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Evaluation of Gelatin Particle Indirect Agglutination Test for Serodiagnosis of Chagas' Disease

HIROMU TOMA[1,3], JUN KOBAYASHI[1,3], MARIA E. GUARIENTO[4], EROS A. DE ALMEIDA[4], YOSHIYA SATO[1,2] AND TAKAO YAMASHITA[5]

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Abstract

The indirect agglutination test using gelatin particles (GPAT) was evaluated for the routine serodiagnosis of Chagas' disease by comparing the results with those of commercially available kits of indirect hemagglutination and enzyme immunoassay. The sensitivity and specificity of the test seemed satisfactory, producing strong positive antibody responses in all patients' sera but not in the control subjects. The agglutination titers were also significantly higher in the GPAT than in the hemagglutination test compared. On the other hand, the cross-reactions with other parasitic infections including *Leishmania* infection were negligibly low. The GPAT seems to have great value for laboratory diagnosis and screening mass survey of Chagas' disease, because of its rapid manipulation without specialized equipment and preservation of antigen-particles for a long period.

Key words: serodiagnosis, Chagas' disease, *Trypanosoma cruzi*, gelatin particle agglutination test (GPAT)

Introduction

American trypanosomiasis, Chagas' disease, is a major public health problem in rural areas of Central and South America. An acute phase of the disease is characterized by high blood parasitemia and extensive tissue parasitism. Following the acute phase, however, the parasitemia usually subsides into a chronic phase in which parasites persist at rarely detectable levels. Thus, the detection of specific antibodies is probably the best method for the identification of such a chronic infection.

There have been many attempts to develop practical serologic techniques for the diagnosis of Chagas' disease. Most of these efforts have employed the complement fixation test (Fife and Kent, 1960; Pereira *et al.*, 1980), indirect immunofluorescence (Fife and Muschel, 1959; Camargo, 1966; Araujo and Batista, 1969; Cerisola *et al.*, 1970), indirect hemaggluti-nation (Cerisola, 1970; Neal and Miles, 1970; Kagan *et al.*, 1978; Schmunis *et al.*, 1980) and enzymatic assay (Spencer *et al.*, 1980). However, the most reliable routine serologic test may be the indirect agglutination, because of its relative simplicity and lack of requirements for special equipment. The hemagglutination technique has been successfully applied to the diagnosis of Chagas' disease. The test, however, has some disadvantages in the preservation of sensitized erythrocytes and in the necessity to absorb natural antibodies to carrier erythrocytes. Recently, the indirect agglutination test using artificial gelatin particles has been successfully used for serodiagnosis of strongyloidiasis and schistosomiasis (Sato and Ryumon, 1990; Sato *et al.*, 1991; Kobayashi *et al.*, 1994). In the application for serodiagnosis of Chagas' disease, the agglutination test is also recognized as sensitive and reliable (Yamashita *et al.*, 1994).

In the present study, the authors further tried to evaluate diagnostic efficacy of the particle agglutination test, in comparing the results with those of commercially available kits of hemagglutination

[1)]*Department of Parasitology, and* [2)]*Research Center of Comprehensive Medicine, School of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan.*

[3)]*Gastrocenter, and* [4)]*Department of Internal Medicine, Faculty of Medical Sciences, State University of Campinas, Campinas, SP, Brazil.*

[5)]*Department of Immunology and Parasitology, School of Medicine, Yamagata University, Yamagata, Japan.*

當眞 弘, 小林 潤, 佐藤良也 *(琉球大学医学部寄生虫学教室; *同地域医療研究センター)

Maria E. Guariento, Eros A. de Almeida (State University of Campinas, Brazil).

山下隆夫 (山形大学医学部免疫・寄生虫学教室)

and enzyme immunoassay which have been used for the routine serodiagnosis of Chagas' disease.

