Welsh Terrier, Rottweiler, Czechoslovakian Wolfdog and Labrador Retriever.

Legible sequences were obtained for 46 DNA samples. The sequences of CPV VP2 gene obtained in the study demonstrated mutual homology of 98.5-100%. The similarity of the nucleotide sequences of VP2 gene fragments obtained in our study to the analogous fragments amplified from vaccine viruses was 98.5-99.3% for Intervet, 98.5-99.3% for Merial, 98.7-99.4% for Pfizer, 98.9-99.4% for Quantum, 98.9-99.4% for Biocan and 98.9-99.4% for Versican.

Computer processing of the sequences CPV VP2 gene from vaccinated dogs using the Lasergene DNA Star software enabled the creation of a phylogenetic tree demonstrating similarities between particular isolates. Differences in primary genetic structure of each isolate made it pos-

sible to distinguish 6 monophyletic groups. Group 1 was formed by isolates No: 11, 24, 25, 27, 34 and 26. It demonstrated the highest homology (99.5%) with CPV-2b strain sequence isolated in Turkey (KF500499). The next group was formed by a single strain, No 22, which demonstrated the highest similarity of the examined gene sequence (99.4%) to CPV-2b isolated in Korea (EU009206). The third group included isolates No 3, 17, 19, 39, which demonstrated the highest homology (99.8-100%) with a Japanese strain (AB054220). The fourth group was formed by a single isolate, No 31, which was homologous (100%) to Greek CPV-2c strains: (GQ865518 and GQ865519), Spanish strain (FJ005214) and American

Fig. 1. Phylogenetic tree of CPV strains isolated from dogs who had received a preventive vaccination

strain (FJ005235). The fifth group consisted of three isolates (No: 13, 14 and 18), demonstrating the highest homology (99.3-99.4%) with Indian CPV-2b strain (DQ182623). The last group was formed by one isolate

(No 15), showing the greatest similarity of the examined gene sequence (99.8%) to Italian CPV-2a isolate (FJ005252). It may be stated that the parvovirus strains isolated from vaccinated dogs in which the disease de-

veloped were classified in most cases (14/16) as CPV-2b, one as CPV-2a and one as CPV-2c (Fig. 1).

Phylogenetic analysis of the VP2 CPV sequences obtained from the group of non-vaccinated dogs demonstrated the presence of 5 monophyletic groups. The first group consisted of 11 isolates (No: 2, 10, 28, 33, 35, 36, 37, 38, 40, 42, 53), demonstrating the highest similarity (99.8-100%) to the CPV-2b virus sequence isolated in Japan (AB054220). The second group was formed by a single strain (No 46) most homologous (99.8%) to CPV-2b isolated in Italy (FJ005263). The next group was formed by isolates 23, 29 and 41, demonstrating the highest homology (99.8%) to the CPV-2b virus isolated in South Korea (EU009206). The fourth group was formed by 8 isolates (No: 5, 7, 8, 9, 20, 21, 30 and 51), demonstrating the highest similarity of the analysed VP2 gene sequence to sequence DQ182623 from India (99.4-99.8%). The last group consisted of isolates No 4, 6, 44, 48, 49, 50 and 52 demonstrating the highest homology (99.6-99.8%) with CPV-2a strains from New Zealand (AY742933), China (EU145958) and Brazil (DQ340434). Similarly, to the group of vaccinated dogs the main CPV strain isolated from non-vaccinated dogs with the symptoms of parvovirus was CPV-2b (23/30). In the remaining seven cases, CPV-2a was the etiological factor of the disease infectious agent. CPV2c was not isolated from any of the dogs in this study group (Fig. 2).

Nucleotide substitutions among VP2 CPV sequences obtained from vaccinated as well as non-vaccinated dogs with parvovirus are presented in Table 2 and 3. All sequences for tested isolates are available in GenBank database.

