Research Note: Development of an ELISA to distinguish between goose parvovirus infection and vaccine immunization antibodies

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ABSTRACT Goose parvovirus (**GPV**) leads to a huge loss in the poultry industry, and early diagnosis is required to prevent the disease from spreading. At present, there are a variety of detection methods for GPV infection, and the ELISA method has the advantages of simple and rapid operation. However, most ELISA methods for detecting GPV can only detect the antibody level of the sample, but cannot distinguish between the GPV infection and vaccine immunization antibodies. Therefore, this study has a wider application value by establishing the detection method based on the structure and non-structural protein of the virus. The GPV non-structural (NS1) and structure (VP3) fusion proteins were used as coating antigens to establish 2 indirect ELISA methods, and the detection conditions were optimized. A series of experiments proved that the detection method has good specificity, sensitivity, and repeatability. The test results of 120 immune sera samples and 145 natural infection serum samples showed that the positive rates of immunized serum were 9.17% (NS1) and 88.33% (VP3), and the positive rates of natural infection were 88.97% (NS1) and 86.21% (VP3), which distinguish between the GPV infection and vaccine immunization antibodies. The establishment of 2 indirect ELISA methods using NS1 and VP3 proteins as inclusion antigens provides a new method for detecting GPV infection and inactivated immune antibodies, which lays a foundation for the serological diagnosis and epidemiological monitoring of GPV.

Key words: goose parvovirus, prokaryotic expression, natural infection, inactivated vaccine, indirect ELISA

INTRODUCTION

Gosling plague, also known as goose flu, goose hepatitis, goose enteritis, ascites, liver and nephritis, infectious myocarditis, is a highly contagious disease of goslings and Muscovy ducks caused by goose parvovirus (**GPV**) infection (Calnek, 1991). The main host of this disease is a gosling within 20 D of age, which spreads rapidly and has a high mortality rate. The disease infected adult geese without clinical symptoms but can be transmitted vertically to the next generation (Diao, 2016). In 1956, Fang Ding Yi (Fang, 1962) first discovered the disease in Yang Zhou and isolated the virus from goose embryos. In the 1960s, similar diseases were reported in Europe, including Poland, Hungary, France, Bulgaria, Federal Republic of Germany, the Netherlands, the former Soviet Union, France, and Former Czechoslovakia (Calnek, 1991). In China, due to the wide demand for goose products, it is important to prevent GPV infection.

The GPV genome is a single-stranded DNA of 5,106 nucleotides long and can encode 5 proteins, including 2 nonstructural proteins (NS1 and NS2) and 3 structural proteins (VP1, VP2, and VP3). The unstructured NS1 protein is produced in the early stage of the virus replication, in which the NS1 gene contains 1,884 nucleotides, encoding 628 amino acids, and NS1 protein is involved in the virus's toxic effect on the cells, the replication of the virus, and the gene expression. The VP3 protein is the most abundant of 3 core proteins (Le Gall-Recule and Jestin, 1994) and can induce neutralizing antibodies in GPV- or Muscovy duck parvovirus (**MDPV**)-infected waterfowl (Le Gall-Recule et al.,

1996; Tian et al., 2002; Wang et al., 2004, 2005; Shang et al., 2010).

The cost of *Escherichia coli*-expressed proteins is relatively low, and the recombinant protein is easily purified by either chromatography or elution. Many reports have demonstrated that *E. coli*-expressed proteins are useful antigens for detecting antibodies against a variety of viral diseases (Crabb and Studdert, 1995; Boshoff et al., 1997; De Diego et al., 1997; Johne et al., 2004).

In this study, an NS1-ELISA test and a VP3-ELISA test were developed to diagnose GPV infection and to monitor serum antibody titers against GPV. It distinguishes between the GPV infection and vaccine immunization antibodies and lays the foundation for the diagnosis of GPV disease and the serological investigation.

MATERIALS AND METHODS

Virus

In this study, we isolated the strains of GPV virus from the liver of goose with GPV in the Anhui province of China. We used 9-day-old goose embryos free of GPV to isolate the virus through the allantoic cavities. We amplified and sequenced the VP2 gene to identify the virus. PCR assay was performed using one pair of specific primers: 5′-GGCCTAGTAGAAGAGCCTRTCA-3′ (position 2,854 and 2,875 bases) for GPV-F and 5′-CGCCAGGAAGTGCTTTATTTG-3′ (position 4,658 and 4,678 bases) for GPV-R. The PCR reactions specifically produced 1,825 bp PCR products. We submitted the sequence to the GenBank database to obtain the GenBank accession number.

