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Research article

DIAGNOSTICS AND GENOTYPING OF CANINE PARVOVIRUS TYPE 2 (CPV-2) FROM DISEASE CASES IN SOUTH-EASTERN POLAND

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Canine parvovirus type 2 is one of the most common causes of death among puppies. Despite preventive vaccination, the disease continues to be diagnosed. The aim of the study was to provide a molecular characterization of CPV-2 isolates found in south-eastern Poland. Genetic CPV-2 material was isolated from the blood (n=10) and feces (n=50) of infected dogs. The presence of CPV-2 was confirmed by amplification of sequences coding both VP1 and VP2 protein. The products of the PCR reaction with primers amplifying VP2 protein were sequenced and used for genotyping. Bioinformatics analysis of the sequenced PCR product was performed to determine the phylogenetic relationships with variants recorded in the public databases. Based on the analysis of polymorphism in the nucleotide sequence 7 nucleotide variants were detected and assigned into four amino acid groups. Representatives of three groups contained asparagine at amino acid position 426 of the VP2 protein, which is characteristic of CPV-2a. The variant from the fourth group belonged to type CPV-2b. CPV-2a is the dominant antigenic type of CPV-2 in Poland. The pathogen's high degree of polymorphism is manifested not only by the presence of numerous variants within the type, but also by the presence of representatives of type CPV-2b. Further studies of the molecular epidemiology of CPV-2 are necessary to optimize the effectiveness of preventive measures.

Key words: canine parvovirus type 2, molecular diagnostics, phylogenetics, VP2 protein

INTRODUCTION

Despite the widespread use of specific immunoprophylaxis, parvovirus infections in dogs are a current problem worldwide. The acute course of the disease and high

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mortality among puppies under the age of six months make the parvovirus infection one of the most dangerous diseases in young dogs. The etiologic agent of the disease is a non-enveloped virus of the genus *Parvovirus*, subfamily *Parvovirinae*, family *Parvoviridae* [1].

The genetic material of CPV-2 is a single-stranded DNA with a length of 5.3 kb. Two open reading frames enable translation of fragments encoding two non-structural proteins (NS1 and NS2) and two structural proteins (VP1 and VP2). During CPV-2 infection, digestion of the VP2 protein by a host protease results in the formation of the VP3 protein [2].

CPV-2 infection was first diagnosed in the 1970s. The virus has since undergone intensive evolution, resulting after three decades in the emergence of three types, CPV-2a, CPC-2b and CPV-2c, [3-5], which supplanted the original variant. Its substantial genetic variation, with a similar substitution rate to that of viral RNA [6], entails difficulty in selecting both diagnostic tests and vaccine antigens. What is more, due to the presence of maternal antibodies, vaccination may be not as effective as it is needed. Therefore, diagnostics plays a crucial role in CPV-2 prophylaxis [7].

Classical diagnostic methods are not always sufficiently sensitive, which makes precise identification of a disease entity difficult [3]. For this reason, molecular methods with a higher sensitivity and specificity are increasingly used for routine diagnostics [8,9].The leading method of canine parvovirus diagnosis is PCR (Polymerase Chain Reaction) and its modifications, such as Real-Time PCR [10,11], multiplex PCR [12], and nested PCR [13]. Owing to the high sensitivity and specificity of the PCR technique, the CPV-2 genetic material can be detected directly at an early stage of infection. An additional advantage of molecular techniques based on amplification of nucleic acids is that the variants obtained can be genotyped by sequencing or using appropriate probes in a qPCR reaction [3, 8]. Knowledge of the dominant CPV-2 variants in a given area makes it possible to select a vaccine antigen that provides effective protection against infection [14].

Poland is a country with a relatively poorly characterized molecular epidemiology. Given the high rate of evolution of the virus, existing research results must be updated and supplemented. The aim of the study was to examine the molecular variability of Polish CPV-2 isolates, determine their antigenic variants, and present their relationship with variants from other countries.

