Polish Journal of Veterinary Sciences Vol. 25, No. 1 (2022), 109–118

DOI 10.24425/pjvs.2022.140847

Original article

Development of an indirect enzyme-linked immunosorbent assay for the detection of novel chicken orthoreovirus

H. Liu[1,2,3], Z. Wei[1,2,3], J. Yang[1,2,3], Y. Wang[1,2,3], J. Hu[1,2,3,*], Y. Tang[1,2,3,*], Y. Diao[1,2,3,*]

[1] College of Veterinary Medicine,
Shandong Agricultural University, No.61 Daizong Street, Tai'an 271018, China
[2] Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention,
Shandong Agricultural University, No.61 Daizong Street, Tai'an 271018, China
[3] Shandong Provincial Engineering Technology Research Center of Animal Disease Control and Prevention,
Shandong Agricultural University, No.61 Daizong Street, Tai'an 271018, China

Abstract

A novel avian orthoreovirus (N-ARV) variant characterized with obvious arthritis and synovial inflammation, was isolated from Shandong, China in May 2016. It caused chicken poor growth and enormous economic losses to the poultry industry of China. However, there are few effective methods for detecting the antibody levels of N-ARV. In this study, a viral structural protein σC was expressed using the prokaryotic expression vector pET32a (+). The target protein was obtained by inducing for 6 hours at an IPTG concentration of 0.6mM. The optimal dilution of the coating antigen and serum antibody were determined to be 1000 fold and 10 fold respectively. A specificity test showed that there was no positive reactivity between N-ARV and other pathogens, and when the positive serum was diluted 100 times detection results were still checkable. The repeatability of this method was determined by the inter assay and intra assay tests with variability ranging from 4.85% to 7.93%. In conclusion, this indirect enzyme linked immunosorbent assay (ELISA) will be useful for large-scale serological surveys and monitoring antibody levels in N-ARV infection.

Key words: novel avian orthoreovirus, σC gene, recombinant plasmid, indirect enzyme linked immunosorbent assay, epidemiological investigation

Correspondence to: J. Hu, e-mail: hjd1968@163.com;
Y. Tang, e-mail: tyck288@163.com;
Y. Diao, e-mail: yxdiao@163.com
H. Liu and Z. Wei made an equal contribution to this article

Introduction

Since 2010, emerging strains of pathogenic avian orthoreovirus (ARV) have become a challenge to the chicken industry in Shandong, Jiangsu, Hebei and Fujian provinces of China. Moreover, in western Canada (Palomino-Tapia et al. 2018), the United States of America (Tang and Lu 2015), Korea (Noh et al. 2018), some poultry infected with avian orthoreovirus (ARV) strains had paralyzed and tenosynovitis and other symptoms.

Avian reovirus (ARV), a member of the *Orthoreovirus* genus, consists of 10 double stranded RNA segments based on the segments electrophoretic mobility (Benavente and Martinezcostas 2007), causes enteric disease (Dutta and Pomero 1967), viral arthritis (Mor Sunil K et al. 2013) and tenosynovitis (Marks and Marks 2016), runting-stunting syndrome (Jones and Savage 1987) in infected poultry, and given serious economic losses to the poultry industry (Ping-Yuan et al. 2006). ARVs are pathogenetic to many animals such as chickens (Dandár et al. 2013), geese (Palya et al. 2003), ducks (Liu et al. 2016), Muscovy ducks (Yun et al. 2015), pheasants (Curtis et al. 1992), pigeons (Vindevogel et al. 1982) and turkeys (Sharafeldin et al. 2015). At the same time, the disease could affect different fowl breeds, including broiler breeders (Ide and Dewitt 1979), broilers (Howell and Walker 1972), domesti-cated chickens and wild commodity chickens, which can cause higher mortality (Jones and Kibenge 1984).

ARV encoded for at least 12 distinct proteins (Rendón-Anaya et al. 2017); all genomic segments except S1 of chicken ARV are monocistronic and encode a single protein, including eight structures (λA, λB, λC, μA, μB, σA, σB and σC) and four non-structural viral proteins (μNS, σNS, p10 and p17) (Bodelón et al. 2001). The S1 segment is tricistronic with three partially overlapping ORFs encoding p10, p17 and σC, respectively (Bodelón et al. 2001). σC is one of the major antigens responsible for producing neutralizing antibodies which may guard against the viral infection for host cells (Shih et al. 2004).

To date, using SPF chicken embryo or chicken hepatoma cells (LMH) for isolation and identification of N-ARV is the classic diagnostic method (Heggen-Peay et al. 2002). However, inevitably there have been some shortcomings, such as being time consuming and laborious. Several molecular biology diagnostic methods have been established, such as reverse transcriptase (RT)-PCR (Lin et al. 2007), nested PCR (Liu et al. 1997), and multiplex PCR (Caterina et al. 2004), with the advantage of high sensitivity and timesaving. However, these methods need expensive instruments and professional operators. Some serological methods such as agar-gel precipitin assay (Adair et al. 1987), fluorescent antibody assay (Adair et al. 1987) have been established to detect ARV, although there have low-sensitivity issues. However, all of these methods were inefficient for accurately detecting N-ARV antigen and antibody. The main reason is the extensive sequence divergence of genome segments between classical ARV and N-ARV. Most of these detection methods were based on the sequences of historical ARV strains, and they were unable to detect various ARV variants from field strains (Guo et al. 2011). Currently, few diagnostic methods have been developed for the detection of antibodies and evaluating antibody titers in N-ARV infection. Therefore, it is urgent to establish a sensitive and specific method to diagnose N-ARV. Enzyme-linked immunosorbent assay has to be considered as an effective diagnostic tool for antibody detection and epidemiological investigation with its many advantages: low-cost, reproducibility and easy performance. In this study, we used the recombinant piroplasm surface protein (σC) to develop an indirect ELISA method for diagnosis of N-ARV infection.

