

Utility of *Schistosoma bovis* Adult Worm Antigens for Diagnosis of Human Schistosomiasis by Enzyme-Linked Immunosorbent Assay and Electroimmunotransfer Blot Techniques

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Immunodiagnostic methods based on the detection of antibodies continue to be the most effective and practical methods for the diagnosis of imported schistosomiasis. *Schistosoma bovis* is a species whose final natural hosts are bovines, ovines, caprines, and small wild ruminants. Different studies have demonstrated the analogies existing between *S. bovis* and other *Schistosoma* species which affect humans. The objective of this work was to evaluate the utility of *S. bovis* adult worm antigens (AWA) for the diagnosis of imported human schistosomiasis by enzyme-linked immunosorbent assay (ELISA) and electroimmunotransfer blotting (EITB) techniques. By detecting eggs, the ELISA for *S. bovis* AWA was able to definitively detect imported cases with a sensitivity of 94%. The specificity of the ELISA for *S. bovis* AWA was 97%. There were no differences between the results of the *S. bovis* AWA ELISA for patients infected with *Schistosoma mansoni* and those infected with *Schistosoma haematobium*. The EITB technique showed bands of 85, 37, and 20 kDa, which are characteristic of infections with *Schistosoma* spp. Specific bands to indicate infection by different species of *Schistosoma* have not been detected. The combined use of the ELISA for *S. bovis* AWA and EITB increased the global sensitivity of the study to 97%. Our findings suggest that the ELISA for *S. bovis* AWA is a useful test for the immunodiagnosis of imported schistosomiasis and that EITB for detecting *S. bovis* AWA permits the confirmation of diagnosis when the ELISA for *S. bovis* AWA is positive.

Human schistosomiasis is endemic in 76 developing countries. More than 190 million people living in these countries suffer from the illness, and about 650 million are at risk (32). In recent years, immigration and the increase in travel from high-risk areas have led to an increase in the number of imported cases. Some studies indicate that 15% of immigrants and 2.5% of the travelers from areas where schistosomiasis is endemic have schistosomiasis (26). The species most commonly implicated are *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma intercalatum*.

Definitive diagnosis of schistosomiasis is carried out by the detection of eggs in feces and/or urine. Nevertheless, the parasitological methods of diagnosis have low sensitivities, especially with patients with the acute phase of the illness or with low-intensity infection (8, 13).

The detection of circulating antigens and copro-antigens allows the diagnosis of active schistosomiasis. The most frequently used methods have been those using cathodic and anodic circulating antigens. These circulating antigens have been correlated clinically with the intensity of the infection and with morbidity (14). Moreover, techniques using these antigens

have demonstrated their utility in posttreatment monitoring (15, 30). Their sensitivities lie between 65 and 95%, according to different studies (10, 31). However, these methods are not readily available, and the use of specific monoclonal antibodies limits their clinical use. Techniques of molecular diagnosis by PCR have also recently demonstrated their utility in the diagnosis of infection with *S. mansoni* (23), although no data are available for other *Schistosoma* spp.

Immunodiagnostic methods based on the detection of antibodies continue to be the most effective and practical methods for the diagnosis of imported schistosomiasis (29). Different methods have been used, with enzyme-linked immunosorbent assay (ELISA) being the most widely developed (13). The use of recombinant and purified antigens does not appear to have important advantages in comparison with the use of complex antigens. These antigens from adult worms and eggs from different species of *Schistosoma* are still the most widely used for the diagnosis of schistosomiasis (3, 13).

Schistosoma bovis is a species whose final natural hosts are bovines, ovines, and caprines and whose secondary hosts are small wild ruminants. It is distributed throughout Africa, Southwest Asia, and Mediterranean Europe. Different studies have demonstrated the analogies existing between *S. bovis* and other *Schistosoma* species which affect humans. The similarities include morphological, ecological, physiological, and genetic aspects (6, 7, 12). Furthermore, a high degree of cross-

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reactivity among *S. bovis*, *S. mansoni*, and *S. haematobium* has been demonstrated (2, 4). In spite of all these homologies, human infection with *S. bovis* has not been reported.