Materials and Methods

Sera

Sera were obtained from 42 patients who were diagnosed positive for Chagas' disease on the basis of clinical and serological (indirect immunofluorescence) evidences in University Hospital, State University of Campinas (UNICAMP), SP, Brazil. These include 9 patients in the indeterminate phase of the disease, showing no symptom attributable to *T. cruzi* infection, 17 with cardiopathy, 4 with megadisease, and 9 with both cardiopathy and megadisease. As a control, 18 sera from presumably noninfected Brazilian adults with no known history of the disease was used for the study. The incidence of anti-*T. cruzi* antibody by the GPAT was determined on 144 serum samples from residents in Maceió, Alagoas State, Brazil and on 100 samples from patients with gastroenterological disorders in the "Gastrocenter", UNICAMP. To determine cross-reaction with other parasitic infections, 186 serum samples from patients with clonorchiasis sinensis in Taiwan, opisthorchiasis viverrini in Thailand, hookworm disease in Thailand, strongyloidiasis stercoralis in Japan and Thailand, schistosomiasis mansoni in Brazil and Central Africa, and leishmaniasis in Ecuador were also tested in the present study.

Antigen of *T. cruzi*

Antigen extracts were prepared from *T. cruzi* epimastigotes cultured in a liquid medium. The same antigen previously prepared as follows by Yamashita *et al.* (1994) was used in the present study. Freshly washed epimastigotes were disintegrated by ultrasonication in PBS containing 1% Triton-X, 1 mM phenylmethylsulfonyl fluoride and 10% glycerol. After centrifuging at 10,000 ×g for 1 hr, the supernatant fluid was used as antigen.

Gelatin particle agglutination test (GPAT)

The test was performed with artificial gelatin particles coated with the above antigens. The particles were kindly supplied by Fujirebio Inc. (Tokyo, Japan). The procedures for the GPAT was the same as those originally described by Sato and Ryumon (1990) for strongyloidiasis and applied for Chagas' disease by Yamashita *et al.* (1994). Briefly, the particles were treated with 0.001% tannic acid solution for 30 min at room temperature. The tanned particles were then sensitized with *T. cruzi* antigens by mixing equal volumes of 3% particle suspension and 200 μg/ml *T. cruzi* antigen for 1 hr at room temperature. After washing, the antigen-particles were finally suspended at 1% in 1% BSA-PBS for use.

For the estimation of agglutination titers, a microtiter technique using a plastic microplate with U-bottomed wells was used. One drop (25 μl) of the antigen-particle suspension was mixed in the wells with an equal volume of test serum diluted in a serial 2-fold dilution. The particles were allowed to settle for 3 hr and the settling patterns at the bottom were read. The antibody titers were determined as the highest serum dilution giving a positive agglutination pattern. The sera which showed positive responses in dilution of 1:16 or more were considered to be antibody positive.

Indirect hemagglutination test (IHAT)

The IHAT kits for Chagas' disease using fixed erythrocytes sensitized with *T. cruzi* antigen was purchased from Polychaco S.A.I.C. (HAI Chagas: Buenos Aires, Argentina) and Wiener Lab. (Chagatest HAI: Rosario, Argentina). The procedures to estimate agglutination titers were almost the same as those of the above GPAT, although serum diluent containing 1.0% 2-mercaptoethanol (2-ME) was used in the case of IHAT to inactivate natural antibodies of IgM class to the carrier erythrocytes. According to the instrument manual of the kit, serum samples which showed agglutination patterns in serum dilution of over 1:16 were interpreted to be positive.

Enzyme immunoassay (EIA)

ABBOTT Chagas enzyme immunoassay (ABBOTT Laboratory, Sao Paulo, SP, Brazil) was used as follows in the present study. The serum samples were incubated with a *T. cruzi* antigen-coated polystyrene bead in wells of a reaction tray at 30C for 30 min. After washing, peroxidase conjugated goat anti-human IgG was added to the

bead in each well and incubated similarly. The bead was then transferred to an assay tube and substrate (O-phenylenediamine) solution was added to the bead. Following the incubation of the bead and substrate, the antibody levels were determined by measuring spectrophotometrically the absorbance value of color which developed in proportion to the amount of antibody bound to antigen on the bead. The specimens with absorbance values of more than the cutoff value (0.500 at 429 nm) which were calculated according to the positive and negative control values were considered to be positive for antibodies to *T. cruzi*.

