Rev. Inst. Med. trop. São Paulo
8(5):227-234, setembro-outubro, 1966

UDC 616.937.3

FLUORESCENT ANTIBODY TEST FOR THE SERODIAGNOSIS OF AMERICAN TRYPANOSOMIASIS. TECHNICAL MODIFICATION EMPLOYING PRESERVED CULTURE FORMS OF *TRYPANOSOMA CRUZI* IN A SLIDE TEST

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SUMMARY

A few modifications introduced in the indirect fluorescent antibody test made it a practical technique for the routine serological diagnosis of American trypanosomiasis. *Trypanosoma cruzi* culture forms were kept as a stable antigen and tests were performed on microscope slides. By running twenty reactions on each slide reading of results was much facilitated and numerous tests could be made at the same time. Comparative evaluation between fluorescent and complement fixation tests in more than a thousand sera indicated a close agreement of results. *Trypanosoma cruzi* culture and blood forms were also compared as antigens in the fluorescent test, and no divergences as to reactivity and non-reactivity were observed, although for most reactive sera tested, higher titers were obtained with blood forms.

To study specificity of the fluorescent test sera from patients with different diseases are being submitted to it and initial results are reported.

INTRODUCTION

An increasing variety of routine serological work can be simplified through the use of immunofluorescent techniques. Examples are tests for *Treponema* [10, 13], *Toxoplasma* [5, 6] or *Schistosoma* [7, 19] antibodies.

In the serodiagnosis of infections produced by haemoflagellates, fluorescent tests have also been reported, both for trypanosomiasis [3, 12, 20] and for leishmaniasis, visceral or cutaneous [11, 17, 22]. However, before such tests can be recommended for general application, their sensitivity and specificity must be thoroughly evaluated. Stable, practical antigens must also be devised and standardized so as to make the tests easy enough to perform in routine work. In this sense, culture forms of haemoflagellates are to be preferred to "vertebrate" forms as antigens. Among such evolutive forms antigenic dissimilarities certainly exist although so far insufficiently determined [23]. Culture forms of leishmania have been successfully employed as antigens in fluorescent tests for Kala-azar [11, 22]. However in fluorescent tests for trypanosomiasis preference has been given to "vertebrate" forms of trypanosomes.

FIFE & MUSCHEL [12] used *T. cruzi* culture forms in a fluorescent test for Chagas disease, but as non-specific staining resulted from fixing this antigen on microscope slides, reactions had to be run in test-tubes. This is not a very practical solution for routine work since it involves much added work and a larger consumption of conjugates. WILLIAMS et al. [25] employed a fluorescent test to detect antibodies to *T. rhodesiense* and *T. gambiense* in experimentally infected animals. As antigens they used

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both blood and culture forms of these trypanosomes, fixed on microscope slides. Higher sensitivity was reported for blood forms and only these were employed in subsequent fluorescent tests for the diagnosis of trypanosomiasis in humans. BIAGI et al.[3] in a fluorescent test for Chagas' disease employed as antigen tissue forms of *T. cruzi* in formalin-fixed, paraffin-embedded myocardium from infected rats. Tests were made on deparaffinized sections on slides and this antigen was reported to keep its activity for at least one year at room temperature. Since slide-fixed culture forms of trypanosomes have been successfully employed in fluorescence studies as referred by VOLLER[24] and SHAW & VOLLER[21], we have turned to such forms when looking for a practical antigen for a routine test. This paper presents technical details of the test as performed in our laboratory as well as comparative results between fluorescent and complement fixation tests in a routine serology. A comparison was also made between culture and blood forms as antigens in fluorescent tests.

Specificity of the fluorescent test is being evaluated in sera from patients with diseases other than trypanosomiasis and results already obtained are presented.

MATERIAL AND METHODS

Sera — One thousand and seventy six sera received for *T. cruzi* complement fixation tests by three different laboratories were later submitted to our laboratory for the immunofluorescent test. Sera were inactivated for 30 minutes at 56°C prior to complement fixation but not reinactivated before fluorescent tests. In the few days between reactions sera were kept frozen at −20°C. Sera from patients presenting other diseases as Kala-azar, cutaneous or mucocutaneous leishmaniasis, toxoplasmosis, syphilis, systemic lupus erythematosus and rheumatic fever were also tested. Blood from cases of tuberculosis, lepromatous leprosy and *mansoni* schistosomiasis collected by finger puncture on filter paper and later eluted in saline solution, as described in a following paper[15] was also submitted to the fluorescent test. Blood smear eluates and most such sera were not inactivated. In order to avoid non-specific fluorescent staining occasionally observed with undiluted sera, dilutions of 1:10 in buffered saline (NaCl 0.15 M; phosphates 0.01 M; pH 7.2) were used. When titrated, sera were diluted threefold from 1:10 on.

