Posttherapeutic Cure Criteria in Chagas' Disease: Conventional Serology followed by Supplementary Serological, Parasitological, and Molecular Tests

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We performed a critical study of conventional serology, followed by supplementary serological, parasitological, and molecular tests, to assess the response to etiologic treatment of Chagas' disease. A group of 94 Chagas' disease patients treated with benznidazole at least 10 years earlier were evaluated from the laboratory and clinical points of view. When conventional serology (enzyme-linked immunosorbent assay [ELISA], indirect immunofluorescence [IIF], and indirect hemagglutination [IHA]) and classic criteria (consistent results with any two of the three tests) or more rigorous criteria (consistent results from the three tests) were used, 10.6% and 8.5% of patients were considered treated and cured (TC) by classic and rigorous criteria, respectively. Patients were then evaluated using supplementary (recombinant ELISA and *Trypanosoma cruzi* excreted-secreted antigen blotting [TESA-blot]), parasitological (hemoculture), and molecular (PCR) tests. The results of recombinant ELISA were similar to those with the rigorous criterion (three consistent test results). The TESA-blot group showed a higher percentage (21.3%) of negative results than the groups defined by either cure criterion. Hemoculture and PCR gave negative results for all treated and cured (TC) patients, regardless of the criterion used. Recombinant ELISA and TESA-blot tests showed negative results for 70% and 87.5% of the patients categorized as TC by the classic and three-test criteria, respectively. For patients with discordant conventional serology, the supplementary serological and molecular tests were the decisive factor in determining therapeutic failure. Clinical evaluation showed that 62.5% of TC patients presented with the indeterminate form of the disease. Additionally, treated patients with negative TESA-blot results should be reevaluated later with all methodologies used here to verify whether TESA-blot is a reliable way to determine early parasitological cure of Chagas' disease.

Chagas' disease, caused by the flagellate protozoa *Trypanosoma cruzi*, is a serious public health problem that occurs predominantly in Latin America, largely linked to limited social and economic conditions. An estimated 10 million people worldwide are infected with *T. cruzi*, mostly in areas of 21 Latin American countries where the infection is endemic (53). Infected people frequently develop serious and complex clinical manifestations, mainly in the heart and gastrointestinal tract (35).

Transmission chiefly occurs through triatomine vectors, blood transfusions, and congenital routes (10). In the last few years, the estimated prevalence of Chagas' disease has progressively declined due to an intensive large-scale program designed to limit domestic populations of insect vectors and to improve blood donor screening. Progress must still be made with individual diagnosis, clinical assistance, and necessary etiological treatment, all of which are huge challenges for health services (26).

Infection with *T. cruzi* progresses through an acute phase characterized by patent parasitemia that is followed by a chronic phase with subpatent parasitemia and scarce tissue parasitism. In the acute phase, diagnosis is primarily based on detection of the parasite by direct parasitological methods. In the chronic phase, diagnosis is typically based on the presence of IgG antibodies in the serum of patients (38). Serological tests currently used are enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF), and indirect hemagglutination (IHA).

To date, only two drugs have been effectively used in Chagas'

disease chemotherapy: a nitrofuran (nifurtimox), which is manufactured by Bayer Health Care as Lampit, and a nitroimidazolic derivative (benznidazole), which was previously manufactured by Roche as Rochagan or Rodanil and now by Laboratorio Farmacêutico do Estado de Pernambuco (LAFEPE), in Brazil, and Laboratorio ELEA, in Argentina. The best chemotherapy results are achieved in acute or early chronic infections, as opposed to those observed in late chronic infections (5, 8, 9, 25, 49, 50). Several laboratory methods are available for monitoring posttherapeutic cure of Chagas' disease. These include two major categories: parasitological (microhematocrit, xenodiagnostic, hemoculture) and molecular (PCR) methods and serological tests. Serological tests fall into either conventional (ELISA, IIF, IHA), alternative (recombinant/synthetic ELISA, among others), or other nonconventional serological (*T. cruzi* excreted-secreted antigen blotting [TESA-blot], immunochromatography, complement-mediated lysis [LMCo],

and flow cytometry analysis of anti-live trypomastigote antibodies [FC-ALTA]) test categories. Several researchers have used recombinant/synthetic and biochemically purified antigens in both the diagnosis and monitoring of posttherapeutic cure (13, 21, 33, 42, 46). Umezawa et al. (45) described new immunodiagnostic techniques employing *T. cruzi* excreted-secreted antigens (TESA). The TESA test is based on ELISA and Western blotting and presents excellent results that are both sensitive and specific. The major advantage of recombinant antigens and TESA-blot for the serodiagnosis of Chagas' disease is the absence of cross-reaction with other parasitic diseases, including leishmaniasis (4, 46).

