Modification of the indirect haemagglutination test for amoebiasis¹

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SYNOPSIS Optimal conditions for carrying out the indirect haemagglutination test with sheep erythrocytes were studied using the microtitre system. The age of the erythrocytes, concentration of reagents, time and temperature of tanning, sensitization, and incubation are described with reference to replication of titres. Sera from patients with amoebic colitis were used to evaluate the test conditions.

The haemagglutination test originally described by Kessel, Lewis, Ma, and Kim (1961), and modified by Milgram, Healy, and Kagan (1966), has been used in our laboratory for over three years with sera from patients with clinical amoebiasis and asymptomatic cyst passers. Persons who were negative for *E. histolytica*, but might have had other diseases and/or parasitic infections and were living in an endemic area, were also tested. Replication of titres was not always obtained when the same samples were titrated at weekly or monthly intervals.

An amoebiasis serology evaluation study has been carried on for several years as an informal co-operative effort between several interested investigators working in this field. A comparison of titres obtained by the various investigators, all using the same serum samples, invariably shows wide discrepancies. A possible explanation for this may be the variations in the technique as used by different investigators. These include reaction volume, method of determining serum dilution, concentration of tannic acid, and concentration of sensitized cells. In view of these facts, studies were set up to investigate the factors that affect replication of results and to determine optimum conditions for standardizing the test procedures.

MATERIALS AND METHODS

SHEEP CELLS Sheep blood² was collected aseptically in Alsever's solution, was received weekly, dispensed into

sterile vials in 5 millilitre volumes, and stored at 4°C. Cells were kept sterile during storage, and were put aside for three days before use; thereafter they were used for periods up to two weeks. Erythrocytes from some sheep spontaneously agglutinated when sensitized with *E. histolytica* antigen; only sheep whose blood cells were found to go through the tanning and sensitization procedures without agglutination were used for this study. In preparation for tanning, erythrocytes were washed three times, by centrifugation, in phosphate-buffered saline at pH 7.2 (380 ml of 0.15M Na₂HPO₄, 120 ml of 0.15M KH₂PO₄, and 1,000 ml of 0.15M NaCl)³, and a 2.5% suspension was made after the final washing.

TANNING A stock solution of tannic acid⁴ (1:1,000 w/v) in saline was prepared weekly and stored at 4°C. Dilutions for reaction with erythrocytes were prepared from the stock solution with phosphate-buffered saline at pH 7.2. Equal volumes of erythrocyte suspensions and tannic acid solutions were combined in Erlenmeyer flasks and gently rotated at 4°C for 20 minutes. After tanning, the cells were washed in phosphate-buffered saline (pH 7.2). After the second washing the cells were diluted to 2.5% in phosphate-buffered saline at pH 6.4 (100 ml of 0.15M Na₂HPO₄, 210 ml of 0.15M KH₂PO₄, and 310 ml of 0.15M NaCl) for sensitization.

SENSITIZATION This was carried out by gently rotating at 37°C, for 20 minutes, 1 volume of antigen dilution in phosphate-buffered saline, pH 6.4, and 1 volume of 2.5% tanned erythrocytes. Agamma rabbit serum⁵ was used as a stabilizer in the test and was activated and absorbed with sheep cells before use as described by Bing, Weyand, and Stavitsky (1967). Sensitized erythrocytes were washed twice in 1% agamma rabbit serum—in phosphate-buffered

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²Bio Labs, Lafayette, Louisiana.

³Demineralized water was used in all solutions.

⁴Eastman-Kodak Company.

⁵Hyland Laboratories.

saline at pH 7.2, and then diluted in the same solution for use on the microtitre plates.

ANTIGEN *E. histolytica* strain DKB, grown in modified Shaffer-Frye medium with *Bacillus symbiosis* (Reeves, Meleney, and Frye, 1957), was provided in lyophilised form by Dr R. Reeves of Louisiana State University Medical School, and was kept stored at -20°C . Before use the amoebae were reconstituted with saline, 1,000,000 amoebae/ml, and stored in 0.5 ml amounts at -70°C . This antigen, reconstituted with saline, was further diluted with phosphate-buffered saline, pH 6.4, for use in the test. It was found that centrifugation at 18,000 rpm for 20 minutes clarified the antigen and improved the detection of antibodies in test sera. Antigen was diluted 1:20, 1:40, 1:60, 1:80, etc, with phosphate-buffered saline, pH 6.4, and combined with equal volumes of a 2.5 % suspension of tanned erythrocytes for sensitization. The sensitized cells were treated with threefold dilutions of reference sera with established titres (1:27, 1:729, and 1:19,700). Serum dilutions were prepared with 1% agamma rabbit serum and phosphate-buffered saline, pH 7.2. The same reference sera were always used for titration, and the dilution of antigen giving the desired titre with all sera was used for subsequent testing.

