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INDIRECT HAEMAGGLUTINATION TEST FOR CHAGAS' DISEASE, WITH A SIMPLE METHOD FOR SURVEY WORK

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SUMMARY

A detailed description is given of an indirect haemagglutination test using sheep red blood cells for detection of antibodies to *Trypanosoma cruzi.* Particular attention was given to the preparation of a standardized stable, water soluble antigen from epimastigotes of *T. cruzi in vitro.*

A series of sera from patients from Brazil and Argentina with chronic Chagas disease were tested and gave titres varying from 1/8,000 to 1/512,000. The titre did not vary when samples of sera were taken over a period of 42 and 20 weeks from two patients.

No cross-reactions were observed with sera from patients suffering from amoebic dysentery, tuberculosis, syphilis, malaria (*Plasmodium falciparum*) and leprosy. A cross-reaction was seen with sera from patients with leishmaniasis.

The indirect haemagglutination technique was adapted for use with finger prick blood dried on filter paper. Antibodies were extracted from the dried blood and gave positive reactions. Using dried blood, positive reactions were obtained from material collected in Venezuela, Peru, Paraguay and Colombia.

INTRODUCTION

During studies of serum antibodies in dog and guinea-pig infections with *Trypanosoma cruzi* using the complement fixation test, it was found that the development of anticomplementary properties by serum hindered the progress of experiments. To avoid drawback, the indirect haemagglutination test was developed following techniques described for *Trypanosoma evansi* (GILL [7]). Since this serological method proved simple to use, its value as a diagnostic test was explored by testing the antigen against sera from patients with diseases other than Chagas' disease. A preliminary note of our result was published earlier (NEAL & MILES [11]).

MATERIAL, METHODS AND PRELIMINARY EXPERIMENTS

The procedure described by GILL [7] for the study of sera from *T. evansi* infections was used as the basic procedure. The main modification of this technique was the preparation of antigen. The details of the methods including preparation of the antigen are as follows:

Buffered salines — 0.15 M phosphate buffers were prepared at pH 7.1 to 7.2 and pH 6.4. For use in the experiments, the phosphate buffers were diluted ten-fold with 0.9% (w/v) sodium chloride solution. All water was double glass distilled. All pH values were checked before use.

Sheep red blood cells — Sheep red blood cells were obtained suspended in Alsevers

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solution saline. The cells were washed three times with pH 7.1 phosphate buffered saline (PBS) and a 10% v/v suspension was prepared in pH 7.1 PBS. The suspension was stored at 4°C for five days during use in haemagglutination experiments, then discarded. Another 10% suspension was then prepared from a fresh supply of sheep red blood cells. On only a few occasions was spontaneous lysis of red blood cells observed, when detected that sample of red blood cells was discarded.

Tannic acid — A stock solution of tannic acid (Mallinckrodt tannic acid, analytical reagent grade was the only sample used) was prepared in distilled water at a concentration of either 1.0 or 3.3 mg/ml. It was stored at 4°C until the stock solution was used or showed signs of deterioration. The deterioration showed either as a deepening brown colour or as a granular precipitate. The deterioration was often observed if the solution had been stored for three to six months at 4°C. Before use, the stock tannic acid solution was diluted appropriately with PBS pH 7.1.

Cultures of Trypanosoma cruzi — Antigen was prepared in various ways from either of two isolates of *T. cruzi*. The majority of the work has been carried out with isolate *Y* (SILVA & NUSSENZWEIG [12]), but a few studies have utilised isolate *Sonya* (GARNHAM [6]). Unless otherwise stated, isolate *Y* was used. Epimastigotes (nomenclature of HOARE & WALLACE [8]) of both isolates were grown *in vitro* in various types of liquid culture media. The medium employed was a peptone-glucose-yeast or liver extract-haemin-serum mixture. The composition was developed during this sorological work (NEAL & MACLAREN, umpublished and BONÉ & STEINERT [3]). Latterly, the liquid media described by BONÉ & PARENT[2] has been used with equal success, either the undefined medium serum or the semidefined medium (T2). *T. cruzi* was grown in bulk using 1 or 2 litres of medium and harvested after seven to ten days growth at 26°C.

The flagellates were washed three times with PBS pH 7.1. The flagellate suspension was used for preparation of antigen.

