Research article

Comparative study on locally produced bivalent inactivated Newcastle disease vaccine in broiler chicks
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Abstract
The study included titration of the Newcastle disease virus strains before preparation of the vaccines used in this experiment, by detection of EID50 for these strains. This study regarded the first one in the country in the preparation double strain oily Newcastle disease virus vaccine. The immune responses for this vaccine were compared with those of single strain NDV vaccine. The same vaccination program was used for all groups included in this study in which live attenuated (Lasota strain) vaccine given at one day old as an installation by eye and nostril, and subcutaneous injection of oily vaccine at the neck region. A total of 120 commercial broiler chicks was divided into fourth groups (1th) group vaccinated with live attenuated vaccine and bivalent (B1, Lasota ) oily vaccine while the (2th) group vaccinated with live attenuated vaccine and (Lasota) oily vaccine and (3th) group vaccinated with live attenuated vaccine and (AG68) oily vaccine, (4th) group C was left unvaccinated as control, the Immune response for all groups was measured at (1) day old, (7), (14), (21), and (35) days old by hemagglutination test (HI) and Enzyme linked immunosorbent assay (ELISA). The results showed gradual recreation of immune response titer at 7, 14 and 21 days post vaccination intervals with non-significant differences (P>0.05) among vaccinated groups at (7, 14) days and significant difference between vaccinated groups and control group (P<0.05) at (21) days old. The third group reflected better titer among vaccinated groups, and the result shows increased antibodies titer at (35) day in comparison with antibodies titer at (14, 21) days intervals. With statistical differences (P<0.01) among (third) group and second, and control group). However all groups gave higher titer than the control group (P<0.01) and the result of (HI) test agreement with (ELISA) test while the protection percentage were measured by challenge test, it was found to be (100%) for group (3th) vaccinated with live attenuated vaccine and bivalent (B1, Lasota) oily vaccine, (92%) for the second groups (2th) (96%) for the first groups (1th) whereas the control group (4th) gave (10%) protection. This study indicates efficacy of prepared ND vaccines (B1, Lasota) as compared with monoclonal vaccines (B1, Lasota) and the high immunosuppressive capacity of the oily vaccine increased with virulence.

Keywords: broiler chicks, bivalent inactivated vaccine, Newcastle.

Introduction
Newcastle disease is a highly contagious disease, affecting domestic poultry, pet birds, wild birds, and the forms of disease vary between the sub-clinical and mild form to the sever and sudden death, therefore this disease considered very important disease around the world due to the significant economic losses in the commercial poultry industry (1). Many of the specialized research laboratories have studied many of the virus strains, which included some isolated strains, and in recent years have developed different strategies for vaccination programs, using The dead vaccine after using
live vaccine in endemic area, in the most susceptible areas of the disease, and made it capable of early resistance to virulence virus (2), many of the world's most developed countries in the poultry industry and under encouraging economic conditions have been able to reduce the spread of the disease to a degree where it can be reduced in severity. But in the less developed countries, the problem is still present, including Iraq, as it is difficult to achieve preventive security against infection by virus, especially in recent years because of the presence the infection in neighboring countries and the ease of transmission of the virus (3), from the foregoing, our current study included the production of a killed vaccine from several strains, and use it experimentally assess its efficiency and the degree of protection it produce.

Materials and Method

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 410

Materials: The study was conducted using:

A-fertilized egg from a (golden nest) hatchery in Al-Mahmudiya area, the eggs were incubate in incubator at a temperature of (37)°C until the process of strains injection

B-A one-day old chick (Hibard) breeds, equipped from (success) hatchery in Abu Graib, and was raised in the experimental unit of the Agriculture College-Baghdad University.

Vaccines:

A- Newcastle oil emulsion vaccines (Lasota) and (AG68) they equipped by Al- kindy company for production of veterinary vaccines and medicines given according to the manufacturer's recommendations.

B- Newcastle oil emulsion vaccine (B1, Lasota). The two strains has been Titrated and propagated in fertilized eggs for vaccine preparation.

C- (Lyophilized) live attenuated ND vaccine (Lasota–strain) is equipped by Al-kindy Company.

3- Blood & Sabroad Dextrose agars

4-Heamagglutination & Heamagglutination inhibition test

5- in direct Enzyme linked immune-sorbent assay kit was manufactured by Proflok Company

6- Solutions and their concentration

A-Phosphate Buffer solution (PBS)

B- Al severs Solution

C-Physiological salt solution.

