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Comparative Evaluation of Enzyme-Linked Immunosorbent Assay for the Diagnosis of Pulmonary Echinococcosis

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An enzyme-linked immunosorbent assay was done for the detection of immunoglobulin G and M (IgG and IgM) antibodies to *Echinococcus granulosus* in surgically proved cases of hydatidosis, especially pulmonary hydatidosis, by use of human hydatid cyst fluid antigen and soluble scolex antigen. This assay was compared with the following standardized techniques: the indirect hemagglutination test, the indirect immunofluorescent antibody test, and Casoni's intradermal test. The enzyme-linked immunosorbent assay, with either of the antigens (human hydatid cyst fluid or soluble scolex antigen), was more sensitive and specific than the other techniques in diagnosing cases of hydatidosis, especially hydatid disease of the lung.

The enzyme-linked immunosorbent assay (ELISA) has recently been used for immunodiagnosis of hydatid disease (1, 4, 5, 14, 20, 22). These reports are, however, conflicting. Some reports suggest that ELISA results are only comparable to results of other, more commonly used, immunodiagnostic tests, such as the indirect hemagglutination (IHA) test and the indirect immunofluorescent antibody (IFA) test (20). Some investigators have advocated the use of purified antigens to improve the results of ELISA in hydatid disease (4, 5, 14). It is now well documented that the existing serological tests, such as Casoni's intradermal (i.d.) test, the IHA test, and the indirect IFA test, are less sensitive in the detection of pulmonary echinococcosis than in the detection of liver hydatidosis or multiple hydatidosis (3, 6, 7, 10, 13, 16, 17, 19). It is still a diagnostic problem. The purposes of this study were (i) to evaluate the sensitivity and specificity of the ELISA for the detection of echinococcal antibodies, especially in patients with pulmonary echinococcosis, (ii) to detect by ELISA the persistence of echinococcal antibodies after surgery, and (iii) to compare the ELISA with other immunological tests, such as Casoni's i.d. test, the IHA test, and the indirect IFA test, in the diagnosis of hydatid disease.

MATERIALS AND METHODS

Specimens. We obtained 296 sera from patients visiting the Sher-i-Kashmir Institute of Medical Sciences, Soura, Srinagar, Kashmir, India. These included 200 sera from patients suffering from a disease other than hydatidosis (viral hepatitis, hepatocellular carcinoma, cirrhosis, chronic bronchitis, bronchiectasis, Hodgkin's disease, and tuberculosis) and 44 sera from normal healthy blood donors. We obtained 52 sera from surgically proved hydatid cases; these included 6 sera from former lung and liver hydatid cases which involved surgery 6 months to 10 years previously.

The cyst localization in 46 hydatid cases was as follows: hepatic, 21 cases; pulmonary, 22 cases; and multiple, 3 cases. All patients were examined for other parasitic infections prevalent in this geographical area that could cross-react in hydatid serology, such as ascariasis and taeniasis. All of the sera were stored at −20°C until use.

Hydatid antigens. (i) HHCF antigen. Human hydatid cyst fluid (HHCF) antigen was prepared from the hydatid cysts obtained postoperatively. Hydatid fluid collected aseptically from fertile hydatid cysts was passed through a Whatman WCN type membrane filter (cellulose nitrate, 47-mm diameter, 0.45-μm pore size; Whatman Limited, Maidstone, England) and dialyzed against distilled water (2). The antigen protein concentration was estimated by the method of Lowry et al. (8), with bovine serum albumin as a reference standard.

(ii) Human hydatid scolex antigen. Scolices obtained from hydatid sand of hydatid cysts were washed five times in normal saline (0.85% NaCl). Whole scolices (20 to 25 scolices, both ortho- and matascolices in 0.5 ml of normal saline) were used as antigen (whole-scolex antigen) after acetone fixation on the slides for the indirect IFA test. Soluble scolex antigen (SSA) was prepared after the disruption of whole scolices by frequent freezing and thawing. Scolices were further ultrasonically disrupted in an MSE Soniprep 150 ultrasonic distintegrator. The suspension was centrifuged at 2,000 × *g*, and the supernatant was collected. The deposit was suspended in phosphate-buffered saline (pH 7.2) and ultrasonically disintegrated, and this supernatant was collected and added to the earlier supernatant. The combined supernatant was used as SSA. Its protein content was estimated by the method of Lowry et al. (8).