ELISA antigen preparation

Primers specific for the partial *NS1*-encoding gene and *VP3*-encoding gene were designed using Oligo 6.24 (Molecular Biology Insights, Inc.) based on conserved nucleotide sequences from previously reported GPV. The *NS1* forward primer was NS1-F: 5′-CGGAATTCATGG-CACTTTCTAGGCCTCTTCAG-3′ (position 541 and 564 bases, EcoR I site is underlined) and the *NS1* reverse primer was NS1-R: 5′-CCCAAGCTTGTTAAGCAG-CAGTGAATAAAGG-3′ (position 1,072 and 1,089 bases, Hind III site is underlined). The *VP3* forward primer was VP3-F: 5′-CGGAATTCATGGCAGAGG-GAGGAGGCGGAG-3′ (position 3,037 and 3,058 bases, EcoR I site is underlined) and the *VP3* reverse primer was VP3-R: 5′-CCCAAGCTTGTTATCGTGCACCG TTCTGGTTGG-3′ (position 3,575 and 3,594 bases, Hind III site is underlined). The sequences of *NS1* and *VP3* genes were amplified by using DNA containing GPV (The Institute of Avian Disease, Shandong Agricultural University, Taian, China) sequence plasmid as a template. The 552 and 561 bp PCR products were cloned into EcoR I (15 U/μL, TaKaRa, Beijing, China) and Hind III (15 U/μL, TaKaRa, Beijing, Chi-na) sites of pET-32a (The Institute of Avian Disease, Shandong

Agricultural University). The correct orientation of the insert was confirmed by nucleotide sequencing. Then, the plasmid was transformed into *BL21(DE3) Competent Cell* (10 tubes, CWBIO, Beijing, China). Positive clones were selected for large-scale production and purification. The expressed NS1 and VP3 proteins were purified by using the gradient urea method. The total amount of protein in the crude extracts was quantified by using the BSA protein content determination kit (500 microplate assays, CWBIO, Beijing, China).

Protein extracts from *BL21(DE3) Competent Cell* were mixed with an equal volume of loading buffer, boiled for 5 min, and separated by SDS-PAGE. The gels were stained by standard methods using Coomassie brilliant blue (1 mL × 2, TaKaRa, Beijing, China). Nitrocellulose (**NC**) membranes (0.45 μm, 14 cm × 20 cm/sheet, CWBIO, Beijing, China) were post-coated with 5% skimmed milk in phosphate-buffered saline (pH 7.4) for 1 h at room temperature (**RT**), and the NC membranes were then probed with GPV-positive serum (1:40, The Institute of Avian Disease, Shandong Agricultural University) overnight. Then, the NC membranes were reacted with phosphatase-labeled rabbit anti-goose IgG (1:100, The Institute of Avian Disease, Shandong Agricultural University) conjugates for 1.5 h at 37°C. In addition, the pET32a vector without parvovirus genes used as a control to prove the accuracy of the protein.

Development of NS1-ELISA and VP3-ELISA

A checkerboard titration was performed to determine the optimal working dilution of the coating antigen and serum using a 96-well ELISA plate (2.2lU, TIANGEN, Beijing, China). Using matrix titration, the purified VP3 and NS1 proteins were diluted laterally in a 96-well ELISA plate with 0.05 mol/L carbonate buffer (pH 9.6) (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200), add 100 μL per well, and incubated at 4°C overnight. The plates were then blocked for 1 h with 5% skimmed milk at 37°C and washed 3 times with phosphate-buffered saline Tween (**PBST**). Subsequently, 100 μL of GPV sera (positive serum and negative serum) was added and incubated at 37°C for 1 h. The samples were washed, and then incubated for 1 h with 100 μL of horseradish peroxidase (**HRP**)-rabbit anti-goose IgG (The Institute of Avian Disease, Shandong Agricultural University) diluted 1:50 in 5% skimmed milk at 37°C, washed again, and detected with 100 μL of 3,3′,5,5′-tetramethylbenzidine (**TMB**) (100 mL, TIANGEN) for 15 min at 37°C and away from light. The reaction was then stopped by the addition of 50 μL of 3 M H_2SO_4. Optical density (**OD**) values were measured at 450 nm. The optimal condition was obtained by comparing the positive/negative ratio (**P/N**) of the samples. Subsequently, optimal antigen coating conditions, optimal closure times, optimal working conditions for enzyme-labeled secondary antibodies, optimal serum response time, and optimal color rendering time were optimized.
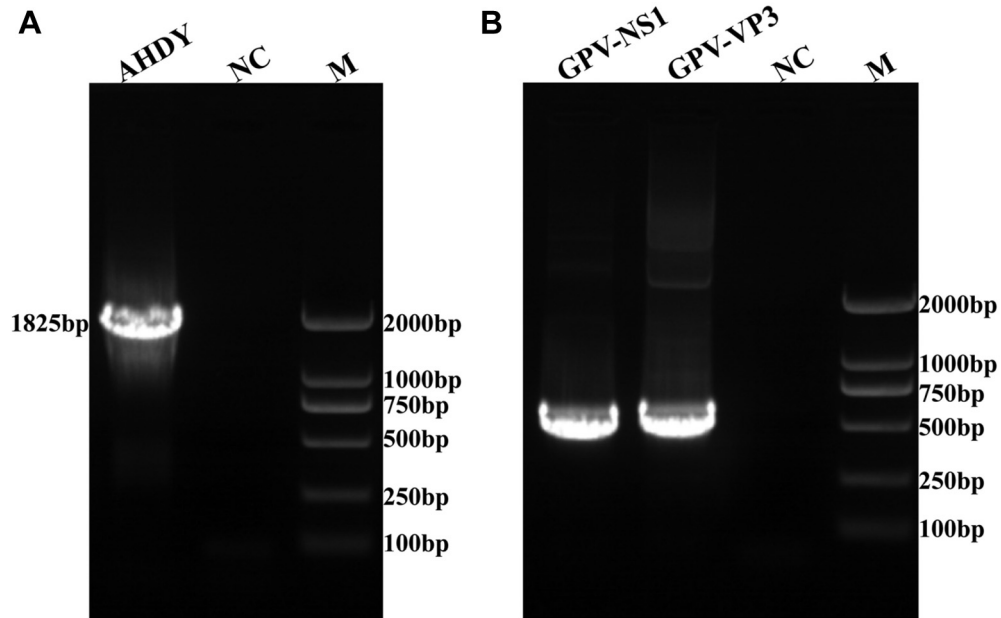
A