Materials and Methods

Virus, bacterial species, plasmids and serum

In this study, the novel avian reovirus strain LY383 (N-ARV-LY383) was isolated and preserved by the Research Institute of Poultry Disease of Shandong Agricultural University. Positive and negative control samples of N-ARV and other virus infectious positive control serum samples involved in infectious bronchitis (IB), infectious bursal disease (IBD), avian influenza virus serotype H9 (H9N2-AIV) and fowl adenovirus (FAV) were prepared and saved in our laboratory. 90 and 363 clinical serum samples used in this study were collected from diseased chickens in Yantai, Weihai, Dongying of Shandong province of China.

RT-PCR, sequence analysis and phylogenetic

According to the genome sequence of the LY383 strain (accession no. MF183217.1), primers were designed based on the sequence of S1 gene by primer premier 5.0 with restriction sites (*BamH* I and *Sal* I), shown underlined.

F:5'-GGATCCATGGACGGATTAACTCAACA-3'
R:5'-AGTCGACTTAGGTATCGATGCCCGT-3'

The total viral RNA from culture supernatant was extracted using MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China). Conventional RT-PCR was carried out using a One-step RT-PCR Kit (TaKaRa,

Dalian, China). The PCR reaction system was as follows: 50°C for 30 min, 94°C for 2 min,30 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s), and extension (72°C for 60 s), and final extension at 72°C for 10 min. 1% agarose gel was used to separate and purify the amplified σC gene. A 981bp fragment was then excised. PCR products were purified using a gel extraction kit (Omega, Georgia, USA). The σC- pMD18-T plasmid was then transformed into *E.coli* DH5α competent cells, and positive clones were sequenced by BGI Tech (BGI Tech, Shenzhen, China). Sequence alignment and phylogenetic tree construction of σC gene S1 segment sequences was performed using MegAlign (DNASTAR, Inc. Madison, WI, USA) and MEGA6.0. For phylogenetic tree analysis, σC gene sequences of reference ARV strains and variant sequences from around the world retrieved from Genbank were included. At the same time, the amino acid (aa) and pairwise nucleotide sequence was performed to examine the degree of sequence identity.

Construction of σc-pET-32a recombinant plasmid

The σC- pMD18-T plasmid and the pET-32a (+) vector were double digested with *BamH* I and *Sal* I. 15 μL σC-pMD18-T plasmid and 2 μL linearized pET-32a were incubated at 16°C for 2 h in the presence of 1 μL T4 DNA ligase and 2 μL buffer in a total volume of 20 μL for construction of the expressed recombinant plasmid σC-pET32a. Recombinant plasmid was confirmed by restriction analysis and sequencing and preserved at -80°C.

Expression and purification of σc-pET-32a protein

The correct orientation of the insert was confirmed by nucleotide sequencing. The plasmid was then transformed into BL21 (DE3) Competent Cell (10 tubes, CWBIO, Beijing, China). The positive bacterial culture was inoculated in 6 mL of 2×YT medium containing ampicilin (100 mg/mL) overnight at 37°C with rocking at 220 rpm (Bocai, Shanghai, China). The 2 mL overnight bacterial culture was respectively inoculated to four culture bottles with 200 mL 2×YT medium under the same conditions. Isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.2 mmol/L, 0.4 mmol/L, 0.6 mmol/L and 0.8 mmol/L respectively, when the optical density (OD) of the culture reached 0.6 to 0.8 at a wavelength of 600 nm. Expression of protein was then measured for several hours at 37°C with rocking, and cultures were harvested every two hours. At the same time the non-carrier of pET-32a vector was used as a control for 6h under the same conditions. All of the bacterial cultures were centrifuged at 8,000×g for 15min, and the supernatant was discarded. The precipitation was resuspended in PBS, and lysed by ultrasonication using an ultrasonic cell disruptor (Ningbo Xinzhi Biological Technology Co., Ltd.).

SDS-PAGE and western blotting

After degeneration, the supernatant and precipitation were analyzed using a 12% SDS-PAGE gel, and the activity of the recombinant protein was analyzed using western blotting. The identified protein was purified sing the different concentrations of urea solution. Western blotting analysis was performed following established procedures. Briefly, the recombinant proteins which were separated by SDS-PAGE were transferred onto nitrocellulose filter membranes (Millipore, USA). The membrane was blocked for 2 h with 5% skim milk powder which was diluted in Tris-buffered saline containing 0.05% Tween 20 (TBST), and incubated at 4°C overnight with His tag mouse Monoclonal antibodies (Biodragon, Beijing, China). After another washing step, anti-His tag rabbit polyclonal antibody at a dilution of 1:12000 was incubated for 2 h, and the result was visualized in using the BeyoECL Plus kit (Beyotime, Shanghai, China).

