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Use of the EIE-Recombinant-Chagas-Biomanguinhos Kit to Monitor Cure of Human Chagas' Disease

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We used the EIE-Recombinant-Chagas-Biomanguinhos kit (EIE-Rec kit) developed by the Oswaldo Cruz Foundation, Brazil, to monitor cure of chagasic patients who were treated during the acute phase of *T. cruzi* infection. Treated patients were previously studied by parasitological and serological tests and classified as cured patients (CP) (n = 10), dissociated patients (DP) (n = 6), and noncured patients (NCP) (n = 6). When sera of these patients were assayed by EIE-Rec kit all sera from NCP and all sera from CP showed positive and negative reactions, respectively. These results were in full agreement with those obtained previously by the classical tests. Two DP showed a positive reaction; the remaining four displayed a negative reaction, similar to that observed in sera from nonchagasic (NCh) individuals, and could therefore be considered CP. Our results suggest that the EIE-Rec kit could be used to monitor the efficacy of Chagas' disease treatment. J. Clin. Lab.Anal. 16:132–136, 2002.

Key words: Chagas' disease; serologic diagnostic follow-up; *Trypanosoma cruzi*; recombinant antigens; ELISA

INTRODUCTION

Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, is a major problem in Latin America, where some 16–18 million people are infected and 90 million are at risk of infection (1). *T. cruzi* induces in humans an acute-phase infection, with patent parasitemia, which is followed by a lifelong chronic phase characterized by subpatent parasitemia and scarce tissue parasitism. In the acute phase, diagnosis is based upon detection of these organisms by direct parasitological methods (thin or thick blood smears, the Strout method, and buffy coat on slide) (2). In the chronic phase, diagnosis is based primarily on conventional serology (CS) (complement fixation reaction (CF), indirect immunofluorescence (IIF), indirect hemagglutination (IHA), and enzyme-linked immunosorbent assay (ELISA)), or indirect parasitological methods (hemoculture (HC) and xenodiagnosis (XD)) (2).

Treatment of patients in the acute phase of the disease with the nitroheterocyclic drugs, benznidazole and nifurtimox, can prevent a fatal outcome and produce some cures. Cure in this phase is followed by CS becoming completely and consistently negative after a period of about 12 months. However, in the chronic phase negative seroconversion is slow, taking years to be completed and requiring a long follow-up to prove cure of chronic disease (3). The effectiveness of the treatment is evaluated using parasitological methods (HC and XD), and CS tests. The complement-mediated lysis-CoML test (4), which detects lytic antibodies against living trypo-

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mastigotes, has been linked to the presence of active infection and is also used for this purpose.

On the basis of the parasitological and serological tests, treated patients are classified as 1) cured patients (CP) when parasitological, CS and CoML tests are negative; 2) dissociated patients (DP) when parasitological and CoML tests are negative but are positive for at least two of the three conventional serological tests; and 3) noncured patients (NCP) when parasitological, CS, and CoML tests are positive. This classification evaluates the presence of active infection. Thus, CP and DP patients do not present active infection, but patients included in the DP group continue to produce antibodies against *T. cruzi* antigens detected by CS (5). A negative CoML predicts elimination of *T. cruzi*; however, this test has practical limitations since it requires live and infectious parasites. Thus, there is an urgent need for the development of serological tests that use purified *T. cruzi* antigens and hence do not recognize cross-reacting antibodies. Such tests should avoid the occurrence of positive conventional serological results after the parasite has been eliminated (5).

Several technical serological approaches using conventional ELISA with purified (6) and recombinant antigens (7), or using flow cytometry technology (8) instead of the CoML test have been evaluated.

