Comparison of a Microneutralization Test in Cell Culture and Virus Neutralization Test in Embryonated Eggs for Determining Infectious Bronchitis Virus Antibodies¹

R. E. WOOLEY,* J. BROWN, R. B. DAVIS, J. L. BLUE, AND P. D. LUKERT

Department of Medical Microbiology and Poultry Disease Research Center, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

Received for publication 6 October 1975

A microneutralization test (MNT) system utilizing cytopathic effect end points was effective in determining neutralization indexes for infectious bronchitis virus antibodies. The system is reproducible within 1 index unit at the 95% level of probability. Comparison of the MNT to tests in eggs resulted in a positive correlation ($B = 0.81$), which was significant ($P > 0.01$). The quantitative dose-response relationship of the MNT is linear ($P > 0.005$), with the 95% prediction limits fitting between one 10-fold dilution.

In recent years, microculture methods for virus titration and serological procedures have come into frequent use. The application of microculture has been reported in the study of arboviruses (2), transmissible gastroenteritis virus (18), measles virus (9), poliovirus (8), Newcastle disease virus (19), respiratory virus seroepidemiology (14), and in other serological investigations (4, 6, 15). Methods utilizing microculture are generally as sensitive and far more economical than macroculture methods (6, 19).

Laboratory procedures used to detect infectious bronchitis virus (IBV) antibodies include neutralization in embryonated chicken eggs (13) and plaque reduction (11). These procedures are both cumbersome and expensive (1).

The objective of this study was to develop a virus neutralization test (VNT) for IBV antibodies utilizing microcultures as the indicator system to detect un-neutralized virus and to compare the micromethod with the standard VNT in embryonated chicken eggs with regard to sensitivity, reproducibility, and correlation of titers.

MATERIALS AND METHODS

Media. 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 (12). 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 chicks 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 chick 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 (10), was used throughout the investigation. Virus stocks maintained at a titer of approximately 10^7 plaque-forming units/ml by frequent passage in chicken kidney cell cultures were stored at -70 C.

Sera. Pooled sera used in the experiments were obtained from 30 flocks. The chickens selected included broilers (6 to 7 weeks old), broiler breeders (22 to 66 weeks old), and commercial laying hens (23 to 63 weeks old). All 30 flocks had been vaccinated against infectious bronchitis by the following methods: 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. Three negative serum samples were obtained from unvaccinated, specific-pathogen-free chickens housed at the Poultry Disease Research Center, University of Georgia. The 33 serum samples were employed in the microneutralization test (MNT) and the VNT in embryonated chicken eggs.

MNT. Microculture plates (IS-FB-96-TC; 0.4 ml/well; Linbro Chemical Co., New Haven, Conn.) were used for determination of neutralization indexes (NIs) (constant serum-virus dilution method). Sera were diluted 1/10 in Hanks solution, and the IBV stock virus (10^7 plaque-forming units/ml) was

¹ Manuscript no. 1283.

used in 10-fold dilutions ranging from undiluted to 10^{-5} . The virus dilutions (0.025 ml) were added to an equal volume of the 1:10 serum dilution and allowed to react in the microculture wells for 30 min at 26 C. Each virus-serum mixture was placed into four replicate wells, and each plate was used for three serum samples (Fig. 1). Virus and cell controls were included in a separate plate (Fig. 2). After addition of the virus and virus-serum mixtures to appropriate wells and completion of the reaction time, 0.20 ml of chicken kidney cell suspension was added per well. The plates were then sealed with a clear mylar sheet with an adhesive back (35 PSM, Linbro Chemical Co., New Haven, Conn.), covered with a clear polystyrene top (53, Linbro Chemical Co., New Haven, Conn.), and incubated at 37 C for 96 h. The incubation period used in the test was determined by preliminary trials to give maximal sensitivity in the shortest period of time. After incubation, the medium was removed and the cells were fixed with 10% neutral formalin for 3 to 5 min. The formalin was decanted, and the fixed cells were stained with 1% crystal violet for 30 min. The stained cells were examined for cytopathic effects by gross examination with an appropriate light source. Control monolayers and virus-negative monolayers appeared solid blue, whereas virus-infected monolayers were mottled and lighter in color (see Fig. 1 and 2). End points were determined by comparing the solid blue virus-negative monolayers and the lighter, mottled virus-infected cells. End points were expressed as the highest dilution of virus or virus-serum mixture that had the mottled, lighter-stained cell monolayer. NIs were calculated by comparing the virus control and virus-serum mixture end points. The NI

ranged from 1 to 5. A NI of 1 was considered ≤ 1 because a 1/10 serum dilution was used. A NI of 5 was considered to be ≥ 5 because it was the maximum reading. Antibody titer is expressed in MNT index numbers where neutralization of 10-fold virus dilutions are represented by the numbers 1, 2, 3, 4, and 5. The reproducibility of the NI was determined by repeating the test with the 33 serum samples three times at separate intervals.