Establishment of the indirect ELISA

The indirect ELISA assay was performed in 96-well microtiter plates. In order to determine the optimal concentration of antigen and serum, a checkerboard titration was performed. The purified σC protein was diluted 500-16000 times with 0.05M carbonate buffer (pH 9.6), and 100 μL of which was added into each well of the plates at 4°C overnight. The plates were washed three times with PBST (0.01 M PBS, pH 7.2, 0.05% Tween 20) and then blocked with 5% skimmed milk in 0.01 M PBST (pH 7.4) for 2 h at 37°C. Both positive and negative serum diluted from 1:10 to 1:80 with blocking solution were added into the plates, which were incubated at 37°C for 1h, and washed for 4 minutes for a total of three times with PBST. 100 μL of rabbit anti-chicken IgG labeled with horseradish peroxidase (HRP) diluted with blocking buffer were added to each well, incubated for 1 mh at 37°C, followed by three times washing with PBST. 100 μL of substrate buffer containing TMB (3,3',5,5'-tetra-methylbenzidine) (TIANGEN biotech, Beijing, China)) was added at 37°C for 15 min followed by 50 μL 2M H_2SO_4 to stop the reaction. The results were determined at a wavelength of 450 nm optical density (OD) values using an microplate reader (Bio Tek Instruments, Inc, USA). In order to confirm the optimal coating duration of σC proteins, three different experiments were carried out: 37°C for 2 h and then 4°C overnight, 4°C overnight, 37°C 2 h. At the

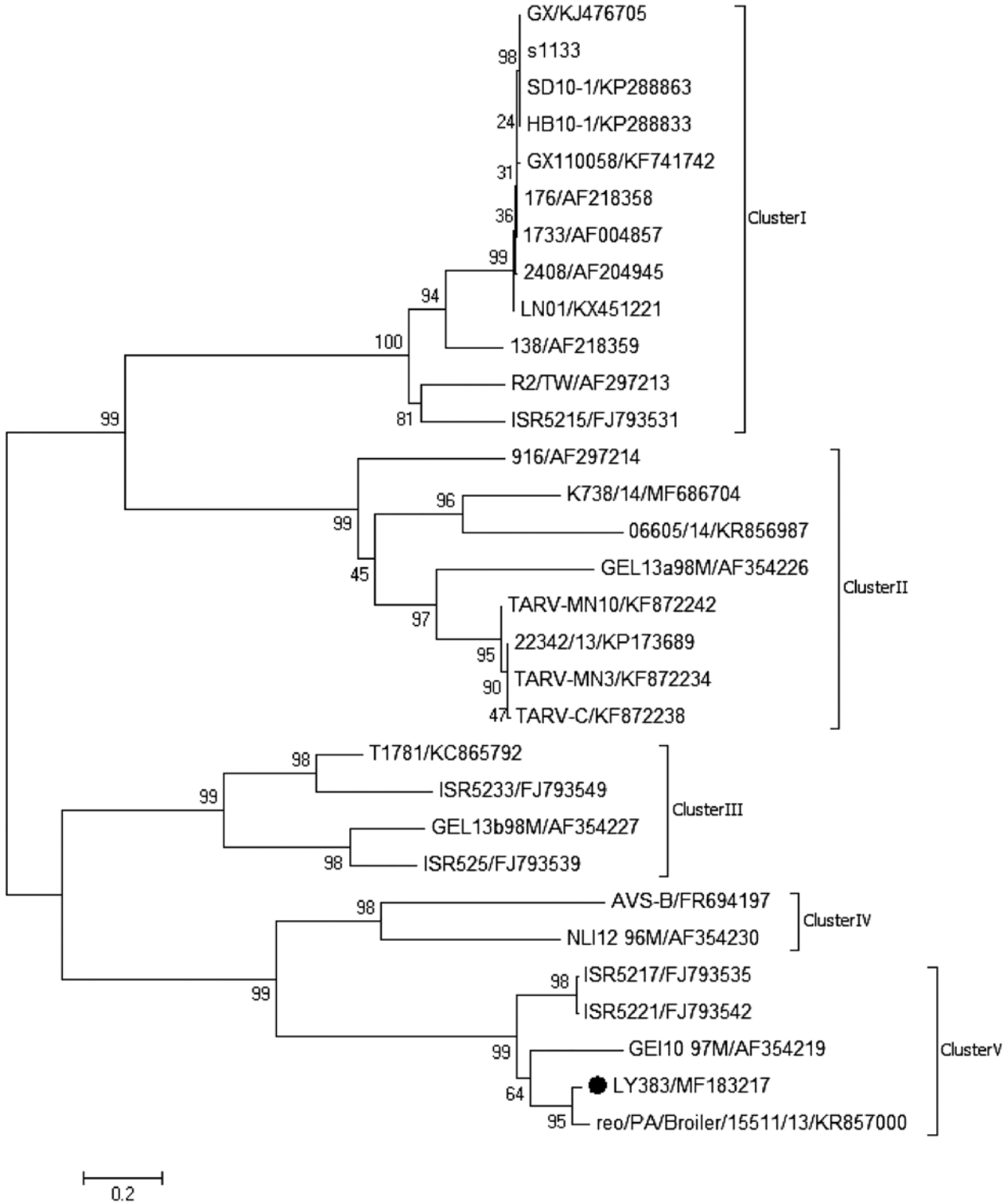

Fig. 1. Phylogenetic trees constructed using the neighbour-joining method by avian orthoreovirus (ARV) based on nucleotide sequences of σC genes. LY383 strains are marked with a black colour dot.

