

Research Article

Effectiveness of Haemagglutination Test in Detection of Newcastle Disease Virus (NDV) AF2240 Strain from Harvested Allantoic Fluids of Infected Chicken Eggs

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Abstract

Haemagglutination (HA) test for the detection of Newcastle disease virus (NDV) is described experimentally using chicken red blood cells (RBCs). The method proved to be reliable and effective, since infectious stocks of AF2240 strains of NDV were detected after the experimental procedure. To detect NDV in chicken allantoic fluids of SPF eggs, 0.1 mL of NDV AF2240 (velogenic) virus stock were inoculated into each egg. The virus was allowed to propagate for production of more stock and the unclarified allantoic fluids were harvested aseptically and tested for confirmation of the virus presence before the stock virus was further clarified and purified using high speed refrigerated centrifuge following sucrose gradients method. This sucrose gradients were later balanced with NTE buffer and sieved using 0.45 µm filter and the purified samples of the virus were collected followed by HA test to detect the presence and to determine the virus titer. The results showed (2⁸ = 256 HAU/mL) virus titer after purification of the allantoic fluids.

Keywords: Chicken red blood cells, Embryonated chickens eggs, Haemagglutination (HA) test, Newcastle disease virus (NDV).

1. Introduction

Newcastle disease virus, also known as avian paramyxovirus type-1, APMV-1^{1,2} causes disease of Newcastle in aneclectic of birds, mostly noticed in chickens³ and the disease is spread worldwide, affecting various species of poultry and other birds^{4,5,6}. The agent that causes the disease is known as Newcastle disease virus (NDV), which is classified in the family of *Paramyxoviridae*^{7,2} of the genus *Avulaviru*^{8,9,10} and subfamily *Paramyxovirinae*¹¹. NDV possesses pleomorphic (an unstable) structures¹² and is encircled by the bilayer of a lipid envelope derived from the host's membrane cell¹³. This disease is often fatal, characterized by some infection of the brain, respiratory and gastrointestinal tracts^{14,15}. NDV can also infect humans, but only causes mild flu-like symptoms; conjunctivitis and/or laryngitis^{16,11,17}.

NDV is classified into three types, based on its virulence strength (i.e. pathogenicity): *Velogenic*, causing gastrointestinal and neurological diseases resulting in high mortality rate of infected birds, *Lentogenic*, causing mild respiratory disease and *Mesogenic*, produces respiratory and nervous signs with moderate mortality¹. The presence of numerous basic amino acids at the cleavage site of fusion protein (F0) precursor designates the molecular basis of virulence in chicken^{5,18}.

A very virulent Malaysian strain of NDV virus known as AF2240 has been shown to be responsible for a very high mortality and morbidity among poultry flocks in Malaysia^{19,20}. The viscerotropic-velogenic NDV AF2240 strain was first isolated from a local field outbreak in the 1960s¹⁹ and it was being used for vaccine trials in Malaysia²¹. This strain is similar to other NDV strains in terms of morphology, cytopathogenicity and replication, but differs from the other NDV isolates by having a different HN protein length²². The genes encoding the HN, F and M proteins of the virus have been sequenced completely and its diameter was ranged from 150 to 600 nm^{13,22}. AF2240 causes haemadsorption, polykaryocytosis and plaque formation¹⁹, as well as agglutination of red blood cells (RBCs), just like other NDV strains tested for Haemagglutination for virus titer in most laboratories of the world.

When diagnosing Newcastle disease, some methods were recommended by OIE Manual²³ and EU Council Directive²⁴ among which include isolation and detection from SPF embryonated chicken eggs, through haemagglutination (HA) inhibition test. Although HA is an old method used in determining the presence of the virus but still being applied in many laboratories of the world for the detection and identification of NDV's titer presence in the samples.

Therefore, the aim of this study was to apply haemagglutination (HA) inhibition test to detect and quantify the titer of Newcastle disease virus (NDV) AF2240 strain in infected, experimental embryonated chicken eggs (SPF) using chicken red blood cells (RBCs).

2. Material and Methods

2.1 Viruses

In this study, NDV AF2240 strains were used and the seed virus stock was kindly provided by Biology section, Unit of Virology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), Serdang, Selangor. Before testing for virus presence using Haemagglutination (HA) test, the strain were propagated in embryonated eggs (SPF) and the allantoic fluids were used for further studies.

