Statistical Evaluation of Diluents and Automatic Diluting and Pipetting Machines in Influenza Serology

THOMAS C. O’BRIEN, SURESH RASTOGI, AND NICOLA M. TAURASO

Laboratory of Virology and Rickettsiology, and Biometrics Section, Division of Biologies Standards, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 31 August 1970

The use of three diluents (i.e., 0.01 m phosphate-buffered saline, PBS; PBS with 0.2% gelatin, PBS/GEL; and PBS with 0.4% bovine plasma albumin) and three methods (i.e., the standard tube macro-procedure, TUBE; the manual microtechnique, MANUAL; and the semiautomatic microtechnique, AUTO) were statistically compared for their reproducibility and sensitivity in determining hemagglutinin (HA) and hemagglutination-inhibition (HI) antibody titers. In the HA test, analyses of between-cell variances of the different methods showed the AUTO microtiter procedure to be more reproducible than the standard TUBE method. The MANUAL microtiter procedure was the least reproducible. In the HI test, the TUBE method was the most reproducible. No significant difference in the reproducibility of the diluents was observed in either the HA or HI test. When a comparison of the sensitivity of test methods and diluents was made for determining HI titers, the AUTO microtiter procedure and PBS/GEL diluent appeared to be the method and diluent of choice. Evaluation of another instrument, the autopipetter, which standardizes the volume of diluent to be added in the microtechnique, suggests that the reproducibility of the AUTO microtiter procedure might be further increased.

The application of microtechniques to serological investigations in virology, i.e., hemagglutination (HA), hemagglutination-inhibition (HI), and complement-fixation (CF) tests, is well known. In recent years, automation of these microtechniques has enabled laboratories to increase their volume of testing. Few investigators, however, have reported on the standardization and comparative reproducibility of the HA and HI tests utilizing these newer methods (1–3). Because of this, and because standardized procedures are necessary for the correct interpretation of serological test results, we investigated the reproducibility of the HA and HI tests for influenza by comparing the standard tube test with the microtechniques performed by hand (7) and by use of semiautomatic equipment (6, 10). This report deals with a statistical analysis of our results.

MATERIALS AND METHODS

Antigens. Influenza virus HA antigens, consisting of infectious allantoic fluids of A0/Swine/1967/31, A0/PR/8/34, A1/FM/1/47, and A2/Japan/170/62 virus strains, were part of the reagent collection maintained in our laboratory.

Antibody. The antibody reagents used in the HI tests consisted of sera from chickens immune to A1/FM/1/47 and A2/Japan/170/62 virus strains. The sera were treated with receptor-destroying enzyme to remove nonspecific inhibitors. [A detailed description of the receptor-destroying enzyme procedure is the subject of a separate report (Tauraso et al., in press).]

Diluents. Three diluents were employed: (i) 0.01 m phosphate-buffered saline, pH 7.2 (PBS; NaCl, 8.5 g/liter; Na2HPO4, 1.089 g/liter; KH2PO4, 0.315 g/liter); (ii) PBS containing 0.2% gelatin, pH 7.2 (PBS/GEL); and (iii) PBS containing 0.4% bovine plasma albumin, pH 7.2 (PBS/BPA).

HA tests. Three HA procedures were evaluated. The standard tube test (TUBE) was performed by use of 1-ml glass pipettes and 13 by 100 mm tubes. To 0.25 ml of serial twofold dilutions of antigen were added 0.25 ml of the same diluent and 0.5 ml of a 0.5% cockerel red blood cell (RBC) suspension. The tubes were sealed with Parafilm (American Can Co., Neenah, Wis.), shaken vigorously, and incubated at room temperature (23 to 25 C) until the RBC settled (approximately 1 hr), at which time the test was read.
The manual microtechnique (MANUAL) was performed as described by Sever (7) except for the use of "tulip-type" loops (0.025 ml; Cooke Engineering Co., Alexandria, Va.) and disposable rigid plastic plates containing 96 U-shaped wells (no. IS-MRC-96; Linbro Chemical Co., New Haven, Conn.). As suggested by Hirata et al. (3), all loops were pre-soaked in the same diluent used in the test. Dilutions were performed by manual twirling of eight loops simultaneously, and all reagents (diluent and RBC suspension) were delivered by hand. To 0.025 ml of serial twofold dilutions of antigen were added 0.025 ml of the same diluent and 0.05 ml of a 0.5% cockerel RBC suspension. After being sealed with plastic tape, the plates were shaken vigorously and incubated at room temperature until the RBC settled.