Preparation of sensitized cells — A 1% suspension of sheep red cells was prepared from the stock suspension of 10% red blood cells. An appropriate volume of the 1% suspension was mixed with an equal volume of the tannic acid solution and incubated for 15 minutes at 37°C. After washing once with buffered saline (pH 7.1), the tanned red cells were centrifuged and the red cell sediment was mixed with the antigen preparation in buffer saline (pH 6.4) and left at room temperature (21 — 23°C) for 15 minutes. The proportions of antigen to tanned red cell suspension were 0.1 ml of antigen for each 0.1 ml of 1% tanned red cell suspension.

The sensitized red cells were then washed three times with buffered saline (pH 7.1) containing 0.4% rabbit serum. They were finally resuspended in buffered saline + rabbit serum to give a 1% red cell suspension.

Sera — All sera were stored at -20°C. They were decomplemented by heating at 56°C for 30 minutes. To remove possible non-specific haemagglutination, all sera were absorbed by adding serum to packed normal sheep red cells in the proportion 0.1 ml of serum to the packed red cells from 0.1 ml of 10% red cell suspension. After absorption overnight at 4°C, 4.9 ml of PBS containing 1.0% rabbit serum were added and the red cell suspension centrifuged. The supernatant was taken as the 1/50 dilution and further dilutions prepared as required with PBS + 1% rabbit serum. The 1/50 absorbed dilution was kept for a limited period at -20°C.

The indirect haemagglutination test

The test was performed in tubes with a uniform round bottom, of the size 81 x 9 mm. The serum dilution was added, 1.0 ml per tube, followed by 0.1 ml of the sensitized red cell suspension. The tubes were inverted twice to ensure complete mixing and then left overnight to allow the cells to settle. The settling patterns were examined the next morning.

Interpretation of results

The degree of agglutination was recorded in a similar manner to that used by GILL [7]. The endpoint was represented by the highest serum dilution to give a ++ reaction, that is a smooth mat of cells with the first sign of a thickened ring at the periphery of the hemisphere at the bottom of the tube.

In every experiment, normal red blood cells were included to act as a control for non-

specific agglutination of the sera under test. The cells were treated in an identical manner to the antigen control cells except that antigen was omitted during incubation with pH 6.4 PBS. As non-specific agglutination was never observed, the results of these control tubes are not included in the description of experiments given below.

Use of blood dried on filter paper

Since the haemagglutination test was very sensitive, attempts were made to use blood dried on filter paper. Using guinea-pig and rabbit blood, it was calculated from measurements of weight of dried blood or from the known volume and the area of spread on the filter paper, that the dilution of serum eluted from a disc punched from the blood blot was approximately equivalent to between 1/17 to 1/400. The wide variation was due to the amount of spreading which occurred with the blood sample. Since it was found that haemagglutinating antibodies were eluted from a dried drop of positive serum, attempts were made to standardize a suitable technique.

The technique finally adopted was as follows. Blood was dropped onto Whatman no. 1 filter paper and allowed to dry. Details of the origin of blood were written in pencil on the paper. The area of the blood blot was such that two discs measuring 6 mm in diameter could be punched from the blot. Discs were punched using a standard office punch (Velos "Easy-punch" no. 4363). Each disc was placed in a test-tube and 1.0 ml of PBS + 1% rabbit serum added. The tube was kept overnight at 4°C to extract the serum. Next morning, the discs of filter paper were removed and the extract heated at 56°C for 30 minutes to remove complement. After centrifuging to remove particles, the supernatant was used as the serum dilution. To the two extracts, either 0.1 ml of sensitized tanned red cells, or 0.1 ml of tanned cells to act as control red cells, was added.

For transport of blood blots from the South American survey, the finger prick blood was blotted on to Whatman no. 1 filter paper circles, 4.25 cm diameter. After drying, they were stored in tins containing silica gel. On arrival in the laboratory, they were stored at -20°C until used.

RESULTS

Before exploring the usefulness of this technique for serological investigations in experimental or human Chagas' disease, it was necessary to standardize the various factors affecting the performance of the test.

