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STUDY SEROLOGIC STATUS OF NEWCASTLE DISEASE IN BROILERS CHIKENS BY HAEMAGGLUTINATION INHIBITION TEST IN SULIAMANIA PROVINCE

Hanan Hasan. Ali
Department of Microbiology / Immunology, Suliamani University, Iraq

ABSTRACT
Newcastle disease (ND) is a common problem in poultry farms of Iraq. Several serological and molecular tools are applied to diagnose the infection and predict its effects. Haemagglutination inhibition (HI) is a serologic test that commonly used in diagnostic laboratories. In order to determine NDV antibody status in broiler flocks of Suliamania province at the end of rearing period, A total of 112 blood samples from different source (Iraq , Iran , Belgium) , different age (20-29 , 30-39 , 40-50 dyes) vaccinated and not vaccinated groups were collected from different Commercial broiler farms, the specific immunity of Chicken source Iraq at different age was more higher than Iran origins, samples were positive for NDV antibodies. High prevalence of NDV infections in poultry flocks indicates that present applied strategies are not fully effective in the case of this infection. Biosecurity measures, vaccination programs and surveillance are altogether effective strategies in prevention of infection.

KEYWORDS: Newcastle disease , Haemagglutination inhibition (HI) , specific immunity ,Broilers chickens.

INTRODUCTION

Newcastle disease (ND) is one of the most serious infectious diseases of poultry , which causes mortalities as high as 100% and causes a great loss in domesticated chickens and poultry industry which are one of the main sources of protein for human consumption (Njagi et al .,2010). The etiological agent is Newcastle disease virus (avian paramyxovirus 1 (PMV1)) which belongs to the genus of Avula virus under the family of Paramyxoviridae in the order of Mononegavirales (Alexander , 2003) .Newcastle disease (ND), this disease is more costly than any other poultry virus because it requires repeated testing for trade purposes and expensive control measures NDV has a large host range and has been reported to infect more than 240 species of birds (Alexander , 2001).The terms velogenic viscerotropic, mesogenic, and lentogenic are applied to Newcastle disease virus strains of high, intermediate, and low virulence respectively. Whereas in the velogenic strains, morbidity and mortality rates near 100% in susceptible chickens, neurological signs or sever depression are the most obvious clinical signs, and some of non vaccinated birds may be found dead with no detectable signs of prior illness Influence of age and sex (Alexander , 2003) .Newcastle disease virus infects most avian species. The serious consequence in the disease (frequently fatal) occurs when virulent strains of the virus infect domestic chickens . The epidemiology of ND in commercial chickens is fairly well understood. Infected chickens are the usual source of virus, which can be transported mechanically by fomites or by people. Preventive measures include vaccination and attention to biosecurity (Alders and Spradbrow, 2001). In Iraq, live and killed vaccines are used for protection of broiler flocks against disease.The best method for diagnosis of the disease is isolation and characterization of agent. Serological tests are useful tools in diagnosis of infection. Hemagglutination inhibition (HI) test is the most commonly used test for detection of immune response in affected birds (Alexandr and Senne, 2008). Value of serology in diagnosis of disease depends on vaccination history of birds and on prevailing disease conditions (OIE Terrestrial Manual 2012). Broilers chickens are susceptible to ND, which, in developing countries is the most important constraint to rural poultry production. Similar epidemiological factors probably apply to the spread of NDV in commercial chickens and village chickens. Both epizootic and enzootic ND are recognized in village chickens. Epizootics occur when the virus is introduced to a susceptible population. Spectacular outbreaks with high mortalities result. Enzootic ND occurs when the virus circulates slowly in a partially immune population. In this case, there are too few susceptible birds to maintain an outbreak and the occasional birds that die do not come to veterinary attention. Possibly a breeding population of as few as 500 birds can sustain the virus, the sex of the birds may influence the morbidity and mortality of Newcastle disease virus (Barman, 2003).People of Sulaimani province from the past, have a history of rearing and breeding chickens and other birds for the purpose of meat production, egg production, and even for pleasure. Inspite of the development of poultry production many diseases were prevalent in Sulaimani province including the Newcastle disease which is the most serious disease and causesa great loss in the poultry production which is a threat for the future rearing of poultry industry by the farmers (Abdull-sattar, 20). The main objective of this study was to

determine antibody titers for non spesific and spesific immunity to Newcastle disease virus in broiler chicken, assess the prevalence of anti-NDV antibodies Titer in Broiler chickens that vaccinated and not vaccinated , to evaluate the effect of age group in anti-NDV Titer in Broiler chickens & to assess comparison of serologic status of Newcastle virus in Broilers chicken seropositive different source (Iraq , Iran , Belgium) , and different age .

