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Automated Microtransfer Technique for the Assay of Poliovirus- and Mumps Virus-Neutralizing Antibodies

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Use of an automated apparatus to quantitate mumps virus- and poliovirus-neutralizing antibody is described. The automated titration equipment affords savings in effort, time, and reagents in conducting large-scale surveys for the determination of mumps- and poliovirus-neutralizing antibodies. This technique has been found to be reproducible and gives results comparable to other antibody assay methods.

The recent development of an automatic programmable machine (Dynatiter, Cooke Engineering Co., Alexandria, Va.) for performing serial dilutions and dispensing reagents in microtitration plates has been found applicable to automated testing for mumps- and poliovirus-neutralizing antibodies. This report describes the necessary modification of this equipment for use in such tests and our experience in comparing these test results with other antibody assay systems.

MATERIALS AND METHODS

Microtitration equipment. Microtitration equipment was obtained from a commercial source (Microbiological Associates, Inc., Bethesda, Md.) and consisted of a Dynatiter diluting and dispensing machine, transfer plates, transfer plate holders, microtissue culture plates and lids, 0.025-ml Go-NoGo diluter testers (blotters), 0.025-ml hand diluters, 0.025-ml disposable pipettes, and Microdel dispensing bottles.

The Dynatiter is an automatic, programmable machine equipped with an 8-channel 0.025-ml microdiluter assembly, three independent 8-channel 0.025-ml dispensing manifolds, a diluting head rinsing module, a diluting head flaming unit, and microplate delivery (down stack) and pickup (up stack) modules. To adapt the Dynatiter for transfer plate operation, the manufacturer modified the diluter head cam assembly so that the microdiluters would lower more gently into the transfer plate wells. This modification prevented the loss of diluent through the transfer plate capillary holes. Transfer plate holders were milled to fit within standard microplate lids. By carrying the transfer plate and holder through the machine on an inverted lid, the stacking modules and plate carriers operated without modification, and, in addition, this lid acted as a transparent undersurface which could be examined to detect possible loss of serum-virus mixtures through the capillary holes of the transfer plate.

Decontamination. Decontamination procedures for microtitration components were as follows: (i) the Dynatiter was exposed to ultraviolet light overnight prior to use and the metal surfaces were sponged with 70% ethanol prior to each test; (ii) microdiluters were flamed before each test and periodically (every 10 plates) during the test; (iii) the diluter rinsing units were flushed with 70% ethanol before priming with sterile phosphate-buffered saline; (iv) blotter and rinsing reservoirs were ultraviolet-irradiated overnight prior to a test; and (v) microdispenser units, transfer plate holders, and micropipettes were autoclaved at 121 C for 30 min. During a test, assembled transfer plates were stacked and the top plate was covered to prevent airborne contamination.

Cell cultures. Vero cells, originally obtained from Y. Rhim (Microbiological Associates, Inc., Bethesda, Md.) in passage 127, were maintained in our laboratory in continuous culture and used for all neutralization tests between passages 357 and 368. Cell cultures were grown in medium 199 (M 199) supplemented with 5% fetal bovine serum; M 199 containing 2% fetal bovine serum was used for maintenance. Penicillin and streptomycin, 100 U/ml and 100 μg/ml, respectively, were added to all media.

Microtissue culture plates were prepared by dispensing 0.075 ml of trypsinized Vero suspension per well containing approximately 4×10^5 cells per ml. When using the Dynatiter for preparing microcultures, cell suspensions were passed through a 13-mm stainless Swinny filter holder (Millipore Corp., Bedford, Mass.) containing surgical steel gauze (no. 50-mesh, 0.003-inch diameter wire, Ethicon, Inc., Sommerville, N.J.) to remove cell clumps. Cell cultures were usually used within 48 h after seeding to eliminate the need for changing media. Occasionally, a medium change was required to maintain cultures until a test was conducted. To conserve lids, plates were stacked in rows of five with a lid covering the top plate.

A darkened, humidified incubator at 35 C in an atmosphere of 5% CO_2 in air was used for all incubation periods.

