Appropriate time of vaccination against infectious bursal disease virus in layer chicks by Elisa in single dilution

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Prevention of Infectious Bursal Disease (IBD) is very crucial in poultry production because the disease makes the birds susceptible to other diseases through immunosuppression. Thus prevention is preferred to treatment in its control. Prevention of the disease has been mainly achieved by active vaccination of layers to facilitate transfer of IBDV maternal antibodies (Mab) to chicks via the eggs, thereby protecting chicks during the first few weeks before active vaccination. But most active vaccination of chicks against the virus had resulted in failure due to interference by maternal antibodies and inability to determine the crucial time for active vaccination of chicks without interference by maternal antibodies. The methods of Enzyme Linked Immunosorbent Assay (ELISA) by titration, virus neutralization or Agar Gel Precipitation Tests commonly used are unreliable as far as generating daily serological data was concerned in collaboration with the Department of Animal Science, Guangxi University, Guangxi Province, Peoples’ Republic of China, as these are long, tedious less sensitive.

ELISA in single dilution has the inherent capacity of overcoming this limitation and therefore was employed in monitoring maternal antibodies to determine the right day to vaccinate chicks without maternal antibody interference. Thus using ELISA in single dilution, a batch of randomly selected 30 chicks and blood samples from 25 vaccinated hens were obtained from a breeder farm, monitored for maternal antibody depletion in comparison to the titres of the hens for four weeks. ELISA in single dilution more specifically, rapidly and accurately measured the antibody titres of the birds from day old to twenty four days. A correlation coefficient of 0.83 (P≤0.05) was obtained by linear regression of ELISA titres and positive/negative ratios. The average Mab of the offspring ($\overline{ET}_{x} = 4068.5$) were higher than the average ET of hens (1983) for the first ten days. A sharp rise in antibody production was observed for the second day ($\overline{ET}_{x} = 7676$), a sharp drop on the third day ($\overline{ET}_{x} = 3547$), followed by a steady decline on to the 24th day with a $\overline{ET}_{x} = 919$, when maternal antibodies were low enough to permit active vaccination of the chicks without interference by the former. The discriminatory level between positives and negatives was obtained as $\overline{ET}_{x} = 709$. The half life of Mab was ascertained as 4 to 5 days ($r = 0.77, |r| > 0.05$). The appropriate time to implement active vaccination without risk of vaccine failure or incidence of infection was determined to fall between 24th and 26th days post hatching.

Key words: Vaccination, layer chicks and Production

INTRODUCTION

Infectious Bursal Disease (IBD) was first recognised in 1962 by Cosgrove as an acute contagious and immunosuppressive disease of young chickens, which makes them highly susceptible to several other
infections: Newcastle disease (Allan et al., 1972, Faragher et al., 1974); Inclusion body Hepatitis (Hiral et al., 1979, Fadley et al., 1976); Coccidiosis (Anderson et al., 1977); Marek’s Disease (Cho, 1970); Hemorrhagic aplastic Anemia and Gangrenous Dermatitis (Rosenberger et al., 1979); Infectious Brochitis (Pejkovski et al., 1979); Infectious Laryngotracheitis (Rosenberger et al., 1979); Salmonellosis and Collibacilosis (Wyeth, 1975); Reovirus (Moradan et al., 1990); Escherichia coli (Nakamura et al., 1990); Avian Nephritis virus (Nantik et al., 1991); Aspergillus flavus (Okoye et al., 1991).

Prevention of IBD in poultry is better than cure to escape higher mortality and severe economic losses.