same time, 0.5% BSA, 1% BSA, 2.5% skimmed milk powder, and 5% skimmed milk powder was used to determine the optimal blocking solution and working concentration.

Determination of the cut-off value

A total of 30 specific pathogen free (SPF) chicken negative serum samples were tested under optimal conditions by indirect ELISA. The cut-off value = mean OD_{450nm} = $\overline{X}$ +3S. All experiments were performed in triplicate.

Table 1. Sequence identities between novel avian orthoreovirus (N-ARV) LY383 strain and members of orthoreovirus genus.

Cluster	isolate	Gennk number	nt	aa	Cluster	isolate	Genbak number	nt	aa
	LY383					LY383			
V	15511/13	KR857000	95.1	95.1	II	GEL13a98M	AF354226	53.9	49.6
	ISR5221	FJ793542	80.6	85.0		916	AF297214	54.5	50.8
	GEI10_97M	AF354219	80.2	84.6		06605/14	KR856987	51.3	43.6
	ISR5217	FJ793535	80.0	84.6	I	SD10-1	KP288863	50.9	44.7
IV	AVS-B	FR694197	62.1	57.5		HB10-1	KP288833	50.9	44.7
	NLI12_96M	AF354230	60.6	60.5		GX	KJ476705	51.1	44.7
III	ISR5233	FJ793549	55.4	50.4		GX110058	KF741742	51.1	44.4
	ISR525	FJ793539	54.6	47.0		LN01	KX451221	51.4	45.5
	GEL13b98M	AF354227	55.7	50.4		ISR5215	FJ793531	53.3	47.4
	T1781	KC865792	55.5	49.6		R2/TW	AF297213	51.9	46.2
II	2234213	KP173689	52.3	48.1		138	AF218359	53.3	46.2
	TARV-MN10	KF872242	51.8	48.5		176	AF218358	51.3	45.5
	TARV-C	KF872238	52.2	48.1		2408	AF204945	51.3	45.5
	TARV-MN3	KF872234	52.0	48.1		1733	AF004857	51.2	45.5
	K738/14	MF686704	52.3	48.1		s1133	MG822668	51.1	45.1

Evaluation of specificity, sensitivity and repeatability of the ELISA assay

In order to determine the specificity of the established ELISA method, other virus infectious positive control serum samples including IB, IBD, H9N2-AIV, FAV were detected as established procedures, while N-ARV positive and negative samples were used as control. Double dilution series dilutions (1:25~1:1400) of positive sera were used to evaluate its sensitivity. Meanwhile, 90 clinical serum samples were tested for the presence of the ARV antibody using an IDEXX Avian reovirus antibody detection kit (IDEXX, Beijing, China) according to the instructions and these results were used as a reference to define the sensitivity and specificity of the established ELISA method.

To evaluate the reproducibility of the ELISA detection method for N-ARV detection, triplicate from each serum were tested in the same ELISA plate, and the intra assay coefficient of variation (CV), CV=SD/$\bar{X}$×100% was calculated. For the inter-assay test, samples were tested in three independent plates. The inter-assay coefficient of variation (CV), CV= SD/$\bar{X}$×100% was then calculated.

Clinical Sample detection

363 serum samples were collected from diseased chickens. All samples were collected as part of routine veterinary procedures.

Results

Phylogenetic and sequence analysis of σC

The σC protein encoding genes of LY383 were phylogenetically analyzed along with gene sequences from previously identified ARV strains. The results indicate that the isolated ARVs share the highest homology with Reo/PA/Broiler/15511, which is a novel strain of American broiler and in the same cluster with Israeli and Netherlands strains, which suggests these viruses might share an evolutionary relationship. The isolates were grouped into genotyping cluster V and distinct from the reference vaccine strains (S1133,1733, and 2048), which revealed newly emerging ARV variants (Fig.1). The aa similarities were less than 60.5% between any 2 of the 5 genotyping clusters except cluster V. The ARV variant shared aa identity was 84.6-95.1% within cluster V but 44.7-47.4% within I cluster and shared 80.0-95.1% of nucleotide identity with cluster V but 50.9-53.3% within the clusters I (Table 1).