Viruses. The Bureau of Biologics reference vaccine viruses for poliovirus type 2 and mumps virus (lot no. 3) were used. The challenge virus preparations were diluted so as to contain approximately 50 mean tissue culture infective doses per 0.025 ml of mumps virus and 100 mean tissue culture infective doses of poliovirus type 2. Actual values obtained ranged from 6 to 50 for mumps virus and from 6 to 200 for poliovirus type 2. Medium and challenge virus were maintained at ice-bath temperatures during titrations. Virus dilutions were prepared in microtest maintenance medium.

Sera. Human sera stored at −20 C were inactivated at 56 C for 30 min prior to use.

Neutralization test. The microtransfer poliovirus neutralization tests were performed as described by Moritsugu et al. (8) with the modification that microcultures were maintained in M 199 and incubated in 5% CO_2 in air. The mumps microneutralization test was performed as described by Kenny et al. (4) with the modifications that M 199 was used in place of Earle balanced salt solution, serum dilutions were made in tubes, and all reagents were transferred to the wells using disposable 1-ml glass pipettes.

In the automated microneutralization test, microdiluters were tested prior to use for proper delivery on Go-NoGo testers. The Dynatiter dispensing units were calibrated to deliver 0.025 ml. Serum (0.025 ml) was manually dispensed into the first wells of the already assembled transfer plates with Microdel dispensing bottles or disposable 0.025-ml pipettes. Rinse and blotter reservoirs were placed so that before each series of dilutions the microdiluters were wetted in phosphate-buffered saline and then blotted. Following the serum dilution step, the microdiluters were rinsed and blotted, and the cycle was repeated for the next plate. The transfer plates were stacked in the down-stacking holder and the machine was cycled to perform the following operations: (i) diluent (microtest maintenance medium) was added in 0.025-ml quantities to all wells; (ii) serial twofold dilutions were made; and (iii) 0.025 ml of virus was added to each well. Appropriate virus titrations, serum controls, and cell controls were manually prepared on separate plates. When these operations were completed for all plates, mixtures were incubated at 35 C for 1 h and then inoculated by the microtransfer technique (1) into previously monolayered Vero microtissue culture plates. Cultures were examined for mumps or poliovirus cytopathic effect at day 6 and 7, respectively. End points were calculated by the method of Kärber (3) and expressed for serum antibodies as $\log_2$ 50% serum neutralizing antibody titers (SNT_{50}) per 0.025 ml or for challenge virus $\log_{10}$ 50% tissue culture infectious doses per 0.025 ml.

RESULTS

In Figure 1, data are presented showing the results of mumps virus microneutralization tests performed by manual and automated techniques on human sera previously classified as antibody negative, or with low or high levels of antibody. Twofold dilution series were made starting at a 1:2 dilution; the autotiter test used a series to two titrations (i.e., two rows of microtiter wells) per serum, whereas three replicate titrations (i.e., three rows of microtiter wells) per serum were done by the manual method. Thiry sera were shown to contain antibodies by both tests. Among this group, antibody levels agreed within a twofold dilution in 25 (83%). Of the remaining five sera, three (10%) gave titers greater than twofold higher in the autotiter system and two (7%) had greater than twofold lower values. Two sera by each test were antibody positive at the 0.5 $\log_2$ titer level and negative by the other. Eighteen sera were negative by both methods. Thus, the tests appeared to be of approximately equal sensitivity.

One variable of concern using the autotiter method was the increased time that the challenge virus was exposed to heat and light during the performance of the test. For example, during a 2-h test, sera titrated on the first plate are in the presence of virus 2 h longer than the sera on the last plate. Repeated mumps and poliovirus type 2 autoneutralization tests were conducted with portions from the same serum to determine the effect of test time on neutralizing antibody titers and challenge virus stability. Data are presented in Table 1 showing SNT_{50} and challenge dose results over a 2-h test period,

