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**Communication**

[*Comunicação*]

# Development of a sandwich enzyme-linked immunosorbent assay based on single-domain antibody for detecting goose parvovirus infection

[*Desenvolvimento de um ensaio imunoadsorvente ligado a enzima sandwich baseado em anticorpo de domínio único para detecção de infecção por parvovírus de ganso*]

X. *Zhao*[1,2,3], M. *Li*[1,2,3], J.P. *Zhang*[1*], T.F. *Yu*[3,4*]

[1] College of Computer Science and Technology, Harbin Engineering University, Harbin, China
[2] College of Computer and Control Engineering, Qiqihar University, Qiqihar, China
[3] Heilongjiang Provincial Key Laboratory of resistance Gene Engineering and Protection of Biodiversity in Cold Areas, Qiqihar University, Qiqihar, China
[4] College of Life Science and Agriculture Forestry, Qiqihar University, Qiqihar, China

Goose parvovirus (GPV) causes goose parvovirus infection in goslings and Muscovy ducklings, with typical symptoms of anorexia, diarrhea, and growth retardation. The disease has high morbidity and mortality, and the mortality rate of goslings or Muscovy ducklings within one week of age infected with GPV can be 100% (Li *et al*., 2021). According to the latest ICTV virus classification, GPV and the closely related Muscovy duck parvovirus are listed as a single species and called *Anseriformes Dependent Parvovirus* 1, in the genus *Dependovirus* and the family *Parvoviridae* (Wang *et al*., 2021b). In 1956, the Chinese scholar Dingyi Fang first reported an unknown infectious disease that killed many goslings in Yangzhou, Jiangsu Province, China. He isolated a new virus from goose embryos in 1961 and suggested that the virus be named gosling plague virus. In 1978, the World Poultry Association recommended that the disease be called goose parvovirus infection. The disease currently occurs in many countries and regions in the world (Isidan *et al*., 2021).

GPV is a single-stranded DNA virus with two open reading frames (left ORF and right ORF) and inverted terminal repeats at both ends of the genome. The left ORF encodes the non-structural proteins (NS1 and NS2 protein) involved in replicating the viral genome and regulating the expression of structural genes. The right ORF encodes the structural proteins (VP1, VP2, and VP3), which play important roles in viral pathogenicity and virulence (Yan *et al*., 2021).

The traditional methods used to detect GPV infection include detecting the GPV antigen or antibodies against GPV. Among GPV antigen detection methods, electron microscopy observations, polymerase chain reaction (PCR), real-time PCR, matrix-assisted laser desorption/ionization-time of flight mass spectrometry, loop-mediated isothermal amplification, and recombinant polymerase amplification combined with the vertical flow have been established to detect GPV (Wan *et al*., 2019). However, these techniques are more appropriate for laboratory analysis and are not relevant for field practice, as they are time-consuming methods that require specialized systems and trained personnel. Consequently, a fast, easy, and convenient detection approach for GPV is needed.

Until now, no commercially available enzyme-linked immunosorbent assay (ELISA) kits are available for detecting the GPV antigen. Therefore, in this study, we developed a novel sandwich ELISA to detect the GPV antigen using an anti-GPV NS1 protein rabbit polyclonal antibody as a coating antibody and a specific heavy chain single-domain antibody against the GPV NS1 protein as a detection antibody. This sandwich ELISA is suitable for detecting GPV and exhibited good specificity and sensitivity.

*Corresponding author: zhangjianpei@hrbeu.edu.cn; yutianfei2001@163.com
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The GPV sandwich ELISA provides support for rapid diagnosis and accurate monitoring of GPV infection.