Recently, we evaluated the performance of the EIE-Recombinant-Chagas-Biomanguinhos kit (EIE-Rec kit), developed by the Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, Brazil), for the diagnosis of *T. cruzi* infection using characterized serum samples from individuals living in Chagas' disease-endemic areas, and from individuals with other infectious diseases (9). This kit uses two recombinant proteins, CRA + FRA, as antigens and the reaction is carried out by a direct ELISA. Cytoplasmic repetitive antigen (CRA) is distributed throughout the cytoplasm and has a 14-amino acid repeat. Flagellar repetitive antigen (FRA) is located in the region of the flagellum that faces the body of the parasite and displays a 68-amino acid repeat (10,11). The EIE-Rec kit showed high sensitivity (100%) and high specificity (100%). In addition, the data obtained were in full agreement with clinical and conventional serology, and no cross-reaction was observed with serum of patients with other infectious diseases, including visceral and cutaneous leishmaniasis. These results led us to evaluate the use of the EIE-Rec kit to monitor cure by specific treatments in human Chagas' disease.

MATERIALS AND METHODS

Twenty-two patients from the Hospital das Clínicas of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, were treated with either 3-methyl-4(5′-nitrofurfurylideneamino)-tetrahydro-4H-1,4-thiazine-1-dioxide (nifurtimox, lampit; Bayer, São Paulo, Brazil) or N-benzil-1-nitro-1-imidazoleacetamide (benznidazole, rochagan; Roche, São Paulo, Brazil). These patients were previously analyzed by parasitological (HC or XD), CS (IIF, IHA, or CF), and CoLM tests (Table 1), with the following results: CP = 10, DP = 6, and NCP = 6 (12). Written informed consent was obtained from patients according to the guidelines of the Ethics Committee of the Fundação Oswaldo Cruz (Ministry of Health, Brazil). We evaluated these characterized sera using the EIE-Rec kit. As negative and positive controls we used sera from nonchagasic (NCh) individuals (n = 10, all of whom were laboratory members of Centro de Pesquisas Aggeu Magalhães, Fiocruz, Recife, Brazil), and chronically infected untreated patients (UTP) (n = 10, all from the Hospital Universitário Oswaldo Cruz, Recife, Brazil), respectively. Two assays were carried out for each individual serum samples.

The enzyme immunoassay, with the EIE-Rec kit, was performed according to the manufacturer's protocols. Microplates sensitized with the recombinant antigens were incubated with undiluted patient sera (50 μl) at 37°C for 30 min. After washes, the plates were incubated for 30 min at 37°C with 50 μl of peroxidase-conjugated antigens. After repeated washing cycles the immune complexes were revealed by the addition of hydrogen peroxide and 3, 3′, 5, 5′-tetramethylbenzidine. The reaction was stopped with 2 M H_2SO_4, and the optical density (OD) at 450 nm was determined in an ELISA reader (Bio-Rad 3550). The cut-off (CO) values and the gray zone were calculated according to the manufacturer. Sera with OD values equal to or greater than the CO value were considered reactive, and consequently considered positive for antibodies against *T. cruzi*. Sera with OD values below CO were considered nonreactive and negative for antibodies against *T. cruzi*.

RESULTS

The levels of antibodies measured by ELISA in individual serum samples from treated patients from different groups, as well as those measured in samples from NCh individuals and chronically infected UTP (control groups) were similar in two assays. These results reinforce the reproducibility of the EIE-Rec kit. Figure 1 shows the results of an assay. All sera from NCP (6/6) and all sera from CP (10/10) showed positive and negative reactions, respectively. These results were 100% in agreement with the classification measured previously by classical diagnosis methods (parasitological, CS, and CoML) (Table 1). Regarding the results from six DP, two patients (33.4%) (codes 14 and 17, Table 1) showed OD values above the CO values (0.580 and 0.265, respectively) and were considered positive (Fig. 1). The four remaining DP (66.6%) showed OD values (0.091, 0.085, 0.170, and 0.107) similar to those observed in NCh sera (Fig. 1) and could be considered CP. The gray zone was established in an interval of 0.210 and 0.252 ODs, but reactivity within this zone was not observed.

