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Application of a Microtechnique to the Agglutination Test for Leptospiral Antibodies[1]

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ABSTRACT

GALTON, MILDRED M. (Veterinary Public Health Laboratory, Atlanta, Ga.), CATHERINE R. SULZER, C. A. SANTA ROSA, AND MICHAEL J. FIELDS. Application of a microtechnique to the agglutination test for leptospiral antibodies. Appl. Microbiol. **13**:81–85. 1965.—A microtechnique has been developed and adapted successfully to the microscopic agglutination test with live antigens for detection of leptospiral antibodies. Simultaneous titrations were performed by the conventional microscopic agglutination test and the microtechnique. When the microtechnique was used to screen 50 unknown leptospiral strains with a battery of hyperimmune sera, 98% agreement was obtained with the conventional procedure. Comparative data on 635 tests on these 50 cultures established the reliability of the microtechnique. Results with the two tests on 46 human sera revealed 93% agreement in the detection of leptospiral antibodies. The validity and reliability of the microtechnique obtained in these comparative studies suggests that it can be used as a valuable screening procedure for the microscopic agglutination test for preliminary cross agglutination studies on unknown strains and for the detection of leptospiral antibodies in human and animal sera.

The microscopic agglutination test with live antigens is the accepted procedure in most laboratories for the identification of unknown leptospiral types and for the detection of leptospiral antibodies in human and animal sera (Wolff, 1954; Galton et al., 1962). This test is tedious, time-consuming, and requires considerable amounts of antigen. To obtain a preliminary classification as to the leptospiral group to which a culture belongs, it is necessary to screen by cross-agglutination tests with a battery of reference rabbit antisera for 12 to 18 known serotypes and a live antigen of the unknown culture. Similarly, a battery of live leptospiral antigens of known types must be used in agglutination tests with human and animal sera to detect leptospiral antibodies.

A microtechnique which was found useful for viral serological investigations (Takatsy, 1955; Sever, 1962) has been adapted to the microscopic agglutination test both for screening leptospiral types and for detection of antibodies in unknown sera. The microtechnique provides a rapid, accurate method for preparing dilutions of sera with calibrated spiral loops, by use of minute quantities of reagents. Sever modified and adapted the original microtitrator system (Takatsy, 1955) to the complement fixation, hemagglutination, and hemagglutination inhibition tests with viral antigens. When this modified microtitration equipment became commercially available, Casey (*personal communication*) compared the technique with standard procedures used routinely in the Communicable Disease Center, Virus Laboratory, Immuno-Serology Unit. The reliability of the microtechnique, as indicated by her findings, resulted in its adoption for routine serological testing in virology at the Communicable Disease Center. In this paper, the results of the microtechnique when adapted to the microscopic agglutination test for preliminary identification of leptospires and for detection of leptospiral antibodies are reported. Comparative data with the two methods indicate the degree of reliability and validity of the microtechnique.

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MATERIALS AND METHODS

Microtechnique equipment. The following equipment was used: (i) disposable plastic plates, 3.25

× 5 in. containing 96 "U" wells (8 rows of 12), to contain 0.125 ml; (ii) spiral steel diluting loops calibrated to deliver 0.025 ml; (iii) loop delivery tester of special blotter material marked with 48 circles (10.5 mm diameter); (iv) plastic pipette droppers calibrated to deliver 0.025 ml; and (v) porous Packlon (Minnesota Mining & Mgf. Co., St. Paul, Minn.) tape, 3.25 in. wide.

Performance of the test. Hyperimmune sera and unknown sera to be tested were diluted twofold from 1:25 to 1:12,800 in a final volume of 0.025 ml. Dilutions were prepared with the calibrated loops with buffered 0.85% of saline in the disposable plates, according to the technique described by Sever (1962).

Antigens consisted of 4- to 5-day-old cultures of the desired leptospiral strain grown in Stuart's medium (without indicator) and standardized to a density of 25 to 30 nephelometer units in a Coleman no. 7 nephelometer. The pipette droppers were used to deliver 0.025 ml of antigen into each dilution of serum, making a final volume of 0.05 ml. Porous Packlon tape was then applied to the surface of the plate to prevent evaporation during the 3-hr period that the tests are incubated at 30 C.