Optimal dilutions of antigen used in the IHA test and the ELISA were determined by block titration with reference sera positive for antibodies to *Echinococcus granulosus*.

Casoni's i.d. test. For Casoni's i.d. test, a 0.1-ml amount of HHCF antigen was injected i.d. into the forearm, and normal saline was injected into the other forearm as a control. The nitrogen content of HHCF antigen was adjusted to 25 to 30 μg/ml, as suggested by Kagan (6). Wheals were measured after 30 min, and a wheal area of more than 1.2 cm^2 was considered positive (19).

IHA test. The IHA test was done in microtiter plates by use of tanned sheep erythrocytes sensitized with HHCF antigen or SSA by the method described earlier (2). The nitrogen content of the antigen was kept between 25 and 30 μg/ml (6), and the working dilutions of the antigens used were 1:4 and 1:2 for HHCF antigen and SSA, respectively, as shown by earlier block titration. Titers more than or equal to 1:80 were considered positive (9, 10).

Indirect IFA test. Whole-scolex antigen was acetone fixed on non-fluorescent-microscope slides at −20°C, and the indirect IFA test was done by the technique described earlier

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TABLE 1. Immunological profile of 46 surgically proved cases of hydatid disease

Cyst localization	No. of cases tested	No. (%) of cases positive in Casoni's i.d. test	No. (%) of cases positive in the IHA test [a]	No. (%) of cases positive in the IFA test [b]	No. (%) of cases positive in the IgG-ELISA[c]
Lung	22	11 (50)	13 (59)	18 (81)	22 (100)
Liver	21	15 (71.4)	16 (76.2)	19 (90)	21 (100)
Multiple cysts	3	3 (100)	3 (100)	3 (100)	3 (100)

[a] Titers ≥ 80.
[b] Titers ≥ 32.
[c] Titers ≥ 160.

(9). Doubling dilutions of serum were made starting from 1:4, and for each dilution one slide was used. Fluorescein-labeled sheep anti-human immunoglobulin G (IgG), IgM, and IgA (Poly-IFA) used for the test was obtained from Wellcome Diagnostics (Dartford, England). Evans blue was used as a counterstain to minimize autofluorescence (7). Slides were examined with an Olympus BHF fluorescence microscope. A titer of ≥1:32 was considered positive (10).

Sera from hydatid cases which involved surgery 6 months to 10 years previously were also tested with fluorescein-labeled sheep anti-human IgM (IgM-IFA), obtained from Wellcome Diagnostics, for the IgM class of anti-echinococcus antibodies.

To ascertain the specificity of immunofluorescence, cross-absorption studies were done with ascaris larval antigen. Since ascariasis is endemic in Kashmir (India), the amount of false-positive fluorescence due to anti-ascaris antibodies cross-reacting with anti-echinococcus antibodies in serum from patients was evaluated. Second-stage (L2) ascaris larvae, obtained after the hatching of ascaris eggs in vitro (21), and whole-scolex antigen were used as antigens for serum absorption. A 0.5-ml serum sample positive for anti-echinococcus antibodies was mixed with L2 larvae or whole-scolex antigen in separate test tubes and incubated at 37°C for 2 h. After incubation, both test tubes were spun at 2,000 × *g* for 10 min, and the supernatant serum was used for the IFA test, separately.

