

Automated, Quantitative Microhemagglutination Assay for *Treponema pallidum* Antibodies

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An automated, quantitative microhemagglutination assay for antibodies to *Treponema pallidum* was developed by using *T. pallidum*-sensitized erythrocytes and an automatic serial-dilution instrument. Reactivity was found in sera from 54 rabbits and 6 chimpanzees infected with *T. pallidum*. Reactivity was also found in sera from animals infected with *T. pertenue*, *T. carateum*, and *T. cuniculi*. No reactivity was found in sera from 75 normal rabbits or from 129 rabbits immunized with cultivatable treponemes or a variety of other bacteria. In approximately 3 min, 13 two-fold serial dilutions of each of 8 preabsorbed sera and the addition of sensitized erythrocytes to each dilution were accomplished automatically. The automated assay can serve as a research tool in quantitating antibodies to pathogenic treponemes, and evaluation of its clinical usefulness seems warranted.

In research studies aimed at the refinement of serological tests for syphilis, it is often useful to determine anti-*Treponema pallidum* antibody levels present in experimental animals. Although serial dilutions of sera can be tested in the ordinarily qualitative *T. pallidum* immobilization (TPI) test (10) and the fluorescent-treponemal antibody-absorption (FTA-ABS) test (3), the preparation and examination of dilutions require a significant amount of time and labor. These limitations have discouraged quantitation of anti-*T. pallidum* reactivity in all but the most special research circumstances.

Since the indirect hemagglutination technique is a widely accepted serological procedure which lends itself to quantitation, we were interested in the experiments of Rathlev (11, 12) and Tomizawa and Kasamatsu (14), in which *T. pallidum*-sensitized erythrocytes were used in hemagglutination assays. In these studies, serum dilutions were prepared manually in macrovolume hemagglutination trays, and the sensitized erythrocytes were added by hand.

We report here the development of an automated, quantitative microhemagglutination assay for *T. pallidum* antibodies. Our general approach was to utilize reagents commercially available for a manual *T. pallidum* hemagglutination test (TPHA, Fuji Zoki Pharmaceutical Co., Tokyo, Japan) and adapt them to a microvolume technique that could be performed on an automatic serial-dilution instrument. A preliminary charac-

terization of the quantitative assay was then performed with sera from animals infected with pathogenic treponemes, from animals immunized with a variety of cultivatable treponemes and other bacteria, and from presumably normal animals.

MATERIALS AND METHODS

Hemagglutination reagents. The hemagglutination reagents used throughout this study were generously supplied by the Fuji Zoki Pharmaceutical Company of Tokyo, Japan. The lyophilized reagents were reconstituted according to the manufacturer's instructions.

The Formol-treated, tanned sheep erythrocytes had been sensitized with cell components of *T. pallidum* (Nichols strain) and were reconstituted as a 2.5% suspension in test diluent. The procedural details have been previously described by Tomizawa and Kasamatsu (14).

The control sheep cells were treated in the same manner as the sensitized cells except that they were not sensitized with *T. pallidum* antigen.

A 10-ml volume of reconstituted absorbent contains: 0.1 ml of packed sheep and ox red cells, 5 mg of acetone-treated powder of normal rabbit testes, 0.05 ml of cell components of Reiter treponemes, 25 mg of gum arabic powder, 0.1 ml of normal rabbit serum, 0.001 ml of polysorbate 80, 1 mg of thimerosal, and the remaining volume of phosphate-buffered saline (PBS), pH 7.2.

A 25-ml volume of test diluent contains: 62.5 mg of gum arabic powder, 0.25 ml of normal rabbit serum, 0.0025 ml of polysorbate 80, 2.5 mg of thimerosal, and the remaining volume PBS (pH 7.2).

Unless otherwise specified, this diluent was used throughout these studies.

Automatic diluting device. The construction, operation, and evaluation of the automatic serial-diluting instrument (Autotiter; Astec, Inc., Orange, Conn.) has recently been described by Goss and Cimijotti (2) in a report of its use for antibiotic sensitivity determinations. The machine used in hemagglutination procedures consists of the following items: a prewet tray; paper blotter; movable carriage containing a forward manifold (injector) with eight matched 20-gauge needles which deliver diluent; a set of eight microtiter loops designed to deliver 0.025 ml; and a rear manifold (injector), which delivers sensitized sheep cells; water (cleaning) bath; burner; injector air cylinders; injector syringe; control switches; and a programing circuit. The machine can serially dilute, and add two different reagents to 120 samples per hr.

