Original Article

Assessment of Different Newcastle Disease Virus Antigens and Inactivators of Binary Ethylene Amine and Formalin for the Hemagglutination Inhibition Assay

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ABSTRACT

Newcastle disease is a severe viral threat to the global poultry industry due to its high prevalence and rapid transmission. Evaluating vaccination timing and effectiveness is crucial, often accomplished through the hemagglutination inhibition (HI) assay. This test relies on the virus's agglutination ability in certain animals, utilizing various inactivated antigens. Our study aimed to assess multiple Newcastle viral antigens (LaSota, clone, thermo-resistant strain, B1, and V4) inactivated by binary ethylene amine (BEA) and formalin, seeking the best antigen and inactivator for the HI assay. We prepared the different ND antigens include; LaSota, Clone, thermo resistant, B1, V4 and the mixture of the antigens then inactivated them using BEA and formalin. The hemagglutination (HA) assay determined mean titers, comparing BEA and formalin inactivation. These antigens were also subjected to the HI test using 112 serum samples from different commercial poultry flocks to assess their performance. BEA-inactivated antigens exhibited significantly higher mean titers in the HA assay than formalin-inactivated antigens. In the evaluation of different antigens in the HI test, the mean titer of antigen B1 followed by clone and LaSota displayed a higher mean titer than others. In conclusion, this study recommends using Hitchner pathotype antigens, specifically the B1 vaccine, for Newcastle disease HI testing. BEA is the preferred inactivator, preserving antigen structure particularly the structure of hemagglutinin antigen while minimizing risks. These findings can enhance serological testing accuracy, contributing to more effective disease control and prevention in the poultry industry.

Keywords: Newcastle viral antigens; Serological tests; Commercial poultry flocks; Hitchner pathotype; Inactivator.

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1. Introduction

Newcastle disease is one of the most serious poultry diseases. In Asia and Africa, this disease is among the most significant issues (1). Many countries spend a remarkable amount of money on Newcastle prevention to reduce the associated economic consequences (2). Newcastle disease virus is a member of the paramyxovirus family and belongs to the genus Avolavirus in avian paramyxovirus serotype 1. This is an enveloped single-stranded RNA virus with a negative sense. Nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and polymerase protein are the six primary structural proteins encoded by the virus genome(3). Disease control demands rapid and accurate diagnosis in the event of an outbreak. Most of the symptoms of this disease are not pathognomonic; therefore, laboratory tests should be used for definitive diagnosis (2, 4). Serological tests, particularly hemagglutination inhibition (HI) assay, are commonly used in laboratories to monitor and evaluate vaccination effectiveness. The ability of the Newcastle virus hemagglutinin antigen for agglutinating red blood cells is the basis of this assay (3). Different Newcastle virus antigens with a strength of 4 or 8 units are utilized in this test (5). These antigens should be inactivated by various inactivators, such as formalin, beta-propiolactone, and binary ethylene amine (BEA) for a variety of reasons, including the risk of the virus becoming zoonotic and spreading to other places. In addition to lowering and eliminating the risks of virus transmission and spread, an appropriate inactivator must also maintain the virus's antigenic structure. In the case of the Newcastle virus, it should also have no negative impact on the hemagglutination activity of the virus (6, 7). Due to variations in the sensitivity of diverse antigens in the HI assay, choosing the right antigen can be crucial in detecting antibodies produced by different viruses in the farm(4). Therefore, due to the possibility of using different antigens, the purpose of the present study is to select and evaluate the most appropriate antigen for this test. In addition, we investigated the effects of formalin and BEA on distinct Newcastle virus antigens because different inactivators have distinct effects on the virus's hemagglutination properties.

2. Materials and Methods

2.1. Preparation of antigens

This test was conducted using five different antigens, including Lasota, Clon, Thermo-resistant, B1 and V4 provided by Razi Vaccine and Serum Research Institute. A dose of 0.2 ml of each antigen was injected into the SPF eggs (9-10 days) after the viral seeds were prepared with an EID50 titer of 4. All these steps were performed next to the flame. Subsequently, glue was applied to the injection site and then placed in an incubator for 72 h at 37˚C. The eggs were candled three times daily throughout this period, and the mortality on the first and second days were discarded. The allantoic fluid from the eggs was collected and poured into sterile containers. The allantoic fluid was centrifuged twice at 2000 rpm for 10 min to remove red blood cells and embryonic cells, and the supernatant was collected (5). The collected fluid was also frozen and thawed three times to eliminate cell debris and clarify it.

