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GELATIN PARTICLE INDIRECT AGGLUTINATION TEST FOR SERODIAGNOSIS OF SCHISTOSOMIASIS: COMPARATIVE STUDY WITH ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY

A new serological test, the gelatin particle agglutination test (GPAT), was used for the serodiagnosis of schistosomiasis mansoni. This technique showed the sensitivity (90.6%) and specificity (97.8%) close to those of enzyme-linked immunosorbent assay. The GPAT can be easily and rapidly performed without specialized equipment, by using lyophilized antigen-coated gelatin particles. The test also seems to be useful for mass screening of *Schistosoma* infection in field conditions.

KEYWORDS: *Schistosoma mansoni*; Serodiagnosis; GPAT (gelatin particle agglutination test); ELISA.

INTRODUCTION

Many attempts have been made to develop a serological test for the diagnosis and epidemiological studies of schistosomiasis; these have included a complement fixation (CF) [13, 19], indirect hemagglutination (IHA) [1, 8, 14, 22] and indirect immunofluorescence (IF) [3, 19, 28, 29]. The enzyme-linked immunosorbent assay (ELISA) is also currently used for the serodiagnosis with reliable results [2, 5, 7, 9, 21, 27]. The ELISA, however, has some disadvantages for field survey and for diagnosis in the laboratory of terminal hospital, because it involves several experimental steps which are difficult to perform under such conditions.

Recently, a simple indirect agglutination test using newly developed gelatin particles has been used to detect antibodies specific for human immunodeficiency virus (HIV) [31], human T cell leukemia virus type-I (HTLV-I) [10] and *Mycobacterium leprae* [11]. The authors have also successfully used the gelatin particles in the indirect agglutination test for strongyloidiasis in Japan [23, 25].

In the present study, we further tried to diagnosis schistosomiasis mansoni by gelatin particle indirect agglutination test (GPAT).

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MATERIALS AND METHODS

Human subjects

Diagnosis of schistosomiasis mansoni in 64 patients was based on stool examinations by the Kato-Katz method, physical examination and on ultrasonographic findings at the Hospital das Clínicas, Universidade de São Paulo. Four groups were included as controls: 46 Brazilian patients with gastroenterological disorders unrelated to schistosomiasis; 10 Japanese patients with strongyloidiasis; 9 clinically healthy Japanese; and 26 Thai villagers with various parasitic infections such as, opisthorchiasis, strongyloidiasis, trichuriasis, taeniasis and hookworm disease.

Antigen preparation

Crude *Schistosoma mansoni* antigen was prepared from adult male and female worms. The worms were recovered from mice experimentally infected with Brazilian *S. mansoni* strain. The worms were ground with a Teflon homogenizer and then further fragmented by sonication (ultrasonic processor VP-5, TAITEC) at 4°C. The worm fragments in suspension were centrifuged at 10,000 rpm for 30 minutes. The supernatant was then passed through a 45μ-filter (Millipore Corp., Bedford, MS), aliquoted and frozen at -80°C. The protein concentration was measured by a dye-binding assay kit (Bio-rad Lab., Richmond, CA) and adjusted to 100 μg/ml with 0.15 M phosphate buffer, pH 7.2, for GPAT and to 10 μg/ml with 0.05 M carbonate buffer, pH 9.6, for ELISA.

Gelatin particle agglutination test (GPAT)

The gelatin particles were kindly supplied by Fujirebio Inc., Tokyo, Japan. The particles were washed four times by centrifugation with an excess of PBS, adjusted to a 3% suspension and mixed with an equal volume of 10^{-5} tannic acid (Merck, Darmstadt, Germany) dissolved in PBS. The mixture was stirred for 30 minutes at room temperature. The tanned particles were then washed three times, suspended in order to provide a 3% suspension and poured into a tube containing an equal volume of antigen solution. The mixture was allowed to stir for one hour at 37°C. After washing four times with PBS containing 1.0% bovine serum albumin, the particles suspension was adjusted to a final concentration of 1%.

