Journal of Animal and Veterinary Advances 13 (4): 236-240, 2014
ISSN: 1680-5593

Influence of the Series Multiepitopes of DNV-HN and IBDV-VP2 on an Immune Enhancement Mediated by Invariant Chain Segments as Carrier

Ran Xiong, Wei Dai, Weiyi Yu and Fangfang Chen
Key Laboratory of Zoonoses of Anhui Province, Anhui Agricultural University, 230036 Hefei, China

Abstract: To know the influence of the series multiepitopes from two chicken pathogens (NDV and IBDV) on their immunogenicity as well as on the boosting this immune response mediated by Ii segments (Cyt/TM) in present research, researchers linked NDV-HN (172-201) and IBDV-VP2 (197-209) in various series and further linked the both hybrids with Ii segments (Cyt/TM) and detected their immune effect in an animal experiment. The results showed that all the recombinant epitopes as single or multiepitopes with or without Ii carrier could induce mice to produce antibodies which specifically bound to VP2 (197-209) or HN (172-201) in Western blotting. The mice immunized by alone epitopes produced antibody titers only from 0.8×10^4-1.25×10^4 while the mice immunized by the single or multiepitopes with Ii segments (Cyt/TM) had antibody titers 3.1~3.5 fold than the titers by those without the Ii segments ($p<0.01$). It was interesting that there was no difference in the antibody titers of groups between the single epitope and series multiepitopes with the Ii segments ($p>0.05$). These suggest that Ii segments (Cyt/TM) as a potential immune carrier could improve the immune response to single epitope as well as multiepitopes.

Key words: NDV-HN, IBDV-VP2, multiepitope, Ii carrier, immune enhancement

INTRODUCTION

Invariant chain (Ii) is a non-polymorphic type II integral membrane protein (Jasanoff *et al*., 1998). It consists of the cytosolic, transmembrane and luminal domains (Ashman and Miller, 1999). Its cytosolic and transmembrane domains contain an endosome-targeting signal that is essential for Ii targeting to the endosomal compartment (Rudensky *et al*., 1994) also the both domains function for a membrane localization (Xu *et al*., 2008). Ii binds MHC class II molecule to form trimers or nonamers (Lindner, 2002) and plays an important role in exogenous antigen presenting (Roche and Cresswell, 2011). In this process, its Class II-Associated Invariant Chain Peptide (CLIP) occupies the peptide binding groove of MGC class II molecules to prevent endogenous peptides (Vogt *et al*., 1995). Recently, it was found that the Cytosolic and Transmembrane domains (Cyt/TM) could bind MHC class II molecules and thereby boost the immune responses (Chen *et al*., 2012).

Newcastle Disease Virus (NDV) and Infectious Bursal Disease Virus (IBDV) are two kinds of highly infectious pathogens for chicken. Hemagglutinin-Neuraminidase glycoprotein (HN) is an important structural protein of NDV (Romer-Oberdorfer *et al*., 2003). It has a decisive influence on the virulence and immune responses (Zhao *et al*., 2013). IBDV-VP2 is mainly structural protein as well as the host protective antigen (Schnitzler *et al*., 1993; Zanetti *et al*., 2012) which can induce birds to produce neutralizing antibodies (Heine *et al*., 1991; Wang *et al*., 2005).

A multiepitope fusion antigen which is from various pathogens has a potential immunogenicity and as a kind of multivalent vaccine against infectious diseases has been reported (Vakharia *et al*., 1994; Li *et al*., 2013; Hashish *et al*., 2013). In the previous research, researchers cloned three gene fragments of epitopes of NDV F protein and linked them with Ii segments (Cyt/TM) and found that this recombination protein had a favorable immunogenicity and Cyt/TM could improve immune response (Chen *et al*., 2012).

To know whether the multiepitopes in series from two chicken pathogens (NDV and IBDV) change their immunogenicity as well as effect of Ii (Cyt/TM) carrier in boosting an immune response, in present research researchers linked NDV-HN (172-201) (Huang *et al*., 2003) and IBDV-VP2 (197-209) (Wang *et al*., 2005; Pradhan *et al*., 2012) in various series, further linked with Ii (Cyt/TM) and detected their immune effect in an animal experiment.