Additional two EIA kits from Wiener Lab. and Polychaco S.A.I.C., Argentina, were also compared in their results with the GPAT. The tests were performed using a microplate, according to the instrument manuals for the kits.

Results

Frequencies of GPAT titers in 42 patients and 18 uninfected controls are shown in Fig. 1. All patients showed positive agglutination response in serum dilution of 1:32 or more, whereas negative results were demonstrated at the lowest serum dilution of 1:8 in all of the control subjects. Log_2 reciprocal titers in the patients ranged from 5 to 19, the majority showing from 9 to 15. On the basis of our criteria titer (log_2 reciprocal titer of 4) for the positive antibody response, it was determined that the GPAT was positive for all of the patients but negative for the control subjects.

Table 1 shows the comparative results between GPAT and the other tests, IHAT and EIA, which are currently used for the routine serodiagnosis of Chagas' disease. Although all patients' sera showed positive antibody response in the GPAT, there were few false-negative results in kits from Wiener Lab. and Polychaco S.A.I.C. As to the control subjects, 15 serum samples showed positive agglutination response in dilution of 1:16 or more in the Polychaco kit. Their titers, however, were extremely low, showing that 11 out of the 15 samples were positive in only 1:16 of serum dilution in the presence of 2-ME. On the other hand, when the kit from Wiener Lab. was tested, 17 out of the 60 samples tested produced non-specific agglutination response of non-sensitized erythrocytes in serum dilution of 1:8. It was necessary for these samples to be absorbed with

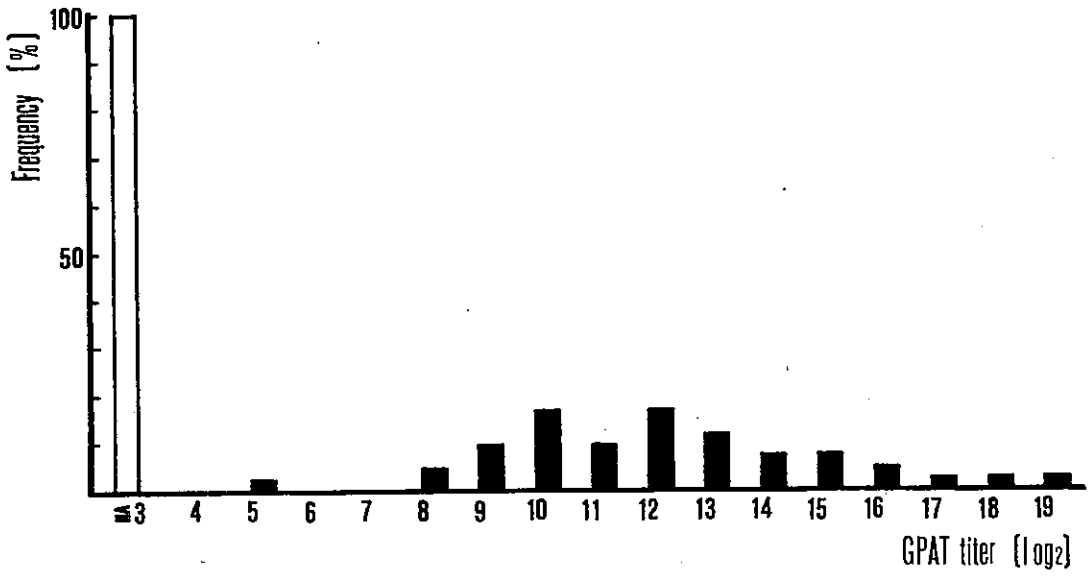

Fig. 1 Percent frequency distributions of agglutination titers with the antigen-coated gelatin particles in 42 patients with Chagas' disease ( ) and in 18 uninfected controls ( ) in Brazil.