Controversy still remains regarding the benefit of chemotherapy for Chagas' disease patients and how to detect parasitological cure after etiological treatment. Some authors consider patients cured only if conventional serology tests are permanently negative (5, 34, 52). However, permanently negative conventional serological tests are normally observed 10 to 25 years following treatment of patients in the late chronic phase, even when nonconventional serology and parasitological methods are consistently negative (27). In addition, other authors interpret accelerated decay of conventional serological titers as indicative of a cure if present with decreasing titers in nonconventional serological tests and persistently negative parasitological tests (15, 22). A third interpretation is that patients with negative results from parasitological methods and nonconventional serology (LMCo or FC-ALTA) are cured, even in the presence of positive results of conventional serology, because the detected antibodies with nonconventional tests are distinct from lytic and anti-live trypomastigote antibodies and are consequently not related to active infection (15, 23).

Taking into account the considerable and persistent controversy regarding the interpretation of serological test results for individuals with Chagas' disease after etiological treatment, the aim of this work is to carry out a careful evaluation of patients at least 10 years posttreatment using several laboratory and clinical methods for better definition of cure assessment. The laboratorial methods include various conventional serological (isolated and/or associated), supplementary serological tests (recombinant ELISA [rec-ELISA] and TESA-blot), and parasitological (hemoculture) and molecular (PCR) methods.

MATERIALS AND METHODS

Patients, materials, and methods. A group of 94 individuals in the chronic phase of Chagas' disease (40 males and 54 females) were evaluated. All individuals were born and live in the Berilo municipality, Jequitinhonha Valley, Minas Gerais State (MG), Brazil, a vector-controlled area of Chagas' disease. Individuals were treated with benznidazole at least 10 years ago at a dosage of 5 mg/kg of benznidazole for 60 days. Before treatment, the Chagas' disease diagnosis was confirmed by two conventional serological tests. Before inclusion in the study, patients or their legal guardians read and signed the consent form approved by the Ethical Committee in Human Research from CPqRR, FIOCRUZ, Belo Horizonte, MG (process 007/2002). Blood samples from each patient were taken with the Vacutainer system (BD) and aliquoted in appropriate volumes for different laboratory assays: 5 ml for serological tests, 5 ml for PCR, and 30 ml for hemoculture (adult patient). For children, the volume of blood taken for hemoculture was proportional to body weight.

Conventional serological tests. The conventional serological tests used were ELISA, IIF, and IHA as recommended by the World Health Organization (52). Positive- and negative-control serum samples were run in parallel.

"In-house" ELISA was performed as described by Voller et al. (51) using 4.5 μg/ml of antigen, a serum dilution of 1:80, and peroxidase-anti-

human IgG conjugate at a dilution of 1:7,500. The cutoff value calculated for each plate was the mean absorbance of 10 negative-control serum samples plus two standard deviations (39). The data that fell within 10% of this cutoff were considered in the "gray zone."

The IIF assay was performed with the Bio-Manguinhos kit according to the manufacturer's instructions.

The IHA assay was performed with the Hemacruzi kit (bioMérieux Brasil) following the manufacturer's instructions.

Supplementary serological tests. ELISA with recombinant antigen (rec-ELISA) and immunoblot assay (TESA-blot) were used as supplementary serological tests.

The rec-ELISA was the Chagatest kit (ELISA recombinant v.3.0; Wiener Laboratorios, Rosario, Argentina) with modifications. Sera were diluted 1/20 in phosphate-buffered saline-Tween 20 (0.05%) containing 1% bovine serum albumin. The peroxidase-labeled anti-human IgG conjugate was used at a 1:40,000 dilution. Plates were read with a 450-nm-wavelength filter in a microplate reader (model 680; Bio-Rad). Positive and negative serum controls were included in parallel. The cutoff was the mean absorbance of 10 negative controls plus 0.3 optical density (OD) units.

The TESA-blot test used was TESA-cruzi (bioMérieux Brasil S.A.). Strips with TESA antigenic proteins of *T. cruzi* that were separated by electrophoresis and fixed in nitrocellulose membranes were placed in a chamber treated with serum samples diluted 1:100. Human anti-IgG conjugate labeled with peroxidase and T Chromogen (4-chloro-α-naphthol) solution, diluted 1:5, were used. Bands of 120 to 200 kDa are observed in positive reactions and are absent in negative reactions.

Parasitological and molecular methods. Hemoculture and PCR were used. Two samples from each patient were examined at an interval of 3 years.

For hemoculture, 30 ml of heparinized venous blood was centrifuged at 3,000 rpm for 10 min and the plasma discarded. The pellet was resuspended in 15 ml of liver infusion tryptose (LIT) medium and centrifuged under the same conditions. After removal of the supernatant, the pellet was resuspended in 15 ml of LIT and distributed into three tubes that were maintained at 28°C and examined at regular intervals of 30 days for 120 days (7).

For PCR, blood samples were collected in equal volumes of 6 M guanidine and 0.2 M EDTA, pH 8, and stored at room temperature. DNA was extracted with a DNA purification kit (Wizard Genomic; Promega). PCR was performed in duplicate according to the method of Gomes et al. (18) with modifications. Negative results were reevaluated using primers for the beta-globin genes PCO3 (ACA CAA ACT GTG TTC ACT AGC) and PCO4 (CAA CTT CAT CCA CGT TCA CC) as amplification controls for the reaction. Positive, negative, and reagent controls were run in parallel. The primers 121 (AAATAATGTACGGGTGAGATGCATGA) and 122 (G GTTCGATTGGGGTTGGTGTAATATA) were used to amplify kinetoplast DNA (k-DNA) minicircles in the presence of *Taq* DNA polymerase (Platinum; Invitrogen). The products were separated on polyacrylamide gels.