Corresponding Author: Fangfang Chen, College of Animal Science, Anhui Agricultural University, Changjiang West Road 130 Hefei, 230036 Anhui Province, China

MATERIALS AND METHODS

Animals: Balb/c female mice (6 weeks old) were obtained from the Animal Centre of Anhui Medicine University and bred under specific pathogen-free conditions at the facility. All experimental procedures were performed following the Anhui Medicine University animal care guidelines under an approved protocol.

PCR primers: According to the gene sequences of mouse Ii chain, NDV-HN (172-201) and IBDV-VP2 (197-209) in GenBank, a series of primers were designed (Table 1).

Cloning and constructing the hybrids: First the gene fragments, Ii segments (cytosolic and transmembrane domains, Cyt/TM) (Chen *et al*., 2012) and epitopes, NDV-HN (172-201) and IBDV-VP2 (197-209) were cloned from the plasmids in Key Laboratory of Zoonoses of Anhui Province by PCR (Table 1). Secondly the gene hybrids, Cyt/TM/VP2 (197-209), Cyt/TM/HN (172-201), Cyt/TM/HN (172-201)/VP2 (197-209) and Cyt/TM/VP2 (197-209)/HN (172-201) (Fig. 1) were constructed with a series of primers (Table 1) by overlap extension PCR, respectively. Finally, all the constructed hybrids were inserted into prokaryotic expression vectors pET-32a for immunization antigen, respectively. Additionally the both fragments, HN (172-201) and VP2 (197-209) also were respectively inserted into pGEX-4T-1 for expression of the coating antigen used in the ELISA. All of the constructed hybrids were identified by sequencing.

Expression and purification of hybrid proteins: All the reconstructed plasmids were transfected into *E. coli* expression strain Rosetta and then were induced by 1 mmol L^{-1} IPTG for expression, respectively. The expressed recombinant proteins were purified with Native-PAGE and 0.25 mol L^{-1} KCl gel cutting and identification by SDS-PAGE, respectively. The products were dissolved in solution buffer and stored at -70°C.

Immunization of mice: About 32 mice were divided into eight groups. Mice were anesthetized and injected intraperitoneally with 50 μg of each protein antigen, respectively. The animals received the protein doses at day 0 with complete Freund's adjuvant as a 1:1 (v/v) emulsion in 100 μL. The second immunization occurred at day 10 in incomplete Freund's adjuvant and the third immunization took place at day 17 in incomplete Freund's adjuvant. One control group of mice was injected as above with normal saline water. The antisera were prepared at day 24 from collected mouse blood, respectively and were stored at -20°C until used for estimation of the antibody titers.

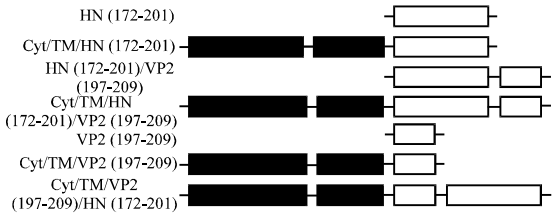

Fig. 1: A schematic diagram of recombinant segments

Table 1: Primers tested in this study

Name of genes	Sequences (5'-3')	For recombinant plasmids
VP2	F:5' cggaattctgtgacagcagtgacaggcc 3'	pET-32a-VP2
	R:5' ccgctcgagagttatggtgtagactctgg 3'	pGEX-4T-1-VP2
HN	F:5' cggaattc tgcactcggataccct 3'	pET-32a-HN
	R:5' ccgctcgaggtgcgagtgatccttg 3'	pGEX-4T-1-HN
HN/VP2	F:5' cggaattctgcactcggataccct 3'	pET-32a-HN/VP2
	R:5'gtcacagcttcctcctccgtgcgagtgatccttgca 3'	
	F':5' acggaggaggaagctgtgacagcagtgacaggccca 3'	
	R':5' ccgctcgagagttatggtgtagactctgg 3'	
Cyt/TM/HN	R:5' cgagtgcagcttcctcctcccttcatgcgaaggctct 3'	pET-32a-Cyt/TM/HN
	F':5' agggaggaggaagctgcactcggataccct 3'	
	R':5' ccgctcgaggtgcgagtgatccttg 3'	
	F:5' cggaattcatggatgaccaacgcgacct 3'	
Cyt/TM/VP2	R:5' ctgtcacagcttcctcctcccttcatgcgaaggctct 3'	pET-32a-Cyt/TM/VP2
	F':5' aagggaggaggaagctgtgacagcagtgacaggcc 3'	
	R':5' ccgctcgagagttatggtgtagactctgg 3'	
Cyt/TM/HN/VP2	F:5' cggaattcatggatgaccaacgcgacct 3'	pET-32a-Cyt/TM/HN/VP2
	R:5'cgagtgcagcttcctcctccagttatggtgtagactctgg 3'	
	F':5' ctggaggaggaagctgcactcggataccct 3'	
	R':5' ccgctcgagagttatggtgtagactctgg 3'	
Cyt/TM/VP2/HN	F.5' cggaattcatggatgaccaacgcgacct 3'	pET-32a-Cyt/TM/VP2/HN
	R:5' cgagtgcagcttcctcctccagttatggtgtagactctgg 3'	
	F':5' ctggaggaggaagctgcactcggataccct 3'	
	R':5' ccgctcgaggtgcgagtgatccttg 3'	