After years of several trials employing various strategies, Hitchner (1976) found that maternal antibodies in the progeny from immunized breeder flocks helped to protect chicks for the first 2-3 weeks. Beard (1985) however recommended that to achieve maximum immunity with the least vaccine reaction at the earliest time would depend on reliable serological data, proper balance of timing, route and selection of vaccine virus strain, which either Virus Neutralization Test (VN), Agar Gel Precipitation Assay (AGP) (Weisman, 1973) or Enzyme Linked Immunosorbent Assay (ELISA) by serial dilution cannot produce (Synder, 1986). This is because VN and AGP tests as well as ELISA by serial titration (Solano, 1985) are slow, less specific and thus less reliable for generating reliable serological data that is required for decision making on vaccinating chicks at the appropriate time. The disadvantages of the above three tests are easily overcome by ELISA in single dilution used and thus is more suited to meet the needs of commercial farms that require results within a day. The test is based on the finding that absorbance values are linearly related to the end point of the antibody titration (Desingviny, 1980; Synder, 1983; 1986).

Prior to this study at Guangxi Agricultural University, IBDV vaccine failure was very common with frequent incidences of infection and very high mortalities in poultry farms in Guangxi province and Sierra Leone, due to improper timing of vaccination of chicks against IBDV as a result of poor serological data for carrying out active vaccination of chicks.

The objective of this study was to determine the proper time for active vaccination using ELISA by single dilution though monitoring maternal antibody depletion pattern in chicks in order to prevent vaccine failure.

**METHODOLOGY**

**Source of biological reagents**

Sera of 38 breeder hens randomly selected and their respective progenies were acquired from a poultry breeder farm in Guangxi province, Peoples Republic of China. Rabbit anti-chicken IgG conjugate was provided by the Institute of Biochemistry in Beijing. The Lucker’s strain of IBDV was produced from the Department of Veterinary Medicine of Guangxi Agricultural University.

**Production of ELISA antigen**

The antigen was cultured on chicken embryo fibroblasts (CEF) at 6.0 × 10⁵ cells/ml in 15 ml amounts of growth medium. The cells were incubated at 37°C for 24 hours. After formation of the monolayer in tightly cocked roux bottles, 100 tissue culture infective dose (TCID)₅₀/0.1 ml of the virus was inoculated in a maintenance medium in 3 ml amounts. After formation of the cytopathic effects (CPE) in 96 h, 400 mls of virus tissue culture fluid was collected by 3 freeze-thaw cycles, stored at -20°C until purified.

**Purification of virus (antigen)**

After 3 freeze-thaw cycles, the culture fluid was centrifuged at 3000rpm, 4700 x g at 4°C for 30 minutes in HIMAC SCR 20 refrigerated centrifuge to concentrate the virus. The supernatant was discarded and the pellets re-suspended in 0.005 M phosphate buffered saline (PBS) at pH 7.2 in ⅓ the original volume. The virus was precipitated with 7% PEG 20,000, 0.3% NaCl after keeping at 4° C overnight. The mixture was centrifuged at 17000 rpm, 4°C for 30 minutes. The supernatant was again discarded, whilst the pellet was re-suspended in one-twentieth the original volume of PBS (20 mls). The solution was dialysed in several changes of 0.005 M PBS, pH 7.2 until purified virus was free from all sulphate ions.

**Determination of working concentrations of antigen, conjugate, positive and negative sera**

**Measurement of virus (antigen) concentration**

The ultra violet spectroscopic reader at 280 nm and 260 nm was used to measure the optical density (OD) of the virus fluid, which was 0.554 mg/ml or 554 ug/ml. The stock was stored at -20°C in 2 ml amounts until used.

**Determination of concentrations of antigen and horse-raddish peroxidise rabbit anti-chicken immunoglobulin conjugate**

The optimum concentrations of antigen and conjugate were determined by the conventional indirect solid phased ELISA in serial dilution. The purpose was to
select a concentration that would discriminate between false positives and false negatives. The use of 0.1 ml of reactants per well, 0.05 micro litres (ul) of 2.0 M H$_2$SO$_4$ at an incubation temperature of 37°C for 2.5 h was selected for the test. With a two-fold dilution of antigen (starting 1:2), conjugate (1:100), 17.33 ug/ml of antigen and 1:200 dilution of conjugate were considered optimal for the quantification of antibodies to IBDV in both the hens and the chicks at absorbance values after 10 to 15 min when the substrate and 85 micro litres of 30% hydrogen peroxide was added.

