Full Length Research Paper

Quantification of antibodies against poultry haemagglutinating viruses by haemagglutination inhibition test in Lahore

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A study was conducted for quantification of antibodies to haemagglutinating viruses such as AIJ (H9N2 and H7N3), NDV and IBV in vaccinated poultry flocks. A total of 198 serum samples from different commercial broiler and layer farms in Lahore, Pakistan, were checked for their serological statuses. Haemagglutination inhibition (HI) test was performed to determine the titer of serum antibodies against the above mentioned viruses. It was observed that 25 and 44% samples were positive for H7N3 and H9N2 in broilers, respectively. The seropositivity of H7N3 and H9N2 in serum samples of commercial layers were 66 and 94%, respectively. It was recorded that 96% of the samples in broilers and 100% of those in layers, were found seropositive for antibodies against NDV. Moreover, 69 and 86% were seropositive for IBV in broilers and layers, respectively. Calculated geometric mean titers for groups of 0-2, 2-4 and 4-6 weeks of age were found to be 36.8, 78.8, and 111.4 for H9N2; 48.5, 157.6, 222.9 for NDV; and 97, 84.4, 111.4 for IBV, respectively. It was concluded that the samples were not having protective antibody titers indicating unsuccessful vaccination practiced in these poultry farms which may lead to significant economic losses.

Key words: Avian influenza virus, Newcastle disease, bronchitis virus, haemagglutinating.

INTRODUCTION

Livestock sector plays an important role in the agricultural economy of Pakistan. It accounts for almost 50% of agricultural value added and about 11.4% of the gross domestic product (GDP) (Economic Survey, 2003-04). In Pakistan, the investment in poultry sector is about 1 billion US $. The importance of poultry industry can be judged from the fact that every family in rural and every fifth family in urban areas are directly or indirectly associated with poultry production (Sadiq, 2004). In Pakistan, poultry industry is facing various diseases such as Newcastle disease (ND), infectious bronchitis (IB), infectious bursal disease (IBD), egg drop syndrome (EDS), hydropericardium syndrome (HPS) and avian influenza (AI). These diseases are causing high economic losses in terms of high mortality, morbidity, stress, decreased egg production and hatchability all over the world including Pakistan (Alexander, 2000). Several viruses have the ability to agglutinate chicken red blood cells and cause ND, EDS, AI and, after special enzyme treatment, IB. The test is serotype-specific for these viruses and different antigenic viral strains of AI, IB occur. Avian influenza viruses are classified in the family Orthomyxoviridae, genus Influenza virus A. The surface is covered by two types of glycoprotein projections, that is, rodshaped trimers of haemagglutinin (HA) and mushroom shaped tetramers of neuraminidase (NA). The HA is the major antigen that elicits antibodies protecting against death and clinical signs. These antibodies are HA subtype specific and can last for periods greater than 35 weeks (Brugh and Stone, 1987). Protection by maternal antibodies to homologous HA last probably for the first
two weeks after hatching (Swayne et al., 2003). AI is a contagious viral disease, worldwide in distribution. It affects chickens of all ages with variable morbidity and mortality. With highly pathogenic AIV, morbidity and mortality rates are very high (50–89%) and can reach 100% in some flocks (Capua et al., 2000). Currently important strains of AIV that are prevalent in Pakistan are H5N1, H7N3, and H9N2. Outbreaks of these strains cause serious economic losses in Pakistan every year. Vaccination against H7N3 and H9N2 are practiced in commercial layer flocks and breeder flocks but less frequently in broiler flocks. But antibodies can be found in unvaccinated flocks suggesting the exposure of poultry birds to these viral strains from time to time.