Preparation of the bivalent Newcastle Disease vaccine (B1, Lasota)

A-Titration was used in the preparation of the lentogenic vaccine strains (B1, Lasota), which was equipped by Al-Kindy company as a seed viruses. The titration done by calculating infected dose of 50% of developing embryos (EID50) for the two strains (B1) and Lasota according to the Sperman-Karber method, AF containing 1010.1ELD50 / 1ml.

B-Propagation of (B1, Lasota) strains

The two vaccine strains were grown and propagation separately. They were inactivated using formalin with a concentration of 40% Formaldehyde, Oil and aqueous phase adjuvant (Span 80 and Tween 80) were used according to the researcher's method (4).

C-Sterility Test & Safty Tes The tests were carried out to determine the efficiency of vaccines and their suitability for vaccination. (5).

D-Final tests of prepared oil vaccines; the efficiency of the vaccine formulation was measured according to the method of the researcher (4).

Experimental design: A total of (120) commercial meat broiler type (Hibard) chick at one day old were used in this study,10 chicks were sacrificed for to measure the level of maternal immunity at one day (zero time) they selected randomly, HI and ELISA tests used for this purpose, The remaining
chicks were divided into (4) equal groups which included
Group1: vaccinated with the live attenuated vaccine containing 9.7 10 EID50 / ml antigen (0.05 mg) by intraocular and intranasal route and(0.1 mg/ml) bivalent(B1,Lasota )oily vaccine by subcutaneous rout of neck area at 1 day old.
Group2: was vaccinated with live attenuated vaccine containing 9.7 10 EID50 / ml of the antigen (0.05 mg) by intraocular and intranasal route and (Lasota) oily vaccine with a dose (0.1) ml / bird s/c the neck area at one day old.
Group3: Vaccinated with live attenuated vaccine containing l 9, 7 10 of the EID50 / ml antigen by (0.05 mg) by intraocular and intranasal route and AG68 oily vaccine with a dose (0.1) ml / bird s/c the neck area at one day old.
Group4: Left without vaccination, considered control group
Blood samples were collected to measure the antibody titers, (10) samples were collected randomly for each group in two ways from the heart directly at (7.1) days and from the femoral vein at the age of (35, 21.14) days, and then placed the tubes in the refrigerator at a temperature of 4 °C for 16 hours the serum separated by using centrifuge 3000 cycles/ minute, the serum kept in the frozen temperature at(-20) C until use in serological tests.
Serological tests
A-Heamagglutination inhibition test:- Tested according to the beta method by Using micro titer plats to see the level of antibody in the chicks throughout the period of the experiment according to the method of researcher (5)
B-In Direct Enzyme linked Immuno-Sorbent Assay (ELISA) he test Used to check for Newcastle disease antibodies during the of the study Challenge Test: according to The method he researchers (6) a local velogenic Newcastle virus (Zahid strain) (Z2003) were used with a dose of 0.5 ml per bird which contain 109.3 EID50/ ml of Ag for all groups at 35 days By intranasal, and intraocular and in the mouth rout then the chicks monitor for 15 days, clinical signs on chicks, percentage of losses and postmortem lesions were recorded. Statistical Analysis: Results were analysis statistically by using the Fractional-Complete Randomized Design (F- CRD), and Dunca test, and the significant level used to determine the difference between these rates was (0.01, 0.05).

