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Expression of goose parvovirus VP1 capsid protein by a baculovirus expression system and establishment of fluorescent antibody test to diagnose goose parvovirus infection

Brief Report

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Summary. The gene encoding VP1 capsid protein of a goose parvovirus was cloned into a baculovirus transfer vector and a recombinant baculovirus was produced. The recombinant virus expressed a protein of 88 kDa corresponding to the molecular weight of VP1 protein and the protein was detected by immunoblotting. By indirect fluorescent antibody (IFA) test, the expressed protein was detected in the nucleus of the insect cells as big granules and electron microscopy also showed several big granules in the nucleus infected with the recombinant virus. The IFA test was developed for screening antibody in Muscovy ducks.

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Goose parvovirus (GPV) infection is a fatal disease affecting goslings and Muscovy ducklings. Occasionally the disease accounts for mortality more than 70% in susceptible flocks [10, 11, 13, 14]. The most typical lesions found in goslings or Muscovy ducklings is myositis in skeletal muscles and myocardium [4, 9], but it is very diffcult to diagnose the diseases pathologically. Diagnosis is based mainly on serological or virological methods because the symptoms and lesions of the disease are not pathognomonic. Diagnosis can be done by isolation of virus in embryonated goose or Muscovy duck eggs and cell cultures prepared from the embryo [1–3, 13], by detection of antibodies using enzyme-linked immunosorbent assay or plaque assay [5, 13], or by polymerase chain reaction (PCR) for rapid detection of GPV DNA [7].

Because GPV is highly contagious, a rapid and simple diagnosis is required to detect the infection. Also parent flocks should be monitored after vaccination for their antibody titer against GPV, whether the titer is enough for protecting offspring from GPV infection. Here we describe the indirect fluorescent antibody (IFA) test to diagnose GPV infection and to monitor serum antibody titer against GPV.

A virulent GPV strain, IH-DE2D3 [7] was used as a template for gene cloning by PCR. Primers VPF and VPR were prepared according a sequence of GPV strain B reported by Zadori et al. [15] from a data base, GenBank U25749. The VPF sequence, 5′-gaa*ggatcc*agatgactcaaagcagatATG-3′ was a 30-base-long oligonucleotide corresponding 5′ upstream of the initiation codon of VP1 gene and the VPR sequence, 5′-gaa*ggatcc*tatttgaatttttacacagaatTTA-3′ was a 34-base-long oligonucleotide corresponding complementary to the 3′ downstream of a stop codon of the VP1 gene. Both primers contained *Bam*HI restriction enzyme site (underlined) and initiation or termination codons (capital). The PCR was carried out using TaKaRa PCR amplification kit (TaKaRa Shuzo Co., Tokyo, Japan). For the PCR the mixture was submitted to 30 cycles of amplification involving heating at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. After phenol extraction and ethanol precipitation, the PCR products were purified by agarose gel electrophoresis. The amplified VP1 gene DNA was digested with *Bam*HI and the resulting fragment was inserted into the *Bam*HI site of the transfer vector pAc YM1 [8] to place the cDNA under the control of the polyhedrin promoter. The resulting recombinant transfer vector was transfected with infectious AcRP23-*lac*Z DNA [6] into *Spodoptera frugiperda* cells using Lipofectin (Bethesda Research Laboratories, MD, USA) as described [12]. Then the culture was harvested, plaque-assayed, and plaques that did not contain blue dye according to β-galactosidase were selected as recombinant viruses. One of the recombinant viruses was designated GPVP1 and passaged in *S. frugiperda* cells to provide high titred virus stocks.

