Detection of specific antigens of Newcastle disease virus using an absorbed Western blotting method

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Summary

Newcastle disease virus (NDV) is an economically important poultry pathogen with a worldwide distribution that may infect a wide range of domestic and wild avian species. The identification of different pathotypes of NDVs plays an important role in the diagnosis and development of vaccines to control and eradicate NDV infections. In our previous study, we showed that mono-specific antibodies can differentiate velogenic and lentogenic strains of NDV in Agar Gel Immuno-Diffusion tests. To evaluate the ability of the specific antibodies to detect NDV specific antigens, this study was conducted with a range of NDV isolates. The samples included 9 NDV neuropathogenic/velogenic isolates from diseased chickens collected from poultry farms in central and northern parts of Iran plus La-Sota and B1 vaccine strains. All samples were propagated in embryonated chicken eggs and concentrated and purified by ultra-centrifugation. All samples were subjected to 12.5% SDS-PAGE and Western blotting using the specific antibodies mentioned previously. In SDS-PAGE all velogenic and vaccine strains showed the same electrophoretic pattern. The detected bands included 15, 38, 46, 48, 53, 55, 68, 74 and 220 kDa proteins. In Western blotting analysis, the mono-specific antibodies reacted specifically to the viral proteins with 15, 38, 48, 55, 74 and 220 kDa and non-specifically to the viral protein with 53 kDa. The results suggest that specific anti-NDV antibodies can react specifically to glycoproteins (haemagglutin-neuraminidase and fusion proteins) but not to internal proteins (nucleoprotein or matrix protein) of NDV strains.

Key words: Newcastle disease virus, Specific antigens, Western blotting method

Introduction

Newcastle disease virus (NDV) is a widespread and economically important poultry pathogen. Although vaccines have long been available and administered to control Newcastle disease, the virus remains an ongoing threat to commercial flocks. The forms of the disease vary and are dependent upon several factors, but mainly on the strain of the virus (Alexander, 1988; Garcia *et al*., 2013).

Newcastle disease virus or avian paramyxovirus type-1 is a member of the Avulavirus genus of subfamily Paramyxovirinae (family Paramyxoviridae, order Mononegavirales) (Maclachlan *et al*., 2010). Depending on the basis of their pathogenicity for chickens, Newcastle disease virus strains are classified into highly pathogenic (velogenic), intermediate (mesogenic) and apathogenic (lentogenic) strains (Römer-Oberdörfer *et al*., 2003).

Newcastle disease virus is an enveloped virus with helical nucleocapsid and a single-stranded, negative sense RNA genome, and is about 15 kb long. The genome comprises of six genes, which in the 3´-5´ orientation encodes a 53-56 kDa nucleocapsid protein (NP), a 56 kDa phosphoprotein (P), a 38-42 kDa matrix protein (M), a 67-68 kDa fusion protein (F), a 74 kDa haemagglutinin/neuraminidase (HN), and a 180-220 kDa large polymerase protein (L). In addition to the mentioned proteins, NDV produces two V and W proteins by an RNA-editing event that occurs during the transcription of the P gene (Steward *et al*., 1993; Dortmans *et al*., 2010).

NDV fusion and entry require two glycoproteins F and HN. Similar to all other paramyxoviruses, the NDV F protein is synthesized initially as a precursor, fusion protein (F_0) (67-68 kDa), which is cleaved into fusion protein (F_1) (56 kDa) and fusion protein (F_2) (12-15 kDa) subunits by a furin-like enzyme of the host cells. The cleavage of the F_0 protein is directly responsible for the hemolysis function, cell-to-cell fusion, and early infection occurrence (Panda *et al*., 2004). Pathotype prediction (Alexander, 2000; Heiden *et al*., 2014) initially involves the NDV inoculation of embryonated eggs to determine the mean time of death of the embryos. The molecular basis for NDV pathogenicity is dependent on the fusion protein cleavage site amino acid sequence (Nagai *et al*., 1976) and the ability of specific cellular proteases to cleave the fusion proteins of different pathotypes (Gotoh *et al*., 1992; Ogasawara *et al*., 1992).

Several hundred strains of NDV have been isolated from around the world and numerous, live and inactivated virus vaccines exist against NDV. Consequently, the need to determine the difference between vaccinal and wild strains of NDV is felt.