B

Figure 1. Agarose gel electrophoresis was used to determine the gene region in the study. (A) The amplification result of VP2 gene about GPV. (B) The amplification result of GPV-NS1(552 bp) and GPV-VP3 (561 bp). M: DL2000 DNA Marker. NC: negative control.

Positive/Negative Cut-Off Value

A total of 30 negative sera from goose were used to determine the cut-off value according to the optimization of the ELISA procedure. The cut-off value at OD_{450} to define a virus positive was calculated based on the formula: positive and negative cut-off value sample mean ($\mathbf{X}$) + 3 $\times$ standard deviations (mean + 3 SD). The serum sample was regarded as positive if the OD_{450}
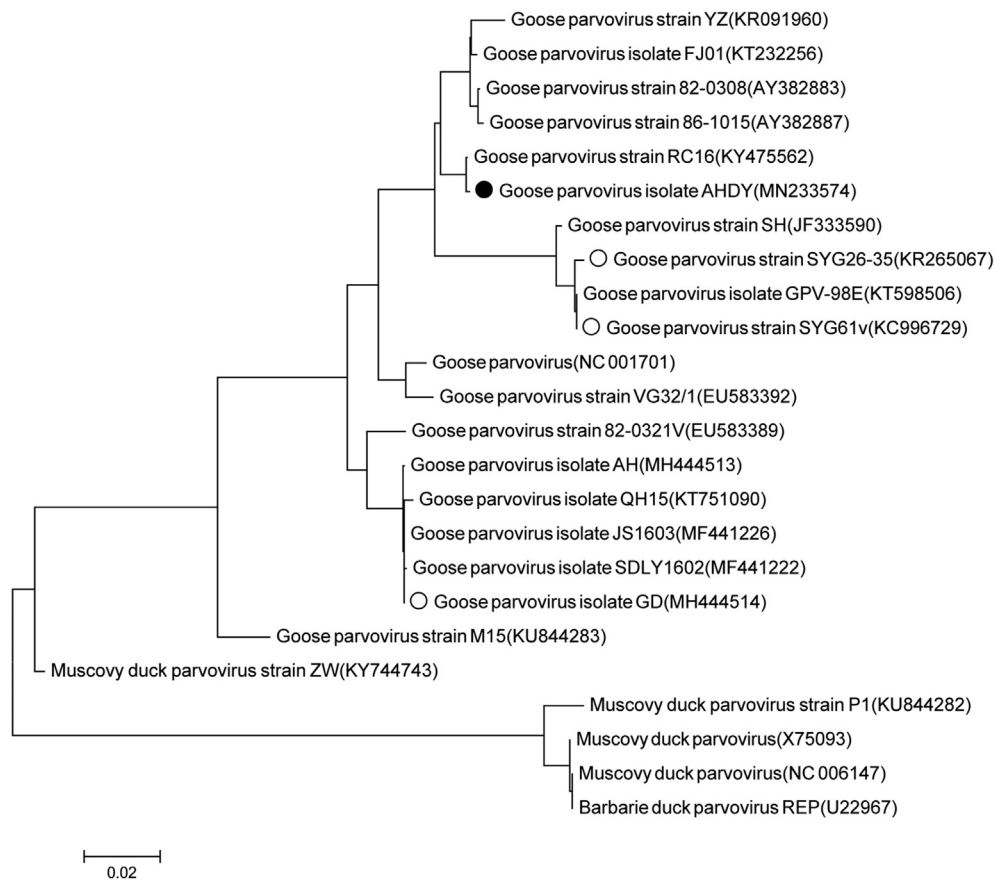

Figure 2. Phylogenetic relationship between AHDY(●) and parvovirus strains based on the VP2 gene in the phylogenetic tree.

value was higher than the cut-off value, or it was considered to be negative.

