CHARACTERISTICS OF GENES ENCODING STRUCTURAL PROTEIN OF POLISH STRAINS OF GOOSE PARVOVIRUS

GRZEGORZ WOŹNIAKOWSKI, ELŻBIETA SAMOREK-SALAMONOWICZ, AND WOJCIECH KOZDRUŃ

Department of Poultry Viral Diseases, National Veterinary Research Institute, 24-100 Pulawy, Poland
grzegorz.wozniakowski@piwet.pulawy.pl

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Abstract

The aim of this study was to come up with molecular characteristics of Polish strains of goose parvovirus (GPV) on the basis of genes encoding their structural proteins. Ten field GPV strains and two vaccine strains: B-38 and MFP were used. The PCR-RFLP method based on three distinct endonucleases: \( HinfI \), \( MboI \), and \( RsaI \), which had restriction sites within the sequences of examined genes, was applied. It was found that the homology of VP1, VP2, and VP3 among the studied strains ranged from 50% to 100%. The major differences in restriction patterns were found after application of \( HinfI \), whereas 100% homology was observed after \( RsaI \) digestion. Significant differences were observed in restriction profiles of vaccine GPV strains. The study revealed that the application of PCR-RFLP for the analysis of VP1, VP2, and VP3 genes allowed for their molecular characteristics and differentiation between the vaccine and field strains.

Key words: Derzsy’s disease, goose parvovirus, restriction analysis.

Derzsy’s disease is an infectious disease of waterfowl, which presents a serious threat for poultry production. An infectious agent is goose parvovirus (GPV) belonging to Dependovirus genus of Parvoviridae family. The envelope-free GPV virion has 20-22 nm in diameter and is assembled from 32 capsomers. Viral genome is represented by a single stranded DNA about 5,106 nt long, with both ends flanked by inverted terminal repeats (ITRs) (3). Non-structural (NS) and structural regulatory proteins (VP) are encoded within two main open reading frames in the genome. The regulatory proteins take part in the virus replication while the structural proteins (VP1, VP2, and VP3) assemble the capsid. These three proteins are encoded by the same sequence and originate from the same site of translation but their 3’ stop codon is different. Genetic variability of viruses that may alter their antigenic features is observed commonly among the structural proteins, which contact with immunological competent cells of a host (3, 9). Subsequently, variability of the nucleotide sequence of GPV regulatory proteins, taking a part in replication, is lower. Within the sequence of the structural proteins several conservative and variable regions were identified (13). Sequencing of PCR products, as well as digestion of total DNA or PCR products (PCR-RFLP - restriction fragment length polymorphism) belong to the most commonly applied methods of molecular analysis of the viral genes. Molecular analysis of the GPV strains using restriction enzymes allows for their differentiation depending on their profiles of restriction fragments. The aim of this investigation was to characterise GPV strains isolated during 2001-2009 from field cases of Derzsy’s disease by restriction analysis, using three different enzymes.

Material and Methods

Strains. For the purpose of this experiment 10 field Polish strains of GPV isolated from field cases of Derzsy’s disease during 2001-2009 (4), as well as two vaccine strains: MFP originated from Palmivax vaccine (Mérial – France) and B-38 isolated from Deparvax vaccine (CEVA-Phylaxia) were used. The numbers of the used field strains were as follows: 14/01, 24/03, 33/03, 232/06, 14/07, 16/07, 27/08, 47/08, 54/08, and 27/09.

Cell cultures. Goose embryo fibroblasts (GEF) were prepared from embryos on the 14th d of incubation according to standard procedure. Negative control comprised non-infected GEF cell culture. Cells were cultured in growth medium MEM with 10% of foetal bovine serum and 0.01% of antibiotics mixture (antibiotic-antimycotic-Gibco), or maintaining medium - MEM with antibiotics. Suspension of fibroblasts of
approximately 0.8 x 10^6 cells/mL density was infected with the examined GPV strains. Infected cell cultures were incubated at 37°C and 5% CO₂ for 7 d. Each day, the occurrence of cytopathic effect (CPE) was controlled under optical microscope. Subsequently, the cell cultures were 3-times frozen and thawed, then the suspension of GEFs was infected (1st passage). By this manner three passages were conducted. Next, the titers of the 3rd passage were determined. The titers ranged from 10^4.1 to 10^5.2 TCID₅₀ per 0.2 ml. The 3rd passage comprised a pool of the virus used for the further studies.

**DNA extraction.** The 3rd passage was 3-times frozen and thawed and then centrifuged at 1,400 x g for 5 min. From the pellet of cells containing viral particles, total DNA was extracted according to manufacturer’s procedure of QIAamp DNA Mini Kit (Qiagen).