Cure criteria. Two cure criteria were used to interpret the results. (i) The classic cure criterion requires that patients present two negative results from two different conventional serology tests. (ii) The more rigorous three-test criterion requires that patients present negative results from three different conventional serological tests.

Patients were considered treated and not cured (TNC) when results were positive by conventional serology, treated under evaluation (TUE) when the conventional serological tests were inconclusive or discordant, and treated and cured (TC) when the conventional serology was negative considering the cure criterion used.

Clinical evaluation. Clinical evaluations were performed through anamnesis, physical examination, conventional electrocardiogram (ECG), thorax X-ray, and esophagus and colon barium-contrasted X-ray (36). Three physicians (clinicians and/or cardiologists) performed these evaluations using blind conditions. A clinical classification was assigned to a

(A) Conventional serology reactivity

ELISA

ELISA

IIF

IHA
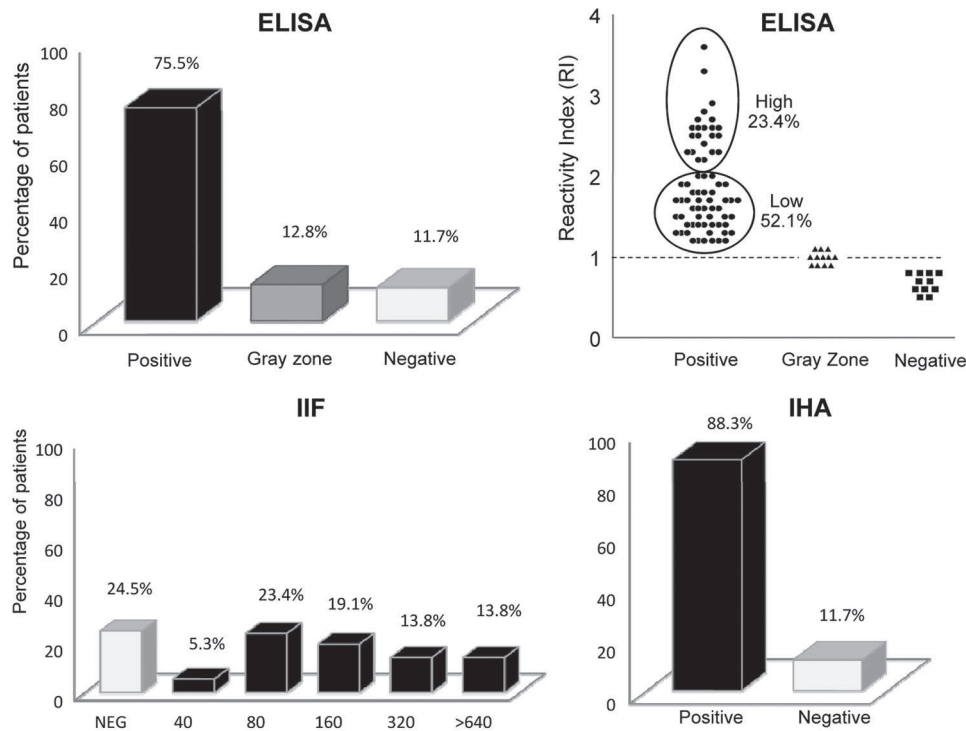
(B) Cure criteria based on conventional serology reactivity

Groups of Patients	Category	Serological Reactivity				
		Classic - Two tests				Three tests
		Any Two Tests[a]	ELISA+IIF[b]	ELISA+IHA[a,b]	IIF+IHA[a,b]	ELISA+IIF+IHA[b]
Treated Not Cured (TNC)	Positive	81.9%	67%	75.5%	73.4%	67%
Treated Under Evaluation (TUE)	Inconclusive	7.5%	23.4%	16%	17%	24.5%
Treated Cured (TC)	Negative	10.6%	9.6%	8.5%	9.6%	8.5%

FIG 1 (A) ELISA "in house," indirect immunofluorescence, and indirect hemagglutination reactivity of Chagas' disease patients evaluated at least 10 years after etiological treatment with benznidazole. (B) Percentages of treated and not cured (TNC), treated under evaluation (TUE), and treated and cured (TC) patients after treatment evaluation with any two or three conventional serological tests. Different letters indicate significant differences.

patient if at least two physicians were in agreement according to standards set by the Brazilian Consensus on Chagas Disease (34).

Statistical analyses. A chi-square test was used to compare the results of conventional serological tests and any supplementary tests. Fisher's exact test was used to compare the percentages of positive PCR results between the TNC and TUE groups categorized by both cure criteria.