MATERIAL AND METHODS

Samples were collected from 116 dogs with gastrointestinal signs during 2008-2016. To identify CPV-2 in the feces of dogs with suspected parvovirus infection, we used the commercial immunochromatographic test Speed Parvo® (Virbac), based on specific monoclonal antibodies. Sixty samples were selected for further tests. The research material consisted of feces (n=50) and blood (n=10) from dogs with acute diarrhea,

vomiting, and fever, in which infection was confirmed by an immunochromatographic test.

To avoid false negative results caused by the presence of enzyme reaction inhibitors in the feces, the virus was isolated from the feces and cultured. Blood samples from dogs with confirmed infection were used directly as the material for further testing. The study was performed according to statutory bioethical standards and approved by the Local Ethics Commission of the University of Life Sciences in Lublin, approval number 83/2009.

Isolation of the virus from feces

The virus was isolated from the feces of dogs with confirmed infection (n=50). Samples of intestinal contents was diluted in PBS in the ratio 1:10 and mixed with chloroform (one part of chloroform with ten parts of sample). The mixture was vortexed and incubated for 12 hours at 4°C and centrifuged at 2000g for 15 min. The supernatant was treated with penicillin, streptomycin, amphotericin and tylosin, incubated and mixed for 30 min at 20°C. After incubation, the supernatant was filtered through a 0.45 μm and 0.2 μm filter (Millipore™). After confirming the hemagglutination activity of the resulting filtrate, it was used to inoculate a culture of the continuous cell line CCC clone 81 (clone 81 of transformer feline kidney cells). A cytopathic effect was obtained after the first blind passage. After five passages, the hemagglutination titre was measured and found to be in the range of 400-3,200, while the cell culture infectious dose ($CCID_{50}ml^{-1}$) ranged from $10^{3.5}$ to $10^{5.5}$. The strains were freeze-dried and pooled for further studies.

DNA extraction and amplification

DNA was extracted from cell cultures showing cytopathic effects (n=50) and from blood samples obtained from dogs with positive results of Speed Parvo test (n=10). Viral DNA was isolated from the cell culture and blood with a QIAmp DNA Mini Kit (Qiagen) and the genetic material was subjected to electrophoretic evaluation. A PCR reaction was conducted to detect the genetic material of the CPV-2 virus in the blood of the animals. Two primers pairs were used for the reaction. The first pair (V1) amplified the coding sequence of a VP2 protein fragment, and the second pair (V2) amplified the entire CPV-2 capsid protein. The primer sequences, reaction conditions and mixture composition are given in Tables 1 and 2. The optimum amplification temperature was established by modifying the reaction conditions using a thermal gradient and a magnesium concentration gradient. PCR products were separated in a 1% agarose gel with ethidium bromide at 60 V.

Table 1. Primer sequences used for PCR, product lengths obtained after amplification, and references to studies in which the primers were first used

Primername		Sequence of Primer	Length of Product	References
V1	F	5'-ATTTCTACGGGTGCTTTC-3'	374 bp	[2]
	R	5'-ACTTTAGTTGGTGGCTGA-3'		
V2	F	5'-CTTACGCTGCTTATCTTCGCTCTGG-3'	2487 bp	[5]
	R	5'-TTTTGGTCCTTAACATATTCTAAGGGCAA-3'		

Table 2. Reaction mix composition and temperature profiles for each primer pair

Reaction mix	Primer V1		Primer V2	
Water	to 25 µl		to 25 µl	
Buffer 10x	1x concentrated		1x concentrated	
GC Enhancer	0.05x concentrated		0.05x concentrated	
Mg2+	3mM		2.5 mM	
dNTP	0.8 mM		0.8 mM	
Starter Forward	0.8 µM		0.8 µM	
Starter Reverse	0.8 µM		0.8 µM	
Polymerase	1.3U		1.3 U	
Thermal Profile	**Temperature**	**Time**	**Temperature**	**Time**
Initial Denaturation	95°C	10 min	95°C	10 min
Denaturation	95°C	45 s	95°C	45 s
Annealing	50°C	45 s	60°C	45 s
Elongation	72°C	45 s	72°C	2.5 min
Final Elongation	72°C	10 min	72°C	10 min