TABLE 1. Efficacy of treatment of the chagasic patients treated during acute phase of *T. cruzi* infection with years of follow-up and the results obtained by use EIE-Recombinant-Chagas-Biomanguinhos kit

Patients (codes)	Age (years)	Follow-up (years)	Tests			Efficacy of treatment (classification)	EIE-rec assay (results)
			Parasitol	CS	CoML		
1	41	23	–	–	–	CP	–
2	22	22	–	–	–	CP	–
3	43	19	–	–	–	CP	–
4	58	20	–	–	–	CP	–
5	38	22	–	–	–	CP	–
6	34	20	–	–	–	CP	–
7	44	17	–	–	–	CP	–
8	40	16	–	–	–	CP	–
9	18	15	–	–	–	CP	–
10	30	24	–	–	–	CP	–
11	28	14	–	+	–	DP	–
12	60	23	–	+	–	DP	–
13	29	22	–	+	–	DP	–
14	43	30	–	+	–	DP	+
15	29	17	–	+	–	DP	–
17	23	20	–	+	–	DP	+
18	24	19	+	+	+	NCP	+
19	19	18	+	+	+	NCP	+
20	41	16	+	+	+	NCP	+
23	75	17	+	+	+	NCP	+
24	26	21	+	+	+	NCP	+
25	40	22	+	+	+	NCP	+

Modified according to Bahia-Oliveira et al. (12).
Parasitol, parasitological; CS, conventional serology; CoML, complement-mediated lysis; CP, cured patient; DP, dissociated patient; NCP, noncured patient; +, positive test; –, negative test.

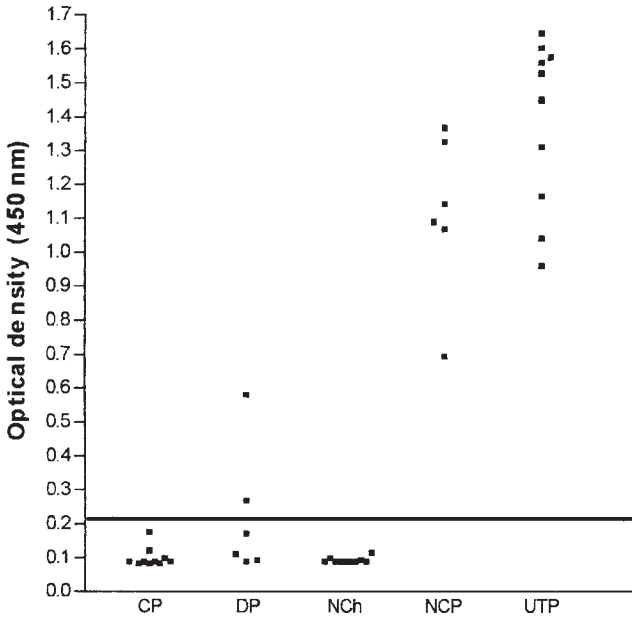

Fig. 1. Distribution of optical density values from treated chagasic patients evaluated by the EIE-Recombinant-Chagas-Biomanguinhos kit. The horizontal line inside the drops represents the cut-off values (0.210). CP = cured patients (n = 10); DP = dissociated patients (n = 6); NCP = noncured patients (n = 12); NCh = nonchagasic individuals (n = 10); UTP = untreated chagasic patients (n = 10).

DISCUSSION

CS tests are very useful in the diagnosis of chronic Chagas' disease, but they are not suitable for monitoring treatment effectiveness in this infection because they have limitations when used as a criterion of therapeutic cure. The CoML test that detects lytic antibodies, which are recognized by epitopes present on the surface of living trypomastigotes, has been used as an alternative method to determine cure. In a 10-year follow-up study of 82 treated patients, only seven patients (8.5%) could have been considered cured on the basis of the negative results of CS, even though 21 (25.6%) of them, consistently positive by CS, had negative CoML results. These patients, who demonstrated a discrepancy between the CoML and CS results, were described as dissociated and were considered cured (5). However, CoML is not a routine test because it requires careful management of living infective trypomastigotes, it has many variables that are difficult to control and standardize (such as the count of live and motile parasites), and it needs human serum as a source of complement, which may vary from one donor to another. In addition, live target cells can be lysed by heterophylic antibodies (13). It should also be stressed that heterologous strains can offer different sensitivities (14). These facts may be responsible for the difficulties reported by some laboratories in relation to reproducibility of CoML.