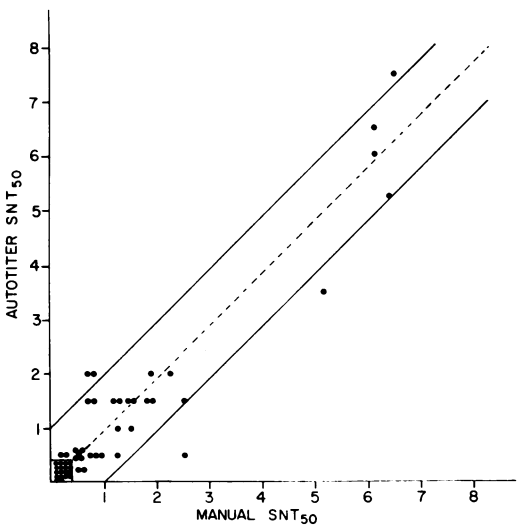

FIG. 1. *Comparison of autotiter and manual techniques for detecting mumps virus-neutralizing antibodies in selected human sera. Titers are expressed as* $\log_2$ *50%* SNT_{50}. *The broken line represents equivalence in titers; the solid lines represent twofold variation from equivalence.*

which is approximately the time required to complete serum dilutions and dispense virus for a test utilizing 60 microplates. Each serum antibody level was calculated from a series of eight titrations. The inherent error of the mumps virus and poliovirus SNT_{50} as shown by the standard error of the means was ± 0.1 and ± 0.2 $\log_2$, respectively. One standard deviation of these replicate tests was ± 0.3 SNT_{50} for poliovirus and ± 0.4 SNT_{50} for mumps virus. These values fall well within a twofold dilution and demonstrate that a 2-h test period has little effect on autoneutralization titers for mumps and poliovirus type 2.

The expected degree of reproducibility of autotiter mumps- and poliovirus-neutralization test results was determined by performing four tests over a 3-month period with portions of the same antibody-positive human sera for mumps and poliovirus. The SNT_{50} was calculated from six to eight titrations as described earlier and was used for analysis. As shown in Table 2, the mean antibody titers in these tests were within a twofold deviation for poliovirus type 2 SNT_{50} (standard deviation ± 0.3; standard error ± 0.1) and mumps virus SNT_{50} (standard deviation ± 0.3; standard error ± 0.2). It should be noted that these values are identical to those of the elapsed time experiment (Table 1) and suggest that test deviations are small and reproducible.

To test the reproducibility of replicate titrations of automated and manual microtransfer methods for poliovirus type 2, groups of 490 and 208 sera, respectively, were compared by the test statistics Z (10). The proportion of replicates showing less than a twofold deviation was 483/490 (0.986) for the autotitration method, whereas 180/208 (0.876) was obtained by manual dilution. The value of Z was 6.953 which gives a P value <0.001. Therefore, automated and manual methods are significantly different, with the automated method demonstrating more reproducible values within a twofold deviation.

Neither contamination nor cell survival has been a problem in the automated microneutralization test. In two typical tests consisting of 8,132 microculture wells in total, only four were contaminated. All were sporadic fungus contaminants that could not be correlated with contaminated sera. Fungal contaminants were easily contained if observed and treated early by adding 1 drop of Thimersol 1:10,000 (Eli Lilly and Co., Indianapolis, Ind.), allowing it to stand for 1 min, and then aspirating well contents. Dehydration of microtissue culture wells was readily prevented over a 7-day incubation period by keeping incubator relative humidity at approximately 92% and weighing the microplates to insure a firm fit between the microplate and lid.

TABLE 1. *Effect of elapsed test time on antibody titers and virus challenge dose in the automated neutralization test*[a]

Elapsed time from start of test (min)	Poliovirus type 2		Mumps virus	
	Antibody titer/ SNT_{50} ($\log_2$)	Virus dose/ $TCID_{50}$ ($\log_{10}$)	Antibody titer/ SNT_{50} ($\log_2$)	Virus dose/ $TCID_{50}$ ($\log_{10}$)
0	5.5	1.8	4.8	1.1
30	4.8	NT	5.3	1.7
60	5.0	1.8	NT	1.5
90	5.0	NT	5.3	NT
120	5.3	2.1	4.5	1.5
Mean ± SD (SE)[b]	5.1 ± 0.3 (0.1)	1.9 ± 0.2 (0.1)	5.0 ± 0.4 (0.2)	1.5 ± 0.3 (0.1)

[a] Calculations are from eight separate dilution series (eight rows of microtiter wells per serum or virus dilution). NT, Not tested; $TCID_{50}$, mean tissue culture infective dose.
[b] Mean ± standard deviation (standard error).

