Detection and Quantification of Antibodies to Newcastle Disease Virus in Ostrich and Rhea Sera Using a Liquid Phase Blocking Enzyme-Linked Immunosorbent Assay

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A liquid phase blocking ELISA (LPB-ELISA) was adapted for the detection and quantification of antibodies to Newcastle disease virus. Sera from vaccinated and unvaccinated commercial flocks of ostriches (Struthio camelus) and rheas (Rhea americana) were tested. The purified and nonpurified virus used as the antigen and the capture and detector antibodies were prepared and standardized for this purpose. The hemagglutination-inhibition (HI) test was regarded as the reference method. The cutoff point for the LPB-ELISA was determined by a two-graph receiver operating characteristic analysis. The LPB-ELISA titers regressed significantly ($P < 0.0001$) on the HI titers with a high correlation coefficient ($r = 0.875$). The two tests showed good agreement ($\kappa = 0.82; P < 0.0001$), relative sensitivity (90.91%) and specificity (91.18%), and accuracy (91.02%), suggesting that they are interchangeable.

Newcastle disease (ND) is caused by an avian paramyxovirus (APMV-1 serotype) that belongs to the genus Rubulavirus of the family Paramyxoviridae (19). Newcastle disease virus (NDV) occurs worldwide and has a considerable economic impact on the world poultry industry, ranging from losses due to disease and the expense of vaccination to the significant cost of diagnostic laboratory investigations (14). The breeding of ratites (ostriches, emus, and rheas) has expanded considerably all over the world in recent years. They are susceptible to several diseases of domestic fowl, including ND (15, 20). Efforts to control and prevent ND through efficient vaccination programs and corresponding serological monitoring are constant.

The hemagglutination-inhibition (HI) test is still the most widely used conventional serological method for measuring anti-NDV antibody levels in poultry sera, and it is considered the standard laboratory test for this disease (30). However, sera from other species tend to give a high incidence of false-positive results. And although the number of nonspecific agglutination reactions can be reduced by pretreatment with heat and kaolin, these procedures decrease the sensitivity of this test (28).

Indirect enzyme-linked immunosorbent assays (I-ELISA) have been developed, evaluated, and well correlated to the HI test for serodiagnosis of NDV in poultry (4, 8, 18). In spite of their high sensitivity, easy standardization, lack of requirement for serum pretreatment, and possible computerization of the system, these assays have the disadvantage of not being applicable to the testing of ratite sera in a single system unless anti-ratite species conjugates are used in place of an anti-chicken conjugate (5, 28).

An APMV-1-specific monoclonal antibody blocking ELISA with the ability to test sera from exotic or wild avian species for NDV-specific antibodies in serial twofold dilutions or a single dilution has been described (9, 13). However, production and maintenance of hybridoma cells are time-consuming and sometimes expensive for laboratories with limited facilities. Moreover, assays with a single serum dilution are faster and more practical than serial dilution assays (7, 25, 26).

Additionally, the determination of a suitable cutoff point in ELISA and other quantitative serodiagnostic tests becomes a useful tool of analysis for better test performance as well as reliable sensitivity and specificity, principally when no specific assumptions are made concerning the distribution of the ELISA data (30). If sensitivity and specificity are equally important, the two-graph receiver operating characteristic (TG-ROC) method is appropriate (11).

In this study a liquid phase blocking ELISA (LPB-ELISA) with polyclonal immunoreagents was adapted for the detection and quantification of antibodies to NDV in sera from vaccinated and unvaccinated commercial flocks of ostriches (Struthio camelus) and rheas (Rhea americana) in a single system. Furthermore, TG-ROC analysis was carried out to determine the optimum cutoff point for the LPB-ELISA. Values obtained in the HI test and LPB-ELISA were compared for relative sensitivity and specificity, predictive values, accuracy, agreement, and likelihood ratio by linear regression analysis and determination of the correlation coefficient.

