Automated Microtitration Test for Antistreptolysin O

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A commercially available instrument that automatically makes serial dilutions and delivers reagents was used for the determination of antistreptolysin O titers in serum. The automated method was compared with tube-dilution and manual microtitration techniques. It gave higher reproducibility of results and was quicker to perform than both the other tests; and it was much more economical in reagents than the tube test. The automated technique is considered to be the best of the three methods when more than a small number of specimens are examined at one time. It is now in routine use in our laboratory.

The antistreptolysin O (ASO) titer of serum is a useful index in the study of infections by hemolytic streptococci and their sequelae, notably rheumatic fever. ASO titers are commonly determined by a tube-dilution method. In recent years, however, microtitration methods for the determination of antistreptolysin O in human serum have been described by Edwards (2) and by Klein et al. (4). Among their advantages are savings in reagents and technical time. Even more time would be saved and perhaps higher accuracy would be achieved, if the microtitrations could be performed on an automatic serial-dilution machine. In this report, the application and use of such a machine is described, and the automated method is compared with a tube-dilution and a manual microtitration technique.

MATERIALS AND METHODS

Specimens. The specimens tested were unselected samples of human serum submitted for ASO determination from various hospitals and private practitioners.

Reagents. Streptolysin and antistreptolysin supplied by Hyland Laboratories, Los Angeles, Calif., were used. Lyophilized Streptolysin O Reagent was reconstituted immediately before use in accordance with the manufacturer’s instructions. Antistreptolysin O Standard (Hyland) was used as a reference standard. The diluent for the automated and manual microtitration methods was the buffered barbital-gelatin diluent (pH 7.4) described by Klein et al. (4). For the tube test, the diluent used was a phosphate buffer, pH 6.5 (7.4 g of NaCl, 2.77 g of KH₂PO₄, 2.23 g of Na₂HPO₄ in 1 liter of water).

Automated microtitration. An automatic serial-diluting instrument (Autotiter; Astec Inc., Orange, Conn.) was used. The machine and its operation were described in detail by Goss and Cimijotti (3), who reported its use for antibiotic sensitivity tests. It consists basically of a moveable carriage that holds eight 0.05-ml microtiter transfer loops. Manifolds that deliver reagents through eight 20-gauge needles are situated both in front of and behind the row of loops. Injector syringes powered by compressed air (30 lb/in²) deliver fluids from reservoirs to each manifold. The program circuit regulated the movements of the loops, the position of the carriage, and the delivery of fluids from the manifolds.

Eight microtitrations were performed simultaneously in disposable plastic trays (Autotray; Astec Inc.) having 8 rows of 15 U-shaped wells. The forward manifold delivered 0.05 ml of barbital-gelatin diluent, and the rear manifold delivered 0.05 ml of streptolysin O at a concentration of 1 unit per ml.

Two rows of seven wells were used for each serum. To the first well of one row was added 0.1 ml of a 1:20 serum dilution, and to that of the other row was added 0.1 ml of a 1:30 dilution. The tray was placed in the machine, and the automated program was begun. In the first two steps of the cycle, the loops were lowered into a prewetting tank containing diluent and then blotted on filter paper. The carriage next stopped above the first column of wells, and the loops were lowered to pick up 0.05 ml of the initial serum dilutions and raised, while 0.05 ml diluent was delivered from the forward manifold to each well in the second column. The carriage moved to the next position in which the loops were lowered to deliver, rotated to mix, and then raised. Simultaneously, diluent was delivered to the third column of wells. The carriage moved forward another step, where the procedures were as described in the last position but, in addition, 0.05 ml of streptolysin solution was added to the second column of wells. The procedure was then repeated until seven cups of the row had been processed, resulting in dilutions of serum in each pair of rows from 1:20 to 1:1,920 by 0.5 log₂ increments. The 1:20 and 1:30 dilutions acted as serum controls, since no streptolysin was added to these cups; the dilutions of serum tested thus began at 1:40. At the end of the
cycle, the loops passed through a cleansing bath of flowing water and then a loop-flaming assembly.

Each tray holds 15 rows of wells and so can be used for a second cycle of the machine for four more specimens. One tray of eight specimens can be processed in about 3 min. The trays were then removed from the machine, gently shaken, covered, and incubated at 37 C for 15 min. An addition to each well of 0.05 ml of 3% trishe washed rabbit erythrocytes in barbital-gelatin diluent was made from a manually operated manifold holding eight needle injectors. The tray was incubated at 37 C for 45 min, being shaken gently after 15 min and at the end of the incubation, covered, and left at room temperature until the red cells settled. The trays were then stored overnight at 4 C. The tubes were gently shaken at 4 C overnight. The ASO test in Todd units was read as the reciprocal of the highest serum dilution that completely prevented hemolysis.

The only requirement for the machine that is not available in most microbiology laboratories is a supply of compressed air at a pressure of 30 lb per in$^2$.

