COMPARISON OF HEMAGGLUTINATION INHIBITION TEST AND ELISA IN QUANTIFICATION OF ANTIBODIES TO EGG DROP SYNDROME VIRUS

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Summary. – A single-serum dilution ELISA for egg drop syndrome (EDS) virus-specific antibodies was developed. In testing 425 chicken sera it was found to have a 93.6% sensitivity and 98.7% specificity relative to a hemagglutination inhibition (HI) test. The correlation coefficient for ELISA and HI titers was 0.793. The ELISA was efficacious in quantification of both vaccinal and infection antibodies and could routinely be used for screening large numbers of field sera.

Key words: egg drop syndrome virus; antibodies; quantification; ELISA; HI test; correlation coefficient

Introduction

EDS, a condition resulting in a drastic reduction in the egg production and deterioration of the egg shell quality has emerged in the mid 1970s throughout the world (Baxendale, 1978). Although the disease affects broiler breeder chicken flocks almost exclusively, the causative agent – the EDS virus – was found to infect commonly ducks (Calnek, 1978). EDS virus (Duck adenovirus 1) is so far classified as an unassigned member of the family *Adenoviridae* (van Regenmortel *et al.,* 2000). Antibodies to EDS virus (EDS virus antibodies) have been found in a number of wild and domestic bird species (Kaleta *et al.*, 1980; Bartha *et al.*, 1982) and this virus is apparently wide spread. Recently, it has also been associated with a severe respiratory disease of geese (Ivanics *et al.,* 2001).

The availability of a standard, sensitive serological test would facilitate EDS diagnosis and accurate monitoring of vaccination programs. Several serological tests for EDS virus antibodies have been elaborated and compared (Adair *et al.*, 1986; Darbyshire and Peters, 1980). HI test has been currently used for detection and quantification of EDS virus antibodies (Adair *et al.*, 1986). HI test, while simple to perform is difficult to standardize and cannot be automated. In contrast, ELISA is easy to perform with large number of samples, can be automated and has been used widely for the preparation of serological EDS kits.

Serological evidence of EDS virus infections in India has been obtained for chickens and quails (Das and Pradhan, 1992; Das *et al.*, 1995; Shaw *et al*., 1995). In some instances the virus has been isolated and characterized genomically and pathotypically (Dhinakar Raj *et al.*, 2001a, b). Earlier, Adair *et al.* (1986) have compared ELISA and HI test qualitatively but not in terms of sensitivity, specificity or correlation coefficient.

The present study describes the development and evaluation of a single-serum dilution ELISA for quantification of EDS virus-specific antibodies and comparison of this assay with a HI test in terms of sensitivity, specificity and correlation coefficient.

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Abbreviations:EDS = egg drop syndrome; HI = hemagglutination inhibition; p.i. = post infection; p.v. = post vaccination

Materials and Methods

Virus. An Indian isolate of EDS virus, TN4 was used for experimental infection and antigen preparation for ELISA (Dhinakar Raj *et al.*, 2001a).

Virus propagation. The virus was propagated in the allantoic cavity of 10–11-day-old embryonated duck eggs obtained from a local source. Random analysis of yolk samples indicated that these eggs were free of HI antibodies to EDS virus. Five days after inoculation, live embryos were chilled; the allantoic fluid was harvested, pooled and after checking for the HA or egg infective titer (EID_{50}) it was used as source of the virus

Virus purification. The allantoic fluid was clarified at 5,000 x g for 15 mins and subjected to ultracentrifugation at 80,000 x g for 2 hrs at 4°C. The crude viral pellet was resuspended in a minimum quantity of TNE buffer (10 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, pH 8.0) and overlaid on a 36% sucrose cushion. Following ultracentrifugation at 100,000 x g for 4 hrs the pellet was re suspended in TNE buffer and used as coating antigen in ELISA.

ELISA. ELISA plates (Maxisorp, Nunc) were coated with 50 µl per well of the antigen diluted in a sodium carbonate coating buffer pH 9.6 overnight at 4°C. Between each step the plates were washed three times with PBS containing 0.01% Tween 20 and incubated for 45 mins at room temperature. The plates were blocked with 100 µl/well of 5% skimmed milk in PBS. After washing, 50 µl/well of a serum sample was added at a pre-determined dilution into duplicate wells. After incubation, an anti-chicken peroxidase conjugate (Bangalore Genei, India) was added at a pre-determined optimum dilution of 1:1000. A substrate solution was prepared by dissolving 30 mg of ortho-phenylenediamine in 75 ml of distilled water. Forty µl of hydrogen peroxide was added to 10 ml of the substrate solution before use. After washing the unbound conjugate, 50 µl/well of complete substrate solution was added and the reaction was left to run at 37°C for 10 mins. The reaction was stopped by adding 25 µl of 0.25 N sulphuric acid and A_{492} nm was read.

