Correlation of Cytopathic Effect, Fluorescent-Antibody Microneutralization, and Plaque Reduction Test Results for Determining Avian Infectious Bronchitis Virus Antibodies

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A microneutralization fluorescent-antibody (MFA) test was effective in determining the level of antibodies to avian infectious bronchitis virus. A comparison of the MFA test with the cytopathic effect microneutralization (MNT) test and 50% plaque reduction (PR) test resulted in positive correlations that were significant ($P < 0.001$). The PR test was more sensitive than either the MFA or the MNT test, but there was no significant difference between the sensitivities of the MFA and MNT tests. The MFA test has advantages over the PR test in the capacity to test large numbers of sera in a shorter period of time. The MFA test also can be completed in one-half the time required for the MNT test.

Microculture techniques have come into wide use and are generally as sensitive and far more economical than macroculture methods (5, 17, 18). Methods used to detect avian infectious bronchitis virus (IBV) antibodies include neutralization in embryonated eggs (12), plaque reduction (10), and microneutralization (17). The microneutralization test utilizing cytopathic effect and points at 96 h was found to be reproducible and had a positive correlation with the virus neutralization test in embryonated eggs (17).

Fluorescent-antibody staining procedures have, until recently, been restricted to acetone fixation of virus-infected cell cultures propagated on glass slides, due to the clouding effect of the fixative on plastic. A recently published report (13) described a procedure for fluorescent-antibody staining of infected cell cultures in plastic microculture plates.

The objectives of this study were to adapt the fluorescent-antibody staining procedure to the IBV microneutralization (MFA) test and compare it with the cytopathic effect microneutralization (MNT) test and 50% plaque reduction (PR) test.

MATERIALS AND METHODS

Media. The growth medium for cell cultures consisted of Hanks balanced salt solution supplemented with 0.25% lactalbumin hydrolysate, 10% fetal calf serum, and 10% tryptose phosphate broth. A solution of methyl- α -D-glucoside, sterilized by filtration, was added to the growth medium to a final concentration of 1.5%; this solution was used to block IBV inhibitors present in bovine serum (11). Gentamicin

was added to a concentration of 100 μ g/ml. Virus dilutions were prepared in Hanks balanced salt solution.

Cell cultures. Chicken kidney cell cultures were used for virus propagation. Minced kidneys from 1-day-old chickens were trypsinized for 15 min at 37°C. Two hundred milliliters of 0.25% trypsin was sufficient to disperse the cells from four to five chicken kidneys. The dispersed cells were filtered through sterile gauze, and the trypsin was then inactivated by the addition of 20 ml of calf serum. The cells were sedimented by centrifugation (International centrifuge, Universal model UV, International Equipment Co., Needham Heights, Mass.) at $500 \times g$ for 20 min, and 1 ml of packed cells was suspended in 800 ml of growth medium.

Source of virus. The Beaudette strain (IBV-42), adapted to chicken embryonic kidney cells (9), was used as the antigen in the serological tests throughout the investigation. Virus stocks maintained at a titer of approximately 10^7 plaque-forming units (PFU)/ml of frequent passage in chicken kidney cell cultures were stored at -70°C .

Sera. Pooled sera used in the experiments were obtained from 18 commercial flocks. The chickens selected included broilers (6 to 7 weeks old), broiler breeders (22 to 66 weeks old), and laying hens (23 to 63 weeks old). All 18 flocks had been vaccinated against avian infectious bronchitis by the following methods recommended by the vaccine manufacturer: broilers received the Massachusetts strain of IBV by the Beak-o-vac method at 1 day of age; broiler breeders and laying hens were administered the Massachusetts and Connecticut strains of IBV in the drinking water at 1, 4, and 14 weeks of age. Two negative serum samples were obtained from unvaccinated, specific-pathogen-free chickens housed at the Poultry Disease Research Center, University of Georgia. The 20 serum samples were employed in

the MNT test, MFA test, and PR test.

To avoid bias, all serum samples were randomly coded before each test. In this manner, the sera were examined in a sequence unknown to the individual conducting the tests. The titration end points were not decoded until the data were ordered for statistical analysis.

MNT. The MNT test, which was previously described (17), was used for one part of the study. IBV antibody levels were determined by using the constant virus-serum dilution method. Twofold dilutions of serum, starting at 1:10, were prepared in tubes, and 0.025 ml was inoculated into the appropriate microculture well. An equal volume of virus containing approximately 250 PFU of IBV-42 was added, and the virus-serum mixtures were allowed to react for 30 min at 26°C. After completion of the reaction time, 0.20 ml of chicken kidney cell suspension was added per well. Appropriate virus and cell controls were included on each plate (Fig. 1). Plates were incubated for 96 h before staining for evidence of infective virus (17). A subjective evaluation of each well was made to determine the percent cell destruction as compared with the virus control (100%) wells.

MFA. MFA tests were done identically to the MNT test, except that results were determined at 48 h. The incubation period and PFU of IBV-42 utilized in the test were determined by preliminary trials to obtain maximal sensitivity in the shortest period of time. Examination of the fluorescent-antibody-

stained microculture plates was done by the method of Pursell and Cole (13). Plates were examined microscopically by inverting them on the stage of a Leitz Ortholux II equipped with a Ploem illuminator, a $\times 10$ long-working-distance objective, and a $\times 10$ eyepiece. The evaluation of wells was done subjectively to establish the percentage of cells infected with virus as compared with the virus control (100%) wells.