According to Cançado (3), negative CoML by itself is not a criterion of cure of Chagas' disease, because the absence of living trypomastigotes does not necessarily mean absence of active infection. The parasite may persist in tissues of the mammalian host in the amastigote form. Even circulating in the peripheral blood, these parasites would not stimulate the production of lytic antibodies, but could be the cause of the uniformly positive CS in the great majority of the treated patients with long-standing Chagas' disease. Furthermore, Cançado (3) emphasizes that the control of cure based on CS is important for several reasons: 1) Parasitological tests (XD and HC) have poor sensitivity, are slow, and are only available in research institutions. 2) It is impossible for a negative CS test to occur with a positive parasitological test, except possibly in the case of immunosuppression. 3) In the acute disease phase, CS is the universally accepted criterion of cure. 4) All research on refined antigens to be used for serological evaluation of chemotherapy use as their gold standard for cure patients who present consistent negative CS tests. Positive CS conclusively demonstrates the presence of *T. cruzi* in the patient's tissues, i.e., Chagas' disease (3).

Why CS remains positive for long periods is not clear; however, some hypotheses are suggested: 1) Mice infected with *T. cruzi*, but parasitologically cured after specific chemotherapy, continue to exhibit positive IIF tests 3–6 months after treatment due to parasite antigens trapped by certain cells in the spleen (15). 2) Studies showed that a high proportion of antibodies detected by CS, in treated chagasic patients, are directed against the carbohydrate residue Galα1->3 Gal, a determinant also recognized by antibodies from noninfected healthy individuals (6). Because of their wide distribution among microorganisms of intestinal and pulmonary microflora, these determinants may keep stimulating lymphocytes previously primed by *T. cruzi* Galα1->3 Gal epitopes, thereby accounting for false-positive results in cured patients. This fact suggests that the use of an antigen recognized by sera of most chronically infected chagasic patients that do not express Galα1->3 Gal residues might be useful in eliminating possible cross-reactive antibodies, and, therefore, false-positive reactions (6).

In the present report we evaluated the use of the EIE-Rec kit, which uses two specific *T. cruzi* recombinant antigens (CRA + FRA), to monitor cure in 22 treated patients, classified as CP, DP, or NCP on the basis of parasitological CS and CoML tests (Table 1). The following results were obtained: 1) All sera from NCP and all sera from CP showed positive and negative reactions, respectively. These results were in complete agreement with the classification previously determined using classical methods (parasitological, CS, and CoML). 2) The analysis of six DP sera showed that two of them (33.4%) displayed positive reaction and can be considered NCP, while the remaining four (66.6%) were negative and can be considered CP. In the light of the specificity (100%) of the EIE-Rec kit (9,11), the positive and negative reactions showed by the DP are not likely to be false reactions. In addition, for the four DP who showed negative reactions, the results were in agreement with previous parasitological and CoML tests that showed negative reactions. Taken as a whole, these results are 90.9% in agreement with the classical diagnosis methods, which paves the way for using the EIE-Rec kit in initial trials to evaluate cure of treated chagasic patients.

In a previous study, a new approach using flow cytometry to detect anti-live trypomastigote antibodies and monitor the efficacy of specific treatment in human Chagas' disease was shown to be more sensitive and accurate than CoML (8). However, that method requires expensive equipment (FACScan) which is not commonly available in laboratories. In contrast, the EIE-Rec kit presents several advantages, including: 1) the use of specific *T. cruzi* recombinant antigens avoids false-positive reactions; 2) the direct ELISA procedure increases the sensitivity of the method, allowing the evaluation of low-titer sera and corroborating its specificity; and 3) the use of undiluted sera samples reduces the possibility of error due to manipulation, requiring as equipment only an ELISA reader.

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