Cross-Reaction Test

The specificity of the ELISA was evaluated by testing antisera to other goose pathogens including H9 avian influenza virus (**H9 AIV**), Tembusu virus (**TMUV**), reovirus (**REO**), Newcastle virus (**NDV**), and goose circovirus (**GoCV**). The specificity of the method was evaluated based on the results.

Duplicability Test

Six serum samples (4 positives and 2 negatives) were detected in the same plate, and 4 wells were repeated for each sample. The intraplate coefficient of variation (**CV**) of the same serum sample was calculated to determine the intraplate repeatability of the tested samples. Six different serum samples (4 positives and 2 negatives) were tested using the enzyme-labeled plates coated at different time intervals. The interplate CV of the same serum samples was calculated to determine the interplate reproducibility of the tested samples. The intra-assay and inter-assay CV were calculated using the following formula: CV = standard deviations $(SD)/\text{mean OD}_{450}$ of samples $(X) \times 100\%$.

Sensitivity Test

In order to determine the sensitivity of this method, sera were diluted 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. The other operating conditions were performed as the optimal working procedure.

Detection of Clinical Samples

To determine the detection efficiency of this method for distinguishing between GPV infection and vaccine immunization antibodies, 145 GPV infection goose serum samples and 120 immunized goose serum samples were collected from Shandong and Henan. Among them, GPV infection serum samples were taken from natural diseased geese, and immune serum samples were obtained from healthy geese that are immunologically commercial gosling antibodies (purified egg yolk antibody against gosling plague virus, Pulike Biological Engineering, Inc., Henan, China). In addition, because MDPV showed 88% identity with GPV (Zhang et al., 2010), we collected 65 MDPV infection duck serum samples and 50 immunized duck serum samples to know the detection efficiency about the ELISA method with MDPV-positive sera.

Statistical Analysis

For determining this method to distinguish between the GPV infection and vaccine immune antibodies, 145 GPV infection serum samples and 120 immunized serum samples were tested. Statistical analysis was performed using the WPS table software.

RESULTS

Virus Identification and Sequence Analysis

Within 5 D, the goose embryos used to isolate the virus died. The dead goose embryos show the symptoms of dysplasia and skin bleeding. The allantoic fluid was positive for GPV by PCR detection. The result is shown in Figure 1A. After sequence alignments and phylogenetic analysis, we determine that the isolate was GPV. The GPV strain was named as AHDY (GenBank accession number: MN233574). Comparing with the reference GPV strains, the AHDY isolated strain shared 91.5 to 99.8% nucleotide similarities. Phylogenetic analysis indicated that the AHDY isolate strain clustered with the reference GPV strains (Figure 2). The result of sequence alignment showed that the amplified NS1 and VP3 gene (Figure 1B) were the same as the AHDY strain. Also, no insertions or deletions can be observed.

Recombinant Protein Expression and Purification

The *NS1* and *VP3* PCR products were cloned into the pET-32a vector. The recombinant plasmids of the correct size were identified by PCR and sequencing. The pET32a-NS1 and pET32a-VP3 expression plasmid was expressed successfully in *E. coli*. The sequence information obtained from recombinant protein expression and purification is given in Table 1. The average yield of

Table 1. Sequence information obtained from recombinant protein expression and purification.

Protein	Protein sequence	Nucleotides position[3]
NS1[1]	5′-MALSRPLQISSDKFYEVIIRLPSDIDQDVPGLSLNFVEWLSTGVWEPTGIWNMEHVNL-PMVTLAEKIKNIFIQRWNQFNQDETDFFFQLEEGSEYIHLHCCIAQGNVRSFVLGRYMS-QIKDSIIRDVYEGKQIKIPDWFAITKTKRGGQNKTVTAAYILHYLIPKKQPELQWAFTN-MPLFTAA-3′	541–1089
VP3[2]	5′-MAEGGGGALGDASGGADGVGNASGNWHCDSQWMGNTVITKTTRTWVLPSYNNHI-YKAITSGTSQDANVQYAGYSTPWGYFDFNRFHCHFSPRDWQRLINNHWGIRPKSLKFK-IFNVQVKEVTTQDQTKTIANNLTSTIQVFTDDEHQLPYVLGSATEGTMPPFPSDVYALP-QYGYCTMHTNQNGAR-3′	3037–3594