The GPAT was a microtiter technique using a plastic microplate with U bottomed wells. A drop (25 μl) of PBS containing 0.6% normal rabbit serum was placed into each well using a calibrated pipette dropper and a test serum was diluted in the wells by a serial two fold dilution.

Then, a drop of antigen-particle suspension was added to each well and the plate was shaken to suspend the particles. The particles were allowed to settle for 3 hours and the results interpreted according to their settling patterns.

Enzyme-linked immunosorbent assay

The ELISA procedure was almost the same as that previously described for strongyloidiasis [24]. The sensitization of plastic plate (MS-3596F/H, Sumitomo Barkelite Co., Tokyo, Japan) wells with antigen was performed for 2 hours at 37°C and subsequently overnight at 4°C. The wells were washed three times with 0.05% Tween 20-PBS and then treated with 1% bovine serum albumin in PBS (BSA-PBS) for 2 hours at 37°C to block free binding sites. After washing, serum samples (0.3 ml) were added to each well and incubated for 2 hours at 37°C. Then, the test was completed using peroxidase-conjugated antiserum (goat anti-human IgG; Capell, East Chestar, PA), diluted 1:400 with BSA-PBS, and 0.01% orthophenylenediamine (Eastman Kodak Co., Rochester, NY) as the substrate. Each serum sample was tested at a single dilution of 1:50 with 1% BSA-PBS and the net absorbancy of each serum sample was read by a spectrophotometer (MPR-A4i, Tosoh, Japan) at 492 nm.

Statistics

Statistical significance of the differences of ELISA values between two groups was analyzed by the Student t-test. Association between ELISA values and GPAT titers was evaluated using Spearman's coefficient of rank correlation. A probability (P) less than 0.05 was considered to be significant.

RESULTS

The results obtained in sera from schistosomiasis patients and on four control groups by ELISA are presented in Fig. 1. The mean ELISA value of the patients serum group was significantly higher (t Student test) than those of the control groups. When the mean value plus 3 standard deviations (S.D.) of the Brazilian controls (Group B) was stipulated as a criterion for cutoff (dash line), 6 sera from schistosomiasis patients (9.4%) were considered as false-negative in ELISA. On the other hand, all sera except one from control groups were negative for anti-*Schistosoma* antibody.

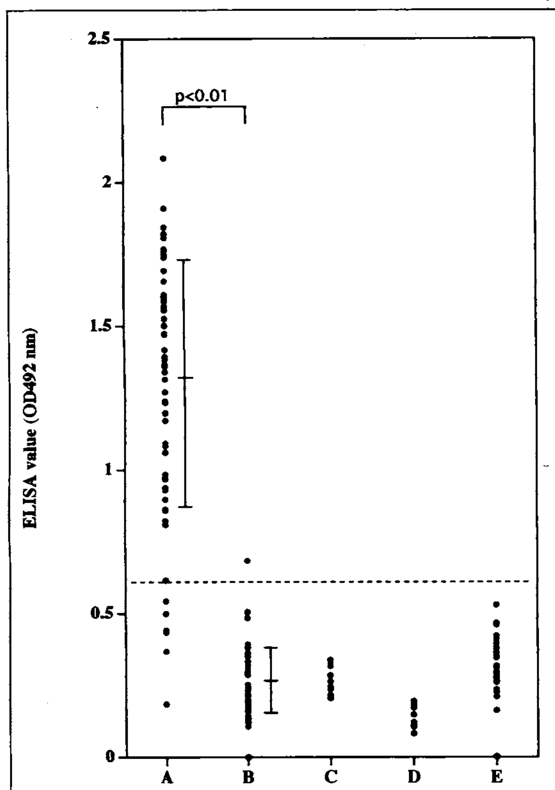

Fig. 1 - Results obtained in patients with schistosomiasis and in four control groups by ELISA.
A: 64 Brazilian patients with schistosomiasis (Hospital das Clínicas, University of São Paulo). B: 46 Brazilian patients with gastroenterological disorders unrelated to schistosomiasis (Gastrocentro, State University of Campinas). C: 10 Japanese patients with strongyloidiasis. D: 9 healthy Japanese. E: 26 Thai villagers with different parasitic infections other than schistosomiasis.
Dashed line: Cutoff value (mean + 3S. D. of Group B)

