Standardization of an Enzyme-Linked Immunosorbent Assay for Detection of Infectious Bronchitis Virus Antibody

Short Communication

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Summery

An indirect enzyme–linked immunosorbent assay (ELISA) was developed for screening of antibody to avian infectious bronchitis virus (IBV). Antigen was prepared from whole-purified IBV Massachusetts serotype (BR 801 strain). Optimum dilution with minimum background for antigen concentration, rabbit anti-chicken conjugate and sera in developed ELISA was determined 0.1µg/ml, 1:3000 and 1:100, respectively. The optical densities (OD) were compared with a commercial ELISA kit. Also, the results of ELISA were compared to hemagglutination inhibition (HI) test. The correlation coefficient of the results of both ELISAs and HI was significant ($P<0.05$). The developed ELISA can detect antibody against IBV and is more sensitive and suitable for screening of samples in diagnostic laboratories.

Key word: infectious bronchitis, ELISA, antibody, Massachusetts serotype

Introduction

Infectious bronchitis virus (IBV) is the cause of a highly contagious respiratory disease that produces high morbidity in all ages of chickens and high mortality in chicks less than 6 weeks old (Cavanagh & Naqi 2003). Immunity to IBV has most often been assessed using traditional serological assays; however, the enzyme-linked immunosorbent assay (ELISA) is used on a more frequent basis to measure IBV

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antibodies (Case *et al* 1982). The technique was initially developed by Engvall and Perlmann (1971) and has since been widely used. The use of ELISA offers a number of advantages compared with traditional serological assays, including increased sensitivity (Garcia & Bankowski 1981) and simplicity of automation (Synder *et al* 1984). The antigen that is used in the test is of one serotype (usually Massachusetts). The cross reactivity of the IB ELISA with several strains of the virus and detection antibodies against other serotypes (Zellen & Thorsen 1986, De Wit 2000) support the idea that ELISA is a promising tool for serological studies, especially for use in evaluating the efficacy of vaccination regimens and monitoring the immune status of birds in a flock (Cavanagh & Naqi 2003, Wing *et al* 2002). Indirect ELISAs were adapted and standardized for detecting antibodies against whole virus, and S1, S2 and N proteins of IBV (Mockett & Darbyshire 1981, Ignjatovic & Galli 1995, Wing *et al* 2002) and compared with other serological tests (De Wit *et al* 1992, 1997, Perrotta *et al* 1988, Mockett & Darbyshire 1981, Thater *et al* 1987).

This paper describes an optimized ELISA for detection of antibody against IBV.

Materials and Methods

Antigen preparation. The isolated strain of Massachusetts serotype (BR801 strain) as described by Ghadakchi *et al* (2003) was propagation in specific pathogen free emberyonated chicken eggs (Cuxhaven, Germany). 700ml allantoic fluid was harvested and clarified at 8000*g* for 30min then EID_{50} was calculated. It was inactivated with 0.05% ß-PL and centrifuged at 90,000*g* for 90min in a sorvall AT-629 (32ml) at 4°C. The pooled result was pelleted twice in TEN buffer (150mM NaCl, 10mM Tris-hydrochloride, and 1mM EDTA, pH7.4). A sucrose gradient (20-60%) at 90,000*g* at 4°C in a sorvall TH-641 (17ml) rotor was used. The fraction was pelleted twice in TEN buffer to remove the sucrose and analyzed for protein content by the method of Lowry. It was finally divided into aliquots and frozen at -70°C.

Conjugate titration. Horseradish peroxidase conjugated rabbit anti-chicken IgG (Sigma) in conjugate buffer (0.15M NaCl, 1mM EDTA, 50mM Tris-hydrochloride, 0.1% bovine serum albumin, 0.02% NaN_3 and 0.05 Tween20, pH7.4) was used. Known positive and negative sera in dilution buffer (1mM EDTA, 0.05M Tris-hydrochloride, 0.1% BSA, 0.02% NaN_3, 0.15M NaCl and 0.05% Tween 20, pH7.4) were added to wells and the last row was considered as conjugate control. After incubation for 30min at 37°C, the wells were washed, and 100µl of serial diluted conjugate beginning from 1:1000 was added to the rows. The plate was washed after incubation at 37°C for 30min. The color reagent was ortho-phenylene diamine was obtained from Sigma chemical Co. The optical densities were measured at 492nm using an automatic ELISA reader (Rosys Anthos 2001) and the signal-to noise (S/N) ratio at the same dilution were evaluated.

ELISA test. The ELISA procedure was standardized on the method developed by Case *et al* (1982) with some modifications. Purified antigen was assayed at concentrations 0.5-1.5µg/ml in carbonate buffer (0.1M Na_2CO_3, 0.02%NaN_3, pH9.6). Flat bottomed micro plates Falcon 3915, Nunc, and Dynatech were coated at same time. Washing buffer (0.5M NaCl, 2.5mM KCl, 1.5mM KH_2PO_4, 9.0mM Na_2HPO_4, and 0.05% Tween 20, pH7.4) was used with soaking for 5min at room temperature in each time and trapped out onto absorbent paper. Serum samples were prepared from chickens that reared under controlled conditions and vaccinated at day 10 with live H120 and at day 110 with a commercial oil-emulsion vaccine (Merial, France). The chickens were bled at days 20, 30, 40, 110, 130 and 140 of age and after challenge with 10^7EID_{50} of the same virus. 240 sera were tested by HI and both experimental and commercial ELISAs. A group of chicken were reared as negative control and bled at the same times. Diluted sera were prepared and 100µl volume was added to each well. 100µl of horseradish peroxidase conjugated rabbit anti-chicken IgG was incubated for 30min. After washing, 100µl of substrate (0.04% OPD, 0.04% H_2O_2, 0.2M Na_2HPO_4, 0.1M Citric acid, pH5) was added, and

