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FURTHER EVALUATION OF THE «I.M.T.-CHAGAS FLOCCULATION TEST». A COMPARISON WITH COMPLEMENT FIXATION, HEMAGGLUTINATION AND IMMUNOFLUORESCENCE TESTS

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SUMMARY

The accuracy of a recently developed rapid test for American trypanosomiasis serodiagnostic, the "I.M.T.-Chagas flocculation test", was evaluated in 1,456 serum samples from blood donors and patients, as compared to complement fixation, hemagglutination and immunofluorescence tests, taken as reference. The high co-positivity and co-negativity ratios obtained indicated the flocculation test as a reliable serologic procedure, being very sensitive and sufficiently specific for screening purposes. Easy to perform and allowing final readings within a few minutes, the flocculation test seems very practical for identification of infected donors in blood banks. The stability of the lyophilized flocculation reagent is long-lasting and the fact that it remains stable even after reconstitution as a liquid suspension for use, makes this test very convenient for routine purposes.

INTRODUCTION

The "I.M.T.-Chagas flocculation test", recently developed in our Laboratory [4], being very sensitive and allowing final readings within a few minutes, seems very practical as a screening procedure. Besides, the lyophilized reagent is stable and can be produced in central laboratories and distributed for general use in blood banks and clinical laboratories.

In this publication we present comparative results with other serologic tests performed in serum samples from 1,132 blood donors, collected in different areas of Brazil, and in 324 samples sent to the laboratory for serologic diagnosis of American trypanosomiasis.

MATERIAL AND METHODS

I.M.T.-Chagas flocculation test

As described elsewhere [4], the flocculation test (FO) is performed on microscope slides or glass-plates. Within a circular area 1.5 cm in diameter, drawn with thickly applied nail polish so as to form a rim, one drop of heat-inactivated serum and one drop of the reconstituted reagent were mixed. After agitation for 10 minutes on a rotator (*) routinely used for the cardiolipin V.D.R.L. test, the mixture was observed for any evident agglutination by slowly tilting the plate against a dark background. Positive tests showed agglutination evaluated according to intensity from 1+ to 4+. In negative tests the mixture remained as a milky homoge-

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(*) Yankee Rotator, Clay-Adams Inc., N.Y., USA.

neous suspension, as in the V.D.R.L. slide flocculation test, or occasionally presented an almost non-identifiable agglutination in very small clumps (Fig. 1). In a previous publication [4] these were considered as doubtful or even as weakly positive, which accounted for a certain percentage of non-specific positive results.

The flocculation reagent was prepared by fixation of *Trypanosoma cruzi* epimastigotes (Y strain), for 18 hours in a 2% formalin solution in 0.15M, pH 8.0 Tris-HCl buffer, followed by ultrasonic desintegration. The final suspension containing the usual stabilizing proteins, aminoacids and preservatives [4], was standardized to give 85% transmittance at 600 nm, 1 cm cuvette, in a Coleman Jr. spectrophotometer, when diluted at 1:40. This suspension was distributed in ampoules and lyophilized. After reconstitution with distilled water, the flocculation reagent could be immediately used or kept at 4°C for at least 4 months without any observed decrease in antigenic activity.

Other serologic tests

Complement fixation tests (CF) were carried out in Micro-titer U-shaped plastic plates (**), with one drop volumes (0.025 ml) respectively of pure or and 1:2 serum, four 50% hemolytic complement units, and antigen dilution giving a maximal fixation. After incubating plates for 18 hours at 4°C and 30 minutes at 37°C, one drop of a 2% suspension of hemolysin-sensitized sheep erythrocytes was added to each well. The plates were left for 1 hour at 37°C with occasional agitation, centrifuged, and the tests immediately read. Controls of complement, standard positive and negative sera were always