For analysis of polypeptides synthesized by the recombinant baculovirus, monolayers of *S. frugiperda* cells in 35 mm tissue culture dishes were infected with GPVP1 or wild type *Autografa californica* nuclear polyhedrosis virus (AcNPV) at a multiplicity of 5 plaque forming units/cell and incubated at 28 °C, then the infected cells and the culture medium were harvested separately at 1, 2, 3, or 4 days post-infection. Samples were dissociated by boiling for 5 min in sample buffer containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), and 0.02% bromphenol blue. Proteins were analyzed on a 10% SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue or detected by immunoblotting analysis using anti-GPV serum which was prepared in rabbit immunized with CsCl purified GPV strain IH [7]. As shown in Fig. 1a, GPVP1 expressed 88 kDa protein corresponded to the molecular weight of VP1 of GPV [15] at 1 day post-infection and the protein was accumulated in high amount till 2 days post-infection. After 3 days post-infection, the amount of the 88 kDa protein was reduced and smaller polypeptides appeared. No specific

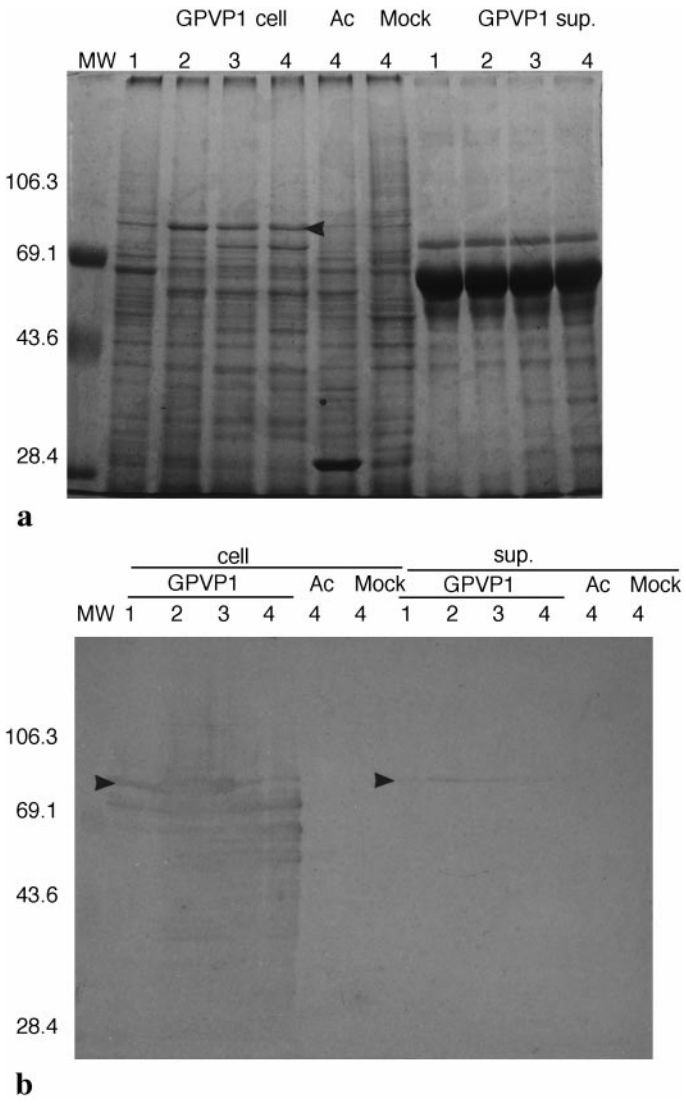

Fig. 1. Time course analysis of proteins expressed by a recombinant virus. *S. frugiperda* cells were infected with a recombinant virus GPVP1 or a wild type AcNPV. The cells were harvested at 1, 2, 3, and 4 days post-inoculation (*1–4*, at 4 days post-inoculation for AcNPV and mock infected cells), lysed, resolved by gel electrophoresis, stained with Coomassie brilliant blue (**a**) or immunoblotting assay (**b**). The position of 88 kDa protein is indicated

band was detected in the culture medium by Coomassie brilliant blue staining. Western blotting also showed similar pattern for cell lysates (Fig. 1b). In the infected supernatants, only the 88 kDa band was detected but no other smaller bands were detected (Fig. 1b).