[1]The recombinant protein of NS1 contains 183 amino acid and no change in amino acids.
[2]The recombinant protein of VP3 contains 186 amino acid and no change in amino acids.
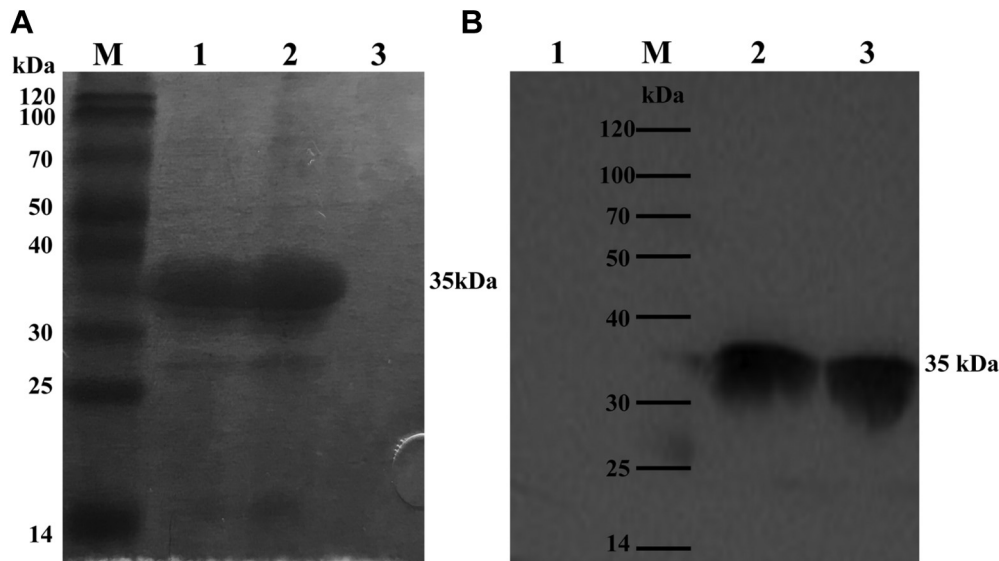[3]The nucleotides position of the recombinant protein in the genome.

Figure 3. (A) Identification of NS1 and VP3 proteins from the pET32a-NS1 plasmid and pET32a-vp3 plasmid by SDS-PAGE. Lane 1: *E. coli* expressing pET32a-NS1 of induction and purification. Lane 2: *E. coli* expressing pET32a-VP3 of induction and purification. Lane 3: *E. coli* expressing pET32a vector. M: molecular weight marker. (B) Detection of recombinant NS1 and VP3 fusion proteins by Western blotting with goose anti-GPV sera. Lane 1: *E. coli* expressing pET32a vector. Lane 2: *E. coli* expressing pET32a-NS1. Lane 3: *E. coli* expressing pET32a-VP3. M: molecular weight marker.

recombinant protein was calculated to be 20.64 (NS1) and 22.4 (VP3) mg/200 mL of culture. After the recombinant proteins were purified, the concentrations of NS1 and VP3 proteins are 2.58 and 2.8 μg/μL, respectively ($Y = 0.1652X + 0.0014$, $R^2 = 0.9923$, Y = Absorbance after removing the background value, X = Protein concentration (μg/μL)). The percentage of purification is about 75%. Protein bands were subsequently visualized by Coomassie brilliant blue staining. Analysis of extracts of pET32a-NS1 and pET32a-VP3-transformed *E. coli* by SDS-PAGE revealed the NS1 and VP3 fusion proteins with an approximate molecular mass of 35 kDa (including the His tag), which was consistent with the expected size of the NS1 and VP3 fusion proteins (Figure 3A). The expressed protein was then analyzed by immunoblotting with an HRP-labeled rabbit anti-goose IgG. Western blotting (**WB**) showed that GPV-

positive sera reacted specifically against the NS1 and VP3 fusion proteins that had an approximate molecular mass of 35 kDa (Figure 3B). The results of WB analysis suggested that the recombinant NS1 and VP3 fusion proteins possessed high levels of antigenicity and immunogenicity. Meanwhile, parvovirus-specific proteins were not detected in lysates derived from pET32a-transformed *E. coli* cells.

Standardization of the NS1-ELISA and VP3-ELISA Procedure

The optimal dilutions of antigen and test sera in the NS1 and VP3-ELISA were determined by using a checkerboard titration. Using the GPV-antibody positive goose, the optimal dilution of the tested sera was found

Table 2. Determination of critical value of indirect ELISA method for NS1 protein.[1]

	OD_{450}[2]			
	0.134	0.12	0.188	0.178
	0.17	0.173	0.167	0.169
	0.228	0.156	0.217	0.158
	0.165	0.194	0.139	0.218
	0.133	0.16	0.1	0.144
	0.15	0.19	0.238	0.168
Mean (X)	0.169			
Standard deviation (SD)	0.0332			
Cut-off value[3]	0.269			

[1]Thirty negative sera from goose were used to determine the cut-off value according to the optimization of the ELISA procedure.
[2]Values within a column represent the optical density of 30 sera samples from goose uninfected with GPV.
[3]Limit of detection (LOD): Cut-off value(LOD) = mean(X) + 3 × Standard deviation(SD).

