Acta virol. 13 : 60—66, 1969

A MICROAGGLUTINATION TECHNIQUE FOR DETECTION AND MEASUREMENT OF RICKETTSIAL ANTIBODIES[1])

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Received August 8, 1968

Summary. — A microagglutination test for Q fever and typhus antibodies is described. The test is an adaptation of the microtiter technique using highly purified rickettsial antigens. With *Coxiella burneti* antigen the test shows a high degree of specificity and sensitivity for detecting phase I and phase II antibodies. With typhus group antigens, the test appears to have the same limits of resolution as the standard agglutination tests. Its advantages are rapidity and ease of performance and reading, and economy of reagents.

Introduction

Rickettsial agglutination techniques have been used for many years and are generally thought to be as sensitive and specific as the complement fixation (CF) test (Plotz *et al.*, 1948; Scoville *et al.*, 1948; Lennette, *et al.*, 1952). However, these techniques have been less popular than the CF test because of the requirement of large amounts of usually unavailable antigen.

The development of procedures which yield large quantities of highly purified preparations (Ormsbee, 1962; Fiset and Silberman, 1966) and the microtechniques described here, which require as little as 8 μg of antigen per tube, make rickettsial agglutination a useful and practical serological procedure.

Materials and Methods

Strains of Rickettsiae. Coxiella burneti. Strain Nine Mile, phase I [307th guinea pig passage, third egg passage, (GP307/EP3)] and strain Nine Mile, phase II EP90. *Rickettsia prowazeki.* Breinl strain (EP152), prior history unknown. *R. mooseri.* Wilmington strain (EP42), prior history unknown.

Sera. A variety of human and animal sera obtained after natural infection, experimental infection, or vaccination were tested. These sera are described under "Results". The sera were usually inactivated at 56° C for 30 minutes. This treatment did not affect agglutinin titers to any significant degree.

Preparation of antigens. It is essential that the antigen suspensions be as free as possible of contaminating material. Gross contamination with host tissue results in agglutination patterns

[1]) This study was carried out under the aegis of the Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board and was partially supported by contract number DA-49--193-MD-2277 with the U. S. Army Medical Research and Development Command.

that are impossible to interpret. Since the organisms are grown in yolk sacs of chick embryos, even the cleanest preparations show some degree of serologic cross reactivity with hyperimmune rabbit anti-egg serum. Although such cross reactions are negligible with *C. burneti* in phase I, they tend to be much stronger with *C. burneti* in phase II and with the strains of *R. prowazeki* and *R. mooseri* used in this study.

The organisms are extracted from yolk sacs and purified either with M/1 KCl according to the procedure of Ormsbee (1962) or with anion exchange celluloses (ECTEOLA or DEAE) according to a modification (Fiset and Silberman, 1966) of the method of Silberman and Fiset (1963). In the final steps of purification, the antigens are treated once or twice with ether or until a clean interface is obtained. With *C. burneti*, the aqueous phase, after evaporation of the ether, represents the final antigen. With *R. prowazeki* and *R. mooseri*, the aqueous phase is centrifuged at 17,000 × g for 30 minutes and the supernatants saved as a source of group "soluble" antigen. The sediments are washed three times and resuspended in saline and represent the final antigen.

Antigens prepared as described are sufficiently clean that they can safely be standardized photometrically. It will suffice to state that, using a Beckman B spectrophotometer with 1.0 cm cuvettes, at a wavelength of 420 nm, a suspension of *C. burneti* containing 100 μg dry weight/ml gives an OD reading of 0.330, whereas 100 μg/ml of *R. prowazeki* or *R. mooseri* give OD readings of 0.440. All antigens are made up in 0.85% NaCl and standardized to a final concentration of 1 mg/ml. Merthiolate, at a concentration of 1 : 10,000 is added as a preservative.

