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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

A Specific Dot-Blot Immunoassay for Newcastle Disease Serodiagnosis

Farhid Hemmatzadeh¹ and Ali Sharifzadeh²

¹Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Iran

²Faculty of Veterinary Medicine, Islamic Azad University of Shahrekord, Iran

Abstract: Dot-blot enzyme immunoassay (DB-EIA) was utilized for detection of Newcastle disease virus (NDV) specific antibodies in infected poultries. Velogenic strain of NDV virus was propagated in embryonated eggs and purified by using ultracentrifugation. Purified whole particle of virus was absorbed by rabbit anti B1 and La-Sota strains antibodies and tested by AGID method. A total number of 140 sera from experimentally infected and 850 sera from vaccinated, healthy and/or diseased chicks tested by HI and ELISA were used for investigating of specific NDV antibodies. HRPO-conjugated anti-chicken antibody was used to detect bound NDV antibodies using TMB as substrate. Statistical analyses were shown significant differences in results of DB-EIA, to HI and ELISA test to detection of specific anti-velogeni NDV antibodies. The assay proved to be a simple, inexpensive reliable and rapid tool for NDV serodiagnosis and differentiation between vaccination and disease.

Key words: Dot-blot enzyme immunoassays, NDV, HI, AGID, ELISA

Introduction

Newcastle disease virus (NDV) is a member of the genus Avulavirus, in the family Paramyxoviridae, which is composed of enveloped helical nucleocapside (Murphy *et al.*, 1999).

Single stranded minus sense RNA genome of ND virus encoded six major proteins. The NP (nucleocapsid protein), P (phospho protein) and L (large protein) are located in nucleocapside. The two glycoproteins, F (fusion) and HN (Hemagglutinin - Neuraminidase) as well as non-glycosylated M (matrix protein) are associated with the viral envelope (Alexander, 1990; Mass *et al.*, 2003).

Fusion and entry of NDV require two glycoproteins F and HN. Like all the paramyxoviruses, the F protein is synthesized initially as a precursor, F₀, and then cleaved into F₁ and F₂ by a furin-like enzyme of the host cells (Panda *et al.*, 2004). Cleavage of F is required for NDV to function in hemolysis, cell-to-cell fusion, and early event in infection. (Panshin *et al.*, 2000; Kianizadeh *et al.*, 1999).

The objective of this study was to develop and evaluate a convenient and inexpensive system for detection of specific antibodies to velogenic strains of NDV and differentiation between vaccination and disease induced antibodies.

Materials and Methods

Sampling: Four different group of sera were selected for the experiment. First group included 321 sera from 22 days old vaccinated chicks that received B1 vaccine strain 10 days before bleeding. 183 sera from a herd with 32 days old that showed clinical signs of Newcastle disease and vaccinated 15 days before bleeding,

Newcastle disease was confirmed by clinical examination, serology and virus isolation in infected chicks. Second group included 286 sera from 48 days old chicks that vaccinated by B1 and La-Sota, this group was received B1 vaccine, 30 days and La-Sota vaccine 13 days before sampling. Third group included 60 sera from non-vaccinated and non-infected 18 days old chicks. Forth group included 140 sera from experimentally infected chicks inoculated by a virulent strain and bleed 15 days after inoculation. All serum samples were tested using HI test and indirect antibody enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's. (Svanova Biotech, Uppsala, Sweden) instruction (Alexander, 2000).

Viruses and Antigen preparation: To obtain pure virus stocks a virulent (velogenic) isolate and two B1 and La-Sota vaccine strains of NDV were used. The virulent strain of ND virus were isolated from an outbreak of Newcastle disease and confirmed by RT-PCR and restriction endonuclease mapping. (Hemmatzadeh *et al.*, 2006).

All the viruses were inoculated into allantoic cavity of 7 days old of chicken embryonated eggs and harvested after 4 days. Following HA examination, the allantoic fluid of eggs was clarified by centrifugation at 600g for 20 min, and 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 then resuspended in 1ml PBS, and stored in aliquots at -80°C. Protein content of purified samples were measured by Bradford method (Hay *et al.*, 2002) and adjusted to 10 mg/ml. (Brian and Hillar, 1996; Tseung *et al.*, 1993).

900 µl of each samples emulsified with an equal volume

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of Freund's incomplete adjuvant (final volume 1800 μ l) and used for immunization of 6 young and apparently healthy rabbits. Four boosts of the samples were injected subcutaneously at two week intervals. Fifteen days before bleeding, rabbits injected intravenously with 900 μ l of the purified viruses in sterile PBS. On the fifteenth day, the rabbits were bled and sera were collected aseptically. 6 different rabbits were selected for immunization by vaccine strains (B1 and La-Sota) and immunized and bleed as same as first group. Antibody level of each rabbit was detected by HI and AGID method weekly till HI titer reached to 1/4000 or more (Casto and Heuschele, 1992).

The sera from rabbits that immunized by virulent strains were absorbed by a mixture of vaccinal strains (B1 and La-Sota). After 1 hour incubation the mixture were centrifuged at 25000g to remove antigen antibodies complex. The remained antigen was tested in AGID method by using anti-vaccinal and anti-virulent antibodies. This antigen did not react with B1 and/or La-Sota vaccinal strains but only reacted with virulent strains.