2.2 Embryonated eggs

Nine to eleven days old embryonated chicken eggs (SPF), were purchased from Malaysian Vaccines and Pharmaceuticals (Co No: 82381-X) SDN BHD. Upon arrival of the eggs to the laboratory, they were immediately sprayed with 70% ethanol to avoid contamination and wiped with dry tissue paper. Each egg was candled using a candling lamp to enable the location of the air sac for markings, and also to detect non-viable eggs for removal. A mark was made just superior to the air sac margin of the eggs using a pencil and then kept in a 37°C humidified incubator for a day. All processes of viral inoculation, propagation, and harvesting were done aseptically under biological safety cabinet.

2.3 Virus Inoculation

Upon removal of the eggs from the incubator they were then sprayed with 70% ethanol and clean up with dry paper towels to clear away the contaminants. The eggs were then placed in the biological safety cabinet. An approximately 1 mm hole in diameter was made by using pin to punch the eggs just above the air sac margin that was marked with pencil the previous day. The seed suspension of 0.1 mL of the virus was inoculated into the punched hole of each egg using 1 mL syringe. The eggs were sterilized with 70% ethanol again prior to sealing the holes with melted candle wax and the eggs were then kept inside the incubator for 24 hours, after which, candling of the eggs were ensured to check for the dead embryos. Any egg that was found with collapsed blood vessel or unmovable embryos was considered dead, which might be due contamination. This has been removed and kept in the refrigerator at 4°C to prevent contaminating others. The remaining eggs were candled on a daily basis until 90% of the embryos were dead. The eggs were then kept in the 4°C refrigerator overnight before harvesting to ensure the constriction of the blood vessels to avoid blood suck while harvesting the allantoic fluid.

2.4 Harvesting

Here, eggs were removed from the refrigerator and were placed in the biological safety cabinet to allow for regain of room temperature for at least 30 minutes to prevent egg shells from excessive condensation. The eggs were sterilized with 70% ethanol and dapped with tissue paper for dryness and then the egg's membrane were punctured using sterile pipette and the clear allantoic fluids was collected in 50 mL sterile tubes. Unclear or bloody-like allantoic fluids were rejected. For confirmation of the stock virus (NDV) from the harvested allantoic fluid, nippy test were performed using chicken red blood cells (RBC).

2.5 Clarification and Purification

The stored allantoic fluid was removed and thawed before it was clarified with high-speed refrigerated centrifuge at 6000 g for 10 minutes at 4°C. The supernatants were collected into sterile bottles and further centrifuged at 20,000 rpm for 3 hours at 4°C using T21 (Beckman, USA) rotor of the ultra-speed centrifuge and the supernatants were discarded. The obtained pellets were dissolved in 1 mL NTE (NaCl, Tris-HCl, EDTA) buffer using a 1 mL syringe (Terumo Co, Japan) and resuspended for a while. Filtered sucrose gradients of 30%, 40%, 50% and 60% were prepared, and then each of the sucrose gradients were put in equal volumes into the ultra-clear tubes (Beckman, USA) in a descending order using a 1 mL syringe and kept at 4°C refrigerator for overnight. After which, the tubes were removed and topped up with virus-NTE buffer using a sterile Pasteur pipette until all the ultra-clear tubes were properly balanced. The tubes were then centrifuged at 40,000 rpm for 4 hours at 4°C using the pre-cooled SW 41 (Beckman, USA) rotor of the ultra-speed centrifuge. The purified virus band was observed under an inverted microscopy and the band was marked with a marker pen. The band was sucked out and transferred using a 1 mL syringe into polyalomer tubes (Beckman, USA) and re-topped up with NTE buffer. Then, it was centrifuged at 20,000 rpm for 2 hours at 4°C using 100Ti (Beckman, USA) rotor of the ultra-speed centrifuge, to discard any stability of sucrose. The supernatants were discarded and the collected pellets were dissolved in NTE buffer, filtered with 0.4 µm filter and kept in Eppendorf tubes at -80°C for further use.