TEST SERA Sera collected from patients with amoebiasis and from uninfected persons were centrifuged at 5,000 rpm for 20 minutes and stored at -20°C until used. Sera were heated at 56°C for 30 minutes to inactivate complement and were tested for heterophile antibody. If positive, sera were absorbed as described by Bing *et al* (1967).

TEST SYSTEM The microtitre system was routinely used (Sever, 1962). Of 1% agamma rabbit serum-7.2 phosphate buffered saline, 0.10 ml was placed into each of the 96 wells on the microtitre plate.⁶ Then 0.05 ml of each serum sample was picked up by Takatsy loops and serially diluted, threefold, in 12 successive wells (1:3–1:532,000) and 0.025 ml of a suspension of sensitized cells added to each serum dilution. The plates were sealed with transparent gummed tape, gently rotated for half an hour and then allowed to pattern for several hours at room temperature. The plates were again shaken, placed in the refrigerator overnight, shaken again in the morning and allowed to pattern at room temperature before recording the titre.

RESULTS

TANNIC ACID Concentrations of tannic acid used were 1:20,000, 40,000, 60,000, 80,000, and 100,000. One volume of cells plus 1 volume of each tannic acid dilution was reacted at 4°C ; one-fourth of the volume was removed at 20, 30, 45, and 60 minutes. Cells were washed and sensitized and then reacted with a reference serum with a titre of 1:6,500. Parallel experiments were set up, keeping all

conditions constant except that tannic acid dilutions were reacted at 37°C instead of at 4°C . Results for cells processed at 4°C are presented in Table I.

TABLE I

EFFECT OF CONCENTRATION AND TIME OF EXPOSURE TO TANNIC ACID ON SENSITIZATION OF SHEEP ERYTHROCYTES USED IN THE HAEMAGGLUTINATION TEST FOR AMOEBIASIS AS REFLECTED BY ANTIBODY TITRE

Time of Exposure to Tannic Acid (min)	Reaction ¹	Concentration of Tannic Acid (Reciprocal)				
		20,000	40,000	60,000	80,000	100,000
20	SE + S	2,000	6,500	6,500	all ++	2,000
	SE + Dil	—	—	—	++	—
	TE + Dil	±	—	—	++	±
30	SE + S	2,000	6,500	6,500	±	2,000
	SE + Dil	—	—	—	±	—
	TE + Dil	±	—	—	±	±
45	SE + S	2,000	6,500	6,500	2,000	6,500
	SE + Dil	—	—	—	—	—
	TE + Dil	—	—	—	—	±
60	SE + S	2,000	2,000	6,500	6,500	6,500
	SE + Dil	—	—	—	—	—
	TE + Dil	—	—	—	±	±

¹SE + S = sensitized erythrocytes + reference serum

SE + Dil = sensitized erythrocytes + diluent (1% agamma rabbit serum-7.2 phosphate-buffered saline)

TE + Dil = tanned erythrocytes + diluent (1% agamma rabbit serum-7.2 phosphate-buffered saline)

Cells tanned for 20 and 30 minutes with 1:20,000, 80,000 and 100,000 dilutions of tannic acid showed some spontaneous clumping and were very difficult to process. Dilutions of 1:80,000 and 1:100,000 consistently showed some clumping after tanning. Dilutions of tannic acid of 1:40,000 and 1:60,000 consistently gave good results. Increasing the time of exposure to tannic acid over 20 minutes did not improve results. With reference to the temperature for tanning there was no difference when cells were tanned at 4°C or at 37°C . The titres of the test serum were similar. However, the patterns of cells tanned at 4°C were easier to read than those tanned at 37°C ; the positive and negative patterns were more distinct. Optimum conditions were found to be 1:60,000 dilution of tannic acid, tanned at 4°C for 20 minutes with constant gentle rotation.