MATERIALS & METHODS

The Study Area and samples collection:

The study was carried out in Suliamania Province from December 2013 to march , 2014 . A total of 112 blood samples from different source (Iraq and Iran), different age , vaccinated and not vaccinated were collected from different commercial broiler farms, For Haemagglutination Inhibition (HI) test.

Laboratory procedures

A. Collection and processing of blood

Blood was collected from by heart puncture directly, and collecting the blood into universal bottles, without anticoagulant. Serum was separated from respective clotted blood samples by centrifugation at 3000 rpm for 10 minutes, , then the sera were collected in the Eppendorf tubes and labeled then stored in deep freeze at - 20˚C for further studies until hemagglutination –inhibition (HI) test was performed.

B . Preparation of washed chicken red blood cells:

Chickens used for the supply of blood for the preparation of red blood cells should be housed separately from chickens used for other purposes. Usually they are not vaccinated with Newcastle disease vaccine. Blood from vaccinated chickens is acceptable if that is all that is available. Collect blood from more than one chicken. A collection of 1.0 mL from each of three chickens will usually give between 8 to 10 mL of a 10 percent solution of washed red blood cells there are Three steps in the preparation of washed red blood cells.

1. Collection of the blood.
2. Washing the red blood cells.
3. Preparation of a 10 percent suspension of red blood cells for storage.

C. Anticoagulant

Anticoagulants are substances that prevent blood from clotting. An ethylenediamine tetraacetic acid (EDTA)anticoagulant, therefore, uses this type of acid to stop the clotting process. Blood is collected from the Heart puncture of three chickens. It is mixed with an anticoagulant.

D. Techniques for collection of blood into anticoagulant

1. Place Special tubes have EDTA.
2. Bleed the first chicken and take 1 mL of blood. Immediately remove the needle from the syringe, gently push down the plunger and transfer the blood to the bottle. Replace the lid on the bottle and rotate it gently to mix.
3. Repeat Step 2 and take 1 mL of blood from a second chicken. Transfer the blood to the bottle of blood and anticoagulant and rotate it gently to mix.
4. Repeat Step 2 and take 1 mL of blood from a third chicken. Transfer the blood to the bottle of blood and anticoagulant and rotate it gently to mix.

MATERIALS

- Blood in anticoagulant
- PBS solution for washing.
- Sterile 20 mL centrifuge tube with a lid or a 20 mL bottle with a lid, to fit the centrifuge bucket.
- Pasteur pipette or 10 mL graduated pipette with pipette filler.

Procedure Technique

1. Transfer the blood to a container suitable for centrifugation.
2. Add PBS to fill the container. Mix gently.
3. Centrifuge at 500 g for 10 minutes.
4. Use a Pasteur pipette or a 10 mL glass pipette to remove the supernatant. Take care not to disturb the pellet of red blood cells.
5. Repeat Steps 2, 3, and 4 twice.

The cells have now formed a pellet after being washed three times and centrifuged.

EQUPEMENTS

1- Precision pipettes (range from 5-200 µl)
2- Disposable pipette tips
3- Distilled water
4- Wash bottles
5- Container: 1 to 2 liters for Washing Buffer .
6- Microplate 96 wells .
3. Serology

Presence of NDV antibody was detected by hemagglutination inhibition test as described by OIE (2000). A cut off titer of 1:4 was considered specific indicating that the birds had been previously exposed to the virus, while titers less that this value were considered non specific (Numan et al., 2005). The validity of the results was assessed against a negative control serum included in the test. The HI titers were determined in all chicken, and the geometric mean titter (GMT) of each group calculated.

A. Haemagglutination test (HA) procedure

1- Twenty five microlitres of normal saline were dispensed into each well of v- shape bottom 96 wells microtiter plate.
2- Twenty five microlitres of virus suspension were placed in the first well.
3- Two fold dilutions of 0.025 ml volumes of the virus suspension were made across the plate.
4- 11th well contains 25 µl normal saline and 0.025 ml of 1% washed RBC, for negative control.
5- 12th well contains 25 µl of the antigen (virus) and 1% of washed RBC, for positive control.
6-Twenty five microlitres of 1% chickens RBCs were dispensed to each well.
7-The suspension was mixed by tapping the plate gently the RBCs were allowed to settle for about 40 minutes at room temperature (OIE, 2004).

When control RBCs were settled to a distinct bottom, HA was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs.

The titration was determined as the highest dilution giving complete HA (No streaming); this represented 1 HA unit (HAU) and was calculated accurately from the initial range of dilutions (OIE, 2004).