The objective of this work was to evaluate the utility of *S. bovis* adult worm antigen (AWA) for the diagnosis of imported human schistosomiasis using ELISA and electroimmunotransfer blotting (EITB) techniques.

MATERIALS AND METHODS

Patients. Two hundred nineteen samples of human sera from immigrants and travelers presenting at the Departments of Tropical Medicine of Hospital Insular (Las Palmas, Gran Canaria, Spain) and Hospital Ramón y Cajal (Madrid, Spain) from 1997 to 2003 were collected. The sera were divided into five groups as described below.

Group 1. Thirty-five sera were collected from patients with definitive diagnoses of schistosomiasis; 20 of these sera were from patients infected with *S. haematobium*, 12 were from patients infected with *S. mansoni*, 2 were from patients infected with *S. intercalatum*, and 1 was from a patient infected with both *S. haematobium* and *S. mansoni*. The diagnoses were made by the detection of parasite eggs in stool, urine, and/or biopsy specimens.

Group 2. Forty-one sera were collected from healthy controls from areas of sub-Saharan Africa where schistosomiasis is endemic. Sera from individuals with schistosomiasis or other parasitic infections were excluded based on parasitological tests.

Group 3. Fifty-two sera from healthy Spanish blood donors were used as controls.

Group 4. Forty-five sera were collected from patients with other isolated helminthic infections, namely, infections with *Fasciola hepatica* (nine specimens), *Echinococcus granulosus* (three specimens), *Taenia* sp. (three specimens), *Cisticercus cellulosae* (two specimens), *Onchocerca volvulus* (two specimens), *Loa loa* (two specimens), *Mansonella perstans* (six specimens), *Ascaris lumbricoides* (two specimens), *Trichuris trichiura* (three specimens), *Ancylostoma duodenale* (four specimens), *Strongyloides stercoralis* (two specimens), *Trichostrongylus* sp. (one specimen), *Toxocara canis* (two specimens), *Trichinella spiralis* (two specimens), and *Gnathostoma* sp. (two specimens).

Group 5. Twenty-seven sera were collected from patients with protozoal infections, namely, infections with *Plasmodium falciparum* (13 specimens), *Plasmodium malariae* (1 specimen), *Plasmodium ovale* (1 specimen), *Trypanosoma cruzi* (2 specimens), *Microsporidium* sp. (1 specimen), *Entamoeba histolytica* (4 specimens), *Entamoeba coli* (1 specimen), *Giardia lamblia* (3 specimens), and *Isoospora belli* (1 specimen).

Group 6. Nineteen sera were collected from patients with bacterial or viral infections, namely, infections with *Rickettsia typhi* (four specimens), *Leptospira interrogans* (one specimen), *Bartonella henselae* (one specimen), *Nocardia asteroides* (one specimen), *Borrelia burgdorferi* (one specimen), *Aeromonas* sp. (two specimens), human immunodeficiency virus (HIV) (four specimens), hepatitis A virus (three specimens), hepatitis B virus (one specimen), and hepatitis C virus (one specimen).

Antigens. *S. bovis* AWA was obtained as described previously by Abán et al. (1). The worms were suspended in sterile phosphate-buffered saline (PBS) at a concentration of 20 worms/ml with 1 mM phenylmethylsulfonyl fluoride, homogenized with a Ten Broeck tissue grinder, frozen and thawed three times, and then sonicated with three cycles at 70 kHz for 1 min each. The suspension was centrifuged at $5,000 \times g$ for 30 min at 4°C. The protein concentration of the supernatant was determined using the Micro-BCA protein assay reagent kit (Pierce).

Whole-worm egg antigen (WWE) of *S. mansoni* was provided by G. V. Hillyer, University of Puerto Rico (25).

ELISA with *S. bovis* and *S. mansoni* antigens. Polystyrene microtiter plates (Costar) were coated with 100 μ l of *S. bovis* AWA per well at a previously determined protein concentration of 5 μ g/ml diluted in carbonate buffer (pH 9.6). Serum at a dilution of 1:100 was added to the wells and incubated for 1 h at 37°C. Horseradish peroxidase goat anti-human immunoglobulin G (Sigma, St. Louis, Mo.) at a dilution of 1:2,000 was added. Washes were performed three times with 200 μ l of PBS-Tween 20 per well. After incubation for 1 h at 37°C, substrate solution (ortho-phenylene diamine plus H₂O₂) was added, and the reaction was stopped at 10 min with 3 N H₂SO₄. This test was performed with all sera described above.