ELISA. The ELISA was done with both antigens, HHCF antigen and SSA, separately, by the method already described (22). Briefly, wells of polystyrene microtitration plates (U bottom) (Dynatech Laboratories, Inc., Alexandria, Va.) were coated by overnight incubation at 4°C with 300 μl of HHCF antigen (25 μg of protein per ml) or SSA (30 μg of protein per ml) in 0.05 M carbonate buffer (pH 9.6). The plates were washed three times in phosphate buffer (pH 7.2) containing 0.5 g of polysorbate-20 per liter (PBS-T) and were postcoated with 1% bovine serum albumin in PBS-T at 37°C for 1 h. After an additional washing, plates were shaken dry and used immediately. The test sera were doubly diluted in PBS-T starting from 1:5. A 300-μl amount of each serum dilution was added into a single well. One row of 12 wells was used for one sample. Plates were incubated at 37°C for 1 h. After the washing, 300 μl of anti-human IgG-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) (IgG-ELISA) was added to each well and incubated at 37°C for 1 h. After incubation and washing with PBS-T, 300 μl of disodium *para*-nitrophenyl phosphate (Sigma), 1 g/liter in diethanolamine buffer (pH 9.8) containing 0.05 M magnesium chloride, was added to all of the wells. The enzyme-substrate reaction was allowed to proceed for 45 min at 37°C, and the A_{405} of the enzymatic product was determined directly in the wells with an automated ELISA reader (Dynatech). Positive and negative serum samples were included in each plate along with substrate and buffer blanks. The reported titer for the ELISA is the highest serum dilution that resulted in an absorbance at least twice the mean absorbance of wells containing negative control samples. The titer taken to be positive in ELISA thus obtained was 1:160 (average A_{405}, 0.120). The sera of patients who underwent surgery 6 months to 10 years previously were also tested for the IgM class of antibodies to *E. granulosus* with anti-human IgM-alkaline phosphatase conjugate (Sigma) in the ELISA (IgM-ELISA).

Control specimens for ELISA. Positive control serum, obtained from a surgically diagnosed patient with multiple hydatidosis, was positive in the Casoni's i.d. (2-cm^2 wheal area), Poly-IFA (1:512), IgG-ELISA (1:2,560), and IHA (1:64) tests. Negative control serum was obtained from an uninfected individual who was negative in the IFA (1:4), IgG-ELISA (1:20), IgM-ELISA (1:5), IHA (1:10), and Casoni's i.d. (<1.2-cm^2 wheal area) tests.

Inhibition ELISA test. The specificity of the ELISA was known by the inhibition ELISA test. Positive serum samples of known A_{405} of the enzymatic product, at 1:320 dilution, were mixed with HHCF antigen (25 μg of protein per ml) or SSA (30 μg of protein per ml). The mixtures were kept at 4°C for 2 h, and the serum-antigen mixture was used as a serum specimen for IgG-ELISA. A fourfold or greater fall in absorbance was considered as satisfactory inhibition of the ELISA.

The precision of the ELISA technique used was known by the intra- and interassay coefficients of variation (CVs) for measurement of anti-hydatid antibodies in three positive hydatid lung sera. Each sample was tested 10 times for each assay, and the percents CV and standard deviations were calculated.

RESULTS

All 296 patients under study were found to be free from other parasitic infections relevant to this geographical area which could possibly interfere in hydatid serology. The results for 46 surgically proved hydatid cases with different immunological techniques are shown in Table 1. Out of 46 cases, the ELISA detected all, irrespective of the type of antigen used (HHCF antigen or SSA) (Table 2 and Fig. 1 and 2), whereas the number of cases positive by the Casoni's i.d., IHA, and IFA tests were 29, 32, and 40, respectively. Out of 22 lung hydatid sera, only 11, 13, and 18 were positive by the Casoni's i.d., IHA, and IFA tests, respectively. Similarly, out of 21 liver hydatid sera, 15, 16, and 19 were positive by the Casoni's i.d., IHA, and IFA tests, respectively. Lung hydatid cases were low reactors as compared with liver echinococcosis cases in the Casoni's i.d., IHA,

TABLE 2. Immunological profile of 46 surgically proved cases of hydatidosis by use of SSA and HHCF antigen in the IHA test and the IgG-ELISA

Cyst localization	No. of cases tested	No. (%) of cases positive by use of the following antigen in:			
		IHA test		IgG-ELISA	
		HHCF	SSA	HHCF	SSA
Lung	22	13 (59)[a]	14 (63.6)[a]	22 (100)	22 (100)
Liver	21	16 (76.2)[a]	17 (80.9)[a]	21 (100)	21 (100)
Multiple cysts	3	3 (100)	3 (100)	3 (100)	3 (100)

[a] Statistically not significant.