In our previous studies on NDV proteins, we used a cross absorption method, Western blotting and dot-ELISA to find particular antibodies that reacted specifically to viral antigens of velogenic and lentogenic

strains (Hemmatzadeh *et al*., 2005, 2006; Hemmatzadeh and Sharifzadeh, 2006). The objective of this study was to determine specific antigen(s) of NDV vaccinal strains and strains from outbreaks of Newcastle disease using the absorbed Western blotting method.

Materials and Methods

Viruses and antigen preparation

The vaccinal strains of NDV used in this study were B1 and La-Sota (Razi Vaccine and Serum Research Institute, Tehran, Iran), commonly used in the poultry industry for NDV vaccination in Iran. The virulent strain of NDVs were isolated from few outbreaks of Newcastle disease in different poultry farms during a 3-year period from 2005 to 2008. The isolated viruses were confirmed by RT-PCR and restriction endonuclease mapping (Hemmatzadeh *et al*., 2006). The isolated viruses used in this study were identified by S1, S4, S5, S6, S12, S44, S23, S16, and S17 codes.

All the viruses were inoculated into the allantoic cavity of 7- to 9-day-old embryonated chicken eggs (Morghak Co., Karaj, Iran) as described previously (Dufour-Zavala, 2008). Five days after incubation or after the embryos died (between 3 to 5 days of incubation), the allantoic fluids were collected from chilled eggs and tested directly for hemagglutination activity with chicken red blood cells as previously described (Dufour-Zavala, 2008). To confirm the existence of ND viruses in the allantoic fluid, the hemagglutination assay (HA) positive samples were examined via hemagglutination inhibition (HI) tests using specific NDV antibodies (Dufour-Zavala, 2008).

Following HA and HI examinations, the harvested allantoic fluid was clarified by centrifugation at 600 g for 20 min to remove debris, and then ultra-centrifuged (Beckman-Avanti J-251, Rotor: JA-25-50) at 70,000 g for 2 h through a 30% sucrose cushion. The pellet was washed, re-suspended in 1 ml PBS, and stored in aliquots at -80°C.

The protein content of purified samples were quantified by the Bradford method using Bradford reagent (Sigma-Aldrich), according to the manufacturer's instructions and adjusted to 10 mg/ml (Tseung *et al*., 1993; Mahy and Kangro, 1996). The purified viruses from each were used in SDS-PAGE and Western blotting tests.

SDS-PAGE

Each purified NDV sample was run in a discontinuous polyacrylamide gel (5% and 12%) under denaturing and reducing conditions in duplicate gels (one being stained and another used for Western blotting) according to standard procedure (Laemmli, 1970). The samples were diluted in Tris/HCl buffer containing 10% SDS, 14 mM β-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue and heated at 100°C for 5 min. To compare the reactivity of specific antibodies to different viral strains, each gel used for Western blotting contained 3 repeats of B1, La-Sota and one of the velogenic strains of NDV with a protein marker (Fermentas, PageRuler™ Prestained Protein Ladder and BioLabs Prestained Protein Marker, Broad Range (6-175 kDa)). The SDS-PAGE gels were stained by coomassie brilliant blue and the molecular weight of the protein bands were determined.

Antibodies

The antibodies used in this study were mono-specific polyclonal antibodies that were raised in rabbits against velogenic, B1 and La-Sota strains of NDV by a previously described method (Hemmatzadeh *et al*., 2005; Hemmatzadeh *et al*., 2006; Hemmatzadeh and Sharifzadeh, 2006). In brief, each purified strain of NDV was used to immunize two rabbits. The rabbits were boosted twice and tested in HI until the titer reached to at least 1/4000.

In order to obtain an antibody specific to velogenic strains, sera of rabbits immune to velogenic strains were absorbed with a mixture of lentogenic (B1 and La-Sota) strains. Cross absorption was also performed for sera immune to B1 and La-Sota with a mixture of velogenic, La-Sota and velogenic and B1 strains, respectively, in order to remove antibodies to velogenic and other lentogenic strains. Therefore, antibodies recognizing cross-reactive antigens were removed from the antisera by cross absorption using purified viruses. The specific reactivity of these antisera were confirmed by Agar gel immunodiffusion, Dot-blot immunoassay and immunofluorescence methods (Hemmatzadeh *et al*., 2005, 2006; Hemmatzadeh and Sharifzadeh, 2006).