To decrease variations between different plates, titers of sera were estimated using the sample to positive sample (SP) ratio calculated as follows:

SP = (sample A_{492} – neg. samples A_{492}) / (pos. samples A_{492} – neg. samples A_{492})

In order to establish a regression equation relating the SP ratio at a fixed dilution directly to the titer, 10 negative serum samples from specific antibody-negative (SAN) chickens were titrated at 8 two-fold dilutions starting with 1:125. A positive-negative threshold (PNT) base line was established following the method of Snyder *et al.* (1983). The mean A_{492} of the negative samples at each dilution was taken and a twice-negative average (TNA) was used to construct the PNT base line. Then the titer of each sample was determined as the inverse of the dilution at which the line relating the absorbance to the dilution intersected the PNT base line. The correlation coefficients of the SP ratio for thirty-seven serum samples tested and calculated at different dilutions against the observed titers were determined. On this basis, 1:1000 was chosen as the optimum dilution of a serum to be tested by the assay since this dilution gave a maximum correlation with actual titers. A regression equation was generated by comparing the log SP ratio of samples tested at 1: 1000 dilution with their actual titers as follows:

$$\log SP = \text{intercept} + \text{slope} \times \log (\text{titer})$$

or rewritten as

$$\log (\text{titer}) = (1/\text{slope}) \times \log SP - (\text{intercept}/\text{slope})$$

Samples were tested in duplicate at this dilution, the log SP ratio was calculated for each sample and using the regression equation obtained their ELISA titers were determined. Positive and negative samples were included in each plate as controls. When the mean absorbance for the negative control exceeded 0.2, the results were rejected.

HI test was performed following the method of Adair *et al.* (1986) using 4 HA units of EDS antigen and 1% chicken erythrocytes.

Experimental design. Four 16-week-old chickens were inoculated with a commercial inactivated EDS vaccine (Ranbaxy, India) and the serum was prepared at weekly intervals from 1 to 10 weeks post vaccination (p.v.) for use in ELISA and HI tests (n = 40).

Twenty 3-week-old chickens were infected orally with 10^4 EID_{50} of EDS TN4 and were given a booster 3 weeks post infection (p.i.). Sera were prepared at weekly intervals from at least five chickens on every occasion for 3 weeks p.i. and for 4 weeks post secondary infection (n = 35). In addition, 350 random field sera samples were checked by HI and the developed single serum-dilution ELISA tests.

Statistical analysis. The specificity, sensitivity and accuracy of the method were calculated as described earlier (Dhinakar Raj *et al*., 1998). The correlation coefficients were determined for each group of sera tested by different tests in MS Excel.

Results

Two-fold average ELISA A_{492} values of 10 different negative (control) serum samples in serial two-fold dilutions starting from 1:125 were 0.673, 0.507, 0.430, 0.364, 0.305, 0.284, 0.266 and 0.267. Using these as cut off values, the PNT line was determined. Actual ELISA titer of a sample was determined as the dilution at which the sample A_{492} intersected the PNT line. The dilution of a serum sample where the SP ratio reflected best different titers determined by serial dilutions (actual titers) was found to be 1:1000 based on the highest correlation coefficient obtained (Table 1). Based on these values the regression equation formulated was 1.452 x log SP ratio + 4.032. Thus the serum samples were tested in duplicate at a single dilution of 1:1000, the log SP ratios were determined and the ELISA titers were calculated.