Table 1 Comparative results among GPAT, IHAT and EIA for Chagas' disease obtained by testing sera from 42 patients with Chagas' disease and 18 uninfected controls in Brazil

Serological test	No. positive case (%)	
	Patients (n=42)	Controls (n=18)
GPAT	42 (100)	0 (0)
IHAT		
Wiener Lab. (Chagatest HAI)	36 (86.7)	0 (0)
Polychaco (HAI Chagas)	41 (97.6)	15 (83.3)*
EIA		
ABBOTT (Chagas EIA)	42 (100)	0 (0)
Wiener Lab. (Chagatest ELISA)	42 (100)	0 (0)
Polychaco (BIOZIMA-Ch)	42 (100)	0 (0)

*Eleven positive sera showed agglutination titers of only $\log_2 4$ and the remaining 4 sera showed titers of $\log_2 5$ and $\log_2 6$ in the presence of 1% 2-ME (see Fig. 3)

non-sensitized erythrocytes before estimation of their antibody titers. The agglutination patterns of these IHAT and the GPAT in a patient and a control subject are shown in Fig. 2. In the case of EIA, all patients' sera yielded a positive response in all the three tests but none of the control subjects gave positive responses, resulting in complete agreement with the GPAT.

Fig. 3 represents the correlation of GPAT titers and IHAT titers on the patients. A significant correlation was found between the titers by the two tests. The IHAT titers, however, were generally lower than those of the GPAT (Fig. 4). The mean titer of the GPAT was as high as two times that of the IHAT from Wiener Lab. On the other hand, when comparing GPAT and EIA (Fig. 5), there was no significant correlation between the two tests. In the case of EIA, however, serum samples from 16 patients showed an absorbance value over the upper limit of the measurable range of 2.0. Excluding the over range values, the correlation coefficient was 0.416 ($P<0.05$, $n=24$).

Fig. 6 represents a comparison of GPAT antibody titers in different clinical forms of Chagas' disease. The antibody levels were quite high in these patients, but there was no significant difference in mean antibody levels among the clinical forms.

The reactivity of sera from patients with other parasitic infections, when tested by the GPAT, is shown in Table 2. The patients with parasitic infections other than schistosomiasis and leishmaniasis did not show any positive response in the GPAT for Chagas' disease. In the cases of schistosomiasis, 7 out of 53 Brazilian patients reacted in the GPAT showing $\log_2$ reciprocal titer of over 6, whereas none of the African patients showed cross-reaction. When 22 samples from patients with leishmaniasis were tested, the positive response with low titer of $\log_2 4$ or $\log_2 5$ was only observed in 5 patients.

The GPAT was performed on 114 sera from Brazilian residents of Maceió, Alagoas State and on 100 samples from patients with any gastroenterological disorders (Table 3). The positive rates were 7.0% in residents in Maceió where Chagas' disease is endemic and 4.0% in patients in the "Gastrocenter" of UNICAMP. Among the 8 persons positive in the GPAT in Maceió, 2 persons were negative in the ABBOTT EIA. In the "Gastrocenter" 4 patients were positive in each test, but one patient was negative with the others showing the concordance of 98.0%. Unfortunately, these positive cases could not be examined to obtain any parasitological information related to *T. cruzi* infection.