Antigens — A suspension of formolized culture forms of *T. cruzi* was used as stock antigen. To prepare this suspension a few milliliters of a three to four days culture of strain Y of *T. cruzi* in Yaeger liquid medium[46], incubated at 28°C and containing about 100 million parasites per milliliter, were centrifuged, the sediment washed twice in buffered saline solution and resuspended in about the initial culture volume of 2 per cent formalin in buffered saline solution. After about 24 hours at room temperature this suspension was transferred to the refrigerator and could be used as a stock antigen for about three months without any observed decrease in antigenic activity. Before using, it was daily diluted in saline solution so as to furnish 5 to 10 parasites per microscopic field in final preparations. A very practical and stable antigen was obtained by freeze-drying formol-fixed trypanosomes. The described stock antigen was centrifuged and the sediment resuspended in about the original volume of 6 per cent dextran in saline solution *. Volumes of 0.5 or 1.0 ml were freeze-dried in ampoules. Even after one year's storage at room temperature, freeze-dried trypanosomes presented unimpaired morphological and antigenic characteristics when reconstituded by adding distilled water to the ampoules. When 1 per cent formalin is distilled water was used to rehydrated antigens resulting suspensions could be kept for three to four months in a refrigerator without evidence of diminished antigenic activity.

In the tests microscope slides were used, on which an area corresponding to a 25 × 40 mm coverslip was marked and divided into twenty 6 × 8 mm areas by means of nail polish traces, in a similar way previously described[4]. A drop of the diluted

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antigen was laid on each such small area, and immediately removed with the help of a hypodermic needle without bevel attached to a pipette and rubber bulb, only a thin film being left. This could be accomplished when perfectly clean slides were used and best results were obtained when, after marking areas on already clean slides, their surfaces were quickly flamed over a burner and rubbed with tissue paper. Drying slides for 30 minutes at about 50°C in an oven or under an infrared lamp and ventilator, plus gentle heating over a flame for a few seconds proved a sufficient procedure to fix parasites. Shorter drying periods or imperfectly clean slides could result in insufficient fixation of smears.

For reactions employing blood forms of *T. cruzi* as antigen, citrated blood from infected mice obtained by heart puncture 4 to 6 days after inoculation was used. Smears on slides were dried for a few minutes at room temperature and divided into small areas by nail polish traces, as indicated above. Slides were kept at −20°C for one month with no signs of diminished antigenic activity. Fixing smears with acids or organic solvents was not necessary.

Anti-human globulin conjugates — Hyperimmune sera were obtained by injecting rabbits with human gamma-globulin in complete Freund's adjuvant. A dyalisis technique slightly modified from CLARK & SHEPARD [9] was used to conjugate fluorescein isothiocyanate * to several batches of rabbit antibodies. Resulting conjugates had F/P ratios (in milligrams per milliliter) varying from 5 to about 8×10^{-3}, specific staining in dilutions up to 1/100 or 1/500 and non specific staining only at very low dilutions (under 1/5) for the employed antigens.

Evans blue as a counterstain was incorporated at 0.1 mg/ml to conjugate dilutions, as modified from NICHOLS & MCCOMB [16].

Immunofluorescent reactions — Diluted sera or eluates of blood smears were pipetted (about 0.005 ml) on the slides, each one being alloted to a different antigenic area, so that twenty tests were run per microscope slide. Slides were then incubated for one hour at 37°C in a wet chamber, washed in two changes of buffered saline solution ten minutes each and dried for a few minutes under a ventilator, blotting with paper being avoided. Conjugate diluted to titer was then pipetted on each area and the slides incubated again for one hour at 37°C, twice washed in buffered saline solution for five minutes and mounted with coverslip and buffered glycerine (pH 8). Reactions were read under a 40 × dry objective binocular microscope, provided with dark-field, HBO200W as the light source, BG12 and 50 (ZEISS) as exciter and barrier filters. For reactive sera culture forms of parasites are seen as red counterstained structures sharply outlined by a peripheric green fluorescence. For non-reactive sera, parasites remain as dim red spots which in occasional reactions may show irregular patches of weak inner fluorescence. Evans blue counterstaining which accounts for the red hue in parasites is a real help in minimizing such non-specific fluorescent reactions and to make specific ones more clearcut. Trypanosome blood forms do not generally show any degree of non-specific staining. For reactive sera these are seen as thoroughly fluorescent structures eventually rimmed in strong reactions by a more intense fluorescent line.