MATERIALS AND METHODS

Virus antigen. The NDV live vaccine strain La Sota was propagated in the allantoic cavities of 9- to 11-day-old embryonated specific-pathogen-free chicken eggs by inoculation with 0.1 ml of infectious allantoic fluid containing $10^{4.4}$ median embryo infective doses (EID$_{50}$). The infected allantoic fluid (IAF) was harvested and clarified by centrifugation at 8,000 $\times g$ for 1 h at 4°C in a Sorvall SLA-1500 rotor (Sorvall Products, Newtown, Conn.). The reciprocal of the hemagglutination (HA) titer of the stock NDV harvested was 2,048. Approximately 800 ml of IAF was subjected to protein precipitation with 8.7% (wt/vol) polyethylene glycol (PEG-8000) (Sigma Chemical Co., St. Louis, Mo.) and 2.7% (wt/vol) sodium chloride (NaCl) under gentle stirring for 18 h at 4°C. The concentrated IAF was centrifuged at 4°C for 1 h at 8,000 $\times g$ in a Sorvall SLA-1500 rotor, and the pellet was resuspended in 30 ml of TNE buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA [pH 7.4]). Next, 10-ml volumes of concentrated virus suspension were layered over a discontinuous 30 to 55% (wt/vol) sucrose gradient.
Viral purification step. The IAF clarified by centrifugation at 8,000 rpm at 4°C for 4 h at 96,000 × g in a Sorvall AH-629 rotor. The 1 ml fractions from each tube which highly adsorbed at 254 nm (viral RNA) and 280 nm (total protein) were pooled and ultracentrifuged through a linear 20% to 55% (wt/vol) sucrose gradient (TNE buffer), and ultracentrifuged at 96,000 × g for 12 h at 4°C in a Sorvall AH-629 rotor. The fractions collected as described above were pooled and centrifuged for sucrose removal. The final pellet was resuspended in 4 ml of TNE buffer, and the protein concentration was estimated by a biichromonic acid (BCA) assay (23). The resultant TNE suspension containing the purified viral antigen was stored at −70°C in 0.30-ml aliquots. The efficiency of all purification processes with regard to viral hemagglutinin reactivity was monitored by an HA test using aliquots from each viral purification step. The IAF clarified by centrifugation at 8,000 × g for 1 h at 4°C in a Sorvall SLA-1500 rotor was used as nonpurified antigen in the LPB-ELISA and the HI test.

Capture antibody. The capture antibody was prepared by the immunization of three guinea pigs with purified NDV (6). Previously the purified NDV was subjected to an adsorption-elution assay with chicken red blood cells (22) in order to achieve a higher purity level by removing undesirable IAF-derived residual protein components from the purified NDV suspension. The resultant guinea pig anti-NDV serum was inactivated at 56°C for 30 min, titrated by an HI test, and stored at −20°C. The specific reactivity of the capture antibody to NDV was tested by checking nonspecific reactions between the guinea pig antiserum and noninfected allantoic fluid at a protein concentration of 1 μg/ml in coating buffer. An antiserum to rabbit anti-guinea pig immunoglobulin G (IgG)-horseradish peroxidase conjugate diluted 1:1,000 in phosphate-buffered saline (PBS).

Detector antibody. The chicken anti-NDV serum was used as the detector antibody as described previously (26). Briefly, the purified NDV was subjected to the procedure described above for enhancing the purity level. The chicken anti-NDV serum obtained was inactivated at 56°C for 30 min, titrated by an HI test, and stored at −20°C. The reactivity of the detector antibody to noninfected allantoic fluid was also determined by an I-ELISA (10) as described above, using a rabbit anti-chicken IgG-horseradish peroxidase conjugate prepared as described below and diluted 1:2,000 in PBS.

Conjugate. A rabbit anti-chicken IgG coupled to horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) was used as the conjugate. Possible reactivity of the conjugate and ratite sera was tested by a double antibody sandwich ELISA (6) using two serum pools consisting of a mixture of three sera, one from each ratite species, showing high HI titters.

Test sera. A total of 78 ratite serum samples from ostrich and rhea breeder farms, divided into four groups, were tested by both the HI test and the LPB-ELISA. Twenty-five of the sera were from a population of unvaccinated 3-month-old ostrich chicks, 11 were obtained from vaccinated 3.5-month-old ostrich chicks, and 31 were from vaccinated 4-month-old rhea chicks. The birds were vaccinated with the La Sota live strain of NDV by eye drop instillation and were bled 20 days after the vaccination. No clinical signs of disease were observed in any of the birds tested. Sota live strain of NDV by eye drop instillation and were bled 20 days after the vaccination. No clinical signs of disease were observed in any of the birds tested.

RESULTS

Capture and detector antibodies and conjugate nonspecific reactivity. The guinea pig antiserum tested by I-ELISA at a 1:8 dilution of 0.088 at 490 nm presented an OD of 0.069; the chicken antiserum at a 1:10 dilution showed a mean OD of 0.040, and the corresponding blank mean OD was 0.035. When the noninfected allantoic fluid was changed to IAF, mean OD readings increased to 1.991 for the guinea pig antiserum and 1.755 for the chicken antiserum; the blank mean OD readings were 0.035 and 0.038, respectively. Similarly, the conjugate at a 1:2,000 dilution exhibited a mean OD of 0.069 at 490 nm to a 1:8-diluted ostrich serum pool and a mean OD of 0.071 to a 1:8-diluted rhea serum pool by a double antibody sandwich ELISA. However, mean OD readings increased to 2.790 when ratite serum pools were replaced by a detector antiserum at a 1:8 dilution.