RESULTS

Patients studied were 2 to 60 years old (32.9 ± 10.9 years) before treatment. The majority (71.2%) were 21 to 40 years old. Elapsed time after treatment ranged from 10 to 36 years (16.9 ± 6.8 years). The majority of elapsed time (59.6%) fell between 10 and 14 years after treatment.

Conventional serology. Serological evaluation of the 94 treated patients with conventional ELISA showed that 75.5% of the patients had positive results, 12.8% had results in the gray zone (within 10% of the cutoff), and 11.7% had negative results. Among the patients with positive results, 23.4% presented a high reactivity index (RI > 2) and 52.1% a low reactivity index (1.2 ≤ RI ≤ 2) (Fig. 1A).

The indirect immunofluorescence test showed that 24.5% of the patients had negative results and 75.4% positive results, with titers ranging from 1:40 (5.3%) to 1:640 (13.8%) (Fig. 1A).

The IHA test revealed that 88.3% of the patients had positive and 11.7% had negative results (Fig. 1A).
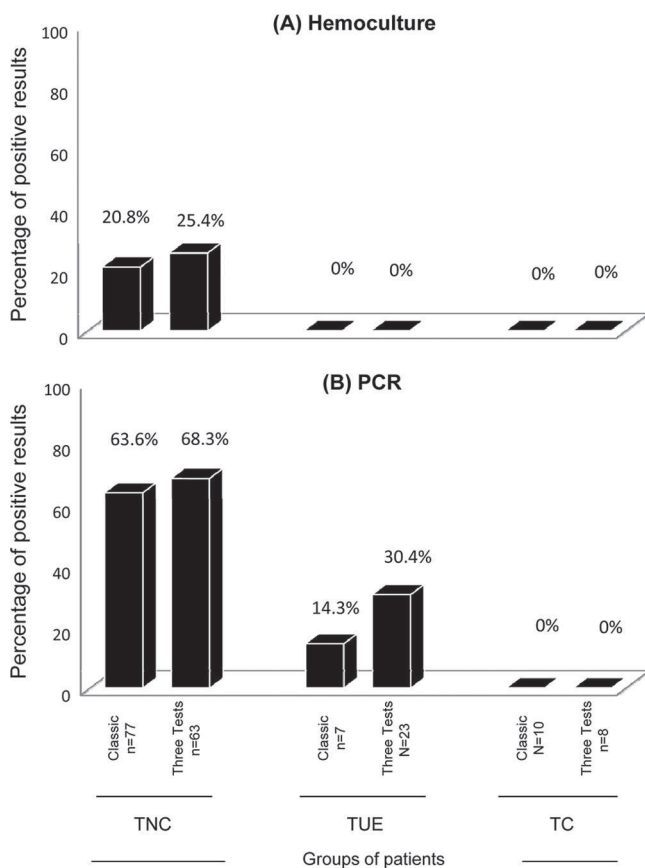
FIG 2 Percentage of positive results from hemoculture and PCR for patients at least 10 years after treatment with benznidazole that were categorized as treated and not cured (TNC), treated under evaluation (TUE), and treated and cured (TC) using the classic criterion and the criterion using three conventional serological tests.

Association of conventional serological tests. Using the classic cure criterion based on the comparison of results from two out of three conventional serological tests, the patients were grouped into three groups: treated and not cured (TNC), treated under evaluation (TUE), and treated and cured (TC) (Fig. 1B). The percentage of patients classified as TC ranged from 8.5 to 9.6%, depending on whether results from ELISA plus IIF, ELISA plus IHA, or IIF plus IHA were in agreement ($P > 0.05$) (Fig. 1B). If the results from any two conventional serological tests were considered, the percentages of TNC, TUE, and TC patients were 81.9, 7.5, and 10.6%, respectively. Comparisons of the results from any

two conventional serology tests were significantly different between ELISA and IIF ($P < 0.05$) and similar between ELISA and IHA and between IIF and IHA (Fig. 1B).

When using the three-test criterion, the percentages of patients in the TNC, TUE, and TC groups were 67%, 24.5%, and 8.5%, respectively. These results are similar to the correspondence between ELISA plus IIF, IIF plus IHA, and ELISA plus IHA ($P > 0.05$) (Fig. 1B).

Conventional serology versus parasitological and molecular methods. Hemoculture examinations of patients that were categorized into the TNC, TUE, and TC groups were performed. Subsequent analysis was performed based on whether patients were categorized using the classic cure criterion or the three-test criterion. The hemoculture tests presented positive results in 20.8% and 25.4% of the patients, respectively. All positive results were from patients in the TNC group. No positive results from hemoculture were observed in the TUE and TC groups (Fig. 2A).

PCR tests were conducted on patients and evaluated based on how patients were previously categorized. These gave positive results for 63.6% (classic criterion) and 68.3% (three-test criterion) of patients in the TNC group and for 14.3% and 30.4% of those in the TUE group. These differences were not significant. PCR was always negative for all TC patients (Fig. 2B).