Occasional doubtful reactions were reported as negative.

RESULTS

a) *Comparison between fluorescent and complent fixation tests* — Sera were divided into two groups according to the complement fixation technique used. Group I comprised 542 sera submitted to a 50 per cent hemolysis fixation technique employing a benzene-treated *T. cruzi* antigen, as described by PEDREIRA DE FREITAS [18]. As shown in Table I, 48 reactive sera in the complement fixation test were also reactive in the fluorescent test. The remaining 494 sera were non-reactive in both tests. No clinical data are available except for twenty reactive sera from American trypanosomiasis patients from whom *T. cruzi* had been isolated.

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TABLE I

Group I sera distributed as to results of complement fixation (benzene-treated antigen) and fluorescent tests

Fluorescent test	Complement fixation test	
	Non-reactive	Reactive
Non-reactive	494	0
Reactive	0	48

Group II comprised 534 sera submitted to a Kolmer complement fixation test with a methanol-extracted *T. cruzi* antigen. In this group 349 sera were non-reactive in the fluorescent test and non-reactive also in the complement fixation test, except for two anticomplementary sera. The remaining 140 sera were reactive in the fluorescent test, but in the complement fixation test only 90 were reactive, 8 anticomplementary and 42 non-reactive. However, when these 42 sera were tested again with a benzene-treated antigen, positive complement fixation results were found for 40, only two sera proving non-reactive (Table II).

TABLE II

Group II sera distributed as to results of complement fixation (with methanol-extracted and/or benzene treated antigen) and fluorescent tests

Fluorescent test	Complement fixation test			Total
	Non-reactive	Reactive	Anticomplementary	
Non-reactive	392	0	2	394
Reactive	2	130	8	140
Total	394	130	10	534

b) *Comparison between culture and blood forms of* T. cruzi *as antigens in the fluorescent test* — Identical results as to reactivity were found in 632 sera submitted to fluorescent tests employing culture or blood forms as antigen. The same 65 sera were reactive in both tests, and 567 sera were non-reactive. However, when titrating 57 reactive sera, in most of them higher titers were found for blood forms than for culture forms, as shown in Table III. The twenty sera referred, from known cases of Chagas' disease, are here included.

TABLE III

Sera distributed according to titers in fluorescent tests employing either culture or blood forms of *Trypanosoma cruzi*

Titers with culture forms antigen	Titers with blood smear antigen						Total
	1/10	1/30	1/90	1/270	1/810	> 1/810	
1/10	—	—	1	1	—	—	2
1/30	—	3	5	6	1	—	15
1/90	—	—	7	8	13	—	28
1/270	—	—	—	4	5	2	11
1/810	—	—	—	—	1	—	1
> 1/810	—	—	—	—	—	—	—
Total	—	3	13	19	20	2	57

c) *Results of the* T. cruzi *fluorescent test in other diseases* — This will be the subject of a later publication and only initial results are here reported.

Sera from 24 cases of Kala-azar were tested and 10 were found reactive, although only in low titers (1/10). Among the remaining 14 sera reported as negative, four doubtful reactions were found.

Five cases of muco-cutaneous leishmaniasis tested gave positive results, titers ranging from 1/10 to 1/30. All five had also been reactive in complement fixation tests with *T. cruzi* antigen.

Eighty-three eluates of blood smears in filter paper from cases of Schistosomiasis, strongly reactive in a fluorescent test with a *S. mansoni* antigen [7], were non-reactive in the *T. cruzi* fluorescent test.

Blood eluates from 54 tuberculosis patients furnished 49 negative and 5 reactive results. Sera obtained from these 5 cases resulted in positive complement fixation and fluorescent tests.