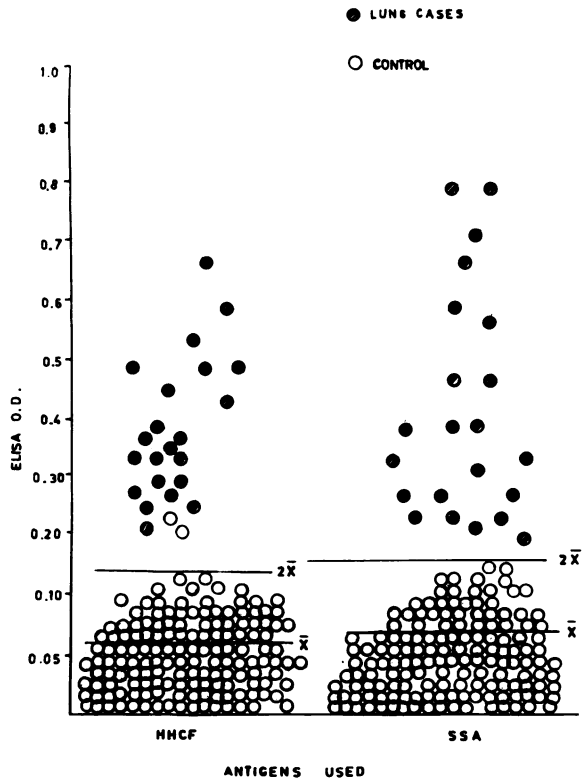

FIG. 1. Distribution of individual results obtained with different hydatid antigens (HHCF and SSA) in IgG-ELISA in nonhydatid (control) and lung hydatid disease cases (mean absorbance control = 0.06 ± 0.012, pulmonary hydatidosis control = 0.548 ± 0.267).

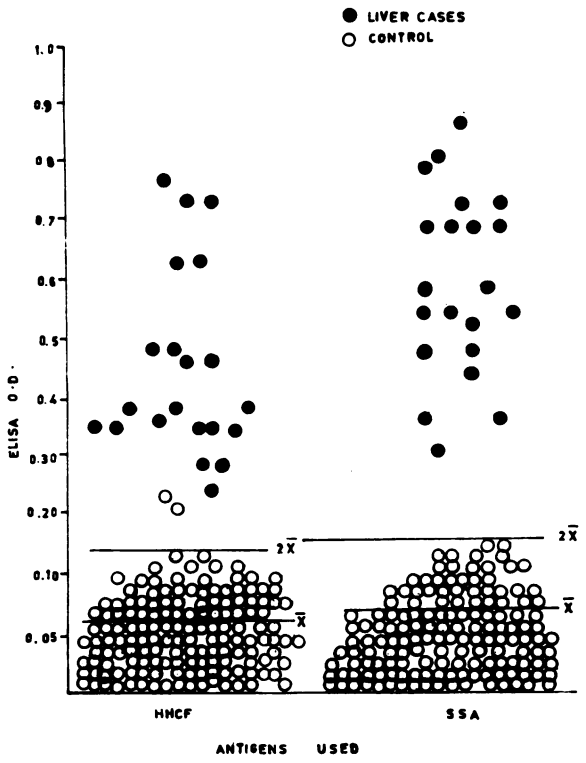

FIG. 2. Distribution of individual results obtained with hydatid antigens (HHCF and SSA) in IgG-ELISA in liver hydatid disease (mean absorbance = 0.683 ± 0.022) against nonhydatid controls (mean absorbance = 0.06 ± 0.012).

and IFA tests. The ELISA was positive in all cases, irrespective of the site of cyst localization, indicating 100% sensitivity.

The antibody level in lung hydatid cases was lower than that in liver hydatid cases (Fig. 1 and 2). Only one hydatid case was negative by the IFA test but positive by the IHA test; otherwise, all IFA test-negative cases were negative by the IHA test also. The corresponding ELISA values, in sera negative by the IHA and IFA tests, were 1:160 and even as high as 1:1,280.

The two antigens, HHCF antigen and SSA, used in the IHA test did not increase the sensitivity of this technique (Table 2). Only two hydatid cases, one each of lung and liver, were positive with SSA but earlier not positive with HHCF antigen, but such a finding was not statistically significant.