For IFA test, *S. frugiperda* cells in 35 mm tissue culture dishes were infected with the recombinant virus GPVP1 or AcNPV. The cells were harvested, washed three times in phosphate buffered saline, and aliquots containing approximately

10^4 cells were spotted onto a heavy Teflon-coated slide (Bokusui Brown, NY, USA). After drying, the cells were fixed in acetone at 4 °C for 10 min, rinsed in water, and air dried to prepare "FA spot slides". A 10 μl aliquot of properly diluted anti-GPV rabbit serum was applied to each spot. The slides were incubated at 37 °C for 1 h, washed with PBS, stained with fluorescein-conjugated goat anti-rabbit IgG antibody for 1 h at 37 °C, rinsed again, and examined under UV illumination. At 12 h post-infection, fluorescence was observed specifically in the nuclei of cells infected with GPVP1 as patches (data not shown). By 48 h post-infection, the fluorescence was seen in granules (around 10 granules per nucleus, Fig. 2a) and maintained until 96 h post-infection. No fluorescence was seen in AcNPV or mock infected cells (Fig. 2b). The observed granular staining in the nucleus is distinct from fluorescence seen in GPV infected Muscovy duck embryo fibroblasts (MDEF) where staining was spread throughout the nucleus (Fig. 2c). Electron microscopy showed several big granules specifically in the nucleus of the cell infected with GPVP1 (Fig. 3). These granules corresponded to the staining detected by IFA.

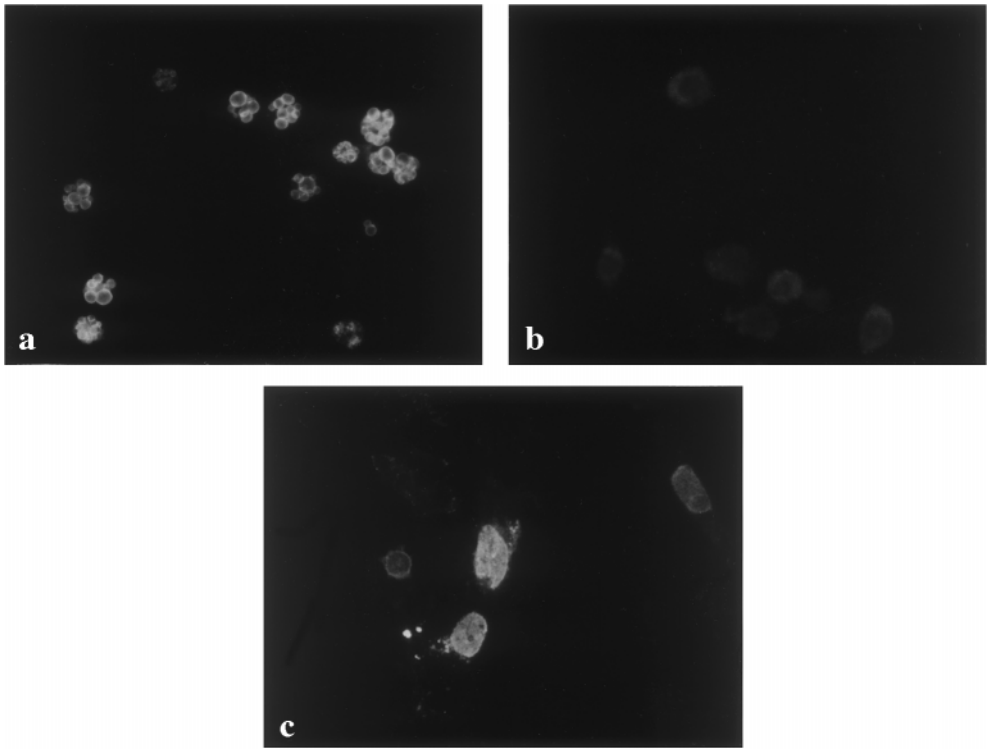

Fig. 2. Immunofluorescence analysis using anti-GPV rabbit serum at 48 h post-inoculation. **a** *S. frugiperda* cells infected with a recombinant virus GPVP1. Intranuclear granules were seen (around 10 granules per nucleus). **b** *S. frugiperda* cells infected with AcNPV. No specific fluorescence were seen. **c** MDEF infected with GPV strain IHC. Specific staining were shown all over the nuclei