PR. Neutralizing activity of sera by PR was determined by the constant virus-serum dilution technique. A dilution of virus to give approximately 100 plaques was mixed with an equal volume of each dilution of serum. Plaques were counted after incubation for 72 h (18).

The neutralization titers (50% end points) from the MNT, MFA, and PR tests were calculated by the Kärber method (8).

Fluorescent-antibody conjugate. Hyperimmune serum was produced in adult chickens raised under filtered-air, positive-pressure conditions (1). Preinoculation serum from each chicken was tested for the presence of IBV antibodies. Five adult chickens were inoculated intravenously with 1 ml (10^7 PFU) and nasally with 0.1 ml (10^6 PFU) of IBV-42 stock virus weekly for 1 month. The chickens were bled by cardiac puncture, the serum was fractionated by salt precipitation (2), and the gamma globulin preparation was conjugated to fluorescein isothiocyanate (3). Portions were frozen at -70°C .

Biometrics. The data were analyzed by using an

FIG. 1. Microculture plate showing position of wells used for serum dilutions and virus mixtures, virus controls (VC), and cell controls (CC).

analysis of variance (two way-one observation per cell). The purpose of this analysis was to determine the feasibility of ascertaining the significance of the differences between the mean neutralizing titers of the MFA, MNT, and PR tests by the method of least significant difference (15). Further analysis was done by calculating the correlation coefficients of all the combinations of test pairs (MFA and MNT, MFA and PR, and MNT and PR).

RESULTS

The neutralizing titers are given in Table 1. An analysis of variance demonstrated highly significant ($P < 0.001$) differences to exist among: (i) the serological tests in question and (ii) the neutralizing capacity of the sera. An inspection of the differences between the pairs of mean neutralization titers demonstrated that the mean PR neutralization titer (2,042) was significantly (5% level) higher than both of the mean neutralizing titers of MFA (812) and MNT (544). No significant difference could be shown between the mean neutralization titers of MFA and MNT.

Correlation coefficients (r) were calculated for every combination of pairs of tests (Table 2). The significances of the correlation coefficient-

TABLE 1. Significance^a of the differences of the mean utilizing neutralizing titers of the MFA, MNT, and PR tests of IBV antisera

Serum	Neutralizing titer		
	MNT	MFA	PR
1 ^b	0 ^c	0	0
2	1,090	1,820	4,310
3	263	380	1,380
4	120	245	475
5	731	1,550	1,800
6	3,390	3,890	8,460
7	1,120	1,920	2,820
8	852	1,120	3,270
9	2,280	3,310	10,600
10	389	851	4,100
11	69	160	384
12	66	129	320
13	113	132	380
14	169	314	1,050
15	0	0	12
16 ^b	0	0	0
17	50	78	234
18	85	174	577
19	85	140	513
20	16	43	160
Total	10,888	16,256	40,845
$\bar{x}$	544	812	2,042

^a As determined by the method of least significant difference.

^b Sera from specific-pathogen-free chickens.

^c Reciprocal of serum dilutions.

TABLE 2. Correlation of the results of the MFA, MNT, and the PR tests for detecting IBV antibodies

Correlation	Correlation coefficient	Significant	95% Confidence limit	
			Lower	Upper
MFA and MNT	0.98	Yes ^a	0.95	0.99
MFA and PR	0.94	Yes	0.85	0.98
MNT and PR	0.92	Yes	0.80	0.97

^a Calculated t exceeds tabular t 0.001 (18 df).

cies ($r = 0.98$ for MFA and MNT; $r = 0.94$ for MFA and PR; and $r = 0.92$ for MNT and PR) were determined by using a t test. In each case, calculated t exceeded tabular t (0.001).

DISCUSSION

The correlation coefficients for each pair of tests (MFA and MNT, MFA and PR, and MNT and PR) demonstrated a high degree of association among these neutralization tests in that a determination of the significance of these coefficients by a t test always resulted in values higher than tabular t at the 0.1% level of probability. This high degree of correlation was not unexpected because in each of the three tests neutralization of infectious virus is being measured. In the MNT and MFA tests, a high degree of correlation was expected since the two tests are conducted in an identical manner, with the same volumes of cell suspension, sera, and virus utilized. Only the time of incubation is altered from 96 to 48 h and also the method for establishing the end points.

Analysis of variance demonstrated that significant differences existed among the neutralization tests. Therefore, the comparison of the mean neutralization titers of the results of the MFA, MNT, and PR tests could be done by the method of least significant difference without question. These comparisons demonstrated that the PR test was more sensitive (5% level of significance) than either the MFA or the MNT tests. No significant difference could be demonstrated between the mean neutralization titers of the MFA and the MNT tests.

Undoubtedly, the PR test has a place in medical situations, where sensitivity of detection of serum-neutralizing antibodies is essential. However, PR methodology entails cell monolayer preparation time and then an additional incubation period before the results of the test can be determined. In a medical situation, where large numbers of sera may need processing, the cumbersomeness of the PR methodology, which encompasses the preparation of large numbers of tissue culture flasks and the

need for agar overlay, would create logistical problems. This is in contrast to the MFA test, which has simpler methodology and a shorter incubation time that would permit more rapid test results.

The MFA and the MNT tests are essentially the same in experimental design. The MFA test decreases the incubation time from 96 to 48 h, which is an obvious advantage.

Microculture has been used in the study of arboviruses (4), transmissible gastroenteritis virus (16), rubeola virus (7), poliovirus (6), respiratory virus seroepidemiology (14), and Newcastle disease virus (18). From our study it would appear that the MFA model could be adapted to these microculture systems.

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