Results
1-Results of the titration of vaccine strains of Newcastle disease in embryonated eggs.
The dosage of the strains vaccine calculated for 50% of embryos according to the method (Sperman-karber) that mentioned by the researchers (7) and the recorded results are as shown in Table 1.
2-The results of the sterility &Safety test after laboratory tests the result of the tests showed that vaccines free from contamination and could be used to safely vaccination without adverse side effects.
3-Results of final tests of oily vaccines the results of the oil vaccine have demonstrated the efficacy of the (adjuvant material) and the persistence and stability of the oily vaccine prepared.
4-The results of inhibition of haematological agglutination (HI) before the vaccination measure titer of the antibodies that inhibit agglutination of the red blood cells of the one-day age chicks, and the general mean rate (115.2). As shown in Table (2) The results of the test showed no significant differences at the level of (P > 0.05) between the experiment groups at (7) days after the vaccination, as it was the mean value of antibodies (60.8) shown by the fourth Group (control group) as the lowest level of the antibodies, and (70.4) shown by the second group, the value of antibodies recorded for the other groups was (67.2) for the first and third groups, At the age of 14 days after vaccination, the results showed significant differences at the (P > 0.05) level
in all experiment groups, as the mean rate was (34.4). For the first group, (35.2) for the second group and (33.6) for the third group, while the control group showed a decrease in the value of antibody that inhibits agglutination was (28.8), the result showed lower value if compared at age (7) days. At age 21, the results showed significant difference at a level of (P<0.05) among the vaccinated groups if compared with the group control where the third group showed the high titer of the antibodies with other groups as the recorded mean value (32.0), while the control group showed the lowest value of the inhibitor antibodies if compared with other groups, the recorded mean value was (8.8) , the first group was taken second ranked at a mean value of (26.4), followed by the second group at a mean value of (25.6). At the age of (35), the results showed varying mean values between vaccinated groups, the third group showing a high of antibodies titer, reached (64.0) while the titration of antibodies converge in first and second groups, at the value of (48.0) and (41.6) respectively, the results showed a level of significant difference (P<0.01) where the third group surpassed the second group. In addition to there are no significant differences among the vaccinated groups, and the first group, and on the other hand, all groups as exceed the control group at a level (P < 0.01).

5-Results of indirect immunological adsorption test (ELISA):
As shown in Table 3. The test results showed their compatibility with the results of the haemagglutination inhibition test, in addition the value of antibodies in the vaccinated chicks groups dropped gradually from what it is before vaccination, the mean of maternal antibodies value recorded at the age of one day (7863.4), and fall back at the age of (7) days and significant differences recorded at the level of (P>0.05) among the experiment groups were the lowest mean value of antibodies is (4549.3) for the control group followed by the third group ascending by mean value of the antibodies reached (5112.7), The first group is at a value of (5245.2), and the second group is at a value (5313.0). The results of the titration of antibodies at the age of 14 days groups after vaccination showed non-significant differences at the level of (P>0.05) among the different experiment With the superiority of the second group at the value (4153.0), then the first group (4064.1), then the third group at the value (3987.8), and the vaccinated groups exceeded the control group, with the recorded antibodies value (3038.4). At the age of 21 days after vaccination, the level of antibodies continued to fall below the level of 14 days, and the third group exceed all groups at the value (3493.4) the first group at value (3041.1), then the second at the value (2870.4), and the results showed significant differences at the level of (P<0.05) among vaccinated groups compared to the control group, which had an arithmetic mean antibody value (1028.9). The results of the antibody titration of the experiment chicks at the age of (35) days continued to exceed the third group, with the value of antibodies that given (5075.8), and the regulate of the groups remained as it age was (21) day after the vaccination with clarity in the high standard of antibodies began to vaccinated groups and significant superiority at the level of (P<0.01) of the third group, on the second group that reached antibody value (3843.9), and there no significant differences among the experiment groups that vaccinated against virus of Newcastle disease and the first group that reached the value of antibodies recorded in this Period (4299.5) all groups showed a level of significant superiority at level (P<0.01) on the control group, which showed a decline in the level of antibodies at an mean value (97.3). The results of the tests of the haemagglutination inhibition (HI) and the indirect immnosorbent assay linked enzyme (ELISA) indicate for all the periods after vaccination rise up antibodies standard at age
(35) day of vaccines administration for all the groups.

6-Challenge Test Results:
The results of the challenge test for the experimental groups after exposed to virulent Newcastle disease virus with a dose of EID50/0.1 ml 8.3 was (10) showed severe respiratory signs at the second and third days of the occurrence then the chicks dead, the p.m. lesion of dead chicks, reveal presence of hemorrhagic ulcers in the lining of proventriculus, swelling and bleeding in cecal tonsils and ulcers in the Bayer batches and intestine congestion, and presence mucous secretions, in addition to petechial hemorrhage in the heart, congestion, liver enlargement, and trachea congestion. The highest rate of mortality in the control group was recorded compared to the other groups and the percentage of protection (10%) The third group gave the highest protection rate (100%) The protection rate for the second group (92%) while the first group reach rate (96%). As shown in Table (4)