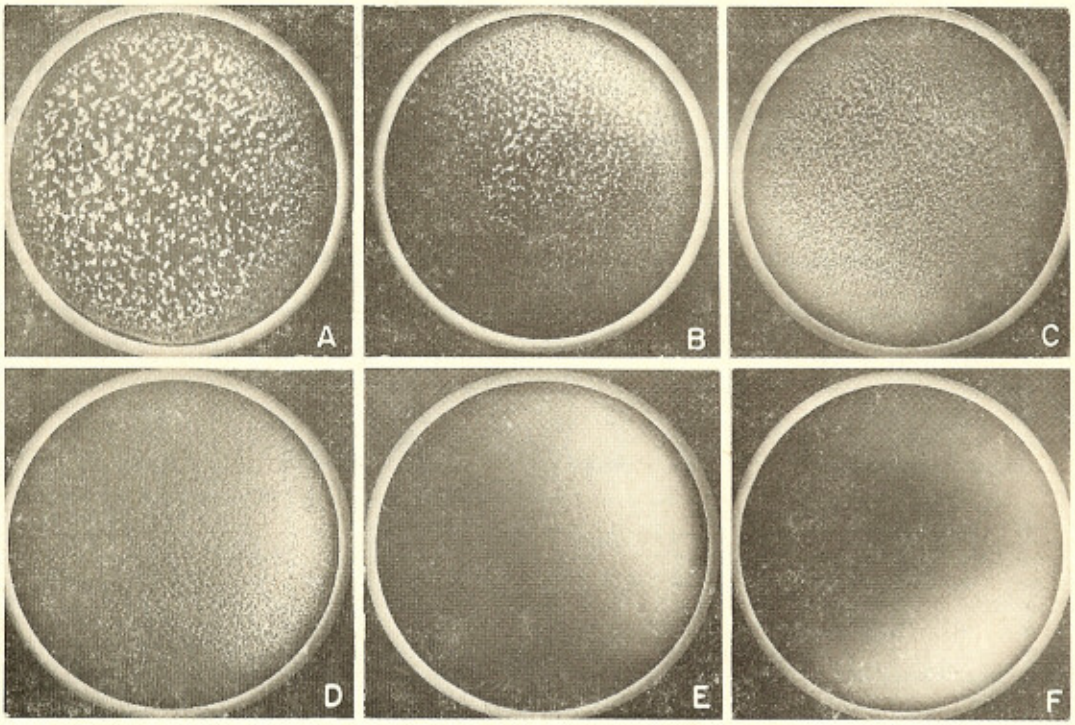

Fig. 1 — Flocculation patterns: A, B, C, D — Positive tests (4+ to 1+); E — Doubtful; F — Negative.

(**) Cooke Engineering Co., USA.

included, as well as controls for anticomplementary activity of serum samples. Hemolysis of 50% or less in the test was taken as positive, for serum or serum dilutions presenting no anticomplementary activity. Antigen was prepared according to MAEKELT [5] from washed and lyophilized *T. cruzi* culture forms obtained in "LIT" medium.

For the hemagglutination test [3] (HA), a preserved antigen was used. This was prepared by lyophilizing formalin-treated human red cells which had been fixed by glutaraldehyde after tannic acid treatment and sensitization with *T. cruzi* extracts.

Immunofluorescence tests (IF) were performed as previously described [2], but with an anti-gamma chain specific (anti-IgG) conjugate (***) diluted for maximal reactivity.

Serum samples

Group I sera consisted of samples collected from 1,132 blood donors in different cities throughout Brazil, frozen at −20°C and sent by air-mail to the laboratory. After thawing, samples were heated at 56°C for 30 minutes and each kind of test performed independently by individual technicians. Group II comprised 324 samples sent to the laboratory for routine testing, usually performed on the same day they were received.

RESULTS

From the 1,132 Group I samples (blood donors), results agreed entirely in the four tests in 1,122 sera (99.1%) with 1,097 non reactive and 25 reactive sera. In serum samples from 324 patients (Group II), there were concordant results in 309 sera (95.4%), with 236 non-reactive and 73 reactive samples. One anticomplementary sample showing positive results in the other tests was included with the reactive samples. Divergent results were seen for the remaining sera, 10 from Group I and 15 from Group II. In 22 out of these 25 samples they were due to positive isolate results, observed in only one test, most commonly to a positive flocculation test, as shown in Tables I and II.

When comparing CF and FO results (Table II), a narrow agreement was observed, with only 1 serum showing a negative FO

TABLE I

Distribution of 1,456 serum samples, according to patterns of results observed in different serologic tests

Pattern of results CF	HA	IF	FO	Group I samples	Group II samples	Total
0	0	0	0 (*)	1,097	236	1,333
+	+	+	+	25	73	98
Total concordant				1,122 (99.1%)	309 (95.4%)	1,431 (98.3%)
+	0	0	0	0	1	1
Ac	+	0	0	1	0	1
Ac	0	+	0	0	1	1
0	+	0	+	1	1	2
0	+	0	0	2	1	3
0	0	0	+	6	11	17
Total divergent				10 (0.9%)	15 (4.6%)	25 (1.7%)
Total				1,132	324	1,456

(*) 0 = non-reactive; + = reactive; Ac = anticomplementary

(***) Hyland, Travenol Laboratories, USA.

TABLE II

Comparative results between flocculation and complement fixation tests in serum samples from Groups I and II

Flocculation test	Complement fixation test			Total
	Inconclusive (*)	Reactive	Non-reactive	
a) Group I (blood donors)				
Reactive	0	25	7	32
Non-reactive	3	0	1,097	1,100
Total	3	25	1,104	1,132
b) Group II (patients)				
Reactive	2	71	12	85
Non-reactive	1	1	237	239
Total	3	72	249	324

(*) Anticomplementary or doubtful results

test and a positive CF test. However, HA and IF were also negative for this sample and the CF titer observed, of 1:2, was low for the usual range of titers found in positive cases, of 1:8 to 1:64.

