Microculture System for Detection of Newcastle Disease Virus Antibodies

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A microculture system utilizing cytopathic effect (CPE) and hemadsorption (HAd) end points was effective in determining the level of Newcastle disease virus (NDV) antibodies. The microculture system was of comparable sensitivity to the plaque reduction test for the detection of NDV antibodies. The standards by which the CPE and HAd microculture tests would be considered reproducible were defined. The results indicate that the CPE and HAd microculture tests are reproducible within one twofold dilution.

In recent years, microculture methods for virus titration and serological procedures have come into more frequent use. The application of microculture has been reported in the study of arboviruses (2), transmissible gastroenteritis virus (11), rubeola virus (6), poliovirus (5), respiratory virus seroepidemiology (8), and in other serological investigations (3, 4, 9). Methods utilizing microculture have been shown to be as sensitive and far more economical than macro cell culture methods (4).

Laboratory procedures used to detect Newcastle disease virus (NDV) antibodies include the hemagglutination-inhibition (HI) test, neutralization in embryonated eggs, plaque reduction, and the egg-bit technique (1). The detection of HI antibodies and virus neutralizing (VN) antibodies has been utilized as an indication of exposure to NDV. Serum neutralization tests are universally accepted as standard quantitative tests for antibody levels. However, the neutralization tests for NDV, by using either embryonated eggs or cell cultures as a virus indication system, are expensive and cumbersome.

The objective of this study was to develop a virus neutralization test for NDV antibodies utilizing microcultures as the indicator system for unneutralized virus.

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. Penicillin and streptomycin were added at concentrations of 100 U/ml and 100 μg/ml, respectively. Virus dilutions were prepared in Hanks balanced salt solution. Agar overlay medium, for plaque enumeration, consisted of Eagle minimal essential medium supplemented with 2% fetal calf serum and 1% purified agar (Difco Laboratories).

Cell cultures. Chicken kidney cell cultures were used for virus propagation. Kidneys from 1-day-old chicks were trypsinized for 30 min, and the dispersed cells were filtered through sterile gauze and sedimented by centrifugation. A 1-ml amount of packed cells was suspended in 200 ml of growth medium.

Source of virus. The Kansas-Manhattan strain of NDV was supplied by P. D. Lukert, University of Georgia. This strain was selected because of its rapid cytopathic effect (CPE) in chicken kidney cell cultures.

Serums. Serums used in the experiments were obtained from 34 individual chickens. Thirty serum samples were obtained from broiler chickens in northern Georgia. The broilers were given NDV, B-1 vaccine at 7 days of age by aerosol; serum samples were collected 42 days later. Four negative serum samples were obtained from unvaccinated specific pathogen-free chickens housed at the Poultry Disease Research Center, University of Georgia.

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.

Virus-neutralization tests: plaque reduction. Neutralizing activity of sera by plaque reduction was determined by the constant virus serum dilution technique. A virus dilution shown to give approximately 100 plaques was mixed with an equal volume of each serum dilution. Plaques were counted after incubation for 48 h, and the highest serum dilution that gave greater than 50% plaque reduction as compared with virus control plates was recorded as the end point.

Microculture cytopathic effects. Microculture plates (IS-FB-96-TC, 0.4 ml/well, Linbro Chemical
Co., New Haven, Conn.) were used for determination of virus neutralization by inhibition of viral CPE. Twofold serum dilutions from 1:10 to 1:1,280 were prepared in tubes. The serum dilutions were added to equal volumes of NDV so that each 0.05 ml of virus-serum mixture contained 1,000 to 2,000 plaque-forming units (PFU) of NDV. The virus-serum mixtures were mixed in a test tube and allowed to react for 30 min at 26°C. Microculture plates were inoculated with 0.05 ml of the virus-serum mixtures. Each virus-serum dilution was inoculated into eight replicate well cultures. Five uninoculated replicate well cultures served as cell controls. Virus controls consisted of inoculating five replicate well cultures (Fig. 1). After the inoculation of the virus and virus-serum mixtures, each well was inoculated with 0.15 ml of the chicken kidney cell suspension. The microculture plates were then sealed with a clear mylar sheet with an adhesive back (35 PSM, Linbro Chemical Co., New Haven, Conn.) and covered with a clear polystyrene top (53, Linbro Chemical Co., New Haven, Conn.). The microculture plates were incubated at 37°C for 48 h. The incubation period, temperature of incubation, and PFU of NDV utilized in the test were determined by preliminary trials to obtain 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 removed, and the fixed cells were stained with 1% crystal violet for 30 min. The stained cells were examined by gross inspection with an appropriate light background. Control monolayers and virus negative monolayers appeared solid blue. Virus-infected monolayers were mottled or had discrete plaques (Fig. 1). The antibody titers obtained in the microculture CPE system were calculated by the Kärber method (7).