Transparent plastic trays containing 8 rows of 15 cups each (Autotray, Astec, Inc.) were used throughout the study. The cups had suitable U-shaped bottoms to allow for proper settling patterns of the erythrocytes.

Technique for an automated microhemagglutination assay by using *T. pallidum*-sensitized erythrocytes (AMHA-TP). The unheated sera are absorbed by mixing 0.38 ml of absorbent and 0.02 ml of serum, incubating at 2 to 6 C for at least 30 min, and by centrifuging at $1,300 \times g$ for 5 min. The supernatant fluid is a 1:20 dilution of absorbed serum. A 0.05-ml sample of each 1:20 dilution is pipetted into the first row, and 0.025 ml into the last row of cups in the plastic tray. The 2.5% *T. pallidum*-sensitized and unsensitized sheep red cells are diluted 1:7.5 in diluent, and kept in suspension with the use of a magnetic stirrer.

When the machine is in operation, the carriage moves forward to a position where the loops are over the first row of cups. The loops are lowered and pick up 0.025 ml of the 1:20 dilution. Concurrently, the forward manifold delivers 0.025 ml of diluent into the second row of cups. The carriage moves forward, and the transfer loops are lowered and rotated in the second row of cups. Simultaneously, the forward manifold delivers 0.025 ml of diluent into row 3, and the rear manifold delivers 0.075 ml of diluted *T. pallidum*-sensitized sheep cells into the first row. The carriage moves forward and continues this operation until 13 twofold serial dilutions are made, and *T. pallidum*-sensitized cells are added to each dilution. The resultant serum dilution in the first cup is 1:80, with subsequent cups containing twofold serial dilutions. As a control, 0.075 ml of diluted unsensitized cells are added to the sera in the last row of cups. Reactive and nonreactive sera are included in each day's testing, as well as controls of sensitized and unsensitized erythrocytes in diluent alone. The trays containing the tests are sealed with plastic film and incubated for 4 hr at room temperature.

The settling patterns are read by using an angled mirror to reflect the patterns from below. Reactivity is scored on a scale of negative to 4+, and the degree of reactivity is judged according to the following criteria: (4+) smooth mat of cells covering entire

bottom of cup, edges sometimes folded; (3+) smooth mat of cells covering less area of cup; (2+) smooth mat of cells surrounded by circle of red; (1+) smooth mat of cells surrounded by smaller circle of red; (\pm) button of cells having small "hole" in center; and (-) definite compact button in center of cup. A serum showing a reaction of 1+ or more at a dilution of 1:80 or greater is considered reactive. Serum titer is taken to be the last dilution having at least a 1+ degree of hemagglutination.

Animal sera. All sera, unless otherwise specified, were collected from previous or continuing studies in this laboratory and were stored at -20 C.

Serum samples taken at regular intervals during the course of infection were obtained from six chimpanzees infected with *T. pallidum* in serous material from human syphilitic chancres, three chimpanzees infected with *T. carateum* in serous material from human pinta lesions (6), and nine rabbits infected intratesticularly with *T. pallidum* Nichols strain (7.5×10^7 organisms). Three of the six chimpanzees experimentally infected with *T. pallidum* had preexisting reactive serology of unknown origin (5). Single serum samples were obtained from 55 rabbits 20 or more days after experimental infection with *T. pallidum*, 10 rabbits infected with *T. cuniculi*, and 9 rabbits infected with the Haiti B strain of *T. pertenuis*.

A group of 67 rabbit antisera to cultivatable treponemes included 44 antisera to Reiter, 11 to Kazan, 6 to mouth treponemes, 2 to cultivatable Nichols, 2 to *T. vincentii*, and 2 to *T. zuelzeri* (7). Fifty-four rabbit antisera to *Neisseria gonorrhoeae*, *N. catarrhalis*, and *N. meningitidis* were produced by intravenous inoculation of gradually increasing volumes of 9×10^8 organisms/ml over a 3-week period. Antisera produced in rabbits to *Leptospira canicola*, *L. copenhageni*, *L. pomona*, *Proteus OX19*, *Salmonella* "H," *Haemophilus influenzae*, *Shigella sonnei*, and *Streptococcus* Group A, were obtained from the Biological Reagents Section, Laboratory Division, National Communicable Disease Center. Additional sera were obtained from four goats immunized with normal rabbit testicular homogenate (4) and from five male rabbits immunized in a similar manner. Sera were also obtained from 6 presumably normal goats and 75 rabbits nonreactive in the FTA-ABS test.