**Determination of antibody titres in test sera**

Immune status determination was based on serum immunoglobulin (IgG) and neutralizing antibody measurements by the Indirect Enzyme Linked Immunosorbent Assay in single dilution. It’s been ascertained that total serum IgG antibody correlates to the protective activity of serum (Howard, 1982). Applying ELISA in single dilution (1:200), individual dams and respective progenies’ antibody activity titles were measured in ELISA reader DG3022 at 280 nm. The average titles and individual titres were taken into consideration as these two are important parameters in determining the effectiveness of a vaccination programme. Negative sera and 6 to 7 test samples at Log$_{10}$ dilution were included in the assay everyday to use in determining a linear regression equation.

**Statistical analysis**

From the means of logarithmically diluted negatives and positives, the Positive/Negative (P/N) ratios were determined. According to Tizsen (1965) on the central limit theorem, the means of individual absorbance values are normally distributed, but the individuals are not. The recorded absorbances for all wells were analysed by regression and T-tests at 95% confidence limits. Based on the exponents of Log$_{10}$ dilution (X) and the P/N ratio (Y), a linear equation: Y = a + bX was established and this formed the basis for determining the actual antibody titres of test samples. By definition, Y is the estimated value of Y, ‘a’ is the intercept or point where the line crosses the Y axis, and b is the slope or regression coefficient (the amount of change in Y, in this antibody activity) associated with a unit change in X (antibody dilution or level (Little et al., 1978).

To distinguish positive from negative samples, a discrimination or cut-off point was set at the mean of negative samples included multiplied by 3 s.d. above the mean (Malvano et al., 1982). The significant cut-off level was thus determined from the relationship: mean absorbance zero dose (negative response) + t$_{0.95}$ df x 2 sd/n. The term negative is used to indicate the absence of specific antibodies. The selection of a suitable cut-off value is important for minimizing false responses.

**Calculation of ELISA titres and half-life of maternal antibody in the chicks**

ELISA antibody titles were determined by following steps:

1. P/N ratio = mean absorbance values/0.36
2. ELISA titres = Substituting in the regression equation the P/N ratios for each sample.
3. ELISA value was also defined graphically by plotting P/N ratios against log$_{10}$ dilution values and the reciprocal of dilution where P/N = 1 gives the ELISA titre (Figure 1).

To prove whether any correlation existed between dilution (antibody level) and antibody activity as indicated by regression equation, the P/N values for each sample OD value were plotted against their respective log$_{10}$ ET. Figure 1 displays the derived regression line. By plotting each serum sample mean P/N value against its respective log$_{10}$ ET, a more correct statistical analysis was performed. The means of daily P/N ratios, log$_{10}$ ET, ELISA values of breeder hens and their progenies on various days of testing were tabulated in order to figure out the pattern in antibody depletion in the progenies relative to that in the hens (Table 2)

**RESULTS AND DISCUSSION**

The linear regression equation (\( \bar{Y} = 2.71 + 0.39\bar{X} \)) was calculated from Table 1. The calculated regression equation gave a coefficient of correlation \( \beta = 0.83 \) between antibody titre and antibody dilution or level at \( \geq \) 95% confidence limits, indicating a high positive correlation between dilution and titre.

The analysis provided evidence that the relationship tested had a good line fit (Figure 2). By incorporating the P/N ratio in the calculations, it was possible to standardize the reporting of results from day to day.