**Microculture hemadsorption.** Hemadsorption (HAd) tests were conducted in the same type of plates used for the microculture CPE test (Fig. 1). In this case, 0.05 ml of chicken erythrocytes were added to three replicate cultures which had been inoculated with virus-serum mixture, to five replicate cultures used for virus controls, and to five replicate cultures used for cell controls. The red blood cells were allowed to settle at 4°C. The wells were washed two times with phosphate-buffered saline and examined microscopically for HAd. The antibody end points were determined as the reciprocal of that serum dilution which completely inhibited the adsorption of chicken red blood cells.

**Reproducibility of antibody titers in microculture systems.** The reproducibility of antibody titers obtained in the microculture systems was determined by repeating the microculture CPE and HAd tests with 25 of the serum samples three times at intervals of 4 months and 1 week.

**Statistical analysis.** Assessment of the accuracy of the CPE and HAd microculture tests requires a knowledge of the true neutralizing antibody titers in CPE and HAd microculture systems. This information is not available. Therefore, the question of accuracy cannot be answered directly. However, if results of a test system are consistently reproducible, accuracy can be inferred. Because the true CPE and HAd microculture titers remain the same, only the variations (human and mechanical) inherent in any test system need to be considered to answer questions concerning reproducibility. The mean and the standard deviation are the "best" estimates of variation (10). By utilizing 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 twofold dilution.

By employing the statistics which can be developed and the presently accepted custom, two hypotheses are advanced. (i) The CPE and HAd microculture tests are reproducible between one twofold dilution (Fig. 2). (ii) The CPE and HAd microculture tests are reproducible within one twofold dilution (Fig. 2).

The criterion of reproducibility revolves around "between" and "within" one twofold dilution. The logarithmic range between one twofold dilution is 0.602 and within one twofold dilution is 1.204 (Fig. 2).

By definition then, the CPE and HAd microculture tests shall be considered reproducible if the calculated
confidence interval of the means of the CPE and HAd microculture titers do not exceed 0.602 (between) or 1.204 (within). From the logarithmic numbers obtained from the end point determination, the mean, standard error, coefficient of variation, and confidence interval for the CPE and HAd microculture titers of each serum sample were calculated (10).

The confidence interval based upon the t-distribution was calculated at the 90 and 95% levels.

**RESULTS**

The neutralizing antibody end points in microculture CPE and HAd systems were comparable to those obtained by the plaque reduction technique. The results are shown in Table 1.

The reproducibility of the microculture CPE and HAd tests was established by utilizing the t-distribution. Confidence limits for serum sample antibody titer means were calculated at the 90 and 95% levels.

At the 90% confidence interval, it was ascertained that the range for the CPE microculture test did not exceed the standard of reproducibility of being between one twofold dilution in 17 of the 25 sera tested. Additionally, all of these confidence intervals were within one twofold dilution (Table 2). It was also determined that at the 95% confidence interval this range for the CPE microculture test exceeded the standard of reproducibility of being between one twofold dilution in 10 of the 25 sera. However, when the standard of reproducibility was to be within one twofold dilution, only 3 of the 25 sera failed to meet this standard.

The coefficient of variability is another method of describing variation in a population.

**Table 1. Comparison of NDV antibody titers by microculture cytopathic effect (CPE) and hemadsorption (HAd) tests and the plaque reduction test**

| Serum sample | 50% Plaque reduction | Microculture | HAd | CPE |
|--------------|----------------------|--------------|-----|-----|
|              |                      |              | 1   | 2   | 3   |
|              |                      |              | 1   | 2   | 3   |
|              |                      |              | 1   | 2   | 3   |
| 2            | 80*                  | 80           | 80  | 390 | 399 | 224 |
| 3            | 80                   | 80           | 160 | 390 | 563 | 502 |
| 4            | 80                   | 160          | 80  | 590 |     |     |
| 5            | 20                   | 20           | 20  | 74  | 56  | 63  |
| 6            | 40                   | 20           | 20  | 195 | 70  | 70  |
| 7            | 320                  | 320          | 20  | 708 | 500 | 563 |
| 8            | 20                   | 20           | 20  | 195 | 178 | 113 |
| 9*           | 0                    | 0            |     |     |     |     |
| 10           | 80                   | 160          | 160 | 447 | 447 | 399 |
| 11           | 160                  | 160          | 160 | 677 | 708 | 708 |
| 12           | 160                  | 320          | 160 | 677 | 795 | 563 |
| 13*          | 0                    | 0            |     |     |     |     |
| 14           | 320                  | 320          | 160 | 813 | 892 | 708 |
| 15           | 320                  | 320          | 160 | 777 | 399 | 355 |
| 16           | 40                   | 40           | 80  | 477 | 178 | 442 |
| 17           | 80                   | 80           |     | 224 |     |     |
| 18           | 80                   | 160          | 80  | 195 | 113 | 178 |
| 19           | 80                   | 20           |     | 37  |     |     |
| 20           | 80                   | 160          | 80  | 224 | 178 | 113 |
| 21           | 160                  | 320          | 160 | 677 | 355 | 224 |
| 22*          | 0                    | 0            |     |     |     |     |
| 23           | 160                  | 320          | 320 | 390 | 708 | 399 |
| 24           | 40                   | 160          | 80  | 148 | 447 | 283 |
| 25           | 320                  | 640          | 640 | 1,030 | 1,280 | 1,130 |
| 26           | 160                  | 160          | 320 | 390 | 447 | 589 |
| 27           | 80                   | 160          | 80  | 224 | 399 | 142 |
| 28           | 80                   | 160          | 80  | 677 | 282 | 317 |
| 29           | 20                   | 20           | 20  | 97  | 45  | 80  |
| 30*          | 0                    | 0            |     |     |     |     |
| 31           | 320                  | 640          | 320 | 777 | 640 | 447 |
| 32           | 80                   | 160          | 320 | 390 | 708 | 355 |
| 33           | 640                  | >1,280       | >1,280 | >1,280 | >1,280 | >1,280 |
| 34           | 80                   | 160          | 80  | 390 | 399 | 224 |
| 35           | 320                  | 160          |     |     |     |     |