The recombinant glutathione S-transferase (GST) fusion protein GST-NS1 at a concentration of 0.48mg/mL was prepared and stored in our laboratory (Yu *et al*., 2021). Briefly, the NS1 gene of GPV strain H1 (GenBank Accession number: JQ409356.1) was inserted into the pGEX-6p-1 prokaryotic expression vector to generate the pGEX-NS1 recombinant plasmid. pGEX-NS1 was transformed in *E. coli* Rosetta (DE3) and induced with IPTG at a final concentration of 1.0mM for 4h at 37℃. The recombinant GST-NS1 protein was purified by elution from a sodium dodecyl sulfate-polyacrylamide gel, as described previously (Yu *et al*., 2016). A 500μg portion of purified GST-NS1 was blended and emulsified with the same quantity of Freund's complete adjuvant or Freund's incomplete adjuvant. Then, two healthy New Zealand white rabbits were injected subcutaneously into the back at multiple sites. The immunization procedure was conducted following a previous study (Yang *et al*., 2021). The rabbits were bled by cardiac puncture seven days after the fourth immunization. Then, the polyclonal antibody (Pab) was purified with a Protein A Antibody Purification Kit (Beyotime Bioteck, Shanghai, China), and protein concentration was determined with a Bradford Protein Assay Kit (Beyotime Bioteck) according to the manufacturer's instructions. An indirect ELISA was performed to measure the unpurified or purified Pab titers as described previously (Yu *et al*., 2021). A specific heavy chain single-domain antibody against the GPV NS1 protein named Trx-VH, which was generated from monoclonal antibody 3D9 (Qiu *et al*., 2012) at a concentration of 0.74mg/mL, was prepared and stored in our laboratory (Yu *et al*., 2021). A checkerboard titration was applied to determine the optimal operating concentrations for the coating antibody (purified Pab) and the detection antibody (Trx-VH). The purified Pab and Trx-VH were diluted from 10 to 0.625μg/mL, respectively. GST-NS1 was diluted to 0.4μg/mL. HRP-labeled 6×His Tag Monoclonal Antibody (Thermo, Shanghai, China) was diluted from 1:500 to 1:4,000. A microplate reader (BioRad, Hercules, CA, USA) was used to measure the absorbance value at 450nm. The coating conditions, blocking conditions, antigen, detection antibody, HRP-labeled monoclonal antibody incubation conditions, and color development conditions were tested to determine the optimal response parameters. To determine the cut-off values between positive and negative samples, 60 GPV-negative samples (cloacal swab) were acquired from healthy goslings (≤3-weeks-of-age). All animals samples were collected in accordance with the standards approved by the Ethics Committee for Animal Welfare and Research of Qiqihar University, Heilongjiang Province, China (Number: 20210316-7). The cloacal swabs samples were scoured fully with 1 mL PBS (0.01 M, pH 7.3). The suspensions were subjected to three freeze-thaw cycles before they were centrifuged at 8000 × g at 4℃ for 30 min. The harvested supernatant was detected with the sandwich ELISA. The X + 3 standard deviations (SDs) formula was used to determine the threshold value between the positive and negative samples. Here "X" represents the mean $OD_{450}$ value of the 60 negative samples, and "3SD" represents three times the SD.

A 100μL aliquot of 400, 200, 100, 50, 25, 10, 5, 2.5, and 0ng/mL diluted GST-NS1 protein with PBS (0.01M, pH 7.3) was added to microplates and detected by the sandwich ELISA to assess the sensitivity for detecting the GST-NS1 protein. To evaluate the sensitivity for detecting the GPV virus, the GPVstrain H1 ($10^{6.7}$ $TCID_{50}$/mL) was diluted to $10^{6.0}$ $TCID_{50}$/mL and then diluted 2- or 10-fold with PBS (0.01 M, pH 7.3) and detected with the sandwich ELISA. Specificity was evaluated in the sandwich ELISA with five common viruses that can infect geese, including Tembusu virus (TMUV), goose circovirus (GoCV), fowl adenovirus (FAdV), Newcastle virus (NDV), and H9 avian influenza virus (H9 AIV). Intra-batch or inter-batch assay variability was evaluated with extraordinary batches of purified Pab using six positive samples in three parallel wells. All duplicability checks were repeated three times. The coefficients of variation (%CV) were measured using the formula: %CV = (SD/mean) × 100%.

A total of 118 cloacal swab samples obtained from goslings revealed diarrhea symptoms of GPV infection at different goose farms in Heilongjiang and Jilin Provinces, China. The samples were processed as described above. The

viral DNAs for PCR detection were extracted from the harvested supernatant using the TIANamp Virus DNA Kit (Tiangen Bioteck, Beijing, China). The PCR primers were selected from our previous study (Yu *et al*., 2016). The sequence of the forward primer was 5'-GAGCGCGGATCCACTTATGACAATTCT ATGGA-3'. The sequence of the reverse primer was 5'-CACCGCTCGAGTTACCACCCATGTTCA TC-3'. The PCR procedure was: 5 min at 95℃, followed by 35 cycles of 15s at 95℃, 15s at 57℃, and 30s at 72℃. Sensitivity, specificity, and accuracy were calculated as described previously (Wang *et al*., 2021a). The consistency between the sandwich ELISA and PCR methods was evaluated with a kappa analysis.