Construction of recombinant plasmid and expression of fusion protein

The σC gene was amplified and confirmed using 1.0% agarose gel electrophoresis. The 981bp target fragment inserted into the pET-32a plasmid was identified by sequencing and double digestion with *BamH* I / *Sal* I. The recombinant expressed plasmid was trans-

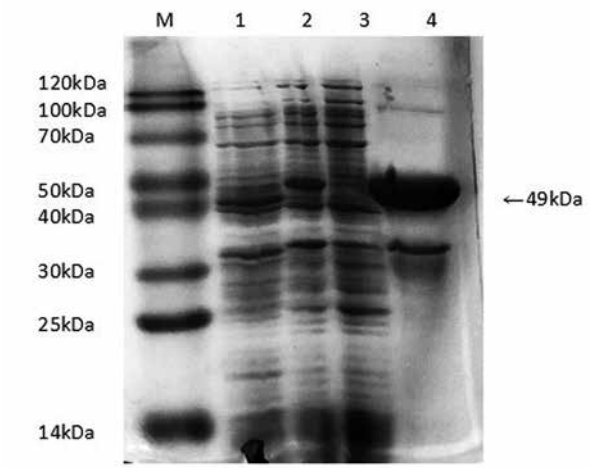

Fig. 2. The SDS-PAGE of recombinant protein. Note: M, Premixed Protein Marker; 1, pET-32a vector; 2, σc-pET-32a without induction; 3, pellet of bacteria σc-pET-32a lysate; 4, pellet of bacteria σc-pET-32a lysate after purification.

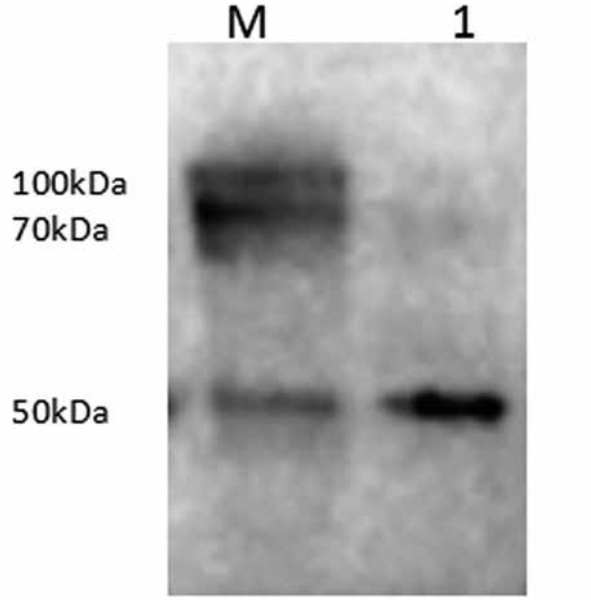

Fig. 3. Western-blot analysis of recombinant protein. Note: M, Blue Plus II Protein Marker (14-120kDa); 1, purified σc protein.

formed into an *E. coli* BL21 (DE3) competent cell and an insoluble 49kDa His-tagged fusion protein was successfully expressed. The fusion protein was purified using different concentrations of urea and analyzed in a 12% SDS-PAGE and empty vector and pre-induction cultures were used as control at the same time (Fig. 2). The western blot analysis showed that the σC protein possessed a high level of antigenicity, and could be used as a candidate antigen for detection of anti-N-ARV antibodies raised in the infected chicken (Fig. 3).

Determination of optimal protein expression conditions

SDS-PAGE electrophoresis analysis showed that 6 hours after induction the expression amount was the highest (Fig. 4). When the OD_{600nm} reached 0.6-0.8, IPTG was added the culture at a final concentration of 0 mmol/L, 0.2 mmol/L, 0.4 mmol/L, 0.6 mmol/L, 0.8 mmol/L respectively, at 37°C with shaken incubation for 6 hours. The results of electrophoresis showed that the expression level was the highest when the induction concentration was 0.6mmol/L (Fig. 5).

ELISA condition optimization

The OD value ratio between the positive serum and negative serum (P/N value of 6.16) is maximum when the final concentration of coating antigen and serum samples were diluted 1000 fold and 10 fold, respectively (Table 2). At the same time, the plates were coated and incubated at 37°C for 2 h and blocked using 5% skimmed milk powder the OD ratio of positive and the negative (P/N) was the greatest. As for the optimal concentration of the second antibody, HRP labeled Rabbit antichicken IgG was diluted 500 fold.

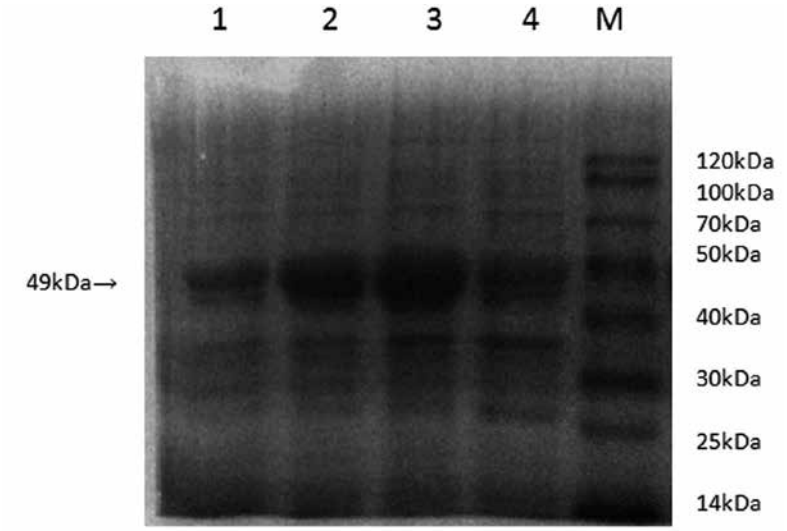

Fig. 4. Influence of expression of restructuring plasmid with differ ent induced time. Note: M, Premixed Protein Marker; 1, Recombinant bacteria σc-pET-32a lysates after 2h; 2, Recombinant bacteria σc-pET-32a lysates with 4h; 3, Recombinant bacteria σc-pET-32a lysates with 6h; 4, Recombinant bacteria σc-pET-32a lysates with 8h.

