A Liquid-Phase Blocking ELISA for the detection of antibodies to Infectious Bronchitis Virus.

SUMMARY

A liquid phase blocking ELISA (LPB-ELISA) was developed to the detection and titration of antibodies to infectious bronchitis virus (IBV). The purified and nonpurified virus used as antigen, capture and detector antibodies, and chicken hyperimmune sera were prepared and standardised for this purpose. A total of 156 sera from vaccinated and 100 from specific pathogen free chickens, that had no recorded contact with the virus were tested. The respective serum titers obtained in serum neutralization test (SNT) were compared with those in LPB-ELISA. There was a high correlation \( r^2 = 0.8926 \) between the two tests. The LPB-ELISA provides a single test suitable for the rapid detection of antibodies against bronchitis virus in the chicken sera, with a good sensitivity (100%), specificity (88%) and agreement (95.31%).

Introduction

Infectious bronchitis virus (IBV) infects the respiratory tract, kidneys or oviduct of chicks of all ages causing retarded growth, mortality, reduced egg production and inferior egg shell quality (1). For the control of virus infection, broilers are vaccinated at one day of age with live attenuated vaccines. Breeders and egg layers are vaccinated at approximatelly 8-weekly intervals with live attenuated vaccines and with inactivated vaccines after commencement of lay (2). IBVs contain four structural proteins: \( S^1 \), \( S^2 \) with Mr of 92K and 84K, respectively. The membrane (M) polipeptide p23 is heterogeneously glycosylated with Mr of 34K. The nucleocapsid (N) protein with Mr of 52K, associated with RNA (3). The role of \( S^1 \) glycoprotein in induction of humoral antibody responses has been best studied to date; and it induces both virus neutralization (VN) and haemagglutination inhibiting (HI) antibodies (1,2). Generally, acute infections are diagnosed by the immunofluorescence test (IFT) or virus isolation. Serological assays like haemagglutination inhibition (HI) test, the agar gel precipitation (AGP) test, and enzyme linked immunosorbent assay (ELISA), are also used (4). Although the serum
neutralization (SN) test is the only technique that could measure the neutralizing antibodies, it is rarely used because it is too expensive and laborious to perform. In broilers, the usually short time between infection and slaughter may hamper the interpretation of serological results. A field study in commercial broiler flocks without clinical infections favoured the use of an indirect and sandwich ELISA for the serological diagnosis of IBV humoral response (4,5,6,7). Therefore, we developed and standardised a liquid phase blocking ELISA (LPB-ELISA) comparing the results with the serum neutralization test, for the detection of post-vaccinal humoral response against IBV.

Materials and Methods

Virus antigen

The Massachusetts strain (Mass 41) of IBV was propagated by infection of 9-11 days old embryonated specific pathogen free eggs and the allantoic fluid was harvest as recommended. (3). The virus was further purified by a modification of methods (5,6). The same IBV strain replicated in embryonated specific pathogen free eggs and clarified at low speed centrifugation was used as nonpurified antigen in the LPB-ELISA.

Capture antibody

The chicken IBV specific γ-globulin was used as the capture antibody. For this purpose, one group of ten specific pathogen free chicks, white Legorns, an inbred C/O Line, obtained from Rhodia Merieux were placed at one day of age into positive pressure isolation units. At 2, 6 and 10 weeks of age, ten chicks were vaccinated intraocularly (i.o) with 10^{4.0} CD_{50} of M_{41} strain in 50 μl of phosphate buffered solution (PBS). After that, all chicks were vaccinated intramuscularly at two weeks of age with 0.5mg of inactivated M_{41} strain in 10μl of PBS mixed with Freund’s complete adjuvant (1:1). At 6 and 10 weeks of age these ten chicks were revaccinated intramuscularly with 0.5mg of inactivated M_{41} strain in 100μl of PBS mixed with Freund’s incomplete adjuvant (1:1). The chicks were bled from wing vein at ten days after the last vaccination. The chicken IBV specific γ-globulin fraction was obtained as described previously (8).

Detector antibody

The detector antibody was prepared by the immunization of three guinea pigs with purified IBV as described previously (6,7).

Serum samples

A total of 156 serum samples collected from different vaccinated commercial breeding, and 100 serum samples from SPF chickens were titrated both in LPB ELISA and SNT. A positive reference serum was obtained as described previously (6). The negative reference serum consisted of a mixture of 10 serum samples collected from SPF chickens.

Serum neutralization test

The reagents and the basic procedures used were described before (6,7).

Development of LPB ELISA assay

Optimal dilution of all reagents (capture antibody, detector antibody, serum samples and nonpurified virus) were determined using chessboard titration (9). Different capture antibody dilutions were tested against several nonpurified viral antigen concentration able to give the best discrimination between the positive and the negative reference sera (10).