Procedures of other serological tests. The other serological procedures used were the VDRL Slide (15), fluorescent treponemal antibody 1:5 (FTA 1:5) (1), FTA-ABS (9, 13), and TPI (15) tests. The VDRL Slide and TPI tests were performed by members of the Testing Unit of the Venereal Disease Research Laboratory.

Inhibition procedure. Serum from a syphilitic primate was selected for use in the inhibition experiments; the serum was reactive in the FTA-ABS, VDRL Slide, TPI, and AMHA-TP assays. The serum was absorbed by using an equal volume of the AMHA-TP absorbent previously described, and twofold serial dilutions were made. Then, 0.09 ml of each dilution was mixed with 0.01 ml of saline, or with the same volume of a stock solution of sonically treated (20 kc for 20 min) *T. pallidum* (5×10^9 organisms/ml, and fivefold dilutions to a final concentration of 1.6×10^6 or-

TABLE 1. Appearance of serum reactivity in the AMHA-TP, FTA 1:5, and VDRL Slide assays of nine rabbits after infection with *Treponema pallidum*

Assay	Animals reactive by postinfection day					
	0	2	5	7	10	14
AMHA-TP.....	0	0	2	8	9	9
FTA 1:5.....	0	0	0	3	8	9
VDRL Slide.....	0	0	0	5	9	9

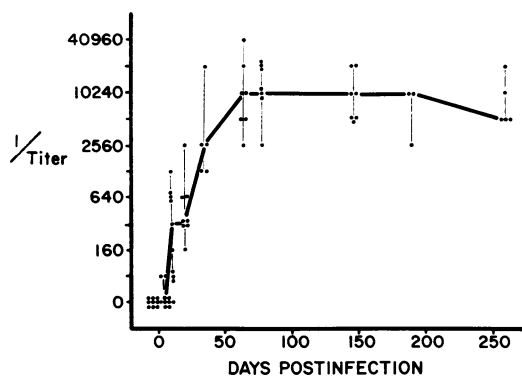


FIG. 1. Range and median AMHA-TP titers in sera taken at intervals from rabbits infected with *Treponema pallidum*. Individual rabbit titers each time a blood sample was taken (•); heavy line connects the median titers. Sera were not available from all animals at all points tested.

ganisms/ml), Reiter treponemes (1.2×10^{11} organisms/ml), *Escherichia coli* (80 mg of protein/ml), or extract of normal rabbit testicular material (60 mg of protein per ml). After a 30-min incubation period at 37 C, 0.01 ml of *T. pallidum*-sensitized sheep cells (2.5%) was added to each dilution. The mixtures were left at room temperature and read in the manner previously described.

Another sample of the reactive serum pool was absorbed with VDRL antigen particles (cardiolipin, cholesterol, and lecithin), incubated at 4 C, and then centrifuged. The serum, which previously had a titer of 1:64 in the VDRL Slide test, was absorbed until it became nonreactive in that test, and the AMHA-TP assay was then performed.

RESULTS

Sera from rabbits infected with pathogenic treponemes. Nine sero-negative rabbits infected intratesticularly became reactive in the AMHA-TP assay as early as or earlier than in the FTA 1:5 and VDRL Slide tests. The hemagglutinating antibody appeared in two rabbits 5 days after inoculation, and by the 10th day all nine animals

were reactive. In comparison, the FTA 1:5 test first detected antibody in three rabbits on the 7th day and in all rabbits by the 14th day. The VDRL Slide test became reactive in five rabbits by the 7th day, and in all rabbits by the 10th day (Table 1). The median and range of the AMHA-TP antibody titers detected at regular time intervals in the course of *T. pallidum* infection are shown in Fig. 1.

The AMHA-TP assay showed reactivity in sera from 54 of 55 rabbits with experimental *T. pallidum* infections of various durations (Table 2). The antibody titers ranged from 1:320 to 1:40,960. Sera from 9 rabbits experimentally infected with *T. pertenuis* and from 10 rabbits infected with *T. cuniculi* were also reactive in the AMHA-TP assay, with antibody titers ranging from 1:160 to 1:20,480.

