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Short communication

Cloning of the genome of a goose parvovirus vaccine strain SYG61v and rescue of infectious virions from recombinant plasmid in embryonated goose eggs

Jianye Wang[a,b,*], Jinkun Duan[a,b], Xia Meng[a,b], Jiansen Gong[c], Zhiwei Jiang[a,b], Guoqiang Zhu[a,b]

[a] Ministry of Education Key Lab for Avian Preventive Medicine, College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China
[b] Jiangsu Co-Innovation Center for Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China
[c] Poultry Institute, Chinese Academy of Agricultural Sciences, Yangzhou 225125, China

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A B S T R A C T

The SYG61v is an attenuated goose parvovirus (GPV) that has been used as a vaccine strain in China. The genome of SYG61v was sequenced to attempt to identify the genetic basis for the attenuation of this strain. The entire genome consists of 5102 nucleotides (nts), with four nt deletions compared to that of virulent strain B. The inverted terminal repeats (ITR) are 442 nts in length, of which 360 nts form a stem region, and 43 nts constitute the bubble region. Although mutations were observed throughout the ITR, no mismatch was found in the stem. Alignment with other pathogenic GPV strains (B, 82-0321, 06-0329, and YZ99-5) indicated that there are 10 and 11 amino acid mutations in the Rep1 and VP1 proteins of SYG61v, respectively. The complete genome of SYG61v was cloned into the pBluescript II vector and an infectious plasmid pSYG61v was generated. Infectious progeny virus was successfully rescued through transfection of the plasmid pSYG61v in embryonated goose eggs and yielded viral titers similar to its parental virus, as evaluated by ELD_{50}.

1. Introduction

Goose parvovirus is the etiological agent of Derzsy's disease, which is referred to as Gosling plaque in China (Fang, 1962; Derzsy, 1967). This disease is characterized by anorexia, prostration and death in 30-day-old goslings, and is a serious threat to the goose industry worldwide (Derzsy, 1967; Hoekstra et al., 1973; Takehara et al., 1995; Jansson et al., 2007). GPV is a member of the *Dependovirus* genus of the *Parvoviridae* (Fauquet et al., 2004). The genome of GPV is a single-stranded ~5.1-kb DNA molecule with equal polarity. Extracted GPV genomic DNA can form double-stranded molecules *in vitro* after annealing (Zadori et al., 1994).

The GPV genome is flanked by identical inverted terminal repeats (ITRs), which in the case of strain B are 444 nts in length (Zadori et al., 1995). The ITR can fold into an energetically favored U-shaped hairpin structure, which serves as the origin of replication and contains *cis*-acting elements required for rescue, excision from cloning vectors, and packaging (Bossis and Chiorini, 2003; Qiu et al., 2005). There are two large open reading frames (ORFs) in the GPV genome. The left ORF encodes the nonstructural proteins (Rep), including the Rep1 protein and presumably a smaller Rep protein (Qiu et al., 2005; Li et al., 2009), which are involved in viral genome replication, excision, and regulation of capsid gene expression (Legendre and Rommelaere, 1994). The right ORF encodes the three capsid proteins (VP1, VP2, and VP3), which constitute the icosahedral capsid with a ratio of about 1:1:8 (Berns, 1990; Gall-Recule and Jestin, 1994).

Live attenuated vaccines are widely used in breeder geese or goslings to control Derzsy's disease (Kisary, 1977; Gough and Spackman, 1982). Viral passages in goose embryo fibroblasts (GEF) or embryonated goose eggs result in decreased GPV virulence. The attenuated viruses lose pathogenicity for goslings, but still remain immunogenicity, and are suitable for active immunization against Derzsy's disease (Gough and Spackman,1982; **Kisary** et al., 1978). When the breeder geese flocks are immunized with the attenuated vaccine before laying, the progeny passively acquires immunity against Derzsy's disease by maternally derived antibodies in the egg yolks (Derzsy, 1967; Kisary et al., 1978; Fang et al., 1981).

Attenuated viruses for vaccines can be obtained through multiple routes. For example, GEF-adapted virus Bav strain was

* Corresponding author at: College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China. Tel.: +86 514 87972590; fax: +86 514 87972218.
E-mail addresses: wangjy@yzu.edu.cn (J. Wang), yzgqzhu@yzu.edu.cn, yzdxwjy@hotmail.com (G. Zhu).

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generated with the virulent B strain isolated originally in Hungary through 42 passages in cell culture. It showed no pathogenicity to both susceptible goslings and embryonated goose embryos, but still could propagate in the latter (Kisary et al., 1978). Embryo-adapted viruses lose pathogenicity for goslings, but can kill embryonated goose embryos. The GPV SYG61 strain was isolated from deceased goslings in 1961 in China. After 27 passages in embryonated goose eggs, it lost pathogenicity both for adult geese and goslings (Fang et al., 1981). The 45th embryo passage of strain SYG61 was designated SYG61v to distinguish it from the virulent parent. SYG61v has been licensed as a vaccine virus used in goslings for preventing Derzsy's disease in China for over three decades.

Although GPV vaccines have been used since the 1970s, the molecular mechanism involved in its attenuation is unknown. To gain an understanding, the complete genome of the vaccine strain SYG61v was sequenced and compared with other virulent strains. Several amino acid substitutions in Rep1 and VP1 proteins were identified that might contribute to the attenuation of virulence. In addition, the infectious clone plasmid pSYG61v was generated and a method was established to rescue progeny virions through transfection in embryonated goose eggs.