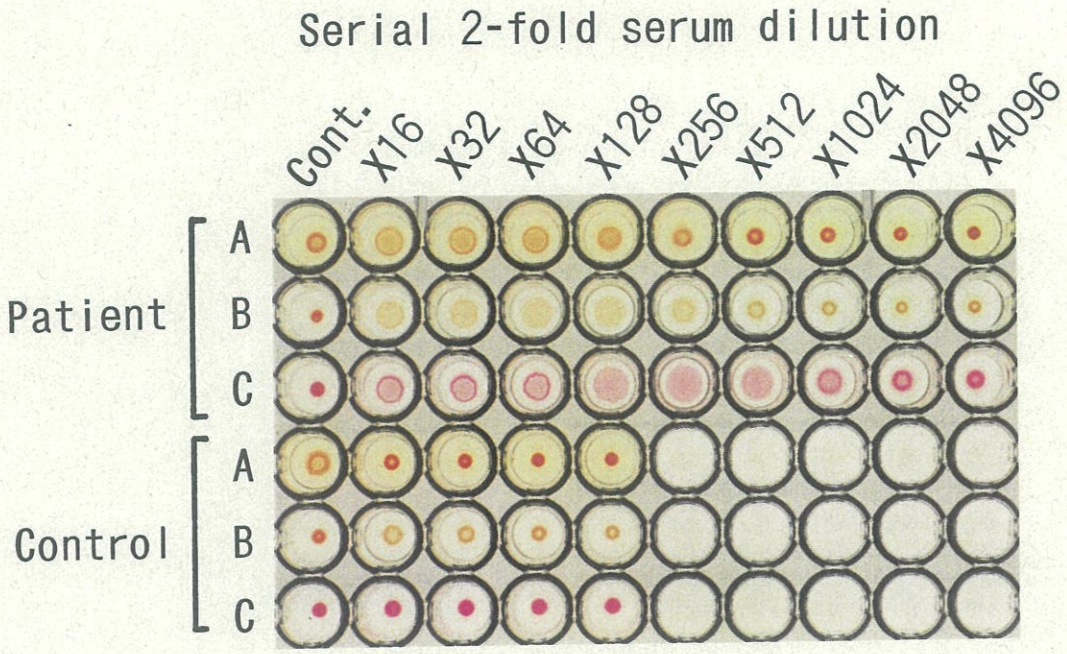

Fig. 2 Comparison of agglutination patterns of the GPAT with commercial IHAT kits from Wiener Lab. and Polychaco SAIC in sera from a patient and a control subject. The highest agglutination response was observed in the GPAT in the patient's serum. On the other hand, equivocally positive patterns were observed in control wells with non-sensitized erythrocytes from Wiener Lab. A weak positive pattern was also observed in control serum with sensitized erythrocytes from Polychaco SAIC kit in the presence of 2-ME.
A: Wiener IHAT, B: Polychaco IHAT, C: GPAT, Cont.: Control well (×8 serum dilution) with non-sensitized particles.

Discussion

The purpose of this study was to compare the sensitivity and the specificity of the GPAT with those of the other ordinary serologic tests for the diagnosis of Chagas' disease.

The sensitivity of the present GPAT was significantly higher than that of IHAT in which few cases produced false-negative results and their mean titer value was extremely low compared to that of the GPAT. On the other hand, the sensitivity did not differ from that of the EIA when the overall rates of positive reactions among patients' sera clinically diagnosed to be Chagas' disease were compared; in sera of 42 patients with Chagas' disease, positive antibody responses were demonstrated in all of them by the GPAT and EIA. When each test was evaluated for specificity by testing sera from individuals presumed to be normal, the results completely agreed by not giving any false-positive reactions in each test, with the exception of the IHAT kit from Polychaco SAIC which caused non-specific weak responses in many samples.

In comparison of GPAT and IHAT responses, the titers of GPAT were well correlated with those of IHAT, although the application of the paired *t*-test to the data showed that the geometric mean titer of reactions in the GPAT was significantly higher than that of reactions in the IHAT. The different agglutination responses in the two tests were probably due to the different antigenic activity of each antigen-particle preparation used. The lower titers in the IHAT might have contributed to the false-negative results produced in a few cases.

On the other hand, a weak but significant correlation was also observed between GPAT and EIA

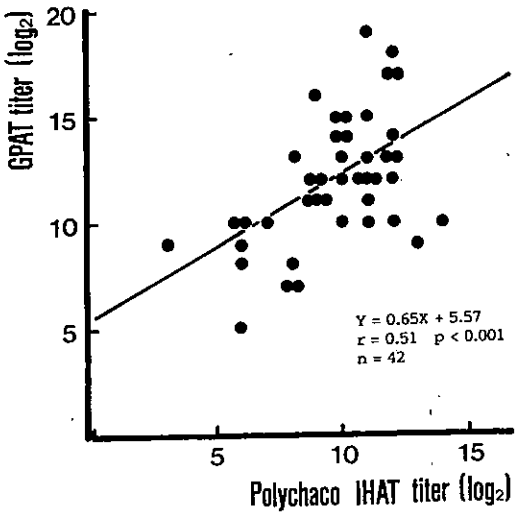

Fig. 3 A scatter diagram of GPAT and IHAT values and correlation between the two tests performed on 42 patients with Chagas' disease. Reciprocal Log_2 GPAT titers were plotted against the Polychaco IHAT titers.