Conventional serology versus supplementary tests. The rec-ELISA gave positive results for 91.5% of patients. When the patients were categorized using the classic criterion, rec-ELISA gave positive results for 98.7% of the TNC patients and 100% of the TUE patients. Of patients categorized as TC, 70% were rec-ELISA negative (Table 1). When the patients were categorized using the three-test criterion, 100% of the TNC patients were rec-ELISA positive and 87.5% of the TC patients were negative (Table 1).

The TESA-cruzi test showed positive results for 78.7% of patients and negative results for 21.3%. When patients were categorized by the classic criterion, negative results from the TESA-cruzi test were observed for 13%, 42.9%, and 70% of patients in the TNC, TUE, and TC groups, respectively (Table 2). Using the three-test criterion, 8%, 34.8%, and 87.5% of patients in the TNC, TUE, and TC groups, respectively, had negative results in the TESA-cruzi test (Table 2). The results of the TESA-cruzi test did not show significant similarity with the results obtained from patients categorized by both criteria (two or three tests negative) and presented a significant increase in the number of negative results.

Of the 10 patients considered negative by the classic criterion, six (60%) were negative by rec-ELISA and TESA-cruzi. Six (75%) out of the eight patients with negative results according to the three-test criterion also had negative results with rec-ELISA and TESA-cruzi. Only one patient (number 1121) had negative results

TABLE 1 Results of the supplementary test rec-ELISA for groups of patients categorized serologically by the classic (any two tests) and three-test criteria

Supplementary test result	No. (%) of patients in group[a]						
	Rec-ELISA	Any two tests			Three tests		
		TNC	TUE	TC	TNC	TUE	TC
Positive	86 (91.5%)	76 (98.7%)	7 (100%)	3 (30%)	63 (100%)	22 (95.7%)	1 (12.5%)
Negative	8 (8.5%)	1 (1.3%)	0 (0%)	7 (70%)	0 (0%)	1 (4.3%)	7 (87.5%)
Total	94 (100%)	77 (100%)	7 (100%)	10 (100%)	63 (100%)	23 (100%)	8 (100%)

[a] TNC, treated and not cured; TUE, treated under evaluation; TC, treated and cured.

TABLE 2 Results of the supplementary test TESA-cruzi for groups of patients categorized serologically by the classic (any two tests) and three-test criteria

Supplementary test result	No. (%) of patients in group[a]							
	TESA-cruzi	Any two tests			Three tests			
		TNC	TUE	TC	TNC	TUE	TC
Positive	74 (78.7%)	67 (87%)	4 (57.1%)	3 (30%)	58 (92%)	15 (65.2%)	1 (12.5%)
Negative	20 (21.3%)	10 (13%)	3 (42.9%)	7 (70%)	5 (8%)	8 (34.8%)	7 (87.5%)
Total	94 (100%)	77 (100%)	7 (100%)	10 (100%)	63 (100%)	23 (100%)	8 (100%)

[a] TNC, treated and not cured; TUE, treated under evaluation; TC, treated and cured.

in all conventional serological tests but a positive rec-ELISA result. One patient (number 759) had negative results in three conventional serological tests but showed a positive TESA-cruzi result.

Our results again verified that supplementary serological tests confirmed as negative a high percentage of patients (70% and 87.5%) categorized as TC using either the classic or the three-test criterion.

Patients categorized as inconclusive by the three-test criterion ($n = 23$) were classified into two categories when evaluated using parasitological (hemoculture) or molecular (PCR) methods and supplementary tests (rec-ELISA and TESA-cruzi): TNC ($n = 17$) when both supplementary tests and/or PCR was positive and TUE ($n = 6$) when supplementary tests showed discordant results and the PCR was negative (Fig. 3).

Clinical data. The patients were classified into the TNC, TUE, and TC groups according to the results of the conventional sero-

logical tests, and parasitological and molecular methods and supplementary tests were evaluated clinically (Fig. 4). The patients in the TNC or TUE group showed 67.5% and 83.3% of cardiac and/or digestive abnormalities, respectively. In the TC group, 62.5% of the patients were in the indeterminate form of Chagas' disease. Furthermore, the TC group had an absence of the digestive form of the disease, a lower percentage of the cardiac form, and a higher percentage of the mixed form (cardiac plus digestive) of the disease (Fig. 4).