The design of all primers was based on the reported cDNA sequences: mouse Ii from GenBank ID: NM_010545, NDV-HN gene from GenBank ID: AY510092, IBDV-VP2 from GenBank ID: AY444873. The reconstructed vectors were used for expression as immunization antigens (pET-32a) or for expression as coating antigen in ELISA (pGEX-4T-1); HN fragment was NDV-HN (172-201), VP2 fragment was IBDV-VP2 (197-209)

Detection of antibody with ELISA and statistical analysis: The 96-well EIA/RIA plates (COSTAR, USA) were coated with 6 μg mL^{-1} GST-HN (172-201) and GST-VP2 (197-209) peptide, respectively and then blocked with 0.05% Tween-20 in PBS (PBST) containing 1% bovine serum albumin. The serum was added to the top row of each plate and serial 1:2 dilutions in PBST were then placed in subsequent rows. The plates were incubated for 45 min at 37°C and washed with PBST for 3 times (5 min $time^{-1}$). A goat anti-mouse IgG HRP conjugate (Zhongshan Golden Bridge Biotechnology, Beijing, China) diluted 1:5000 was used as a secondary antibody and incubated for 45 min followed by addition of OPD peroxidase (Sigma, USA) used as a substrate. After 15 min of incubation at room temperature, the absorbance was measured at 492 nm. Repeat the process above for three times and put all the data in the SPSS Software for statistical analysis.

Western blotting: The purified hybrid proteins were separated by SDS-PAGE and transferred onto the polyvinylidene fluoride membrane (Millipore, Schwalbach, Germany) then blocked for an hour with 10% bovine serum albumin. After that the antisera were added into the reaction plate, respectively and a goat anti-mouse IgG HRP-conjugate (Zhongshan Golden Bridge Biotechnology) diluted 1:5000 was used as a secondary antibody. Finally, the results were observed with DAB color.

RESULTS AND DISCUSSION

Identification for the recombinant plasmids containing Ii segment/epitope in series: All the single epitope or recombinant genes were first identified by agarose electrophoresis. Figure 2 showed the results of the cloned DNA fragments and the recombinant plasmids after a restriction enzyme digestion: VP2 (197-209), 39 bp; HN (172-201), 90 bp; HN (172-201)/VP2 (197-209), 141 bp; Cyt/TM/VP2 (197-209), 288 bp; Cyt/TM/HN (172-201), 339 bp; Cyt/TM/HN (172-201)/VP2 (197-209), 390 bp and Cyt/TM/VP2 (197-209)/HN (172-201), 390 bp, respectively. The results of DNA sequencing in parallel showed that all the expected hybrids were gotten.

Identification for the expressed and purified fusion proteins: After all the target genes were respectively induced to express fusion proteins, the latter was then purified. The results in Fig. 3 exhibited their bands in SDS-PAGE: His-VP2 (197-209), 21.4 kDa; His-HN (172-201), 3.5 kDa; His-HN (172-201)/VP2 (197-209), 25.2 kDa; His-Cyt/TM/VP2 (197-209), 30.6 kDa; His-Cyt/TM/HN (172-201), 32.4 kDa; His-Cyt/TM/HN (172-201)/VP2 (197-209); His-Cyt/TM/VP2 (197-209)/HN (172-201), 34.6 kDa; GST-VP2 (197-209), 27.4 kDa; GST-HN (172-201), 29.3 kDa in which the tagging molecule His was 20 kDa, GST was 26 kDa and the epitope VP2 (197-209) was 1.4 kDa, HN (172-201) was 3.3 kDa, Cyt/TM was 9.1 kDa, respectively. In addition, the fusion proteins with his were used as antigen for immunization.

