

Laboratory diagnosis of Chagas' heart disease

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The laboratory diagnosis of Chagas' disease is a complex one. Factors relating to the host immune response and the antigenic variability of *T. cruzi* must be considered in the final interpretation of test results. Parasitologic methods for detecting *T. cruzi*, immunologic methods for detecting *T. cruzi* antigens in different biological fluids and serologic tests for detection and quantification of different classes of immunoglobulins are well standardized and used in the diagnosis of the acute or chronic phase of the disease. Xenodiagnosis is the most common parasitologic test employed, although it detects only 50% of infections in the chronic phase. Indirect immunofluorescence for detecting IgG and IgM antibodies, hemagglutination and enzyme immunoassay are the serologic tests most frequently employed for diagnosis, to screen blood donors and for seroepidemiologic studies. An important caveat to be remembered is that serologic tests provide only a probable diagnosis, which depends on the prevalence of Chagas disease, as well as on the sensitivity and specificity of the test employed. The use of well defined specific antigens, obtained through recombinant methods or chromatography, opens an important field for the development of very specific tests, without significant loss of sensitivity.

UNITERMOS: Chagas. Laboratory. Diagnosis.

INTRODUCTION

Trypanosoma *cruzi*, the etiologic agent of Chagas' disease, is a protozoan found in different animal species. Human disease is acquired through insect vectors belonging to the subfamily *Triatomina*, family *Reduviidae*, order *Hemiptera*.

In Latin America, the most important vectors implicated in the transmission of the rural form of the disease are *Triatoma infestans*, *Pastrongylus megistus*, *Rhodnius prolixus*, *Triatoma dimidiata* and *Triatoma brasiliensis*. *T. cruzi* belongs to the *Stercoraria* session of the family *Trypanosomatidae*, which includes

trypanosomes which develop in the digestive tract of the vectors and are transmitted through direct contact with the feces of the triatoma bug. *T. cruzi* has great antigenic variation, which makes difficult the study of the epidemiological, clinical, pathological, laboratory and therapeutic aspects of Chagas' disease. In urban areas, transmission of the disease through blood products is of great concern and results from the migration of infected individuals from endemic areas to industrialized centers. Congenital and accidental transmission are also been described in the literature.

LABORATORY DIAGNOSIS

The laboratory diagnosis of Chagas' disease is accomplished by methods which demonstrate the presence of the parasite in the blood, directly or indirectly (parasitologic methods) and through the detection of serum anti-*T. cruzi* antibodies.

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PARASITOLOGIC METHODS

These methods can be employed to diagnose either the acute or chronic phase of disease.

In the acute phase of Chagas' disease (first 6 weeks), methods which demonstrate the presence of trypanosomes in the bloodstream are employed. This can be accomplished through the examination of a thin peripheral blood smear under a microscope. Other variations of this method, such as preparing thick blood smears or concentrating the parasite, increase the likelihood of detection. A promising technique called Quantitative Buffy Coat (QBC method), which is widely used to diagnose infections with plasmodia, has been successfully applied to detect trypanosomes, especially in patients with very low levels of parasitemia. In newborns with congenital *T. cruzi* infection, many authors recommend the search for trypanosomes in bone marrow and spinal fluid. Amastigote forms have also been demonstrated in the chronic phase of the disease, mainly in muscle tissue. The sensitivity of the methods which directly demonstrate the presence *T. cruzi* in the bloodstream ranges from 50 to 95% and is influenced by several factors, ranging from the quality of the microscopic equipment to the expertise of the observer.

In the chronic phase of the disease, xenodiagnosis and blood culture (indirect methods) are employed.

Xenodiagnosis is done through the use of nymph vectors, bred in laboratory and fed with blood from fowl resistant to *T. cruzi* infection. In this technique, the nymphs are allowed to have a blood meal through the patient's skin. About 40 nymphs are put in 4 boxes and kept at 28°C, with 85% air humidity. After 4 to 6 weeks, the gut of the insect and its contents are examined for the presence of *T. cruzi*, under a microscopy. The sensitivity of this method is 85-100% in the acute phase and about 50% in the chronic phase. A variant of the classical method (artificial xenodiagnosis) was introduced to avoid hypersensitivity reactions in susceptible patients. Nymph vectors are allowed to feed on blood from the suspected patients, which is kept in small dialysis bags, usually made from the intestine of pigs. Many authors report that sequential readings, at 30, 60, 90 days increase positive results in the chronic phase of the disease.

Blood culture, though an insensitive technique, is useful in isolating *T. cruzi* strains for studies of biochemical and immunochemistry typing. When used for diagnostic purposes, it detects approximately 50% of cases in the chronic disease.

Other diagnostic methods such as animal inoculation (mice, guinea pigs) and in vitro cell culture are seldom employed (7).