DISCUSSION

The purpose of this work was to use several laboratory analyses to conduct an evaluation of 94 patients at least 10 years posttreatment for Chagas' disease. This study used conventional serological tests, parasitological and molecular methods, and supplementary serological tests. Additionally, this investigation included a punc-
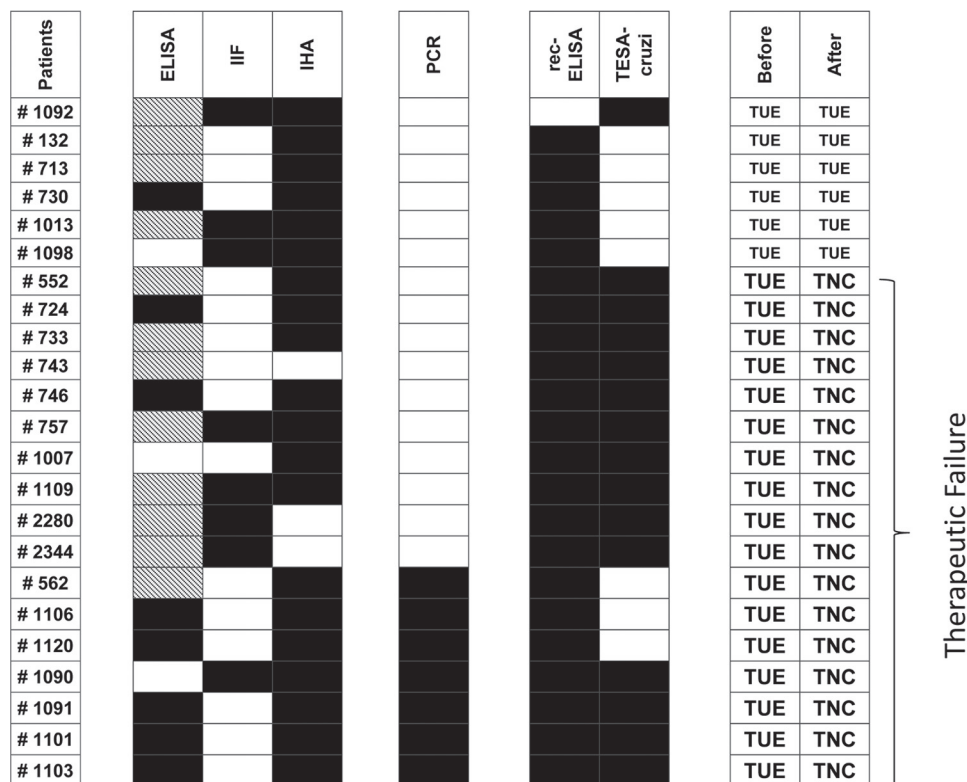

FIG 3 Results of conventional serological tests, supplemental tests, and parasitological methods for patients categorized as treated under evaluation (TUE) by the three-test criterion. Black box, positive; white box, negative; hatched box, gray zone. TNC, treated and not cured.
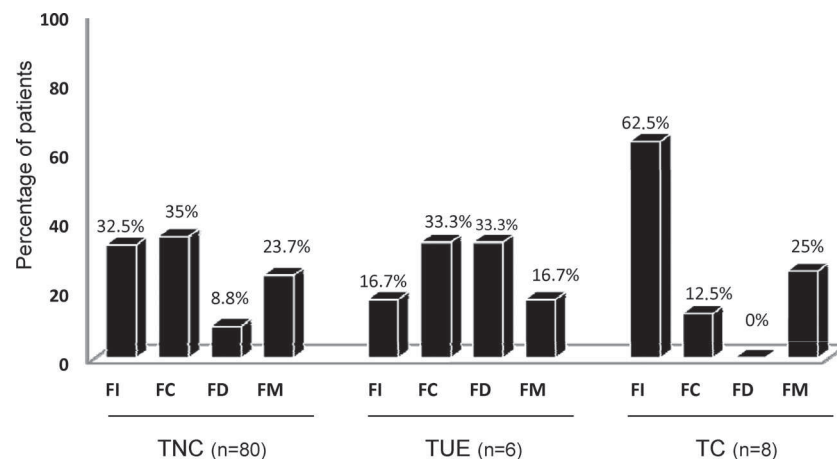
FIG 4 Percentage of different clinical forms of Chagas' disease observed in treated patients categorized as treated and not cured (TNC), treated under evaluation (TUE), and treated and cured (TC) based on three conventional serological tests followed by PCR and supplementary serological tests. FI, indeterminate form; FC, cardiac form; FD, digestive form; FM, mixed form.

tual, global, and critical analysis of different associations between the results of any two or three conventional serological tests or any supplementary serological tests.

Studies evaluating Chagas' disease after specific treatment are controversial and conflicting because of differences in the methods used for evaluation, the time elapsed, and the cure criteria adopted (8).

In this study, the ELISA showed that 75.5% (71/94) of the patients were positive, of which 23.4% (22/94) were highly reactive (RI > 2) and 52.1% (49/94) had low reactivity (1.2 < RI < 2). Low reactivity was most likely due to decreasing serological titers after treatment. Of the patients, 12.8% (12/94) were in the gray zone and 11.7% (11/94) were negative. Subsequently, an IIF test showed 24.5% (23/94) with negative results, and the IHA test was negative for 11.7% (11/94) of the patients.

Despite the recommendation to compare the results from two conventional serological tests that differ in principle to diagnose and/or monitor etiological treatment of Chagas' disease (34, 52), it is not apparent which test comparisons are most effective. Because there are three conventional serological tests in frequent use, three combinations of two serological tests are possible (ELISA plus IIF, ELISA plus IHA, and IIF plus IHA). This study performed the tests in these combinations and analyzed the results. The percentage of negative patients was 9.6% for ELISA plus IFI and for IIF plus IHA and 8.5% for ELISA plus IHA. The differences between the combinations were not significant.