The specificity of all four immunodiagnostic tests was judged from the study of 244 nonhydatid cases, as shown in Table 3. False-positive reactions obtained in the Casoni's i.d., IHA, and IFA tests constituted 16, 5.7, and 3%, respectively. The wheal area in the Casoni's i.d. test in all of the 32 false-positive cases (16%) exceeded 1.2 cm^2, and in three cases it measured up to 2 cm^2. These 32 cases also included 6 healthy blood donors. The IHA and IFA titers in false-positive sera varied from 1:160 to 1:320 and from 1:32 to 1:64, respectively. Among sera from 44 healthy blood donors, none was positive by the IHA or IFA test. The ELISA had a seropositivity of 98.5 to 100%. Two sera, one each from a patient with Hodgkin's disease and hepatocellular carcinoma, showed a titer of 1:160 and 1:640, respectively, in the IgG-ELISA test with HHCF antigen. When the ELISA test was done with SSA, titers in these two cases were 1:10 and 1:80, respectively. Hence, the false-positivity in these two cases disappeared when SSA was used. The false-positivity of the IHA test did not decrease when SSA was used. It should be mentioned that sera showing false-positivity were not always the same in different immunodiagnostic tests.

Altogether, six sera from cases of lung or liver hydatidosis or both, involving surgery 6 months to 10 years previously, were subjected to different immunodiagnostic tests. The Casoni's i.d. reaction was a 1.2-cm^2 wheal area in all of these cases, except for one patient operated upon 10 years previously. The IHA test was positive in three patients (titers,

TABLE 3. Immunological profile of nonhydatid cases

Immunological technique	No. of cases tested	No. of cases positive	% Cases false-positive
Casoni's i.d. test	200	32	16
IHA[a]	244	14	5.7
Poly-IFA[b]	202	6	3.0
IgG-ELISA			
HHCF[c]	174	2[d]	1.5
SSA	174	0	0

[a] The IHA test with both antigens (HHCF and SSA) showed no statistically significant difference in results; figures shown here are with the HHCF antigen. Titers were 1:160 to 1:320.
[b] Poly-IFA (IgM + IgG + IgA), titers 1:32 to 1:64.
[c] ELISA with the HHCF antigen, titers 1:160 and 1:640.
[d] Cases of Hodgkin's disease and hepatocellular carcinoma.

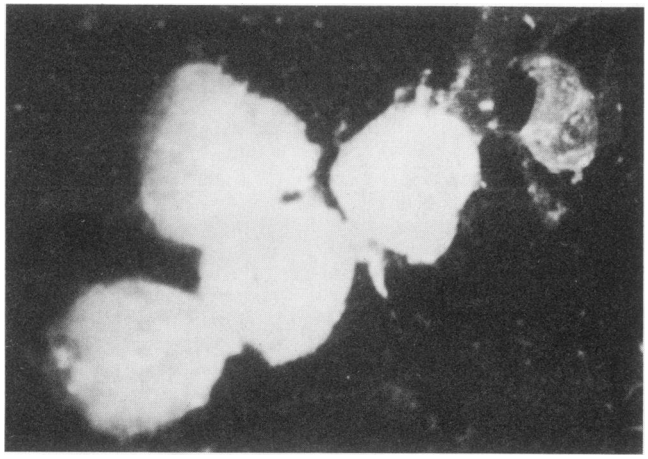

FIG. 3. Fluorescence of whole scolices in the IFA test with serum positive for *E. granulosus* antibodies. Magnification, ×200 (four-plus fluorescence).

1:320 and 1:640). Two of these patients were operated upon 6 months earlier, and the third was operated upon 5 years previously. In the Poly-IFA test, two sera were positive (titers, 1:32 and 1:64). These sera were from patients operated upon 6 months and 5 years previously, respectively. All of the six sera were negative in the IgM-IFA test. Only one serum sample, obtained from a patient operated upon 6 months previously, was positive (titer, 1:320) in the IgG-ELISA test, whereas all were negative in the IgM-ELISA test. The number of cases being less, its use in the follow-up of postoperative cases is difficult to comment upon.