2. Materials and methods

2.1. Virus propagation

The SYG61v is a vaccine virus used widely in China (Fang et al., 1981). The viral stock was diluted 1:100 with a sterile phosphate buffer saline containing penicillin (1000 U/ml) and streptomycin (1000 mg/ml) and inoculated into the chorioallantoic cavity of 12-day-old embryonated goose eggs. The eggs were incubated until the embryos died from propagation of GPV. The dead embryos were kept at 4 °C for 6 h, then their allantoic fluids were pooled and stored at −70 °C.

2.2. Virus purification and DNA extraction

Approximately 400 ml of allantoic fluid was centrifuged at 11,000 *g* for 20 min. Chloroform (1/3 v/v) was added to the supernatant, shaken intensively, and then subjected to centrifugation at 11,000 *g* for 20 min. The upper aqueous phase containing virus was transferred and pelleted by ultracentrifugation at 150,000 *g* for 3 h (SW32Ti rotor, Beckman), and the virus-containing pellet was resuspended in 5 ml of a TE buffer (50 mM Tris, 20 mM EDTA, pH 8.0). The viral DNAs were extracted as previously described (Bossis and Chiorini, 2003). The genomic DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and then annealed by heating to 95 °C for 5 min followed by slow cooling to 65 °C. The annealed double-stranded DNAs of approximately 5.1 kb were observed in a 0.8% agarose gel by electrophoresis after staining with ethidium bromide.

2.3. Genome sequencing and infectious plasmid generation

To facilitate cloning, the plasmid pBluescript II SK (Agilent, Santa Clara, USA) was modified with the original EcoRV site replaced by an NcoI site, resulting in plasmid pBSKN. GPV dsDNA was digested with NcoI, which cuts the genome at nucleotide position 3833 according to the reported genome sequence of the virulent B strain (GenBank Acc. no., U25749). The digested fragments (3.8 and 1.3 kb) were separated by electrophoresis and purified with a gel extraction kit (Tiangen, Peiking, China). The 3.8-kb fragment was ligated into the HincII-NcoI site of the pBSKN plasmid, generating plasmid pBSKNL. The 1.3-kb fragment was ligated into the NcoI-SmaI site, generating the plasmid pBSKNR. The pBSKNL plasmid was then cut with XhoI and NcoI, thus generating a 3.8-kb fragment with XhoI/NcoI sites at either end. The 3.8-kb fragment was then ligated into the XhoI/NcoI-digested pBSKNR, resulting in the plasmid pSYG61v that contained the entire genome of the SYG61v strain.

The cloned gene fragments were sequenced using an ABI-PRISM3730 automated sequencer and BigDye terminators v3.1 (Applied Biosystems, Foster City, USA). To overcome the difficulties in sequencing regions with secondary structures, the ITR was digested with SphI or HincII, which cut the ITR in the middle loop region, and the resulting fragments were subcloned into the pBSK plasmid or pUC18 plasmid for sequencing. The sequences were assembled using SeqManII software included in the Lasergene package 5.0 (DNASTAR, Madison, USA). The complete genome sequence was submitted to GenBank.

2.4. Genome sequence comparison

For comparative studies, the complete genome sequences of four virulent strains (strain B, 82-0321, 06-0329, and YZ99-6) and a vaccine strain VG32-1 were retrieved from GenBank (Table 1). The virulent strain B was isolated in Hungary in 1967. The 82-0321 and 06-0329 strains were isolated in Taiwan in 1982 and 2006, respectively. YZ99-6 was isolated from Yangzhou, China in 1999. A vaccine strain VG32-1 from Germany was also used. The genome sequences of strain 82-0321, 06-0329 and VG32-1 were obtained by PCR amplification (Shien et al., 2008), while genome sequences of strain B and YZ99-6 were obtained by the similar method as described in this report. Sequence comparison was performed with the MegAlign program packaged in the Lasergene package 5.0.

2.5. Transfection in embryonated goose eggs

Transfection of the pSYG61v plasmid was conducted in 10-day-old embryonated goose eggs. Preparation of the transfection mixture was performed according to the specification of the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, plasmid (μg) was premixed with transfection reagent (μl) at a ratio of 1:2.5. The transfection mixture was injected into the chorioallantoic cavity or into the chorioallantoic membrane with 2.0 μg of plasmid per egg, and the eggs were continually incubated. The embryos died after 48 h post-transfection and their allantoic fluid was harvested.

An aliquot of 100 μl allantoic fluid containing the rescued virions was treated with DNaseI (Promega, Madison, WI, USA) to remove the remaining plasmid, and then 1:50 diluted with sterile phosphate buffered saline to inoculate another five 12-day-old embronated goose eggs. The rescued virus was continually passaged in 12-day-old embryonated goose eggs for 12 times. The presence of the rescued virions was detected by PCR using a pair of primers targeting the VP1 gene of GPV and by electron microscopy.

2.6. Genetic marker analysis of the rescued and parental virus

To exclude the possibility that the rescued virus was originated from contamination during the course of transfection and passage, a single nucleotide mutation (C → T) was introduced into the VP1 gene of the pSYG61v plasmid by overlap PCR. The nt mutation is synonymous, but results in loss of the SacII restriction endonuclease site in pSYG61 plasmid. The SacII site exists in the parental strain SYG61v and other virulent strains of GPV. A 1252-bp gene fragment containing the mutation site was amplified by PCR from DNAs extracted from the passaged allantoic fluid. The amplified gene fragments were further digested with SacII.