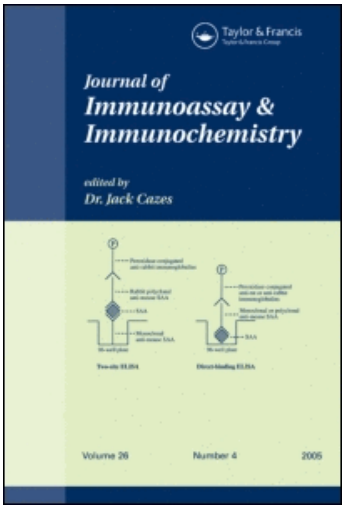

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AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF SPECIFIC IgG ANTIBODY TO *MYCOPLASMA GALLISEPTICUM* IN SERA AND TRACHEOBRONCHIAL WASHES

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KEY WORDS: Indirect IgG ELISA, regression analysis, standard curve, temporal appearance of IgG.

ABSTRACT

A sensitive indirect ELISA is reported for the detection and quantitation of specific IgG to *Mycoplasma gallisepticum* (MG) in sera and tracheobronchial washes (TBW) of MG-infected chickens. The sensitivity of the assay was ensured by the use of mouse monoclonal antibody to chicken IgG bound to a prospective anti-MG containing sample that was complexed with MG antigen immobilized on a solid phase. The level of specific IgG antibody in a test sample was detected by using peroxidase-conjugated goat anti-mouse IgG. Serum samples with various levels of anti-MG IgG activity were used to construct a standard curve response at a single working dilution by using Logit-Log curve fitting. The assay was simple, reliable, and specific and was used to monitor the appearance of specific anti-MG IgG in chicken sera and TBW at various intervals after the onset of mycoplasma-induced respiratory disease. The IgG response reached a plateau at 2 and 4 weeks postinfection in TBW and sera, respectively; then the response waned but still was detectable at a significant level for up to 25 weeks postinfection.

INTRODUCTION

Mycoplasma gallisepticum (MG) is the major cause of chronic respiratory disease in chickens (25,33) and infectious sinusitis in turkeys (12). Due to the danger of vertical and horizontal transmission of mycoplasma-induced respiratory disease, rapid surveillance programs on status of flocks is necessary to control epidemics of the disease. The most commonly used methods for serodiagnosis of MG infection are the serum plate agglutination (SPA) and hemagglutination inhibition (HI) tests. Although these tests are specific and convenient for rapid serodiagnosis, they are not suitable for quantitative measurement of humoral responses, which was needed in our research. With the advent of enzyme-linked immunosorbent assay (ELISA) (14), the contribution of a host's response to infection can be evaluated and monitored after the onset of illness. Recently, several investigators (1,26,27,31) have reported on the development of ELISA for MG and *Mycoplasma synoviae* (MS) infection. None of the reports has addressed the kinetics of the appearance of specific anti-MG IgG in simultaneously obtained samples of sera and TBW at various intervals after exposure of chickens to MG. This study specifically addressed this point and furthermore provides a statistical model for both optimization of various ELISA reagents and prediction of antibody titer from absorbance value recorded at a single working dilution.

MATERIAL AND METHODS

Antigen Preparation

The mycoplasma antigen was prepared from an overnight culture of MG, R strain, organisms grown in 5 liters of sterile Frey's medium (17). At the peak of the logarithmic phase of bacterial growth, the organisms were harvested by centrifugation at 10,000 X g_{max} for 30 min at 4°C. The pellets were washed 4 times with sterile 0.01 M phosphate buffered saline solution (PBS), pH 7.2, and resuspended in 100 ml of PBS. Protein concentration was determined by the Bradford method (7) using reagent obtained from Bio-Rad Lab (Richmond, CA) and adjusted to contain 5 mg protein per ml. This fraction was then aliquoted in 5 ml tubes and sedimented at 1000 X g_{max} for 10 min at 4°C. The pellets then were solubilized by addition of an equal volume of a detergent mixture of TDSET [10mM Tris-hydrochloride (pH 7.8), 0.2% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate, 10 mM tetrasodium EDTA and 1% (vol/vol) Triton X-100] (24) containing 1 mM of phenylmethylsulfonyl fluoride (PMSF). The preparation was incubated for 60 min in a shaking water bath at 37°C and then centrifuged at 1000 X g_{max} for 10 min to remove insoluble debris. A pale yellow supernatant solution was collected and subsequently dialyzed against 3 changes of 10 mM Tris-HCl buffer, pH 7.8, for 3 days at 4°C and then dialyzed twice against 0.05 M CO_3-HCO_3 buffer, pH 9.6, at 4°C. The dialysate was collected, sterilized through 0.22 μm membrane filters, and

adjusted with buffer to contain 2.0 mg protein per ml. This stock antigen was finally divided into 1 ml aliquots and stored at -70°C.

Positive and Negative Reference Sera

Chicken anti-MG sera were obtained from five 10-week-old, specific-pathogen-free (SPF) white leghorns that had been infected with MG, R strain, at 6 and 8 weeks of age by aerosol exposure with 4.0×10^8 CFU/ml. Sera were tested for anti-MG and anti-MS antibodies by SPA and HI methods (4) before and after exposure to confirm absence and presence of specific antibodies. Specific hyperimmune antisera were pooled and used as MG-positive reference serum in the ELISA.

Negative antisera (normal chicken sera) were obtained from 10-week-old SPF chickens, field chickens, and from the National Veterinary Services Laboratory, Ames, Iowa. None of the negative antisera had developed a positive reaction to MG by either SPA or HI tests and accordingly they were selected as MG-negative reference sera in the ELISA.