Such antigens, except for those prepared from phase II *C. burneti* (EP90), may be employed for both CF and agglutination reactions. Suspensions of phase II antigens tend to agglutinate spontaneously and are strongly agglutinated by normal serum. However, in the course of studies on the antigenic structure of *C. burneti*, Silberman, Fiset and Ormsbee (unpublished data) observed that when phase I strains were treated with trichloroacetic acid (Brezina and Urvölgyi, 1962; Anacker *et al.*, 1962) a soluble complex was obtained which showed phase I activity in the CF test, while the residual particulate antigen had acquired phase II specificity in both CF and agglutination reactions. Such trichloroacetic acid-extracted antigen suspensions are stable and are agglutinated only by specific antisera. These antigens will be referred to as TCA-phase II. They are prepared in the following way: All reagents are chilled in an ice bath. To a suspension of 1 mg/ml of phase I rickettsiae, trichloroacetic acid is added to a final concentration of 10%. The mixture is held in an ice bath for 3 hours, then centrifuged at 17,000 × g for 30 minutes. The sediments are washed three times and resuspended to the original volume in 0.85% NaCl.

Staining of antigens. The use of stained antigens makes the reading of agglutination patterns much easier. Unfortunately, purified rickettsiae obtained from the thoroughly egg-adapted strains which were used in this study (*C. burneti* phase II, *R. prowazeki* and *R. mooseri*) absorb stain with great avidity resulting in unstable preparations. Such antigens settle out of suspension rapidly and are unsuitable for agglutination reactions.

Strains of *C. burneti* phase I and *C. burneti* TCA-phase II, however, are easily stained in suspension and such preparations are stable and retain their activity for several months when stored at 4° C. The staining procedure adopted is a modification of the method described by Luoto (1953) for staining of *C. burneti* antigen used in the capillary agglutination (CA) test for *Q* fever. Hematoxylin, without acetic acid (Harris, 1900), is added to a suspension of *C. burneti* antigen (1.0 mg/ml) to a final concentration of 10%. The mixture is incubated at 56° C for 48 hours with occasional shaking. The antigen is washed three times and resuspended in 0.85% NaCl to the original volume. Merthiolate (1 : 10,000) is used as preservative.

With *R. prowazeki* and *R. mooseri*, a different staining procedure is used as described under "Agglutination test procedure".

Agglutination test procedure. The method used is an adaptation of the micro-titration method of Takátsy (1955) as modified by Sever (1962). Serial two-fold dilutions of serum are prepared in disposable hard plastic plates with round bottom cups (Linbro Chemical Co., Inc., New Haven, Connecticut) of 0.3 ml capacity with micro-diluters calibrated to transfer 0.025 ml. Antigen, diluted 1 : 3 (333 μg/ml) is added to each cup with a dropper calibrated to deliver 0.025 ml (Cook Engineering Co., 735 North St. Asaph St., Alexandria, Virginia). The plates are sealed with translucent pressure tape and incubated overnight at room temperature. The test is read by the pattern method, i.e., a tight, regular button indicates a negative reaction whereas a film covering the bottom of the cup indicates a positive reaction. There are also intermediate patterns of partial agglutination. The highest serum dilution showing partial agglutination is taken as the endpoint (Fig. 1).

The patterns are more clear-cut and easier to read when the serum and antigen dilutions are made up in 0.85% NaCl solution containing 0.5% normal human serum (Plotz *et al.*, 1948). It is important to avoid applying pressure on the microdiluters as scratching of the plastic cups interferes with the formation of reproducible patterns. The test should be read from the bottom with a viewer after covering the plate with a glass or plastic light diffuser.

With *R. prowazeki* and *R. mooseri* antigens which cannot be stained prior to reaction with serum, the patterns can be defined more clearly if, after overnight incubation, a drop of aequeous acridine orange, 1 : 5000, is added to each cup and left at room temperature for an hour or so. The antigen stains deep orange making the test extremely easy to read.

Complement-fixation test procedure. The micro-technique described by Sever (1962) and modified by Fiset (1964) was used throughout with concentrations of antigen of 250 μg/ml.