The unpurified Pab titer to GST-NS1 was 1:25,600 after four immunizations. After purification, the Pab titer to GST-NS1 was 1:204,800. The concentration of the purified Pab was 2.54mg/mL as measured by the Bradford method. The optimal coating concentration for the purified Pab in the sandwich ELISA was 1.25μg/mL. The optimal Trx-VH working concentration was 2.5μg/mL, and the optimal dilution of the HRP-labeled 6×His Tag Monoclonal Antibody was 1:2,000. Bovine serum albumin (5%) was used as the blocking buffer, and the optimal blocking conditions were 2 h at 37℃. The optimal incubation conditions for the antigen were 60 min at 37℃. The optimum reaction time for TMB was 10min. The cut-off value was determined in 60 GPV-negative cloacal swab samples. The mean (X) was 0.173, and the SD was 0.011; thus, the cut-off value (X + 3SD) was 0.206. When a sample reached an $OD_{450}$ value ≥ 0.206 (mean OD + 3SD), it was considered positive. Otherwise, it was negative. The GST-NS1 detection limit in the sandwich ELISA was approximately 5 ng/mL, and the GPV detection limit was $10^{2.9}$ TCID50/mL (Table 1).

Table 1. The sensitivity of the sandwich ELISA

| Specificity to GST-NS1 | | Specificity to GPV | | |
|---|---|---|---|---|
| Protein Concentration (ng/mL) | $OD_{450}$ Value | Dilution Fold of Virus | Virus Titer ($TCID_{50}$/mL) | $OD_{450}$ Value |
| 400 | 3.596±0.096 | 1:10 | $10^{5.0}$ | 3.402±0.093 |
| 200 | 3.515±0.088 | 1:20 | $10^{4.7}$ | 3.515±0.094 |
| 100 | 3.302±0.091 | 1:40 | $10^{4.4}$ | 2.836±0.087 |
| 50 | 1.717±0.066 | 1:80 | $10^{4.1}$ | 1.634±0.065 |
| 25 | 0.934±0.043 | 1:160 | $10^{3.8}$ | 1.397±0.051 |
| 10 | 0.452±0.018 | 1:320 | $10^{3.5}$ | 0.767±0.027 |
| 5 | 0.267±0.008 | 1:640 | $10^{3.2}$ | 0.438±0.016 |
| 2.5 | 0.183±0.006 | 1:1280 | $10^{2.9}$ | 0.278±0.007 |
| 0 | 0.023±0.003 | 1:2560 | $10^{2.6}$ | 0.127±0.005 |
| Negative control | 0.021±0.002 | Negative control | - | 0.022±0.002 |

Note: The cut-off value was 0.206.

The specificity of the sandwich ELISA was evaluated by testing five other viruses, including TMUV, GoCV, FAdV, NDV, and H9 AIV. The results are shown in Table 2. GPV showed the only positive signal, and the other viruses were negative, suggesting no cross-reactivity among these viruses in the sandwich ELISA. These results demonstrate that the sandwich ELISA had high specificity for GPV.

Table 2. The specificity of the sandwich ELISA

| Virus | GPV | TMUV | GoCV | FAdV | NDV | H9 AIV |
|---|---|---|---|---|---|---|
| $OD_{450}$ Value | 1.873±0.056 | 0.037±0.004 | 0.029±0.003 | 0.059±0.008 | 0.046±0.007 | 0.062±0.006 |

Note: The cut-off value was 0.206.

The results of duplicability testing showed that the %CVs of intra- and inter-batch duplicability tests were 0.11%–5.21% (Table 3) and 0.24%–6.44% (Table 4), respectively. Both were less than 10%, indicating that the sandwich ELISA had good repeatability and was stable for detecting GPV.

Table 3. The intra-batch duplicability of the sandwich ELISA

| Assay Time | No. and $OD_{450}$ Values of GPV Positive Cloacal Swab Samples | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| First | 1.429 | 1.868 | 1.147 | 1.339 | 2.823 | 1.227 |
| Second | 1.281 | 1.839 | 1.141 | 1.024 | 2.471 | 1.342 |
| Third | 1.126 | 1.604 | 1.202 | 0.997 | 2.106 | 1.338 |
| X | 1.279 | 1.770 | 1.163 | 1.120 | 2.467 | 1.302 |
| SD | 0.023 | 0.021 | 0.001 | 0.036 | 0.129 | 0.004 |
| CV | 1.80% | 1.18% | 0.11% | 3.23% | 5.21% | 0.33% |