1a — *Optimum concentrations of tannic acid and antigen*

The initial experiments were carried out with the tannic acid concentration used by GILL [7] that is 33 μg/ml. However, when the method of antigen preparation had been investigated and the water-lysate technique employed as routine, the effect of varying tannic acid and antigen concentrations was reinvestigated.

A checkerboard titration was carried out with serum from a chronic Chagas' disease patient, and with antigen prepared from strain *Y*. The titre of the serum was determined with each variable. The results (Table I) show that the optimum concentration of tannic acid was 17 to 33 μg/ml, with an antigen dilution of 20 or 30 fold. Therefore the use of tannic acid in the concentration of 33 μg/ml was used in other experiments. Before a new batch of antigen was employed in routine tests, it was titrated against a standard serum to determine the optimum dilution in combination with tannic acid at the standard concentration (33 μg/ml).

1b — *Antigen preparations, potency and stability*

In the first preparation, the washed flagellates were freeze-dried in bulk. The dry powder was stored at -20°C. When required for use, a suspension was prepared at a concentration of 1 mg/ml in PBS pH 6.4. The suspension was ground by hand and the cellular debris removed by centrifugation. For use, this clear supernatant was diluted 4 times (equivalent to 250 μg dry weight/ml). This method was time consuming with variable degree of antigen extraction and did not provide sufficient antigen of known potency for many tests. Moreover, the antigen varied in potency from batch to batch. Other methods of antigen preparation from the suspension of flagellates were therefore investigated.

TABLE I

Determination of the optimum dilution of tannic acid and antigen

Tannic acid concentration µg/ml	Reciprocal of the titre (x 10^3) of serum obtained with various dilutions of the sensitizing antigen				
	10x	20x	30x	40x	0
100	64	16	8	2	0
33	> 256*	128	128	16	0
17	> 256*	128	128	64	0
1	256	64	64	32	0
0	0	0	0	0	0

* Titre was uncertain owing to false positives due to high antigen concentration

TABLE II

Influence of method of preparation of antigen on potency. A single human Chagas' serum was used as antiserum

Treatment of cells	Method of rupturing cells	Method of preserving cell extract	Optimum dilution
Killed with formalin	lysed in distilled water	Frozen Freeze-dried	40x 40x
	Sonic rupture	Frozen Freeze-dried	100x 100x
Living flagellates	Lysed in distilled water	Frozen Freeze-dried	40x 60x
	Sonic rupture	Frozen Freeze-dried	80 to 100x 100x

The effect of different methods of preparation is shown in Table II. The results show that an active cell extract was obtained from *T. cruzi* cells after rupture by ultrasonic oscillations or by lysis in distilled water, and that the antigenic potency of the cell extracts was not changed after freeze-drying. Potent antigen was prepared from *T. cruzi* which had been killed with formalin (0.1% v/v formalin overnight at 4°C). Although the most potent extract was obtained after ultrasonic oscillation, it was more convenient to prepare antigen from a *T. cruzi* epimastigote suspension by killing the organisms with distilled water. This was the method adopted as routine.

The standard method for antigen preparation, involved harvesting the *T. cruzi* flagellates from a 7 to 10 day culture. The pellet of *T. cruzi* cells was then washed three times with PBS, pH 7.1. The suspension was adjusted to contain 5 x 10^8 flagellates/ml. The cells were then sedimented by centrifugation and distilled water was added, equal in volume to the original 5 x 10^8 cells/ml suspension. The distilled water suspension was left at 4°C overnight (about 16-18 hrs.). Next morning the suspension was centrifuged to remove all particulate material. The clear supernatant was ampouled in 0.125 ml amounts and freeze-dried. The whole technique

including centrifugation was carried out at 0-4°C. The powder after freeze-drying constituted the antigen. It dissolves instantly on the addition of PBS pH 6.4 to give a clear solution. The optimum dilution of antigen was obtained by dissolving contents of each ampoule in the appropriate volume of PBS pH 6.4 (usually 20 or 30 ml). Nitrogen and protein determinations of two batches of antigen (nos. 300 and 301) gave 312 μg nitrogen/ml for both batches and 1664 and 1728 μg protein/ml for each batch respectively. Treatment of the T. *cruzi* flagellates with distilled water effectively killed all flagellates, and no viable organisms were demonstrated by subculturing in a suitable medium. The ampoules of dried antigen were stored at -20°C until used.