The ELISA for *S. mansoni* WWE was performed as described above with modifications in order to correlate the results with those of the ELISA for *S.*

bovis AWA. The *S. mansoni* WWE ELISA was carried out using 46 sera: 28 sera from patients with schistosomiasis (group 1), 7 sera from healthy controls (group 3), and 11 sera from patients with other infections (groups 4, 5, and 6). All the sera were analyzed in triplicate.

EITB with *S. bovis* AWA. Proteins of *S. bovis* AWA were separated by electrophoresis under reducing conditions by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described by Laemmli (16). One hundred thirty micrograms of *S. bovis* AWA was boiled for 3 min and applied to the 15% polyacrylamide gel with a Mini Protean III apparatus (Bio-Rad). The separated proteins were electrotransferred to nitrocellulose filters using a Trans Blot transfer cell apparatus (Bio-Rad), and 5-mm-wide strips were cut. The transference was verified with Ponceau solution. Nonspecific sites were blocked with a solution of PBS-0.05% Tween and 5% bovine serum albumin and incubated for 30 min at room temperature. Serum diluted 1:50 in a solution containing PBS-0.05% Tween and 5% bovine serum albumin was added and left overnight at 4°C. Horseradish peroxidase goat anti-human immunoglobulin G (Sigma) was added at a dilution of 1:500 and incubated for 2 h at room temperature. After being washed, the bands were visualized with substrate solution (methanol plus 4-chloro-1-naphthol plus H₂O₂). The reaction was stopped by washing the strips in distilled water. This test was performed on all sera from positive controls, all sera shown by ELISA to be positive for *S. bovis* AWA, 31 randomly selected sera from patients with other infections, and 12 sera from healthy controls.

Statistical analysis. Statistical tests were carried out using the SPSS 11.5 statistical package. The level of significance accepted was a *P* value of <0.05. The results are expressed as means and standard deviations. The analysis of variance test and SDS post hoc test were used for comparison of the means of the groups. The Kruskal-Wallis test was used if the variances were not homogenous. The receiver operating characteristic curve was used for establishment of the cutoff of the ELISA. In order to compare the mean optical densities (ODs) of different species of *Schistosoma*, the Mann-Whitney U test was used. Wilcoxon's matched-pair signed-rank test and the Pearson correlation coefficient were used to compare the results for *S. bovis* AWA and *S. mansoni* AWA. The kappa test was used for the establishment of reliability.

RESULTS

ELISA for *S. bovis* AWA. The ODs of the 218 sera analyzed are presented in Fig. 1. The mean OD \pm standard deviation for the group of patients with confirmed schistosomiasis was the highest (0.972 ± 0.307) of those calculated. The remaining groups presented lower mean ODs: 0.195 ± 0.119 for healthy patients from zones of endemicity, 0.289 ± 0.06 for healthy Spanish blood donors, 0.338 ± 0.133 for patients with other isolated helminthic diseases, 0.293 ± 0.112 for patients with protozoan infections, and 0.347 ± 0.121 for patients with other infections. Comparative analysis of the mean ODs detected significant differences between values for group 1 and those for the remaining groups ($P < 0.0001$). A receiver operating characteristic curve was established from these results to define the sensitivities and specificities at different cutoff OD values. A cutoff of 0.500 with a 94% sensitivity and a 97% specificity was established. The positive predictive value was 89%, and the negative predictive value was 99%. None of the sera from healthy Spanish donors or from patients with protozoan infections presented ODs higher than this cutoff value.

Only one serum specimen from the group of healthy patients from an area of endemicity presented an OD higher than the cutoff value. Among the sera from patients with helminthiasis, only the serum from one patient (of nine sera analyzed) with *Fasciola hepatica* infection had an OD higher than the cutoff value. Among the sera of patients with other infections, only that of one patient with HIV and another with *Nocardia* infection had ODs higher than the cutoff value.