Table 4. The inter-batch duplicability of the sandwich ELISA

| Assay Time | No. and $OD_{450}$ Values of GPV Positive Cloacal Swab Samples | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| First | 1.392 | 1.755 | 1.133 | 1.432 | 2.774 | 1.377 |
| Second | 1.274 | 1.847 | 1.146 | 1.098 | 2.369 | 1.289 |
| Third | 1.217 | 1.594 | 1.259 | 0.895 | 2.502 | 1.395 |
| X | 1.294 | 1.732 | 1.180 | 1.142 | 2.548 | 1.354 |
| SD | 0.008 | 0.016 | 0.005 | 0.036 | 0.043 | 0.003 |
| CV | 0.62% | 0.95% | 0.41% | 6.44% | 1.67% | 0.24% |

A total of 118 cloacal swab samples were assayed for the GPV antigen using the sandwich ELISA and PCR (Table 5). The sandwich ELISA had 91.6% sensitivity (76/83) and 91.4% specificity (32/35) relative to PCR. The consistency of these two methods was (108/118) = 91.5%. Furthermore, the kappa value was 0.803, which is regarded as the best consistency between the sandwich ELISA and the PCR assay.

Table 5. Comparison of sandwich ELISA and PCR for the detection of GPV

| | | Sandwich ELISA | | |
|---|---|---|---|---|
| | Cloacal Swab | Positive | Negative | Total |
| PCR | Positive | 76 | 7 | 83 |
| | Negative | 3 | 32 | 35 |
| | Total | 79 | 39 | 118 |

The emergence and rapid development of antibody engineering have enabled researchers to expand the field of antibody application by designing and manufacturing transforming antibodies (Hanning *et al*., 2022). The VH and VL of the antibody together constitute the antigen-binding site, and the specificity of the antibody is determined by these parts. Genetically engineered recombinant antibodies are most used to construct VH and VL fusion single-chain antibodies (single-chain Fv). Some studies have shown that VH fragments play a major role in the specific binding of the antibody to the corresponding antigen. It has also been reported that VL alone can bind to the antigen. Even a single variable region CDR3 fragment can specifically bind to the antigen (Ou *et al*., 2022). These active small-molecule antibody fragments have many advantages in clinical application and are promising new antibodies to replace traditional polyclonal antibodies or monoclonal antibodies. As the individual VL or VH fragments do not contain Fc fragments, they are not easily combined with conventional enzyme-labeled secondary antibodies, making it inconvenient to detect activity. To overcome this shortcoming, a tagged protein was used as the detection marker in this sandwich ELISA.

We detected clinical samples with the sandwich ELISA and PCR. A total of 10/118 cloacal swab samples gave inconsistent outcomes, of which seven were GPV-positive by PCR but GPV-negative in the sandwich ELISA. Understandably, the sandwich ELISA may not recognize the pathogens in samples with a very low number of GPV copies. Three other samples were GPV-negative by PCR; however, they were GPV-positive in the sandwich ELISA. This result probably occurred because of nucleic acid

degradation during extraction of the nucleic acids from the samples; hence, affecting PCR accuracy. The kappa value between the sandwich ELISA and PCR was 0.803, suggesting excellent consistency between the two methods. This sandwich ELISA has high sensitivity and specificity and presents the correct and reliable approach for detecting the GPV.

Keywords: goose parvovirus, NS1 protein, ELISA, single-domain antibody, PCR

## RESUMO

*A infecção por parvovírus de ganso (GPV) é uma doença infecciosa altamente patogênica em gansinhos e patinhos de Muscovy. Para detectar o antígeno GPV, desenvolvemos um novo ensaio imunoenzimático (ELISA) sanduíche usando um anticorpo de domínio único de cadeia pesada específico contra a proteína GPV NS1 como anticorpo de detecção. Os limites de detecção da proteína GST-NS1 e do título do vírus da cepa GPV H1 foram 5 ng/mL e $10^{2,9}$ $TCID_{50}$/mL, respectivamente. O ELISA sanduíche foi específico para GPV sem reatividade cruzada com outros vírus comuns de ganso, incluindo vírus Tembusu, circovírus de ganso, adenovírus de aves, vírus Newcastle ou vírus H9 da gripe aviária. Um total de 118 amostras de swab cloacal foram usadas para detectar o antígeno GPV usando o ELISA sanduíche e reação em cadeia da polimerase com uma taxa de coincidência de 91,5%. A sensibilidade e especificidade do ELISA sanduíche foram de 91,6% e 91,4%, respectivamente. Esses resultados sugerem que este ELISA sanduíche pode ser aplicado para detectar GPV.*

*Palavras-chave: parvovírus de ganso, proteína NS1, ELISA, anticorpo de domínio único, PCR*

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