TABLE 2. *Reproducibility of poliovirus- and mumps virus-neutralizing antibody titers assayed using the automated procedure*[a]

Test no.	Poliovirus type 2		Mumps virus	
	Antibody titer/ SNT_{50} ($\log_2$)	Virus dose/ $TCID_{50}$ ($\log_{10}$)	Antibody titer/ SNT_{50} ($\log_2$)	Virus dose/ $TCID_{50}$ ($\log_{10}$)
1	7.0	2.1	5.1	1.4
2	7.0	2.3	5.1	1.2
3	6.5	1.7	5.7	0.8
4	7.0	0.8	4.6	1.1
Mean ± SD (SE)[b]	6.9 ± 0.3 (0.1)		5.1 ± 0.4 (0.2)	

[a] SNT_{50} calculated from six to eight separate dilution series (six to eight microtiter wells per serum). $TCID_{50}$, mean tissue culture infective dose.
[b] Mean ± standard deviation (standard error).

Several problems were encountered in early autotitration experiments. Loss of serum-virus mixtures through the capillary holes during autotitration was corrected by the manufacturer's modification of the diluter head cam assembly, proper centering of the dispenser manifolds over the microtiter wells, and adjustment of the dispenser injection system. Before each test, a plate was run through the machine to assure proper alignment. Another problem encountered was saturation of diluter blotters causing inaccurate dilution series. This was corrected by using two rinse-blotter reservoirs, as described above, and changing reservoirs every 30 plates.

DISCUSSION

The amount of effort, time, and reagents has limited laboratory ability to perform neutralization tests on large numbers of sera. The advantages of the microculture method have been described previously (2, 4–9). The development of an automated microneutralization test was of great value. In our laboratory, the autotiter method enables two technicians to perform a 60-microculture plate test in 4 to 5 h, including the time required for setting up, incubating, and inoculating. In addition, the autotiter equipment provides a further saving in time and manpower in the processes of dispensing cells and changing nutrient media.

Replicate comparisons of automated and manual microtransfer neutralization techniques indicate that the autotitration procedure gives more reproducible results. Autoneutralization tests for mumps and poliovirus has little day-to-day or within test variation.

Moritsugu et al. (8) compared poliovirus microtransfer neutralization titers assayed in Vero cells with conventional tube neutralization titers assayed in primary cynomolgus monkey kidney cells. Their results indicate that both systems give comparable results. Our observation that microtransfer by autotitration is more reproducible extends these observations to the use of the autotitration system. The observations reported here concern poliovirus type 2. Parallel experience involving fewer tests with poliovirus types 1 and 3 have given comparable results in our hands. Kenny et al. (3) compared mumps serum neutralization titers by Vero microneutralization with the primary chicken embryo-hemadsorption test performed in tube cultures and found excellent correlation if the tests were read on day 5 or 6 after inoculation. The comparison of mumps virus autoneutralization titers with manual microneutralization titers shows a high degree of correlation. As with all procedures for the determination of mumps antibodies, a few variations were seen in sera containing low levels of antibody. These titer deviations, however, fall well within day-to-day test variance; their frequency in this study was magnified by the intended selection of a large number of antibody-negative sera and sera thought to contain low levels of antibody. A major factor influencing the application of autotiter to other virus microneutralization tests (4–9) is the stability of the challenge virus for the time period required to complete autotitrations. Currently, automated tests for the detection of antibodies to herpes simplex, varicella-zoster, and cytomegaloviruses using the autotiter are being evaluated.

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