2.2. Inactivation of the propagated NDV

2.2.1. Inactivation by Formaldehyde

The harvested infected allantoic fluids were treated with formaldehyde (Merck, Germany) at a concentration of 0.1% and incubated for 16 h at 37°C (6, 8).

2.2.2. Inactivation by binary ethylene amine

The harvested infected allantoic fluid was treated with BEA prepared from Razi Vaccine and Serum Research Institute at a final concentration of 0.001 M (1% v/v) and incubated at 30°C for 21 h. After the complete inactivation of the virus, the inactivation was stopped in each virus sample by adding 20% sodium-thiosulphate solution to a final concentration of 2% (6, 8).

2.3. Evaluation of the correctness of the inactivation process of antigens

Afterward, a passage was given to ensure the inactivation of our antigens. To this aim, 0.2 of each antigen was injected into the eggs, and then their allantoic fluid was removed and the HA test was taken (5). If the sedimentation of red blood cells occurred in all wells, the viruses were properly deactivated and could not reproduce in the eggs. As a result, the allantoic material of the Newcastle virus was not found.

2.4. Preparation of mixed inactivated antigens

To make a mixture of five formalin-inactivated strains, equal amounts of each were taken and combined in a container. We also prepared such a mixture with five BEA-inactivated antigens in separate glasses (Table 1).

2.5. Preparation of dilution

For standardization, we diluted all antigens to 8 HA units (HAU). Next, back titration was performed to confirm the number of HAU.

2.6. HI assay

For evaluating different antigens, the HI test was performed on 112 serum samples from different commercial poultry flocks with a strength of 8 HAU. Furthermore, to compare the antigens prepared with standard antigen (catalog no: VLDIA039, product code: HAG-NDL), an HI test was also performed on 112 serum samples.

Table 1. Inactivated antigens

Antigen 1	Formalin-inactivated LaSota
Antigen 2	Formalin-inactivated Clone
Antigen 3	Formalin-inactivated thermo resistant
Antigen 4	Formalin-inactivated B_1
Antigen 5	Formalin-inactivated V_4
Antigen 6	Formalin-inactivated mixture of antigens
Antigen 7	Binary ethylene amine-inactivated LaSota
Antigen 8	Binary ethylene amine-inactivated Clone
Antigen 9	Binary ethylene amine-inactivated thermo resistant
Antigen 10	Binary ethylene amine-inactivated B_1
Antigen 11	Binary ethylene amine-inactivated V_4
Antigen 12	Binary ethylene amine-inactivated mixture of antigens

2.7. Statistical analysis

One-way analysis of variance and the Tukey post hoc test were used to examine the mean of antigens obtained. In addition, at a significance level of $P \leq 0.05$, the one-sample t-test was employed to compare the mean titers obtained from distinct antigens with the standard antigen.

3. Results

The mean titer of antigens inactivated by BEA in the HA test was significantly higher than the mean titer of antigens inactivated by formalin ($P \leq 0.05$) in this study (Table 2). (Figure 1).

The mean titer of antigens inactivated by formalin in the HI test was significantly higher than the antigens inactivated by BEA ($P \leq 0.05$) (Table 3) (Figure 2).

In the evaluation of different antigens in the HI test, the mean titer of antigen B_1 was higher than the mean titer of other antigens (Table 4) (Figure 3).

Table 2. HA test

Inactivators	Average	Geometric average
Formalin	7.833±0.16667	7.823
Binary ethylene amine	8.833±0.16667	8.825

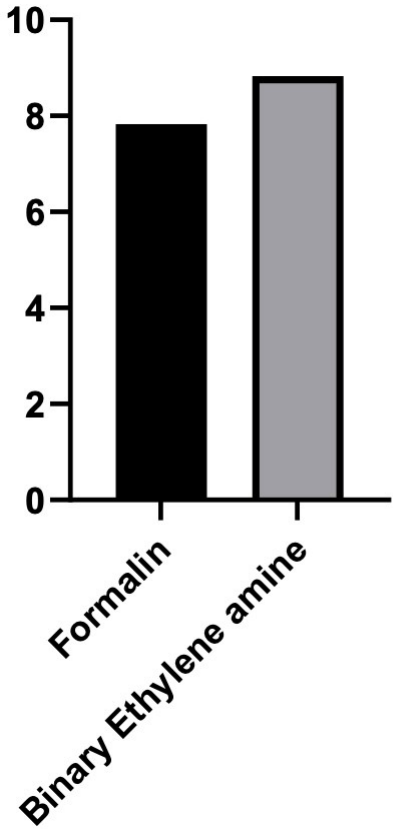

Figure 1. The mean titer of HA test of inactivated antigens by BEA and formalin

Table 3. HI test

Inactivators	Average	Geometric average
Formalin	7.509±0.0952	7.120
Ethylamine binary amine	6.894±0.0967	6.493