VNT. This test in embryonated chicken eggs is the standard reference for the examination of poultry biologics (13). Procedures for performing the test are well documented (1). In this test, the NI is the difference between the log titer of the virus control end point and the titer of the virus-serum mixture end point. This difference represents the logarithm of the NI of the serum.

Statistical analysis. Assessment of the accuracy of the MNT requires a knowledge of the true neutralizing antibody titer in the cytopathic effects microculture system (19). Since this information is not available, the question of the accuracy of the test system cannot be answered directly. However, if results of a test system are consistently reproducible, accuracy can be inferred. This is because the true MNT titer remains the same, and only the human and mechanical variations inherent in any test system need to be considered to answer questions concerning reproducibility. The "best" estimates of variation are the mean and the standard deviation of the mean (16). With these statistics, a "reproducibility" level can be defined. An acceptable level of reproducibility of many serological tests is, by custom, commonly referred to as being "within" one 10-fold dilution.

FIG. 1. Microculture plate showing position of wells used for the virus dilutions and sera for the MNT.

FIG. 2. Microculture plate showing position of wells used for the virus and cell controls in the MNT.

Accepting this empirical value as a standard of reproducibility two hypotheses are advanced: (i) IBV antibody titers as measured by the MNT are reproducible "between" one 10-fold dilution (Fig. 3); (ii) IBV antibody titers as measured by MNT are reproducible "within" one 10-fold dilution (Fig. 3).

By definition, then, the MNT will be considered reproducible if the calculated confidence interval of the means of the various MNT does not exceed 2 (between) or 4 (within). The confidence interval is based upon the *t* distribution calculated at the 95% level.

The definition of "between" one 10-fold dilution is that it must be less than two 10-fold dilutions, i.e., plus or minus less than one 10-fold dilution. In a similar fashion, "within" one 10-fold dilution means that it must be less than four 10-fold dilutions, i.e., plus or minus one 10-fold dilution but not two 10-fold dilutions.

After the completion of the first three trials, a fourth trial was conducted. The purpose of the fourth trial was to test the hypothesis that the IBV antibody titer expressed by MNT was not reproducible within 1 index unit of any of the indexes of the first three trials for the sera in question. The hypothesis was tested with a goodness-of-fit test (16).

To establish and estimate the direct association between the MNT and the VNT, the product-moment correlation coefficient was calculated (3, 17). In this test, the null hypothesis is that correlation is zero.

To establish and estimate the dependence of antibody titer calculated by either the MNT or VNT

FIG. 3. Schematic representation of virus dilution tubes illustrating the range between and within one 10-fold virus dilution utilizing NI numbers from 1 to 5.

methods upon serum dilution, a test of regression was reckoned for each (17). This test for regression included an analysis of variance, calculation of the 95% confidence limits around the regression line, calculation of the 95% prediction limits for a sample of size 1, and a determination if the calculated 95% prediction limit for a sample size of 1 was either "between" or "within" one 10-fold dilution.

Both the MNT and VNT reaction systems consist of decreasing virus and constant serum. For the test of correlation (regression), the reaction system of diluting virus and constant serum is maintained. In this test of regression, one serum with a NI in MNT that equaled 5 was chosen randomly for dilution. However, the test serum was diluted twofold from 1/10 to 1/1,280. Each dilution (eight samples) was employed as the constant serum.

RESULTS

The NIs for the 33 sera are given in Table 1. The sera from the specific-pathogen-free chickens were negative. Seventeen of the sera completely neutralized the IBV at index number 5 (undiluted virus) in the three trials and were therefore not amenable to further analysis. The determination of reproducibility was accomplished with the remaining 13 sera. As shown in Table 2, the MNT was reproducible for each of these sera within 1 index unit at the 95% level of probability.