Sera from chimpanzees infected with pathogenic

TABLE 2. AMHA-TP results on 278 rabbit sera

Infected or immunized with	No. tested	No. reactive	No. non-reactive
<i>Treponema pallidum</i>	55	54	1
<i>T. cuniculi</i>	10	10	0
<i>T. pertenuis</i>	9	9	0
Cultivable treponemes	67	0	67
Other bacteria	62	0	62
Sera from presumed normal rabbits	75	0	75

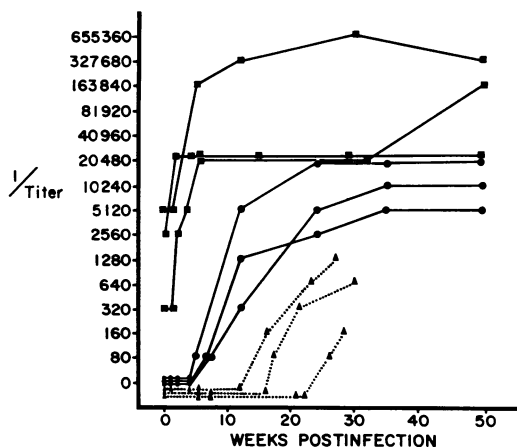


FIG. 2. Increase of AMHA-TP titers in nine chimpanzees after inoculation with pathogenic treponemes. Three animals (▲) were inoculated with *Treponema carateum*. Six animals were inoculated with *T. pallidum*. On the day of inoculation, three of these (●) were non-reactive in the AMHA-TP assay, and three (■) were already reactive.

treponemes. The AMHA-TP antibody titers of three seronegative chimpanzees subsequently infected with *T. pallidum* were followed over a 50-week period. The hemagglutinating antibody appeared in one animal after 5 weeks of infection, and in all three by the 7th week. By comparison, the FTA-ABS procedure detected antibodies in two animals by the 3rd week, and in all three by the 7th week. The VDRL Slide test was reactive in one animal by the 3rd week, and in all animals by the 7th week. The AMHA-TP titers increased to 1:5,120, 1:10,240, and 1:20,480, and remained at those levels through 50 weeks postinfection (Fig. 2).

Preinoculation sera from three additional chimpanzees were FTA-ABS-reactive and demonstrated antibody levels of 1:320, 1:2,560, and 1:5,120 in the AMHA-TP assay. At 50 weeks after infection with *T. pallidum*, the hemagglutinating antibody titers in the same animals increased to 1:163,840, 1:20,480, and 1:327,680, respectively (Fig. 2).

Another group of three chimpanzees, initially AMHA-TP and FTA-ABS nonreactive and subsequently infected with *T. carateum*, first showed reactivity in both assays at 16, 17, and 26 weeks post-infection. After 30 weeks of infection, the hemagglutinating antibody titers increased to 1:160, 1:640, and 1:1,280 (Fig. 2).

Sera from presumed normal rabbits and rabbits immunized with nonpathogenic treponemes and other bacteria. The AMHA-TP assay was nonreactive in 67 sera from rabbits immunized with various nonpathogenic treponemes, including Reiter, Kazan, mouth treponemes, Nichols, *T. Vincentii*, and *T. zuelzeri*. No AMHA-TP reactivity was found in 75 sera from presumed normal rabbits or in 62 rabbit antisera to *Neisseria*, *Leptospira*, *Proteus*, *Salmonella*, *Hemophilus*, *Shigella*, and *Streptococcus* bacterial categories (Table 2).

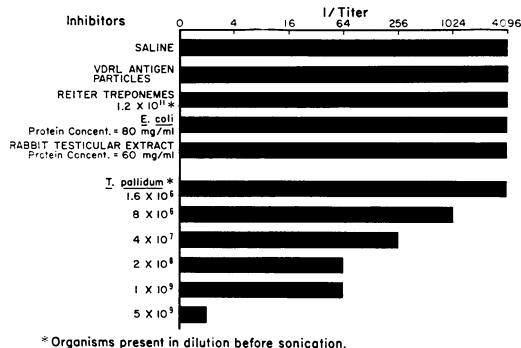


FIG. 3. Effect of absorption with various materials on the AMHA-TP titer of an anti-*Treponema pallidum* serum from a primate.

Sera from animals immunized with rabbit testicular material. Five male rabbits immunized with normal rabbit testicular homogenate did not show serum reactivity. However, AMHA-TP antibody titers of 1:640 and 1:1,280 were found in two out of four goats immunized in the same manner. The two goat antisera were also reactive in the TPI test. Sera from six presumed normal goats were nonreactive.