**PCR.** Amplification of VP1, VP2, and VP3 was carried out using primers designed on the basis of complete sequence of B standard strain of GPV (accession number: U25749 Genebank). Primer sequences and exact complementary regions of GPV genome are listed in Table 1. PCR conditions and composition of reaction mixture were described elsewhere (11).

**Purification of products.** After electrophoresis in 1.5% agarose gels with ethidium bromide (0.5 µg/mL), PCR products were cut from gel and purified using QIAquick Gel Extraction Kit according to manufacturer’s procedure (Qiagen).

**RFLP.** Purificated PCR fragments were digested with three different enzymes: HinfI, MboI, and RsaI (Fermentas). Digestion was carried out in total volume of 30 µl and contained: 3 µl of 10 x concentrated Red+ buffer, 15 µl of DNA (3 µg), 0.5 µl of enzyme (5 U), and 11.5 µl of MiliQ water. Digestion mixtures were incubated in water bath at 37ºC for 3 h.

**Electrophoresis.** Reaction mixtures containing restriction fragments were separated in 1.5% agarose gels as in the case of PCR products. To each well, 15 µl of reaction mixture and 3 µl of loading buffer (6 x loading dye, Fermentas) were loaded, and then the fragments were separated under the voltage of 120 V for 50 min. The analysis of restriction fragments was done in Biogene software ver. 95.16 (Vilber-Lourmat). Phylogenetic trees were drawn with 2% homology coefficient.

**Results**

The CPE exhibited by the focuses of roundly shaped cells reflecting light waves was observed in the infected cell cultures after 3-4 d of incubation. No presence of CPE was observed in mock-infected GEF cell cultures. Seven days after infection of GEFs, total DNA was extracted. PCR conducted for the VP1, VP2, and VP3 regions of GPV revealed the presence of products of expected lengths as follows: 2,198 bp for VP1, 1,763 bp for VP2, and 1,604 bp for VP3. Three restriction enzymes: HinfI, MboI, and RsaI with at least three cleavage sites within the examined sequences were chosen based on the complete sequence of GPV. In case of digestion of VP1 gene with HinfI, strains 24/03, 33/03, and 14/07 fell into to the same clade with 95% homology of restriction profiles, whereas vaccine strain B-38 and field strain 16/07 presented 85% homology with the previous clade (Fig. 1).

Another clad was created by field strains 54/08, 27/08, and 27/09, which VP1 sequence had over 60% homology with other examined strains. The distinct pattern of restriction fragments was exhibited by MFP vaccine and 232/06 field strain. Simultaneously, 47/08 strain had 85% homology with this clade. The digestion of VP2 fragment with the same enzyme allowed for identification of three clades. B-38 vaccine strain and two field strains 27/08 and 232/06 fell into the first clade, while the rest of strains fell into the second clade, except of vaccine MFP strain, whose VP2 restriction profile presented 80% homology with the other strains.

**Table 1**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’-3’</th>
<th>Complementary region</th>
<th>PCR product length</th>
</tr>
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<tbody>
<tr>
<td>VP1 F</td>
<td>CGT GGC GGA ATC TGA AAG</td>
<td>2,491-4,674</td>
<td>2,198 bp</td>
</tr>
<tr>
<td>VP1 R</td>
<td>CGC CAG GGA AGT GCT TTA TTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP2 F</td>
<td>GAG CCA TTT GGC CTA GTA</td>
<td>2,841-4,590</td>
<td>1,763 bp</td>
</tr>
<tr>
<td>VP2 R</td>
<td>CAT ATC CAC CAG TTT CAT TAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP3 F</td>
<td>GTG CCG ATG GAG TGG GTA AT</td>
<td>3,076-4,675</td>
<td>1,604 bp</td>
</tr>
<tr>
<td>VP3 R</td>
<td>GCG CCA GGA AGT GCT TTA T</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Meanwhile, after the cleavage of VP3 gene, identical restriction profiles were observed in case of vaccine strain B-38 and two field strains - 47/08 and 27/09, while the strains 14/01, 24/03, 33/03, 14/07, and 16/07 fell into the second clade. The third clade was created by MFP, 232/06 and 54/08 strains, and was homological in 60% with the both previous clades. The most different VP3 restriction profile showed the 27/08 field strain, which was in 50% coincident with other examined strains. The characteristic feature of all restriction patterns given after digestion of VP1, VP2, and VP3 genes with \textit{Hinf}I enzyme was the significant differences between the length of obtained fragments between vaccine MFP and B-38 strain and examined field strains. After the use of \textit{Mbo}I for digestion of VP1 gene, the presence of four main clades was found. The first one was represented by 47/08 field strain, which had 70% homology with other strains, while the second clade was generated by vaccine B-38 and field 54/08 strain. The strains 27/08, MFP, and 232/06 fell into the third clade. The greatest fourth clade included six field strains: 14/01, 24/03, 33/03, 14/07, 16/07, and 27/09. Similarly, like in case of \textit{Hinf}I endonuclease, after digestion of VP1 with \textit{Mbo}I, the major differences in length of the bands were observed between the vaccine and field GPV strains. Digestion of VP2 and VP3 genes of the examined strains caused no differentiation of restriction profiles. The total lack of differentiation of strains after digestion of the three GPV genes was found after applying \textit{Rsal} enzyme.

