JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1995, p. 1034–1035 Vol. 33, No. 4
0095-1137/95/$04.00+0

Diagnosis of American Trypanosomiasis (Chagas' Disease) by the New Complement Fixation Test

ESPERANZA GARCIA,[1] LUIS E. RAMIREZ,[2] VICTOR MONTEON,[3] AND JULIO SOTELO[1]*

National Institute of Neurology and Neurosurgery of Mexico[1] and National Institute of Cardiology of Mexico,[3] Mexico City, Mexico, and Faculty of Medicine of Triangulo Mineiro, Uberaba, Brazil[2]

Received 20 July 1994/Returned for modification 8 November 1994/Accepted 28 December 1994

A new immunodiagnostic method of complement fixation was used for serodiagnosis of American trypanosomiasis; 92% sensitivity and 99% specificity were obtained, for an overall accuracy of 97%. This test can be used in field studies, obviating the use of most laboratory equipment and imported reagents; places where economic limitations hinder the use of other immunodiagnostic procedures; and in association with other tests for confirmation of the diagnosis.

The complement fixation test (CFT) is a classical means for the immunological diagnosis of American trypanosomiasis (4, 6, 12). Developed 80 years ago, it has been widely used since then because of its low cost and high level of accuracy (1, 2, 8–10). However, the major drawback to the CFT resides in the technique itself, because it requires freshly prepared reagents and their standardization every time the test is applied. Consequently, it is time-consuming and its reliability largely depends on the skills of the technician who performs the test. Also, although it uses inexpensive reagents of animal origin, they are short-lived and have to be constantly replaced by fresh reagents.

The new CFT (NCFT) developed at our laboratory greatly simplifies the technical difficulties of the classical CFT, yet still retains a high rate of accuracy (5). Our experience with its routine use during the last 5 years has confirmed its reliability for immunodiagnosis of another parasitic disease, neurocysticercosis, in over 10,000 cerebrospinal fluid samples studied at the National Institute of Neurology of Mexico. Here, we report the adaptation of the assay for use with serum samples for the diagnosis of Chagas' disease.

Sixty-six serum samples from patients with confirmed diagnosis of Chagas' disease were tested: 47 of them were Brazilian and 19 were Mexican, and all had chronic trypanosomiasis, most with chronic cardiopathy. A total of 181 serum samples were used as controls: 57 of them were from healthy blood donors, 24 were from Brazilian patients with a diagnosis of schistosomiasis, and 100 were from Mexican patients with a comprehensive variety of neurological ailments. All samples had been kept frozen at −70°C for periods which varied from 1 month to 1 year. All samples were tested blindly by the NCFT at least twice. After the results of the test were obtained, a correlation with the group origin of the sample was made.

Human erythrocytes and hemolysin-complement reagent were prepared as described previously (5). Antigen was prepared as follows. Epimastigotes of *Trypanosoma cruzi* NINAO were cultured and harvested (3), cultures rich in parasites were washed by centrifugation three times at 2,795 × *g* for 15 min at 4°C, and the pellets containing the parasites were pooled until 10 to 15 ml of parasite-rich suspension was obtained. This suspension was spread in a petri dish 50 mm in diameter, acetone was added to cover the parasites, and then the dish was placed at 37°C until the acetone had evaporated. The procedure was repeated three times until total desiccation; 100 mg of this powder was then placed in an opaque flask, and 1 ml of absolute ethanol was added. This suspension was gently agitated for 15 days; afterwards, it was centrifuged at 2,795 × *g* for 15 min at 4°C. The supernatant was kept at room temperature in an opaque flask and was titrated (5) and used as the concentrated antigen of *T. cruzi*.

The NCFT was carried out as follows. Thirty microliters of undiluted, inactivated serum to be tested was placed in each well of a microtiter plate with 96 V-shaped wells. A total of 25 μl of antigen diluted 0.1:9.5 in saline solution and 50 μl of complement-hemolysin reagent diluted 0.5:7 (each ampoule contained 0.5 ml of lyophilized guinea pig hyperimmune serum against human erythrocytes) in saline solution were added. This mixture was gently agitated and left for 45 min at 37°C. Afterwards, 8 μl of a 2% suspension of human erythrocytes in saline solution was added. The new mixture was again gently agitated and placed back at 37°C for 60 min. Finally, the plate was centrifuged (Beckman Instruments) at 1,400 × *g* for 10 min. A given sample was considered positive when a button of erythrocytes was observed; it was considered negative in the absence of a button due to lysis of the erythrocytes. To exclude the samples with anticomplementary activity, the test was also performed with each serum sample but with the antigen omitted.