Western blotting

The gels were transferred to a nitrocellulose (NC) membrane (Schleicher and Schuell BA-S 83, 0.2 µm, Keene, NH), blocked at room temperature (3% BSA, 0.05% Tween-20 in PBS) for 1 h and washed 3 × 5 min in PBS-T (0.5% Tween-20 in PBS). Since the membranes contained 3 repeats of B1, La-Sota and one of the velogenic strains of NDV, they were cut in three strips, each overlaid with a 1/20 dilution of the three previously described specific antibodies including anti B1, anti-La-Sota (Anti L) and anti-velogenic (Anti V) antibodies, incubated for 1 h at 37°C and washed 3 × 5 min in PBS-T. A dilution of 1/1000 of horseradish peroxidase (HRP) conjugated anti-rabbit antibody was used as secondary antibody for 1 h. The membranes were washed and developed using a 3,3´-Diaminobenzidine (DAB) substrate solution (Sigma, Chemical. Co., St Louis, Mo.) (Sambrook *et al*., 2001). The color development was allowed to proceed for 5-10 min followed by several washes with distilled water. The membranes were air dried between filter paper in the dark before being read. They were then scanned and the molecular weight of the proteins was measured according to a pre-stained MW marker.

Results

Following the propagation of viruses into the

allantoic cavity, all of the isolates were found to have an HA titre over 128. The purified viruses in the SDS-PAGE method showed a pattern of 9 bands of 15, 38, 46, 48, 53, 55, 68, 74, and 220 kDa. Electrophoretic patterns of major proteins demonstrated no variation between velogenic and vaccinal strains (data not shown).

Western blot analysis was performed for the 9 isolates as well as two B1 and La-Sota vaccine strains of NDV by anti B1, Anti L and Anti V antibodies. As shown in Fig. 1, there was a nonspecific reaction in the 53 kDa molecular weight that seems to be a nucleoprotein in all antibodies. Specific reactions were seen in HN with the 74 kDa molecular weight in the Anti V reaction with velogenic strains and the B1 vaccine strain to some extent. A reaction was also observed in the 68 kDa molecular weight that seems to be the precursor F_0 in Anti V reaction with velogenic strains that had no reaction with others. Other specific reactions were in F_1 with the 55 kDa molecular weight in velogenic strains and to a certain extent the B1 strain with specific antibodies (Fig. 1). It must be noted that two lentogenic strains only reacted with anti B1 antibody in F_1 (Fig. 2). In addition, there were reactions in the 15 kDa molecular weight that could be F_2 in non-purified anti B1 and Anti V reactions with the B1 vaccine strain (Fig. 3).

Figure 4 shows the results of the Western blotting of three lentogenic strains by Anti La, Anti B1-La and Anti V antibodies. As shown, the best reaction is in NP with the 53 kDa molecular weight in Anti La and weakly in Anti B1-La, but there is no reaction in Anti V antibodies.

There were reactions in the 38 kDa molecular weight that seems to be the matrix protein in only three isolates with the anti B1 antibody only (Fig. 5).