Table (1) reveal the infected dose of 50% of developing embryos EID50 for vaccines strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Strains infected dose of 50% of developing embryos EID50</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG68</td>
<td>10^{8.3} EID&lt;sub&gt;50&lt;/sub&gt;/0.1 ml 10^{-9} EID&lt;sub&gt;50&lt;/sub&gt;/ml</td>
</tr>
<tr>
<td>Lasota</td>
<td>10^{8.9} EID&lt;sub&gt;50&lt;/sub&gt;/0.1 ml 10^{-9} EID&lt;sub&gt;50&lt;/sub&gt;/ml</td>
</tr>
<tr>
<td>Lasota</td>
<td>10^{9.1} EID&lt;sub&gt;50&lt;/sub&gt;/0.1 ml 10^{-10} EID&lt;sub&gt;50&lt;/sub&gt;/ml</td>
</tr>
<tr>
<td>B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10^{9.3} EID&lt;sub&gt;50&lt;/sub&gt;/0.1 ml 10^{-10} EID&lt;sub&gt;50&lt;/sub&gt;/ml</td>
</tr>
</tbody>
</table>

Table (2) reveal ND antibody titers mean for different periods measured by a HI test

<table>
<thead>
<tr>
<th>Groups</th>
<th>ND antibody HI titers (mean ± SE) Zero Time( )</th>
<th>ND antibody HI titers (mean ± SE) (7 days)</th>
<th>ND antibody HI titers (mean ± SE) (14 days)</th>
<th>ND antibody HI titers (mean ± SE) (21 days)</th>
<th>ND antibody HI titers (mean ± SE) (35 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>18.59±115.2 A 11.13±67.2 B 2.93±26.4 B</td>
<td>BD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>18.59±115.2 A 13.32±70.4 B 3.2±35.2 B</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>18.59±115.2 A 11.13±67.2 A 5.56±33.6 B</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>18.59±115.2 A 8.86±60.8 A 5.08±28.8 A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± S.E.
Different letters refer to significant differentiations between groups.
Similar letters denote differences between the groups.

Table (3) reveal ND antibody titers mean for different periods measured by ELISA test

<table>
<thead>
<tr>
<th>groups</th>
<th>ND antibody titers (mean ± SE) Zero Time( )</th>
<th>ND antibody titers (mean ± SE) (7 days)</th>
<th>ND antibody titers (mean ± SE) (14 days)</th>
<th>ND antibody titers (mean ± SE) (21 days)</th>
<th>ND antibody titers (mean ± SE) (35 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>7863.4± 426.05 A 5245.2± 560.45 AB</td>
<td>4064.1± 380.67 BD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>7863.4± 426.05 A 5313.0± 533.39 B</td>
<td>4153.0± 383.46 BD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>7863.4± 426.05 A 5112.7± 391.89 AB</td>
<td>3987.8± 408.0 AB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>7863.4± 426.05 A 4549.3± 323.35 A</td>
<td>3038.4± 415.34 A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± S.E.
Different letters refer to significant differentiations between groups.
Similar letters denote differences between the groups.
Table (4) reveal the result of challenge test as compare with the result of HI and ELISA test at (35) days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>HI titer before challenge test</th>
<th>ELISA titer before challenge test</th>
<th>Total count / mortality</th>
<th>protection</th>
<th>mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>48.0</td>
<td>4299.5</td>
<td>25/1</td>
<td>96%</td>
<td>4%</td>
</tr>
<tr>
<td>Group2</td>
<td>41.6</td>
<td>3843.9</td>
<td>25/1</td>
<td>92%</td>
<td>8%</td>
</tr>
<tr>
<td>Group3</td>
<td>64.0</td>
<td>5075.8</td>
<td>250/</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Control group</td>
<td>1.2</td>
<td>97.3</td>
<td>10/9</td>
<td>10%</td>
<td>90%</td>
</tr>
</tbody>
</table>