Preliminary evaluation of CPV-2 variation by RAPD

RAPD method was used for the initial determination of variation in samples successfully isolated from cell cultures (n=50) and to select samples for sequencing. All DNA samples isolated from the blood were directly sequenced, without preliminary selection. RAPD reaction was carried out in a total volume of 25 µl using the following temperature-time profile: initial denaturation at 94°C – 2 min, 45 cycles – denaturation (94°C – 60s), primer annealing (36°C – 60s), thread elongation (72°C – 60s), final elongation– 10 min. The PCR reaction was carried out using the Taq PCR Core Kit (Qiagen). Three primers were used: OPA 7 – 5'GAAACGGGTG 3', OPA 13 – 5' CAGCACCCAC 3'and OPH 12 – 5' ACGCGCATGT 3'. The reaction was carried out in a Sensoquest thermocycler. The reaction products were separated by electrophoresis in a 2.5% agarose gel with ethidium bromide in 1xTBE buffer, at a constant voltage of 70 V for 210 minutes. The lengths of the bands were determined by comparison to size markers.

Sequencing

A representative of each of the variants obtained during RAPD amplification (n = 16) was sequenced and all isolates from the blood of animals with infection confirmed by PCR (n=10) were sequenced (in total 26 samples were sequenced). Products for the V2 primers were purified using the ExoSAP-IT kit (Affymetrix). Sequencing PCR was performed with the same primers as for the original amplification, using a BigDye® Terminator 3.1 CycleSequencing Kit (Applied Biosystems) as recommended by the manufacturer. Sequencing PCR products were purified with a DyeEx Spin Kit (Qiagen) on a QIAcube BioRobot (Qiagen). Samples were subjected to thermal denaturation in formamide. The sequencing reaction was carried out in a 3100 Avant Genetic Analyser (Applied Biosystems).

Bioinformatic analysis

Sequencing results were analyzed using DNA Baser software. The sequences used in the alignment were taken from the NCBI bioinformatic database. Editing and alignment of sequences were performed in MEGA6 software. The similarity between isolates was assessed in Bioedit software. Phylogenetic analysis was performed for the amino acid sequence obtained after translation of the nucleotides encoding the VP2 protein fragment in MEGA6 software. Maximum likelihood estimation at 1,000 bootstrap replications was used to construct a tree. Visualization of the tree was prepared in Figtree software.

RESULTS

A group of 116 dogs was tested with Speed Parvo test, and infection was confirmed in the case of 60 individuals. Feces from 50 of them were used for cell culture and virus isolation. The typical parvovirus associated cytopathic effects appeared in the cell cultures inoculated with the isolated virus. The specificity of the isolated virus was also confirmed by the hemagglutination test. The ability to agglutinate pig erythrocytes was confirmed in the case of each sample. Presence of viral DNA in the blood and cell cultures was confirmed with PCR with primers VP1 and VP2.

Optimization of PCR was aimed at the determination of the optimum annealing temperature and concentration of the reaction mixture components for both sets of primers. In the case of the VP1 primers, the greatest reaction efficiency was achieved at 50°C (Figure 1), which was confirmed by the presence of a bright band of 374 bp. A clear PCR product was obtained for each isolate from the blood, as well as from the cell cultures. The reaction protocol can be used to diagnose CPV-2 infections.

The optimum annealing temperature for the VP2 primer pair was 60°C. Due to the longer product length, the elongation time had to be extended to 2.5 minutes. For the optimized protocol, a single specific product of approximately 2,487 bp (Figure 2) was obtained for each isolate and was the template in the sequencing reaction.