Study on the depletion pattern of the maternal antibody in the progeny saw a sharp rise the second day and also dropped sharply on the third day, but maintained a steady decline towards negative over time. From studies on the ontogeny of the chicken humoral immune mechanism, it has been reported that the predominant Ig of yolk is IgY and that at 11 days of embryonation, the transmission of yolk IgY to the embryo commenced; at 15 days IgG level is about 2-4%, while no traces of IgM or IgA is found; the final 5-6 days of embryonation to hatching, most of the IgY is already absorbed into the embryonic circulation. Possibly, therefore, egg-laying may be responsible for
some of the IgG drop by transmission. However it is not very obvious what determines the amount of transmission. Further, according to the same study, IgM is detected only 3 to 4 days after hatching; IgG is also detected in the egg according to the report, but is insufficient in the serum of newly hatched chicks, so that most likely this may account for the rise in the IgG level on the second day.

The phenomenon of the half-life in addition to the ontogeny theory is also capable of making reliable explanations. The concentration of circulating immunoglobulin in serum depends on both the synthetic and the catabolic rates, and when the animal is in a steady state, the two are assumed to be equal; that is, the concentration remains at a constant level (Figure 3; Day 10 and 14). When the synthetic rate is less than the catabolic rate, the serum concentration of the antibody falls.

In the absence of additional antigenic stimulation the concentration may reach undetectable levels (Day 3, 7,

![Figure 1. Regression line showing P/N ratio and Log10 dilution.](image)

Table 1. Log 10 dilution of positive and negative reference sera and their positive/negative ratios.

<table>
<thead>
<tr>
<th>Log10 dilution (X)</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive/Negative ratio (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-1}</td>
<td>0.84</td>
<td>0.42</td>
<td>2.0</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>0.74</td>
<td>0.30</td>
<td>2.37</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>0.47</td>
<td>0.29</td>
<td>1.62</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>0.24</td>
<td>0.23</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Table 2. ET of hens and progenies determined on selected days.

<table>
<thead>
<tr>
<th>Post hatching days of testing progenies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statistic</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>P/N</td>
</tr>
<tr>
<td>Log10 ET</td>
</tr>
<tr>
<td>ET</td>
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<tr>
<td>Sd</td>
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</tbody>
</table>
The half-life of maternal antibody ascertained as 4 to 5 days by the regression equation \( Y = 5048.58 - 192.38X \) with post hatching days of testing as \( X \) and predicted ET as \( Y \) (Figure 3) and Coefficient of correlation as, \( r = 0.77 \), \( r_{0.05} = 0.71 \), \( |r| > 0.05 \), is also suggestive of high correlation.

The half-life (the period required for the antibody to deplete by half) from the highest ET 7676 to the cut-off point (709) was \( 4192.5 \) (7676 - 709/2 + 709).

The half life was calculated as 4 to 5 days approximately. This contrasts with the half-life of IgM, while that of the IgM is short (3 to 5) days in most animals and independent of the concentration in serum; the half-life of IgG is longer (6 to 23 days) and is dependent upon the serum concentration. When the IgG concentration is low, the animal is hypo-gammaglobulinemic, the half-life is longer and the catabolic rate decreases (Sell, 1964). This is regarded as regulatory.

The Fc of the heavy chain appears to be the portion of the molecule determining the catabolic rate, as the metabolism of the Fc piece is similar to the metabolism of the whole molecule (Waldman et al., 1971).

On the basis of the data collected was analyzed and discussed the following conclusion was made:

1. ELISA in single dilution is an appropriate and reliable diagnostic tool for rapid serological profiling of flocks.
2. The vaccination programme of this farm can be evaluated as good, since the maternal antibody of the progeny was high enough to confer immunity to the progeny for the first three weeks as recommended for the control of IBDV in poultry.

Therefore, since immunization is the main method of prevention in poultry, and as this to be successful also depends on balance of timing based on a reliable serological data, it is highly necessary that the tool for immune-surveillance of poultry flocks for IBDV, be rapid, cost effective and accurate. This technique has proven beyond all doubts its capability to reveal a reliable immunological data with respect to antibody response to IBDV in poultry.

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