Various series of multiepitopes in the hybrids had the same immunogenicity: The process in which the multiepitopes were linked in series could potentially results in changing structure and losing or lowering the antigenicity due to interaction of amino acids. To know whether the epitopes, VP2 (197-209) and HN (172-201) in the fusion proteins change their immunogenicity after linking tagging protein His and Ii-segments, the Western blotting were carried out with the antisera from the immunized mice. As indicated in Fig. 4, the both epitopes as single or hybrids could induce mice to produce specific antibodies which could bind VP2 (197-209) or HN

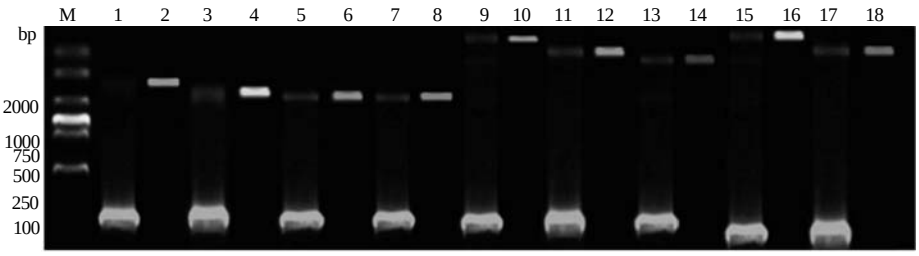

Fig. 2: Products of recombinant genes and plasmids digested with EcoR I and Xhol; M: DNA Marker; the products digested with EcoR I and Xhol; 1: pET-32a-Cyt/TM/VP2 (197-209); 3: pET-32a-Cyt/TM/HN (172-201); 5: pET-32a-Cyt/TM/HN (172-201)/VP2 (197-209); 7: pET-32a-Cyt/TM/VP2 (197-209)/HN (172-201); 9: pET-32a-VP2 (197-209); 11: pET-32a-HN (172-201); 13: pET-32a-HN (172-201)/VP2 (197-209); 15: pGEX-4T-1-VP2 (197-209); 17: pGEX-4T-1-HN (172-201). The PCR products; 2: Cyt/TM/VP2 (197-209); 4: Cyt/TM/HN (172-201); 6: Cyt/TM/HN (172-201)/VP2 (197-209); 8: Cyt/TM/VP2 (197-209)/HN (172-201); 10: VP2 (197-209); 12: HN; 14: HN (172-201)/VP2 (197-209); 16: VP2 (197-209); 18: HN (172-201)

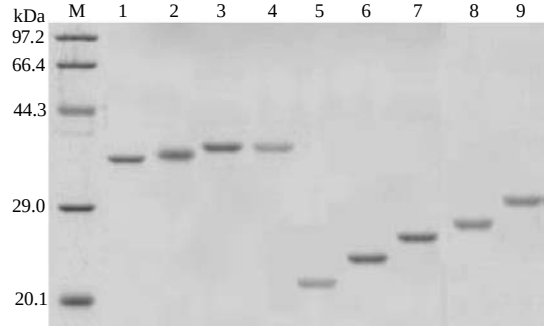

Fig. 3: Expressed and purified products. M: Protein marker; 1: His-Cyt/TM/VP2 (197-209); 2: His-Cyt/TM/HN (172-201); 3: His-Cyt/TM/HN (172-201)/VP2 (197-209); 4: His-Cyt/TM/VP2 (197-209)/HN(172-201); 5: His-VP2 (197-209); 6: His-HN (172-201); 7) His-HN (172-201)/VP2 (197-209); 8: GST-VP2 (197-209); 9: GST-HN (172-201)