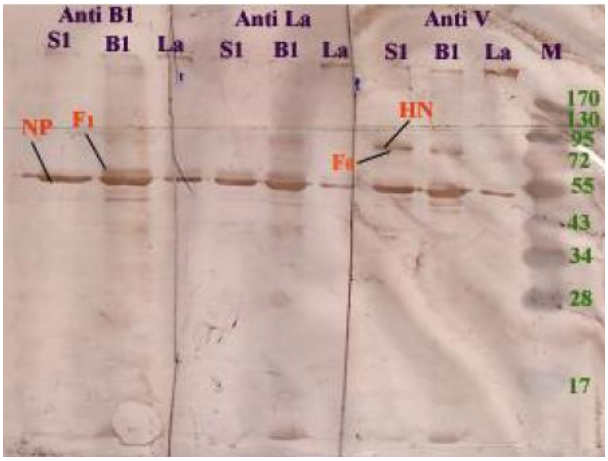

Fig. 1: Western blot analysis of the one virulent isolate (S1) and two B1 and La-Sota (La) vaccine strains of NDV by Anti B1, Anti La and Anti V antibodies. Specific reactions were in 74 kDa in Anti V reaction with velogenic strains and to some extent the B1 vaccine strain. Also, there was a reaction in the 68 kDa molecular weight in Anti V reaction with velogenic strains that had no reaction in others. Other specific reactions were in 55 kDa in velogenic strains and somehow B1 vaccine strain with specific antibodies. However, there was a nonspecific reaction in molecular weight 53 kDa. M: Matrix protein, NP: Nucleocapsid protein, HN: Haemagglutinin/ neuraminidase, F1: Fusion protein, and F0: Fusion protein

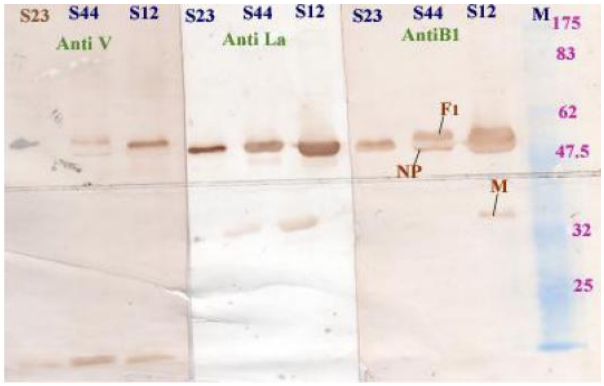

Fig. 2: Western blot of S12, S44 and S23 samples with Anti B1, Anti La and Anti V antibodies, two lentogenic strains (S12 and S44) reacted with just Anti B1 antibody in 55-56 kDa (F1). M: Matrix protein, F1: Fusion protein, and NP: Nucleocapsid protein

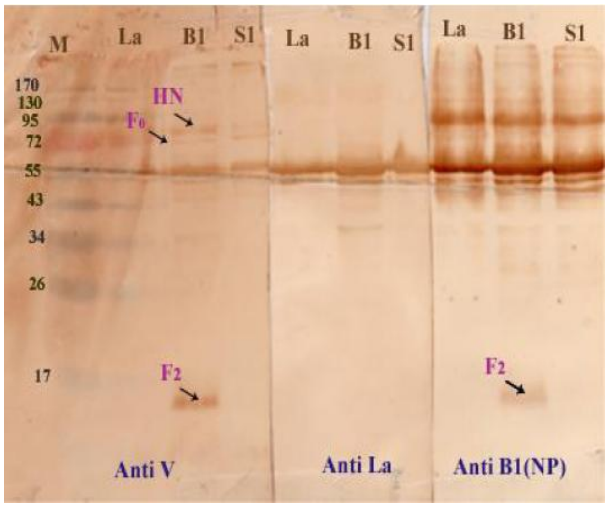

Fig. 3: Western blot of B1, La and S1 samples with Anti V, Anti La and non-purified anti B1 antibodies. There were reactions in molecular weight 15 kDa that may be fusion protein (F_2) in non-purified anti B1 and Anti V reaction with the B1 vaccine strain. M: Matrix protein, HN: Haemagglutinin/ neuraminidase, and F0: Fusion protein

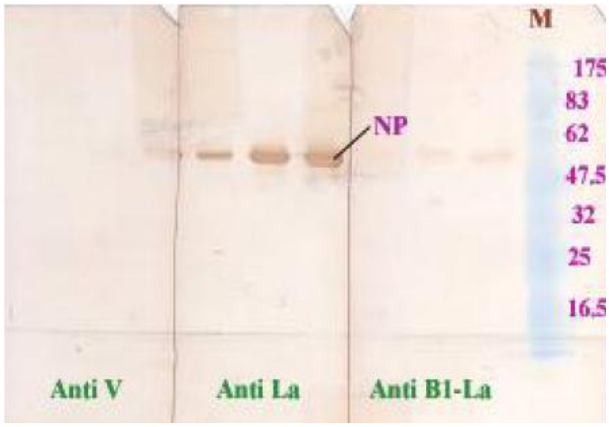

Fig. 4: Western blot analysis of the three lentogenic strains by Anti La, Anti B1-La and Anti V antibodies. M: Matrix protein, and NP: Nucleocapsid protein. As shown, the best reaction is in NP (53 kDa) in Anti La and weekly in Anti B1-La, but there is no reaction in Anti V