There were no significant differences between the results of the ELISA for *S. bovis* AWA with the sera of patients infected

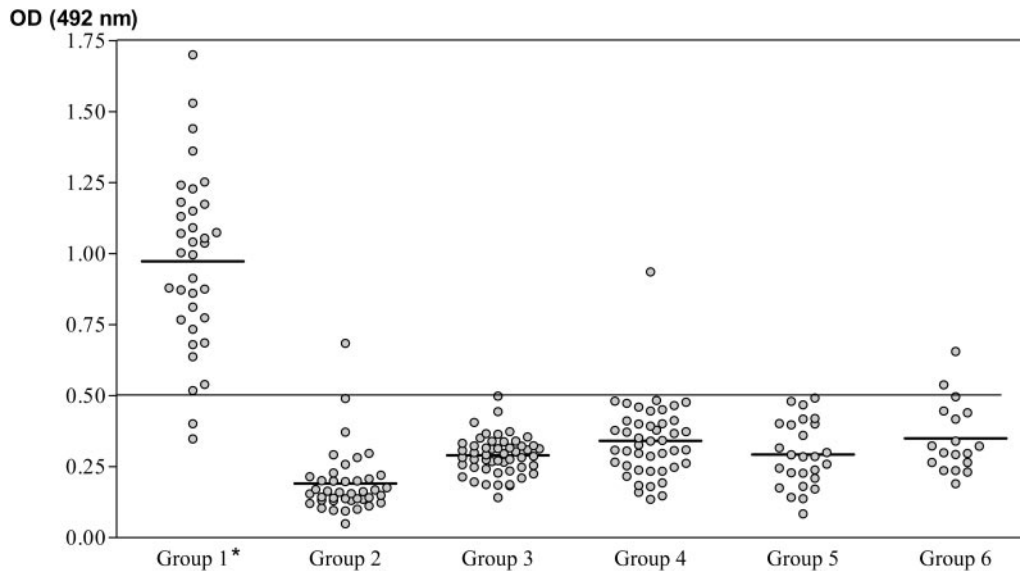


FIG. 1. Values of OD at 492 nm determined by the *S. bovis* AWA ELISA with sera from patients included in this study. We included sera from patients diagnosed with schistosomiasis by the detection of eggs (group 1), sera from healthy controls from areas of schistosomiasis endemicity (group 2), sera from healthy Spanish blood donors (group 3), sera from patients with other helminth infections (group 4), sera from patients with protozoan infections (group 5), and sera from patients with viral and bacterial infections (group 6). The horizontal line represents the cutoff value. *, significant differences were found between group 1 and the other groups.

with *S. mansoni* and *S. haematobium*. We analyzed the reliability of the test on 45 sera, finding a kappa index of 0.86.

Correlation between the *S. bovis* AWA ELISA and the *S. mansoni* WWE ELISA. The sera from the patients with a definite diagnosis of schistosomiasis exhibited, in the *S. bovis* AWA ELISA, a higher mean OD than the sera used in the *S. mansoni* WWE ELISA (1.04 ± 0.347 versus 0.793 ± 0.244). This difference was statistically significant ($P < 0.001$).

The remainder of the sera analyzed presented a mean OD in the *S. bovis* AWA ELISA lower than that obtained in the *S. mansoni* WWE ELISA (0.284 ± 0.104 versus 0.382 ± 0.039), and the difference was statistically significant ($P = 0.028$).

We detected a strong correlation ($r = 0.856$) between the ODs of the sera analyzed with the two antigens (Fig. 2).

EITB with *S. bovis* AWA. The patterns of bands of antigenic recognition of sera from patients infected with *S. mansoni*, *S. haematobium*, and *S. intercalatum* and the percentages of sera that exhibited each band are shown in Fig. 3. The five principal protein clusters recognized were 85, 65, 37, 29, and 20 kDa. Of the sera analyzed, 86% exhibited the band of 85 kDa, 96% exhibited the band of 65 kDa, 83% exhibited the band of 37 kDa, 55% exhibited the band of 29 kDa, and 44% exhibited the band of 20 kDa. No differences were detected in the profiles of bands among the different species of *Schistosoma*.