The results of cross-absorption studies for the IFA test with L2 and whole-scolex antigen (see Fig. 3 to 5) indicate that immunofluorescence was specific for echinococcus antibodies. The four-plus fluorescence of scolices with a positive serum is shown in Fig. 3. The fluorescence of scolices was almost absent when positive serum absorbed with whole-scolex antigen was used (Fig. 4). There was no inhibition of immunofluorescence after absorption with L2 (Fig. 5). These results indicate that the endemicity of ascariasis in Kashmir does not interfere in the IFA test for antibodies to *E. granulosus*.

In inhibition ELISA, a fourfold fall in absorbance ($A_{0.432}$ to $A_{0.023}$ at a 1:320 dilution) of the positive serum was present,

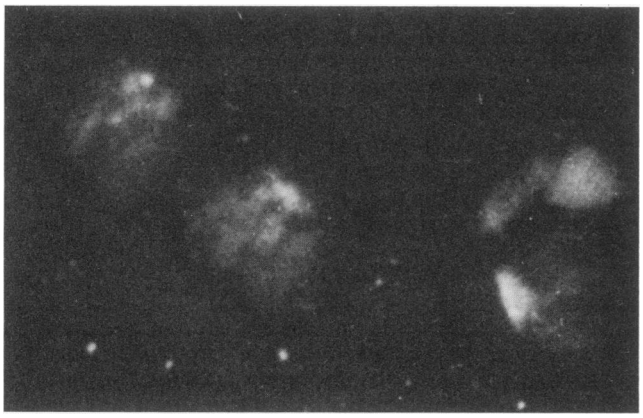

FIG. 4. IFA with hydatid-positive serum previously absorbed with whole-scolex antigen. Magnification, ×200 (fluorescence nil).

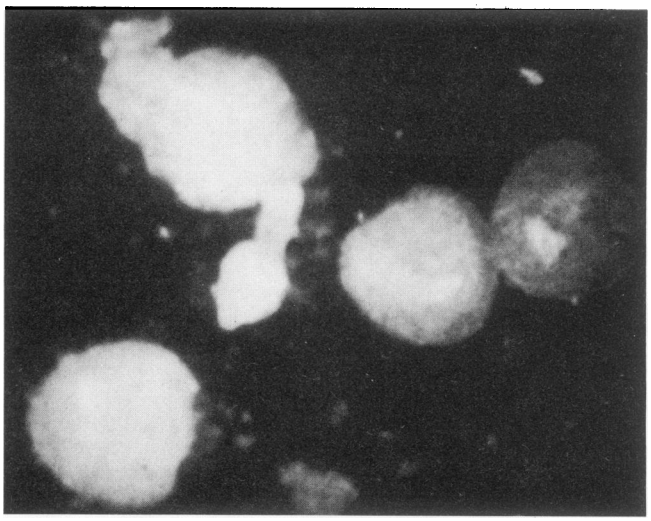

FIG. 5. IFA with hydatid-positive serum previously absorbed with L2. Magnification, ×190 (four-plus fluorescence).

indicating that the present assay system (ELISA) for the measurement of anti-hydatid antibodies is highly specific. CVs in the ELISA precision test are shown in Table 4. These values are much below 10% and are consistent with criteria laid down for quality control in the ELISA (11).

DISCUSSION

Our results indicate that the ELISA is highly sensitive and specific in detecting anti-echinococcus antibodies irrespective of the site of the cyst localization. We found the ELISA useful in the diagnosis of pulmonary hydatid disease. This is in contrast to the lower sensitivity and specificity of the IHA, double diffusion, IFA, and Casoni's i.d. tests (6, 7, 13, 20) in diagnosing pulmonary hydatidosis. Todorov et al. (16) compared complement fixation, latex agglutination, bentonite flocculation, IHA, and Casoni's i.d. tests and did not find them to be significantly sensitive for the diagnosis of pulmonary hydatidosis.