Sera for Temporal Studies

Twenty-four 7-week-old SPF white leghorns were divided into 2 equal groups and maintained separately. One group, was infected by aerosol exposure with an overnight culture of MG organisms (R strain) containing approximately 3.0×10^{10} CFU ml. The other group, housed separately in a room supplied with filtered air under positive pressure, served as uninfected controls. Serum

samples were collected from both groups for analysis of anti-MG IgG at various times for up to 25 weeks after exposure to MG. All serum samples were heat-inactivated in a water bath at 56°C for 30 min to inactivate complement and were stored at -20° until assayed.

Collection of Tracheobronchial Washes

Thirty-six 7-week-old SPF white leghorns were infected as described above with an overnight culture of MG organisms. Four birds were killed at a time for the collection of TBW for analysis of anti-MG IgG for up to 25 weeks postinfection. Tracheae and bronchi were exposed and removed as described by Ewert *et al.* (15) for trachea. Wash samples were obtained by flushing the tracheobronchial tree with 2.0 ml of PBS containing 1 mM PMSF via a canula with gentle tissue squeezing for 3 min. Subsequently, the wash samples were emptied into 3 ml tubes. Samples were held on ice and sedimented at 600 X g_{max} for 20 min at 4°C; the supernatant solutions were collected, passed through 0.45 μm and 0.22 μm sterile membrane filters, and stored at -20°C until assayed.

ELISA Reagents

Mouse IgG_1, Kappa, monoclonal anti-chicken IgG (9) (0.1 mg/ml) was obtained from Fisher Scientific (Orangeburg, NY), diluted 1:100 in PBS/T, pH 7.2, and stored in small aliquots at -20°C until used. Horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-mouse IgG (heavy and light chain

specific) was obtained from Pel-freeze Biologicals (Rogers, AR) and used as a marker for the detection of specifically bound antibodies. The substrate solution consisted of 0.5% ortho-phenylenediamine (OPD) (Sigma Chemical Co., St. Louis, MO) in 0.1 M citrate buffer, pH 6.0, and stored at -20°C in small aliquots for a maximum period of 1 month. The working substrate solution was 2.2 mM OPD and 6.15 mM fresh H_2O_2. The washing buffer consisted of 0.01 M sodium phosphate, 0.14 M sodium chloride, and 0.05% Tween 20 (PBS/T), pH 7.2. The PBS/T was used both as a diluent for test samples, monoclonal antibody, and conjugate, and as a wash solution between the various steps of the assay. The blocking buffer consisted of 3% nonfat dry milk in PBS/T and was used to prevent nonspecific binding of immunoglobulin to the solid phase.

ELISA Reagent Concentrations

The optimum concentrations for the ELISA reagents were determined by the method of Voller and Bidwell (35). Briefly, MG antigen, antisera/TBW, IgG monoclonal antibody (MoAb), and conjugate were all tested in checkerboard titrations to determine their range of activities. Various dilutions of MG antigen were titrated against the positive and negative reference sera while the IgG MoAb and conjugate were added at constant dilutions. Then, various dilutions of the IgG MoAb were tested against various dilutions of the conjugate with the antisera added at constant dilutions to antigen-coated plates. The rationale was to determine the most economical concentration of each reagent that

would give a reading of > 1.0 with the positive serum and < 0.2 with the negative serum.

ELISA Protocol

Flat-bottomed polystyrene microtiter plates (No. 439454, Nunc, Roskilde, Denmark) were coated with detergent-solubilized MG membrane antigen (0.5 μg/well) in 100 μl of 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated for 1 hr at 37°C and overnight at 4°C. The wells were then washed 3 times (3x) with the washing buffer by running 200 μl of PBS/T down the side of each well, flicking the liquid out of the wells over a sink, then blotting the plate gently against absorbent paper. To reduce nonspecific binding to the solid phase, 150 μl of 3% nonfat dry milk in PBS/T solution was delivered to each well and the plates were incubated for 1 hr at room temperature (RT). After the wells were washed 3x as above, 100 μl aliquots of serially diluted sera or tracheobronchial wash samples in PBS/T were pipetted into the appropriate wells in triplicate and the plates were incubated for 1 hr at RT. Subsequently, the wells were washed 3x as above and 100 μl of mouse monoclonal anti-chicken IgG (2 ng/100 μl) was added to each well and the plates were incubated for 1 hr at RT. The wells then were washed 3x as above and 100 μl aliquots (0.06 μg/100 μl) of enzyme conjugated anti-mouse antibody were added to appropriate wells for detection. After 1 hr of incubation at RT, the wells were washed 3x as above to remove excess conjugate. Next, 100 μl of substrate solution was added to each well and allowed to react for 10 min at RT in the dark. The yellow product

reaction was stopped with 2 N H_2SO_4 (100 µl/well) and the optical density (OD) of each well was read at 490 nm with a microplate reader (Molecular Devices, San Francisco, CA). Each assay included antigen control (PBS/T substituted for antisera), positive and negative serum control, MoAb, conjugate, and substrate control. The PBS/T controls were included for identification of non-specific color development while negative controls were for measurement of background. The enzyme activity of the monoclonal control, conjugate control, and substrate control was consistently < 0.1 OD units while the background controls had an OD of < 0.15 for a 10 min reaction with substrate.

Expression of ELISA Results

The mean OD values of test samples per plate were compared with similar values of reference positive serum control diluted from 1/200 to 1/12,800 in triplicate (8). Background binding by normal chicken serum was subtracted from each dilution of unknown test samples and from dilutions of the positive reference control to obtain absolute OD values. To minimize the effect of day-to-day variations, the absolute OD values at each dilution were multiplied by the OD value of the standard reference positive serum control and divided by the OD value of a reference positive control determined in that particular assay on that day. The OD value of the standard reference positive serum control was chosen as the mean of 10 assays performed on 10 different days (28).

The ELISA endpoint (titer) was defined as the point where a titration curve transected a horizontal line (cut-off level) set

at 0.244 absorbance, which was twice the OD value of the negative reference serum control. The point of transection was calculated by interpolation between the two points closest to either side of the cut-off line, and recorded as reciprocal of the derived dilution.