The procedure for the semiautomatic microtechnique (AUTO) was similar to that described for the manual procedure except that all dilutions were made with a semiautomatic autodiluter (Fig. 1), and all other reagents (diluents and RBC suspension) were delivered by hand. [The autodiluter model 4-2410 (American Instrument Co., Inc., Silver Spring, Md.) was fabricated according to the design of a prototype autodiluter developed by the Biomedical Engineering and Instrumentation Branch, Division of Research Services, National Institutes of Health, Bethesda, Md.]

A 1:10 and a 1:15 dilution of each antigen were initially prepared. A sample of each of these two stock dilutions was employed as the starting dilution for each HA test, and both starting dilutions were further diluted in quadruplicate. For each antigen, the three HA tests were performed on the same day and the same starting stock dilutions were used. This enabled us to compare the different diluents and procedures without the bias which might be introduced if tests were performed on different days with different RBC suspensions and different starting dilutions. The HA end point was the highest antigen dilution causing \( \geq 50\% \) of the RBC to form an agglutinated pattern by visual estimation, and was recorded as the \( \log_2 \) value of the reciprocal of the end point.

HI tests. Two homologous antigen/antibody systems were employed (i.e., A1/FM/1/47 and A2/Japan/170/62) in evaluating the different diluents and titration procedures. The HI tests were performed as previously reported (9) except for modifications necessary to adapt to the three different procedures described above. Essentially, to one volume (0.25 ml for tube test, 0.025 ml for microtechnique procedures) of serial twofold dilutions (starting at 1:8 and 1:12) of serum was added one volume of diluent containing 4 to 8 antigen units. After incubation of this mixture at room temperature for 1 hr, two volumes of 0.5% cockerel RBC suspension was added. The tubes of plates were sealed, shaken vigorously, and incubated at room temperature until the RBC settled. The HI end point was the highest serum dilution completely inhibiting hemagglutination as determined visually. Other test details (such as quadruplicate titrations, recording of end points, etc.) were essentially similar to those described for the HA tests.

RESULTS

Evaluation of the reproducibility of diluting procedures and diluents to measure HA antigen titer. Two-way analysis of variance (8) was performed on the observed variation within each diluting method and each diluent (Table 1). No significant difference in the reproducibility of the HA tests was observed when different diluents were used. A significant difference \( (P < 0.01) \) was observed among the various methods used. When the means of the various methods from Table 1 were analyzed by Tukey's honestly significant difference (HSD) test (4), the TUBE and MANUAL methods differed significantly \( (P < 0.01) \), and the AUTO and the MANUAL procedures differed significantly \( (P < 0.05) \); however, no significant difference between the TUBE and AUTO methods was detected. When the within-cell variances of the different methods in Table 1 were compared (Table 2), the TUBE procedure appeared to be the most reproducible method and the MANUAL microtechnique to be the least reproducible. However, the between-cell variances (Table 2) of the individual cell means of the different methods used to measure HA antigen titer show the AUTO microtiter system to be the most reproducible from test to test and the MANUAL microtiter method again to be the least reproducible.