Avian IB is caused by a Coronavirus. IB is an acute, highly contagious viral respiratory disease of chickens, characterized by tracheal rales, coughing, and sneezing. In addition, the disease may affect kidneys, and in laying flocks there is usually a drop in egg production and egg quality. Mortality may occur in young chicks due to respiratory or kidney infection (Cavanagh and Naqi, 1997). The respiratory infection is usually mild and self-limiting in chickens. A serologic test such as haemagglutination inhibition (HI) is commonly employed (DeWit et al., 1997). The flocks in Pakistan are routinely vaccinated with Massachusetts-41 (M-41) strain of IBV, the problem still exists and the disease prevalence is routinely observed in vaccinated flocks (unpublished data). Interestingly, the highest antibody titers in nonvaccinated flocks (8.7%) are those against M-41 strain as well (Muneer et al., 1987), thereby suggesting the presence of this and possibly other IBV strains in Pakistan. There are age and seasonal associations with the IBV infections reported in Pakistani flocks (Javed et al., 1991). There are more than 20 serotypes within IBV known so far worldwide (Lee and Jackwood, 2000).

ND is also considered as one of the major threats to the poultry raisers in Pakistan. Its high morbidity, mortality and reduced productivity, resulting in remarkable economic losses every year. ND is caused by Newcastle disease virus (NDV), and characterized by sudden appearance and rapid spread of the virus within the flock with high morbidity and mortality. NDV is synonymous with avian paramyxovirus type 1 (APMV-1) and is an important member of the family Paramyxoviridae (Alexander, 1997, 1998; Lamb et al., 1996). Clinically, NDV can be categorized into three main pathotypes, that is, lentogenic, mesogenic and velogenic strains (Alexander, 1997).

Most of fatal avian viruses are known to have haemagglutinating activity (Sarker, 2006). As such this property is being used for the preliminary screening of the poultry farm for the presence of any such devastating viruses such as AI, ND, EDS etc. (OIE, 2006). Serological examinations are of major importance for monitoring vaccination response and to detect field infections in poultry flocks. Many serological tests are easy to perform, relatively cheap and can be performed in smaller labs. In contrast, other methods like virus isolation; histopathology and molecular biological examinations (for example, PCR techniques) require special equipment and trained lab technicians. Serological test are used to detect antibodies against various viruses and bacteria relevant for poultry. Depending on the test system different antibody types are detected (IgG and/or IgM) (Voss, 2006).

The objectives of current study were to determine the efficacy of the program of vaccination practiced. For this purpose, antibodies were quantified against haemagglutinating viruses (AIV, subtypes H7N3 and H9N2; NDV and IBV) by HI test in commercial broilers and layer flocks in Lahore District and the antibody titers of AIV H9N2 subtype, NDV and IBV in different age groups of broiler birds.

MATERIALS AND METHODS

Study area and season

The study was conducted at the University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences Lahore, Pakistan, during the months of June until August 2009.

Sources of blood samples

The blood samples were brought by the local farmers of broiler and layer flocks located in the vicinity of Lahore. Each sample contained about 3-5 ml of blood in 5 ml sterilized disposable syringes. The blood was collected from the wing vein of bird. A total of 198 serum samples were brought by different commercial broiler and layer farmers for serological analyses of their poultry status during summer 2009. Out of a total of 198 sera, 96 samples were of broiler origin and the remaining 102 samples were from commercial layer farms. These serum samples were checked for the presence of antibodies against H7N3, H9N2, NDV and IBV.

Samples from broiler farms were divided into three groups according to their age, that is, 0-2, 2-4 and 4-6 weeks. HI test was performed to determine the serum antibody titers against H7N3, H9N2, NDV and IBV.

Collection of sera

The samples were further processed to obtain sera for HI test. The blood was kept in slant position at room temperature, overnight. After 24 h, the sera were transferred to 1 ml sterile, properly labeled Eppendorf tubes and placed at ~20°C until further used. Prior to conducting HI test, the serum samples were thawed and placed in a water bath at 56°C for 30 min to destroy heat labile non-specific agglutinins. For each sample, duplicate HI tests were conducted.

Source of antigens

The antigens of AIV (H7N3 and H9N2), NDV and IBV were obtained from UDL, UVAS, Lahore. Four HA units of each of these antigens were used in HI test. Atlantic fluid harvested from AIV inoculated eggs, separately for each of two H7N3 and H9N2 subtypes were used. Commercially available NDV vaccine (NDV-Lasota) was used as antigen to titrate antibodies against NDV. The IBV harvested fluid was first trypsinized followed by trypsin induced
haemagglutination. Whereas the activity of AIV subtypes and NDV was confirmed by HA test and Agar Gel Precipitation test.