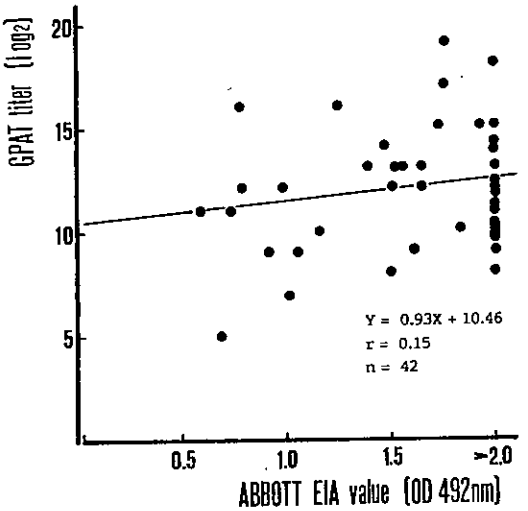

Fig. 5 A scatter diagram of GPAT and EIA values and correlation between the two tests performed on the same patients in Fig. 2. The GPAT titers (Log_2) were plotted against ABBOTT EIA values.

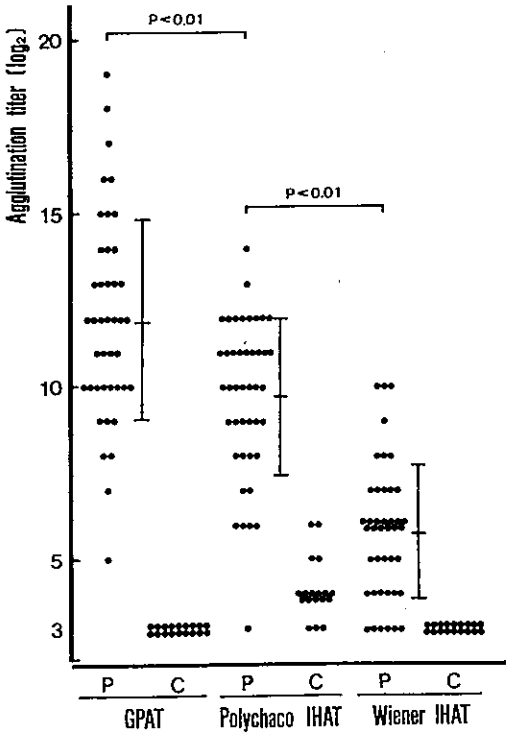

Fig. 4 Comparison of agglutination titers in the GPAT and IHAT. The GPAT titers were compared with those of two commercial IHAT kits from Wiener Lab. and Polychaco SAIC. Vertical line represents mean±SD.
P: Patients; C: Controls

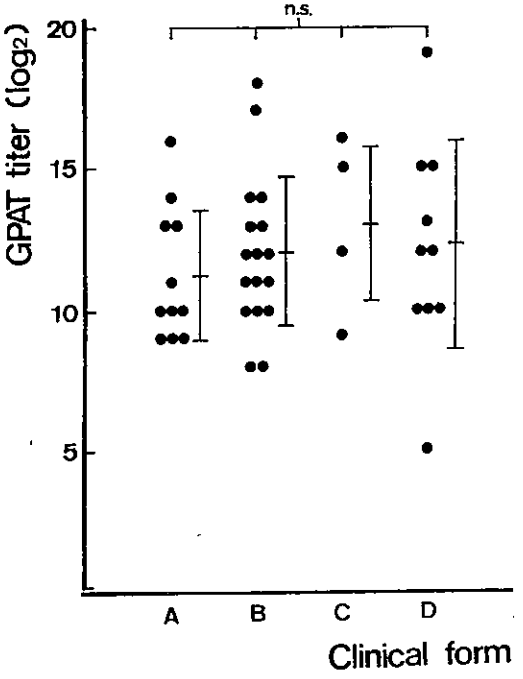

Fig. 6 Comparison of the GPAT titers between four clinical forms with Chagas' disease. Vertical line represents mean±SD.
A: Indeterminated; B: Cardiopathy; C; Megadisease; D: Cardiopathy and megadisease

Table 2 Reactivity in other parasitic infections tested by the GPAT for Chagas' disease