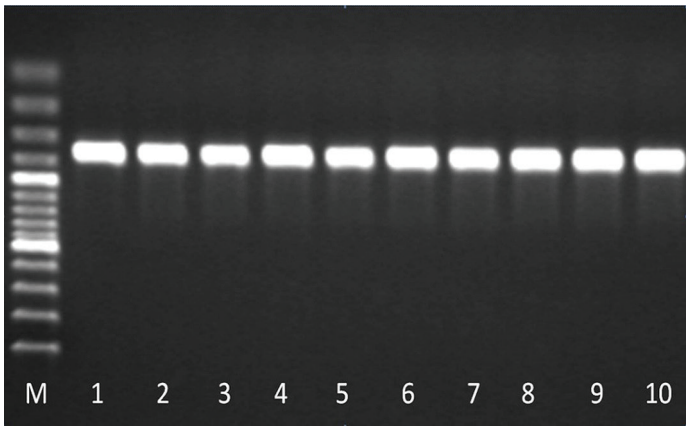

Figure 1. Electrophoretic separation of PCR products with VP1 primers in 1% agarose gel. A distinct band of about 380 bp was obtained. M – size marker 100bp (with brighter bands 500 bp and 1000bp) , 1-10 – test samples

RAPD analysis was prepared for the samples that were successfully isolated from the cell cultures (n=50). Results indicated that all isolates were grouped into 16 RAPD variants (Figure 3).

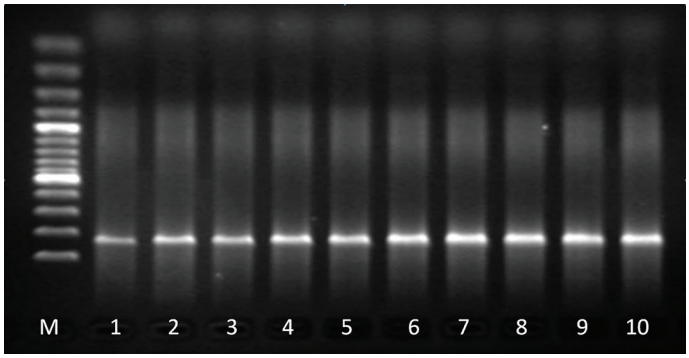

Figure 2. Electrophoretic separation of PCR products with VP2 primers in 1% agarose gel. A distinct band of about 2,487 bp was obtained. M – size marker 100bp (with brighter bands 500bp and 1000bp), 1-10 – test samples

We sequenced 16 samples (representatives of each variant obtained from RAPD analysis) from cell cultures, and 10 from blood (26 samples in total). Based on the polymorphism in the nucleotide sequence, seven genetic variants were identified, with a similarity of over 99%. The most common were variants IV (n=9) and V (n=5), remaining variants were detected with less frequency (I – n=3, II – n=2, III – n=3, VI – n=2 and VII – n=2). The sequencing results were used to determine the similarity between the obtained variants. Based on polymorphism in the nucleotide sequence, seven genetic variants were identified, with similarity of over 99%. Analysis of polymorphisms in the amino acid sequence indicated 100% similarity between variants I, IV, V and VI, in which the differences in the nucleotide sequence were

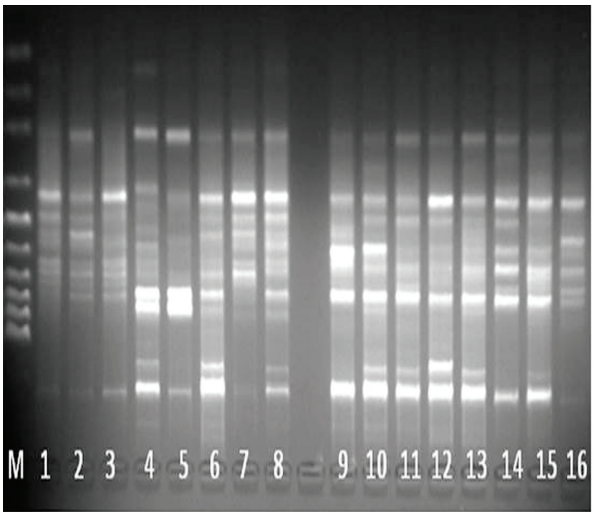

Figure 3. Electrophoretic separation of RAPD products with primers OPA 13 in 2,5% agarose gel. M – size marker, 1-16 – test samples