DB-EIA: Viral antigen prepared as described above was spotted onto of 1 \times 1cm nitrocellulose (NC) membrane (Schleicher & Schuell BA-S 83, Keene, NH) at 20 μ l per spot and then allowed to air dry. Each NC membrane was transferred into a well of a 6 well flat bottom plate and incubated in blocking solution (3% BSA/0.05% Tween-20/PBS) for 1 h at room temperature with occasional shaking. The membranes were then washed 3 \times 5 min in PBS-T, overlaid with two-fold serial dilutions from 1/2 to 1/1024 of 50 positive and 50 negative sera that previously tested by ELISA and HI tests and incubated for 1 h at room temperature and washed 3 \times 5 min in PBS-T. All the membranes were overlaid with horseradish peroxidase (HRP) conjugated anti-avian antibody incubated and washed as described before and developed using TMB (3,3', 5,5'-tetramethylbenzidine) as substrate solution (Sigma, Chemical. Co. St Louis, Mo.). The color reaction was allowed to proceed for 5-10 min and then stopped with several washes with distilled water. The membranes were air dried in the dark before being read. Positive samples were visually determined by appearance of blue spot (Fig. 1A) at the site where the antigen was spotted while a minor/no color change (Fig. 1. B, C and D) were considered as negative results. (Cesar *et al.*, 2000; Chang *et al.*, 1998).

For optimizing the DB-EIA was performed blindly, and thus the results from the ELISA and HI were not made available to the person performing the assay until after all the analyses had been carried out. Each dot was scored independently as being reactive or non-reactive and was subsequently compared with values of ELISA and HI tests for analysis (Cesar *et al.*, 2000).

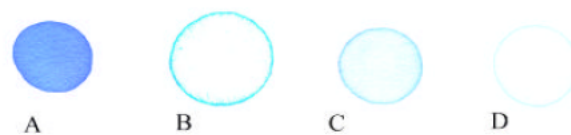


Fig. 1: Examples of DB-EIA results: (A) undiluted positive serum with purified NDV antigen, (B) undiluted positive serum with negative control antigen, uninfected allantoic fluid (C) undiluted negative serum with NDV antigen and (D) undiluted negative serum with negative control antigen.

Statistical analysis: Chi-square and Fisher's exact tests and Kappa values were used to compare sensitivity and specificity of ELISA, HI and DB-EIA tests. (Enoe *et al.*, 2000)

Results

Following optimization of DB-EIA the optimal concentrations for HRP-conjugated anti-avian antibody and sera were 1:20000 and 1:20 respectively. The maximum sensitivity and specificity were achieved when undiluted antigen (10mg/ml) was used, higher dilutions of antigen resulted in reduced sensitivity of the test. In contrast, no significant staining was observed in the negative control (non-infected Allantoic fluid), even in the undiluted and different dilution of positive and negative sera samples. In addition, no staining was appeared when negative sera were applied onto spotted antigen. Unlike HI and ELISA method, DB-EIA described here, does not require pretreatment of samples or expensive supplies and equipment. We have also observed that antigen spotted membranes is highly stable at -20°C for up to 8 months, and it is not necessary to use freshly prepared antigen spotted membranes.

Out of 321 sera samples from first group (vaccinated by B1) 4 were positive in DB-EIA. All of the experimentally infected chicks and 153 of the vaccinated and diseased chicks had positive in DB-EIA but all of these 3 groups had positive reaction in ELISA and HI tests with different titers. From 286 sera samples from second group (vaccinated by B1 and La-Sota) 9 were positive reaction in DB-EIA. All of the 60 non-vaccinated apparently healthy chicks had negative reaction in 3 mentioned tests. Chi-square statistical analysis showed a very highly significant association among DB-EIA and HI and ELISA test for detection of actually diseased chicks by virulent strains of NDV but HI and ELISA tests had positive results in vaccinated chicks as same as diseased birds.

Discussion

The most important factor influencing the specificity is antigen specification and concentration (Enoe *et al.*, 2000). Because non-purified antigens in HI and ELISA

could not differentiate infection from vaccination antibodies response, but the DB-EIA by using purified specific antigen can differentiate between infection and vaccination. As a conclusion, DB-EIA is powerful laboratory test for detection infection by virulent strains. The specificity level of the DB-EIA assay found in this study allows its usage as an alternative method for virus isolation. High sensitivity of DB-EIA dose makes it as a suitable diagnostic test to evaluate NDV infection caused by virulent strains. In contrast to HI test, the evaluation of DB-EIA in heat-inactivated sera (56°C; 30 min) showed no difference between heated and unheated sera (results not shown). In addition to its superior performance with the panel of tested sera used in this study, the DB-EIA was much easier to read and interpret to virus isolation and pathogenicity measurement (Mass *et al.*, 2003).

A possible disadvantage of DB-EIA, compared to HI or ELSA tests, is that it does not detect antibody titer or optical density as a document.

In conclusion, the present study provides evidence that it is still possible to implement simple techniques for the sero-monitoring of NDV which offer high sensitivity and specificity. Where costs are an issue, and in the absence of more elaborated diagnostic tests in many developing countries, simple tests such as DB-EIA could still deliver reliable information that can be used to monitor individuals and/or herds that have problems with NDV infections.

The goal of this work was to develop a simple test that could replace the ELISA system for use at locations where the appropriate equipment is not easily available. This method was a simple protocol and could be a good candidate for a dot blot system in field trials for the diagnosis of NDV infections. To our best knowledge this is the first report of a DB-EIA for the detection of NDV .

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