This work also adopted a more rigorous cure criterion (three tests) to categorize the treated patients into three distinct groups: TNC ($n = 63$) when the three tests were positive, TUE ($n = 23$) when the results were inconclusive or discordant between the three tests, and TC ($n = 8$) when all three tests were negative. Using these criteria, it was possible to verify negative results in 8.5% of the patients. This result corroborates the findings of several authors who found a cure index of 5% to 19.1% in posttreatment evaluations of patients etiologically treated in the chronic phase of Chagas' disease in Brazil and Argentina (5, 12, 49, 50). Moreover, the eight TC patients were the same across the different combinations of conventional serological tests (two or three tests). It is important to note that in the TUE group, some patients

were negative by IIF, were in the gray zone by ELISA, or showed low reactivity (data not shown). This indicates a considerable decrease in reactivity, suggesting the need of further evaluation of these patients, because a decrease in serological reactivity is also considered indicative of cure (15, 22, 27).

The evaluation of different combinations of two tests (ELISA plus IIF, ELISA plus IHA, and IIF plus IHA), three serological tests (ELISA plus IIF plus IHA), or any two tests showed that seroconversion rates were similar and ranged from 8.5% to 10.6%. We emphasize that the comparison of two serological tests (ELISA plus IIF, IIF plus IHA, or ELISA plus IHA), as recommended by the classic cure criterion (34, 52), shows results similar to those found with the more rigorous cure criterion (three tests negative). These results indicate that a comparison between two serological tests is sufficient as a cure criterion in posttreatment evaluation of Chagas' disease. Moreover, the results of any two tests showed significant statistical differences when compared with the more rigorous criterion and the comparison between ELISA and IIF. These differences are explained by the higher number of patients categorized as inconclusive using the three-test criterion.

When the hemoculture technique was used for patients characterized by the classic criterion or the three-test criterion, therapeutic failure occurred for 20.8% or 25.4% of the patients in the TNC group, respectively. No positive hemoculture was detected in the TUE and TC groups as defined by either criterion. Hemoculture is the only method with unquestionable specificity but simultaneously with low sensitivity and has been used in several studies as evidence of therapeutic failure. However, when used alone, its negative results do not suggest parasitological cure but may indicate low or intermittent parasitemia. Moreover, if the test is repeated for the same patient and performed with a larger volume of blood centrifuged at 4°C, the sensitivity of this method increases. Other factors, such as time between the collection and processing of blood samples, are also important (6, 7, 28).

PCR showed different percentages of positive results in the TNC and TUE groups that were categorized using either criterion, and these differences were not significant. It is interesting that no positive PCR results were observed for TC patients. Several authors have described positive PCR or low reactivity with conven-

tional serology for TUE patients (2, 3, 19). This reinforces the importance of PCR due to its high sensitivity (18). Other authors have suggested that positive PCR results are due to intact extracellular parasites or recently lysed parasites that are still present in the bloodstream (44). Future studies using PCR in the context of cure criteria are necessary. The results presented here suggest that PCR, along with serological evaluations, especially for TUE patients, should be repeated because of the possibility of identification of more TC or parasitologically cured patients.

The literature shows that PCR is a very useful tool for the confirmation of diagnoses of patients with questionable serology (29) and for monitoring therapeutic response (3, 16, 37, 40). However, further studies are needed to better elucidate the real role of PCR in the context of cure criteria. Indeed, a workshop and symposium with the purpose of standardizing and validating the protocols and improving the results of this technique for the diagnosis of Chagas' disease have occurred (41).

In the second part of this study, supplementary serological tests (rec-ELISA and TESA-cruzi) were used to evaluate the same groups of patients categorized as TNC, TUE, and TC. The rec-ELISA results were similar to those found with the three-test criterion, and they confirmed that all patients previously categorized as TNC by either criterion were also positive, with the exception of one patient categorized as TNC by the classic criterion. All patients categorized in the TUE group by either criterion were positive, with the exception of one patient who showed a negative result and was classified as TUE by the three-test criterion. The rec-ELISA confirmed the TC classification of 70% (7/10) of the patients categorized by the classic criterion and 87.5% (7/8) of the patients grouped by the three-test criterion. The results show a better agreement between the three-test criterion and rec-ELISA, suggesting that this test can be used for monitoring the cure of patients treated for Chagas' disease. Caballero et al. (4) showed that rec-ELISA is a test with high sensitivity and specificity that reduces false positives, inconclusive results, and cross-reactions with other pathogens. Currently, this test has been very useful in monitoring parasitological cure, with good results (11, 12, 38, 54). In addition, several other serological tests, including conventional ELISA with purified antigens (17, 20), ELISA with recombinant antigens (21, 33, 42), flow cytometry (31, 32), and the complement-mediated lysis test (15), have been used for diagnosis and cure control of Chagas' disease. However, these techniques show poor reproducibility with divergent results; they are expensive and difficult to handle, and the preparation of the antigens is complex.