2.6 Collection of Chicken Red Blood Cells (RBCs)

Vogel and Shelokov²⁴ were the first to discovered haemagglutination (HA) assay test for detection of virus with haemabsorption properties. In this study, HA test was carried out to determine the amount of NDV titer required for inhibition test on haemagglutination, neuraminidase and haemolysis. Chicken red blood cells (RBCs) were prepared from its blood drawn via wing vein into K2EDTA coated tubes (Germany), with PBS and EDTA composition to prevent the blood from clotting. These compositions (mixture of blood, PBS and EDTA) were then transferred into a 15 mL centrifuge tube (TPP, Switzerland) and topped up with PBS before it was centrifuged at 1500 rpm for 10 min at room temperature. The supernatants were discarded and the pellets were resuspended with PBS and centrifuged again, this step was repeated three times. For virus titration, 500 µL of RBCs were diluted in 100 mL PBS to get 0.5% suspension of the RBCs which can later be used for HA test.

2.7 Haemagglutination (HA) Test

100 µL of purified virus was put in the first well of the V-bottom 96-well plate (Nunc, Denmark). Next, 50 µL of PBS was put into the second till the 24th well of the 96-well plate. Then, 50 µL of purified virus was taken from the first well and put into the second well and resuspended to make a two-fold dilution of the virus, 50 µL of from the second well was taken and put in the third well and resuspended and this step was repeated till the 23rd well. After that, 50 µL of 0.5% RBC suspension was added into each of the 24 wells and left at room temperature. The first well, which contains the purified virus and RBC acts as the positive control, whereas the 24th well which contains PBS and RBC acts as the negative control. After 30 to 60 minutes, red buttons appear on the bottom of the well-plate and the last well where the red button does not appear represent the HA titre of the virus.

3. Results

The haemagglutination (HA) test used in our study with NDV AFF2240 strain showed effective, as the virus titer were obtained serologically using chicken red blood cells (RBCs) Fig. 1A. The highest titre of the purified NDV AF2240 obtained was 2⁸ or 256 HA units after propagation and purification of the virus.

Figure 1: 96 well (V-bottom) plate and Ultra-clear tubes (containing the virus)

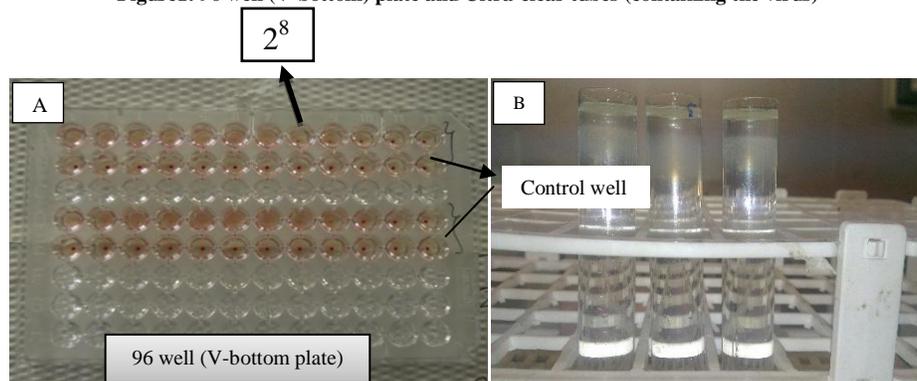


Fig. 1A, is the 96 well plate containing both the agglutinated and unagglutinated RBCs. Note that the reading were done in duplicate. It showed the agglutination and position of the purified virus (NDV AF2240) titer at 2⁸ = 256HAU/ml. For fig. 1B, are the ultra-clear tubes containing the virus bands as seen above just closer to the opening of the tubes.

Figure 1 shows the results of virus propagation (Fig.1B) after clarification and purification, the band of the virus can be observed above (just closer to the opening of the tubes) and this band expressed itself between 40% and 30% of the sucrose gradient, since the sucrose was placed in descending order starting with 60% followed by 50% to 30% concentrations, likewise, nor any other fraction of the virus was observed apart from the one seen above (i.e. only single band was form from the gradient concentration of the sucrose for each tube seen above).

For the Haemagglutination (HA) test (Fig. 1A), virus, PBS and RBCs were seeded as stated in methodology section and we determined the virus titer as seen above (Fig. 1A). By critically observing the well-plates, agglutination was presence starting from 1st well plate until 8th well plate, while the rest of the plates contain no lattices form of RBCs indicating red button appearance of the RBCs at the bottom of the plates, signifying that there is no agglutination of the red blood cells in those plates. This was due to the facts that, unagglutinated RBCs will sink down to the bottom of the well plate and form a very well defined pellet of the RBCs, while the agglutinated RBCs will structurally become lattice in form, which coats the well plate's side²⁵. The two morphological appearances (pellet and lattice) were easily noticeable with naked eye before taking the reading.