The results of GPAT in the study of the same sera are shown in Table 1. The results were similar to those of ELISA, showing that more than 90% of patient sera gave positive agglutination results at dilutions higher than 1:16. Whereas in the control group, 5 sera (one from Brazil and 4 from Thailand) showed positive results at lower dilutions, not exceeding 1:16 and 1:32.

The distribution of the frequency of antibody titers found in sera from patients and control subjects by GPAT are shown in Fig. 2. Two distinct serologic profiles with respective peaks were observed. It can be seem that the majority of sera from control subjects comprised the first peak, whereas those of the patients comprised the second peak. These results allowed to establish the criteria for the GPAT as follows: $\geq \log_2 5$: positive; $\log_2 4$: equivocal; and $\leq \log_2 3$: negative

In Fig. 3, the association between antibody levels assessed by ELISA and GPAT is represented based on data obtained from 64 patients with schistosomiasis. The results of the GPAT agreed precisely with those of ELISA, since 6 patients were negative and the remaining 58 patients were positive in both tests. There was a significant correlation between the results given by these tests.

Finally, the specificity of the GPAT was determined with the sera of Thai villagers who had various helminthic infections other than schistosomiasis. As seen in Table 2, four subjects with *Opisthorchis* (liver fluke), *Trichuris* and hookworm infection showed posi-

TABLE 1

Distribution of GPAT titers in patients with schistosomiasis and in controls.

GPAT titer($\log_2$)	≤3	4	5	6	7	8	9	10	11
Patients with schistosomiasis (n=64)	6 (9.4%)	3 (4.7%)	3 (4.7%)	5 (7.8%)	12 (18.8%)	13 (20.3%)	15 (23.4%)	5 (7.8%)	2 (3.1%)
Patients with gastroenterological disorders (n=46)	45 (97.8%)	1 (2.2%)							
Patients with strongyloidiasis (n=10)	10 (100.0%)								
Healthy Japanese (n=9)	9 (100.0%)								
Thai villagers with other parasitic infections (n=26)	22 (84.6%)	3 (11.5%)	1 (4.4%)						

( ):% frequency

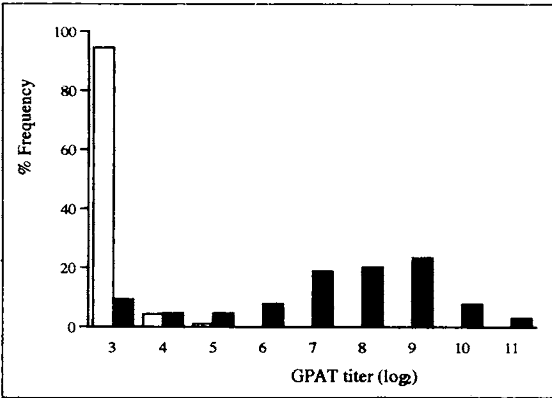

Fig. 2 - Frequency of the distribution of GPAT titers from 64 patients with schistosomiasis (■) and from 91 controls (□).

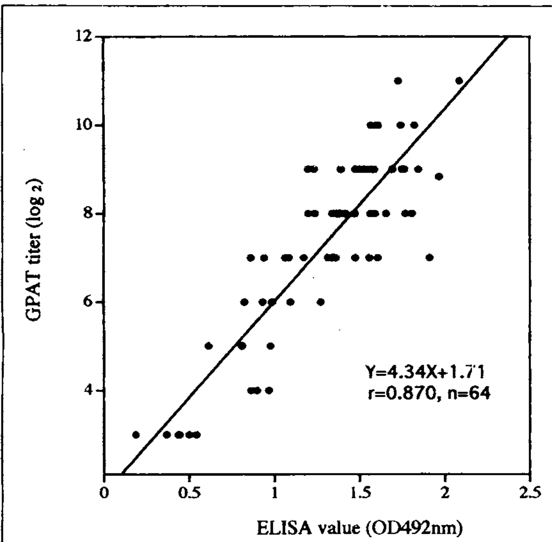

Fig. 3 - Correlation between GPAT titers and ELISA values in the studied 64 patients with schistosomiasis.

tive agglutination results, but their titers were only $\log_2 4$ and $\log_2 5$, close to the cutoff titer $\log_2 3$.