The use of a second supplementary serological test based on an immunoblot assay (TESA-cruzi) showed that 78.7% of the patients were positive and 21.3% were negative. TESA-cruzi gave 13% of negative results in the TNC group, 42.9% in the TUE group, and 70% in the TC group when patients were categorized according to the classic cure criterion. The patients categorized with the three-test criterion into TNC, TUE, and TC groups showed 8%, 34.8%, and 87.5% of negative results, respectively. The higher percentage of negative patients in the TESA-cruzi test may be due to the specific anti-*T. cruzi* antibodies that recognize epitopes found only in the trypomastigote forms of *T. cruzi* present in the antigens used (47) versus the blood trypomastigote forms present in humans. This explanation is consistent with the negative TESA-cruzi results for patients with at least one positive serological test. This TESA-cruzi test is now commercially available, presents high indices of sensitivity and specificity in the di-

agnosis of acute and chronic *T. cruzi* infection (45, 47, 55), does not present cross-reaction with other pathogens (45, 47), is able to elucidate inconclusive cases (14, 43), and shows equivalent sensitivity with tests employing recombinant antigens (48). Zarate-Blades et al. (55) evaluated the performance of the TESA-cruzi test for patients from an area of Bolivia in which Chagas' disease is endemic and verified high indices of sensitivity and specificity. These authors recommended this test for various applications such as blood bank screening, diagnosis of the acute or chronic phase of Chagas' disease, and confirmation of epidemiological and clinical suspicion of Chagas' disease.

The three-test cure criterion revealed only eight TC patients, and six of them were also rec-ELISA and TESA-cruzi negative. One patient showed a positive rec-ELISA result and the other a positive TESA-cruzi result. Both patients were negative in the conventional serological tests. We believe that the contradictory results among the available tests were not surprising because the antigenic preparations were different and consequently had different affinities for both specific and nonspecific antibodies. These differences in specificity would give rise to false-negative or inconclusive results.

The overall results obtained in this study using all methodologies employed in posttherapeutic evaluation of Chagas' patients indicate that it still remains impossible to consider supplementary serological tests (rec-ELISA and TESA-cruzi) alone for monitoring the cure of Chagas' disease. However, we showed that rec-ELISA and TESA-cruzi tests confirmed the majority (six out of eight cases) of TC patients as categorized by the three-test cure criterion. Thus, rec-ELISA and TESA-cruzi may be appropriate for verifying the status of a great number of treated patients previously evaluated but not as unique tests for Chagas' disease cure control.

Interestingly, the TESA-cruzi test was negative for a higher number of patients relative to the other serological tests (conventional serology and rec-ELISA). If other authors confirm our results in similar studies, this method (TESA-cruzi) could be used as an effective early serological marker of parasitological cure in Chagas' disease under the classic criterion used (5, 52).

Despite persistent negative results from parasitological methods such as hemoculture and xenodiagnosis that are used in parallel with conventional serological tests, accurate posttherapeutic evaluation as a follow-up for treated patients in the chronic phase of Chagas' disease still constitutes a major challenge due to the long-term persistence of antibodies used in conventional serology (1, 5, 15, 22). Several factors contribute to enduring positive results from conventional serological tests for patients that are parasitologically cured, including mechanisms of autoimmunity in Chagas' disease, the long-term presence of antibodies due to parasitic antigens present in dendritic or cardiac cells, anti-idiotypic antibodies, anti-laminine antibodies, and anti-epitopes of sugar residues in *T. cruzi* membranes and others (23).

Here, the use of supplemental tests contributed to a better definition of therapeutic failure for patients previously categorized as TUE by conventional serology. Rec-ELISA and TESA-cruzi gave positive results for two patients considered TC by the classic cure criterion.

In contrast with the preconized procedures for the diagnosis of Chagas' disease, our main conclusion is that the assessment of cure definition for etiologically treated patients requires a more rigorous laboratory evaluation. The use of parasitological and

supplementary tests is also necessary because several patients show discordant results with the conventional serological tests.

This study also evaluated the effect of benznidazole treatment on the morbidity of Chagas' disease. The effect of benznidazole on Chagas' disease is the subject of much discussion and constitutes the objective of the cardiopathy BENEFIT Project funded by WHO (30). The clinical evaluation of the patients in this study revealed that 66% (62/94) presented some cardiac and/or digestive abnormalities. However, it is not possible to infer that the etiological treatment of the patients in this study led to better clinical evolution. This uncertainty is due to several factors, including the fact that this study is transversal, the unknown clinical status of the majority of the patients at the time of treatment, the absence of a control group in our study, and the fact that the differences observed were not significant due to the small number of TC patients grouped using each set of criteria. However, the high percentage of patients with the indeterminate form of Chagas' disease, the lower percentage with the cardiac form, and the absence of the digestive form of Chagas' disease in the TC group suggest a beneficial effect of benznidazole on the prognosis of the disease, although other factors, such as the age and stage of the infection when the patient was treated and the host immune response of the patient, must be taken into account. Moreover, in a previous study using some of these methods, we verified a better clinical evolution of Chagas' disease morbidity in patients (21/22) who received treatment while having the indeterminate form of the disease (24).

ACKNOWLEDGMENTS

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