The results of the fourth trial were similar to the first three trials in that the three sera from the specific-pathogen-free chickens remained negative and the 17 sera that completely neutralized the IBV at NI 5 continued to

TABLE 1. IBV antibody titer expressed by the NIs of the MNT and the VNT

Serum	MNT replicate			Mean	VNT ^a
	1	2	3		
0 ^b	1	1	1	1	0
1	5	5	5	5	5.5
2 ^b	1	1	1	1	0
3	5	5	5	5	5.5
4	5	5	5	5	5.5
5	5	5	5	5	5.5
6	5	5	5	5	3.8
7	5	5	5	5	5.8
8	5	5	5	5	5.8
9	5	5	5	5	5.8
10	5	5	5	5	5.8
11	5	5	5	5	5.8
12	5	5	5	5	5.8
13	5	5	5	5	5.8
14	5	5	5	5	5.8
15	5	5	5	5	5.8
16	5	5	5	5	4.1
17	5	5	5	5	5.8
18	2	2	2	2	3.4
19	5	5	4	4.7	5.8
20	5	5	4	4.7	4.7
21	4	4	4	4	4.2
22	4	4	4	4	5.8
23	4	4	4	4	5.8
24 ^b	1	1	1	1	0
25	4	4	4	4	4.0
26	2	2	3	2.3	2.6
27	2	2	3	2.3	2.6
28	4	4	4	4	4.4
29	4	4	4	4	4.3
30	5	5	5	5	5.8
31	4	4	5	4.3	5.8
32	4	4	5	4.3	5.8

^a Logarithmic number. The NI is more properly expressed as the antilog of the number in question, but by common usage the log number is usually described as the NI.

^b Samples from specific-pathogen-free chickens.

do so. The NIs for the remaining sera of trial 4 are given in Table 3. In this instance, a hypothesis has been proposed that the NI of any of the sera of trial 4 would not be within 1 index unit of any of the indexes of the first three trials. None of the sera met this criterion. A χ^2 of 13.0 was calculated from these results. As compared with a tabular χ^2 0.005 (1) of 7.879, the null hypothesis was rejected. Determination of correlation between MNT and VNT was done utilizing the 13 sera with NIs of less than 5. The results of this test are given in Table 4. In testing the hypothesis that the correlation between MNT and VNT was zero, it was determined that the correlation was 0.81. This positive correlation is significant ($P > 0.001$).

IBV antiserum no. 3 was chosen randomly to test the effect of serum dilution upon the two tests. The results of three trials for each test are presented in Table 5. The quantitative dose-response relationships of the MNT is given in Table 6 and graphically in Fig. 4. First, the dose-response relationship was linear ($P > 0.005$), and the calculated F for deviations from linear regression was less than 1. Therefore, the hypothesis that deviation from regression is less than zero is accepted. The 95% prediction limits if the sample size was 1 was calculated. All of the calculated 95% prediction limits fit inside "between" one 10-fold dilution. Since calculated $\chi^2 = 11.09$ as compared with tabular χ^2 (0.005) = 7.879, that this could have happened by chance alone seems improbable.

In the calculations concerned with the dose-response relationships of the VNT, it was established that the response was not linear. Therefore, the hypothesis that deviation from regression is less than zero was rejected.

DISCUSSION

The laboratory procedures presently used to detect IBV antibodies are cumbersome, expensive, and time-consuming. The development of a microculture method for determining the NI of IBV-vaccinated birds has eliminated these disadvantages. With the use of microculture, samples from individual birds, rather than pooled-flock samples, can be used for determination of NI, due to the small sample volumes required. With the use of multistrain vaccines in the poultry industry, it may be desirable to use strains other than the test strain used in this study. Presently, numerous other IBV strains have been adapted to chicken kidney cells (7) and chicken embryo kidney cells (5) and may be amenable to microculture methods.

It was stated that for the MNT to be consid-

TABLE 2. *Determination of reproducibility of MNT by *t* distribution to calculate a 95% confidence interval around mean NI*

Serum sample	Mean index unit ^a	Standard error of mean	Confidence interval ^b	Confidence interval range	Reproducibility level	
					Between 1 index unit ^c	Within 1 index unit ^d
18	2	0			Yes ^e	
19	4.6667	0.3333	1.4342	2.8684	No	Yes
20	4.6667	0.3333	1.4342	2.8684	No	Yes
21	4	0			Yes	
22	4	0			Yes	
23	4	0			Yes	
25	4	0			Yes	
26	2.3333	0.3333	1.4342	2.8684	No	Yes
27	2.3333	0.3333	1.4342	2.8684	No	Yes
28	4	0			Yes	
29	4	0			Yes	
31	4.3333	0.3333	1.4342	2.8684	No	Yes
32	4.3333	0.3333	1.4342	2.8684	No	Yes