Type of infection	Titer (log_2) in Chagas GPAT	No. serum tested
Clonorchiasis sinensis (Taiwan)	≦3	24
Opisthorchiasis viverrini (Thailand)	≦3	20
Opisthorchiasis & hookworm disease (Thailand)	≦3	12
Hookworm disease (Thailand)	≦3	4
Strongyloidiasis stercoralis (Japan)	≦3	20
Strongyloidiasis (Thailand)	≦3	8
Strongyloidiasis & opisthorchiasis (Thailand)	≦3	12
Schistosomiasis mansoni (Africa)	≦3	4
Schistosomiasis & hookworm disease (Africa)	≦3	7
Schistosomiasis (Brazil)	≦3	46
	6	1
	7	1
	8	4
	9	1
Leishmaniasis (Ecuador)	≦3	17
	4	4
	5	1

Locality where sera were collected was parenthesized.

Table 3 Incidence of anti-*T. cruzi* antibody by GPAT and ABBOTT EIA for Chagas' disease in sera from two groups of Brazilian adults

Subjects	No. exam.	No. positive (%) by		Concordance
		GPAT	ABBOTT EIA	
Residents in Maceió	114	8 (7.0)	6 (5.3)	98.2%
Patients in "Gastrocenter"	100*	4 (4.0)	4 (4.0)	98.0%

*Patients with gastroenterological disorders in "Gastrocenter", State University of Campinas (UNICAMP), Brazil

titers, when serum samples with unmeasurable EIA value over 2.0 were excluded. In addition to the probable difference in antigenic activity of antigen preparations used, the assay system is very different in these tests. For example, the responses in IgG and IgM classes can be detected by the GPAT but the EIA detects only IgG response. Nevertheless, the results were well consistent in both tests.

Sera from patients with other parasitic infections were also examined by the GPAT to determine the specificity of the test. Strong positive responses were observed among few patients with schistosomiasis in Brazil. The positive responses, however, were unlikely to be cross-reaction because the majority of other Brazilian patients did not show such a positive response and also because none of

the African patients who live in nonendemic area for Chagas' disease reacted in the GPAT. The positive responses in these patients were suspected to be due to mixed *leishmania* infection. The weak cross-reactive positive reactions also occurred in sera from few patients with leishmaniasis in Ecuador. The responses, however, were negligibly low in the GPAT. The sera from patients with various other parasitic infections showed no particular pattern that would suggest consistent cross-reactivity. The cross-reactions in sera from leishmaniasis patients seem to correlate with the antigen preparation used rather than the assay itself. By using a purified specific antigen, the cross-reaction can be expected to decrease (Camargo and Rebonato, 1969).

The results here presented indicate that the GPAT can thus be recommended as a reliable serologic test for Chagas' disease, as has already been suggested by Yamashita *et al.* (1994). The indirect agglutination test is technically simple to perform, requires no specialized equipment and/or facility, and can be completed within a few minutes. Therefore, the test using antigen-coated erythrocytes has been used for routine serodiagnosis of Chagas' disease in ordinary laboratories in South America. The test, however, suffered initially from the unstable and variable nature of the antigen-coated erythrocytes. The limited period of storage of antigen-carrier particles has imposed some practical limitations on the use of the test for routine serodiagnosis and several attempts have been made to improve the stability and storage life of the carrier particles, including the use of fixed erythrocytes (Camargo *et al.*, 1971; Hoshino-Shimizu *et al.*, 1978; Camargo *et al.*, 1973) and inert artificial particles (Pellegrino and Katz, 1970). In the present study, recently developed artificial spherical particles were used as the antigen carrier. Because the particles are non-antigenic, they can be used without prior absorption of test serum or addition of 2-ME in diluted serum, which are essential to prevent a nonspecific response by a natural antibody in the case of IHAT. Moreover, the particles are colored and therefore convenient for reading the settling pattern, compared to the other artificial particles, such as latex beads. The particles can also be lyophilized without visible morphological change and the sensitized particles can be stored lyophilized for long periods without detectable loss of activity. Thus, the indirect agglutination test using gelatin particles can be applicable not only in parallel testing with any other examinations for the diagnosis of *T. cruzi* infection but also in a screening field survey.

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