CPV VP2 is the main structural protein, which determines the major mutations during the evolution of CPV. VP1 and VP2 proteins are formed by alternative splicing from

Fig. 2. Phylogenetic tree of CPV strains isolated from dogs who had not received a preventive vaccination

Tab. 2. Genetic sequence variability in strains isolated from vaccinated animals

Position	62	102	266	428	443	494	500
Nucleotide in standard sequence	T	A	T	T	T	A	A
Group I			C	C			C
Group II				C			
Group III					C		
Group IV	A						
Group V		G					
Group VI						G	

Tab. 3. Genetic sequence variability in strains isolated from non-vaccinated animals

Position	102	266	407	428	443	479
Nucleotide in standard sequence	A	T	C	T	T	T
Group I					C	
Group II		C		C		
Group III			T			
Group IV	G					
Group V						C

the same RNA (28). VP1 contains the 584 amino acid residues of VP2 and 143 unique amino terminal residues (25). VP2 is the largest viral antigen component (90%) and plays an important role in its tissue tropism (14). It is where most amino acid substitutions and genetic mutations occur, which can cause changes in viral antigen properties. This protein is responsible for the interaction with the host's transferrin receptor (TfR). Because transferrin receptors are especially and significantly expressed in mitotically active cells, such as rapidly dividing intestinal epithelial cells or cardiomyocytes in puppies, these are the cells for which the canine parvovirus has the highest affinity (14).

CPV has some persistent genetic variations and at present CPV-2a, CPV-2b, and CPV-2c are the three major antigenic variants of origin CPV type 2 (2, 9). The phylogenetic analysis performed here has showed that the main strain of parvovirus isolated in dogs with clinical parvovirus in Poland (both vaccinated and non-vaccinated) was CPV-2b (detected in 14/16 vaccinated dogs and 23/30 non-vaccinated dogs). This variant of the virus, alongside CPV-2a, is considered to be the major pathological factor of parvovirus in Asia and Europe (33).

The domestic CPV-2b strains isolated from the dogs with parvovirus included in our study demonstrated relatively high variability. This indicates considerable diversity of CPV occurrence in nature and an expanding range of occurrence of particular CPV viral strains in the world. So far, CPV-2a was considered to be the main causative factor of parvovirus in dogs in the territory of eastern Europe and Asia (33), while in our study it was detected in only one dog in the group of vaccinated dogs and 7 non-vaccinated dogs.

In 1995 Mizak and Plucienniczak examined CPV strains isolated between 1982-1993 and showed that CPV-2a strain is dominant within the dog population in Poland. On the other hand, in 2011 Majer-Dziedzic et al. and in 2019 Kowalczyk et al., showed that the CPV-2c, and CPV 2a respectively are dominant among other strains. In our research, in which material was collected between 2017-2019, we showed that the dominant variant in the dog population in eastern Poland is CPV-2b. The results of all above mentioned results indicates the dynamic changes in the dominant parvovirus strains (17, 19, 21). Phylogenetic analysis demonstrated that the recent CPV-2c isolate shares a common evolutionary origin with CPV-2c from Greece: (GQ865518 and GQ865519), Spain (FJ005214) and the USA (FJ005235). CPV-2c has been detected also in dogs from the United Kingdom, Greece and Bulgaria, where there was a higher frequency of CPV-2a/2b infections (7, 10, 24). Outside Europe, type 2c isolates were detected in North America (12, 16), in South America (4, 26), in India (23), in Vietnam (22) and in Taiwan (6). Two different European epidemiological surveys (7, 8) showed that CPV-2c is now predominant in Italy, Germany and Spain and is also widely distributed in Portugal and France.

It should be kept in mind that in our study, the 2c strain was detected in only 1 out of 46 dogs with the symptoms of parvovirus. Thus, we cannot talk about a widespread presence of this pathogen in the population of dogs in Poland. Nevertheless, the fact of recording its occurrence in our country is disturbing and may explain the increased incidence of the disease in older animals which have undergone vaccination with traditional anti-CPV vaccines. It may be presumed with a high degree of probability that the number of CPV-2c infections will increase in Poland, as has been happening in other European countries, where, in Italy for example, within 10 years from 1995 to 2005, CPV-2c very rapidly superseded CPV-2b.

The occurrence of parvovirus in regularly vaccinated dogs (as in our own study) poses intriguing questions about the real efficacy of the vaccines although there are some reports about cross-protection within different strains of canine parvovirus (18, 31). Because of this, the isolation of new CPV circulating variants is important in order to be used more effectively in the manufacture of vaccines, from an immunogenic point of view (27).

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