

APPLICATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) METHOD TO THE DIAGNOSIS OF HUMAN HYDATIDOSIS¹

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An investigation was conducted to evaluate the usefulness of a special ELISA method for diagnosing human hydatidosis. The results suggest the test may prove useful in seroepidemiologic studies and that it can make a valuable contribution to diagnosis of pulmonary hydatidosis.

Introduction

For some time immunologic methods such as indirect hemagglutination, latex agglutination, electrosyneresis or counterimmunoelectrophoresis, double diffusion in gel, immunoelectrophoresis, and indirect immunofluorescence have been widely used in diagnosing human hydatidosis. More recently, immunoenzymatic (ELISA) methods have been introduced into immunologic diagnosis. These methods were used first to detect intracellular antigens and antibodies in tissue sections, but this use was soon broadened to include detection of circulating antigens and antibodies, and in this manner the methods were rapidly and effectively incorporated into the procedures for immunodiagnosis of infectious and parasitic diseases.

At present, the ELISA methods' sensitivity is believed similar to that of radioimmunoassay; but they are cheaper and simpler to use than radioimmunoassay, and they permit complete quantification of the antigens and antibodies involved. They have therefore been readily accepted, and the number of research

workers using them for diagnosis of infectious and parasitic diseases is increasing steadily.

Underlying the successive emergence of different immunologic methods for diagnosing hydatidosis has been the desire to achieve several aims—including reduced performance time, more economical use of reagents (especially antigen), better reproducibility, greater sensitivity and specificity, and applicability to seroepidemiologic studies.

It appears that the ELISA methods serve all of these purposes. Certainly with regard to diagnosis of hydatidosis the initial results have appeared promising (1,2). Therefore, in view of such findings and the good results obtained in diagnosing other parasitic diseases, we felt it would be of interest to evaluate a new indirect ELISA method for diagnosing human hydatidosis. Accordingly, we subjected sera from hydatidosis cases and control sera to this ELISA test, the latex agglutination test, and the immunoelectrophoresis test commonly employed as the diagnostic reference method.

Materials and Methods

Antigen for the three tests was obtained from the hydatid fluid of fertile hepatic cysts in sheep. The fluid was dialyzed, lyophilized, and standardized by immunoelectrophoretic analysis (3), a procedure demonstrating the presence of antigen 5 (see Capron et al.—4) together with other antigenic fractions.

For the purpose of evaluating test sensitivity

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and specificity, 172 human sera were employed. These included:

1) 76 preoperative sera from patients with surgically confirmed hydatidosis (41 with hepatic hydatidosis, 23 with pulmonary hydatidosis, and 12 with multiple hydatidosis or hydatidosis at other locations).

2) 96 control sera consisting of:

(a) 30 sera from healthy donors;

(b) 30 sera from patients with various noninfectious and nonparasitic diseases—including diseases such as cirrhosis of the liver, hepatic and pulmonary neoplasms, and collagenopathies—that are capable of producing cross-reactions in serologic tests for hydatidosis.

(c) 36 sera from patients with various infectious and parasitic diseases—including amebiasis, ascariasis, brucellosis, distomatosis caused by *Fasciola hepatica*, giardiasis, leprosy, malaria, oxyuriasis, teniasis, toxoplasmosis, trichomoniasis, tuberculosis, and typhoid fever.

All the sera were stored for varying lengths of time at -20°C until used.

ELISA Test

The ELISA test employed goat sera anti-human-immunoglobulins conjugated with radish peroxidase from the Cappel Laboratories in Cochranville, Pennsylvania, U.S.A. The optimum dilution of the conjugate, determined by prior titrations, proved to be 1:800 in phosphate-buffered saline (pH 7.2) with 4 per cent bovine seroalbumin and 0.05 per cent Tween 20.

The technique used was the indirect method described initially by Engvall and Perlmann (5), employing the micro-technique proposed by Ruitenberget al. (6), with some modifications designed to adapt it to the antigen-antibody system under study.

Phosphate-buffered saline (pH 7.2) with 0.02 per cent sodium azide, was used for preparing the antigen solution. The optimum antigen concentration (5 μg dry weight per ml) was determined by prior titrations.

Polystyrene microtiter plates with flat bottom wells (Microtiter Brand, Dynatech Companies) were used in the test. Each well was

sensitized with 0.1 ml of the antigen solution and incubated for 3 hours at 37°C . The sensitized wells were then washed three times with distilled water containing 0.05 per cent Tween 20 before sera were added to them.