Evaluation of the reproducibility of diluting procedures and diluents to measure HI antibody. Two-way analysis of variance (8) was performed on the observed variation within each method and each diluent (Table 3). No significant difference in the reproducibility of the diluents was observed. However, the analysis of variance showed a difference among the three methods. When the means

![Fig. 1. Semiautomatic microdiluter.](http://aem.asm.org/)
TABLE 1. Variances of hemagglutination titers and analysis of variance of these values

| Diluent         | Method   | Sum of diluents |
|-----------------|----------|-----------------|
|                 | TUBE     | AUTO | MANUAL |       |
| PBS             | 6.60     | 6.69 | 7.09   | 20.38 |
| PBS/GEL        | 6.46     | 6.56 | 6.79   | 19.81 |
| PBS/BPA        | 6.49     | 6.93 | 7.00   | 20.42 |
| Sum of methods  | 19.55    | 20.18| 20.88  | 60.61 |

Source of variation | Degree of freedom | Sum of squares | Mean square | F | P | %  |
|--------------------|------------------|----------------|-------------|---|----|----|
| Method             | 2                | 0.0737         | 0.0369      | 11.53 | 1 | NS |
| Diluent            | 2                | 0.0194         | 0.0097      | 3.03 | NS |    |
| Interaction        | 4                | 0.0128         | 0.0032      | 1.00 | NS |    |
| Replication        | 3                | 0.0236         | 0.0079      | 2.47 | NS |    |
| Error              | 24               | 0.0787         | 0.0032      |     |    |    |
| Total              | 35               | 0.2082         |             |     |    |    |

* Abbreviations: PBS = phosphate-buffered saline; GEL = gelatin; BPA = bovine plasma albumin; TUBE = standard macro-tube test; AUTO = semiautomatic microtechnique; MANUAL = manual microtechnique; F = F ratio; P = probability; NS = not significant.

The value in each cell was calculated as follows: for practicability in mathematical calculations, the four variances obtained from the HA titers (see Materials and Methods) were multiplied by 1,000 and summed; the log₁₀ of that value was calculated, and then its square root was taken. The values for that particular cell for each replication were summed. Mathematically, the values can be expressed as:

\[
\sum_{j=1}^{\eta} \sqrt{\log_{10} \left(1,000 \sum_{i=1}^{4} \left(\frac{y}{\sqrt{y}}\right)\right)}
\]

where \( \eta \) = number of replications.

TABLE 2. Within-cell variances and between-cell variances of individual cell means of the different methods used to measure HA antigen

| Method   | Within-cell variance | Ratio | Between-cell variance | Ratio |
|----------|----------------------|-------|-----------------------|-------|
| TUBE     | 0.228                | 1.00  | 0.247                 | 1.84  |
| AUTO     | 0.351                | 1.54  | 0.134                 | 1.00  |
| MANUAL   | 0.651                | 2.86  | 0.300                 | 3.73  |

* See key to abbreviations in Table 1.

of the various methods from Table 3 were analyzed by Tukey's HSD test (4), both the MANUAL and AUTO microtiter procedures approached borderline significance at the 5% level but did not differ significantly from the TUBE procedure. Neither the MANUAL nor the AUTO microtiter methods differed significantly from each other. Both the within- and between-cell variances (Table 4) of individual cell means of the different methods in Table 3 show the TUBE method to be the most reproducible, and the MANUAL microtiter procedure is only slightly more reproducible than the AUTO microtiter method.

Evaluation of differences in HA titer with different diluents and different diluting procedures. Since the two-way analysis of variance (8; Table 5) of the sums of the mean HA titers from four replications for each cell revealed significant interaction (P < 0.01) between the methods and the diluents, the means of the sums of the mean HA titers of individual cells for each particular diluent and method were compared by Tukey's HSD test. Analysis of these means revealed the AUTO microtechnique and PBS/GEL to be the method and diluent of choice for accuracy in the determination of HA titers.

Evaluation of differences in HI titer with different diluents and different diluting procedures.