Table 1. Antibody response in guinea pigs immunized with Nine Mile phase I or Nine Mile phase II

Days post vaccination	Guinea pig immunized with 20 μg Nine Mile phase I					Guinea pig immunized with 20 μg Nine Mile phase II				
	MA		CF		AR	MA		CF		AR
	Ph I	TCA-PhII	Ph I	PH II	Ph I	Ph I	TCA-PhII	Ph I	Ph II	Ph I
0	0	0	0	0	0	0	0	0	0	0
10	0	256	0	32	0	0	16	0	16	0
20	8	256	0	128	4	0	64	0	64	0
31	8	128	0	128	8	0	32	0	64	0
42	8	64	2	64	8	0	16	0	64	0
52	8	32	4	32	16	0	16	0	8	0
63	8	16	8	32	16	0	16	0	4	0
73	8	16	16	16	8	0	8	0	0	0
83	8	16	16	16	8	0	8	0	0	0
94	8	8	8	8	8	0	8	0	0	0
104	8	8	8	8	8	0	8	0	0	0

Titers are expressed as the reciprocal of the initial serum dilution; 0 = less than 1 : 2. CF = complement fixation; MA = microagglutination; AR = agglutination resuspension.

Results

Coxiella burneti

Antibody response in guinea pigs. Table 1 summarizes the results of comparative serological tests performed on serial bleedings of a representative guinea pig from each of two large groups immunized with a single 20 μg subcutaneous inoculation of either Nine Mile phase I or Nine Mile phase II purified vaccine.

In the animals immunized with phase I vaccine, there was close agreement between the phase I microagglutination (MA) and the agglutination resuspension (AR) (Ormsbee, 1964) tests. Both tests detected phase I antibodies three weeks before positive reactions could be obtained by CF test. Phase II antibodies were detected by both MA and CF tests at 10 days, but somewhat higher titers were obtained by MA. In the animals immunized with phase II vaccine, phase II antibodies were first detected on the tenth day by both MA and CF tests, but agglutinins were detectable for a much

Table 2. Comparison of microagglutination (MA) and capillary agglutination (CA) tests with Phase I antigens on samples of cows' whey

Whey sample	MA	CA
1	4	16
2	2	8
3	8	16
4	2	8
5	16	16
6	8	16
7	32	64
8	8	32
9	8	16
Control:	0	0

Cow's whey had been stored at −25° C for approximately five years. CA tests (Luoto, 1953) were carried out at the Rocky Mountain Laboratory by the late Edgar G. Pickens.

longer period than were the corresponding CF antibodies. None of these animals developed any detectable phase I antibodies.

Detection of antibodies in cows' milk. Tables 2 and 3 illustrate comparative titrations of phase I agglutinins in cows' milk by MA and CA tests. Titrations on samples of "CA-positive whey" stored at −25° C for over 5 years (Table 2) showed good agreement between the MA and CA tests although CA titers were usually slightly higher. Titrations on fresh whole milk samples obtained from a dairy in Hamilton, Montana, indicated that both tests were equally sensitive in detecting phase I antibodies (Table 3).

Table 3. Detection of phase I antibody in fresh whole milk by microagglutination (MA) and capillary agglutination (CA) tests

Milk sample	MA	CA
1–14	0	0
15	32	64
16	4	16
17	16	64

Antibody response in man. Table 4 summarizes results of serological tests in three volunteers with different experiences with *C. burneti.*

Subject E was representative of 66 volunteers immunized with a single 30 μg subcutaneous inoculation of Henzerling strain, phase I vaccine (Hornick, unpublished data). TCA-phase II agglutinins appeared at approximately the same time as phase II CF antibodies, but were usually of higher titer. Subject E, like most other volunteers in this group, developed no phase I CF antibodies. Phase I agglutinins by contrast, were detected as early as 2 weeks after immunization.