synonymous. The variant that differed most from the remaining isolates was variant III; the difference was particularly pronounced in comparison to variants II and VII (the differences in the amino acid sequence exceeded 1%) (Table 3).

Table 3. The similarity of nucleotide and protein sequences between obtained isolates (%)

Polish variants	Nucleotide sequence						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
I	ID	99.50	99.30	99.70	99.70	99.50	99.50
II	99.50	ID	99.30	99.30	99.70	99.50	99.50
III	99.30	99.30	ID	99.10	99.50	99.30	99.30
IV	99.70	99.30	99.10	ID	99.50	99.30	99.30
V	99.70	99.70	99.50	99.50	ID	99.70	99.70
VI	99.50	99.50	99.30	99.30	99.70	ID	99.50
VII	99.50	99.50	99.30	99.30	99.70	99.50	ID
Polish variants	**Protein sequence**						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
I	ID	99.30	99.30	100.00	100.00	100.00	99.30
II	99.30	ID	98.70	99.30	99.30	99.30	98.70
III	99.30	98.70	ID	99.30	99.30	99.30	98.70
IV	100.00	99.30	99.30	ID	100.00	100.00	99.30
V	100.00	99.30	99.30	100.00	ID	100.00	99.30
VI	100.00	99.30	99.30	100.00	100.00	ID	99.30
VII	99.30	98.70	98.70	99.30	99.30	99.30	ID

In the polymorphism analysis, a particular focus was placed on the sequence encoding the main antigenic region in the capsid protein. Most of the tested isolates had asparagine at amino acid position 426 (Table 4), which is a crucial position for division into strains, indicating that they belonged to the CPV-2a strain, whereas the N426D substitution, common for CPV-2b was detected only in the case of variant number III. Substitution N426E, specific for CPV-2c, was not detected in any of the tested samples.

The presence of valine at position 555 made it possible to distinguish the new CPV-2a variants from the original type CPV-2a, which has isoleucine at this position.

The antigenic variant of each of the isolates was determined by comparing the sequences obtained with sequences from the NCBI database. The isolates represented by variants I, II, IV, V, VI and VII belonged to type CPV-2a, while variant III had theN426D substitution characteristic of type CPV-2b. In addition, in the case of variants II and VII, polymorphisms were noted that had not previously been observed – H543P (variant II) and N554D (variant VII). Analysis of polymorphisms in the amino acid sequence indicates the presence of four major groups in the parvovirus population. The first group consists of variants I, IV, V and VI, which are identical in terms of amino acid sequence and highly similar in nucleotide sequence (differences amounting to 0.3%–0.5%); the second group consists of variant II (H543P); the third group is represented by variant VII (N554D); and the fourth group by variant III, belonging to type CPV-2b.