TABLE 3. Variances of hemagglutination-inhibition antibody titers and analysis of variance of these values

| Diluent         | Method   | Sum of diluents |
|-----------------|----------|-----------------|
|                 | TUBE     | AUTO | MANUAL |       |
| PBS             | 3.21     | 3.36 | 3.40   | 9.97  |
| PBS/GEL        | 3.26     | 3.39 | 3.37   | 10.02 |
| PBS/BPA        | 3.14     | 3.79 | 3.17   | 10.64 |
| Sum of methods  | 9.61     | 10.54| 10.48  | 30.63 |

Source of variation | Degree of freedom | Sum of squares | Mean square | F | P | %  |
|--------------------|------------------|----------------|-------------|---|----|----|
| Method             | 2                | 0.0903         | 0.0452      | 5.02 | 5 |    |
| Diluent            | 2                | 0.0464         | 0.0232      | 2.58 | NS|    |
| Interaction        | 4                | 0.0503         | 0.0126      | 1.40 | NS|    |
| Replication        | 1                | 0.0006         | 0.0006      | 0.07 | NS|    |
| Error              | 8                | 0.0721         | 0.0090      |     |    |    |
| Total              | 17               | 0.2597         |             |     |    |    |

* See footnotes to Table 1.
### Table 4. Within-cell variances and between-cell variances of individual cell means of the different methods used to measure HI antibody

| Method     | Within-cell variance | Ratio | Between-cell variance | Ratio |
|------------|----------------------|-------|-----------------------|-------|
| TUBE       | 0.332                | 1.00  | 0.168                 | 1.00  |
| MANUAL     | 0.504                | 1.52  | 0.361                 | 2.51  |
| AUTO       | 0.625                | 1.88  | 0.430                 | 2.56  |

*See key to abbreviations in Table 1.*

### Table 5. Sums of the mean hemagglutinin titers and analysis of variance of these values

| Diluent      | Method | Sum of diluents |
|--------------|--------|-----------------|
| PBS          | TUBE   | 32.83           |
| PBS/GEL      | AUTO   | 33.84           |
| PBS/BPA      | MANUAL | 36.27           |
| Sum of methods | AUTO   | 108.47 |

| Source of variation | Degree of freedom | Sum of squares | Mean square | P | % |
|---------------------|-------------------|----------------|-------------|---|---|
| Method              | 2                 | 3.6319         | 1.8159      | 10.6380 | 1 |
| Diluent             | 2                 | 1.2712         | 0.6356      | 3.7235 | 5 |
| Interaction         | 4                 | 4.2492         | 1.0623      | 6.2232 | 1 |
| Error               | 27                | 4.6075         | 0.1707      |       |   |
| Total               | 35                | 13.7598        |             |       |   |

*See key to abbreviations in Table 1.*

Each entry represents the sum of the mean HA titers of four replications. The mean HA titer per cell for 1 replication was calculated from 16 determinations.

Two-way analysis of variance (8) of the sums of the mean HI titers of two replications for each cell showed no significant differences among the three methods or the three diluents.

Another instrument, the Autopipetter (Fig. 2), which will automatically add either 0.025 ml of undiluted antigen or 0.05 ml of RBC suspension, was evaluated with three different diluents (PBS, PBS/GEL, and PBS/BPA) by our laboratory to determine whether antigen carryover with the row of droppers attached to the Autopipetter occurred. The microtiter system was used to determine the HA titers of two influenza antigens (A2/Japan/170/62, 1:640; A0/PR/8/34, 1:80). The Autopipetter added 0.025 ml of a particular diluent to all wells of U-shaped disposable plastic trays. The diluent in the first row of eight wells was removed, and 0.025 ml of undiluted antigen was added by dropper. In the first of two procedures, the second 0.025 ml of diluent was added by the Autopipetter, and the 0.05 ml of 0.5% RBC suspension was added manually by dropper. In the second procedure, the second 0.025 ml of diluent was added manually by dropper, and the 0.05 ml of 0.5% RBC suspension, maintained in suspension by a magnetic stirrer attached to the Autopipetter, was added by the Autopipetter. The remainder of the HI test was performed as described in Materials and Methods. With both procedures, the average carryover after eight replications of the antigens with PBS/GEL and PBS/BPA as diluents was one well into the second row. With PBS, the average carryover was seven of eight wells in the second row.