Table 4. Comparison of serological response in three volunteers having had different experiences with C. burneti

Weeks after exposure	Subject E: Immunized with 30 μg Henzerling strain phase I vaccine				Subject G: Immunized with live Nine Mile phase II				Subject M: Experimentally infected with 3000 guinea pig ID_{50} by aerosol with AD strain of *C. burneti*			
	MA		CF		MA		CF		MA		CF	
	Ph I	TCA-Ph II	Ph I	Ph II	Ph I	TCA-Ph II	Ph I	Ph II	Ph I	TCA-Ph II	Ph I	Ph II
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0
2	8	32	0	2	0	0	0	0	0	2	0	0
3	8	64	0	4	0	32	0	0	0	128	0	8
4	16	—	0	8	0	32	0	0	2	256	2	16
5	16	64	0	8	0	32	0	8	4	256	2	16
6	16	64	0	16	0	32	0	16	—	—	—	—
8	16	64	0	16	0	32	0	32	16	128	2	128
10	16	64	0	16	0	32	0	32	—	—	—	—
12	32	128	0	32	0	16	0	32	8	128	2	128
20	128	128	0	32	0	8	0	32	8	128	8	64
24	64	128	0	32	—	—	—	—	—	—	—	—

Subject G was representative of 6 volunteers who were given 1 ml of a 10^{-4} dilution of yolk sac suspension containing live Nine Mile phase II, (EP90) (Hornick, unpublished data). This suspension had an ID_{50} for guinea pigs, inoculated intraperitoneally, of $10^{-8.5}$. Subject G, like other members of this group, never developed phase I antibodies detectable by MA, CF or radioisotope precipitation (Tabert and Lackman, 1965). However the MA test with TCA-phase II antigen proved to be as sensitive as the CF tests for the detection of phase II antibodies.

Table 5. Comparison of complement fixation and microagglutination tests in the study of the antibody response in guinea pigs infected with R. prowazeki or R. mooseri

Days after infection	Guinea pig No. 8 infected with *R. prowazeki*				Guinea pig No. 11Y infected with *R. mooseri*			
	MA		CF		MA		CF	
	Epidemic	Murine	Epidemic	Murine	Epidemic	Murine	Epidemic	Murine
0	0	0	0	0	0	0	0	0
7	256	32	0	0	64	512	0	0
14	512	32	512	64	128	512	256	256
21	256	8	512	64	64	256	256	256
30	128	8	256	64	32	256	128	256
45	32	8	256	64	32	256	128	128
60	32	8	128	32	16	128	64	128
90	32	8	64	8	16	128	64	128

Subject M was one of ten volunteers who served as controls in a challenge experiment with the AD strain of *C. burneti* (GP3/EP11), which was primarily in phase I (Huebner *et al.*, 1948; Ormsbee *et al.*, 1964). Each volunteer received 3000 guinea pig ID_{50} by aerosol and each developed clinical Q fever. Phase II agglutinins appeared earlier and were of higher titer than phase II CF antibodies. Phase I agglutinins appeared at approximately the same time as CF phase I antibodies but tended to be of higher titer.

R. prowazeki and R. mooseri

Table 5 illustrates serological results obtained in representative guinea pigs from two groups of animals inoculated with 10^7 egg ID_{50} of *R. prowazeki* or *R. mooseri* respectively. The same antigens were used for MA and CF titrations.

In both groups, the agglutinins appeared earlier than the CF antibodies and the titers were consistantly and significantly higher with the homologous antigens. In both groups, also, the MA test was more reliable than the CF test in distinguishing between homologous and heterologous infections. These findings are in general agreement with the earlier observations of Plotz *et al.* (1948) and Scoville *et al.* (1948).

Discussion

The MA test offers several advantages, the most important of which are economy of time in performing and reading the test and economy of reagents. It should be stressed, however, that the antigens must be of high purity.

The *C. burneti* phase I MA showed the same sensitivity and specificity as Luoto's (1953) CA test and Ormsbee's (1964) AR test. Since naturally occurring cases of Q fever are, in all probability, caused only by phase I organisms (Stoker and Fiset, 1956), the use of phase I MA as a diagnostic procedure seems justifiable. However, in the unusual circumstances where individuals are immunized or infected with phase II organisms, phase I agglutinins may not develop (Tables 1 and 4).

The tendency of purified phase II organisms to agglutinate in the presence of normal serum precludes their use in agglutination reactions. However, the fact that trichloroacetic acid-treated phase I organisms acquire phase II specificity eliminates this problem. Such preparations are stable and serve as excellent agglutinogens. The data in Tables 1 and 4 indicate that the MA test with TCA-phase II antigens is as sensitive and as specific as the CF test with standard phase II antigens.