Table 1. Constants of the regression line for selected dilutions of serum tested by ELISA

Serum dilution	Correlation coefficient	Intercept (C)	Slope (m)
1:500	0.910	-2.16052	0.551848
1:1,000	0.920	-2.77701	0.688694
1:2,000	0.912	-3.49204	0.837786

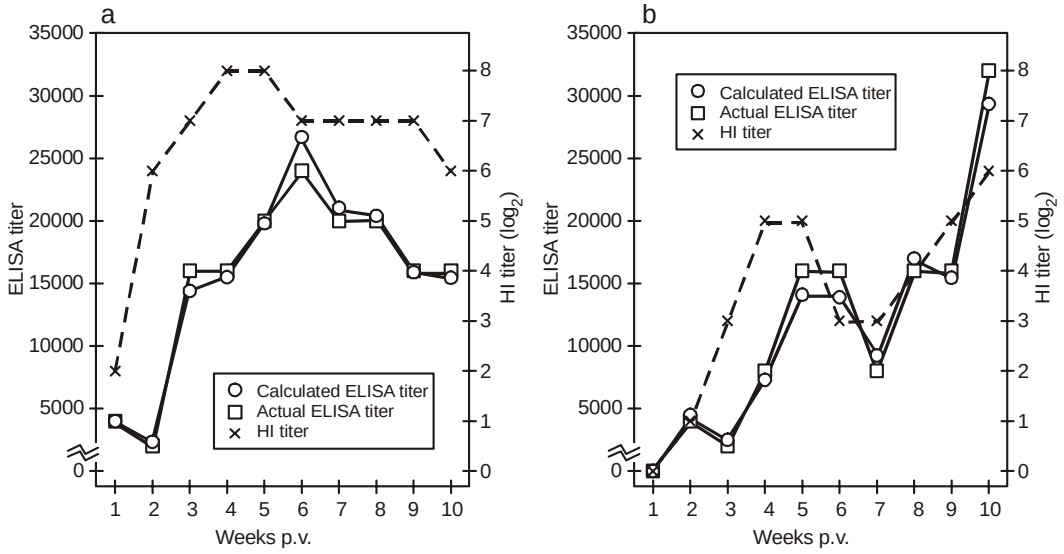

Fig. 1

Actual and calculated ELISA and HI titers of EDS virus antibodies in two EDS virus-vaccinated chicken (a, b)

For 75 serum samples tested initially after experimental vaccination or infection, the differences between actual and calculated ELISA titers were never higher or lower than one twofold dilution. The correlation coefficient for the calculated SP ratio and the actual ELISA titer was 0.985.

The sensitivity, specificity and accuracy of the ELISA and HI tests are shown in Table 2. This was a qualitative comparison taking $\log_2$ HI titers higher than 3.0 and calculated ELISA titers higher than 3000 for positive. The sensitivity, specificity and accuracy of the ELISA relative to the HI test employed in this study were 93.6%, 98.7% and 97.4%, respectively.

The results of the ELISA and HI tests for two vaccinated chickens are shown in Fig. 1. Concerning ELISA titers there was a rise starting 3–4 weeks p.v., while a high level was maintained for 10 weeks p.v. After oral infection of chickens, there was a rise during the first 3 weeks p.i. and after a small 'dip' 1 week post reinfection the rise continued again for 4 weeks post secondary infection (Fig. 2). The HI titers of both vaccinated and infected chickens started rising from 2–3 weeks p.v. and were maintained at moderate levels ($\log_2$ titers of 5–7). The regression line for calculated ELISA and HI titers of EDS virus antibodies for the sera from vaccinated, experimentally infected and non-vaccinated non-infected chickens (n = 425) is shown in Fig. 3. The respective correlation coefficient for all 425 sera was 0.793, which means that 62.9% (r^2 x 100) of the variation in ELISA titers accounted for corresponding variations in HI titers. However, the correlation coefficients for actual or calculated ELISA titers and HI titers for the 75 vaccinated and experimentally infected chickens were 0.561 and 0.586, respectively.

Table 2. Data demonstrating relative sensitivity, specificity and accuracy of ELISA in detecting EDS virus-specific antibodies using HI test as reference

Technique	HI-positive	HI-negative	Total
ELISA-positive	103 (a)	4 (b)	107
ELISA-negative	7 (c)	311 (d)	318
Total	110	315	425

The sensitivity [a/(a+c)], specificity [d/(b+d)] and accuracy [(a+d)/(a+b+c+d)] of ELISA in relation to HI test were 93.6%, 98.7% and 97.4%, respectively.