DISCUSSION

There have been many reports on serological test for the detection of antibodies directed against *Schistosoma* [18]. ELISA is one of the currently evaluated test by its very practical procedure for routine serological diagnosis [4, 6, 20, 26], for both clinical and seroepidemiological purposes [15, 30]. However, this test still presents difficulties for routine medical examination in terminal hospital and for epidemiological studies in field, because it involves time-consuming laborious steps and requires specialized equipments.

On the other hand, the conventional hemagglutination technique is a sensitive and rapid method for detection of antibodies and it has been successfully applied for serodiagnosis of schistosomiasis in several laboratories [1, 8, 14, 22]. However, this technique had initially disadvantage due the use of fresh red cells, providing unstable and variable nature of antigen-coated erythrocytes. Thus, new batches of sensitized erythrocytes were daily needed, thereby limiting this technique for routine serodiagnosis. The difficulty has been overcome by fixing erythrocytes with glutaraldehyde or formaldehyde [12, 16] and by using artificial particles, such as latex beads [17]. In the present study, the authors tried to use gelatin particles as antigen carriers for indirect agglutination test in the diagnosis of schistosomiasis. The results here obtained were quite sensitive and specific, showing comparable serodiagnosis performance to ELISA. The procedure is much simpler than that of the ELISA,

TABLE 2

Cross-reactivity assessed by the GPAT for schistosomiasis in Thai villagers with different parasitic diseases other than schistosomiasis.

Parasitic disease	GPAT titer ($\log_2$)	Number tested
Opisthorchiasis	≤3	10
	4	1
	5	1
Opisthorchiasis & other parasitic disease*	≤3	6
	4	1
Hookworm disease	≤3	2
Strongyloidiasis	≤3	3
Trichuriasis	4	1
Trichuriasis & Taeniasis	≤3	1

*Strongyloidiasis; Hookworm disease; Taeniasis; Strongyloidiasis & Hookworm disease

because it can be performed by one-step reaction with antigen-particles and the test serum. Furthermore, the test requires no specialized equipment other than a microplate, small pipette dropper and calibrated diluter. Additionally, the processing time is much shorter than standardized for other tests; it can be accomplished in few minutes and results obtained 3 hours later.

The employed particles are artificial spherical matrices made of gelatin and arabic gum. The particles are currently being employed as antigen carriers in various serodiagnosis kits [10, 11, 31]. Because they are inert, the test can be performed without a previous inactivation and absorption of patient serum, as recommended for erythrocyte carriers. Moreover, the gelatin-particles have advantages over latex beads and intact erythrocytes, since they can be colored, making easier the reading of the agglutination patterns, as well as be stored lyophilized for long period of time with no decrease in antigenic activity. Thus, this rapid test is by far the simplest serological test for use in the field.

RESUMO

Teste de aglutinação indireta com partículas de gelatina: estudo comparativo com enzima imunoensaio.

Um novo teste sorológico, o teste de aglutinação com partículas de gelatina (TAPG), foi utilizado para o diagnóstico sorológico de esquistossomose mansônica. Esta técnica mostra que a sensibilidade (90.6%) e especificidade (97.8%) chegam próximas do teste de enzima imunoensaio.

O TAPG pode ser fácil e rapidamente realizado sem equipamentos especializados usando partículas liofilizadas sensibilizadas com o antígeno.

O teste mostrou ser também útil em condições de campo para triagem em massa da infecção pelo *Schistosoma mansoni*.

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