Usually, there are two methods to interpret end point titration of the virus;(1) the last dilution showing complete agglutination, (2) the dilution which shows 50% agglutination of the end point titration²⁵. Many of the laboratories use the first method rather than the second one. By considering the first method, means, this dilution contain 1HA unit of the virus. Therefore, from the figure above, the dilution of the virus capable of making complete agglutination is 1/256. Since 0.1 ml (100 µL) of the virus were added to the first well prior to titration, we can conclude that the HA titer of the NDV AF2240 virus was 2⁸ or 256 HA units.

4. Discussion

Newcastle disease is one of the most economically important infectious diseases of avian species, thus, quick detection and identification of the virus is of important to effectively control the disease. To confirm the presence of the virus rapidly in the allantoic fluid²⁶, haemagglutination inhibition test were used in this study. This test is commonly used by many laboratories in the world to determine the virus presence and measure the titer of infectivity of the virus from allantoic fluids or tissue culture.

There are few studies on how to determine the titer of the NDV AF2240 Malaysian strain using haemagglutination assay, thus, this is the first study been conducted so far, to clearly defined the protocols involved for determination of the virus presence by HA test covering inoculation, propagation of the virus, harvesting the allantoic fluids, clarification and purification of the virus to haemagglutination (HA) test.

Haemagglutination (HA) test for the detection of NDV was first described by Vogel and Shelokov²⁴ for detection of virus with haemabsorption properties and to date, it has been successfully utilized in different modifications by many laboratories across the world. We applied HA test in this study, to determine the titer of NDV AF2240 (virulence strain) from the purified allantoic fluids of infected embryonated chicken eggs experimentally.

In our study, the HA titer obtained for Newcastle disease virus AF2240 was 2⁸ or 256 HA units. Almost similar result was obtained by Anushia²⁷, who reported the agglutination effect of NDV AF2240 as 2⁶ or 64 HA units. On the other hand, Zolkapli²⁸, reported the titer of the same virus strain to be 2¹⁴ or 16,384 HA units, whereas another research by Madihah²⁹ had nearest result, 2¹¹ or 2,048 HA units with the previously stated result above using the same virus strain, NDV AF2240. Better results could be obtained by ensuring accuracy when reading the bottom of the 96-well plate where real agglutination is observed because many researchers mistakenly include the red button of the red blood cells when reading the well-plates that are considered agglutination occurred. Hence, the variations in the titer despite the same virus strain were tested by different authors.

5. Conclusion

Many of the NDV strains have been serologically tested for virus presence in Europe, USA, Hungary, Israel, and Asia and, most of the studies involved the use of HA assay virus titer to compare which method is effective or most sensitive in detection of the virus in both allantoic fluids as well as the tissue culture. The NDVA AF2240 has also shown its effects on chicken red blood cells (RBCs) by its ability to form lattice-form of the red blood cells, known as agglutination and it's a good indication that the method is proved correct since stock of the virus is re-discovered after purification process.