^a Mean of three trials.^b 2 Degrees of freedom (4.303).^c Plus or minus less than one dilution. The calculated confidence interval range must be less than 2 to be considered reproducible (see Fig. 3).^d Plus or minus one dilution but not two dilutions. The calculated interval range must be less than 4 to be considered reproducible (see Fig. 3).^e The confidence range does not exceed the appropriate stated reproducibility level. Therefore, the MNT is reproducible.TABLE 3. *Testing hypothesis that IBV antibody titer as expressed by MNT is not reproducible within 1 index unit (goodness-of-fit test)*

Serum sample	MNT index trial			MNT index trial 4	Within one dilution ^a
	1	2	3		
18	2	2	2	2	Yes
19	5	5	4	5	Yes
20	5	5	4	5	Yes
21	4	4	4	4	Yes
22	4	4	4	5	Yes
23	4	4	4	5	Yes
25	4	4	4	5	Yes
26	2	2	3	3	Yes
27	2	2	3	3	Yes
28	4	4	4	5	Yes
29	4	4	4	5	Yes
31	4	4	5	5	Yes
32	4	4	5	4	Yes

^a Is the NI of trial 4 within one dilution of any of the NIs of the first three trials of the serum in question?

Within one dilution	b	Hypothesis	$\bar{b}$
Yes	13	$\hat{p} = 0.50$	$\hat{p}n = 6.5$
No	0	$\hat{q} = 0.50$	$\hat{q}n = 6.5$
n = 13			

Calculated χ^2 is 13.0 as compared with tabular χ^2 0.005 (1) 7.879. The evidence is sufficient to reject the null hypothesis and accept the alternative that MNT is reproducible within 1 index unit.

TABLE 4. *Relationship (product-moment correlation) of MNT and VNT for IBV serum antibody titer^a*

Serum	Mean MNT NI ^b	VNT NI
18	2	3.4
19	4.7	5.8
20	4.7	4.7
21	4	4.2
22	4	5.8
23	4	5.8
25	4	4.0
26	2.3	2.6
27	2.3	2.6
28	4	4.4
29	4	4.3
31	4.3	5.8
32	4.3	5.8

^a Correlation coefficient = 0.81 ($P > 0.01$); 95% confidence limits, L1 = 0.47 and L2 = 0.94.^b Three trials.

ered reproducible, the standard of reproducibility must be "within" one 10-fold dilution. At the 95% confidence interval range, all of the sera examined had NIs that met this criterion (Table 2).

Only three replications were used in arriving at a sample mean and standard deviation. This was a deliberate vigorous assessment of reproducibility. Slight variation in any of the NI end points either sequentially (2, 3, 4) or differing by a 100-fold dilution (2, 2, 4) would have

TABLE 5. Effect of serum dilution on antibody titer for IBV when titers are expressed as NIs of MNT and VNT^a

Serum ^b dilution	MNT NI in trial:			VNT NI in trial:		
	1	2	3	1	2	3
1/10	5	5	5	5.1	3.9	4.9
1/20	5	5	5	4.2	3.0	4.0
1/40	5	4	4	0	1.9	3.0
1/80	4	3	3	0	0	1.8
1/160	3	2	2	0	0	0
1/320	2	2	2			
1/640	ND ^c	1	1			
1/1,280	ND	1	1			

^a Three trials for each test.^b IBV antiserum no. 3 was chosen randomly for these trials.^c ND, Not determined.

meant that the standard of reproducibility could not have been met. These deviations did not occur, so it would appear that extraneous variation is minimal in the MNT.

Underlying the establishment of a standard of reproducibility is the requirement of virologists and serologists that reliable results, within a certain defined range, can be ascertained by conducting a test once. For this reason, a fourth trial was conducted to answer the hypothesis that the antibody titer would not be within 1 index unit of any of the three indexes previously determined for each serum. In each instance, this hypothesis could not be upheld. Since the probability that this could have happened by chance alone ($P > 0.005$) was remote, the alternative that the antibody titer was within 1 index unit of any of the three indexes previously determined was accepted.