LPB-ELISA cutoff point. The proportion of HI-seropositive (HI titer, ≥3 log$_2$) ratites was 56%. The sensitivity and specificity curves of the LPB-ELISA as functions of the cutoff points used are shown in Fig. 1. By TG-ROC analysis, the interception point of the two curves indicates that with a cutoff point of 29.00% (PI value), the LPB-ELISA presents a relative Se and Sp of approximately 0.93, or 93% (point of equivalence). The selection of this cutoff point is based on count data (nonparametric approach), because TG-ROC indicated deviations from a normal distribution. The accuracy level for this analysis was 95%. Thereafter, a PI of ≥29.00% was regarded as indicating an LPB-ELISA-positive serum, and a PI of <29.00% was considered as indicating an LPB-ELISA-negative serum.
Relationship between the LPB-ELISA and the HI test. The relationship between the LPB-ELISA and the HI test is shown in Table 1. Of all the sera tested, 40 (51.28%) were positive and 31 (39.74%) were negative with both the LPB-ELISA and the HI test. Three sera (3.85%) were positive only with the LPB-ELISA, whereas four (5.13%) were positive only with the HI test (Table 1). The relative sensitivity of the LPB-ELISA was 90.91%, and the specificity was 91.18% ($P < 0.0001$); the accuracy between the two tests was 91.02%. The positive predictive value (93.02%) and the negative predictive value (88.57%) are also shown.

There was good agreement between the two serological methods ($\kappa = 0.82$) ($P < 0.0001$). By convention, kappa values of 0.8 to 1.0 express almost perfect agreement between tests (24).

A close correlation ($r = 0.875$) was found between the LPB-ELISA and HI titers ($P < 0.0001$) by a linear regression analysis (Fig. 2).

The likelihood ratio for a positive test (LR+) was 10.30 (95% CI, 3.94 to 29.94), meaning that an LPB-ELISA titer of $\geq 29.00%$ was 10 times as likely to have come from an HI-positive bird as from an HI-negative bird. The likelihood ratio for a negative test (LR−) was 0.1 (95% CI, 0.04 to 0.23), meaning that an LPB-ELISA titer of $< 29.00%$ was 1/10 as likely to have come from an HI-positive bird as from an HI-negative bird. LR+ and LR− are graphically displayed in Fig. 3.

### DISCUSSION

In order to overcome the problems of routine NDV serology for ratites species, an LPB-ELISA was used and evaluated for the detection and titration of NDV-specific antibodies. The basic procedure for the LPB-ELISA was that used by Hamblin et al. (12) and Cardoso et al. (7), with the optimal concentrations of reagents being determined by checkerboard titration.

The usefulness of the ELISA based on the indirect method has been evaluated and correlated to the HI test many times. Brown et al. (4) obtained a correlation coefficient of 0.85 and a kappa value of 0.84 as well as high relative sensitivity (98.2%) and specificity (91.7%). A similar correlation coefficient ($r = 0.85$) was reported by Cvelic-Cabrilo et al. (8); this was somewhat better than the 0.75 calculated by Adair et al. (1). However, in both cases, the authors tested only chicken sera, using a commercial anti-chicken conjugate.

Cadman et al. (5) compared the reactivity of ostrich sera to NDV by an I-ELISA and an HI test. Using a peroxidase-labeled goat anti-ostrich IgG, a sigmoidal relationship ($r = 0.612$) (3rd-degree polynomial) was found when serum samples from vaccinated and naturally infected birds were tested.

![FIG. 1. Curves of relative Se and Sp of the LPB-ELISA using TG-ROC analysis. The intersection point of the two curves indicates the cutoff point (PI value = 29.00%) at which Se = Sp = 0.93 (dotted and dashed horizontal line). The accuracy level used was 95%.

![FIG. 2. Correlation between serum titers obtained by the LPB-ELISA (PI) and the HI test (log2).](image)

| LPB-ELISA result | No. of specimens with the indicated result(s) | HI test |
|------------------|-----------------------------------------------|--------|
|                  | Positive | Negative | Total |
| Positive         | 40       | 3        | 43    |
| Negative         | 4        | 31       | 35    |
| Total            | 44       | 34       | 78    |

* Relative sensitivity = 40/44 × 100 = 90.91%. Relative specificity = 31/34 × 100 = 91.18%. Accuracy = (40 + 31)/78 × 100 = 91.02%. Positive predictive value = 40/(40 + 3) × 100 = 93.02%. Negative predictive value = 31/(4 + 31) × 100 = 88.57%.
Nevertheless, this I-ELISA could not be used for sera from other species, limiting its applicability to laboratory routine. Our significant correlation result also confirms the report of Jestin et al. (13) that a PMV-1-specific monoclonal antibody from vaccinated specific-pathogen-free chickens and Muscovy ducks obtained a high coefficient correlation ($r = 0.90$) ($P < 0.001$). Czifra et al. (9) described a B-ELISA to test sera from chickens experimentally infected with NDV, chickens vaccinated against NDV, and field samples from chickens and turkeys; high sensitivity was found relative to either the HI test or the I-ELISA (mean, >90%), and the B-ELISA was consistently more sensitive than the HI test.