The antigen was stable and retained its potency for prolonged periods at -20°C. Freeze-dried whole flagellates were used for 27 months, while the water lysate antigen was used for 17 months. In each case the batch of antigen was used up, before its potency had declined.

1c — *Comparison of antigen from isolates* Y *with isolate* Sonya

The titre of one serum with antigen prepared from isolate *Y* was compared with that prepared from isolate *Sonya*. The reciprocal of the titre was 64,000 with antigen from isolate *Y* and 128,000 from isolate *Sonya*.

2 — *Titres observed with sera from patients with chronic Chagas' disease*

A series of sera from patients suffering from chronic Chagas' disease were tested by the haemagglutination test. The reciprocal of titres (Table III) usually varied between 8,000 and 32,000, but was occasionally up to 256,000. Titres of sera taken at different times from two patients were found to be constant, in one patient for a period of 42 weeks (Table IV).

A series of five sera from Brazil were tested at low and high dilution. The reaction was highly positive at the lowest dilutions, starting at 1/50. At low dilutions, the mat of agglutinated cells was usually crumpled, as though whole segments of the mat had slipped, but this appearance could be readily distinguished from a negative reaction.

TABLE III

Titres of sera from different patients with chronic Chagas' disease

Source of sera (number)		Reciprocal of titre x 10^3
Argentina series	1	8
	2	32
	3	4
	4	16
	5	16
	6	512
Brazil series (1)	1	8
	2	8
	3	16
	4	8
	5	8
	6	64
Brazil series (2)	1	16
	2	16
	3	16
	4	64
	5	64
	6	16

TABLE IV

Lack of variation of haemagglutination titre of sera taken from two Brazilian patients with chronic Chagas' disease

Patient	Time after first sample weeks	Reciprocal of titre x 10^3
MAO	0	64
	3	
	5	
	20	
	30	
	42	
VMS	0	64
	20	

3 — *Cross-reaction of* T. cruzi *antigen with sera from patients with diseases other than Chagas' disease*

The haemagglutination test was performed with a series of sera which were available from other sources. These sera from diseases listed in Table V, were all negative.

TABLE V

Negative sera from patients with diseases other than Chagas' disease

Disease	Number of sera tested
Amoebic dysentery	5
Tuberculosis	9
Syphilis	5
Malaria (*Plasmodium falciparum*)	24
Leprosy	2

An exception was observed with sera from patients with leishmaniasis (Table VI). Cross reactions were observed with sera from Kala-azar patients whom contracted the disease in Sudan and Iraq. A positive reaction was also observed with sera from a patient with Kala-azar in Brazil and with sera from two Costa Rican patients with cutaneous leishmaniasis.

4 — *Use of dried blood blots on filter paper*

The results of indirect haemagglutination tests on extracts of dried guinea-pig blood are shown in Table VII. The guinea-pigs were infected with *Trypanosoma cruzi* and at the time of preparation of blood blots, were ne-

TABLE VI

Cross reaction of sera from patients suffering from leishmaniasis, with *T. cruzi* antigen

Source of serum	no. tested	Titre of sera
Kala-azar (Sudan)	2	a) 100 b) Negative
Kala-azar (Aden)	1	Negative
Kala-azar (Brazil)	1	1000
Cutaneous leishmaniasis (Costa Rica)	2	a) 2000 b) 2000
Infantile Kala-azar (Baghdad)	5	a) Negative b) 1000 c) Negative d) Negative e) Negative

TABLE VII

Use of dried blood on filter paper for haemagglutination tests for *T. cruzi* antibodies

Origin of blood, no. of guinea-pig	Titre of serum x 10^3	Results of test, weeks of storage of filter paper			
		1	11	13	22
3/11	2	++	—	0	—
3/13	16	+++	—	++	+++
3/14	4	+++	+++	++	+

0 = negative
— = not tested

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gative microscopically for trypanosomes. Extracts made after storage for 1 week at about 20-23°C, were all positive. The dried blood extracts from one guinea-pig (no. 3/11) was negative when next tested at 13 weeks whereas the extracts from the other two guinea-pigs were still positive after the blood blots had been stored for 22 weeks.