Blood eluates were obtained from 42 patients with lepromatous leprosy. Only 2 reactive and 1 weakly reactive cases were found. Complement fixation tests also resulted positive in these three sera. Although these five tuberculous and three leprous patients came from areas where American trypanosomiasis is highly endemic, cross-reactivity with antibodies for Mycobacteria remains a possibility, as indicated by ALMEIDA [1]. Negative results were found for sera from 8 cases of toxoplasmosis (titers above 1/16,000 in toxoplasma fluorescent test [5]), 9 cases of systemic lupus erythematosus (titers above 1/1,000 in fluorescent antinucleus antibodies test [2]) in 12 cases of syphilis (reagent in *T. pallidum* fluorescent test — FTA —200[10]) as well as in 24 sera from active rheumatic fever cases.

Table IV summarizes such results. Thirteen sera from megaesophagus and megacolon, frequent digestive manifestations of Chagas' disease [8, 14] were submitted to complement fixation and fluorescent tests. Five

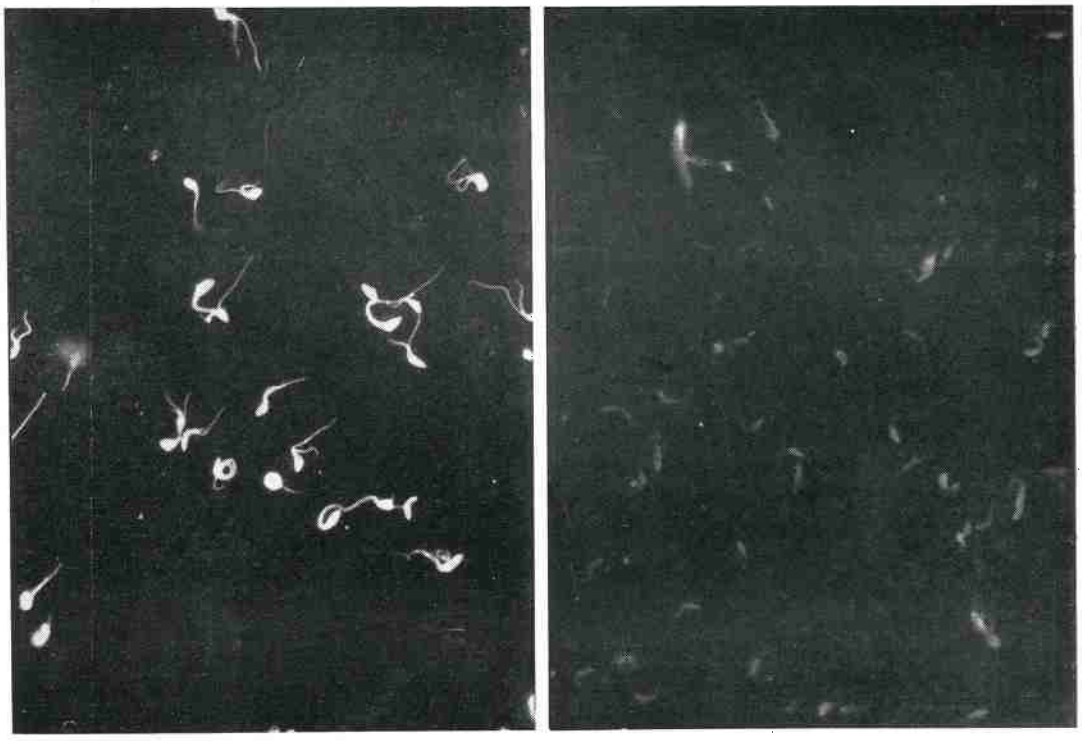

Fig. 1 — Culture forms of *T. cruzi* as seen in reactive and non-reactive fluorescent tests (Obj. 40×,oil)

were non-reactive in both tests, and from the eight sera which reacted in the fluorescent test 5 were positive and 3 negative in the complement fixation test.

TABLE IV

Results of *T. cruzi* fluorescent test in sera or filter paper blood eluates from patients affected by other diseases

Cases of	No. of cases	*T. cruzi* fluorescent tests	
		Reactive	Non-reactive
Kala-azar	24	10	10
Muco-cutaneous leishmaniasis	5	5	0
Schistosomiasis ...	83	0	83
Tuberculosis	54	5	49
Lepromatous leprosy	42	3	39
Systemic lupus erythematosus	9	0	9
Syphilis	12	0	12
Toxoplasmosis	8	0	8
Rheumatic fever .	24	0	24

DISCUSSION

Serological immunofluorescent techniques are basically very simple procedures as the direct visualization of antigen-antibody reactions dismisses such revealing phenomena as haemolysis and derived complexities. Further, as fluorescent techniques have great sensitivity and can be performed with minute volumes of sera or blood eluates from filter paper smears [15, 20] they constitute ideal procedures for large serological surveys.