Trypsinization

Working solution of reagent grade Trypsin (Sigma Chemical Company St. Louis, MO, USA) was prepared containing 2% Trypsin in phosphate buffer saline (PBS), with a pH adjusted at 7.2. Allantoic fluid from inoculated dead and live embryos was collected 72 hours post-inoculation (PI) and treated directly by mixing 0.25 ml of allantoic fluid (AF) from each set of inoculated embryos with 50 µl trypsin working solution. After the addition of Trypsin, all samples were held at 37°C for 30 min then placed at 4°C for 5 min.

Washing of RBCs

About 5-6 ml of blood was collected from unvaccinated broiler chicken through wing vein in a sterile syringe containing EDTA as anticoagulant. The blood and anticoagulant were gently mixed. The blood was centrifuged 1500-2000 rpm for 15 min. The plasma layer and buffy coat were withdrawn with the help of sterile Pasteur pipette and replaced with equal amount of normal saline and centrifuged again. This process was repeated twice. The volume of erythrocytes was measured and 10% stock erythrocyte suspensions were prepared. The working suspensions of 1% RBCs were prepared from the stock solution using PBS as diluent.

Haemagglutination test (HA)

The HA and HI tests were performed in 96 well microtiter plates and all the titrations were made using multi-channel microtiter pipettes. In each of the 12 wells of micro titer plate in a series, 50 µl of PBS was added. In the first well of each series, 50µl of antigen was added with the help of microtiter pipette and mixed well giving twofold dilutions (1:2). Afterwards, 50 µl of the suspension was transferred from the first well to the 2nd well and mixed thoroughly. Process was repeated up to the 11th well. Then 50 µl of the material was discarded from the 11th well. Fifty µl of the 1% washed chicken RBCs were added to each well from 1-12. Well No 12 in each series acted as erythrocyte suspension control as no antigen was added. The plates were incubated at room temperature 37°C for 30 min. The HA test will be positive if the bottom of the well covered with film of finely clumped RBCs, which did not flow when the plate was tilted. A sharply outlined button of RBCs on the bottom of well indicates negative test.

The AIV subtypes and NDV were subjected to haemagglutination titration using chicken RBCs. The reciprocal of the highest dilution indicating haemagglutination, was considered 1HA unit while 4HA units of the antigens were used in the HI test. The 4HA units of AIV antigen was calculated by dividing the AF viral suspension dilution factor (for 1HA) by 4. If the AF viral suspension dilution factor were 128 for 1HA, the 4HA will be located at the dilution factor of 32 and 1 ml of viral suspension will be mixed with 31 ml of PBS to achieve the 4HA units of antigen.

HI test

HI test was performed as discussed by Olsen et al. (2003). In a 96 well microtiter plate, 25 µl of diluents was added in each of the 12 wells in a row. In the 1st well of each series, 25 µl serum was added and thoroughly mixed. Then 25 µl of the diluted serum was transferred from the 1st well to the 2nd well and mixed. This process was repeated till the 10th well from which 25 µl of the diluted serum was discarded. Thus, no serum was added to the 11th and the 12th well. From the first to the 11th well, 25 µl of antigen containing 4HA units were added and mixed well. The 11th well contained no serum and acted as antigen control. To the 12th well, 25 µl of the diluents was added. It acted as diluents control. The plates were incubated at 37°C for 20 min to allow the completion of antigen-antibody reaction. To each well, 50 µl of 1% washed chicken RBCs were added and the plates were further incubated for 30 min. There was complete haemagglutination in well No 11 (antigen control) and no haemagglutination in well No 12 (diluents control). Clear button formation on the bottom of the well indicates positive test and formation of a uniform thin layer of finely clumped RBCs indicates negative test. Reciprocal of the highest dilution of the serum completely inhibiting haemagglutination was recorded as the HI titer (Allan et al., 1974). The data thus obtained was analyzed statistically by measuring the Geometric Mean Titers (GMT) of various treatments (Steel and Torrie, 1982).