Table 3. Determination of critical value of indirect ELISA method for VP3 protein.[1]

	OD_{450}[2]			
	0.112	0.11	0.134	0.19
	0.177	0.104	0.147	0.205
	0.098	0.24	0.175	0.113
	0.1	0.12	0.171	0.152
	0.118	0.16	0.128	0.108
	0.185	0.168	0.103	0.136
Mean (X)	0.144			
Standard deviation (SD)	0.0376			
Cut-off value[3]	0.257			

[1]Thirty negative sera from goose were used to determine the cut-off value according to the optimization of the ELISA procedure.
[2]Values within a column represent the optical density of thirty sera samples from goose uninfected with GPV.
[3]Limit of detection (LOD): Cut-off value(LOD) = mean(X) + 3 × Standard deviation(SD).

Table 4. Indirect ELISA method specificity test results.

	ND	H9	TMUV	REO	GoCV	GPV(+)	GPV(−)
OD value (NS)	0.175	0.187	0.158	0.1	0.093	0.421	0.097
OD value (VP)	0.181	0.223	0.202	0.124	0.108	0.368	0.081
Results	N	N	N	N	N	P	N

Abbreviations: N, negative, the OD450 value was lower than the cut-off value; P, positive; the OD450 value was higher than the cut-off value.

to be 1:40 and the plates were incubated for 1.5 h at 37°C. NS1 and VP3 proteins were used at final concentrations of 0.806 and 0.875 μg/mL for coating antigen, and the plates were incubated at 4°C overnight. A dilution at 1:100 for the HRP-rabbit anti-goose IgG conjugate was determined. Then, the plates were incubated for 1 h at 37°C. The best closure time for 5% skimmed milk powder is 37°C, 1 h, and the best color time is 37°C, 15 min. A good positive/negative (P/N) ratio was obtained by the positive OD value divided by the negative OD value. All experiments were performed in duplicate.

The Positive/Negative Cut-Off Value

A total of 30 sera samples from goose uninfected with GPV were selected randomly in order to calculate cut-off values. The means (X) of the $OD_{450\ nm}$ values for these sera were 0.169 (NS1) and 0.144 (VP3), with SDs of 0.0332 (NS1) and 0.0376 (VP3). The cut-off values of the NS1-ELISA and VP3-ELISA were calculated (Tables 2, 3). If the OD value of the sample was ≥0.269 (NS1) or 0.257 (VP3), the result was positive; if not, it was considered to be negative.

Cross-Reaction Test

No cross-reactions were detected by the NS1-ELISA and VP3-ELISA using antisera against H9 AIV, TMUV, REO, NDV, and GoCV, with the OD values ranging from 0.093 to 0.202 (Table 4), demonstrating an excellent specificity of the NS1-ELISA and VP3-ELISA for detection of the GPV antigen.

Duplicability Test

For the 6 selected sera (4 positives and 2 negatives), the intra-assay CV and the inter-assay CV were both lower than 10% (Tables 5–8). The results showed that the NS1-ELISA and VP3-ELISA assays were highly reproducible and stable.

Sensitivity Test

The sensitivity of a panel of diluted sera was evaluated, and a minimum detection limit of 1:320 (Table 9) was obtained according to the endpoint cut-off value (0.269 (NS1) and 0.257 (VP3)), but the blank control did not yield a positive result.

Detection of Clinical Samples

Two indirect ELISA methods were used to detect 145 goose sera from natural infection. The positive rates were 88.97% (NS1) and 86.21% (VP3), respectively (Table 10). Then, 2 indirect ELISA methods were used to detect 120 immunological goose sera. The positive rates were 9.17% (NS1) and 88.33% (VP3), respectively (Table 10). These results fully show that the indirect ELISA method can effectively distinguish between GPV infection and vaccine immunization antibodies. Another aspect, for 60 MDPV infection duck serum samples, the positive rates were 83.08% (NS1) and 81.54% (VP3), respectively (Table 11). For 50 immunized duck serum samples, the positive rates were 8% (NS1) and 84% (VP3), respectively (Table 11). It is worth noting that the positive rates of MDPV were lower than the positive rates of GPV due to use of different bird conjugates (HRP-rabbit anti-goose IgG).

Table 5. Intra-assay repeatability test results of NS1 protein indirect ELISA.

	OD value						
Samples no.	1	2	3	4	Mean (X)	Standard deviation (SD)	Coefficients of variation (CV%)
P 1	0.48	0.482	0.483	0.47	0.479	0.0052	1.09
P 2	0.379	0.361	0.375	0.378	0.373	0.0072	1.93
P 3	0.48	0.472	0.492	0.481	0.481	0.0071	1.48
P 4	0.367	0.385	0.378	0.377	0.377	0.0064	1.70
N 1	0.106	0.114	0.111	0.11	0.11	0.0029	2.64
N 2	0.154	0.16	0.162	0.175	0.163	0.0077	4.72

Abbreviations: N, negative, the OD_{450} value was lower than the cut-off value; P, positive; the OD_{450} value was higher than the cut-off value.