Reproducibility of the Assay

The reproducibility of the ELISA assay was assessed by performing 10 replicate determinations of the reference positive serum control, using freshly prepared reagents each day. For each determination the anti-MG IgG titer, the geometric mean titer, the standard deviations and the regression slopes were determined. Determinations carried out on the same day as well as on successive days were examined for within and among assay variations.

Forty-six MG positive sera containing various levels of anti-MG IgG were assayed using dilutions from 1/100 to 1/25,600. The slopes, mean slope and 95% confidence interval were calculated. A few data points were above 2.5 absorbance and they were excluded from the calculations. Only the first points below the 0.244 absorbance cut-off level were used. Five to 7 data points were used for most calculations but for the samples with low titer, only 3 to 4 points were used.

Standard Curve for Anti-MG IgG

The 46 MG positive sera containing various levels of anti-MG IgG were used to derive a standard curve at a predetermined

working dilution. Three working dilutions were examined; 1/100, 1/400, and 1/800. Regression analysis by the non-linear least-squares (19) and by the Logit-Log transformation (2) methods were compared for deriving a reliable equation that would predict antibody titer from absorbance recorded at a single working dilution. All statistical calculations were performed on a Zenith computer (Zenith Data Systems Corporation, Franklin Park, IL) with the SAS/STAT™ software (SAS Institute, Inc., Cary, NC).

Test for Specificity

To assess the specificity of anti-MG IgG ELISA, the anti-MG reference serum was absorbed with various soluble antigen inhibitors according to established methods (6,10,18,23). Antigens were added to antisera at a concentration of 10 μg/ml and incubated in a 37°C water bath for 1 hr followed by an overnight incubation at 4°C. The antigens used were *Brucella abortus* and TDSET-solubilized antigens of *Mycoplasma gallisepticum*, *M. synoviae*, *M. pullorum*, *M. gallinaceum*, and *M. pneumoniae*. After incubation, protein complexes were pelleted by centrifugation and serum was filter sterilized through a 0.22 μm membrane filter. Antisera were then analyzed for MG-specific IgG by ELISA as described above. The percent inhibition was calculated by the following equation (18):

% inhibition = 100 [(OD100-OD0) - (ODx-OD0)]/ (OD100-OD0)

where OD100 represents the OD value of no inhibition obtained from antiserum preincubated without soluble protein inhibitors, ODx

represents the inhibited OD value of test samples, and OD0 represents the OD value of the background.

Hemagglutination-inhibition test

The HI test was performed as described by Vardaman and Yoder (34).

RESULTS

Standardization of the ELISA Procedure by Regression Analysis

The dependency of observed absorbance values on the parameters being tested at each checkerboard titration was examined by multiple regression analysis after some transformational changes. Subsets of predictor variables were incorporated in the model to improve the reliability of the model in prediction. Criteria for selecting subsets of predictors and consequently the best fit model (20) for titration results included: (i) the sample squared multiple correlation, R^2, which measures the proportion of variation explained by the model. The closer R^2 is to 1, the better the data fit the model, (ii) the F test statistic for comparing the full and reduced model, (iii) the mean square of error (MSE) for the predictor variable in the model, and (iv) Mallow's Cp statistic which involves error sum squares of predictor(s) variable(s). Mallow's Cp was calculated by the following equation:

$$\text{Mallow's } Cp = [RSS_p - RSSk'/2] + [P-(k'-p)]$$

where RSS_p and $RSS_{k'}$ denote the residual sum squares from predictors in the reduced and full model, respectively. The above

criteria were used as robust measures of the dependent variable and inevitably provided us with confidence in the optimization of various ELISA variables. Inclusion or deletion of any subsets of the predictor variables in the final model was initially examined, at each step, by stepwise regression procedure (13,22). Consequently, the variables Log_{10} reciprocal serum dilution (LRSD), reciprocal antigen concentration (RAC), and ratio of RAC to LRSD were included as predictors for absorbance reading in the multiple regression model, controlling for monoclonal and conjugate concentrations. This relationship can be expressed mathematically by the following regression equation:

$$\text{Absorbance (A)} = 5.620 - 3.111\ X_1 - 1.377\ X_2 + 0.749\ (X_1/X_2)$$

where X_1 denotes RAC; X_2 denotes LRSD; the proportion X_1/X_2 denotes the ratio of RAC to LRSD; 5.620, 3.111, 1.377 and 0.749 were the regression coefficients that corresponded to $\hat{B}_0$ (intercept), $\hat{B}_1$, $\hat{B}_2$, and $\hat{B}_3$, respectively. As shown in Table 1, the coefficients for each of the predictor variables (X_1, X_2 and X_1/X_2) incorporated in the model were highly significant ($P < 0.0001$), indicating their best fit to the model. This was further supported by the high R^2 value in the above model which accounted for 95% of the variability in absorbance readings for the MG positive sample. When the above regression equation was used to select, in terms of prediction reliability, for the appropriate antigen concentration to be used, it was evident that both 5 μg and 10 μg per ml behaved similarly (Fig. 1) on the basis

TABLE 1.

Regression Analysis for the Effect of Various Concentrations of *Mycoplasma gallisepticum* Antigens and Various Dilutions of Positive Antiserum on Absorbance Values Measured by Enzyme-linked Immunosorbent Assay

Variable	Estimated coefficient	Standard error	Model selection criteria
Intercept (B_0)	5.62	0.280	F statistic = 128.4, prob>F = 0.0001
Reciprocal antigen concentration (RAC)	-3.11	0.474	R^2 = 0.951
Log_{10} reciprocal serum dilution (LRSD)	-1.38	0.090	MSE = 0.042
Ratio of RAC/LPSD	0.75	0.151	Mallow's Cp = 2.07

of the above selection criteria. This was further demonstrated when logistic regression was performed on each antigen response separately (output not shown).