The overall accuracy of the NCFT for the diagnosis of Chagas' disease was 97% (Table 1). One sample from the control group showed anticomplementary activity; it was excluded from the study, leaving a final number of 180 control samples. The test maintained its results even when performed by different laboratory workers. In no case did serum from patients with schistosomiasis cross-react with *T. cruzi* antigens. Most serum samples were from well-characterized cases of Chagas' disease confirmed previously by two or more immunodiagnostic tests; thus, a direct comparison of the NCFT versus enzyme-linked immunosorbent assay, immunofluorescence, CFT, or hemagglutination alone was not possible. The sensitivity in detecting well-characterized chronic cases of Chagas' disease was 92%. The specificity in excluding negative controls was 99%. For only two cases, both among healthy controls, were false positives obtained.

The NCFT uses an ethanol extract of highly concentrated (9.2 mg of protein per ml), highly reactive *T. cruzi* antigen (0.00026 ml of concentrated antigen for each sample studied).

* Corresponding author. Mailing address: Neuroimmunology Department, Instituto Nacional de Neurologia y Neurocirugia, Insurgentes Sur 3877, 14269 Mexico, D.F., Mexico. Phone and fax: (525) 606-4040.

TABLE 1. Overall correlation between NCFT results with serum from Chagas' disease patients and controls

Serum group	No. of NCFT results[a]		
	Positive	Negative	Total
Chagas' disease patients	61 (*a*)	5 (*c*)	66
Controls	2 (*b*)	178 (*d*)	180
Total no. tested	63	183	246

[a] Sensitivity, $a/(a + c) = 61/66$ (92%); specificity, $d/(b + d) = 178/180$ (99%); precision, $(a + d)/(a + b + c + d) = 239/246$ (97%); positive predictive value, $a/(a + b) = 61/63$ (97%); negative predictive value, $d/(c + d) = 178/183$ (97%).

The antigen might be kept for more than 1 year at room temperature, with the reactivity obtained at the initial titration maintained for the entire time. Therefore, the NCFT can be routinely performed without further titration of the antigen, in contrast with the traditional CFT, which uses lyophilized antigen that, once diluted, soon becomes inactive. The NCFT uses human erythrocytes obtained from any anticoagulated human blood, regardless of group and Rh factor (5), in contrast with the traditional CFT, which uses fresh sheep erythrocytes, which are difficult to obtain. The NCFT uses a novel hyperimmune serum from guinea pigs as a simultaneous source of hemolysin and complement. Small aliquots of this serum are lyophilized in ampoules to be used once each time the assay is performed. Each ampoule maintains the same reactivity of the lot (made with a pool of the serum samples from five guinea pigs immunized against human erythrocytes); in this way, the antibodies against erythrocytes are incorporated into the complement reagent, thus obviating the preparation of two separate reagents, in contrast with the traditional CFT, which uses hemolysin antiserum from a horse or rabbit and complement from guinea pig serum. The amount of hemolysin antibodies contained in the hemolysin-complement reagent is large; however, this did not alter the reliability of the test, which largely depended on the very careful titration of complement (5) made when a new lot of complement-hemolysin reagent was prepared, as well as the precise addition of 8 μl of a 2% erythrocyte suspension. The NCFT diminishes the use of dilutions of samples, because the whole procedure is done with precise quantities of each reagent; the antigen, erythrocytes, and hemolysin-complement are initially diluted only once to obtain the stock of reagent which will be directly used in the reaction, in contrast to the traditional CFT, which uses several dilutions of most reagents, particularly for titration and standardization, every time the assay is performed. Even when the amount of antibodies to *T. cruzi* varies between patients, the cutoff reactive value obtained with 30 μl of neat serum sample in the NCFT was found to be the precise amount needed to retain the equilibrium between maximum specificity and maximum sensitivity; further titration of positive samples is also possible. The NCFT can be performed in less than 2 h by any laboratory worker, whereas the traditional CFT takes 4 to 5 h in the hands of skillful technicians. The NCFT can be used in field studies without sophisticated equipment, because even the final step, centrifugation of the 96-well plate, can be replaced by passive sedimentation. Less than 2 h after the end of incubation, passive sedimentation of erythrocytes to the bottom of the plate is obtained, and in this way, the results may be observed by visual inspection.