* Figures are expressed as a reciprocal of the highest serum dilution giving virus neutralization.

* Serum samples from specific pathogen-free chickens.
### TABLE 2. Analysis of the reproducibility of the microculture CPE test by using t-distribution to calculate a 90% confidence interval

| Sample | Logarithmic standard error of mean (s₂)* | Confidence interval (t 0.10 s₂)* | Confidence interval range | Reproducibility level |
|--------|--------------------------------------|----------------------------------|--------------------------|-----------------------|
| 2      | 0.0805                               | 0.235                            | 0.470                    | **                    |
| 3      | 0.0480                               | 0.140                            | 0.280                    | *                     |
| 5      | 0.0399                               | 0.089                            | 0.198                    | *                     |
| 6      | 0.1460                               | 0.426                            | 0.852                    | Yes                   |
| 7      | 0.0443                               | 0.129                            | 0.258                    | *                     |
| 8      | 0.0733                               | 0.214                            | 0.428                    | *                     |
| 10     | 0.0170                               | 0.050                            | 0.100                    | *                     |
| 11     | 0.0070                               | 0.020                            | 0.040                    | *                     |
| 12     | 0.0424                               | 0.124                            | 0.248                    | *                     |
| 14     | 0.0295                               | 0.086                            | 0.172                    | *                     |
| 15     | 0.1059                               | 0.309                            | 0.618                    | Yes                   |
| 16     | 0.1335                               | 0.350                            | 0.700                    | Yes                   |
| 18     | 0.0733                               | 0.214                            | 0.428                    | *                     |
| 20     | 0.0873                               | 0.255                            | 0.510                    | *                     |
| 21     | 0.1383                               | 0.404                            | 0.808                    | Yes                   |
| 23     | 0.0854                               | 0.249                            | 0.498                    | *                     |
| 24     | 0.1389                               | 0.408                            | 0.816                    | Yes                   |
| 25     | 0.0260                               | 0.076                            | 0.152                    | *                     |
| 26     | 0.0435                               | 0.127                            | 0.254                    | *                     |
| 27     | 0.1453                               | 0.424                            | 0.848                    | Yes                   |
| 28     | 0.1188                               | 0.347                            | 0.694                    | Yes                   |
| 29     | 0.1035                               | 0.302                            | 0.604                    | Yes                   |
| 31     | 0.0690                               | 0.201                            | 0.402                    | *                     |
| 32     | 0.0930                               | 0.272                            | 0.544                    | *                     |
| 34     | 0.0820                               | 0.239                            | 0.478                    | *                     |

*Logarithms of n to base 10.

* 2 degrees of freedom (2.926).

* Plus or minus one dilution. Since the logarithm of two to the base 10 is 0.301, the calculated confidence interval range cannot exceed 0.602 in order to be considered reproducible.

* Plus or minus two dilutions. The calculated confidence interval range cannot exceed 1.204 in order to be considered reproducible.

** = Equals no; the confidence interval range does not exceed the approximate stated reproducible level.

In this case, in the three CPE microculture titers of each serum sample, the average coefficient of variability was 6%. It should be noted that sera 6, 24, and 27 had coefficients of variability of 12.7, 9.9, and 16.1%, respectively. These were the three largest coefficients of variability, and in each case the confidence interval (95%) exceeded the standard set forth for reproducibility (Table 3).