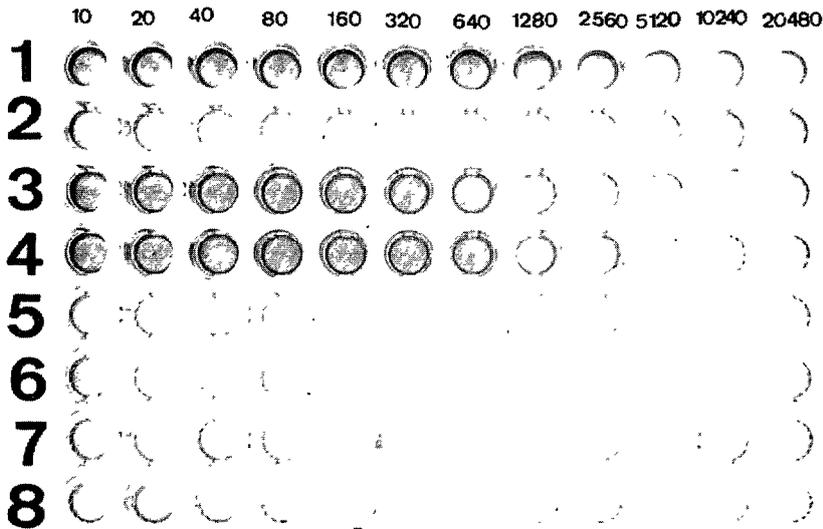
The sera were inactivated for 30 minutes at 56°C and were then diluted with phosphate-buffered saline (pH 7.2) containing 0.5 per cent bovine seroalbumin (Sigma Chemical Co., Missouri, U.S.A.). The initial dilution was 1:10 and double dilutions ranging up to 1:20,480 were made in successive wells (see photograph).

The plates thus prepared were incubated for 1 hour at 37°C . They were then emptied and washed three times with distilled water containing Tween 20. Next, 0.1 ml of the peroxidase conjugate was placed in each well and the plates were again incubated for 1 hour at 37°C . Following a further washing, 0.1 ml of substrate—a solution of 5-aminosalicylic acid (5AS) and H_2O_2 —was placed in each well. The substrate was prepared by dissolving 80 mg of 5AS in 100 ml of warm distilled water and adjusting the pH to 6.0 with NaOH (1N). Immediately before using the substrate, 0.05 per cent H_2O_2 was added to the 5AS solution at a rate of 1 ml for every 9 ml of 5AS solution.

The plates were then maintained at room temperature for 1 hour, after which the reaction was stopped by adding 0.025 ml of NaOH (1N) to each well. The results were then read visually; the final dilution that showed a color other than the color of negative sera was taken to be the highest titer yielding a positive response. Wells containing both positively and negatively responding sera are shown in the accompanying photograph.

Latex Agglutination Test (LA)

The method described by Guisantes and Picardo (7), a technical variant of the Varela-Díaz and Coltorti method (3), was employed. The hydatid antigen, standardized by immunoelectrophoretic analysis, was used at a concentration of 2 mg dry weight per ml of gly-



An ELISA microtitration plate with positive and negative sera. The wells in rows 1, 3, and 4 contain sera from hydatidosis patients, while those in the other rows contain sera from healthy subjects or from disease patients with cirrhosis of the liver or collagenopathy.

cine buffer (pH 8.2). This optimum concentration was determined by prior titrations with sera from a group of known hydatidosis cases and healthy donors.

Immunoelectrophoresis Test (IEP)

The IEP method of Capron et al. (4), as described by Guisantes et al. (8), was used for this test. Those sera that gave rise to diagnostic arc 5, as described by Capron et al. (4), were considered positive.

Results

Table 1 summarizes the ELISA test results obtained with the 96 control sera. These results show that 84 (87.5 per cent) of the 96 nonhydatid sera yielded a negative response, while 10 yielded a positive response at a 1:10 dilution. These latter sera came from one healthy donor, one patient with chronic rheumatoid polyarthritis, and eight patients with communicable or parasitic diseases (one case of teniasis due to *Taenia saginata*, two cases of

Table 1. ELISA test results obtained with 96 control sera from subjects without hydatidosis—including subjects with various other kinds of diseases.