Reading of tests. The tests could not be read satisfactorily in the plates on the standard microscope with dry dark-field condenser and 10× objective. To avoid transfer of a drop from each well to a slide for reading, as is the practice for the microscopic agglutination test, these tests were read in the plates on a Zoom dissecting microscope equipped with 20× wide-field oculars and a 2× supplemental lens. The magnification provided by this system ranged from 40× to 120×. The most satisfactory magnification was found to be 42× to 45×. The black glass stage was used on the microscope and the lamp was adjusted so that light would strike the top surface of the plate at an approximate 45° angle. The degrees of agglutination were recorded as 1+ with 25% of the leptospires clumped, 2+ with about 50% clumped, 3+ with approximately 75% clumped, and 4+ when 75 and 100% agglutination occurred. The end point was taken as the highest dilution, showing a 2+ reaction. The agglutinated organisms in this test closely resemble clear lipid droplets on a black background. Since the work reported here was completed, it was found that the tests can be read in the plates on a binocular microscope equipped with a dry dark-field condenser, a 3.5× objective, and 10× widefield oculars (Fig. 1).

Simultaneous titrations, for comparative purposes, were performed by the conventional microscopic agglutination test, as previously described (Galton et al., 1962).

RESULTS

The reliability of the microtechnique was first evaluated as a method of screening unknown leptospiral isolations to determine the group to

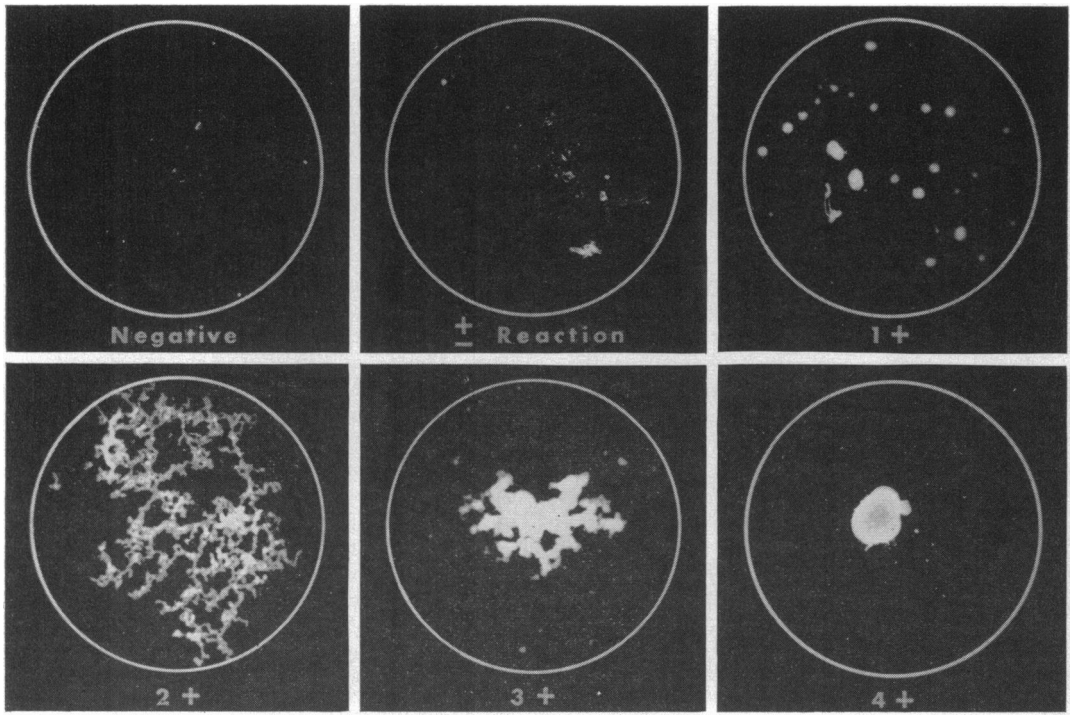

FIG. 1. *Leptospiral agglutination by microtechnique; negative reaction and degrees of positive reactions.*

which they belonged. Comparative findings with antigens of each of 50 leptospiral strains, when examined by the microtechnique (MT) and microscopic agglutination (MA) test with a battery of 12 to 18 leptospiral antisera, are given in Table 1. Positive reactions were obtained in one or more sera by both methods with 42 of the 50 antigens, and seven strains were negative with all sera. The one culture found positive by MT and negative by MA reacted in a 1:100 dilution with serotype *australis* antisera and the 1:50 dilution with serotype *borincana* antisera. Thus, with these 50 strains, there was 98% agreement between the two methods.

TABLE 1. *Findings on 50 unknown leptospiral cultures when screened against multiple leptospiral antisera by microscopic agglutination test and by microtechnique**

Microtechnique	Microscopic agglutination		Total
	Positive	Negative	
Positive	*42*	1	43
Negative	0	*7*	7
Total	42	8	50

* Agreement of tests: 49/50 = 98%.

TABLE 2. *Comparative findings on 635 tests with antigens of 50 unknown leptospiral cultures screened against multiple antisera by microscopic agglutination test and by microtechnique**

Microtechnique	Microscopic agglutination		Total
	Positive	Negative	
Positive	*198*	18	216
Negative	13	*406*	419
Total	211	424	635

* Agreement on tests: 604/635 = 95%.