Sera from 38 patients with a negative ELISA result for *S. bovis* AWA were analyzed with EITB (Fig. 4). Only one serum sample, from a patient infected with *P. falciparum*, exhibited a band close to 85 kDa. No sera exhibited bands in the regions of 20 and 37 kDa. Moreover, 31% of the sera that were *S. bovis* AWA ELISA negative exhibited bands in the region of the 65-kDa cluster, and 26% exhibited bands in the region of 29 kDa. Only two of the four sera that were *S. bovis* AWA ELISA positive from HIV patients and healthy individuals from an area of endemicity exhibited bands of 85, 65, 29, and 20 kDa.

Estimation of the sensitivities of the ELISA and EITB for *S. bovis* antigen. Considering the sera that exhibited the band of 85, 37, or 20 kDa, we found that EITB presented sensitivities of 94% for *S. haematobium* and 100% for *S. mansoni* and *S. intercalatum*. The utilization of ELISA and Western blotting in parallel increased the global sensitivity of the study to 97% for the patients with schistosomiasis, as confirmed by the detection of eggs (Table 1).

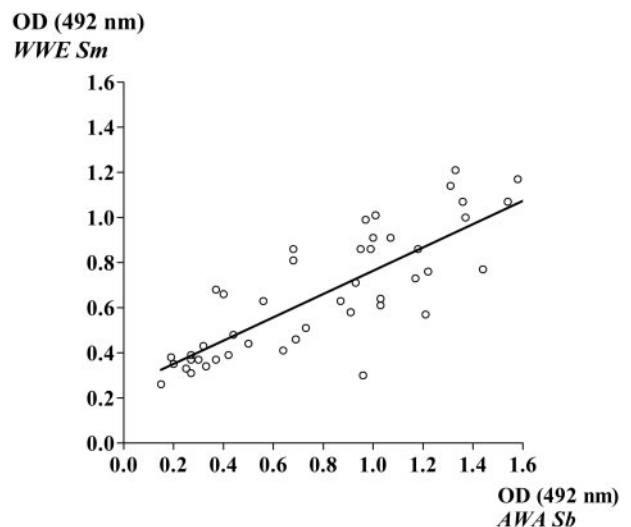


FIG. 2. Correlation among OD values of the sera analyzed with the *S. bovis* AWA (*AWA Sb*) and *S. mansoni* WWE (*WWE Sm*) ELISA using 46 sera: 28 sera from patients with schistosomiasis (group 1), 7 sera from healthy controls (group 3), and 11 sera from patients with other infections (groups 4, 5, and 6).