References

- Alexander, D.J. Newcastle disease and other avian Paramyxoviridae infections. In B.W. Calneck (Ed.), *Diseases of Poultry* 10th edition. Ames, IA: Iowa State University Press. 1997; 541-569.
- Van Regenmortel, Ranquet CM, Bishop DHL. 8th Report of International Committee on Taxonomy of virus. San Diego, Wien, New York. Academic Press. 2000; p. 1024.
- Csatary LK, Moss RW, Beuth J, *et al.* Beneficial treatment of patients with advanced cancer using a Newcastle disease virus vaccine (MTH-68/H). *Anticancer Res.* 1999;19 (1B): 635-8
- Alexander D. J. The epidemiology and control of avian influenza and Newcastle disease. *J Comp Pathol.* 1995; 112, 105-126.
- Alexander D.J. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In: *Diseases of Poultry*, Iowa State Press, 11th edition, 2003; pp. 63-99.
- Kouvenhoven B. Newcastle disease. In: *Virus Infection of Birds*, Elsevier Science Publishers B.V. 1993; pp. 341-361.
- Rima BK, Alexander DJ, Billeter MA, Collins PL, Kingsbury DW, Lipkind MA, *et al.* The Paramyxoviridae. In: Murphy FA, Fouquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Sumner MD eds. *Virus Taxonomy, Classification and Nomenclature of Viruses*. Sixth report of the International Committee on Taxonomy of Viruses. New York: Springer Verlag. 1995
- Mayo MA. A summary of taxonomy change recently approved by ICTV. *Arch Virology* 2002a; 147: 1656-1656.
- Mayo, M. A. A summary of taxonomic changes recently approved by ICTV. *Arch Virol* 2002b; 147, 1655-1663.
- Mayo MA, Sumner MD eds. *Virus Taxonomy, Classification and Nomenclature of Viruses*. Sixth report of the International Committee on Taxonomy of Viruses. New York: Springer Verlag. 1995.
- Fauziah O, Aini I, Motalleb G, Zulkapli E, Asmah R. Onlytic effect of Newcastle Disease Virus (NDV) AF2240 Strain on the MCF-7 Breast Cancer Cell Line. *Yakhteh.* 2010; 12(1): 17-24.
- Nagai Y, Klenk HD, Rott R. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* 1976; 72: 494-508.
- Yusoff K, Tan WS. Newcastle disease virus: macromolecules and opportunities. *Avian Path* 2001; 30: 439-455.
- Hanson RP: The reemergence of Newcastle disease. *Adv Vet Sci Comp Med* 1974; 18 (0): 213-29.
- Nelson NJ: Scientific interest in Newcastle disease virus is reviving. *J Natl Cancer Inst* 1999; 91 (20): 1708-10
- Csatary LK, Eckhardt S, Bukosza I, *et al.* Attenuated veterinary virus vaccine for the treatment of cancer. *Cancer Detect Prev* 1993; 17 (6): 619-627.
- Moss R. W. Alternative pharmacological and biological treatments for cancer: ten promising approaches. *J Naturopathic Med* 1996; 6 (1): 23-32.
- Ravindra PV, Tiwari AK, Ratta B, *et al.*: Newcastle disease virus-induced cytopathic effect in infected cells is caused by apoptosis. *Virus Res* 2009; 141 (1): 13-20.
- Lai, M.C. and Ibrahim, A. Velogenic viscerotropic Newcastle disease virus. In *Newcastle disease in poultry-a new food pellet vaccine*, ed. J.W. Copland. Brisbane: Etching Press. 1987; 33-34.
- Ibrahim, A. L., U. Chulan and A. M. Babjee. The immune response of chicken vaccinated against Newcastle disease with Newcastle diseases V4 vaccine. *Australia Vet. J.* 1980; 56: 29-33.

21. Tan, W.S., Lau, C.H., Ng, B.K., Ibrahim, A.L. and Yusoff, K. Nucleotide sequence of the haemagglutinin-neuraminidase (HN) gene of a Malaysian heat resistant viscerotropic-velogenic Newcastle disease virus. *DNA Sequence*. 1995; 6, 47–50.
22. OIE .Manual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition Chapter 2.1.15. *Newcastle disease*. 2004; pp. 270-282.
23. Council Directive. Introducing Community measures for the control of Newcastle disease. *Official J Europ Commun*, L260. 1992; pp.1-20.
24. John Vogel and Alexis Shelokov. Adsorption-Hemagglutination Test for Influenza Virus in Monkey Kidney Tissue Culture. *Science*. 1957; 358-359.
25. Hierholzer J, Brian Many WJ, Hillar Kangro O. *Virology Methods Manual*. 1st Edition .London: *ACADEMIC PRESS*. 1996; 309-312.
26. M. Meyyappan. Oncolytic effect of Newcastle disease virus on the MCF-7 and MDA-231 breast cancer cell lines, Master Thesis, *Universiti Putra Malaysia*, Malaysia. 2003
27. Anushia Swaminathan. Effect of Newcastle Disease Virus AF2240 On Allografted 4T1 Breast Cancer Cells In Balb/c Mice. Master Thesis, *Universiti Putra Malaysia*. 2010.
28. Zolkapli Eshak. Molecular and Cytoskeletal Changes in Breast Cancer Cell Lines Treated with Velogenic Newcastle Disease Virus Strain AF2240. Master Thesis, *Universiti Putra Malaysia*. 2006.
29. Mahani Mahadi. Cellular apoptosis of 4T1 breast cancer cells induced by V4-UPM NDV. Master Thesis, *Universiti Putra Malaysia*. 2007.