Discussion

Newcastle disease is a dangerous disease in Iraq and the infection consider problem until now in spite of using different types of vaccines it is remarkable that a number of Iraqi researchers have demonstrated the efficacy of locally manufactured vaccines in the challenge of the infection such as a researcher (8), in this study, the bivalent oily vaccine, which contains more than strain of Newcastle disease virus , is the first attempt in the country to study the immunity efficiency of such a bivalent vaccines compared to single vaccines, and the study adopted a unified vaccination program of subcutaneous injection of the oil-emulsion vaccine preceded by a live-in-the-eye and nose using (Lasota strain), where the live vaccine stimulates local immune response and at the same time the slow spread of the killed antigen from injection site by the continuous stimulation of the immune system (9), after measuring the immune response of the groups by using heamagglutination inhibition tests and immunosorbent assay enzyme (ELIZA) the standard of antibodies measured by the age of 7 days after vaccination, showed a significant reduction in the antibodies compared with the one-day age. The reason for the constant decline of the standard of maternal antibodies and their depletion as age progresses, as do neutralize antibodies partial equivalence of the killing viral antigen this line agree with the researchers (10) The results showed no-statistically significant differences at the level of P> 0.05 at (14, 7) days among the groups this is in line with what the researchers said (11) because giving the living vaccine intraocular method stimulates a localized immune response despite maternal immunity while the present. Differences in the standard of antibodies are caused by the individual differences in the immune response among the birds of and different the values of maternal antibodies titration in each group and the age of (21) days, the results showed a continued decline the mean of antibodies compared with the value of antibodies at the age of (7, 14) days to reach the lowest level of this age and this is consistent with the mention of (12) that the antibodies formed after the vaccination of the live vaccine may reach the lowest level of (14-21) days of vaccination depending on the amount of the martial antibodies and comparison with the control group the results showed a Statistically significant differences at the level (P<0.05). The third group provides the remainder of groups after (21) days of vaccination indicating the preference of this group and the efficacy of the composition of the vaccine from the rest of the vaccines, the results for the age of (35) and compared to the mean of antibodies titers measured by the age of (14-21) day showed a significant increase, indicating to increase the efficiency of inactivated vaccine over time to stimulate a higher level of antibodies, and the results showed statistical significant differences at the level of (P<0.01) among the fourth group in comparison with the first and second groups and control groups, which are attributable to the effecting of the type of vaccine strain in Level of immune response this is consistent with (13), that the mean of antibodies that is produced by the (AG68) strain is compared to Lasota strain it will be higher, For the vaccinated group by oily
vaccine (B1, and Lasota strain), the mean of antibodies recorded is higher than that of vaccinated group by oily inactivated vaccine of Lasota strain is not a statistic significant difference, that same with researchers (14), that there is no interfere between vaccine strains if multiple of the case separately, also the inactivated strains cant multiplication into the infected cell is the same as that of the living attenuated vaccine strains, which may interfere with each other at Give it at the same time. The high titer of antibodies in the bivalent vaccine is due to an increase in the amount of antigen, as the absence of reproduction for the killed viral antigen is the titer of the measured antibodies depending on the number antigen units (viral particles) in the dose of the vaccine, which is subsequently found in the tissues to stimulate the production of antibodies (15). The results of the Eliza Test, however, concurred with the results of the tests of agglutination, with a significant increase in the titer of the measured antibodies as compare to the last Test, and the reason is that the Eliza test is more accurate, and is sensitive to measuring the few levels of antibodies that inhibit the agglutination, and neutralization of the virus, fixation the compliment and sedimentation of viral antigen while heamagglutination inhibition test measuring the only antibodies that inhibit the agglutination. This is in agreement with researcher (16) in the present similarity between both tests. As the results showed, the titer of antibodies of the non-vaccinated control group was decline gradually, depending on the amount of maternal antibodies transmitted from mothers to hatched chicks and for the challenge test that is an important test that determines the efficacy of the vaccine and the vaccination program has shown the results are an agreement with the test results of the a HI and Eliza tests, and this strain caused the mortality rate of 90% of non-vaccinated control group due to the depletion of maternal antibodies by passage of Time (8) The high level of protection in the third group is due to the fact that (Ag68) is highly capable of the production of neutralizing antibodies of viral antigen, it has been isolated from field infection in Iraq and is considered to be antigenetical similarity among the strain (AG68).and Virulence field strain has a role in the high immunity protection ratio. (17) The reason for the high protection ratio in first Group as compare with second group, which confirms what researchers have pointed out (18) in that the efficacy of the viral vaccines by induce immunity against the disease varies depending on the used strain, the amount of antigen in the vaccine, and for the used vaccination program, it has proved the efficacy of the challenge at the age of (35) day, because the vaccination of the live vaccine is working on the definition of the immune system to the viral antigen and then stimulates it, and stimuli the memory cell of killed vaccine, which is subsequently given, and many studies have demonstrated the efficacy of vaccination by intraocular for a long period of time and a high degree of immunity against the disease. (19).

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