A similar regression model was established to study the effect of MoAb and conjugate concentrations on absorbance readings, controlling for the antigen and serum dilutions. As a result, an optimum concentration of 1/5000 dilution (20 ng/ml) and 1/2000 dilution (0.565 μg/ml) were selected for the IgG monoclonal and conjugate, respectively. For the titrations of TBW, an optimum concentration of 1/5000 dilution and 1/1000 dilution were found satisfactory for the IgG MoAB and conjugate, respectively.

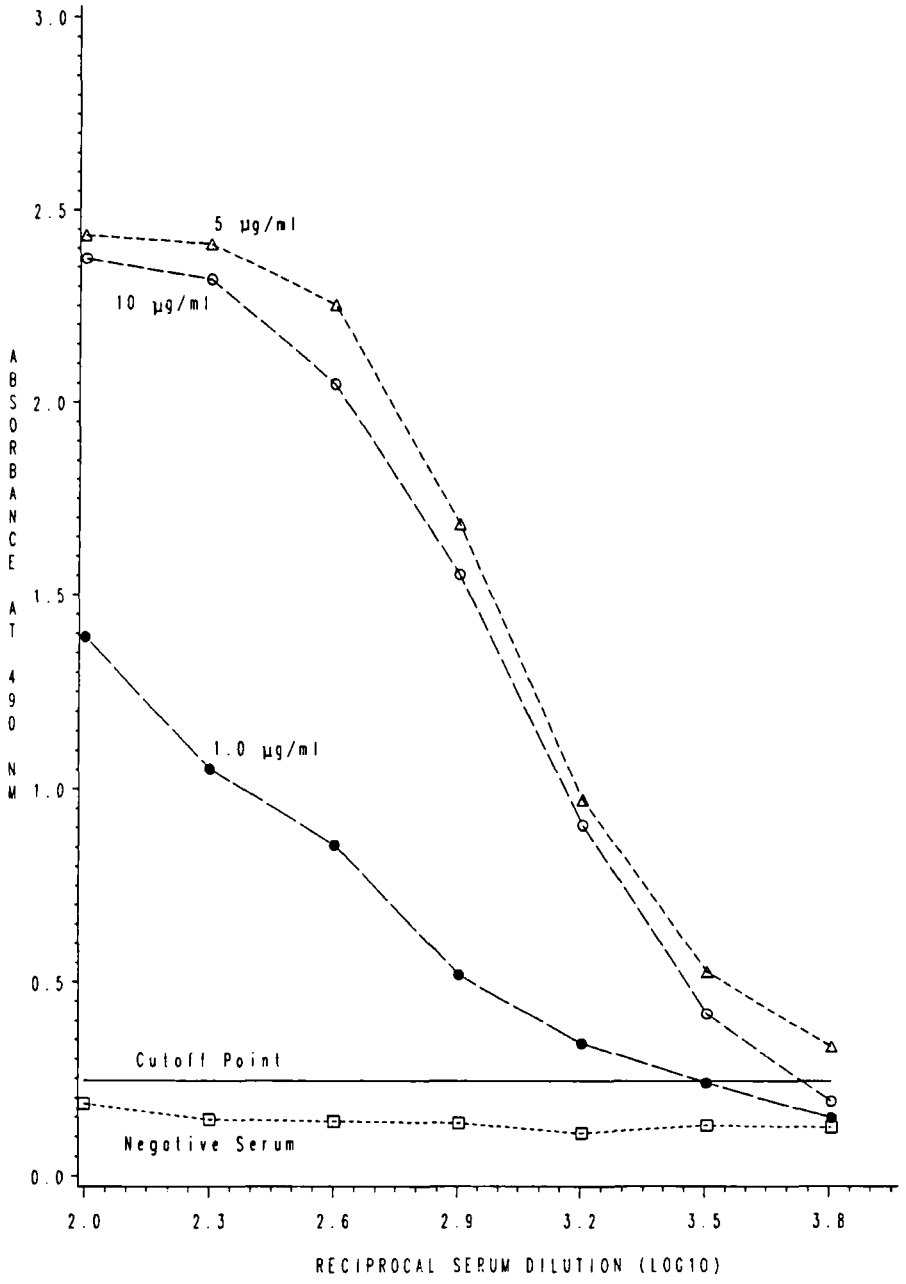

FIGURE 1. Titration of TDSET-solubilized MG antigen against known positive and negative antisera with use of fixed concentrations of mouse monoclonal anti-chicken IgG (1/5000) and conjugate (1/2000). Absolute absorbance at 490 nm after 10 min substrate incubation is noted on ordinate.

From series of assays, it was concluded that coating the microtiter wells with 5 μg per ml of MG membrane protein and using 1/5000 IgG MoAb and 1/2000 conjugate diluted in PBS/T should be the standard used for all subsequent tests to detect serum anti-MG IgG activity after substrate incubation for 10 min.

Titrations of reference positive and negative antisera against various concentrations of MG antigen with use of optimal concentrations of IgG MoAb and conjugate demonstrated that the positive serum had significantly greater absorbance readings than the negative serum at all antigen dilutions tested (Fig. 1). In addition, no specific IgG antibodies were detected at any dilution of negative antisera even when reacted with the highest antigen concentration.

Test for Specificity

Adsorption of anti-MG reference serum with homologous MG antigen significantly ($P < 0.0001$) abolished antibody binding by approximately 82% in the IgG assay at 1:400 dilution of the antiserum (Fig. 2). In contrast, adsorption of the positive antisera with heterologous antigens from different avian mycoplasmas reduced the antibody binding by 14.7, 7.1, and 2.6 percent for *M. gallinaceum*, *M. synoviae*, and *M. pullorum*, respectively. Adsorption of positive antiserum with *M. pneumoniae* membrane antigens did not inhibit antibody activity due to the absence of shared epitopes and, consequently, lack of immune complex formation. *Brucella abortus*, however, inhibited the antibody activity by 5%.