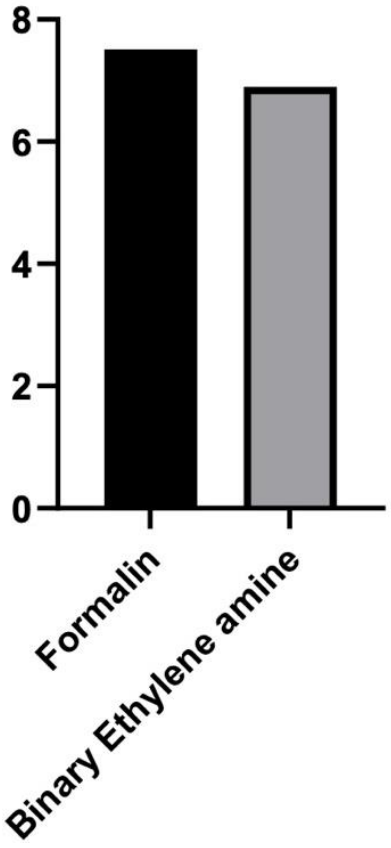

Figure2. The mean titer of HA test of inactivated antigens by BEA and formalin.

Table 4. The mean titer of different antigens in HI

Antigens	Number	Average
1. Formalin-inactivated LaSota	112	7.616±0.2243
2. Formalin-inactivated Clone	112	7.79±0.2167
3. Formalin-inactivated thermo resistant	112	6.634±0.2177
4. Formalin-inactivated B_1	112	7.875±0.1808
5. Formalin-inactivated V_4	112	7.629±0.2049
6. Formalin-inactivated Mixture of antigens	112	7.478±0.2167
7. Binary ethylene amine-inactivated LaSota	112	7.259±0.2047
8. Binary ethylene amine-inactivated Clone	112	7.013±0.2144
9. Binary ethylene amine-inactivated thermo resistant	112	6.152±0.2215
10. Binary ethylene amine-inactivated B_1	112	7.272±0.2101
11. Binary ethylene amine-inactivated V_4	112	6.772±0.2157
12. Binary ethylene amine-inactivated Mixture of antigens	112	6.71±0.2206
13. Standard Antigen (LaSota)	112	7.07±0.1924

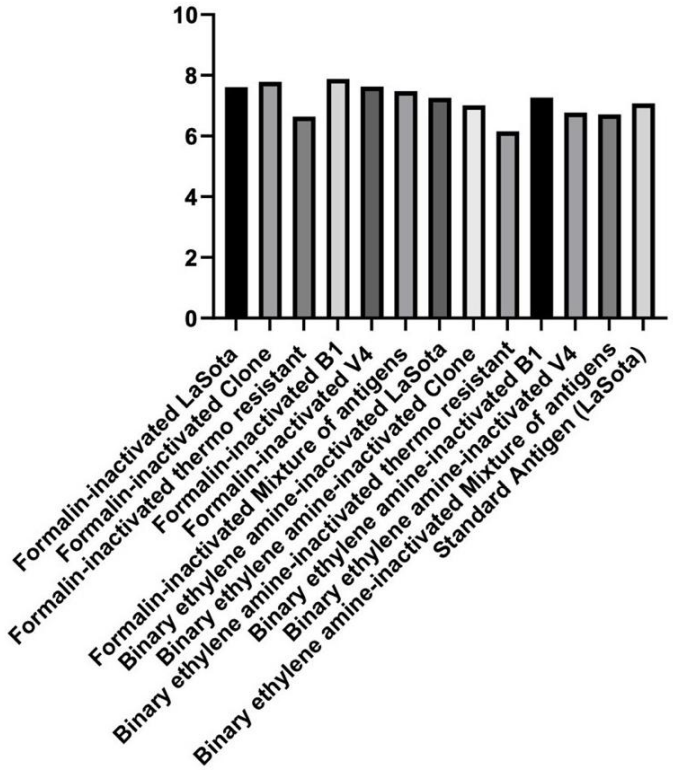

Figure 3. The mean titer of different antigens inactivated by BEA and formalin in HI

The latter difference was statistically significant with thermo-resistant antigens inactivated with formalin and BEA, V_4 inactivated with BEA, and a mixture of inactivated BEA antigens. Statistical analyses of 12 antigens revealed that some of them had a significant difference ($P \leq 0.05$) (Table 5).