Manual microtitration. This was performed in similar trays, using 0.05-ml spirally wound loops (Cooke Engineering Co., Alexandria, Va.) operated by hand. Six titrations were performed simultaneously. Volumes and reagents were identical with those used in the automated test.

Tube-dilution technique. This was performed as described by Rantz and Randall (5), except as noted below. Sera were tested at dilutions from 1:100 to 1:833. Streptolysin O was reconstituted at a concentration of 2 units per ml, and 0.25-ml volumes were added to the serum dilutions in tubes. After being shaken, the tubes were incubated at 37 C for 15 min, and 0.5 ml of a 3% suspension of trishe washed rabbit erythrocytes was added. After further incubation for 45 min and storage at 4 C for 18 hr, end points were determined visually. The reciprocal of the highest serum dilution showing no hemolysis gave the number of Todd units of ASO in the specimen.

Control tests. In each set of tests, the reference standard ASO and a serum of known unitage were included as controls. Serum control tests were done on the lowest dilution of serum tested, and each batch of tests included streptolysin and red cell controls.

RESULTS

Reproducibility of the automated test. A total of 692 titrations was performed in duplicate by the automated method. The tests were generally done on successive days, the sera being stored at 4 C overnight. Of these, 599 (86.6%) gave identical results in both tests, and 91 (13.1%) gave results different by only a single dilution. Thus, 99.7% of the tests were reproducible within one dilution step. The remaining two gave a two-dilution difference on duplicate testing. Corresponding figures for duplicate testing by the other methods were as follows. By manual microtitration, 67.2% gave identical results and 96.7% agreed within one dilution; and by tube dilution, 60.9% gave identical results and 99.4% agreed within one dilution step.

Comparison of the automated test with the tube-dilution method. Three hundred and twenty sera were examined by the automated and tube tests. The titrations were performed by different technologists, often on successive days, with sera being stored at 4 C overnight between tests. The dilutions made, and hence the titers given, in the two tests were not identical. However, the differences were small (e.g., 160 and 166; 240 and 250) and for purposes of comparison were disregarded.

Identical results by the two methods were obtained in 211 tests (65.9%), and there was one dilution step difference in 109 (34.1%); thus there was complete agreement between the techniques within one dilution step.

Economic comparisons. A comparison was made of the technical time and quantities of reagents needed for each method.

(i) Time. The automated technique proved to be simple to perform and took much less time than the tube method when batches of 40 to 60 specimens were tested, numbers which are routinely examined twice weekly in this laboratory. After the initial dilutions of the specimens had been made manually and appropriate volumes were added to the wells in a tray, the procedures whereby serial dilutions were made and the streptolysin was added to eight specimens (16 titrations) were completed in about 3 min. Tests were set up ready for incubation on 48 specimens in the following times: automated microtitration, 45 min; manual microtitration, 70 min; tube dilution, 105 min.

(ii) Reagents. The automated method required less than half the quantity of streptolysin needed for the tube method. Each well in the automatic titration required 0.05 ml of a solution of lysin containing 1 combining unit per ml, compared with 0.25 ml of a solution containing 2 units per ml that was needed for each dilution of the tube test. The manifold and tubing of the Autotiter machine hold about 12 ml of lysin which is not used. Consequently the automated technique uses somewhat more lysin than does manual microtitration. The greater the number of sera tested on a single occasion, the smaller is the proportion of unused lysin. The quantities of streptolysin required to test 48 specimens were: automated microtitration, 46 units; manual microtitration, 34 units; tube-dilution, 192 units. A saving of 292 ml (about $23 at present prices) on 100 tests can result from use of the automated technique in place of the conventional tube test. Allowing for the cost of the disposable microtitration plates,
there is a net saving of about 20\$ per test in lysin alone compared with the tube method; and there is almost no glassware to wash. Because of unused reagents in the machine, the cost of streptolysin for 100 tests by automated microtitration would be about 1\$ per test more than by manual microtitration, but if the saving of technical time is also considered the automated method would allow a net saving of 3\$ per test.

DISCUSSION

The determination of the ASO titer by a tube method is a time-consuming procedure. Efforts have recently been made to simplify the test either by reducing the number of dilutions to a minimum (1) or by employing a manual microtitration technique (2, 4). The application of automation to the microtitration technique reduces the technical time required and provides accurate and highly reproducible quantitative results by greatly reducing dilution errors.

High reproducibility of the results is the major advantage of the automated method. When large numbers of sera are examined, as in serological surveys, the method allows a considerable saving in the cost per test compared with the tube method, and a much smaller but useful saving compared with manual microtitration. The Autotiter costs approximately $3,000, and this expense must be considered; however, a laboratory possessing such a machine would almost certainly use it for other tests in addition to ASO determinations. The machine is simple to operate, and the technician need not have the skill and dexterity necessary to perform accurate manual microtitrations.

In our laboratory, an average of 50 sera are examined twice weekly, and, for this number of tests, we consider that the automated test is the best. It gives highly reproducible results and a worthwhile saving of technical time. It is now in routine use in this laboratory.

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