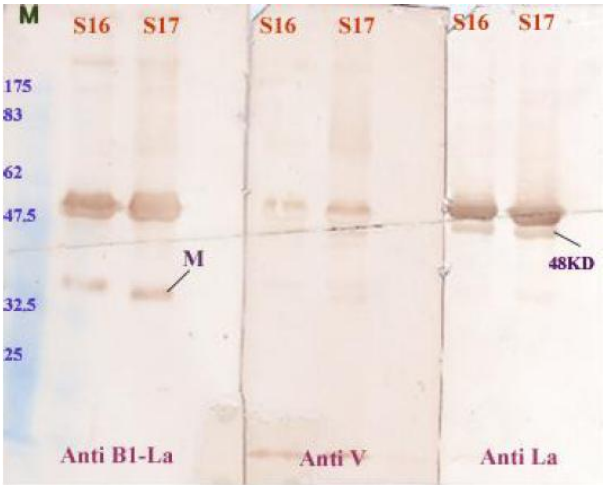

Fig. 5: Western blot of S16 and S17 samples with anti B1, Anti La and Anti V antibodies. There were reactions in molecular weight 48 kDa that may be a different fusion protein with Anti La. M: Matrix protein

Also there were reactions in the 48 kDa molecular weight that may be a different fusion protein in only two isolates with Anti La (Fig. 5).

Discussion

Determining specific antigen(s) of velogenic and lentogenic strains is the basis of most studies aiming at identifying protective antigens and designing serological diagnostic tests.

Our results support our previous study (Hemmatzadeh and Alinejad, 2003) in that electrophoretic patterns demonstrate no variation between velogenic and vaccinal strains.

This study was carried out to identity antigens detected by specific antibodies. The results show that the specific Anti V antibody can react specifically to glycoproteins (haemagglutin-neuraminidase and fusion proteins) of NDV strains but not specifically to nucleoproteins. Although we used specific antibodies in Western blotting, the sera from rabbits immunized by velogenic strains were absorbed by a mixture of both lentogenic strains (B1 and La-Sota) to produce the previously mentioned specific antibodies. We proposed that all cross reactive antibodies be removed from the hyper immune sera in cross absorption step. Specific reactivity of these antisera to velogenic and lentogenic strains was confirmed by Agar gel immunodiffusion, dot-blot immunoassay and immunofluorescence methods. These methods can detect specific antisera that react with the whole virion particles. Matrix and nucleo proteins as internal and considerably large proteins can be exposed to the antibodies during Western blotting as a consequence of denaturation of the whole virus particles in the SDS-PAGE method. This process can explain why the cross reactive antibodies did not react with the whole virus particles in AGID, IFT and the dot-blot immunoassay but reacted with separated antigens in Western blotting. There were 53 kDa bands in almost all lanes of the Western blot analysis. This 53 kDa protein is believed to be the nucleoprotein (Fig. 1). Thus we suggest that in order to producing more specific antibodies, the sera from rabbits immunized by velogenic strains be absorbed by a mixture of virion particles of lentogenic strains (B1 and La-Sota) at denatured conditions.

Similar to previous studies, our results suggest that matrix protein is less immunogenic and that there were 53 kDa bands with anti B1 antibody only in just three lanes of the Western blot analysis.

F_2 was only detected in the B1 vaccine strain using non-pure anti B1 and Anti V antibodies. This could be due to the denaturing condition of the SDS-PAGE sample preparation buffer, hence the F_0 of B1 may be cleaved easier than the others under this condition. Anti V antibody that was also involved in non-pure anti B1, could react with F_2. This finding suggests that F_0 of velogenic strains of NDV was easier cleaved to F_1 and F_2 than lentogenic strains (B1 and La-Sota) in the *in vivo* condition, therefore the antibody against F_2 was raised.

Another finding of our study is the detection of the 48 kDa protein in only two isolates with Anti La which seems to be a different fusion protein than that reported in the previous study (Hemmatzadeh *et al*., 2006). The results presented here demonstrate the differences between the specific protein patterns of velogenic and lentogenic strains NDV using specific antibodies.

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