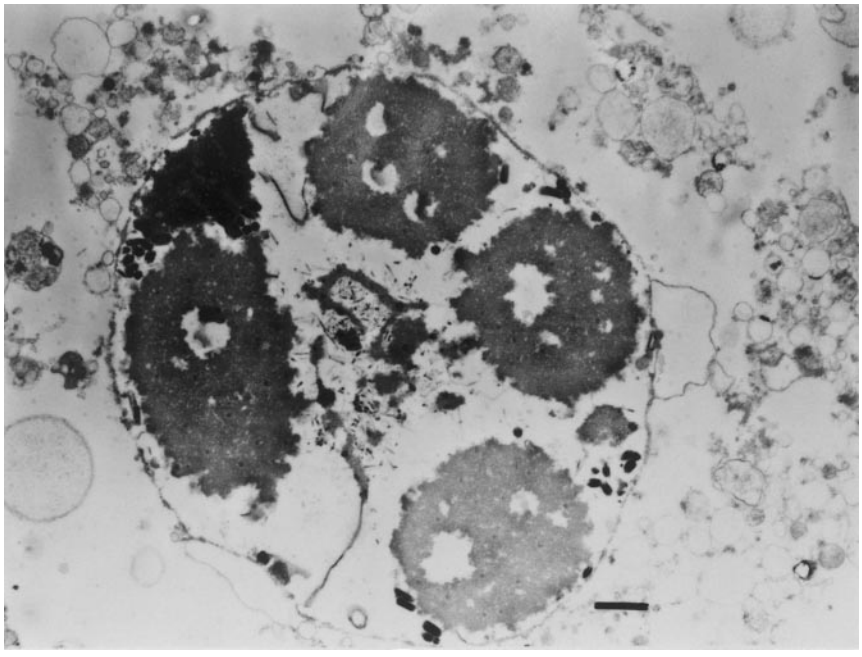

Fig. 3. Electron micrograph of a recombinant virus GPVP1 infected *S. frugiperda* cell. Four specific big granules were shown in nucleus. Bar: 1 μm

With this specific staining, an IFA test for field Muscovy duck sera was developed. First, anti-Muscovy duck IgG rabbit serum was prepared, because no anti-Muscovy duck serum was available. Briefly, immunogloblin from Muscovy duck sera was precipitated with 30% ammonium sulfate and purified by passing through an ion exchange chromatography column. The resulted IgG fraction was mixed with an equal amount of Freund's complete adjuvant and immunized to a rabbit 3 times at 2 week intervals. Two weeks after third immunization, serum was collected and used as anti-Muscovy duck IgG rabbit serum. Field Muscovy duck sera were diluted in phosphate buffered saline, then incubated on an FA spot slide at 37 °C for 30 min. After washing with PBS, 1:5000 diluted anti-Muscovy duck IgG rabbit serum was loaded on the slide and incubated at 37 °C for 30 min. Finally the slide was stained with fluorescein-conjugated goat anti-rabbit IgG antibody at 37 °C for 30 min, rinsed again, and examined under UV illumination. The antibody titer was shown as reciprocal of the highest serum dilution that gave specific fluorescent granules in nucleus. At the same time, virus neutralization test was performed to titrate the field sera as described [14]. Briefly, the cell adapted GPV strain IHC (50 plaque forming units/0.1 ml) [13] was mixed with an equal amount of the serially diluted serum and incubated at 37 °C for 1 h, then the remained virus was plaque titrated on MDEF. The virus neutralization titer was a reciprocal serum dilution giving 50 per cent plaque reduction.