When 12 antigens were compared to the standard antigen (7.07), the titers of antigens 1, 2, 4, and 5 were significantly higher than the standard, whereas antigens 3 and 9 were significantly lower than the standard (Table 6).

Table 5. Multiple Comparisons (Antigens P-value)

Antigens	1	2	3	4	5	6	7	8	9	10	11	12
1	-	1	0.05*	0.999	1	1	0.99	0.691	0.005*	0.993	0.179	0.106
2	1	-	0.007*	1	1	0.997	0.836	0.290	0.0005*	0.858	0.035*	0.018*
3	0.05*	0.007*	-	0.002*	0.045*	0.179	0.638	0.984	0.908	0.606	1	1
4	0.999	1	0.002*	-	1	0.976	0.66	0.155	0.005*	0.691	0.014*	0.006
5	1	1	0.045*	1	-	1	0.986		0.0005*	0.66	0.99	0.16
6	1	0.997	0.179	0.976	1	-	1	0.928	0.001*	1	0.444	0.308
7	0.99	0.83	0.63	0.66	0.98	1	-	1	0.013*	1	0.902	0.804
8	0.691	0.29	0.984	0.155	0.66	0.928	1	-	0.155	0.999	1	0.998
9	0.0005*	0.0005*	0.908	0.0005*	0.0005*	0.001*	0.013*	0.155	-	0.011*	0.649	0.786
10	0.993	0.858	0.606	0.691	0.99	1	1	0.999	0.011*	-	0.885	0.777
11	0.179	0.035*	1	0.014*	0.16	0.444	0.902	1	0.649	0.885	-	1
12	0.106	0.018*	1	0.006	0.094	0.308	0.804	0.998	0.768	0.777	1	-

*$P \leq 0.05$ is statistically significant

1. Formalin-inactivated LaSota 2. Formalin-inactivated clone 3. Formalin-inactivated thermo-resistant 4. Formalin-inactivated B_1 5. Formalin-inactivated V_4 6. The formalin-inactivated mixture of antigens 7. Binary ethylene amine-inactivated LaSota 8. Binary ethylene amine-inactivated clone 9. Binary ethylene amine-inactivated thermo-resistant 10. Binary ethylene amine-inactivated B_1 11. Binary ethylene amine-inactivated V_4 12. Binary ethylene amine-inactivated mixture of antigens.

Table 6. One-Sample Test

Antigens	P-value
1. Formalin-inactivated LaSota	0.017*
2. Formalin-inactivated Clone	0.001*
3. Formalin-inactivated thermo resistant	0.048
4. Formalin-inactivated B_1	0*
5. Formalin-inactivated V_4	0.007*
6. Formalin-inactivated Mixture of antigens	0.063
7. Binary ethylene amine-inactivated LaSota	0.358
8. Binary ethylene amine-inactivated Clone	0.792
9. Binary ethylene amine-inactivated thermo resistant	0*
10. Binary ethylene amine-inactivated B_1	0.338
11. Binary ethylene amine-inactivated V_4	0.170
12. Binary ethylene amine-inactivated Mixture of antigens	0.105