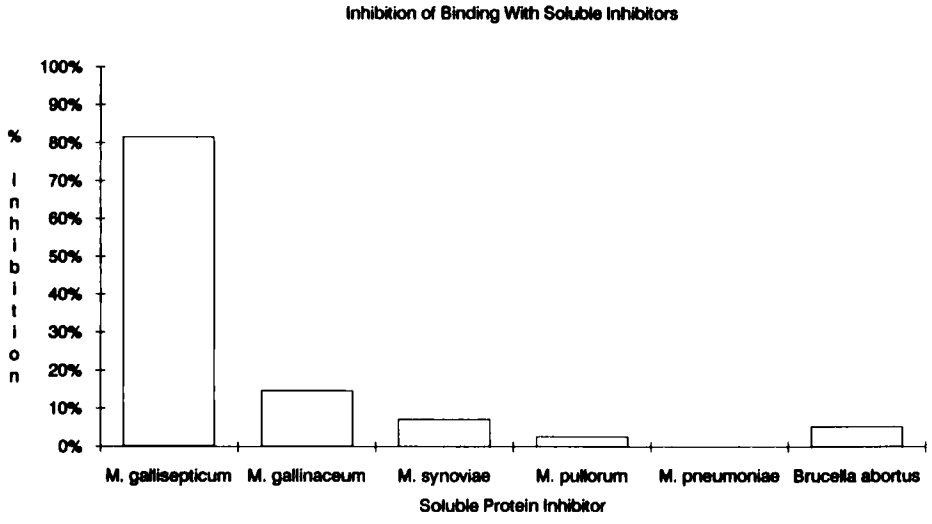

FIGURE 2. Inhibition of binding with soluble inhibitors. Pooled high-titer chicken anti-MG antiserum was diluted 1:400 and preincubated with different soluble inhibitors at a concentration of 10 μg/ml for 1 hr at 37°C and overnight at 4°C before the antibody assay. The soluble inhibitors included *Brucella abortus* and TDSET-solubilized antigens extracted from *Mycoplasma gallisepticum*, *M. gallinaceum*, *M. synoviae*, *M. pullorum*, and *M. pneumoniae*.

Reproducibility of the ELISA Assay

The reproducibility of the assay was based on the performance of the standard reference positive serum control in various independent assays. When determinations were performed on the same day, the variance of anti-MG IgG titer ranged from 0.017 to 0.114 for within-assay variation. When determinations were carried out on different days, the variance was 0.043 for between-assay variation (Table 2).

The regression equation for the replicate assays of the reference positive serum control was $Y= 2.63-0.86X_i$ and for the 46

TABLE 2

Reproducibility of Anti-MG Standard Reference Positive Serum Control Assayed by IgG ELISA

Assay	Log_{10} geometric mean titer	Standard deviation	Number of replicates
1	3.86	0.162	10
2	5.03	0.238	10
3	4.43	0.171	10
4	4.20	0.129	10
5	3.79	0.338	10
Among Assays	4.30	0.208	50

positive sera containing various levels of anti-MG IgG was $Y=2.22-0.97X_i$. The individual slopes of all but 2 of the 46 samples and all of the replicate assays of the reference positive serum were within the 95% confidence interval of the mean slope.

Standard Curve for Anti-MG IgG

A plot relating the absorbance value to Log_{10} titer was examined at the 3 single working dilutions; 1:100, 1:400, and 1:800. Regression analysis of each plot by the non-linear least-squares method revealed the reliability of 1:400 plot in predicting absorbance from antibody titer (Fig. 3). The general form of the logistic equation used for the plot in Fig. 3 is given by:

$$\text{Absorbance } (Y) = \frac{a}{1 + e^{-c(x-b)}}$$

where a is the maximum value of response recorded, b is the

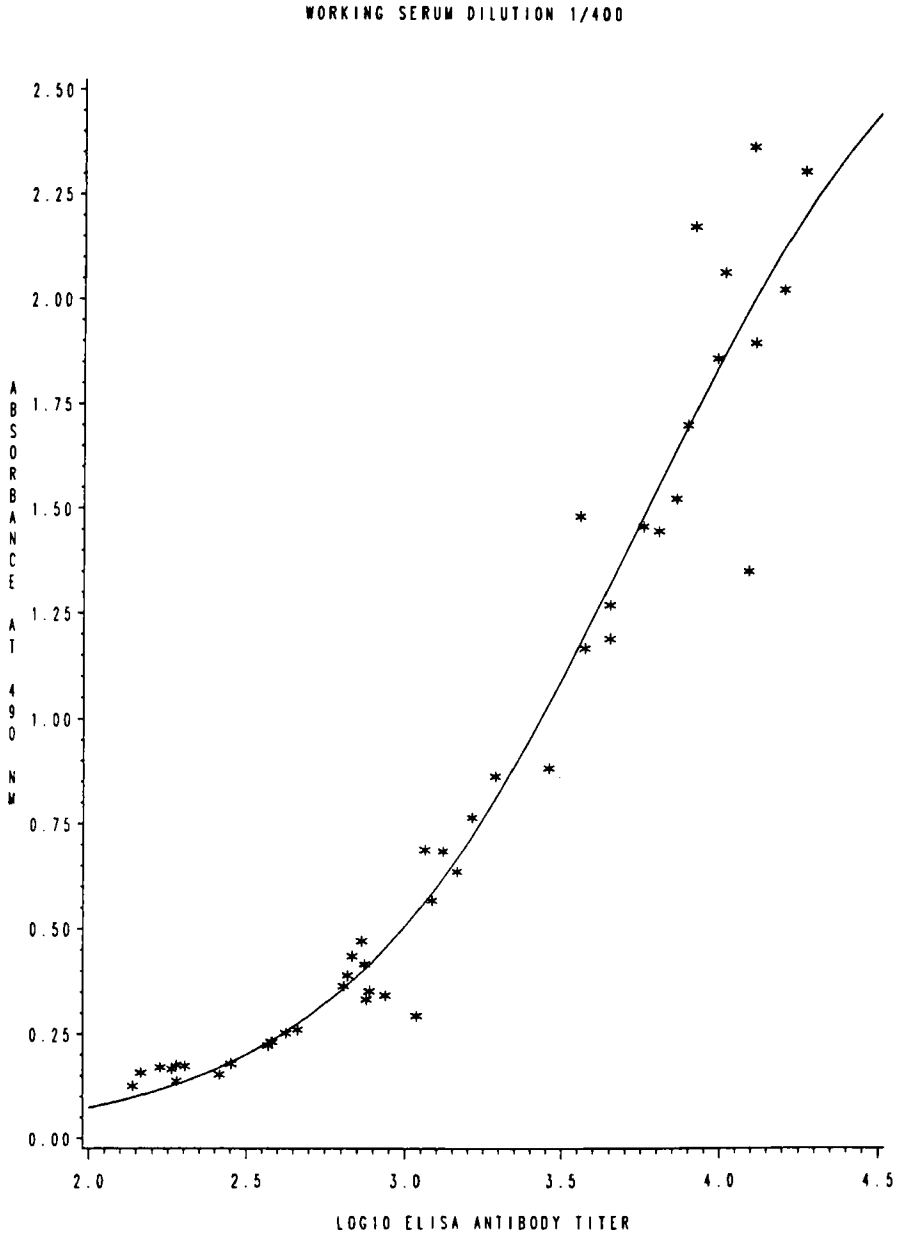

FIGURE 3. Absorbance values recorded at a serum working dilution of 1/400 plotted against their respective end-point titer for 46 sera having various levels of anti-MG IgG titer. Data points (*) are fitted by the logistic regression curve.

inflection point of the curve, c is the slope factor, x is the independent variable, and e is the exponential function which was 2.7183. Although we were interested in prediction of antibody titer from absorbance, we found that the application of logit transformation on the 1:400 plot provided a satisfactory curve that could be fitted by simple linear regression (13,22,37). The fitted line (Fig. 4) demonstrated a significant correlation coefficient ($r = 0.97$, $n = 46$; $P < 0.001$), indicating close association between titer and absorbance measured. The general form of the logit equation that could be used to predict anti-MG IgG titer for a sample whose absorbance value was recorded at a single working dilution is:

Logit Absorbance (Y) = -7.582 + 2.009 (titer)

Hence,

titer = Logit Y + 7.582/2.009

where Logit $Y = \log_{10} [Y/(1-Y)]$, and -7.582 and 2.009 are the regression coefficients which correspond to $\hat{B}_0$ (intercept) and $\hat{B}_1$, respectively.

The HI test was performed on the panel of 46 MG-positive serum samples. The correlation coefficent of HI with ELISA was 0.65.

Temporal Appearance of Anti-MG IgG

The appearance of anti-MG IgG in samples of sera and TBW obtained simultaneously at various intervals after exposure of chickens to MG are demonstrated in Fig. 5. Antibody titer rose dramatically from the first to the second week, peaked on the