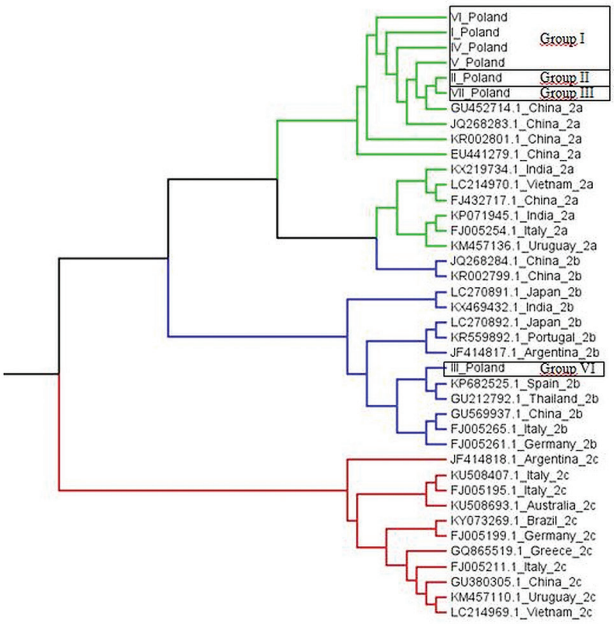

Figure 4. Phylogenetic analysis of strains available in the database and the Polish variants. Maximum likelihood estimation at 1,000 bootstrap replications was used to construct a tree (green – clad containing CPV-2a, blue – CPV-2b, red – CPV-2c)

Table 4. Alignment of the Polish variants of CPV-2 with variants from the database. with underscored amino acid 426 enabling differentiation of CPV-2a, CPV-2b and CPV-2c (Polish variants – bolded. New amino acid substitution – italic)

Variant	426	434	439	440	543	554	555	560	562	564	568	573	579	580
I	**N**	**D**	**K**	**T**	**H**	**N**	**V**	**N**	**V**	**S**	**G**	**Y**	**A**	**P**
II	**.**	**.**	**.**	**.**	***P***	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
III	**D**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
IV	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
V	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
VI	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
VII	**.**	**.**	**.**	**.**	**.**	***D***	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
KM457102.1_Uruguay_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
KR002800.1_China_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
KR002801.1_China_2a	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LC214970.1_Vietnam_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
KP071945.1_India_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
FJ432716.1_China_2a	.	.	.	.	.	.	.	.	.	.	.	.	.	.
FJ432717.1_China_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
FJ005254.1_Italy_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
FJ005257.1_Italy_2a	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GU362932.1_Italy_2a	.	.	.	.	.	.	.	.	.	N	A	.	.	.
KX219734.1_India_2a	.	.	.	A	.	.	.	.	.	.	.	.	.	.
JQ268284.1_China_2b	D	.	.	A	.	.	.	.	.	.	.	.	.	.
LC270892.1_Japan_2b	D	.	.	.	.	.	.	.	L	.	.	.	.	.
FJ005261.1_Germany_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
FJ005262.1_Italy_2b	D	V	N	.	.	.	.	.	.	.	.	.	.	S
FJ005263.1_Italy_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
GU212792.1_Thailand_2b	D	.	.	.	.	.	.	.	.	.	.	F	.	.
GU569937.1_China_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
JF414817.1_Argentina_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
KR559895.1_Portugal_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
KX469432.1_India_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
KP682525.1_Spain_2b	D	.	.	.	.	.	.	.	.	.	.	.	.	.
KY073269.1_Brazil_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
KU508407.1_Italy_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
KM457110.1_Uruguay_2c	E	.	.	.	.	.	.	.	.	.	.	.	V	.
KU508691.1_Australia_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
LC214969.1_Vietnam_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
FJ005195.1_Italy_2c	E	.	.	.	.	.	.	K	.	.	.	.	.	.
FJ005196.1_Germany_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
GQ865518.1_Greece_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
GU380303.1_China_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.
JF414818.1_Argentina_2c	E	.	.	.	.	.	.	.	.	.	.	.	.	.

The phylogenetic analysis indicates that the isolates from groups I-III belong to type CPV-2a, together with the variants recorded in China (Figure 4). A separate branch in the same clade is occupied by isolates from Italy, India and Vietnam. The Polish CPV-2a variants constitute a monophyletic group within an antigenic type. A representative

of the third group clearly belongs to type CPV-2b, together with isolates from Italy, Spain and Germany. None of the variants obtained was matched to type CPV-2c.