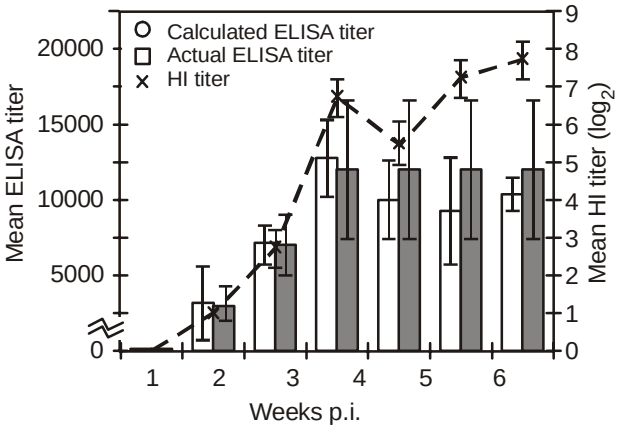

Fig. 2

Actual and calculated ELISA and HI titers of EDS virus antibodies in chickens infected with EDS virus orally and reinfected three weeks later (n = 5)

Mean (± SD) values from 5 chickens.

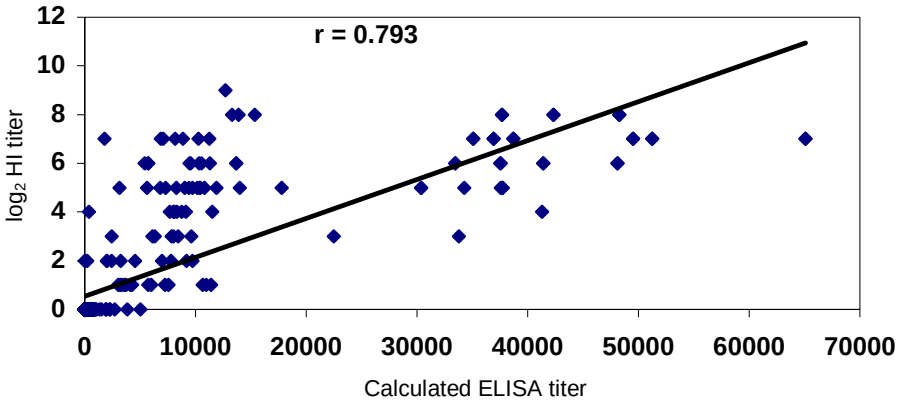

Fig. 3

Regression line of calculated ELISA and HI titers of EDS virus antibodies in the sera of vaccinated, experimentally infected and non-vaccinated non-infected chickens

Alltogether 425 chickens tested.

Discussion

ELISA is an excellent method for rapid serological diagnosis of various viral diseases. In areas where EDS vaccination is not being performed, the EDS antibody detection is of diagnostic value and this method is more useful because EDS virus isolation is more difficult. This study revealed that results of various comparisons of the EDS ELISA and standard HI tests indicate that ELISA is highly efficacious.

Using the two-fold serial dilution ELISA, only about 5–6 serum samples can be evaluated in single ELISA plate using dilutions from e.g. 1:125 to 1:16,000. However, using the single serum dilution ELISA at least 42–44 serum samples can be analyzed and their EDS virus antibody titers determined with sufficient accuracy.

Using the developed regression equation and testing the sera at the single dilution of 1:1000, the ELISA titers calculated were very close to those obtained with classical serial dilutions of sera. The relative sensitivity and specificity of the single serum-dilution ELISA test represented 93% of those of the HI test.

However, when the calculated ELISA titers were compared with the HI titers, the respective r-value was 0.793. In a similar study with Newcastle disease virus (Brown *et al.*, 1990) the correlation coefficient between ELISA and HI titers was found to be 0.85. We obtained a lower value when serum samples only from EDS vaccinated or infected chickens were tested. This shows that although most of the samples positive by HI test were also positive by ELISA (qualitatively) their titers were not in perfect agreement. This may also be partly attributable to inherent variability and subjective reading of the HI titer or to different types of antibodies being determined by both tests. Whereas the HI test expresses the titer of only one fraction of antibodies present in the serum, ELISA expresses the titer of antibodies specific to all virus antigens (including HI-negative and HI-positive).

A difficulty with the EDS virus-specific antibody assayed by ELISA, which was observed earlier, was the occurrence of false positivity due to cross-reactions of the antigen with other fowl adenovirus-specific antibodies. However, although the HI test may not have this inherent cross-reactivity, it is laborious to perform and cannot be automated. Only 3 of 200 HI-negative samples tested by Adair *et al.* (1986) were positive by ELISA. Despite this small number of false positives, ELISA may be preferred to HI test in the programs where ELISA is used for a number of other avian viruses. To further confirm the small number of ELISA-positive serum samples they can be counterchecked by HI test.

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