Nine of the sera examined in the HAd system had the same titer in each of the three trials. Each of the remaining 16 sera examined had two identical titers, and the third differed by only one twofold dilution (Table 1). Of the sera which had the same titers for each trial, the standard error of the mean titration was, of course, zero and all met the reproducibility levels at any of the standards previously described. For the other 16 sera the calculated logarithmic standard error of the mean was 0.1003. Therefore, the confidence interval at the 90% level was 0.2356 and the confidence range was 0.5870 (see legend, Table 2). At the 90% confidence level these sera did not exceed the reproducibility standard either between or within one twofold dilution. Similarly, the confidence interval at the 95% level was 0.4316, and the confidence range was 0.863 (see legends, Tables 2 and 3). At the 95% level, all of these sera failed to meet the reproducibility level of being between one twofold dilution, but all met the standard of being within one twofold dilution. The average coefficient of variation of the 25 titers in the HAd system was 4.8%.

### DISCUSSION

The results presented indicate that the microculture CPE and HAd test systems were of comparable sensitivity to the 50% plaque reduction test for detecting neutralizing antibodies to...
NDV. Plaque reduction end points were read as that serum dilution that decreased the plaque numbers greater than 50% as compared with the virus control count. The microculture CPE antibody end points were determined by the Kärber method, in which titers are interpolated between the serum dilutions showing greater than and less than 50% of the virus control CPE. Antibody end points in the HAd system were determined as that serum dilution which completely inhibited the adsorption of chicken red blood cells. The small variation in the sensitivities of the microculture CPE and HAd tests and the plaque reduction test may be due to the method of reading the end points.

The specificity of the microculture system was tested by the blind inclusion of four sera derived from specific pathogen-free chickens, in which cases all tests were negative.

The microculture method, in addition to being very sensitive in detecting NDV antibodies, has the advantage of being economical, because the quantity of kidney cells harvested from a single 1-day-old chicken is sufficient to establish microcultures in approximately 300 microculture wells. Because CPE are not measured by microscopy examination, but by gross visual inspection of plates, the microtiter CPE method requires less time, and there is less chance for subjective error in reading CPE end points.

It was stated earlier that knowledge of the true CPE and HAd microculture titers of any serum is not available. The probability statements state that we are either 90 or 95% confident that we have included the true mean within the confidence limits outlined.

For the CPE microculture test to be considered reproducible, the standard is that the calculated confidence interval range must either be between or within one twofold dilution. At the 95% confidence interval range, 22 of the 25 confidence interval ranges fell within one twofold dilution. This assessment of reproducibility seems rigorous since only three replicates were employed in arriving at a sample mean and standard deviation. Significant variation in any one of the calculated CPE microculture titers was enough to extend the confidence interval beyond the criteria stated to be acceptable as being reproducible. It would appear then that the human and mechanical variation inherent in any serological test is minimal in the CPE microculture test. This is further borne out by the calculated coefficients of variation which describe the amount of variation in a sampled population. The average titer variation of the sera sampled utilizing the CPE microculture test was 6%.

Because individual investigators have varying requirements for reproducibility levels, no criteria for accepting or rejecting the two hypotheses stated earlier were delineated. However, in the case of the CPE microculture system, it would seem that the first hypothesis concerning reproducibility between one twofold dilution should be rejected. In this case, the calculated confidence interval range at 90 and 95% exceeded the reproducibility standard in 8 and 15 sera, respectively. The second hypothesis concerns reproducibility within one twofold dilution. At the 90% level, the confidence interval met the reproducibility standard in all cases. At the 95% level, the confidence interval met the reproducibility standard 22 out of 25 times. The chi-square criterion (corrected for continuity) was applied to determine if this result could have happened by chance alone. The calculated $P > 0.005$ made this seem unlikely. It would appear then that the second hypothesis can be accepted conditionally upon the standards of reproducibility required.

For the HAd test, assessment of reproducibility was again rigorous because of the minimal number of replications employed. This situation is further complicated because the accepted method of measuring titers is crudely quantitative. Therefore, in a set of three titration end points, if two of the end points are the same and the third is different only by one twofold dilution, only the 95% confidence limits of being reproducible within one twofold dilution are easily met. Slight deviation of end points either sequentially (1:20, 1:40, 1:80) or differing by a fourfold dilution (1:20, 1:20, 1:80) lowers this estimation of the 90% confidence limits of being reproducible within one twofold dilution. These deviations did not occur, and it would appear that extraneous variation is minimal in the HAd test. This statement is supported by the average coefficient of variation of the titers which is 4.8%.

At the 90% confidence level, the HAd test met the reproducibility standard in all cases. Therefore, the first and second hypotheses can be accepted if this level of confidence is acceptable. At the 95% confidence level, only 9 of the 25 sera met the reproducibility standard of being between one twofold dilution, whereas all were reproducible within one twofold dilution. At this level of confidence it would seem that the first hypothesis should be rejected and the second accepted.

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