incubated at room temperature for 10min then stopped by 1M H_2SO_4. Optical density (OD) of each serum was determined. The specificity of the developed ELISA was calculated as the percentage of negatives in non vaccinated group and the sensitivity was calculated as percentage of positives in vaccinated group. The results obtained by the ELISA were compared with those obtained by a commercial kit (IDEXX) and HI.

HI test. HI test was conducted by M41 HI antigen (HI Veterinary Laboratory Agency-New haw, Addlestone) as described by King and Hopkins (1983) and Alexander *et al* (1983).

Statistical analysis. The correlation coefficients between data of experimental ELISA, IDEXX kit and HI titer were analyzed by ANOVA test.

Results and Discussion

The protein content in prepared antigen was 0.2 mg/ml according to Lowry procedure and EID_{50} was calculated 10^7. Optimum dilutions were determined by evaluation of S/N ratio and were determined 0.1µg/well antigen ($0.5\times10^{5.2}$ EID_{50}/well), 1:100 chicken serum dilution and 1:3000 conjugate dilution. Nonspecific attachment was observed in control wells at 1:1000 dilution of conjugate. Low absorbency values with control were observed at 1:4000 conjugate dilution, but it has no significance S/N ratio. Falcon 3915 has significant differences between S/N ratio of the known positive and negative sera. The cut off for the standardized ELISA test was determined as mean of negative sera plus twice the standard deviation 0.196[0.136+(2×0.03)].

The OD obtained by the experimental and commercial ELISAs were measured (Table 1). The specific antisera against several viral poultry disease (AI, ND, EDS) were tested to determine the developed ELISA has not significant reaction with antibodies against the viruses. The specificity and the sensitivity of the developed test were 94% and 89%, respectively.

Table 1. *The result of 180 positive and 60 negative sera tested for IB antibody using experimental and standard ELISAs*

OD	Positive sera							Negative sera						
	20	**30**	**40**	**110**	**130**	**140**	**AC**	**20**	**30**	**40**	**110**	**130**	**140**	**AC**
Exp.	0.133	0.213	0.305	0.203	0.357	0.487	0.657	0.119	0.115	0.114	0.121	0.136	0.145	0.119
Stan.	0.063	0.105	0.216	0.047	0.355	0.602	0.887	0.056	0.050	0.050	0.062	0.071	0.077	0.055

AC: after challenge

The HI titers (log_2) of the positive sera were 4.15, 5.25, 5.16, 4.76, 6.51, 7.93 and 10.2 respectively. In non-vaccinated group, HI titers at first and the end of test period were 3.94 and 3.42, respectively. The peak titer in vaccinated group was recorded at day 140 in ELISAs and HI. There were significant correlation coefficients between the results of experimental ELISA and IDEXX ELISA ($P<0.05$). Also, there were significant correlation coefficients between the results after challenge. There were no significant correlation coefficients between data in non-vaccinated group.

In our trial, an ELISA test for measuring antibody levels against IBV was developed. The test was standardized in term of reagent to obtain the significant S/N ratio. ELISA test has been developed to monitor antibody responses to vaccination against IB (Monreal *et al* 1985, Mockett & Darbyshire 1981, Soula & Moreau 1981, Feng & chen 1992, Xu-LanJu *et al* 1997). Case and colleagues (1982) optimized the parameters of ELISA for detecting antibody against IBV. They obtained minimal nonspecific binding and very high sensitivity using purified IBV as antigen at 50ng protein/well and final NaCl concentration 1.0M in dilution buffer.

In this trial, we obtained minimal nonspecific binding at 100ng protein/well (0.1µg/ml). According to titration result, by using 100ng protein/well with 1:3000 conjugate similar significant differences in S/N ratio was shown but for expensiveness of conjugate we chose the first item. To eliminate nonspecific

binding, we used dilution buffer in final NaCl concentration of 0.15M however, higher concentrations of NaCl was not tested.

The experimental ELISA was detected low to moderate level after live vaccination and moderate to relatively high levels of antibody against IBV after injection of inactivated vaccine as well as commercial one. These data was agreed with the HI results. In non-vaccinated group OD was lower than cut off (0.196) in experimental ELISA. Also, the titer of HI test was parallel with the ELISAs results and was negative in this group during the observation period. The OD of ELISAs and HI titer increased slowly following live H120 vaccination and more during the first 3 weeks following oil vaccine injection. No immediate humoral immune response was detected following vaccination at 10 day of age, may be to neutralize of the vaccine virus by maternal antibody but detected around day 30 of life. Decrease of antibody titers occurred over the following weeks until the second vaccination. The results of comparison of ELISAs and HI tests were similar to the result of Mockett and Darbyshire (1981) and Monreal *et al* (1985).

The results of antibody titration showed that the developed ELISA could be reliable, repeatable and sensitive for monitoring of vaccination schedules and detection of early rising of antibodies against IB rapidly.

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