Table 6. Intra-assay repeatability test results of VP3 protein indirect ELISA.

Samples no.	OD value				Mean (X)	Standard deviation (SD)	Coefficients of variation (CV%)
	1	2	3	4			
P 1	0.488	0.502	0.504	0.484	0.495	0.0087	1.76
P 2	0.377	0.383	0.38	0.369	0.377	0.0052	1.38
P 3	0.484	0.468	0.477	0.48	0.477	0.0059	1.24
P 4	0.37	0.377	0.373	0.381	0.375	0.0042	1.12
N 1	0.12	0.101	0.097	0.103	0.105	0.0088	8.38
N 2	0.16	0.157	0.166	0.173	0.164	0.0061	3.72

Abbreviations: N, negative, the OD_{450} value was lower than the cut-off value; P, positive; the OD_{450} value was higher than the cut-off value.

DISCUSSION

Gosling plague is caused by goose plague virus (GPV), and it is mainly responsible for an acute, highly contact, septic nature of goslings and young Muscovy ducks within 1 mo of age. This disease is highly contagious, has a short course of the disease and a high mortality rate (Yin and Liu, 1985). The full-length genome of GPV is 5,106 bp, and it mainly contains coding regions and non-coding regions (Hu, 2014). The 2 ends of the genome contain an inversion sequence of 444 bp in length and are non-coding regions. The first 407 bp of this sequence forms a hairpin structure with a 44-bp vesicular structure in the middle (Wang et al., 2014). The center point of the structure has a restriction endonuclease site SphI (Yang, 2009). The non-coding region is a palindrome sequence at both ends, that is, the hairpin structure; the coding region is located in the middle, including 2 open reading frames (**ORF**), including the left ORF and right ORF, and the 18 bp fragments between the 2 reading frames, and the left ORF mainly encodes the non-structural protein, including NS1 and NS2; the right side ORF mainly encodes structural protein, including VP1, VP2, VP3 (Li and Pintel, 2012). The NS1 and NS2 proteins were produced early in the virus replication. The NS1 gene contained 1,884 nucleotides and encoded 628 amino acids. The NS1 protein was involved in virus-induced cytotoxicity, virus replication, and gene expression (Cotmore et al., 1995; Tian et al., 2002). The VP gene is an important gene of GPV, which is related to the capsid protein of the virus, but it can also be used as an antigen to induce the body to produce antibodies. At the same time, the VP protein is also related to virulence and pathogenicity of the virus. The VP3 in VP protein is most conserved and stable, and the related sequencing results can confirm this characteristic.

At present, there are a variety of detection methods for GPV infection, in which the ELISA method has the advantages of simple and rapid operation, and can detect a large number of samples at the same time. It can be widely used in clinical detection. However, most ELISA methods for detecting GPV can only detect the antibody level of the sample, but cannot distinguish between the GPV infection and vaccine immunization antibodies. Therefore, this study has a wider application value by establishing the detection method based on the structure and non-structural proteins of the virus. NS1 protein is a non-structural protein of GPV, so it can be used to detect the GPV infection in actual production, so as to determine the GPV infection status of waterfowl, but not to detect the immune sample of inactivated vaccine. VP3 protein is a structural protein of GPV, so the ELISA method using VP3 protein as a coating antigen can detect the GPV infection or inactivated vaccine (Yu et al., 2000). In this study, NS1 protein and VP3 protein were used as inclusion antigens to establish an indirect ELISA detection method to distinguish between the GPV infection and vaccine immunization antibodies, and the method was optimized.

In this study, GPV NS1 and VP3 partial proteins were successfully expressed through a prokaryotic expression system, and the expressed proteins were

Table 7. Inter-assay repeatability test results of NS1 protein indirect ELISA.

Samples no.	OD value				Mean (X)	Standard deviation (SD)	Coefficients of variation (CV%)
	1	2	3	4			
P 1	0.492	0.47	0.465	0.482	0.477	0.0105	2.20
P 2	0.392	0.38	0.37	0.368	0.378	0.0095	2.51
P 3	0.352	0.384	0.369	0.383	0.372	0.013	3.49
P 4	0.396	0.371	0.385	0.359	0.378	0.0111	2.94
N 1	0.114	0.115	0.13	0.122	0.12	0.0064	5.33
N 2	0.172	0.175	0.183	0.173	0.176	0.0043	2.44

Abbreviations: N, negative, the OD_{450} value was lower than the cut-off value; P, positive; the OD_{450} value was higher than the cut-off value.