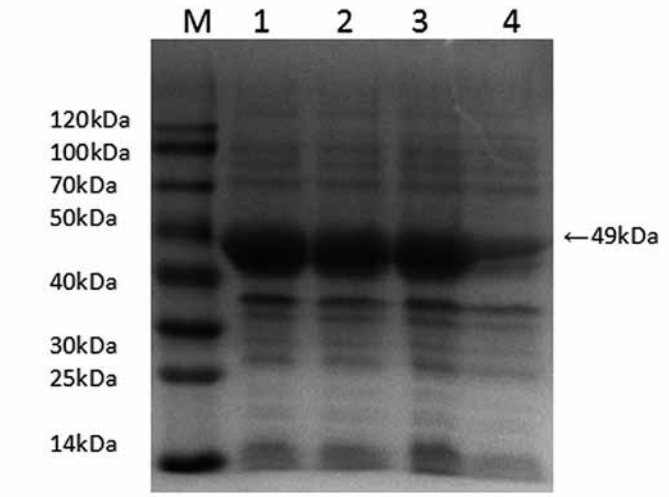

Fig. 5. Influence of expression of restructuring plasmid with different IPTG concentration. Note: M, Premixed Protein Marker (Low); 1, Recombinant bacteria σc-pET-32a lysates with 0.2mM IPTG; 2, Recombinant bacteria σc-pET-32a lysates with 0.4mM IPTG; 3, Recombinant bacteria σc-pET-32a lysates with 0.6mM IPTG; 4, Recombinant bacteria σc-pET-32a lysates with 0.8mM IPTG.

Cut-off value

The average value of OD_{450nm} was 0.0595 and the standard deviation is 0.004. The cut-off value = mean OD_{450nm}=X+3S=0.071. Thus, a serum sample was considered positive if its OD_{450nm}value ≥ 0.071.

Sensitivity of the ELISA assay

Ninety clinical serum samples were used to compare the sensitivity between the IDEXX Avian reovirus antibody detection kit and this method. The detection results of the commercial kit and this method showed that 41 and 80 samples were ARV positive, respectively; of all the 90 serum samples, 41 were judged to be positive and 8 negative with both methods. When the positive serum was diluted 100 fold, the cut off value is still greater than 0.071, which indicates that the results are still positive.

Specificity of the ELISA assay

The results showed that only the OD_{450nm} value of N-ARV positive serum exceeded the cut -off value, while others were negative. Furthermore, the negative control serum (NC) was also negative, which demonstrates that the developed ELISA assay has a great specificity for detection of N-ARV antibodies.

Repeatability of the ELISA assay

In the intra-assay test, four N-ARV positive sera were tested in a plate, and the coefficient of variation (CV) ranged from 5.74% to7.93%. In the inter-assay

Table 2. Best dilution proportion of σc protein and serum.

Serum dilution factor (P/N)	Protein dilution factor (P/N Ratio)					
	1: 500	1: 1000	1: 2000	1: 4000	1: 8000	1: 16000
1: 10	5.25	6.16	5.68	4.08	3.56	3.08
1: 20	5.48	5.60	4.17	4.05	3.06	2.24
1: 40	5.79	4.76	3.95	2.90	2.85	2.08
1: 80	5.02	4.61	4.83	4.09	3.31	2.33

Table 3. Detection of clinical samples from incidence farms.

Farm	Number positive/number tested	Positive rate (%)
1	80/90	88.9%
2	87/90	96.7%
3	83/90	92.2%
4	87/93	93.5%
total	337/363	92.8%

test, the coefficient of variation values varied from 4.85% to 7.62%. These results indicate that this method has great repeatability and excellent stability in detecting N-ARV.

Clinical Sample detection

The indirect ELISA assay established in this study was performed using 363 clinical samples of suspected cases of N-ARV. The positive rate of N-ARV detection was up to 92.8% (337/363) (Table 3).

Discussion

ARV was first isolated from a wild chicken by Fahey and Crawley in 1954 and is confirmed as a highly pathogenic poultry disease characterized as arthritis and tenosynovitis (Fahey and Crawley 1954). Since then, reovirus was widespread worldwide, with high rates of infection in flocks, some attaining 50%, which results in serious economic losses. Formerly, viral arthritis in chickens was generally controlled by vaccines in the serotype of S1133, which have proven effective in most parts of the world. Recently, these vaccines have conferred partial protection against the different antigen in ARV strains, and autogenous ARV vaccines are used in Europe and Israel (Ayalew et al. 2017) (Vasserman et al. 2004). Some research findings of sequencing studies showed significant genetic divergences between newly emerging ARVs and historic strains or vaccine strains (Mor et al. 2014). In addition, research indicates rapid accumulation of point mutations, which is a representative characteristic of RNA viruses, is common and antigenic shift because of the reassortment of genomic ion also occurs in ARV as in other segmented genome viruses, resulting in new strain generate and vaccine failure (Tang et al. 2016). Furthermore, the current epidemic strain of ARV differs greatly from the standard strains S1133 and 1733; perhaps this is one of the reasons why the domestic S1133+1733 viral arthritis inactivated vaccine has lost effective protection against flock and the high infectious ratio of N-ARV can have severe consequences.

σC protein is one of the important structural proteins on the surface of the N-ARV coat, which can induce the body to produce specific antibodies (Shien et al. 2000). Although the σC protein between the vaccine and the mutant strain has a certain degree of conservation, the highly variable regions exposed on the surface of the σC protein determined pathogenicity and antigenic escape. In this study, σC protein expression was induced and had good reactivity after western blotting identification; therefore, the recombinant protein prepared in this experiment can be used for coating proteins as an indirect ELISA method. The assay has no cross-reactivity with IB, IBD, H9N2-AIV, FAV positive serum, and has good repeatability, the intra and inter-assay variations, in the range of 4.85%~7.93%. 90 clinical serum samples were detected for comparison of sensitivity of two tests – commercial and developed ELISAs, while in 41 and 80 samples presence

of ARV antibodies was confirmed respectively. The results indicate the commercial ELISA kit was based on the sequences of historical ARV strains and has the risk of false negative results. The antigen used in the commercial kits is not able to detect anti-N-ARV antibodies, so its use in the current epidemiological situation will not be satisfactory and misleading for veterinary control. This also indicates that previous kits have a low detection rate in this novel reovirus and there is a great need to effectively detect the new disease. Based on the results appear above, we collected 363 serum samples from other infected poultry farms in all, the detection rate reach up to 92.8%.

Adult flocks often remain asymptomatic during reovirus infection, while young flocks often show obvious clinical symptoms such as leg disease, spasm, joint swelling, diseased chickens, and slow growth. Therefore, development of new vaccines tailored against the emerging pathogenic ARV variant strains is essential to minimize economic losses to the broiler chicken industry. Avian reoviruses are resistant to heat, proteolytic enzymes, various disinfectants and a wide pH spectrum, so it is challenging to maintain poultry farms free from ARV infection. Currently, early detection and use of suitable vaccines are still effective ways to control the disease. As far as we know, there are no commercial vaccines or antiviral drugs available to fight N-ARV infection; currently the most successful measures for control are mainly based on serological screening or molecular methods and subsequent quarantine. It is necessary to established the method to detected the serum antibodies. In general, using this method to detecting a large number of samples has high application value and can be widely applied to epidemiological investigation of antibodies, and also lays a foundation for developing an indirect ELISA kit for detecting N-ARV antibodies.

Conclusion

The developed indirect enzyme linked immunosorbent assay (ELISA) will be useful for large-scale serological survey and monitoring of antibody levels in N-ARV infection.

Acknowledgements

This work was supported by the National Key Research and Development Program (2018YFD0500106-3); Tai'shan Industry Leader (LJNY201610); and Funds from the Shandong "Double Top" Program.

References

Adair BM, Burns K, McKillop ER (**1987**) Serological studies with reoviruses in chickens, turkeys and ducks. Comp Pathol 97:495-501.

Ayalew LE, Gupta A, Fricke J, Ahmed KA, Popowich S, Lockerbie B (**2017**) Phenotypic, genotypic and antigenic characterization of emerging avian reoviruses isolated from clinical cases of arthritis in broilers in saskatchewan. Scientific Reports 7: 3565.

Benavente J, Martinezcostas J (**2007**) Avian reovirus: structure and biology. Virus Res 123:105–119.

Bodelón G, Labrada L, Martønez-Costas J (**2001**) The avian reovirus genome segment S1 is a functionally tricistronic gene that expresses One structural and two nonstructural proteins in infected cells. Virology 290:181-191.

Caterina, KM, Frasca S, Girshick T, Khan M (2004) Development of a multiplex pcr for detection of avian adenovirus, avian reovirus, infectious bursal disease virus, and chicken anemia virus. Molecular and Cellular Probes 18: 293-298.

Curtis PE, Al-Mufarrej SI, Jones RC, Morris J, Sutton PM (1992) Tenosynovitis in young pheasants associated with reovirus, staphylococci and environmental factors. Veterinary Record 131: 293.

Dandár E, Bálint Á, Kecskeméti S (**2013**) Detection and characterization of a divergent avian reovirus strain from a broiler chicken with central nervous system disease. Arch Virol. 158: 2583-2588.

Dutta SK , Pomeroy BS (**1967**) Isolation and characterization of an enterovirus from baby chicks having an enteric infection II. Physical and chemical characteristics and ultrastructure. Avian Dis 11: 9-15.