*$P \leq 0.05$ is statistically significant

4. Discussion

The HI assay is a commonly used test for detecting antibodies against viruses containing surface hemagglutinin protein (9). The HI assay for Newcastle disease is widely used by veterinarians and farm health managers to evaluate vaccine response, level of protection, and diagnosis of disease in poultry flocks due to the high importance of Newcastle disease in the country (10). In addition to the difference in the strength and level of immune stimulation by the antigens in these vaccines, different Newcastle virus antigens can also have variable sensitivities in detecting antibodies produced by different viruses in the field (4). According to Table 4, antigen B1 has the highest titer, followed by clone and LaSota. The proximity of the titers of these antigens could be due to the same pathotype (lentogenic), and their greater titers may result from the widespread usage of Hitchner pathotype vaccines, such as B_1, clone, and LaSota. In this study, examining the titer of mixed antigens showed that, , it can be a good option for use in HI tests if flocks are naturally infected or vaccinated with all five strains listed in Table 1, or if no information is available on the type of used vaccine. In a study by Ghalyanchi Langeroudi et al. (2020), the HI test was performed on 41 serum samples using five different antigens. The LaSota clone antigen was shown to be the best antigen in terms of the consistency of responses in the HI test in the latter research. Although the aim of the experiment by Ghalyanchi was to assess the uniformity of results from different antigens in distinct laboratories, the findings demonstrated that the LaSota clone had the best uniformity due to the widespread usage of Hitchner vaccines in the country. As a result, the highest serum titers were found in Hitchner strain antigens, such as B_1 and LaSota in the current study. The higher mean titer of HA test in BEA-inactivated antigens in this study, compared to formalin-inactivated antigens, indicates that BEA could better preserve the structure of Newcastle virus antigen, particularly the structure of hemagglutinin antigen, which is consistent with the results of other studies(11,12). Although formalin-inactivated antigens had greater titers than BEA in the HI assay (Table 4), formalin is not necessarily a more proper inactivator than BEA. BEA inactivates the Newcastle virus primarily through its action on nucleic acid. Therefore, there is no significant effect on the virus's structural antigens, allowing it to maintain its hemagglutination activity (11, 12). BEA preserves the virus's structure better than

formalin and offers more intact antigens, necessitating the usage of more antibodies to neutralize the antigens. As a result, in the HI assay, the diluted antibodies are unable to neutralize all the existing antigens. Therefore, red blood cells attach to free antigens, and hemagglutination takes place instead of precipitation.Therefore, there will be lower but more realistic titer when using BEA compared to formalin in HI assay. Various investigations compared the effect of formalin and BEA on several Newcastle antigens and their hemagglutination activity. The impact of these inactivators on the synthesis of inactivated Newcastle vaccine virus antigen and the amount of antibody titer achieved in birds has also been studied (13, 14). Newcastle virus was inactivated using a variety of techniques in the study by King et al. (1991). The inactivation effects of formalin, beta-propiolactone, and BEA were examined in the mentioned study. They found that in terms of maintaining hemagglutination activity, BEA was the most suitable inactivator (6). In a study on two experimental vaccines inactivated by BEA and formalin, Razmaraii et al. (2012) discovered that the vaccine inactivated by BEA could produce more humoral titers in pathogen-free chickens than the vaccine inactivated with formalin, owing to the inefficacy of BEA compound on the virus's structural proteins. These authors also demonstrated the lack of antigenic structure change in the Newcastle virus in the HI assay and showed that viruses inactivated by BEA had more hemagglutination activity than viruses inactivated by formalin(8) . Akhtar et al. developed two Angara disease vaccines, one of which was inactivated by formalin and the other by BEA. They reported a similar result to the study conducted by Razmaraii on the production of the experimental Newcastle vaccine and chickens vaccinated with the BEA-inactivated vaccine had much higher serum titers than chickens vaccinated with the formalin-inactivated vaccine (15). Mudasser et al. (2006) found similar results when comparing the Gumboro virus vaccine inactivated by BEA with the formalin-inactivated vaccine, and the vaccine inactivated by BEA produced a significantly higher serum titer than the formalin-inactivated vaccine (16). According to our findings, the antigens of the Hitchner pathotype, such as B1, Lasota, and cloned Lasota, especially B1, can be used for the HI test. Due to the possibility of antigenic alteration of the circulating strains, testing for other strains of Newcastle virus and retesting these strains regularly are recommended. In addition, BEA is recommended as an inactivator for the Newcastle virus for the HI assay rather than formalin because it does not affect the virus's antigenic structure and has fewer hazards.

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Authors' Contribution
Study concept and design: F.A.N. and K.P.A.
Acquisition of data: F.R., F.A.N. and K.P.A.
Analysis and interpretation of data: K.P.A. and A.B.
Drafting of the manuscript: F.R. and K.P.A.
Critical revision of the manuscript for important intellectual content: K.P.A.
Statistical analysis: A.B.
Administrative, technical, and material support: F.A.N., F.R. and K.P.A.
Study supervision. K.P.A.

Ethics
Not applicable.

Conflict of Interest
The authors declare that they have no conflict of interest.

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Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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