RESULTS

Seroquantification of haemagglutinating viruses

The positive samples for AIV subtypes H7N3 and H9N2 in broilers were 25 and 44%, respectively, while the seropositivity of AIV subtypes in serum samples of commercial layers was 67% for H7N3 and 94% for H9N2. It was recorded that 96% of the broiler samples and 100% of the layer samples were found seropositive for antibodies against NDV. Out of all serum samples from broiler farms 69% were seropositive for IBV and 86% were positive in layer samples (Figure 1).

Age related seroquantification in broilers

The HA titers against H9N2 in broilers of age 0-2 weeks, 2-4 weeks and 4-6 weeks were log2 5.2 (GMT 36.8), log2 6.4 (GMT 78.8) and log2 6.8 (GMT 111.4), respectively (Table 1). The serum samples showed NDV HI titers log2 5.1 (GMT 48.5), log2 7.3 (GMT 157.6) and log2 7.8 (GMT 222.9) in birds of 0-2, 2-4 and 4-6 weeks, respectively (Table 2). The antibody titers of IBV in 0-2, 2-4 and 4-6 weeks, were log2 6.6 (GMT 97), log2 6.4 (GMT 84.4) and log2 6.8 (GMT 111.4), respectively (Table 3). The GMT of haemagglutinating viruses H9N2, IBV and NDV is shown in Figure 2.

DISCUSSION

AIV antibodies

Antibody titers with GMT value of 67.29 and higher were considered as protective for AIV vaccinated birds (Trani et al., 2002). All vaccinated age groups except 0-2 weeks were having GMT values higher than suggested. This showed that they fall in the protective antibody titer range against AIV-subtype H9N2. This conclusion is supported by a number of unpublished observations in this country about the benefits of AIV killed vaccines. Muneer et al. (2002) indicated that H9N2-AIV was associated with the
Table 1. Age wise seroquantification of avian influenza virus (AIV; subtype H9N2) in broiler serum samples found seropositive by haemagglutination inhibition (HI) test.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Total No. of samples</th>
<th>No. of positive samples</th>
<th>Minimum titer log₂</th>
<th>Maximum titer log₂</th>
<th>Average titer log₂</th>
<th>Geometric mean titer (GMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>38</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>5.2</td>
<td>36.8</td>
</tr>
<tr>
<td>2-4</td>
<td>40</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>6.4</td>
<td>78.8</td>
</tr>
<tr>
<td>4-6</td>
<td>18</td>
<td>10</td>
<td>6</td>
<td>8</td>
<td>6.8</td>
<td>111.4</td>
</tr>
</tbody>
</table>

Table 2. Age wise seroquantification of Newcastle disease virus (NDV) in broiler serum samples found seropositive by haemagglutination inhibition (HI) test.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Total No. of samples</th>
<th>Minimum titer log₂</th>
<th>Maximum titer log₂</th>
<th>Average</th>
<th>Geometric Mean Titer (GMT)</th>
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</thead>
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<td>6</td>
<td>5.1</td>
<td>48.5</td>
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<tr>
<td>2-4</td>
<td>40</td>
<td>6</td>
<td>8</td>
<td>7.3</td>
<td>157.6</td>
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<tr>
<td>4-6</td>
<td>18</td>
<td>6</td>
<td>9</td>
<td>7.8</td>
<td>222.9</td>
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</tbody>
</table>