Chagas' disease is among the most frequent parasitic diseases of humans (7). In areas in which Chagas' disease is endemic, *T. cruzi* may infect as much as 74% of the general population (11). Current economic difficulties in several areas in which Chagas' disease is endemic pose new risks for the spread of the parasite and leave many initial and subclinical cases undiagnosed because of the lack of systematic screening studies. The high level of precision and low cost of the NCFT suggest that it can be incorporated into the immunodiagnosis of Chagas' disease and screening studies.

This work was partly supported by National Council of Science and Technology of Mexico (CONACYT) grant 5146-M9407.

REFERENCES

1. **Camargo, M. E.** 1988. American trypanosomiasis (Chagas' disease), p. 744–753. *In* A. Balows, W. J. Hausler, Jr., and E. H. Lennette (ed.), Laboratory diagnosis of infectious diseases: principles and practice, vol. 1. Springer-Verlag, New York.
2. **Carvalho, M. R., M. A. Krieger, E. Almeida, W. Oelemann, M. A. Shikanai-Yassuda, A. W. Ferreira, J. B. Pereira, A. Saez-Alquezar, P. E. Dorlhiac-Llacer, and D. F. Chamone.** 1993. Chagas' disease diagnosis: evaluation of several tests in blood bank screening. Transfusion **33:**830–834.
3. **Chiari, E., and E. P. Camargo.** 1984. Culturing and cloning *T. cruzi*, p. 23–27. *In* C. M. Morel (ed.), Genes and antigens of parasites, laboratory manual, 2nd ed. World Health Organization, Rio de Janeiro, Brazil.
4. **de Andrade, A. L., C. M. Martelli, A. O. Luquetti, O. S. de Oliveira, S. Almeida e Silva, and F. Zicker.** 1992. Serological screening for *Trypanosoma cruzi* among blood donors in central Brazil. Bol. Oficina Sanit. Panam. **113:**19–27.
5. **Garcia, E., and J. Sotelo.** 1991. A new complement fixation test for the diagnosis of neurocysticercosis in cerebrospinal fluid. J. Neurol. **238:**379–382.
6. **Kerndt, P. R., H. A. Waskin, L. V. Kirchhoff, F. Steurer, S. H. Waterman, J. M. Nelson, G. A. Gellert, and I. A. Shulman.** 1991. Prevalence of antibody to Trypanosoma cruzi among blood donors in Los Angeles, California. Transfusion **31:**814–818.
7. **Kirchhoff, L. V.** 1993. American trypanosomiasis (Chagas' disease)—a tropical disease now in the United States. N. Engl. J. Med. **329:**639–644.
8. **Kirchhoff, L. V., A. A. Gam, R. A. Gusmao, R. S. Goldsmith, J. M. Rezende, and A. Rassi.** 1987. Increased specificity of serodiagnosis of Chagas' disease by detection of antibody to the 72- and 90-kilodalton glycoprotein of *Trypanosoma cruzi*. J. Infect. Dis. **155:**561–564.
9. **Knight, R. A., H. Rocha, and D. Kaye.** 1976. Comparison of the complement fixation test and counterelectrophoresis test for the detection of antibodies in Chagas disease. J. Clin. Microbiol. **3:**67–69.
10. **Pan, A. A., G. B. Rosenberg, M. K. Hurley, G. J. Schock, V. P. Chu, and A. Aiyappa.** 1992. Clinical evaluation of an EIA for the sensitive and specific detection of serum antibody to *Trypanosoma cruzi* (Chagas' disease). J. Infect. Dis. **165:**585–588.
11. **Pless, M., D. Juranek, P. Kozarsky, F. Steurer, G. Tapia, and H. Bermudez.** 1992. The epidemiology of Chagas' disease in a hyperendemic area of Cochabamba, Bolivia: a clinical study including electrocardiography, seroreactivity to *Trypanosoma cruzi*, xenodiagnosis, and domiciliary triatomine distribution. Am. J. Trop. Med. Hyg. **47:**539–546.
12. **Schattschneider, W., E. R. Lopes, J. E. De Alencar, U. Bienzle, and H. Feldmeier.** 1992. A comparative study of four serological methods for diagnosis of acute and chronic Chagas' disease in Brazilian patients. Trop. Geogr. Med. **44:**210–218.