Table 8. Inter-assay repeatability test results of VP3 protein indirect ELISA.

| Samples no. | OD value | | | | Mean (X) | Standard deviation (SD) | Coefficients of variation (CV%) |
	1	2	3	4			
P 1	0.491	0.474	0.461	0.472	0.475	0.0107	2.25
P 2	0.374	0.378	0.393	0.387	0.383	0.0074	1.93
P 3	0.468	0.483	0.471	0.492	0.479	0.0096	2.01
P 4	0.382	0.379	0.36	0.367	0.372	0.0089	2.39
N 1	0.096	0.103	0.108	0.111	0.105	0.0057	5.43
N 2	0.18	0.141	0.166	0.154	0.16	0.0144	9

Abbreviations: N, negative, the OD_{450} value was lower than the cut-off value; P, positive; the OD_{450} value was higher than the cut-off value.

purified by different concentrations of urea solution. If the purity of the coating antigen is not high, the sensitivity, specificity, and reproducibility of the detection method will be seriously affected. The purified NS1 and VP3 protein content and purity were very high, which laid the foundation for the successful establishment of the ELISA detection method. The specificity and sensitivity of ELISA assay are closely related to the concentration of antigen and the dilution of serum. If the antigen coating concentration is too high, frequent interactions between the antigen protein molecules can easily cause the multiplication of protein molecules, so that the coating antigen is easily washed off during washing, resulting in non-specific detection results; if the concentration is too low, the amount of antigen adsorbed on the surface of the carrier is not enough, and false negatives may also occur, which affects the sensitivity of the ELISA test results (Zhang, 2012). In this research, the best dose of antigen was screened out by a square array test. The optimal contents of NS1 protein and VP3 protein were 80.6 ng/pore and 87.5 ng/pore, respectively. The antigen is moderately coated and meets the requirements of antigen coating.

At the same time, other conditions of the established ELISA test methods, such as the coating time and temperature of the antigen, the blocking time of the blocking solution, the conditions of the secondary antibody, the coloration time, the sensitivity, the specificity, and the reproducibility, were also optimized and identified. By optimizing the coating condition of the antigen and the condition of the secondary antibody, the defect that the background value of the goose serum was detected was successfully solved. The detection method has good specificity, sensitive to the positive serum reaction of GPV,

and no cross-reaction with positive serum of NDV, H9 AIV, TMUV, REO, and GoCV. The sensitivity of the method is very high. When the positive serum is diluted to 1:320 times, the detection result is still larger than the critical value. The method is very reproducible, and the CV between the plates is less than 10%.

Two indirect ELISA methods were used to detect clinically 265 samples of goose serum (145 GPV infections and 120 inactivated vaccines). The results showed that the positive rates of GPV infection samples was 88.97% (NS1) and 86.21% (VP3), and the positive rates of inactivated vaccines were 9.17% (NS1) and 88.33% (VP3), respectively. It can be seen that the 2 indirect ELISA methods established can distinguish serum samples from GPV infection and vaccine immunization. Meanwhile, the 2 indirect ELISA methods were used to detect 115 samples of Muscovy duck serum (65 MDPV infections and 50 inactivated vaccines). The positive rates of MDPV infection samples were 83.08% (NS1) and 81.54% (VP3), and the positive rates of inactivated vaccines were 8% (NS1) and 84% (VP3), respectively. The results show that the ELISA methods were used to detect MDPV-positive sera which is also effective.

In conclusion, the 2 indirect ELISA methods can be used to detect and measure anti-GPV and MDPV antibodies with high levels of sensitivity, specificity, and reproducibility. It provides an alternative, inexpensive, and rapid serological detection method that would be suitable for epidemiological surveys that trace the spread of GPV and MDPV, and the assay can monitor anti-GPV and MDPV antibody titers on a large scale. Most importantly, the method can distinguish between GPV or MDPV infection and vaccine immunization antibodies. It provides a sensitive, specific, and reproducible

Table 9. Indirect ELISA method sensitivity test results.

| Dilution times | OD_{450} | | | | | |
	1:40	1:80	1:160	1:320	1:640	1:1280
OD_{450} (NS)	0.537	0.426	0.312	0.276	0.17	0.095
OD_{450} (VP)	0.557	0.435	0.301	0.268	0.142	0.109
Results	P	P	P	P	N	N

Abbreviations: N, negative, the OD_{450} value was lower than the cut-off value; P, positive; the OD_{450} value was higher than the cut-off value.

Table 10. Detection of goose sera samples.

| | NS1-ELISA | | | VP3-ELISA | | |
	N	I	Total	N	I	Total
Positive	129	11	140	125	106	231
Negative	16	109	125	20	14	34
Total	145	120	265	145	120	265
Positive rate (%)	88.97	9.17		86.21	88.33	

Abbreviations: I, immunological sera; N, natural infection sera.

Table 11. Detection of Muscovy duck sera samples.

	NS1-ELISA			VP3-ELISA		
	N	I	Total	N	I	Total
Positive	54	4	58	53	42	95
Negative	11	46	57	12	8	20
Total	65	50	115	65	50	115
Positive rate (%)	83.08	8		81.54	84	

Abbreviations: I, immunological sera; N, natural infection sera.

method for better detection and prevention of the disease.

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