\section*{Discussion}

In the presented study, PCR-RFLP method was applied in order to characterise Polish field strains isolated in the last decade. Due to the different restriction profiles of the examined strains, it was found that their sequence of VP1, VP2, and VP3 genes had 50% to 100% homology. Previously Sirivan et al. (8) in their study conducted by the same technique found that two from 15 GPV strains isolated in Thailand had no cleavage sites for \textit{Hinc}II and \textit{Bgl}I endonucleases. They claimed that PCR-RFLP is sufficient for the differentiation of GPV from the Muscovy duck parvovirus (MDPV). Similarly Zádori et al. (12), comparing the restriction profiles of GPV and MDPV after digestion with \textit{SacI}, \textit{KpnI}, and \textit{XbaI}, identified considerable differences between both species of parvoviruses. In another study, Chang et al. (2) have...
found a major difference in fragment of sequence encoding VP3 gene between GPV and MDPV strains.

In the presented study other three enzymes: 

HinfI, MboI, and Rsal, chosen on the basis of sequence of GPV standard strain, were used for the restriction analysis of Polish GPV strains. Because of the limited breeding of Muscovy duck in Poland, no isolates of MDPV were tested. The method described in this study, used for differentiation of GPV vaccine strains from the field strains on the basis of restriction profiles, has not been previously applied. Tatár-Kis et al. (9) differentiated field strains isolated in Hungary from vaccine B42, GPV486, and VG32 strains on the basis of the 5'- terminal VP1 sequence analysis. Likewise, Kozdruń et al. (4) distinguished the clade of vaccine and low pathogenic strains by the analysis of VP1 sequence. RT-PCR RFLP method was also used by Kataria et al. (5) and Torogh et al. (10) for the analysis of VP2 gene of Gumboro disease virus. They differentiated vaccine strains from field strains of the virus on the basis of the restriction profiles. Similarly, Andreasen et al. (1) and Kirkpatrick et al. (6) distinguished vaccine and field strains of infectious laryngotracheitis virus by RFLP and PCR-RFLP. Meanwhile, Nanthakumar et al. (7) applied this method for pathotyping Newcastle disease virus strains.

In the conducted study, it was possible to differentiate B-38 and MFP vaccine strains from the field GPV strains on the basis of restriction fragment analysis, after digestion of VP1 gene with HinfI endonuclease. Interestingly, 232/06 strain previously assigned as a field GPV strain was found to be a vaccine strain. Indeed, the VP1 restriction profile of this strain was identical with MFP vaccine strain, which was also confirmed after digestion of VP3 gene with the same enzyme (Fig.1). Moreover, on the same phylogenetic tree, field strains fell into two main clades depending on their homology of restriction sites. On the other hand, three clades of strains were distinguished after VP1 digestion with MboI. One of these clades was created by vaccine MFP strain as well as 232/06 and 27/08 strains, which presented 95% VP1 homology. High homology of B-38 vaccine strain with 232/06 and 27/08 strains was confirmed after digestion of VP2 gene with HinfI endonuclease, whereas MFP vaccine strain had approximately 80% homology with previous group, and its restriction profile was distinct from the field strains.

After digestion of VP1 gene with MboI endonuclease, it was found that B-38 vaccine strain was closely related to 54/08 strain, whereas other field strains fell into the common clade except 47/08, which had about 70% VP1 homology. Likewise, in the case of vaccine and field strains, the restriction patterns were different. Cleavage of VP3 gene caused differentiation of strains only after applying HinfI. In this case, the major diversity of the restriction profile, that reached 50%, was found in 27/08 strain, while the other strains fell into three main clades with homology range from 60% to 85%. The total absence of differentiation of restriction profiles was observed after Rsal digestion, which suggests that this enzyme cleaves the GPV sequences in the conserved sites.

Summarising the obtained results, it was found that the best choice of enzyme for the molecular characterisation of Polish field strains, as well as for the differentiation between vaccine and field strains of GPV was HinfI. The conducted study has shown that PCR-RFLP of structural GPV genes can be used for the molecular characteristics of field and vaccine strains, and their differentiation.

References