A total of 635 comparative tests were performed on these 50 cultures against 12 to 18 antisera. Of these, 198 tests were positive and 406 were negative by both methods, an agreement of 95% on the 635 tests (Table 2). To examine the relationship of titers with the two tests, the MT reactions were plotted against the corresponding titers obtained by MA (Fig. 2). Although a fourfold or greater difference in titer was observed in a few scattered instances, reactions were the same or within 1 dilution in 580 of the 635 tests (91%). There were 18 tests positive only by MT, compared with 13 positive only by MA. The titers of these tests in which disagreement occurred were 1:100 or lower, with one exception.

The favorable results obtained with the microtechnique for preliminary identification of unknown leptospiral isolations led to trials of the usefulness of this test to detect leptospiral antibodies in sera from suspected human and animal cases. Forty-six human sera were examined by both tests. Except in a few samples where the quantity was not sufficient, a battery of 12 antigens was used, including serotypes of *ballum, canicola, icterohaemorrhagiae, bataviae, grippotyphosa, pyogenes, autumnalis, pomona, wolffi, australis, hyos,* and *mini georgia* (Galton, Gorman, and Shotts, 1960). Positive reactions were obtained with one or more antigens by both methods with 33 of the 46 samples, and 10 sera were negative with all antigens (Table 3). Of the two samples found positive by MT and negative by MA, one reacted in a 1:100 dilution with *L. autumnalis* antigen and the other at 1:200 with

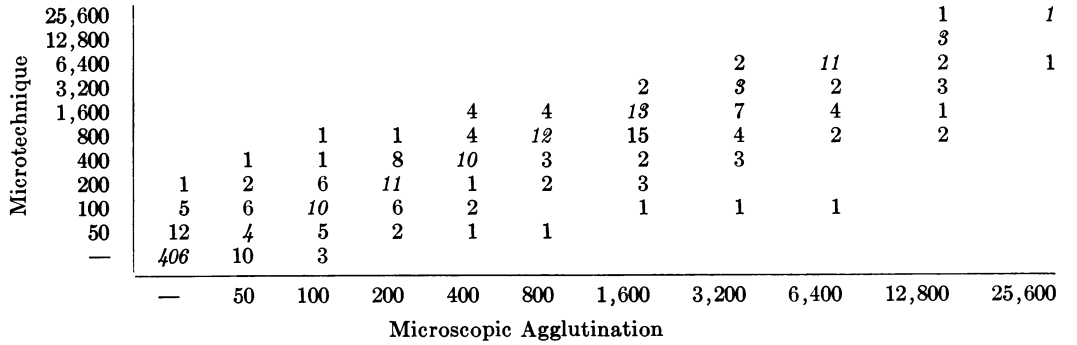

FIG. 2. *Comparative reactions of 635 tests with antigens of 50 unknown leptospiral strains screened against multiple antisera by microscopic agglutination and by microtechnique. Tests with same reaction: 484/635 = 76% agreement. Tests with same reaction or within one dilution: 580/635 = 91% agreement. Titers are expressed as reciprocal of serum dilution.*

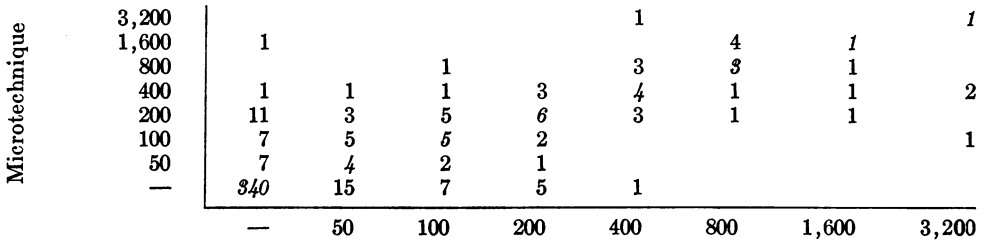

FIG. 3. *Comparative reactions on 462 tests on 46 human sera by microscopic agglutination and by microtechnique with leptospiral antigens. Tests with same reaction: 364/462 = 78% agreement. Tests with same reaction or within one dilution: 415/462 = 89.8% agreement. Titers are expressed as reciprocal of serum dilution.*