Application of LPB-ELISA

The test was performed as described before with some modifications (10,11). The microplates (NUNC) wells were coated with the capture antibody diluted at 1:250, in carbonate bicarbonate buffer (0.05M pH 9.6) for over night at 0°C. After five washings with 0.05% tween 20 (PBSST), this buffer containing 15% skim milk (PBSST) was added, and after incubation for 45 min at 37°C, the plates were ready for use. Sera to be tested were treated
with 1% of trichloroacetic acid before mixture with a fixed concentration of nonpurified virus dilution (1:5), also diluted in phosphate-buffered saline solution with 0.5M of NaCl. Each serum test was run in duplicate. After incubation at 37°C for 90 min, the virus antibody mixture was transferred at 37°C for 60min. IBV antigen was stored at -70°C and used at a concentration of 1:5 which gave an optic density of 1.5 at 492nm. So, the plates were washed as before. Optimal dilution, 1:4000 of guinea pig detector anti-IBV serum in PBSSTM was added. After that, the plates were incubated for 60min at 37°C. An optimal dilution, 1:16,000 of rabbit anti guinea pig IgG (commercial conjugated to horse-radish peroxidase SIGMA) in PBSSTM was added. After incubation for 60 min at 37°C, the plates were again washed as described previously. A mixture of 0.006% H2O2 and 0.4 mg of o-phenylenediamine/ml of 0.1M Na2HP04 and 0.1M citric acid buffer, pH5.0 was used as substrate and chromogen. After 15 min of incubation at room temperature, 2MHC1 was added to block the enzimatic reaction, and the optic density of the plate was read at 492nm in a Titertek multiscan reader. In each plate, 22 wells were used for antigen control with no test sera added, and 2 wells were used of the reciprocal serum dilution that inhibited color development in relation at 22 antigen control wells. For this determination, we used the formula:

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T = \frac{(X - A) \log Y + (B - X) \log Z}{B - A}
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where X=50% of the media optic density (OD) at 492nm in 22 antigen control wells; A= media of OD in the wells the OD values of which are immediately lower than X; B= media OD in the wells the OD values which are immediately higher than X; Y= the reciprocal of the serum test dilution at which B was determined; Z= the reciprocal of the serum test dilution at which A was determined; and T= antibodies titers expressed as the logarithm of the reciprocal serum dilution.

Statistical analysis

The correlation coefficient (r) between LPB-ELISA and SNT was determined for serological analysis as described before (10). The cut off point was determined by graphic analysis of copositivity and conegativity of LPB-ELISA and SNT obtained by intercept of the copositivity and conegativity curves projected on the ordinate axis (11).

Reproducibility of the assay LPB-ELISA

The reproducibility of the LPB-ELISA for antibody detection was determined by using the OD values for serum dilutions of negative and positive controls, tested on twenty different days. These values were subjected to statistical analysis by Student's t-test. The LPB-ELISA assay detected Newcastle disease virus, reovirus or influenza A virus (results no given).

RESULTS

The chicken IBV specific γ-globulin used as the capture antibody showed higher SN titer \( \geq \log_{10} 8.0 \). The trichloroacetic acid used for serum precipitation at 1% of concentration eliminated the inespecific reaction of the some components of the positive and negative avian sera (Fig 03). In order to determine the best dilution for the capture
antibody used to provide trapping, the γ-globulin was titrated in a sandwich ELISA and the optimal dose was 1:250.

The correlation coefficient between the LPB-ELISA and SNT for a total of 256 serum samples were $r^2 = 0.8926$ p<0.0005 (Fig 01). The copositivity and conegativity results were determined with the cutoff recorded as ≥ 0.6 (Fig 02). Table 01 showed the specificity (88%) and sensitivity (92.85%) of LPB-ELISA for antibody detection. The agreement among the LPB-ELISA and SNT was 95.31%. The reproducibility of LPB-ELISA for antibody detection demonstrated the coefficient of variation of 2.40%.

DISCUSSION

Antibody response to the $S_1$, $S_2$, N and M virus proteins were also detected in vaccinated chicks with inactivated IBV strains. The $S_1$, $S_2$ and N proteins all induced cross reactive antibodies which were detected in ELISA (1). In poultry, high specificity of serological tests is more important than high sensitivity, since low sensitivity can be compensated for by using a higher number of blood samples (4). This confirming that LPB-ELISA developed here demonstrated 88% of specificity and 95.31% of agreement, when used the same cut off level ≥ 0.6 reported before (4,9,11,12,13). The determination of 50% competitive antibody titres in LPB-ELISA by use of the mathematical interpolation procedure, on the basis of a larger number of antigen control wells than those described (11,13) allowed a more precise estimate of these titres, considerably reducing the intertest variation (2.40%). The use of chicken IBV specific γ-globulin as capture antibody has several advantages over the use of rabbit polyclonal antisera. Using the polyclonal antibody amplify the non-specific reactions with the allantoid fluid proteins, results also found in others studies (6,7). In this present study, it was necessary to precipitating the chicken serum proteins with 1% of trichloroacetic acid to avoid the inespecific protein binding (15). This was confirmed by the results presented by the figure 03. In fact, it was the first time that LPB-ELISA was applied in chicken sera. It has not been used to measure anti-IBV antibodies in broilers chickens as frequently as the commercial indirect ELISA. In spite of the relatively higher correlation coefficients recorded between indirect ELISA test, agar precipitating gel test, HI test and SN test, it is important to emphasize that is different intrinsic properties of each serological test (4). On the other hand, the LPB-ELISA has been compared with the
serum neutralization test, specially the liquid-phase, where specific antibodies in the test sera effectively "block" the antigen and prevent it from reacting in the sandwich ELISA. Thus the blocking ELISA developed here presented high sensitivity, when it was compared with those reported before (9,10,11,13). However, LPB-ELISA has been used to measure anti-foot-mouth and rabies virus antibodies in cattle and human sera, respectively (9,10,11,13). Our results showed a significant correlation ($r^2=0.8926$) among the LPB-ELISA and SN, similar to the results obtained in others studies (10,11,13). Certain ELISAs in particular “blocking” ELISA may have a number of advantages over the serum neutralization test, but no serological test which has been analysed gave results that interpreted indicated virus protection. Therefore, the LPB-ELISA is considered an useful tool for routine laboratory diagnosis of IBV antibodies, thereby eliminating the need for cumbersome serological monitoring.

References