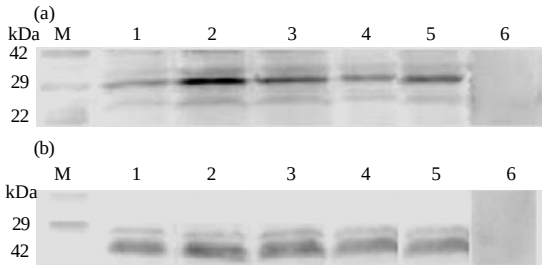

Fig. 4: Specific binding of antibodies to IBDV-VP2 (197-209) or NDV-HN(172-201); a) M: Protein marker; antisera from mice immunized with 1: His-HN (172-201); 2: His-HN (172-201)/VP2 (197-209); 3: His-Cyt/TM/HN (172-201); 4: His-Cyt/TM/HN (172-201)/VP2 (197-209); 5: His-Cyt/TM/VP2 (197-209)/HN (172-201); 6: Control serum (His); b) M. Protein marker; anttisera from mice immunized with 1: His-VP2 (197-209); 2: His-HN (172-201)/VP2 (197-209); 3: His-Cyt/TM/VP2 (197-209); 4: His-Cyt/TM/HN (172-201)/VP2 (197-209); 5: His-Cyt/TM/VP2 (197-209)/HN (172-201); 6: Control serum (His)

(172-201). These proved that the series multiepitopes, HN (172-201)/VP2 (197-209) or VP2 (197-209)/HN (172-201) could well remain the immunogenicity in the hybrids.

No influence of series multiepitopes on the immune enhancement mediated by Ii-segments: In specific immune process for vertebrate Ii plays an important role in antigen peptide presenting (Roche and Cresswell, 2011). Ii segments (Cyt/TM) can could facilitate process of antigen presenting and thereby improve animal specific association with MHC II molecular (Lindner, 2002; Xu *et al*., 2008). To evaluate an influence of the series multiepitopes on the effect of Ii-segment carrier, researchers detected the specific antibody titers of the immunized mice with ELISA. The purified GST-HN (172-201) or GST-VP2 (197-209) protein was used as a coating antigen. As indicated in Table 2, His-VP2 (197-209), His-HN (172-201), His-VP2 (197-209)/HN (172-201) or His-HN (172-201)/VP2 (197-209) could induce the animals to produce specific antibody titers from 0.8×10^4-1.25×10^4. The groups immunized by six hybrids containing Ii segments Cyt/TM had antibody titers 3.1~3.5 fold than the titers from the animals immunized by hybrids without Ii-segments ($p<0.01$). It was interesting that no difference between the series multiepitopes and the single epitope when they were linked with Ii-segment were found in the production of specific antibodies ($p>0.05$). This suggests that Ii segments (Cyt/TM) as a potential immune carrier could increase the immune response to single epitope as well as multiepitopes.

Table 2: Specific antibody titers secreted by the mice immunized with various fusion proteins in ELISA

Coating antigens	Groups immunized with antigens (N = 4)	Antibody titers	Ratio (sample/VP2 or HN)
GST-VP2	Negative control	-	-
	His-VP2	$0.8\pm0.04\times10^4$	1.00
	His-HN/VP2	$1.0\pm0.02\times10^4$	1.25
	His-Cyt/TM/VP2	$2.6\pm0.12\times10^4$	3.25
	His-Cyt/TM/HN/VP2	$2.8\pm0.08\times10^4$	3.50
	His-Cyt/TM/VP2/HN	$2.7\pm0.14\times10^4$	3.37
GST-HN	Negative control	-	-
	His-HN	$1.0\pm0.08\times10^4$	1.00
	His-HN/VP2	$1.1\pm0.07\times10^4$	1.10
	His-Cyt/TM/HN	$3.1\pm0.05\times10^4$	3.10
	His-Cyt/TM/HN/VP2	$3.1\pm0.07\times10^4$	3.10
	His-Cyt/TM/VP2/HN	$3.3\pm0.05\times10^4$	3.30

CONCLUSION

In this research demonstrated that the multiepitopes, HN (172-201)/VP2 (197-209) and VP2 (197-209)/HN (172-201) in various series in hybrids had the same immunogenicity and Ii segments (Cyt/TM) could improve an immune response to single as well as multiepitopes. The Ii segments as an immune carrier potentiated an immune response to multiepitopes.

ACKNOWLEDGEMENT

This research was financially supported by a grant from the National Natural Science Foundation of China under the award No. 31172306 and 31201888.

REFERENCES

Ashman, J.B. and J. Miller, 1999. A role for the transmembrane domain in the trimerization of the MHC class II-associated invariant chain. J. Immunol., 163: 2704-2712.

Chen, F., F. Meng, L. Pan, F. Xu, X. Liu and W. Yu, 2012. Boosting immune response with the invariant chain segments via association with non-peptide binding region of major histocompatibility complex class II molecules. BMC Immunol., Vol. 13. 10.1186/1471-2172-13-55.