SENSITIZATION All factors of the test were kept constant with the exception that the time and temperature of sensitization of tanned cells were varied from 37°C to room temperature. When cells were tanned with a 1:60,000 dilution of tannic acid (for 20 minutes, at 4°C), the optimal temperature of sensitization was found to be 37°C . At this temperature titres were reproducible on subsequent days and titres were higher than they were when cells were

⁶Linbro Chemical Company U-shaped, wells of 0.3 ml capacity.

sensitized at room temperature. Concerning the time of sensitization, the time was varied from 15 to 45 minutes and it was found that the optimum time of sensitization was 20 minutes. When cells were sensitized for a longer period of time, the antibody titres of reference sera decreased.

AGE OF SHEEP CELLS All factors in the test were kept constant. The time of storage of the sheep cells (age of cells) used in the test was the only variable. Table II gives the antibody titres obtained with erythrocytes stored under sterile conditions for varying periods of time. Antibody titres were lower when cells older than 20 days were used. A further decrease in titre was noted at 100 days.

TABLE II

EFFECT OF TIME OF STORAGE OF SHEEP ERYTHROCYTES USED IN THE HAEMAGGLUTINATION TEST

Age of Cells (days)	Reference Serum Titre		
	1:320	1:640	1:5,120
10	320	640	5,120
20	320	640	5,120
30	160	320	5,120
40	160	160	5,120
50	160		5,120
60	160		5,120
70	160		5,120
80	160		
90	160		2,560
100	80		2,560
110	80		2,560

Two-fold serum dilutions used.

TABLE III

HAEMAGGLUTINATION TITRES OBTAINED IN THE MICRO-TITRE SYSTEM USING THE SAME VOLUME (0.025 ml) BUT DIFFERENT CONCENTRATIONS OF SENSITIZED ERYTHROCYTES WITH SERA FROM PATIENTS WITH AMOEBIASIS

Serum Sample	Concentration of Sensitized Cells	
	1%	0.5%
1	729	6 500
2	2,000	19,700
3	59,000	177,000
4	2,000	6,500
5	59,000	532,000
6	177,000	532,000
7	19,700	177,000
8	59,000	177,000
9	729	6,500
10	2,000	6,500

Three-fold serum dilutions used.

EFFECT OF NUMBER OF CELLS ON TITRE Table III presents titres obtained with two cell suspensions, 1% and 0.5%, with 10 serum samples chosen at

random. Results demonstrate that the number of cells is important in replicating antibody titres. The titre is higher when fewer cells are used. The agglutination pattern was easier to read and replication of titres was more consistent when a 0.5% suspension was used.

OPTIMUM CONDITIONS These have been found to be as follows:

Sheep cells These should be stored at 4°C for not longer than 20 days.

Solutions All solutions should be prepared with glass-distilled or demineralized water.

Tanning A 1:60,000 tannic acid dilution, 20 minutes at 4°C with gentle rotation: both tannic acid dilution and cell suspension were precooled to 4°C before combination.

Sensitization A cell suspension of 2.5% in phosphate-buffered saline, pH 6.4, with an equal volume of antigen, gently rotated at 37°C for 20 minutes. Wash sensitized cells in 1% agamma rabbit serum (absorbed with sheep cells) and use the same 1% agamma rabbit serum as a stabilizer.

Microtitre system Each well contained 0.05 ml serum or serum dilution, in 0.01 ml of 1% agamma rabbit serum-phosphate-buffered saline and 0.025 ml of a 0.5% suspension of sensitized cells. Plates are sealed with transparent gummed tape, rotated gently on a serological rotator for half an hour, allowed to remain at room temperature for one to two hours and put into the refrigerator overnight. Plates were shaken again in the morning, allowed to repattern at room temperature, and the titres were recorded.

Sera were tested from persons living in Durban, South Africa, where amoebic disease is endemic, with the following results:

Diagnosis	No. of Persons	Percentage Positive	Percentage Negative
Amoebiasis	200	84	16
Cyst passers	75	9	91
Uninfected	117	7	93

COMMENT

In order to replicate results in the haemagglutination test all of the steps involved must be carefully regulated. Concentration of reagents, time and temperature of tanning, and sensitization were extremely important and variation in any one of these might result in a decrease in antibody titre observed. One of the most important factors is the type of distilled water used. Only glass-distilled or demineralized water was suitable. Other types of distilled water

(metal still or reionized water) resulted in spontaneous agglutination of cells after sensitization.

The number of cells used with each serum dilution was also very important in replication of results. Optimal concentration of cells was 0.5% (v/w). More cells per well produced a pattern more difficult to read and the titres were often lower than those obtained for a 0.5% suspension. The use of fewer cells produced patterns that were difficult to read.

Standardizing all the factors described here produced a sensitive test in which titres were reproducible within one dilution.

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