B. Haemagglutination inhibition test (HI) procedure

The serum samples were tested to determine the antibodies against NDV, using the standard HI method (Allan and Gough, 1974). The antigen used was reconstituted commercial NDV LaSota vaccine .

1- Twenty five microlitres of normal saline were dispensed into each well of a plastic U-shape bottom well microtiter plate.
2- Twenty five microlitres of serum were placed into the first well of the plate.
3- Two fold dilutions of 25 µl volumes of the serum were made across the plate.
4- Four (HAU) virus/antigen in 25 microlitres were added to each well and the plate was left for a minimum of 30 minutes at room temperature, and 25 µl of 1% (v/v) chicken RBCs was added to each well.
5- 11th well contains 25 µl normal saline and 25 µl of 1% washed RBC, for positive control.
6- 12th well contains 25 µl of the antigen (virus) and 25 µl of 1% washed RBC, for negative control.
7- After gentle mixing, the RBCs were allowed to settle for about 40 minutes at room temperature, with checking every 5-10 minutes. (OIE, 2004).

Result: The HI titer was the highest dilution of serum causing completeinhibition of 4 HAU of antigen. The agglutination was assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.025 ml normal saline only) was considered to show inhibition (Grimes, 2002). A titer greater or equal to 4Log2 was taken as positive (Allan and Gough, 1974; Wang and Iorio, 1999 OIE, 2000 ; Faris, 2006).

Statistical analysis

Statistical analysis was performed using SAS . Data were analyzed by using One Way Analysis of Variance (ANOVA). The titers were transformed tologarithm to estimate the geometric means. T-test was used to assess significant difference among geometric means of antibody titers. $P < 0.05$ was consideredstatistically significant (Numan et al,. 2005 ; Kemboi et al ., 2013) .The geometric mean titers were compared across the various age groups (vaccinated and non vaccinated) and compared also between Iraq and Iran.

RESULTS & DISCUSSION

HI Test to Screen Level of Antibodies in Blood Sera HI test was performed in V–bottomed microtitration plates. Test procedure was conducted according to the methodology of OIE Manual (2002). Serum was tested in two fold serial dilutions upto 10th well in microtiter plate. For virus suspension 4 Haemagglutinating Units (HAU) Type B1 Lasota antigen lentogenic strain was added upto 11th well. After keeping the plates at room temperature for 30minutes 1% chicken RBC suspension was added to each well. The 11th well contains antigen and RBCs as the positive control and the 12th well contains only RBCs as the negative control. After gentle mixing, the RBCs were allowed to settle at room temperature for 40minutes and agglutination was assessed by tilting the plates. Finally, at the end of 40minutes samples were showing central button shaped settling of RBCs which were recorded as positive and maximum dilution of each sample causing Haemagglutination Inhibition (HI). This was used to estimate the HI titer (Figure 1). The HI titer of each serum sample was expressed as reciprocal of the serum dilution.

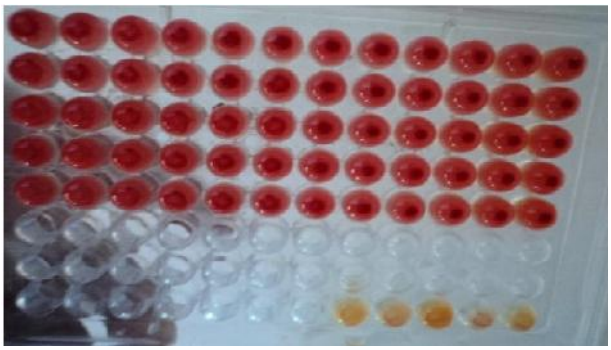

FIGURE 1: Haemagglutination Inhibition test (HI test) (Buttoning)

From the total 112 serum samples of two different Broiler source farms Iraqi source 50 serum samples were ranging from 20–29 days and 30-39 days of age, 50 samples and other 62 samples were taken from Iranian source at same age. Vaccinated Birds of all ages were found positive for specific immunity with positive percentage of 100% for Iraqi source (Table 6). The HI antibody titer for non vaccinated Iraqi source varied from log2 to log7 with a GMT of log14.49 , 13.12 at the age of 20–29days and 30-39 days with a GMT of 14.49 and 13.12 respectively (Table 1) , with specific immunity 92.85% and 93.33% respectively (Table 2). While the broilers Chicken from Iranian source show geometrical mean less than Iraqi chicken at 6.56 and 7.99 respectively at different age (Table 3) with specific immunity 93.33 , 68.75 respectively (table 4) There was a significant difference between the higher geometric mean antibody titers in Iraq (14.49), and that in Iran (6.56) repectively , while with vaccine also showed there was a significant difference between the higher geometric mean antibody titers in Iraq (21.11) , (Iran 10.47) (Table 1,3 ,5 ,7) .