Muscovy duck sera of more than 1-year-old adult collected from farms that had experienced the outbreak of GPV infection showed wide range IFA titers

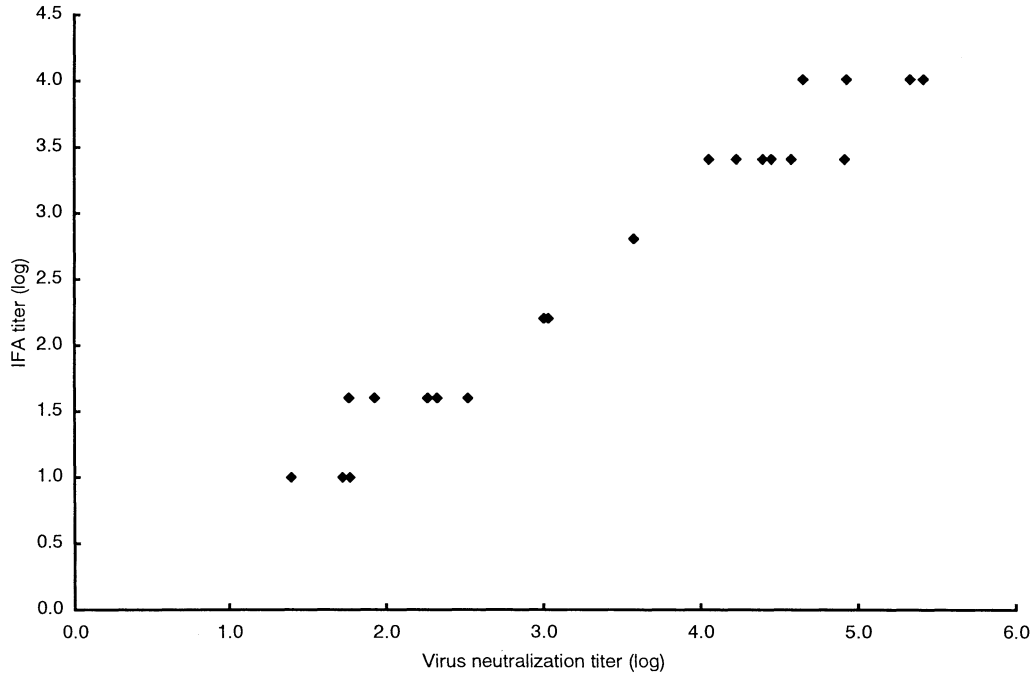

Fig. 4. GPV antibodies in field Muscovy ducks. Sera were collected from the farm that had experienced outbreaks of GPV infection. IFA titer was shown in vertical axis and virus neutralization titer was shown in horizontal axis. The value is shown in $\log_{10}$. Individual birds were shown in dots (n=21)

(10–10 240, representative data are shown in Fig. 4). The virus neutralization titer of these sera against GPV strain IHC are also shown in Fig. 4. The coefficient of the titers of the IFA test and the virus neutralization test was calculated 0.98 (n=21). When sera of 1.6-year-old Muscovy ducks from a GPV free farm were tested, titers in both tests were less than 10 (n=20). We also tested maternal antibodies in egg yolk. The IFA titers ranged from 8 to 512 and the coeffcient of the titers between IFA and virus neutralization tests was caluculated 0.80 (n=15, data not shown).

As shown above, the putative GPV VP1 capsid gene was cloned and expressed by a baculovirus system. The reason why the protein accumulated in granules is not known but this character seems to be specific to the recombinant GPV VP1 capsid protein.

Using these granules as IFA antigen, serum titer can be shown as reciprocal of the highest serum dilution that gave specific fluorescent granules in nucleus. Detection of the granules by IFA test is very easy and specific. The coefficient of the titers of the IFA test and the virus neutralization test was ranged between 0.80 to 0.98. To monitor parent stock immunization condition, virus neutralization tests must be best. But it is time consuming and laborious. Our IFA can be used to replace the virus neutralization test. Therefore this test seems to be a rapid and simple serum diagnostic test for GPV infection.

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