When CF was taken as reference test, FO showed co-positivity ratios of 1.000 for group I and 0.9863 for group II. Co-negativity ratios were respectively 0.9937 and 0.9516. Correspondent ratios for FO when HA or IF were taken as reference tests are given in Table III.

TABLE III

Evaluation of sensitivity and specificity of the "I.M.T.-Chagas flocculation test" in relation to CF, HA and IF tests, through the respective co-positivity and co-negativity ratios, for Groups I and II of sera

Reference test	co-positivity ratio		co-negativity ratio	
	Group I	Group II	Group I	Group II
CF	1.0000	0.9863	0.9937	0.9516
HA	0.9000	0.9867	0.9955	0.9558
IF	1.0000	0.9865	0.9937	0.9520

DISCUSSION

For the evaluation of serologic tests, sensitivity and specificity are fundamental parameters which can be established with certainty when a true diagnosis is available. This is not always the case for American trypanosomiasis, in view of latent forms which correspond to the majority of infected patients. With the help of xenodiagnosis, a group of known infected cases can be obtained. However, since the sensitivity of this parasitological technique is limited, a bias can be introduced through selection of patients with higher parasitemias. Such a possibility is not to be rejected when a large number of Triatomae are used, as in the sensitized test [7], but should be considered especially for the technique still usually performed, with a smaller number of bugs.

Individuals from areas with no possibility of infection could provide a control group for evaluation of specificity of the test. However, as demonstrated by BUCK & ANDERSON [1], specificity of serologic tests can vary widely for different populational groups, which can be subject to different factors influencing the immune response, such as prevalence of other infections, age, nutritional status, etc.

In this way, the accuracy of any new serologic test can be judged by measuring the association with another well-established test, whose results are taken as the reference diagnosis. This is done through co-positivity and co-negativity indices and the nearer the results of the reference test do approach a true diagnosis, the nearer will such indices translate the sensitivity and specificity of the new test.

In the evaluation of new tests for Chagas'disease CF should be considered for reference, when carried out with antigens considered as satisfactory and recommended by the PAHO Study Group on the Serology of Chagas'Disease [8], since it is the most extensively studied serological procedure to the present.

The occurrence of inconclusive results due to anticomplementarity of serum samples, plus the possibility of false positive results as in cross-reactions due to *Leishmania* infections and of false negatives as occasionally found in acute and also chronic forms of Chagas'disease, may represent drawbacks for CF as reference test. However, in our material, both from blood donors and patients, the high co-positivity and co-negativity ratios between FO and CF indicate the narrow agreement of these tests. When HA or IF were taken as reference, high indices were also observed for the FO test, as it would be expected from the close agreement shown by the results of CF, HA and IF. These tests gave entirely concordant results in 99.4% of all sera included. It is to be remarked, in relation to the IF as reference test, that in our experience anti-IgG specific conjugate should be used, so as to avoid doubtful or false positive results frequently related to non-specific reactions of IgM globulins. This observation applies especially when testing samples from patients with different infectious diseases.

Higher co-negativity indices were found for the present series of sera than for a group of samples previously studied [4]. This former group included sera from patients presenting high-titered antibodies against infectious agents or auto-antibodies, as well as from blood donors and individuals with negative clinical and laboratory check-up. However, scoring of flocculation patterns was more strict in the present study, since only an evident agglutination of particles was considered as positive, whereas for the previous series, sera were taken as reactive even when exhibiting very weak agglutination patterns represented by fine particles.

The present results indicate the "I.M.T.-Chagas flocculation test" as a very practical serologic procedure for rapid screening of *T. cruzi* infections. The test should be of value especially in blood banks to identify risky donors, since trustful results can be obtained within a few minutes. Through production in central laboratories standardization of different lots can insure reproducibility of results. Also, stability of the flocculation reagent through lyophilization makes possible its distribution for routine use, with no danger of a progressively decreased reactivity, as observed nowadays for some commercial serologic reagents. Such a

decrease in reactivity was not observed also for suspensions of the reconstituted flocculation reagent when maintained for several weeks in the refrigerator, which makes its use even more practical.

RESUMO

Prosseguimento da avaliação do "Teste I.M.T. de Chagas-floculação". Comparação com as reações de fixação do complemento, hemaglutinação e imunofluorescência

O "Teste I.M.T. de Chagas-floculação", processo rápido recentemente desenvolvido para o diagnóstico da infecção pelo *T. cruzi*, foi avaliado por comparação com as reações de fixação do complemento, hemaglutinação e imunofluorescência. Estudaram-se soros de 1.132 doadores de sangue e de 324 pacientes, no total de 1.456 amostras. Os altos índices de co-positividade e de co-negatividade do teste de floculação, com referência aos demais testes, traduzem o elevado grau de sensibilidade e de especificidade da prova rápida. Pela facilidade de execução, rapidez de resultados e estabilidade do reagente, o "Teste I.M.T. de Chagas-floculação" poderá constituir processo sorológico adequado para fins de rotina, especialmente em bancos de sangue, na triagem de doadores infectados.

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