Inhibition experiments. The reactive pool of primate serum used in the inhibition study had an antibody titer of 1:4,096 in the AMHA-TP assay. The antibody titer remained the same after absorption of the serum with VDRL antigen particles or incubation with sonic-treated material of Reiter treponemes, *Escherichia coli*, and normal testicular material (Fig. 3). However, incubation with *T. pallidum* sonicate (5×10^9 organisms/ml) reduced the titer far below the 1:80 dilution considered significant in routine testing. Inhibition by *T. pallidum* was also shown to be a quantitative phenomenon. It can be seen (Fig. 3) that as the concentration of added *T. pallidum* sonic-treated material was increased, there was greater inhibition of reactivity.

DISCUSSION

The AMHA-TP assay detects substantial antibody titers in chimpanzees and rabbits experimentally infected with *T. pallidum*, and the assay becomes reactive at approximately the same time after infection as do other currently used serological tests for syphilis. In *T. pallidum*-infected rabbits, the hemagglutination assay became reactive as early or earlier than the VDRL Slide or FTA 1:5 procedures, whereas in *T. pallidum*-infected chimpanzees the AMHA-TP response appeared at approximately the same time as the FTA-ABS and VDRL Slide test reactivity. In addition, because of the quantitative nature of the hemagglutination assay, changes in the antibody levels over time were readily observed.

The AMHA-TP assay, like the TPI and FTA-ABS tests, also detects antibodies in animals that have been experimentally infected with *T. pertenue*, *T. cuniculi*, and *T. carateum*. Since these pathogenic treponemes cannot be morphologically or immunologically distinguished at present, it was not unexpected that *T. pallidum*-sensitized erythrocytes reacted with antisera to them.

The AMHA-TP assay appears to be relatively specific for antibodies to pathogenic treponemes, for no reactivity was observed between *T. pallidum*-sensitized erythrocytes and antibodies to a variety of bacteria or to cultivatable treponemes reported to be immunologically related to *T.*

pallidum (7). In addition, no reactivity was observed in the 75 presumed normal rabbit sera.

The results of the inhibition experiments, although performed on only one *T. pallidum* antiserum, tend to confirm the specificity of the AMHA-TP assay. The reaction between *T. pallidum*-sensitized erythrocytes and antibodies to *T. pallidum* could be inhibited with *T. pallidum* but not with Reiter treponemes, VDRL antigen, *E. coli*, or normal rabbit testicular extract.

One should be cautious, however, in the interpretation of AMHA-TP data in situations where antibodies might be directed to antigens present in rabbit material. It has been reported (4, 8) that immunization of goats with a homogenate of normal rabbit testes induces reactivity in the TPI test. Since the organisms used in the TPI procedure are obtained from rabbit testicular syphilomas, it was postulated that the observed reactivity could have been caused by rabbit constituents closely associated with the harvested *T. pallidum*. The *T. pallidum* antigen used to sensitize the erythrocytes for the AMHA-TP assay is also prepared from an extract of rabbit testicular syphilomas. Therefore, it is possible that, in the process of sensitizing the tanned erythrocytes with *T. pallidum*, antigens of rabbit origin may also be present and may attach to the red cells. For example, we found that two goats immunized with normal rabbit testicular homogenate were reactive in the AMHA-TP assay, as well as in the TPI test. However, five rabbits immunized in the same manner were nonreactive.

The AMHA-TP procedure appears to have two main advantages as a research tool. First, the microvolume method minimizes consumption of reagents, a factor which may be important when scarce antigens such as *T. pallidum* are used. The AMHA-TP procedure uses 20% of the amount of *T. pallidum*-sensitized erythrocytes required for the manual, macrovolume hemagglutination technique described by Tomizawa and Kasamatsu (14). Second, the automatic diluting device facilitates the quantitation of antibody; within 3 min, the instrument prepares 13 twofold serial dilutions of eight preabsorbed sera, including the addition of both diluent and sensitized erythrocytes.

Since the AMHA-TP assay was shown to be relatively specific in detecting anti-*T. pallidum* reactivity in animals, and can produce quantitative data quickly and easily as compared to manual tests with *T. pallidum* antigen, it appears to warrant evaluation on sera from patients with syphilis and other treponemal infections.

The automated dilution device has been used in antibiotic sensitivity studies (2), but to our knowledge this is the first report of its use in a hemagglutination procedure. It is also possible to perform complement-fixation assays in a quantitative fashion on the instrument. Automated, quantitative microhemagglutination procedures would seem to have applicability in many areas of microbiology and immunology.

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