DISCUSSION

The molecular epidemiology of CPV-2 in Poland is still poorly understood, and research in this area has previously been undertaken only by Mizak and Rzeżutko [15] and by Majer-Dziedzic *et al.* [16]. The authors performed a molecular characterization of CPV-2 variants circulating in Poland, contributing to the global map of CPV-2 epidemiology [17-23]. The usefulness of the PCR method and its modifications in the diagnosis of parvoviral diseases has been confirmed by numerous researchers [24-26]. The technique has enabled the detection of viral material from both blood and cell cultures. The V1 primers may be particularly important in routine diagnosis of parvovirus infection in dogs; the short amplification product allows for analysis of highly degraded samples, which is particularly important when DNA is to be isolated from difficult biological material, including feces or rectal swabs in the case of CPV-2.

RAPD method may be used for the initial selection of samples to further analysis. Some variants obtained after RAPD analysis, showed identical nucleotide sequences coding the VP2 protein. This fact may be caused by the variation in the parts of genomes which were not amplified during PCR analysis. RAPD method generates more variants than PCR as the technique targets the whole genome. Therefore, RAPD may be especially useful for the initial selection of samples to NGS analysis.

The V2 primers flanking the entire VP2 protein can be used for genotyping and phylogenetic analyses of CPV-2. Bioinformatic analysis confirmed the high variability at the nucleotide level, and seven variants were distinguished. The synonymous nature of polymorphisms in the nucleotide sequence was the basis for including variants I, IV, V, VI in one group. Isolates included in groups II and III differed in single substitutions in the amino acid sequence and belonged to type 2a (which in previous studies was considered the main antigenic variant in Poland in the 1980s [16, 27]) and 2b respectively.

The CPV-2 virus exhibits high variability, which entails changes in its pathogenicity and antigenic structure. The high adaptability of the pathogen is evidenced by the fact that by the late 1980s the parent variant had been completely supplanted by the new CPV-2a and CPV-2b variants [3, 28], which differed in a single amino acid change in the VP2 protein sequence. At the start of the 21st century, the occurrence of another variant was confirmed in Italy and was designated CPV-2c [17]. It differed from the previous 2b strain in a single amino acid change in the main epitope region. In Italy and Argentina the CPV-2c strain is becoming the dominant variant, supplanting the 2a and 2b isolates previously circulating in the environment [21]. Zhao *et al.* point out the importance of amino acid position 440 [5], which is part of the major antigenic region [29]. Nearly 80% of isolates obtained by researchers have alanine in this position.

This mutation was not noted in Polish isolates, as each of the variants obtained had threonine in position 440. Xu *et al.* believe that the T440A mutation may play a role in the process of local adaptation, making it easier for the pathogen to remain in the population [2]. Position 555 also plays an important role, with isoleucine present in the case of the old CPV-2a, while the I555V substitution appears in the new type.

For Poland, there are few data available on the molecular epidemiology of CPV-2. The analyses of the amino acid sequence and phylogenetic relationships are partially confirmed by results obtained by Majer-Dziedzic *et al.* [16] and by Mizak and Pluciennicza k [30], who assigned Polish variants to the CPV-2a strain. In contrast to the results obtained by Majer-Dziedzic *et al.* [16], the present study did not reveal the presence of type CPV-2c, and type CPV-2a was clearly dominant.