SEROLOGIC DIAGNOSIS

Serologic tests are widely used to: screen suitable blood donors, as markers to monitor therapy, to confirm or exclude clinical suspicion of Chagas' disease, in epidemiologic studies and to screen infected industry workers. Serologic tests give only an estimate of probability of disease and their final interpretation is influenced by numerous factors, like the sensitivity and specificity of the test and the prevalence of Chagas' disease in the population being tested.

Many standardized tests have been used to diagnose Chagas' disease. It is important to bear in mind the great antigenic complexity of *T. cruzi*. This characteristic dictates the host's immune response to the disease and has led to researchers for different markers of disease. Through peptide technology and molecular biology techniques, many investigators are attempting to identify highly specific antigenic epitopes.

Stolf, in 1992, described the ideal antigen as the one which would be present in all strains from different endemic areas, highly immunogenic, not present in other pathogenic microorganisms, stable and easy to be obtained for use in serologic tests (19). Next, we will make a discussion of the different tests employed in the serologic diagnosis of Chagas disease. It is worth remembering that, for the different serologic tests, there is an overlap in the reactivity curves of infected and non-infected individuals. By changing the cut-off point of the test, one can achieve maximum sensitivity or specificity. The point where the curves intersect defines the values of sensitivity and specificity (18).

1) Complement fixation

Introduced by Guerreiro and Machado in 1913, the complement fixation test has only historic value, though it is still used by some to screen blood donors and for diagnostic purposes. The technical complexity of this test, which requires the daily standardization of its components - antigen, hemolytic system and complement - interferes in its reproducibility. Its low level of sensitivity and specificity have also contributed to the low popularity of this test.

2) Precipitation test

The different variations of this test, though highly specific, have poor sensitivity.

It is mainly used in the study of the different antigenic components of *T. cruzi*. Counterimmunoelectrophoresis has been the preferred method for diagnosis and seroepidemiologic studies. Breniere, using serum with the component 5 of *T. cruzi* obtained from rabbits, obtained a sensitivity of 85% and specificity of 100%, even when testing sera from patients with leishmaniasis (1). Requejo et al. recently standardized and tested the DIG-ELISA assay, which is an association between the immunoassay and the agar diffusion test. The authors found the test to have high sensitivity and specificity and recommended it for screening and for seroepidemiologic surveys. Multicenter studies are necessary to validate this test (18).

3) Agglutination tests

This test, with its variants, has been widely used in the diagnosis of Chagas disease.

3a) Direct Agglutination

Several authors have described the use of this test, comparing it to immunofluorescence. Vattuone and Yanowsky, employing a suspension of epimastigote forms of *T. cruzi*, treated with enzymes and fixed with formalin, obtained good sensitivity in the detection of antibodies in the acute phase of Chagas' disease (20). Harith et al. standardized the microagglutination test, which employed epimastigote forms of *T. cruzi*, treated with trypsin and stained with Coomassie-Blue. Treatment of the 2 sera with mercaptoethanol was critical for the detection of specific antibodies. The sensitivity and specificity of the test were very high. A drawback of the agglutination test is the large amount of parasites necessary to prepare the antigenic suspension (12).

3b) Hemagglutination

This test is widely used for diagnosis, screening and seroepidemiologic studies. In the test, erythrocytes from mammals or fowl are treated with formalin and sensitized with antigenic components, partially or completely soluble. The product, lyophilized or in suspension, has excellent stability in adverse temperature conditions (5). The test, either quantitative or qualitative, is performed in microtiter plates (13), with the use of different antigenic extracts. The best results are obtained with alkaline and sonicated extracts. The treatment of the 2 sera with mercaptoethanol increases the specificity of the test. The hemagglutination test was studied by Neal and Miles, who used the Y strain of *T. cruzi*, grown in LIT media. They tested diluted blood, collected in paper filter, from different populations from Latin American countries (16).

No regional differences in antibody response were observed. Because of its simplicity and low cost, the hemagglutination test is recommended for screening blood donors. In Brazil, well standardized kits from different manufacturers are commercially available.

3c) Latex agglutination

Though a very promising test, the latex agglutination test was released in Brazil without proper standardization. False-positive and false-negative results, coupled with poor reproducibility, led to the discontinuation of this test by the manufacturer. With the current possibility of creating covalent bonds between *T. cruzi* antigens and free radicals present in the latex particles, new perspectives for obtaining a new test with good stability, low cost, ease of use and reliable sensitivity and specificity have appeared.

4) Immunofluorescence

The indirect immunofluorescence test is usually performed with epimastigote forms of the Y strain of *T. cruzi*, obtained from cultures of the parasite in LIT medium. The formalin treated trypanosomes are then fixed in glass slides and incubated in diluted serum for 30 minutes at 37°C. After proper washings, the slides are incubated with fluorescent conjugate (sheep or goat serum anti-human IgG or IgM, labeled with fluorescein isothiocyanate). After another incubation and washings, the slide is read with the use of a fluorescence microscope (6). The immunofluorescence test for the detection of IgG anti-*T. cruzi* antibodies is considered to be the gold standard in the serologic diagnosis of Chagas' disease (3).