TABLE 1: Hemagglutination Inhibition titers of different age group of chicken without vaccine in Iraq

Age Group/day	No. of Sample	Antibody Titer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	GMT
20-29	14	1	2	3	3	2	3	-	-	14.49
30-39	15	1	3	1	2	6	2	-	-	13.12

TABLE 2: Serum samples of broiler chicks showing immune response of chickenwithout vaccine in Iraq

Age/day	No. of sample	Specific immunity	Non specific immunity	Specific immunity%
20-29	14	13	1	92.85
30-39	15	14	1	93.33

TABLE 3: Haemagglutination Inhibition titers of different age group of chicken without vaccine in Iran

Age Group/day	No. of samples	Antibody Titer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	GMT
20-29	15	1	4	6	1	2	1	-	-	6.56
30-39	16	5	2	1	1	2	3	-	2	7.99

TABLE 4: Serum samples of broiler chicks showing immune response of chicken without vaccine in Iran

Age/day	No. of sample	Specific immunity	Non specific immunity	Specific Immunity %
20-29	15	14	1	93.33
30-39	16	11	5	68.75

Poultry viral diseases constitute one of the major problems facing the rapidlyn expanding poultry industries, these diseases cause considerable economic looses, such as ND. For this reason, veterinary authorities rely fairly on vaccination. The ideal vaccination regimen is depending basically on selecting the type of vaccination method (Hafer, 2005). An effective vaccination plan should result in a general improvement of the health status and the productive performance of the vaccinated population .The efficacy of vaccine administration and the level of immunological response in vaccinated birds can be serologically monitored. Two methods are used to measure antibody titers: the HI (Alexander, 2008). Table 5 .6 showed the immune response against NDV vaccines which have been administered via different age in this study as detected by HI . It is important to mention that the non vaccinated chicken had a highly significant decreased (GMT) in antibody titer from 20-39 day, whereas the vaccinated groups showed an opposite trend as indicated in (Table 4, 6) These findings were in agreement with those of Ali et al.(2004)

TABLE 5: Haemagglutination Inhibition titers of different age group of chicken with vaccine in Iraq

Age Group/day	No. of samples	Antibody Titer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	GMT
20-29	7	-	-	-	1	4	-	2	-	21.11
30-39	14	-	7	1	-	-	4	2	-	15.22

TABLE 6: Serum samples of broiler chicks showing immune response of chicken with vaccine in Iraq

Age/day	No. of sample	Specific immunity	Non specific immunity	Specific Immunity %
20-29	7	7	-	100
30-39	14	14	-	100

TABLE 7: Haemagglutination Inhibition titers of different age group of chicken with vaccine in Iran

Age Group/day	No. of samples	Antibody Titer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	GMT
20-29	18	-	5	5	4	4	-	-	-	10.47
30-39	13	2	5	2	-	3	1	-	-	13.45

All samples irrespective of age represents positive for specific immunity with a positive percentage of 100 where Tariq and Taib (2010) found 26%, 54% and 75% positive for specific immunity at 24–36, 36–48 and more than 48 weeks in chicken which is quite lower than this one. HI test has been widely used for the estimation of titers of specific antibody in the sera of individuals infected with certain viruses including NDV, Influenza virus etc as these viruses can agglutinate erythrocytes (Serrão et al., 2012). The present study, the birds in two diffrent age showed the highest level ofantibody titers in vaccinated and Iraqi source than Iranian with high spesific immunity (GMT 21.11, 15.22) and showed relatively low susceptibility to clinical infection. The HI titers obtained in the present study were higher than those reported by Biswas et al., 2006 who recorded that 64%, 47.4%, 62.6% and 56.3% of sonali chickens in southern part of Bangladesh had protective HI titers against NDV in autumn, winter, summer and rainy seasons respectively. Other factors like poor vaccine quality is a common problem in developing countries and results poor manufacturing standards, lack of adequate storage facilities, application of expired vaccine batches, faulty administration and handling during transportation (Vui et al., 2002). Heat stress and water deprivation also lead to production of steroids and thus result an immunesuppression. Quality of water which is offered to the birds was also found questionable which might hinder the development of specific immunity. Inappropriate vaccination schedule also leads to the neutralization of maternally derived antibodies and resultantly making the birds more susceptible to the infection (Njag et al .,2012).

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