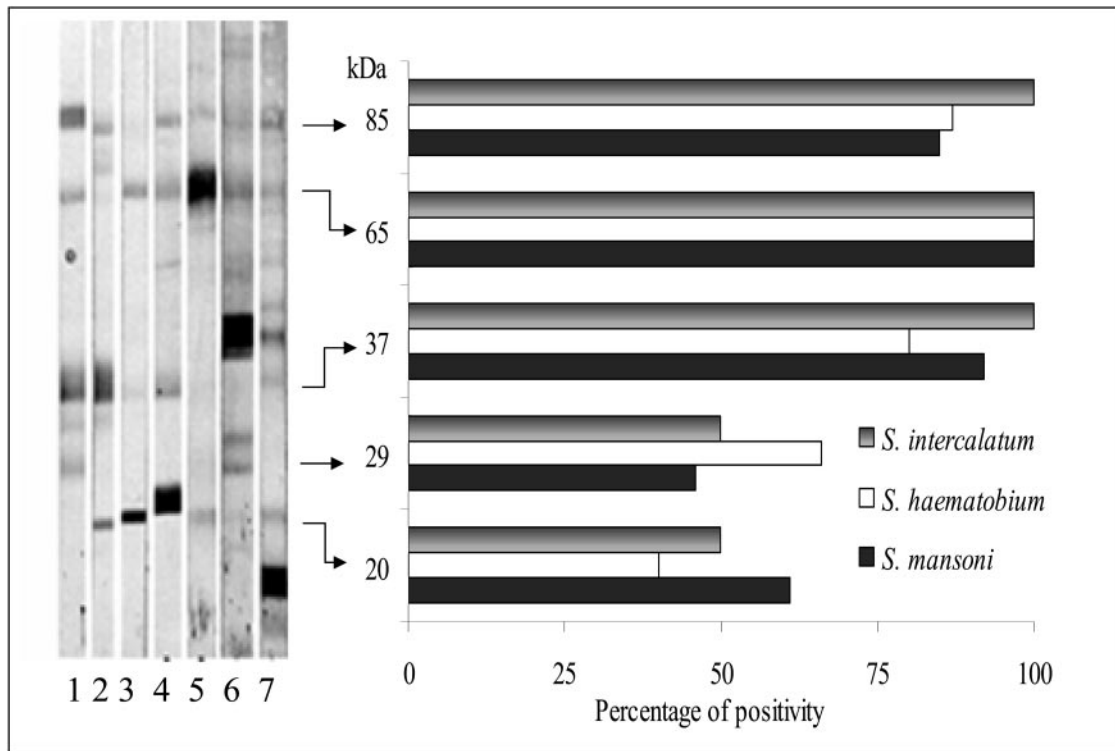


FIG. 3. EITB of sera from patients diagnosed with schistosomiasis, including one to three sera from patients with infections with *S. mansoni*, four to five sera from patients with infections with *S. haematobium*, and six to seven sera from patients with infections with *S. intercalatum*. The percentages of positivity of the principal bands detected in the sera of patients infected with *S. intercalatum*, *S. haematobium*, and *S. mansoni* are shown.

DISCUSSION

The diagnosis of imported schistosomiasis is habitually carried out by detecting eggs of *Schistosoma* in samples of feces and urine. Nevertheless, the low and fluctuating excretion of eggs makes the diagnosis of many cases difficult. The method of immunodiagnosis based on the detection of antibodies in-

creases the detection of cases and constitutes an especially useful method for the study of imported schistosomiasis. Although different techniques, such as those utilizing hemagglutination and immunofluorescence, and the circumoval precipitin test have been used, ELISA has been the technique most widely developed. A great variety of antigens have been employed.

The crude antigens from adult worms and eggs have a high sensitivity for the diagnosis of infection (22); however, they present a specificity lower than 80%, giving false positives for infections with intestinal nematodes and especially with hookworms (21). Moreover, it is common to find polyparasitism in patients from areas of endemicity (27). The fundamental advantage of using complex antigens from adult worms over using antigens from eggs is their greater facility and their high yield of antigenic material (13). Tests using the purified anti-

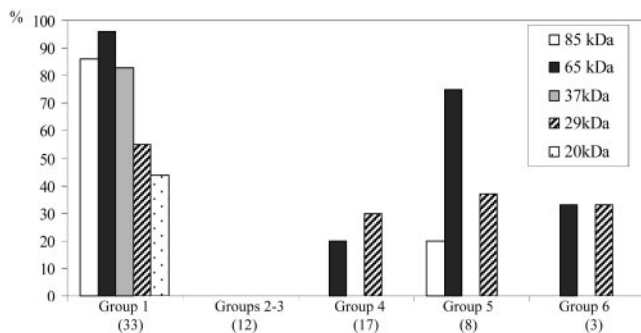


FIG. 4. Percentages of positivity of the bands detected by EITB with *S. bovis* AWA. Group 1 consisted of sera from patients with a definitive diagnosis of schistosomiasis by detection of eggs, groups 2 and 3 consisted of sera from healthy patients from areas of endemicity and nonendemicity, group 4 consisted of sera from patients with isolated helminthic infections, group 5 consisted of sera from patients with protozoan infections, and group 6 consisted of sera from patients with viral and bacterial infections. Numbers in parentheses indicate the number of sera in each group.