Hashish, E.A., C. Zhang, X. Ruan, D.E. Knudsen and C.C. Chase *et al*., 2013. A multiepitope fusion antigen elicits neutralizing antibodies against enterotoxigenic escherichia coli and homologous bovine viral diarrhea virus *in vitro*. Clin. Vaccine Immunol., 20: 1076-1083.

Heine, H.G., M. Haritou, P. Failla, K. Fahey and A. Azad, 1991. Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strains. J. Gen. Virol., 72: 1835-1843.

Huang, X., W.Y. Yu and D.L. Zhong, 2003. Prediction of latent antigenic epitopes for NDV F48E9 and LaSota HN structure protein. Anhui Agri. Sci. Bull., 14: 101-103 (In Chinese).

Jasanoff, A., G. Wagner and D.C. Wiley, 1998. Structure of a trimeric domain of the MHC class II-associated chaperonin and targeting protein Ii. EMBO J., 17: 6812-6818.

Li, W., Q. Chen, Q. Lin, Y. Lv and J. Feng *et al*., 2013. Immunogenicity of a multiepitope plasmid DNA encoding T and B lymphocyte epitopes from latent membrane protein 2 (LMP2) of epstein-barr virus as a vaccine in mice. Protein Peptide Lett., 20: 1136-1143.

Lindner, R., 2002. Transient surface delivery of invariant chain-MHC II complexes via endosomes: A quantitative study. Traffic, 3: 133-146.

Pradhan, S.N., P.R. Prince, J. Madhumathi, P. Roy, R.B. Narayanan and U. Antony, 2012. Protective immune responses of recombinant VP2 subunit antigen of infectious bursal disease virus in chickens. Vet. Immunol. Immunopathol., 148: 293-301.

Roche, P.A. and P. Cresswell, 2011. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. Proc. Natl. Acad. Sci. USA. 1991. 88: 3150-3154. J. Immunol., 187: 1076-1080.

Romer-Oberdorfer, A., W. Ortrud, V. Jutta, M. Teshome and C.M. Thomas, 2003. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. J. Gen. Virol., 84: 3121-3129.

Rudensky, A.Y., M. Maric, S. Eastman, L. Shoemaker, P.C. DeRoos and J.S. Blum, 1994. Intracellular assembly and transport of endogenous peptide-MHC class II complexes. Immunity, 1: 585-594.

Schnitzler, D., F. Bernstein, H. Muller and H. Becht, 1993. The genetic basis for the antigenicity of the VP2 protein of the infectious bursal disease virus. J. Gen. Virol., 74: 1563-1571.

Vakharia, V.N., D.B. Snyder, D. Lutticken, S.A. Mengel-Whereat, P.K. Savage, G.H. Edwards and M.A. Goodwin, 1994. Active and passive protection against variant and classic infectious bursal disease virus strains induced by baculovirus-expressed structural proteins. Vaccine, 12: 452-456.

Vogt, A.B., L.J. Stern, C. Amshoff, B. Dobberstein, G.J. Hammerling and H. Kropshofer, 1995. Interference of distinct invariant chain regions with superantigen contact area and antigenic peptide binding groove of HLA-DR. J. Immunol., 155: 4757-4765.

Wang, X.N., G.P. Zhang, J.Y. Zhou, C.H. Feng and Y.Y. Yang *et al*., 2005. Identification of neutralizing epitopes on the VP2 protein of infectious bursal disease virus by phage-displayed heptapeptide library screening and synthetic peptide mapping. Viral Immunol., 18: 549-557.

Xu, F.Z., H. Ye, J.J. Wang and W.Y. Yu, 2008. The effect of site-directed mutagenesis of the ambient amino acids of leucine-based sorting motifs on the localization of chicken invariant Chain. Poult. Sci., 87: 1980-1986.

Zanetti, F.A., M.P.D.M. Zajac, O.A. Taboga and G. Calamante, 2012. Evaluation of modified vaccinia virus Ankara expressing VP2 protein of infectious bursal disease virus as an immunogen in chickens. J. Vet. Sci., 13: 199-201.

Zhao, W., Z. Zhang, L. Zsak and Q. Yu, 2013. Effects of the HN gene C-terminal extensions on the Newcastle disease virus virulence. Virus Genes, 47: 498-504.