A requirement of any test is specificity. The specificity of the MNT was tested by the blind inclusion of three sera derived from pathogen-free chickens. All were negative.

The MNT and VNT are positively correlated ($B = 0.81$, $P > 0.01$). This is not surprising since the same antiserum is used to neutralize the same strain of IBV. The difference between the tests consists of the indicator systems used. In the MNT, this is a monolayer of chicken kidney cells, and in the VNT it is an entire chicken embryo.

In both of the test systems, specificity was evidenced by the fact that none of the sera from specific-pathogen-free chickens gave neutralization.

The dose-response relationship of the two test systems indicated great differences in sensitivity. First, the dose-response relationship of the MNT is linear ($P > 0.005$). Data of the NI in Table 5 indicate that at the extremes of serum

TABLE 6. Quantitative dose-response relationships of MNT for IBV

Analysis of Variance ^a			
Source of variation	df	F	Probability
Among groups (dilutions)	5	32.7	
Linear regression	1	161.5	0.005
Deviations from regression	4	1	Not significant
Within groups	11		
Total ^b	16		

^a Regression coefficient (B) = -2.6574; 95% confidence limits for B: L1 = -3.08 and L2 = -2.23.^b Total represents degrees of freedom among groups (dilutions) and within groups.

95% Prediction Limits if Sample Size is One

Logarithm of dilution reciprocal	Standard error $\hat{S}_Y$	95% Prediction limits $t_{0.05} \hat{S}_Y$	"Between" one dilution
1.2	0.4511	0.9613	Yes
1.4	0.4435	0.9451	Yes
1.5	0.4404	0.9385	Yes
1.6	0.4378	0.9330	Yes
1.8	0.4345	0.9259	Yes
1.9	0.4335	0.9238	Yes
2.05	0.4332	0.9231	Yes
2.2	0.4342	0.9253	Yes
2.4	0.4374	0.9321	Yes
2.6	0.4428	0.9436	Yes
2.8	0.4503	0.9596	Yes

"Between" one dilution ^a	f	Hypothesis	$\bar{f}$
Yes	11	$\hat{p} = 0.50$	$\hat{p}n = 5.5$
No	0	$\hat{q} = 0.50$	$\hat{q}n = 5.5$
n = 11			

^a $\chi^2 = (11 - 5.5)^2 / (0.50 \times 0.50 \times 11) = 11.09$ (significant, tabular $\chi^2 [0.005] = 7.879$).

dilution some sigmoidal tendency in the regression is evident; therefore, the analysis of variance excludes the responses for serum dilutions 1/10 and 1/1,280. Since no evidence of deviation from linearity is found, the mean square actually being less than that for within dilutions (error), the linearity over a dilution range of 1/20 to 1/640 is effectively linear (Table 6).

In test systems of this type, one primary interest of virologists and serologists is what sort of credence does one give to the results of a single test. For this reason, the 95% prediction limits for a sample the size of 1 were calculated. In turn, the question was asked if these prediction limits fit inside the criterion of being "between" one 10-fold dilution. All of the calculated 95% prediction limits fitted inside of the

FIG. 4. Quantitative dose-response relationship of the NI including the 95% confidence limits of the regression line, the 95% prediction limits if the sample size was 1, and the range of "between" one 10-fold dilution.

"between" one 10-fold dilution. To determine the significance of this finding, a goodness-of-fit test was done. Calculated χ^2 was 11.09 as compared with tabular χ^2 (0.005), which was 7.879 (Table 6 and Fig. 4). That this difference could have resulted from chance alone is improbable. Therefore, the credence that a biologist can give to the results of a single test of this type should be high.

The dose-response relationship of the VNT was not linear. Therefore, the issue of reproducibility could not to be examined. However, the lack of sensitivity of this test is consistent with the findings of other investigators (1).

In previous studies (10), the NIs were much higher when embryonated eggs were used for the detection of IBV-neutralizing antibodies as compared with chicken embryo kidney cell monolayers. However, when the serum was diluted, the embryonated egg was affected adversely as an indicator. It was believed that the virus-serum mixtures continued to react in cell cultures forming a more stable union. This is in

contrast to the technique wherein the virus-serum mixture is inoculated into the allantoic cavity of the embryonated egg; the allantoic fluid acts as a diluent (approximately 1/100) and stops the reaction, favoring dissociation (11).

In summary, the MNT for the detection of IBV antibodies is simple to perform, specific, and reproducible within a defined criterion.

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