A further study with this technique was carried out with the assistance of Dr. W. E. Ormerod. During a tour of South America, samples of dried blood were prepared from finger prick blood from different individuals. These samples were tested at Beckenham approximately 5 months later. It was found that out of the 56 blood blots tested, 43 were positive for *T. cruzi* antibodies (Table VIII).

TABLE VIII

Results of dried blood extracts from South America

Geographical Source	Results of haemagglutination test		
	Positive	Doubtful	Negative
Valencia, Venezuela	19	0	0
Arequipa, Peru	3	2	3
Paraguay	4	0	0
Tibu, Colombia	15	2	9

The geographical source of these materials is given in Table VIII. Control tests on normal filter paper and on the extracting fluid were negative.

DISCUSSION

This work has led to the development of a stable antigen which is simple to prepare and to its use in a simple routine technique. Its high sensitivity makes it suitable for use in work where a quantitative response is required. The technique is similar in principle to that described by CERISOLA et al. [5], KNIERIM & SAAVEDRA [9] and other workers.

The water lysate antigen has also been used for the complement fixation test, according to the technique of BRADSTREET & TAYLOR (1962). Sera were positive by this technique giving titres up to 1/1000. Detailed comparison with results obtained by the complement fixation technique have not been included in this paper since such comparisons have been made by MONTAÑO & UCROS [10]. However, our results confirm these workers conclusions in that all sera were positive by both techniques, but with the haemagglutination test, the titres were at a greater dilution.

No cross reactions were observed with sera from patients with other diseases except leishmaniasis. While it may be argued that dual infections with *Leishmania* and *T. cruzi* were responsible for cross reactions with sera from Brazil and Costa Rica, this cannot be the case with sera obtained from Syria and the Sudan. The latter sera were from patients with visceral leishmaniasis. These studies make it clear that *T. cruzi* antigen can react with leishmaniasis, though with a lower titre than that observed with Chagas' sera. These observations add to those of ARAUJO & MAYRINK [1] and CAMARGO & REBONATO [4] using the immunofluorescent test, who found that cross reactions do occur between *T. cruzi* and *Leishmania* antigen with sera from patients with Chagas' disease and leishmaniasis.

The high sensitivity of the haemagglutination technique enables the detection of small amounts of antibody such as those likely to be eluted from dried blood. Since the antibody in dried blood appears stable when kept dry for several months, the technique seems ideally suited for survey work. SOUZA & CAMARGO [13] have shown that the immunofluorescent technique will also detect *T. cruzi* antibody extracted from dried blood.

All the dried blood samples in the present experiments were obtained from areas where infections with *T. cruzi* occurred. It was not surprising therefore to find a high percentage of positives ranging from 38 to 100%.

It is encouraging that antigen prepared from a single isolate of *T. cruzi* originating from São Paulo, Brazil, gave positive reactions with sera or blood from other parts of Latin America, including Brazil, Argentina, Venezuela, Peru, Paraguay and Colombia.

RESUMO

Teste de hemaglutinação indireta para a doença de Chagas, como método simples de inquérito sorológico

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O trabalho apresenta uma descrição detalhada de um teste de hemaglutinação indireta, usando glóbulos vermelhos de carneiro, para a dosagem de anticorpos do *Trypanosoma cruzi*. Foi dada atenção especial ao preparo de um antígeno padronizado estável, hidrossolúvel, a partir de formas epimastigóticas do *T. cruzi in vitro*.

Uma série de soros de pacientes do Brasil e Argentina com doença de Chagas crônica foi testada e a titulagem obtida variou de 1/8.000 a 1/512.000. O título não variou com amostras de sôro tomadas durante um período de 20 e 42 semanas, de dois pacientes.

Não foram observadas reações cruzadas com soros de pacientes portadores de disenteria amebiana, tuberculose, sífilis, malária (*Plasmodium falciparum*) e lepra. Foi observada reação cruzada com sôro de pacientes portadores de leishmaniose.

A técnica de hemaglutinação indireta foi adaptada para ser usada com sangue obtido pela picada da polpa digital e sêco em papel de filtro. Os anticorpos foram extraídos do sangue dessecado e deram reações positivas. Usando sangue sêco, foram obtidas reações positivas de material coletado na Venezuela, Peru, Paraguai e Colombia.

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