An increasing number of studies confirm the emergence of new variants belonging to types CPV-2a and CPV-2b [31, 32], which exhibit changes in the amino acid sequence in relation to the original types. Polish isolates, containing H543P and N554D substitutions seem to support this hypothesis. Poland is not the only country with recurrence of CPV-2a infection. A return of the CPV-2a variant has also been noted in Argentina, where although CPV-2c is still the dominant type, the presence of type 2a has been confirmed for the first time since 2008 [33]. Filipov *et al.* [34], in a study on the epidemiology of canine parvovirus infection in Bulgaria, confirmed the dominance of CPV-2a, and the same antigenic type was detected in isolates from Hungary [35]. A similar trend was observed in Turkey by Timurkan and Oguzoglu [36], who noted variant 2a in almost 70% of infected animals, while CPV-2c was not detected. However, in the studies of Purpari *et al.* [37] conducted in Italy, the CPV-2c type was dominant (almost 80%). Dominance of CPV-2a and CPV-2b is also noted in Asian countries such as China [38] or India [7], as well as in Australia [39].

The Polish isolates, despite 99% nucleotide similarity, display differences in the VP2 protein sequence which may affect its antigenicity. The fact that four groups were distinguished, including one belonging to CPV-2b, indicates that the pathogen is continually adapting to local conditions and evolving in different directions. The dominant group I, consisting of four variants differing in synonymous substitutions, may be the original starting pool that forms the basis for new genetic variants of CPV-2 in Poland. In view of the high evolutionary dynamics of CPV-2, continual research on the molecular epidemiology of the canine parvovirus is essential to obtain the most accurate possible understanding of the molecular structure of CPV isolates occurring in Poland.

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Authors' contributions:

KM-PCR optimization, amplification and electrophoresis of obtained fragments, bioinformatic processing obtained results, preparing and formatting the manuscript to publication. MDB-samples collection, cell culture preparation. KK-Co-author of research hypothesis, preparing and formatting the manuscript to publication. SA-Amplification and electrophoresis of obtained fragments. ZJ -Samples collection. GM-Preparation of the manuscript for publication. JA-Authorship of the research hypothesis (the idea of the experiment), a lead role in planning the experiment, PCR optimization, sequencing alanalysis of obtained fragments, bioinformatic processing obtained results, preparing and formatting the manuscript to publication. All authors have approved the final version of the manuscript.

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DIJAGNOSTIKA I GENOTIPIZIRANJE PARVOVIRUSA PASA TIP-2 (CPV-2) KOD OBOLELIH PASA U JUGOISTOČNIM REGIONIMA POLJSKE

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Parvovirus pasa tip-2 je jedan od najčešćih uzročnika uginuća štenadi. Uprkos postojanju preventivne vakcinacije, oboljene nastavlja da se dijagnostikuje. Cilj studije je bio da se obavi molekularna karakterizacija CPV-2 izolata iz slučajeva obolele štenadi u jugoistočnim regionima Poljske. Genetski materijal CPV-2 je izolovan iz krvi (n=10) i fecesa (n=50) zaraženih pasa. Prisustvo CPV-2 je dokazano amplifikacijom sekvenci virusa koje kodiraju VP1 i VP2 proteine. Proizvodi PCR reakcije sa prajmerima za amlifikaciju VP2 virusnog proteina su sekvencionisani i upotrebljeni u cilju genotipiziranja. Bioinformatička analiza sekvencionisanih PCR produkata je obavljena sa ciljem da se ustanovi filogenetska povezanost sa varijantama virusa koje se nalaze u dostupnim bazama podataka. Na osnovu analiza polimorfizima u nukleotidnim sekvencama, pronađeno je sedam nukleotidnih varijanti koje su svrstane u četiri aminokiselinske grupe. Predstavnici tri grupe su sadržavali asparagin na aminokiselinskoj poziciji 426 VP2 proteina, što je karakteristika CPV-2a. Varijanta iz četvrte grupe je spadala u tip CPV-2b. U Poljskoj, dominantno se nalazi CPV-2a tip virusa. Visok nivo polimorfizma ovog virusa se manifestuje ne samo u odnosu na veći broj varijanti u okviru tipa već i prisustvom predstavnika CPV-2b. Neophodno je da se obave dalja molekularno epizootiološka istraživanja CPV-2, a radi dobijanja optimalnih rezultata i efekata preventivnih mera parvovirusne infekcije pasa.