TABLE 1. Sensitivity of ELISA and EITB with *S. bovis* AWA

Organism in or characteristic of serum	No. of serum samples positive/total no. tested (%) by:		
	ELISA	EITB	ELISA plus EITB
<i>S. mansoni</i>	11/12 (91.6)	11/11 (100)	12/12 (100)
<i>S. haematobium</i>	19/20 (95)	18/19 (94.7)	19/20 (95)
<i>S. intercalatum</i>	2/2 (100)	2/2 (100)	2/2 (100)
All from patients with definitive schistosomiasis	33/35 (94.2)	32/33 (96.9)	34/35 (97.1)

gens, such as CEF6 (11), cathodic circulating antigen (24), and the recombinant antigens rTEG of *Schistosoma japonicum* (17) and r22 kDa of *S. mansoni*, have demonstrated acceptable sensitivities, although they are less than those of tests using the crude antigens from adult worms and eggs.

Heterologous antigens have been used in the immunodiagnosis of different parasite infections. Their clinical yield has been demonstrated using antigens of *Strongyloides ratti* in the diagnosis of human strongyloidiasis (20) or antigens of *Dirofilaria immitis* in the diagnosis of tropical filariasis (18). In this study, we used complete *S. bovis* AWA for the detection of antibodies by ELISA and EITB in sera from patients with a definitive diagnosis of schistosomiasis. Using the *S. bovis* AWA ELISA, we detected 94% of the cases of confirmed schistosomiasis. Moreover, the specificity achieved was 97%. Of the 185 sera analyzed, only 4 for which a diagnosis of schistosomiasis was discounted were positive by the *S. bovis* AWA ELISA. Two of these patients, infected with *Nocardia asteroides* and *Fasciola hepatica* (5), may represent cases of cross-reactivity. The other two patients with positive ELISA results for *S. bovis* AWA came from an area of endemicity, and it is probable that these test results occurred in both cases due to cryptic schistosomiasis, given the high prevalence of infection with *Schistosoma* in immigrants from areas of endemicity.

The purified antigens of adult worms have allowed the obtainment of microsomal antigens of *S. mansoni* (MAMA) and of *S. haematobium* with sensitivities of 96 and 98% for the diagnosis of schistosomiasis due to *S. mansoni* and *S. haematobium*, respectively (3, 19). Nonetheless, the diagnostic sensitivity for cases produced by other species is lower. The ELISA for MAMA permits the diagnosis of only 55 to 83% of the cases caused by *S. haematobium* (3, 19). This fact limits the potential of using only one of these ELISAs for the diagnosis of imported schistosomiasis, especially among travelers and immigrants coming from Africa (9, 26). In contrast with ELISAs using microsomal antigens, the ELISA in our study revealed no differences in the diagnostic results for patients infected with *S. mansoni* and *S. haematobium*.

The performance of an ELISA using sera from patients both with and without schistosomiasis and using the complete *S. bovis* AWA and the *S. mansoni* WWE allowed us to prove a high degree of correlation between the two tests. This assay further supports the utility of the *S. bovis* AWA ELISA in the diagnosis of human schistosomiasis.

EITB has been used as a method for confirming infections by *Schistosoma* (22, 29). Performing the ELISA and EITB in parallel can increase the sensitivity to 97%. The use of EITB with microsomal antigens allows a species diagnosis. The detection of the 30-kDa band in MAMA EITB is highly specific for infection with *S. mansoni*, and the detection of the 23-kDa band in EITB for microsomal antigens of *S. haematobium* is specific for infection with *S. haematobium* (3, 28).

In this study, we analyzed sera of patients from the different groups by EITB with *S. bovis* AWA. We found that the sera of patients with confirmed schistosomiasis exhibited a pattern of five principal protein clusters. The bands of 85, 37, and 20 kDa were characteristic of infection with *Schistosoma*. Only two *S. bovis* AWA ELISA-positive sera from patients from areas of endemicity exhibited a pattern of antigenic recognition similar to that of sera from patients with confirmed schistosomiasis.

This fact supported the suspicion of cryptic schistosomiasis. In our study, in contrast with what was found by EITB with microsomal antigens, we did not detect specific bands of infection for different species of *Schistosoma*.

In summary, we can conclude that the *S. bovis* AWA ELISA is a useful test for the immunodiagnosis of imported schistosomiasis, comparable with other habitually used techniques which permit the diagnosis of different species of *Schistosoma*. Moreover, the utilization of EITB with *S. bovis* AWA permits the confirmation of diagnosis when the ELISA for *S. bovis* AWA is positive.

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