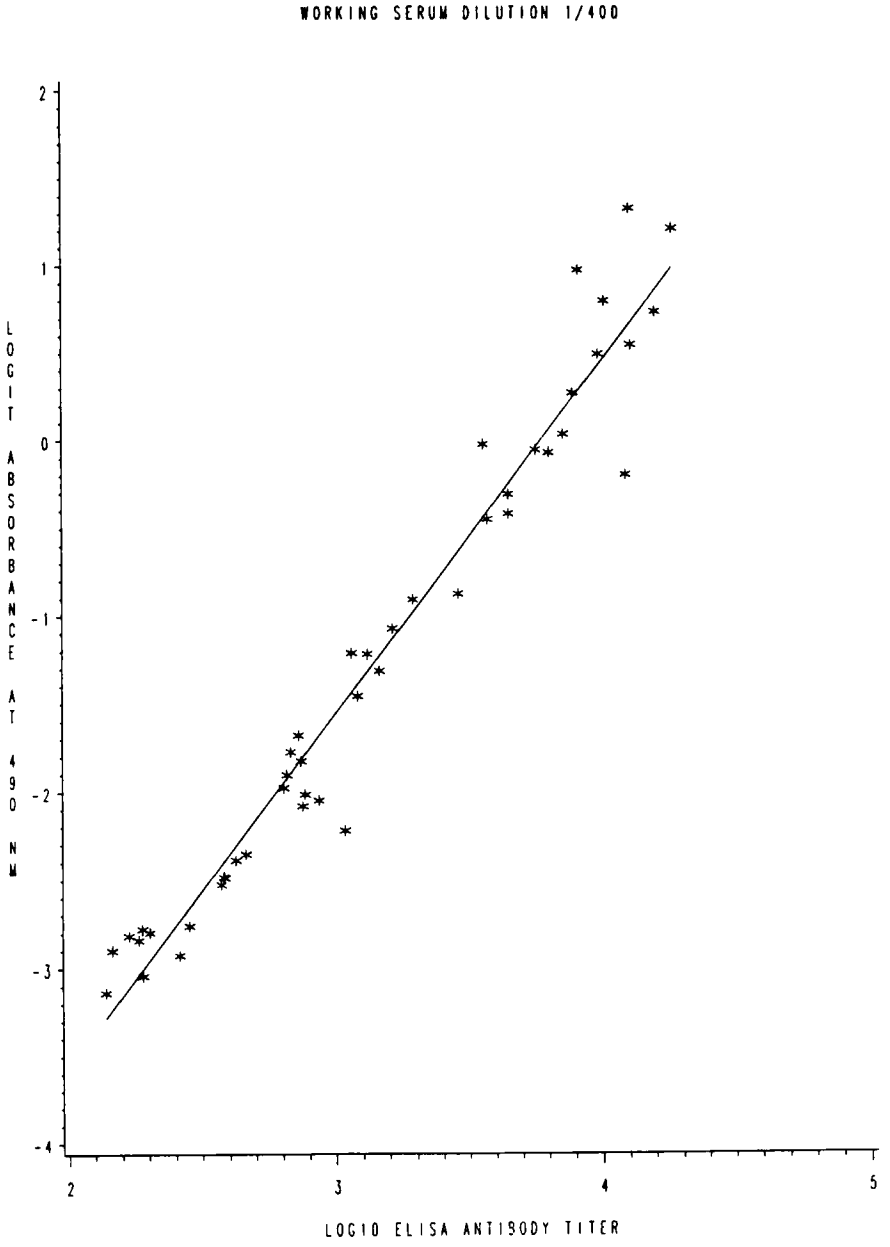

FIGURE 4. Logit transformation of absorbances from Fig. 3. The line describing the relationship between antibody titer and absorbance value recorded at a single working dilution (1:400) was obtained by linear regression analysis of 46 serum samples (*). The correlation coefficient for this line is 0.97.

fourth week, and declined progressively to the tenth week when the titer was the same as in the second week and remained at that level throughout the remainder of the 25 wk. Measurements of geometric mean titer (GMT) in sequential serum samples revealed that the GMT did not vary between samples obtained at 2, 10, 15, and 25 wk or between samples obtained at 6 and 8 wk postinfection (PI). Significant differences ($P < 0.05$) in the GMT, however, were observed when serum samples collected at 1, 2, and 4 wk PI were compared with samples collected at all other intervals. At 1 wk PI, the GMT was very low and sometimes undetectable, with GMT ± SD (standard deviation) of 0.68 ± 0.113 and 0.73 ± 0.021 in sera and TBW, respectively. At 4 wk PI, all the exposed birds had significantly higher titers ($P < 0.05$) in their sera when compared with samples collected at all intervals studied. After 4 wk PI, the serological response waned slowly but was maintained at a detectable level for up to 25 wk PI. Antibodies for MG were not detected in the uninfected controls.

Anti-MG IgG antibody was found more frequently and in significantly higher titers ($P < 0.01$) in TBW at 2 and 4 wk PI when compared with samples obtained at all other intervals. The GMT did vary in TBW collected at 6 to 15 wk after the onset of illness. For all intervals studied, comparison of GMT in sera and TBW revealed major discordances in serum and mucosal responses to mycoplasma. This was particularly noticeable at the peak response of anti-MG IgG in each system. The difference in titer of anti-MG IgG in serum was significantly greater than that in the TBW

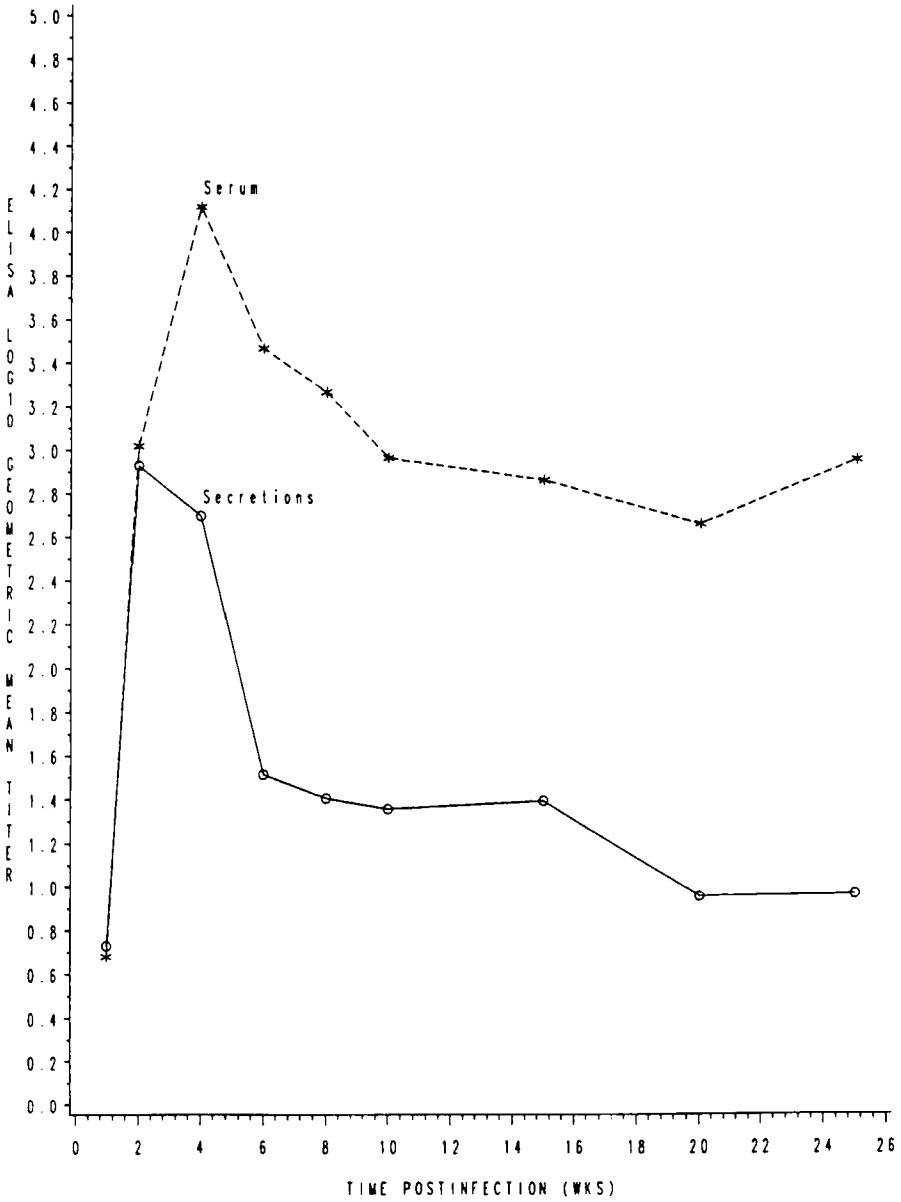

FIGURE 5. Temporal appearance of anti-MG IgG antibody in simultaneously obtained samples of sera (*) and tracheobronchial washes (0) at various intervals after exposure of chickens to *Mycoplasma gallisepticum*, R strain.