Source of sera	No. of sera tested	No. negative	No. positive	
			1:10	≥ 1:20
Healthy donors	30	29	1	0
Subjects with infectious and parasitic diseases	36	26	8	2
Subjects with other diseases	30	29	1	0
Total	96	84	10	2

oxyuriasis, two of distomatosis due to *Fasciola hepatica*, one of amebiasis, and two of tuberculosis). In all, 94 of the 96 control sera yielded responses that were negative, or else were positive only at a titer of 1:10.

The only two sera positive at a titer greater than 1:10 came from two patients with teniasis caused by *Taenia saginata*. One of these was positive at a titer of 1:80 and the other at a titer of 1:160. The latter also gave a positive response to the LA test. Both sera yielded negative IEP results.

In the ELISA test, as in the indirect hemagglutination (3) and other quantitative tests, it is necessary to establish a titer of diagnostic significance, that being a dilution at which positive responses from nonhydatid sera are nonexistent or very rare (9). The data reported here show that we can establish a diagnostic titer of 1:20 for the antigen used, with a probability of error (false positive diagnosis) of $p < 0.025$.

Table 2 summarizes the ELISA test results obtained with 76 sera from hydatidosis cases. Of these, all but three (96 per cent) were positive at the diagnostic (1:20) titer, and two of those three were positive at 1:10. Sensitivity to sera from pulmonary hydatidosis patients appeared slightly less than sensitivity to sera from hepatic hydatidosis patients, but the difference observed was not statistically significant.

Table 3 shows the IEP and LA test results obtained with the 76 sera from hydatidosis cases, according to the location of the cyst. As may be seen, the overall sensitivity of the IEP

test was 68.4 per cent, ranging from 47.8 per cent in cases with pulmonary cysts to 83.3 per cent in cases with multiple cysts or nonhepatic and nonpulmonary cysts. Its sensitivity to hepatic cysts (75.6 per cent) was substantially higher than its sensitivity to pulmonary cysts.

Regarding the LA test, its overall sensitivity was 80.2 per cent, and like the IEP test it showed greater sensitivity to hepatic cysts (90.2 per cent) and to multiple or nonhepatic and nonpulmonary cysts (91.6 per cent) than it did to pulmonary cysts (56.5 per cent).

Table 4 compares the ELISA and LA test results obtained with sera from hydatidosis patients. It should be noted that 12 sera yielding negative LA test results gave a positive response to the ELISA test. In contrast, none of these 76 sera yielded a negative ELISA response and a positive LA response, all three ELISA-negative sera yielding negative LA results.

Only one of the 96 nonhydatid sera (from a teniasis case) yielded positive LA test results, so that the rate of false positive response was 1.04 per cent. None of the 96 control sera yielded a positive IEP response; that is, none gave rise to diagnostic arc 5 (4).

Table 2. ELISA test results obtained with 76 preoperative sera from hydatidosis patients, by cyst location.

Location of cyst	No of sera tested	No. negative	No. positive	
			1:10	≥ 1:20
Hepatic	41	0	1	40(97.5%)
Pulmonary	23	1	1	21(91.3%)
Other location or multiple cysts	12	0	0	12(100%)
Total	76	1	2	73 (96%)

Table 4. A comparison of the ELISA and LA test results obtained with preoperative sera from 76 hydatidosis patients. Only ELISA titers of 1:20 or greater were considered positive.

No. of sera	Test results			
	ELISA + LA +	ELISA + LA -	ELISA - LA +	ELISA - LA -
	61	12	0	3

Table 3. Immunoelectrophoresis (IEP) and latex agglutination (LA) test results obtained with preoperative sera from 76 hydatidosis patients, by cyst location.

Location of cyst	No. of sera tested	Positive by IEP		Positive by LA	
		No.	%	No.	%
Hepatic	41	31	75.6	37	90.2
Pulmonary	23	11	47.8	13	56.5
Other location or multiple cysts	12	10	83.3	11	91.6
Total	76	52	68.4	61	80.2

Discussion

Sensitivity and Specificity

The results obtained indicate that the ELISA method used was highly (96 per cent) sensitive at titers of diagnostic significance. They also show it to be highly specific, only two of the 96 sera from healthy subjects and nonhydatid disease patients having yielded a positive response of diagnostic significance. These two latter sera, it should be noted, came from patients infected with *Taenia saginata*. