Preparation of a novel monoclonal antibody against Avian leukosis virus subgroup J Gp85 protein and identification of its epitope

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ABSTRACT Avian leukosis virus subgroup J (**ALV-J**) is an avian oncogenic retrovirus that has caused huge economic losses in the poultry industry due to its great pathogenicity and transmission ability. However, the continuous emergence of new strains would bring challenges to diagnosis and control of ALV-J. .This study focuses on preparing the monoclonal antibody (**MAb**) against ALV-J Gp85 and identifying its epitope. The truncated ALV-J *gp85* gene fragment was amplified and then cloned into expression vectors. Purified GST-Gp85 was used to immune mice and His-Gp85 was used to screen MAb. Finally, a hybridoma cell line named J16 that produced specific MAb against the ALV-J. Immunofluorescence assay showed that MAb J16 specifically recognized ALV-J rather than ALV-A or ALV-K infected DF-1 cells. To identify the epitope recognized by MAb J16, fourteen partially overlapping ALV-J Gp85 fragments were prepared and tested by Western blot. The results indicated that peptide 150-LIRPYVNQ-157 was the minimal epitope of ALV-J Gp85 recognized by MAb J16. Alignment analysis of Gp85 from different ALV subgroups showed that the epitope keep high conservation among 36 ALV-J strains, but significant different from that of ALV subgroup A, B, C, D, E and K. Overall, we prepared a MAb specific against ALV-J and identified peptide 150-LIRPYVNQ-157 as a novel specific epitope of ALV-J Gp85, which may assist in laying the foundation for specific ALV-J detection methods.

Key words: ALV-J. Gp85 protein. monoclonal antibody. epitope

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INTRODUCTION

Avian leukosis virus subgroup J (**ALV-J**) is an oncogenic exogenous retrovirus first isolated from white Meat-type chickens in the UK in 1988 (Payne et al., 1992). According to the characteristics of host range, viral envelope protein, and cross-neutralization patterns, ALVs can be classified as endogenous or exogenous viruses. Exogenous ALVs can be classified into subgroups (A, B, C, D, J and K) in the chicken, which can cause different pathological lesions in chickens (Liang et al., 2019; Chang et al., 2020). Compared with other subgroups of ALVs, ALV-J mainly causes hematopoietic malignancy with myeloid leukemia and hemangioma in the chicken (Cheng et al., 2010). It is known that ALV-J has been bringing enormous economic loss in poultry industries of the developing countries (Payne et al., 1993). Unfortunately, to date, there are still no vaccines or drugs which can effectively protect against ALV-J infection (Feng et al., 2019). In addition, detection of exogenous ALVs is becoming increasingly difficult due to their high variability and the continuous emergence of new subgroups or strains (Yan et al., 2019). Thus, it has become a major challenge in the poultry production to control and eradicate ALV-J (Payne and Nair, 2012; Dai et al., 2020).

The genome of ALV-J is comprised of 5′-*LTR-UTR-gag-pol-env-UTR-LTR*-3′. The *gag, pol* and *env* genes encode the group-specific antigen, integrase and reverse transcriptase, and envelope glycoproteins **(Gp85** and **Gp37***)*, respectively (Li et al., 2015). The Gp85 of ALV-J located on the viral surface mediates viral binding to cellular receptor on host cell membranes (Venugopal et al., 1998) and can determine the specificity of different subgroups and the host range. Moreover, the sequence of ALV-J *gp85* has a low homology rate with that of other exogenous subgroups (Chang et al., 2020),thereby subgroups of ALV can be distinguished according to the sequence and antigenicity of *gp85*.

Monoclonal antibody (**MAb**) has been widely developed and effectively applied in the detection of

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pathogenic microbes (Garcia-Lunar et al., 2019; Chaudhari et al., 2020). MAbs applied against ALV-J have been successfully prepared (Qin et al., 2001; Sun et al., 2012; Li et al., 2015; Chang et al., 2020) and used to establish some rapid and specific methods for the detection of ALV-J (Liu et al., 2018). Although previous research reported that MAbs against Gp85 of ALV-J that could be used for identifying the antigen, there is still no commercial testing kits for ALV-J appeared in the clinic. Furthermore, the *env* gene of ALV-J is highly variable (Bai et al., 1995; Payne and Nair, 2012), which brings huge challenges to the prevention and control of ALV-J. Up to now, eradication of infected chickens is the most effective way to control ALV-J infection (Sun et al., 2019). Therefore, it is urgent to develop convenient and efficient detection methods of ALV-J.

In this study, we successfully prepared a novel monoclonal antibody against Gp85 of ALV-J using hybridoma technology. Moreover, its epitope was identified by Western blot analysis of a series of overlapping fragments, and the homology of the amino acids of the epitope was compared with other subgroups of ALV. The novel specific MAb could serve as a useful reagent for approaches of ALV-J detection and diagnosis.

MATERIALS AND METHODS

Viruses, Cells, Animals, Plasmids and Main Reagents

The ALV-A (HB2015012), ALV-J (HB2015029), ALV-J(HB2010001) and ALV-K (HB2015032) strains were isolated and identified by our laboratory. In addition, DF-1 cell/ chicken embryo fibroblast (P3, GNO30, Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) and SP2/0 myeloma cells (TCM18, Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) were stored in our laboratory. BALB/c mice were purchased from Hubei Disease Control Center, Wuhan, China. *E. coli* strains DH5α and BL21, prokaryotic expression vectors pGEX-6P-1 and pET-28a were stored by our laboratory. ALV P27 Antigen Detection Kits (IDEXX, Yuangheng, Beijing, China), RPMI-1640 medium (SH30809.01; GE Life Sciences, Pittsburgh, PA) and Dulbecco's modified Eagle medium (C11995500, Gibco, Grand Island, NY) were offered by our laboratory.

Expression and Purification of the Recombinant ALV-J Gp85

According to the published sequence of the ALV-J *gp85* gene (GenBank: HB2015029), the specific primers (F, 5′- CCGGAATTCCCTTGGGACCCCCAAGAATTG-3′, containing *Eco*R I restriction endonuclease site; R, 5′- CCGCTCGAGCTTAGCAGTCCCGTTATTCCAC -3′, containing *Xho* I restriction endonuclease site.) were designed. The size of amplified target fragment was 507 bp. Next, the purified PCR product was cloned into prokaryotic expression vectors pGEX-6P-1 and pET-28a by double enzyme digestion to obtain recombinant clone vector pGEX-6P-1- ALV-J-*gp85* and pET-28a-ALV-J-*gp85*. These 2 recombinant plasmids were confirmed through DNA sequencing and then expressed in BL21 cells, finally the products were harvested and purified.

Immunization of Mice

The 6-week-old female BALB/c mice were inoculated subcutaneously with 100 μg of purified recombinant GST-ALV-J-Gp85 antigen emulsified with the same amount of complete Freund's adjuvant (F5881, Sigma, Steinheim, Germany). Two booster immunizations, which contained the purified GST-Gp85 and an equal amount of incomplete Freund's adjuvant (F5506, Sigma), were given at 2-wk intervals. Two weeks after the second booster immunization, the mice with higher antibody titers were boosted with 50 μg of purified GST-Gp85 antigen and were used to prepare the antibody-secreting splenic lymphocytes three days later.

Preparation of the Monoclonal Antibody

According to the published method (Zhang et al., 2019a), the splenic lymphocytes from the inoculated mice were fused with SP2/0 myeloma cells at a ratio of 10:1 using polyethylene glycol (PEG) (P7306, Sigma). The fused cells were first cultured in a HAT medium for 7 days and then cultured in an HT medium. The supernatants of survival hybridoma cells were detected using an Indirect ELISA with the purified His-GP85 applied as a coating antigen. The positive serum from the inoculated mice and the supernatant of SP2/0 cells were used as positive and negative controls, respectively. The hybridoma cells secreting MAb were subcloned three times by limiting the dilution. The ascetic fluid of the MAb were then prepared, and 8-week-old female BALB/c mice were inoculated with positive hybridoma cells. The titers of ascetic fluid were measured using an indirect ELISA. The subclass of the MAb were identified using a commercial monoclonal subtype identification kit (KMIA-2; Proteintech, Rosemont, IL) according to the manufacturer's protocol.

Enzyme-linked Immunosorbent Assay (ELISA)

Polystyrene microtiter plates (96-well) were coated overnight at 4℃ with 2 μg purified recombinant protein His-Gp85 diluted in carbonate-bicarbonate buffer (pH 9.6). Then the plates were washed 3 times using phosphate buffered solution (**PBS**), and the plates were blocked with 5% skim milk for 2 h. After washing 3 times, diluted positive mouse serum (1:1000), negative mouse serum (1:1000) and undiluted cell supernatant were added to the antigen-coated wells at 37℃ for 2 h. After washing five times again, the horseradish peroxidase (**HRP**)-conjugated goat anti-mouse IgG (D110087,

Sangon Biotech, Shanghai, China) was added to the plates at a dilution of 1:5000 at 37℃ for 1 h. Next, the plates were washed for5 times with PBS, 100 μL 3,3′,5,5′, -tetramethylbenzidine (**TMB**) solution (PA107, Tiangen, Beijing, China) was added. After 15 min of incubation at room temperature in the dark, 50 μL 2M H_2SO_4 was added to stop the reaction. Finally, the optical density (**OD**) was measured at 450 nm using an automated reader (BIO-RAD 680, Hercules, CA).

Indirect Immunofluorescence Assay (IFA)

The ALV-A(HB2015012), ALV-J(HB2015029), ALV-J (HB2010001) and ALV-K (HB2015032) strains were inoculated into DF-1 cells cultured in 24-well plates, and uninfected cells served as a negative control . At 5 days post inoculation, the supernatant of cells was detected using an ALV P27 Antigen Detection Kit. DF-1 cells with a positive supernatant were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min. Next, the fixed cells were washed with PBS three times and treated with 0.5% Triton X-100 for 15 min. After washing with PBS three times, cells were blocked with 5% skim milk powder in PBS at 25°C in the dark for 1 h, followed by washing with PBS, and MAb J16 from the ascetic of the mice diluted at a ratio of 1:200 was added to cells for incubation at 25°C for 45 min. Subsequently, after washing with PBS three times, the cells were incubated with a fluorescein isothiocyanate (**FITC**)-conjugated goat-anti-mouse antibody (D110090, Sangon Biotech) at a dilution of 1:150 at 25°C for 30 min. Finally, the cells were observed under a fluorescence microscope after 3 washes with PBS.

Analysis of the MAb's Epitope

To locate the epitope on the ALV-J Gp85 antigen, a series of overlapping truncated ALV-J *gp85* gene segments (Figure 4 C) were designed and amplified by PCR in this study, and their primers are listed in Table 1. These products of PCR were cloned into prokaryotic expression vector pET-28a and expressed in *E. coli* strain BL21 as fusion proteins containing His-tag. The expression of the recombinant protein was induced by 1 mM IPTG at 37 ℃ for 4 h. After purification, the abilities of these proteins to react with the prepared MAb were determined using Western blot analysis.

To further verify the accuracy of the epitope, three epitope peptides (primers are listed in Table 1) with GST tag in Table 2 were expressed, and their reactions against the prepared MAb were analyzed using Western blot.

Western Blot

Western blot was performed to test the reactivities of the recombinant overlapping proteins with monoclonal antibody. The expressed proteins mixed with a loading

Table 1. Primers for *gp85* gene amplification and epitope identification.

Primers	Sequences
FL-F	5′-CCGGAATTCCCTTGGGACCCCCAAGAATTG-3′
FL-R	5′-CCGCTCGAGCTTAGCAGTCCCGTTATTCCAC-3′
F1-F	5′-GGACAGCAAATGGGTCGCGAACCTTGG-GACCCCCAAGAAT-3′
F1-R	5′-TGTCGACGGAGCTCGAATTCGCTTGGTTGACA-TAGGGCCTTATAAG-3′
F2-F	5′-GGACAGCAAATGGGTCGCGAACGTGACTTCA-TAACAAAATGGA-3′
F2-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F3-F	5′-GGACAGCAAATGGGTCGCGAAAGATATTGTG-GATTCACCAGC-3′
F3-R	5′-TGTCGACGGAGCTCGAATTCGGCTTAG-CAGTCCCATTATTCCA-3′
F4-F	5′-GGACAGCAAATGGGTCGCGAACCTTGG-GACCCCCAAGAAT-3′
F4-R	5′-TCGACGGAGCTCGAATTCGCATAGGGCCTTA-TAAGATGGTCATCA-3′
F5-F	5′-GACAGCAAATGGGTCGCGAAATAACAAAATG-GAAAGGTGATGA-3′
F5-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F6-F	5′-GACAGCAAATGGGTCGCGAATGGAAAGGTGAT-GACCATCT-3′
F6-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F7-F	5′-GACAGCAAATGGGTCGCGAAGATGACCATCT-TATAAGGCCCT-3′
F7-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F8-F	5′-GGACAGCAAATGGGTCGCGAACCTTGG-GACCCCCAAGAAT-3′
F8-R	5′-TCGACGGAGCTCGAATTCGCGACATAGGGCCT-TATAAGATGGTC-3
F9-F	5′-GACAGCAAATGGGTCGCGAAGACCATCTTA-TAAGGCCCTATG-3′
F9-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F10-F	5′-GGACAGCAAATGGGTCGCGAACCTTGG-GACCCCCAAGAAT-3′
F10-R	5′-TCGACGGAGCTCGAATTCGCGTTGACA-TAGGGCCTTATAAGATG-3′′
F11-F	5′-GACAGCAAATGGGTCGCGAACATCTTA-TAAGGCCCTATGTCA-3′
F11-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F12-F	5′-GACAGCAAATGGGTCGCGAACTTATAAGGCCC-TATGTCAACC-3′
F12-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F13-F	5′-GACAGCAAATGGGTCGCGAAATAAGGCCC-TATGTCAACCAA-3′
F13-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
F14-F	5′-GACAGCAAATGGGTCGCGAAAGGCCCTATGT-CAACCAATC-3′
F14-R	5′-TGTCGACGGAGCTCGAATTCGTAAACCCA-TATGCATAATAATTCC-3′
E-F	5′-GCATCGTGACCTTATAAGGCCCTATGTCAAC-CAATGACTGACGATCTG CCTCGCGCGT-3′
E-R	5′-TCGTCAGTCATTGGTTGACATAGGGCCTTA-TAAGGTCACGATGCGGCCGCTCGAGTC-3′
E-L-F	5′-GCATCGTGACATAAGGCCCTATGTCAACCAAT-GACTGACGATCTGCCT CGCGCGT-3′
E-L-R	5′-TCGTCAGTCATTGGTTGACATAGGGCCT-TATGTCACGATGCGGCCGCTCGAGTC-3′
E-Q-F	5′-GCATCGTGACCTTATAAGGCCCTATGTCAACT-GACTGACGATCTGCCT CGCGCGT-3′
E-Q-R	5′-TCGTCAGTCAGTTGACATAGGGCCTTA-TAAGGTCACGATGCGGCCGCTCGAGTC-3′

Table 2. Amino acid sequence and reaction with J16 of the GST fusions.

Peptide	Amino acid sequence	Reaction*
E	LIRPYVNQ	+
E - L	IRPYVNQ	-
E - Q	LIRPYVN	-

+, positive reaction; -, no reaction; *Reaction between GST fusions and MAb J16.

buffer were boiled for 10 min and subjected to 12% SDS-PAGE and then transferred to 0.22 μm nitrocellulose membranes. The membranes were blocked for 2 h with 5% skim milk diluted in Tris Buffered saline Tween (**TBST**) at room temperature and washed three times with TBST. The membranes were then incubated with the monoclonal antibody diluted in 5% skim milk for 2h at room temperature. After washing three times with TBST, the membranes were treated for 1h at room temperature with diluted goat anti-mouse IgG-HRP as the secondary antibody. Following three washes with TBST, the membranes were visualized with the Ultra ECL substrate chemiluminescence detection kit (PP24, Adlai, Hangzhou, China).

Alignment Analysis

To analyze whether the identified epitope was conserved among different ALV strains, the Gp85 amino acid sequence of ALV-A, ALV-B, ALV-C, ALV-D, ALV-E, ALV-J and ALV-K strains were compared using BioEdit7.0.9.0 software (https://bioedit.software.informer.com). To predict secondary structures and biological characteristics of the epitope, a partial ALV-J Gp85 amino acid sequence containing the epitope was analyzed with Protean program of Lasergene (version 7.10). Reference sequences used in this study were downloaded from the GenBank (https://www.ncbi.nlm.nih.gov/) and listed in Table 3.

RESULTS

Expression and Purification of the Recombinant ALV-J Gp85

The ALV-J *gp85* gene with a size of 507 bp was amplified by PCR (Figure 1). The target gene was cloned into prokaryotic expression vector pET-28a and pGEX-6P-1. The results of the restriction endonuclease digestion (Figure 1) and sequencing confirmed that these two recombinant plasmids containing the target gene were successfully constructed. The recombinant ALV-J *gp85* plasmids were successfully expressed after the induction with 1mM IPTG in *E. coli* strain BL21, and the expressed products were purified and confirmed through SDS-PAGE and WB (Figure 2).

The MAb Recognizes ALV-J Strain, but not ALV-A/K Strains in IFA

To obtain the monoclonal antibody against ALV-J, after HAT/HT selection, the hybridoma cells were subcloned three times by limiting dilution and production of Gp85-reactive antibodies were screened by

Table 3. Reference strains used in this study.

Subgroup	Strain	Accession No.	Subgroup	Strain	Accession No.
A	HB2015012	KY612442	J	JS13LHAJ2	KR049172
A	DPRE32	KM434201	J	LYJ195	KF218957
A	GD08	HM775328	J	MRL905	JF951728
A	GX14DJ3-18	MH213216	J	NG-VX32	MH669346
A	Jilin3	MH186087	J	NGA-VX23	MK104146
B	Anhui23	MH186088	J	PDRC-59831	KP284572
B	Guangdong22	MH186089	J	PL09DP5-2	JN378892
B	Heibei17	MH186090	J	QL1	KF218958
B	Heilongjiang20	MH186092	J	SD09TA04	JN378893
B	Henan21	MH186093	J	SD13QJ01	KJ922510
C	RSV-Prague	J02342	J	SG-1	KF218958
D	RSV-S-R-D	D10652	J	Sree-380	MH023420
E	GDFX0603	KP686144	J	SVR807	HM776937
E	GDFX0602	KP686143	J	SZ-08	HM775329
E	JS14CZ02	KY490696	J	WA1112	KJ631315
E	DT190901	MT319756	J	WC512	KJ631316
E	SDAU121E	KJ018762	J	WF13	KJ631314
J	2921/00	HM775330	J	WFMC1	HM017827
J	ADOL-7501	AY027920	J	WGD13	KJ631312
J	AN-1	KY379033	J	WJ612	KJ631317
J	BR2	KF201289	J	WL12	KJ631318
J	DJ146	KF218959	J	WLY13	KJ631311
J	FJ201308	KM655822	J	WSC112	KJ631322
J	GD1403J	KP317566	J	XG-09	HM775332
J	GL09DP02	JN378887	K	GDFX0601	KP686142
J	GM0209-6	MH379647	K	HB2015032	KY581580
J	GX14DJ45	KY983565	K	ALV-K-env-J	MK638922
J	HRPS103	Z46390	K	JS13LY19	MG770235
J	HuB09XZ01	JN378890	K	TW-3593	HM582658

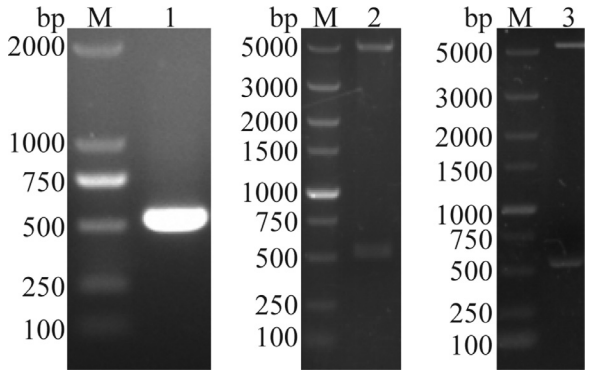

Figure 1. Identification of the amplified products of ALV-J *gp85* gene and construction of prokaryotic expression plasmids containing ALV-J *gp85* gene. Lane M, DNA marker (bp); Lane 1, *gp85* gene of ALV-J; Lane 2, the products of pGEX-6P-1-ALV-J-*gp85* after digestion using double *E*coRI and *X*hoI enzymes; Lane 3, the products of pET28a-ALV-J-*gp85* after digestion using double *E*coRI and *X*hoI enzymes.

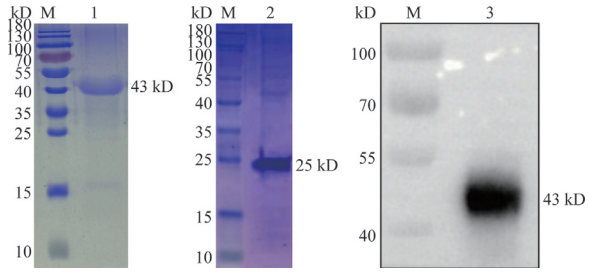

Figure 2. Identification of the expressed and purified products of ALV-J Gp85 by SDS-PAGE and Western blot.Lane M, protein marker (KDa); lane 1, purified recombinant GST-J-Gp85 protein, 43 KDa; Lane 2, purified recombinant His-J-Gp85 protein, 25 KDa; Lane 3, Western blot mediated by anti-GST MAb, 43 KDa.

indirect ELISA and IFA. Finally, one hybridoma cell line secreting monoclonal antibody was obtained and named J16. The subclass of the secreted monoclonal antibody was identified as IgG1. The MAb from the ascites of the mice inoculated with hybridoma cells produced a Gp85-reactive MAb, and its titers were detected as 1:64,000.

To analyze the specificity of the MAb, 3 different subgroup strains including ALV-A(HB2015012), ALV-J (HB2015029), ALV-J(HB2010001) and ALV-K (HB2018032) strains were used to infect DF-1 cells and DF-1 cells with a positive supernatant were detected using IFA mediated with the prepared MAb. The results indicated that green fluorescence was observed in DF-1 cells infected with ALV-J strain (Figure 3A and 3B), but not in cells with ALV-A/K strains (Figure 3C and 3D), suggesting that the MAb J16 could recognize ALV-J strain in IFA, but not ALV-A/K strains.

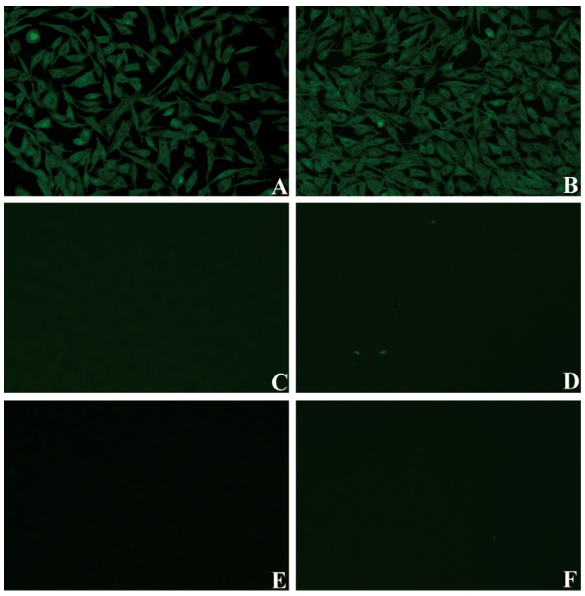

Figure 3. Identification of the specificity of the prepared MAb against different subgroups of ALV strains using IFA in DF-1 cells.(A) ALV-J-HB2015029 strain; (B) ALV-J-HB2010001 strain; (C) ALV-A-HB2015012 strain; (D) ALV-K-HB2015032 strain; (E) negative control with negative mouse serum; (F) negative control without virus.

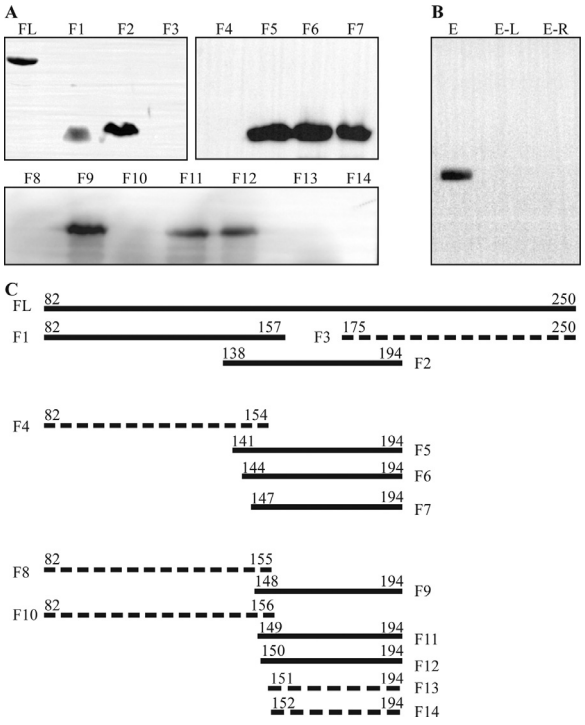

Figure 4. Identification of the epitope recognized by the MAb using Western blot. (A) the results of 14 Gp85 protein segments in Western blot mediated with the MAb. (B) the WB results of three GST fusions contained different amino acids of the peptide. (C) ALV-J Gp85 protein was divided into 14 overlapping fragments and expressed separately, which were identified by Western blot.

Identification of the Epitope in the Gp85 Protein Recognized by the MAb

To further determine the epitope recognized by MAb J16, fourteen overlapping truncated ALV-J Gp85 segments (Figure 4 C) with His-tag were expressed for subsequent Western blot analysis, and the His-Gp85 protein was used as a positive control. The results in Figure 4 A show that the F1 and F2 segment reacted with MAb whereas the F3 segment did not. These

results suggest that MAb's epitope lie in the overlapping region of the F1 and F2 segments. Next, four overlapping truncated protein segments from this overlapping between F1 and F2 protein were expressed for further use in a Western blot analysis. The results in Figure 4A show that F5, F6, and F7 protein segments reacted with MAb, whereas F4 did not. Third, seven truncated protein segments from F4 and F7 proteins were expressed for a Western blot analysis. The results of the Western blot analysis in Figure 4A show that only F9, F11, and F12 segments reacted with MAb. These results indicate that the minimal epitope of MAb exists from 150aa to 157aa (150-LIRPYVNQ-157) on the Gp85 protein.

In addition, three GST fusions contained different amino acids of epitope in Table 2 were expressed, and the WB results in Figure 4 B indicate that the peptide containing the complete 150-LIRPYVNQ-157 could react with prepared MAb J16. These results further confirm that 150-LIRPYVNQ-157 is an optimum liner epitope of the MAb.

Alignment Analysis

To better understand secondary structures and homology of the epitope recognized by MAb, its secondary structural domains in the ALV-J Gp85 protein were predicted using Biological Information Analysis Software. The results in Figure 5A show that the epitope contains one α-helix and one β-folder and has a high hydrophilicity and antigenic index. To analyze the variation in the epitope of MAb in different ALV strains, alignment analysis was performed to evaluate the conservation of the identified epitope among 58 representative ALV strains, including ALV-A, ALV-B, ALV-C, ALV-D, ALV-E, ALV-J, and ALV-K strains, extracted from GenBank. The result in Figure 5B showed that the epitope is completely within all 36 representative ALV-J strains. However, the epitope was not conserved among ALV-A, ALV-B, ALV-C, ALV-D, ALV-E, and ALV-K strains. These data suggest that the epitope differs significantly from four of the different ALV subgroups but has a high homology in the ALV-J strains.

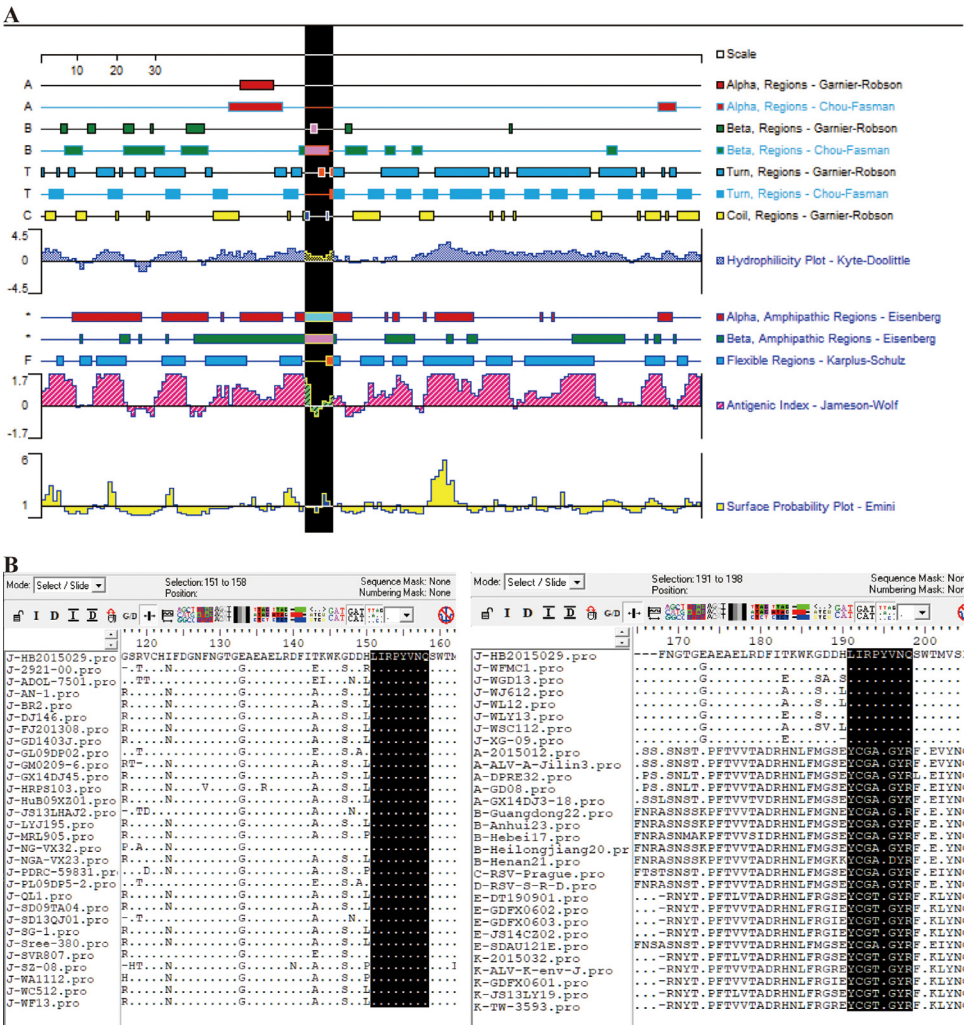

Figure 5. Prediction on secondary structures and homology of the epitope recognized by MAb.(A) the predicted secondary structures and biological characteristics of epitope. (B) amino acid sequence alignment of the epitope of MAb in different subgroups of ALV strains. 36 ALV-J strains, 5 ALV-A strains, 5 ALV-B strains, 1 ALV-C strain, 1 ALV-D strain, 5 ALV-E strains and 5 ALV-K strains were used for comparison. The Gp85 amino acid sequence of HB2015029 was shown in top. The same amino acid is represented by ".", and "-" indicates the amino acid deletion at this position.

DISCUSSION

Based on its pathogenicity and antibody neutralization characteristics, the avian leukosis virus (**ALV**) is divided into seven subgroups (ALV-A/B/C/D/E/J/K) in chickens (Payne et al., 1991; Dong et al., 2015). Since ALV-J was first isolated from White Meat-type chickens in the UK in 1988, it has been reported that ALV-J can infect both layers and indigenous chickens in China (Payne and Nair, 2012; Meng et al., 2018; Su et al., 2018; Wang et al., 2018). The *gp85* gene of ALV determines the properties of the antibody neutralization and becomes one of the main references to classify the ALV subgroups (Payne and Nair, 2012). Currently, there are some specific diagnosis methods for the ALV subgroups, such as RT-PCR, ELISA, and IFA, which were set up according to the ALV-J *gp85* gene or protein sequence in clinical detection (Rajabzadeh et al., 2010; Liu et al., 2018; Zhang et al., 2019b). In this study, two prokaryotic expression plasmids pET28a-ALV-J-*gp85*(His-tag) and pGEX-6P-1-ALV-J-*gp85*(GST-tag) were constructed containing the ALV-J *gp85* gene. The purified GST-Gp85 was used to immunize mice and the purified His-Gp85 was used to detect antigens to avoid false positives caused by tag protein.

In this study, we prepared a novel monoclonal antibody, J16, which specifically reacted with the ALV-J Gp85. Moreover, it was found that IFA mediated with the prepared MAb could detect the ALV-J strain, but not the ALV-A and ALV-K strains. Thus, the monoclonal antibody might be useful for the development of a specific diagnostic method used to detect ALV-J infection and for the analysis of the interaction between the antibody and antigen. Owing to the continuous emergence of new ALV subgroups or new ALV strains, it would be better to determine the specificity of the new MAb using more ALV strains in future study (Yan et al., 2019).

Identification of specific epitopes of MAbs has become a useful method applied to disease diagnosis (Van Regenmortel, 2014). The epitope of MAb was determined by a western blot analysis. As described in previous reports, the ALV-J Gp85 is main envelope protein and the most variable structural protein of the virus (Pandiri et al., 2010). Although one study has been performed to analyze the epitopes of the ALV-J envelope protein and one ALV-J Gp85 MAb has been reported for detection of ALV-J antigen by IFA (the lengths of the epitopes were 91 and 77 amino acids) (Qin et al., 2002), the epitopes have yet not been precisely defined. In this study, to analyze an epitope recognized by MAb J16, the ALV-J Gp85 was artificially divided into 14 different overlapping truncated segments. These segments were expressed in *E. coli* stain BL21 for Western blot analysis to test their reactivity with MAb J16. These results demonstrate that the epitope located in the peptide 150-LIRPYVNQ-157 (Figure 4). A previous study indicated that the majority of epitopes are less than 20 amino acid residues in lengths (Li et al., 2013). In this study, the identified epitope was 8 amino acids, which was consistent with the previous study.

Furthermore, the variation of the epitope in 49 representative ALV strains was analyzed. Alignment of amino acid sequences showed that the epitope was highly conserved among 36 ALV-J strains from different countries or regions in the world but not present in ALV-A, ALV-B, ALV-C, ALV-D, ALV-E and ALV-K strains (Figure 5B). These results indicate that epitope of MAb J16 is unique to the ALV-J Gp85 protein. There are 4 serotypes in chickens infected with ALV including viremia without antibody (V+A-), viremia with antibody (V+A+), no viremia with antibody (V-A+) and no viremia and no antibodies (V-A-) (Tsukamoto et al., 1991), which brings difficulty to monitor ALV infection by serologic testing. Although many methods have been explored to diagnose ALV infection, such as IFA, detection of the group-specific antigen p27 using antigen-capture ELISA, reverse transcription PCR and viral isolation (Rajabzadeh et al., 2010; Zhang et al., 2010), to the best of our knowledge, there is still no commercialized ELISA test kits to detect an ALV-J specific antigen. The ELISA method has the advantages of simplicity, accuracy, and suitability for high throughput detection of clinical samples (Lequin, 2005), thus the prepared MAb J16 and its epitope would be useful for the development of a diagnostic method using antigen-capture ELISA to detect ALV-J infection.

In conclusion, a new monoclonal antibody against ALV-J was successfully prepared, and the IFA mediated by MAb can detect ALV-J strains, but not the ALV-A/K strains. In addition, the epitope of MAb consists of eight amino acids in the region of the ALV-J Gp85 protein and has a complete homology within the 36 representative ALV-J strains. This MAb and the identified linear epitope might be deeply helpful for the understanding of the protein structure and function of ALV-J, as well as offer valuable tools for the development of new immunodiagnostic approaches for specific ALV-J detection methods.

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Ethics Statement: The animals used in this study were approved by the Institutional Animal Care and Use Committee of Yangtze University, Jingzhou, China. All animal experiments conformed to the principles established by this committee and international accepted standards for animal welfare.

DISCLOSURES

The authors declare no competing interests.

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