Fahey JE , Crawley JF (**1954**) Studies On Chronic Respiratory Disease Of Chickens II. Isolation of A Virus. Can J Comp Med Vet Sci 18: 13-21.

Guo K, Dormitorio TV, Ou SC Giambrone JJ (**2011**) Development of TaqMan real-time RT-PCR for detection of avian reoviruses. Virol Methods 177: 75-79.

Heggen-Peay CL, Qureshi MA, Edens FW (**2002**) Isolation of a reovirus from poult enteritis and mortality syndrome and its pathogenicity in turkey poults. Avian Dis 100: 102-105

Howell SH, Walker LL (**1972**) Synthesis of DNA in toluene-treated Chlamydomonas reinhardi (DNA replication-chloroplast DNA-cell cycle-electron microscopy). Proceedings of the National Academy of Sciences of the United States of America 69: 490-494.

Ide PR, Dewitt W (**1979**) Serological incidence of avian reovirus infection in broiler-breeders and progeny in Nova Scotia. Canadian Veterinary Journal La Revue Vétérinaire Canadienne 20: 348.

Jones RC, Kibenge F (**1984**) Reovirus-induced tenosynovitis in chickens: The effect of breed. Avian Pathol 13: 511-528.

Jones RC, Savage CE (**1987**) Effects of experimental immunosuppression on reovirus-induced tenosynovitis in light--hybrid chickens. Avian Pathol 14: 51-58.

Lin FQ, Qi-Lin HU, Chen SY (**2007**) Diagnosis of Muscovy Duck Reovirus Disease by Semi-Nested RT-PCR. Journal of South China Agricultural University 16: 61-68.

Liu HJ, Giambrone JJ, Nielsen BL (**1997**) Molecular characterization of avian reoviruses using nested PCR and nucleotide sequence analysis. J Virol Methods 65: 159-167.

Liu Y, Wan W, Gao D (**2016**) Genetic characterization of novel fowl aviadenovirus 4 isolates from outbreaks of hepatitis-hydropericardium syndrome in broiler chickens in China. Emerg Microbes Infect 5: 117.

Marks M , Marks JL (**2016**) Viral arthritis. Clinical Medicine 1: 16-19.

Mor SK, Sharafeldin TA, Porter RE (**2014**) Molecular characterization of L class genome segments of a newly isolated turkey arthritis reovirus. Infect Genet Evol 27: 193-201.

Mor SK, Sharafeldin TA, Porter RE (**2013**) Isolation and characterization of a turkey arthritis reovirus. Avian Dis 57: 97-103.

Noh JY, Lee DH, Lim TH, Lee J H, Day JM, Song CS (**2018**) Isolation and genomic characterization of a novel avian orthoreovirus strain in Korea, Arch Virol 163: 1-10.

Victor PT, Darko M, Tom I, Frank V, Faizal A (**2018**) Molecular characterization of emerging avian reovirus variants isolated from viral arthritis cases in western canada 2012-2017 based on partial sigma (σ)c gene. Virology 522: 138-146.

Palya V, Glávits R, Dobos-Kovács M, É Ivanics, Nagy, E, K Bányai (**2003**) Reovirus identified as cause of disease in young geese. Avian Pathol 32: 129-138.

Xie L, Xie Z, Wang S, Deng X, Xie Z (**2020**) Study of the activation of the pi3k/akt pathway by the motif of σa and σns proteins of avian reovirus. Innate Immunity 26: 4.

Rendón-Anaya M, Montero-Vargas JM, Saburido-Álvarez S, Vlasova A, Herrera-Estrella A (**2017**) Genomic history of the origin and domestication of common bean unveils its closest sister species. Genome Biology 18: 60.

Sharafeldin TA, Mor SK, Bekele AZ, Verma H, Noll SL, Goyal SM (**2015**) Experimentally induced lameness in turkeys inoculated with a newly emergent turkey reovirus. Veterinary Research 46: 11.

Shien JH, Yin HS , Lee LH (**2000**) An enzyme-linked immunosorbent assay for the detection of antibody to avian reovirus by using protein σ B as the coating antigen. Res Vet Sci. 65: 60

Wen L, Hwh B, Ming H, Long H, Hjlb C (**2004**) Avian reovirus σc protein induces apoptosis in cultured cells. Virology 321: 65-74.

Tang Y, Lu H (**2015**) Genomic characterization of a broiler reovirus field strain detected in pennsylvania. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases 31: 177-182.

Tang Y, Lin L, Sebastian A, Lu H (**2016**) Detection and characterization of two co-infection variant strains of avian orthoreovirus (ARV) in young layer chickens using next-generation sequencing (NGS). Sci Rep 6: 24519.

Vasserman Y, Eliahoo D, Hemsani E (**2004**) The influence of reovirus sigma C protein diversity on vaccination efficiency. Avian Dis 47: 28-30

Vindevogel H, Meulemans G, Pastoret PP (**1982**) Reovirus infection in the pigeon. Ann Rech Vet Rinaires Ann Vet Res 13: 149.

Yun T, Chen H, Yu B (**2015**) Development and application of an indirect ELISA for the detection of antibodies to novel duck reovirus. Journal of Virological Methods 220: 55-59.