However, fluorescent techniques hitherto described for Chagas' disease serodiagnosis were somewhat cumbersome when used on a large scale. So, when culture forms were employed as the antigen, this technique involved the use of test-tubes [12]. When tests were carried out on microscope slides, antigens used were not practical either to preserve (blood forms) [20] or to handle (tissue forms) [3]. By employing a stable antigen easily obtained from *T. cruzi* culture forms, we have succeeded in performing fluorescent tests on microscope slides. Since many tests were run on a single slide, microscope reading — the test's only arduous step — was much facilitated and a large number of reactions could be carried out simultaneously.

Reading and reporting results was also made easy by the marked fluorescence observed for reactive sera and the almost total absence of non-specific staining. Although formolized *T. cruzi* suspensions keep well for a few weeks in a refrigerator, the lyophilized antigen here described is more stable, morphological and antigenic characteristics of the parasites are perfectly preserved and, after reconstitution with distilled water, resulting suspensions may be used at once or kept for many days in a refrigerator. Slides then prepared may be preserved at —20°C for many months.

Initial results here reported show a close agreement between complement fixation tests using a benzene-treated *T. cruzi* antigen and the described fluorescent technique. Although somewhat higher titers are obtained when blood forms of the parasite are used as antigen, in the fluorescent test culture forms are sufficiently sensitive to detect reactivity of sera.

Although non-reactivity was the rule for sera from patients with diseases other than trypanosomiasis, some cross-reactivity was found for other haemoflagellate infections. However, only low titers (1/10 to 1/30) were found for such reactions. On the contrary, high titers were the rule for sera from cases of Chagas' disease (up to 1/810). Non-specific reactions in cases of tuberculosis and lepromatous leprosy could not be excluded, in the limited number of patients here referred. However, reactive sera were found only in patients coming from areas where Chagas' disease is prevalent. Technical modifications here described make the *T. cruzi* fluorescent antibody test a simple serological procedure for routine work, easier to perform and at least as sensitive and specific as the elaborated PEDREIRA DE FREITAS' complement fixation test [18] which, after a large use in our country, is generally recognized as a very reliable test.

CAMARGO, M. E. — Fluorescent antibody test for the serodiagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of *Trypanosoma cruzi* in a slide test. *Rev. Inst. Med. trop. São Paulo* 8:227-234, 1966.

RESUMO

Reação de imunofluorescência indireta para o diagnóstico sorológico da tripanosomíase americana. Técnica em lâminas de microscopia com formas de cultura, preservadas, de Trypanosoma cruzi

A reação de imunofluorescência para o diagnóstico sorológico da doença de Chagas mostrou-se bastante prática para a utilização em rotina, depois da introdução de algumas modificações técnicas descritas na presente publicação. Como antígeno empregaram-se formas de *T. cruzi* obtidas em culturas, fixadas e liofilizadas.

As reações foram feitas em lâminas de microscopia, o que tornou possível a realização concomitante de numerosos testes. A comparação dos resultados das reações de imunofluorescência e de fixação do complemento pela técnica de PEDREIRA DE FREITAS, em mais de 1.000 soros, mostrou estreita concordância. Não houve diferenças, entre reações de fluorescência utilizando como antígeno formas de cultura ou formas sanguícolas de *T. cruzi*, no que concerne a positividade ou negatividade dos soros. Entretanto, diante de formas sanguícolas, em geral os títulos obtidos foram mais altos do que com formas de cultura. Soros de pacientes com outras doenças, além da tripanosomíase americana, estão sendo submetidos à reação de fluorescência para avaliação de sua especificidade. Alguns resultados iniciais são aqui apresentados.

ACKNOWLEDGMENTS

Special thanks are due to Prof. Vicente Amato Neto for providing sera from known cases of Chagas' disease, to Dr. C. Salles Cunha, Mr. Gilberto V. de Siqueira and Mr. V. Salcedo Vegas, for supplying most of the sera and the complement fixation results; to Drs. L. Souza Lima, F. Gicovate and Prof. José V. Torrealba respectively for leprosy, tuberculosis and Kala-azar sera. We wish to thank Profs. J. Ferreira Fernandes and L. M. Deane for *T. cruzi* cultures and *T. cruzi* infected mice blood; also our technician Miss Cacilda Rebonato for the skillfull collaboration which made this work possible.

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Recebido para publicação em 2/6/1966.