**DISCUSSION**

Comparison of the reproducibility of HA titers by analyses of between-cell variances of the different methods showed the AUTO microtiter method to be more reproducible, by a factor of 1.84, than the standard TUBE method. The MANUAL microtiter method was the least reproducible. In the HI test, both the within- and between-cell variances showed the TUBE method to be the most reproducible. Although the TUBE method was the most reproducible, only a borderline significant difference between the MANUAL and AUTO microtiter methods and the TUBE method was observed. The additional number of replications one is able to perform with the MANUAL method and especially the AUTO microtiter method would rapidly negate the reproducibility ratio differences observed in the
comparison of within- and between-cell variances in the HI test. No significant difference in the reproducibility of the diluents was observed in either the HA or HI test.

When a comparison of the sensitivity of test methods and diluents was made for determining HA titers, the AUTO microtiter method and PBS/GEL diluent appeared to be the method and diluent of choice. Although other investigators (5) observed lower HI titers with the microtechnique as compared to the standard TUBE test, no significant difference in HI titers could be observed when all methods and diluents were statistically compared.

As emphasized by Leideman and Mogabgab (5), the savings gained by the use of microtechniques should not justify their use in place of the standard TUBE method unless sufficient statistical data on the reproducibility and sensitivity of these newer methods warrant it. Although only a limited number of replications were performed, our statistical analyses indicate that the AUTO microtechnique is a highly reproducible technique for the determination of HA and HI titers. Furthermore, this reproducibility can be increased with the use of the Autopipetter which standardizes the volume of diluent in each well, reducing errors in performing the serial twofold dilutions. Our tests with the Autopipetter revealed minimal antigen carryover when PBS/GEL and PBS/BPA were the diluents. Statistical analyses on the three diluents in the HA and HI tests showed PBS/GEL, closely followed by PBS/BPA, to give the most accurate and reproducible results in the determination of both HA and HI titers.

The conservation of time and reagents, as well as the practicability and reproducibility of the AUTO microtechnique, particularly when used in conjunction with the Autopipetter for large-scale influenza HA and HI testing, as well as its application for CF tests, supports its use. However, since the results obtained by using the MANUAL microtechnique are the least reproducible, the conservation of time and reagents may not justify the use of this procedure in place of the standard TUBE test.

ACKNOWLEDGMENTS

We thank Rachel Yahwak, William Barthlow, and Eldridge Staton for technical assistance. We also express our appreciation to Sidney S. Spindel, Biometrics Section, for assistance in the statistical analysis of the data.

LITERATURE CITED

1. Hierholzer, J. C., and M. T. Suggs. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions. Appl. Microbiol. 18:816-823.
2. Hierholzer, J. C., M. T. Suggs, and E. C. Hall. 1969. Standardized viral hemagglutination and hemagglutination inhibition tests. II. Description and statistical evaluation. Appl. Microbiol. 18:824-833.
3. Hirata, A. A., D. S. Grant, and L. R. Draper. 1969. Factors affecting the passive hemagglutination titrations: dilution loops, titration trays, vibration, diluents. Appl. Microbiol. 17:563-567.
4. Kirk, R. E. 1968. Experimental design: procedures for the behavioral sciences, p. 88-90. Brooks/Cole Publishing Co., Belmont, Calif.
5. Leideman, E., and W. J. Mogabgab. 1969. Antigenicity of influenza vaccine from bovine cell cultures. Appl. Microbiol. 18:596-600.
6. MacLowry, J. D., and H. H. Marsh. 1968. Semi-automatic micro technique for serial dilution-antibiotic sensitivity testing in the clinical laboratory. J. Lab. Clin. Med. 72:685-687.
7. Sever, J. L. 1962. Application of a microtechnique to viral seriological investigations. J. Immunol. 88:320-329.
8. Snedecor, G. W. 1956. Statistical methods, 5th ed., p. 291-328. Iowa State Univ. Press, Ames.
9. Tauraso, N. M., R. Glickman, F. A. Pedreira, J. Sabbaj, R. Yahwak, and M. A. Madoff. 1969. Effect of dosage and route of inoculation upon antigenicity of inactivated influenza virus vaccine (Hong Kong strain) in man. Bull. World Health Organ. 41:507-516.
10. Vargoško, A. J. 1968. Developments in automation of microbiology. Lab. Manage. 6:23-27.