Antigenic variation has been observed in the different parasitic stages of *T. cruzi*. Camargo found higher antibody titers with the tripomastigote than with epimastigote forms (2). Primavera et al. compared epimastigote with tripomastigote forms of *T. cruzi* and concluded that the amastigotes are more reactive for the detection of IgA antibodies, especially in patients with the digestive form of the disease (17). Levy standardized the in situ immunofluorescence test with tripomastigote forms, in order to detect membrane epitopes and to follow persistent infections. The test has effectively substituted the complement lysis reaction (15).

Different factors may affect the results of the immunofluorescence test: the quality of the optical equipment and of the antigens, the definition of what constitutes a positive test. These factors should be taken into account and the test should be rigorously standardized in order to obtain reliable results (10).

5) Enzyme immunoassay

In 1975, Ferreira standardized the immunoperoxidase test, using formalin fixed epimastigote forms of *T. cruzi*, formalin fixed in glass slides as antigen and enzymatic conjugate (sheep or goat serum conjugated to peroxidase).

After incubation with diluted serum and conjugate, the complex is revealed with substrate and hydrogen donors. The method had the same sensitivity and specificity of the immunofluorescence test. The advantage is that the resulting color development may be visualized with an optical microscope, thus reducing the cost of the test (11).

The use of the enzyme linked immunosorbent assay (ELISA) for the diagnosis of Chagas disease was described by Voller et al. The test was standardized in microtiter plates, adsorbed with soluble *T. cruzi* antigens. After incubation with serum and enzymatic conjugate, color develops in the supernate after addition of substrate and hydrogen donors. The intensity of color development is measured by spectrophotometry (21).

Due to its good sensitivity and specificity and because it is automated, the standardized enzyme immunoassay has opened new perspectives in the serologic diagnosis of Chagas' disease. The possibility of employing very specific antigenic components, obtained through physico-chemical or recombinant methods, is under investigation by several scientists around the world (4). Like the hemagglutination test, well standardized reagents are commercially available and are especially useful for screening blood donors (9).

METHODS FOR DETECTING ANTIGEN IN BODY FLUIDS

The detection of *T. cruzi* antigens in body fluids is of great importance in the confirmation of infection, especially when the serologic tests or parasitologic methods are negative.

Tests based on precipitation, counterimmunoelectrophoresis or immunodiffusion, though highly specific, have low sensitivity, becoming useful in the confirmation and prognosis of disease. The enzyme immunoassay, capture or double sandwich, has proved useful in the detection of *T. cruzi* antigens in blood or urine, confirming a suspected diagnosis and monitoring the efficacy of anti-Chagas therapy. Some authors believe that the detection of *T. cruzi* antigens in urine is useful in the diagnosis of congenital Chagas' disease. Katzin et al. have described a quick agglutination test to detect *T. cruzi* antigens in patients in the chronic phase of the disease. In this test, horse serum with anti-*T. cruzi* antibodies linked to nitrocellulose micelles reacts with small amounts of antigens present in biologic fluids. Though the test appeared to be very sensitive, further studies are necessary to confirm the results found in the article (14).

RESUMO

O diagnóstico de laboratório da doença de Chagas apresenta elevado grau de complexidade. Fatores relacionados com a resposta imune do hospedeiro e fatores relacionados com as diferenças antigênicas do parasita devem ser considerados para a avaliação final do resultado. Métodos parasitológicos ou imunológicos para a pesquisa de *T. cruzi* ou de seus componentes em diferentes fluidos biológicos e testes sorológicos para a pesquisa e quantificação de diferentes classes de imunoglobulinas têm sido padronizados visando o diagnóstico das diferentes fases, aguda, intermediária ou inaparente e crônica da doença. Dos métodos parasitológicos, o xenodiagnóstico, natural ou artificial, é o mais utilizado, embora na fase crônica somente 50% dos casos sejam detectados. Nos testes sorológicos, ênfase tem sido dada a imunofluorescência indireta para a pesquisa de anticorpos IgG e IgM, ao teste de hemaglutinação, pelo baixo custo e ao teste imunoenzimático, pela possibilidade de automação. Dado que os resultados obtidos nos testes sorológicos fornecem diagnóstico de probabilidade, fatores como a prevalência da doença e a sensibilidade e especificidade dos testes devem ser considerados. A possibilidade da utilização de antígenos definidos, obtidos por métodos físico-químicos, biologia molecular ou síntese, abre perspectivas para a padronização de testes com alta especificidade, sem perda significativa da sensibilidade.

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