Although these investigators found the Casoni's i.d. test to be positive in 83.3% of lung hydatid cases, we found only 50% of such cases to be positive. These findings for the Casoni's i.d. test are also corroborated by the results of other investigators (10, 19). Our results regarding the lower sensitivity of the IHA test in lung hydatidosis are similar to those of Afferni et al. (1) and Pini et al. (13). Like others (3,

TABLE 4. Precision profiles for anti-hydatid antibody determination of three positive hydatid lung sera by IgG-ELISA

Absorbance (×10) of sera ± SD	CV (%)
Intraassay variation[a]	
6.5 ± 0.15	2.3
5.4 ± 0.12	2.2
6.5 ± 0.21	3.2
Interassay variation[a]	
6.533 ± 0.273	4.1
6.140 ± 0.2777	4.5
5.30 ± 0.24	4.5

[a] Number of samples analyzed in each case was 10.

10), we also detected false-positive reactions in the Casoni's i.d., IHA, and Poly-IFA tests (Table 3). They were, however, extremely low in the IgG-ELISA in which HHCF antigen was used; only 1.5% of cases showed a false-positive reaction. This was completely absent when SSA was used in the IgG-ELISA (Fig. 1).

Results of the Poly-IFA test were closer to those of the ELISA than other tests. However, the Poly-IFA test was more false-positive and less specific and sensitive in detecting cases of pulmonary and hepatic echinococcosis (Table 1) than was the IgG-ELISA.

To confirm the earlier observations that the use of purified echinococcal antigens is not essential for immunodiagnosis (2), we used two types of antigens, HHCF antigen and SSA. Both of these antigens have been good as such in the ELISA. The reproducibility of the ELISA also proved satisfactory, as shown by the intra- and interassay variation tests. The values of CVs and standard deviations were low (Table 4).

The measurement of hydatid-specific IgG by the ELISA seems superior to and better than even the detection of hydatid-specific IgE by the ELISA (1) with 5 A- and B-echinococcal antigens (12). With the latter method, only 77% of cases could be diagnosed, and the false-positivity was 25% (1). These investigators (1) also did not find purified echinococcal antigen superior to crude concentrated hydatid fluid in the double dilution test.

We found that the IgM-ELISA and IgM-IFA tests become negative within 6 months after surgical removal of the patient's hydatid cyst. This is in contrast to the IHA and Casoni's i.d. tests, which remain positive for several years after this operation (18, 19). It seems, therefore, that a seroconversion or a rising titer of IgM antibodies to echinococcal antigens, either in the IgM-ELISA or IgM-IFA tests, may act as a pointer to the recurrence of disease in surgically treated cases.

Our observation that the IgG-ELISA detects all cases of pulmonary hydatidosis excludes the possibility that decreased seropositivity in tests, other than the ELISA, is due to interference by circulating immune complexes (13, 15). It is difficult to say how far the decreased production of antibodies, consequent to less damage to the cyst germinal membrane and the condition of the cyst fluid, may be held responsible for decreased seropositivity (17). It may be that the IgG-ELISA is sensitive enough to detect such a low level of antibodies produced in pulmonary hydatidosis. Our results are in contrast to those of other investigators (5), who found the ELISA for echinococcal antibodies to be positive in only 72.5 and 45.1% of cases by use of purified echinococcal antigens and whole-hydatid-fluid antigen, respectively. They obtained 9.1% false-positivity with purified echinococcal antigens but none with whole-hydatid-fluid antigen. These investigators (5) have not found the ELISA to be good enough to detect all cases of hydatidosis, irrespective of the site of the cyst localization, and believe that it is better than neither the IHA test nor even the double dilution test. Under their laboratory conditions, the ELISA could detect only 48.5% pulmonary, 70.5% liver, and 78.6% multiple hydatid cyst cases out of 33 pulmonary, 44 hepatic, and 14 multiple echinococcosis cases. The lower sensitivity and specificity of the ELISA reported by these investigators could be due to the poor-quality enzyme-conjugated anti-immunoglobulin. We also tried to make our own enzyme-conjugated anti-immunoglobulin earlier, but the results were not satisfactory. Hence, we used the conjugate obtained from Sigma. Moreover, the optimal sensitizing concentration of antigen used by us was higher. The above-mentioned investigators used 12 μg of antigen protein per ml for coating the plate wells.

It is difficult to interpret the results of these immunodiagnostic tests in cases of multiple hydatid cysts, as their number is small. However, such cases do not appear to be low reactors, and as such their immunodiagnosis should not present any difficulty.

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