Results obtained using the MA test with antigens of the typhus group are in general agreement with the earlier findings of Plotz *et al.* (1948) and Scoville *et al.* (1948) using a test tube agglutination technique (0.25 ml volumes of reagents). Agglutinins appear earlier than CF antibodies and the agglutination test seems to be more sensitive in distinguishing between murine and epidemic typhus infections.

Acknowledgement. The authors wish to express their sincere thanks to Mrs. Hazel Kraemer and Mr. George Tallent for excellent technical assistance.

References

Anacker, R. L., Lackman, D. B., Pickens, E. G., and Ribi, E. (1962): Antigenic and skin-reactive properties of fractions of *Coxiella burneti*. *J. Immunol.* **89**, 145—153.

Brezina, R., and Urvölgyi, J. (1962): Study of the antigenic structure of *Coxiella burneti*. I. Extraction of phase I antigenic component by means of trichloroacetic acid. *Acta virol.* **6**, 84—88.

Fiset, P. (1964): Serological techniques, p. 225—255. R. J. C. Harris (Ed.): *Techniques in Experimental Virology*, Academic Press, London.

Fiset, P., and Silberman, R. (1966): Purification des rickettsies au moyen d'échangeurs d'ions. *Arch. Inst. Pasteur Tunis* **43**, 231—236.

Harris, H. F. (1900): On the rapid conversion of haematoxylin into haematein in staining reactions. *J. appl. Micr.* **3**, 777—780.

Huebner, R. J., Jellison, W. L., Beck, M. D., Parker, R. R., and Shepard, C. C. (1948): Q fever studies in Southern California. I. Recovery of *Rickettsia burneti* from raw milk. *Publ. Hlth. Rep.* (*Wash.*) **63**, 214—222.

Lennette, E. H., Clark, W. H., Jensen, F. W., and Toom, F. W. (1952): Q fever studies. XV. Development and persistence in man of complementfixing and agglutinating antibodies to *Coxiella burneti*. *J. Immunol.* **68**, 591—598.

Luoto, L. (1963): A capillary agglutination test for bovine Q fever. *J. Immunol.* **71**, 226—231.

Ormsbee, R. A. (1962): A method of purifying *Coxiella burneti* and other pathogenic rickettsiae. *J. Immunol.* **88**, 100—108.

Ormsbee, R. A. (1964): An agglutination-resuspension test for Q fever antibodies. *J. Immunol.* **92**, 159—166.

Ormsbee, R. A., Bell, J. E., Lackman, D. B., and Tallent, G. (1964): The influence of phase on the protective potency of Q fever vaccine. *J. Immunol.* **92**, 404—412.

Plotz, H., Bennett, B. L., Wertman, K., Snyder, M. J., and Gauld, R. L. (1948): The serological pattern in typhus fever. I. Epidemic. *Amer. J. Hyg.* **47**, 150—165.

Scoville, Jr., A. B., Bennett, B. L., Wertman, K., and Gauld, R. L. (1948): The serologic pattern of typhus fever. II. Murine. *Amer. J. Hyg.* **47**, 166—176.

Sever, J. L. (1962): Application of a microtechnique to viral serological investigations. *J. Immunol.* **88**, 320—329.

Silberman, R., and Fiset, P. (1963): Purification of psittacosis agent with anion exchange cellulose — Ecteola. *Nature* (*Lond.*) **198**, 699—700.

Stoker, M. G. P., and Fiset, P. (1956): Phase variation of the Nine Mile and other strains of *Rickettsia burneti*. *Canad. J. Microbiol.* **2**, 310—321.

Tabert, G. G., and Lackman, D. B. (1965): The radioisotope precipitation test for study of Q fever antibodies in human and animal sera. *J. Immunol.* **94**, 959—965.

Takátsy, G. (1955): The use of spiral loops in serological and virological micromethods. *Acta microbiol. Acad. Sci. hung.* **3**, 191—201.