Table 3. Age wise seroquantification of infectious bronchitis (IBV) in broiler serum samples found seropositive by HI test.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Total No. of samples</th>
<th>Minimum titer log₂</th>
<th>Maximum titer log₂</th>
<th>Average</th>
<th>Geometric mean titer (GMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>38</td>
<td>6</td>
<td>8</td>
<td>6.6</td>
<td>97</td>
</tr>
<tr>
<td>2-4</td>
<td>40</td>
<td>6</td>
<td>7</td>
<td>6.4</td>
<td>84.4</td>
</tr>
<tr>
<td>4-6</td>
<td>18</td>
<td>6</td>
<td>7</td>
<td>6.8</td>
<td>111.4</td>
</tr>
</tbody>
</table>

epidemic that has occurred in the area of Karachi; inactivated alum precipitated and oil based vaccines developed from local isolates, induced a GM-titer (range) 64-256 in vaccinated broilers. Naeem et al. (2003) also described that avian influenza virus vaccines (H9N2) have been employed during the 1st week of age in broilers and broiler breeders, followed by two more vaccinations at the 8th and 18th weeks in the breeder flocks. Vaccination must be done to protect flocks from AIV subtypes.
NDV antibodies

In the present study, serum samples from broilers and commercial layers showed a seropositivity of 96 and 100%, respectively. This high incidence of antibodies against NDV is due to regular vaccination schedules observed in the commercial poultry farms. The age specific prevalence of antibodies against NDV showed that HI titer increases with age with much higher titers in birds of 4-6 weeks of age. This increase in HI titer is due to booster dose of vaccine often given at the third week of age. Similar findings were shown by Tewari et al. (1992). He has tested 200 serum samples from unvaccinated indigenous chickens and observed that the prevalence rate was higher (46.9%) in adult chickens than in young chickens of less than 12 weeks (23%). Presence of HI antibodies in unvaccinated indigenous chickens indicates that these birds had contracted infection and recovered thereafter. Numan et al. (2005) determined the serum antibodies against NDV. Calculated geometric mean titers for broiler groups of 0-3, 3-5 and 5-7 weeks of age were found to be 11.91, 10.01 and 15.85, respectively. These values for layer groups of 17-27 weeks, 27-37 weeks and 37-47 weeks of age were found to be 134.89, 153.46 and 149.62, respectively. The results showed that the level of protection in vaccinated birds was unsatisfactory in broilers, whereas it was satisfactory in layers. Siddique et al. (2005) also showed that the level of protection of vaccinated birds was unsatisfactory. One of the major causes of outbreaks in vaccinated chickens is due to the emergence of new virus strain resulting in failure of vaccine because of the low immunity of birds. Poor vaccine quality is a common problem in most of the developing countries and it is due to the poor manufacturing standards, application of expired vaccine batches, faulty application and adequate storage facilities.

IBV antibodies

Seroquantification of the IBV was found to be 69% and 86% in broiler and layers, respectively. An incidence of 2.63% has been reported by Ahmad et al. (1985). His findings were based on the presence of anti IBV antibodies in non-vaccinated flocks while the present work indicates the high prevalence of antibodies in flocks vaccinated against IBV. With age, chicken becomes more resistant to the IBV and mortality becomes lower (Smith et al., 1985; Albassam et al., 1986). The seroquantification of 56.5% for IBV was noted by Gutierrez-Ruiz et al. (2000). Some of the findings noted by Hussaini et al. (2005) showed that agar gel precipitation and trypsin induced HI tests revealed 2.22% seroprevalence of IB with significantly higher IB seroprevalence in 1-2 week old broilers (5.36%), while the lowest prevalence was found in older birds.

Conclusions

AI, ND and IB are the major viral diseases affecting the commercial and non-commercial poultry flocks in various regions of Lahore. Commercial poultry farmers adopt different vaccination practices for controlling these diseases. Serum samples brought by local poultry farmers from various regions of Lahore showed a seropositivity of 96% and 100% against NDV and 69% and 86% against IBV, respectively.
farmers to UDL, UVAS, Lahore were checked for protective antibodies titer against above mentioned poultry disease causing viruses. It was concluded that almost all of the samples were not having protective antibody titer against these viruses as detected by HI test, which indicate that vaccination has not been practiced successfully. The unsatisfactory vaccination may lead to significant losses due to the above mentioned fatal viral diseases not only in the farms sampled but also in the surrounding areas. Therefore, it is suggested that regular vaccination against these viruses must be strictly observed in all types of poultry flocks and antibody titers be checked at regular intervals to monitor successfulness or failure of vaccines.

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