($P < 0.05$) at each interval studied except for samples obtained at 1 and 2 wk PI.

DISCUSSION

In the present report we describe the development of an indirect ELISA for the detection and quantitation of specific anti-MG IgG in sera and tracheobronchial washes. The assay was different from the conventional ELISA (14) method in that we used a MoAb as a bridge between test sample and conjugate to improve the sensitivity of the assay. The assay demonstrated high specificity for the detection of anti-MG IgG and low nonspecific binding to the antigen immobilized on a solid phase. Nonspecific reactions were circumvented by the use of partially purified MG antigen and monoclonal antibody. Specificity and sensitivity have been a problem with ELISA described for MG (1,27,31). The monoclonal antibody was used as a bridge to specifically bind to the FC portion of IgG on the test sample and thus resolved part of the issue. The specificity of the assay was determined by cross reaction tests with other protein antigens, including those from other mycoplasma species. The data from inhibition assays demonstrated the specificity of the IgG ELISA in the detection of anti-MG IgG. Thus, the ELISA described herein was not only sensitive but was specific. Nevertheless, more work needs to be done before specificity can be asserted unequivocally. Positive serum samples from chickens infected with known *Mycoplasma species* need to be examined similarly on MG-coated plates. Although this

test was developed for a specific research application, it may be possible to adapt it for diagnostic settings.

The solubilization of MG cell proteins with TDSET detergent followed by extensive dialysis against Tris-HCl buffer and carbonate-bicarbonate buffer resulted in an acceptable antigen for use in ELISA. The antigenicity of the above protein was verified by radial immunodiffusion and immunoblotting before use in the ELISA system (data not shown). Quantitative comparison of various concentrations of MG antigen reacted with the reference positive MG antiserum demonstrated that both 5 and 10 μg conformed to the regression model. In our experience, sensitization of microtiter wells with 5 μg/ml of MG antigen gave satisfactory results and was economically feasible for large scale application. The selection of other ELISA components was approached by regression analysis to determine the optimal concentrations involved in the assay. As a result, an optimum concentration of 20 ng/ml and 0.6 μg/ml were used for IgG monoclonal and conjugate, respectively.

Data in Table 2 demonstrate that levels of precision are adequate for this assay. Repeated assays of the positive reference serum demonstrated reliability between assays. Regression slopes of reference serum and assays of sera having various antibody levels were the same. Therefore, the assay has good reliability for the detection of anti-MG IgG and can be used to measure increases in specific IgG following vaccination and infection.

The use of standard curve methods to predict antibody titer from absorbance value recorded at a single working dilution has

been introduced by several investigators (11,21,30). Disagreement still exists as to the appropriateness of computational methods used by these investigators because the sigmoidal nature of the titration plot introduces significant error at the extremes where inflection of the slope occurs (8). Most of these points fell below the cut-off level in our assays and thus were not included in our calculations. Since antigen-specific ELISA are characterized by the sigmoidal nature of their titration plot, the logistic regression analysis is the method of choice for curve fitting prior to any transformational methods. Logistic-curve fitting is reliable to predict absorbance from antibody titer. However, when the curve is reversed to predict antibody titer from absorbance recorded at a single working dilution, the logistic function is no longer satisfactory to obtain precise estimates. Therefore, for a reliable estimate, we used logit-log transformation (2). The latter method linearizes the sigmoidal curve to be fitted by simple linear regression (13,22,37). The basis of logit transformation in fitting bioassay data has been detailed elsewhere (5,32,36). When observed titers were compared with those calculated after logit curve-fitting, the results obtained showed close similarities, indicating the reliability of logit curve-fitting for the estimation of anti-MG IgG titer by the standard curve method.

More work is needed to explain unequivocally the relationship between absorbance and titer on the basis of current statistical models. Although several models have been described for antigen dose response curves (32), none of them were

applicable to our data. The logistic regression equation described by Ritchie *et al.* (29) for the relationship between absorbance and $\log_{10}$ dilution of fibrinogen was found inappropriate for our data. This raises a question as to how many formulas are applicable to indirect ELISA curves.

Detection of increased levels of anti-MG IgG in TBW coincided with the acute phase of illness when extensive tissue damage was evident. This IgG may have been synthesized locally in the lamina propria of the upper respiratory tract or it might have transduced from serum to mediate local resistance (15,38).

In conclusion, the technique described in this report has several merits over conventional HI testing. First, it is amenable to detect specific IgG. In addition, it can detect other classes of antibody as well if one alters the specificity of monoclonal antibody and conjugate used. In contrast, the HI test is not designed to differentiate between class of antibody present in the sample. Second, the technique is sensitive to detect anti-MG IgG antibody at low levels in specimens that would otherwise be negative by conventional HI test. Although the HI test for MG is a highly specific test, its sensitivity is low (3), and the poor correlation of HI with ELISA is not surprising. Third, the standard reagent used in IgG ELISA can be stored whereas for conventional HI testing, the target red blood cells must be fresh. Fourth, the technique is invaluable in monitoring the appearance of specific anti-MG IgG in sera and TBW of experimentally infected chickens. The kinetics of the appearance of class-specific immunoglobulin can be further exploited in the development of

vaccination schedules if resistance to the mycoplasma is mediated by antibody.

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