Recently, Koch et al. (15) used a similar B-ELISA to test 211 ostrich sera in a twofold dilution series; a kappa value slightly greater ($k = 0.85$) than that calculated in the present study was found, but a lower correlation was found ($r = 0.71$); sensitivity relative to the HI test was 91%, and relative specificity was 96%. The positive predictive value (97%) was greater than that described here, but a similar negative predictive value (87%) was observed, although predictive values are dependent on disease prevalence. The false-positive rate among the sera examined was lower (1.42%) than that found in our study (3.85%), which included two ostrich sera and one rhea serum, while the false-negative rate (5.21%) was similar to our findings (5.13%), including two sera for each ratite species.

Polyclonal immunoreagents (capture and detector antibodies) are cheaper than monoclonal antibodies for the B-ELISA, and the LPB-ELISA is easy to perform, dispensing with special skills and showing adequate applicability, as observed in our study, for testing sera from different avian species, while excluding possible nonspecific reactions to allantoic fluid components or reactivity between the sera tested and the conjugate. Probably the further purification of purified NDV by red-cell adsorption-elution (22) before the production of capture and detector antisera enhanced the effectiveness of antigen purification. Furthermore, the use of a single untreated serum dilution, as described, in our test system is more practical than serial dilution (28) because it decreases the preparation time and the number of microtiter plates required. The working test serum dilution of 1:8 was chosen as being the minimum serum dilution at which no gelation was observed during the liquid phase incubation for rhea serum-virus mixtures, thus enabling these suspensions to be transferred to solid phase microplates more easily. Probably this phenomenon is related to high-fat or low-protein diets offered to the ratites surveyed (16, 17); it continued even when the respective sera were submitted to clarification by centrifugation (10,000 × g for 10 min at 4°C). Koch et al. (15) used a starting ostrich serum dilution of 1:10 for B-ELISA, while Schelling et al. (21) assayed poultry sera for NDV antibodies by a similar B-ELISA using a sample dilution of 1:10.

On the other hand, the determination by TG-ROC analysis of a suitable cutoff point for obtaining balanced Se and Sp or a specific Se or Sp provided a useful tool for obtaining better performance of the test, particularly with data deviating from a normal distribution. The TG-ROC analysis used to compare the Se and Sp of an I-ELISA with those of an HI test using sera from vaccinated and unvaccinated ostriches showed that the I-ELISA was superior to the HI test in both Se and Sp, with both Se and Sp equaling 97.2% at the cutoff point for the I-ELISA (28). Moreover, because likelihood ratio determination is derived from the test Se and Sp only, it is unaffected by disease prevalence, making it an especially stable expression of test performance as found here.

In a previous study (26) we used a similar LPB-ELISA to detect antibodies against NDV in sera from vaccinated and unvaccinated partridges. High relative sensitivity (96.82%) and specificity (90.54%), as well as good accuracy (94.5%), were found. The correlation coefficient ($r = 0.8217; P < 0.0005$) was lower than that described here. However, a cutoff point determination was derived from the mean titer of control birds plus two times the standard deviation, a procedure widely used for this purpose (11). In fact, this approach leads automatically to an Sp of ≈97.5%, and this assumption holds true only in the case of a normally distributed test variable, as shown by Barajas-Rojas et al. (3). Thus, this procedure, without any indication of the resulting test Se, does not reflect the major function of a cutoff value, which is to distinguish between seropositive and seronegative individuals (11). In contrast, TG-ROC analysis provides an appropriate selection of cutoff values for obtaining Se and Sp for both parametric and nonparametric approaches.

The LPB-ELISA described above, together with TG-ROC analysis for cutoff determination, showed statistically significant test indices compared with those from earlier studies, particularly a close correlation coefficient and a high level of agreement with the HI test, emphasizing the diagnostic validity of the LPB-ELISA. Therefore, LPB-ELISA was demonstrated to be a very useful method and able to replace the B-ELISA,
the I-ELISA, and the HI test in seroepidemiological surveys or vaccinal monitoring for the determination of antibody levels to NDV in sera from ratites without a serum pretreatment requirement.

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