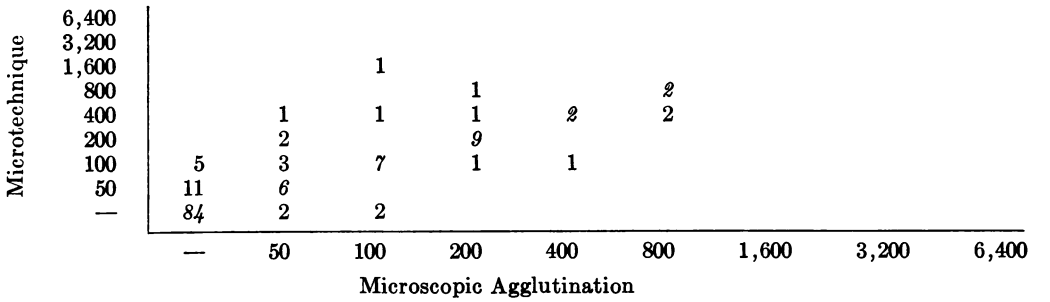

FIG. 4. *Comparative reactions on 145 tests performed on 19 animal sera by microscopic agglutination and by microtechnique with a battery of leptospiral antigens. Tests with same reaction: 110/145 = 75% agreement. Tests with same reaction or within one dilution: 130/145 = 89.6% agreement. Titers are expressed as reciprocal of serum dilution.*

TABLE 3. *Comparative findings on 46 human sera examined by microscopic agglutination test and by microtechnique with leptospiral antigens**

Microtechnique	Microscopic agglutination		Total
	Positive	Negative	
Positive	*33*	2	35
Negative	1	*10*	11
Total	34	12	46

* Agreement on tests: 43/46 = 93.4%.

TABLE 4. *Comparative findings on 462 tests on 46 human sera examined by microscopic agglutination and by microtechnique with leptospiral antigens**

Microtechnique	Microscopic agglutination		Total
	Positive	Negative	
Positive	*67*	27	94
Negative	28	*340*	368
Total	95	367	462

* Agreement on tests: 407/462 = 88%.

TABLE 5. *Comparative findings on 19 animal sera examined by microscopic agglutination and by microtechniques with leptospiral antigens**

Microtechnique	Microscopic agglutination		Total
	Positive	Negative	
Positive	*17*	1	18
Negative	0	*1*	1
Total	17	2	19

* Agreement on tests: 18/19 = 94.7%.

TABLE 6. *Comparative findings on 145 tests on 19 animal sera examined by microscopic agglutination and by microtechnique with leptospiral antigens**

Microtechnique	Microscopic agglutination		Total
	Positive	Negative	
Positive	*41*	16	57
Negative	4	*84*	88
Total	45	100	145

* Agreement on tests: 125/145 = 93%.

L. wolffi antigen. The sample positive only in the MA test agglutinated in a 1:100 dilution with *L. australis* antigen.

A total of 462 comparative tests were performed on these 46 sera. Of these, 67 tests were positive and 340 were negative by both methods, an agreement of 88.0% (Table 4). When the titers of these 462 tests with each of the two methods were plotted for comparison, it was found that the reactions were the same or within one dilution in 415, or 89.8% (Fig. 3).

A similar evaluation was made on 19 animal sera from bovine, canine, and equine sources. Seventeen of the 19 sera were positive by both tests and one sample was negative by both, which made 94.7% agreement between the tests (Table 5). With the single sample in which there was disagreement, titers of 1:50 only were observed with *ballum* and *canicola* antigens. Approximately the same level of agreement (93%) was obtained on individual tests (Table 6). When the MT titers were plotted against corresponding titers by MA, 90% (130/145) of the antibody titers were the same or within one dilution (Fig. 4).

DISCUSSION

These preliminary studies on the application of the microtechnique to the agglutination test for leptospiral antibodies suggest that it can be a valuable tool for screening unknown cultures, particularly in laboratories identifying large numbers of leptospiral isolations. The technique also shows promise as a rapid method of screening large numbers of human and animal sera for leptospiral antibodies. The primary advantages are a 75 to 80% saving in the time required to perform the conventional MA test and an eightfold saving of serum and antigen.

To obtain accurate results, equipment must be scrupulously cleansed. The plastic pipette droppers should be washed first in a solution of sodium hypochloride, then with detergents, followed by repeated rinses in tap and demineralized water. The wells in the Lucite plates were found to be extremely difficult to clean sufficiently for satisfactory reading of the test. Even minute scratches that could not be eliminated caused difficulties. For this reason, the disposable plastic plates were used throughout the study.

It is highly important, also, to check the delivery of the diluting loop. To do this, the loop must be gently passed through a flame, with care taken not to heat to incandescence. The loop is cooled and touched to the surface of a beaker of 0.85% buffered saline. To test delivery, the loop is pressed gently onto the center of the testing circle of a special blotter material marked with circles 10.5 mm in diameter. If the loop is delivering properly, the saline will flow onto the circle, wetting the entire diameter.

For reliable results with the microtechnique, serum must be clean and clear. The greater validity of the microtechnique observed in comparative tests, when hyperimmune sera was used with unknown antigens than when human and animal sera were used with known antigens, was probably because of the better condition of the former sera.

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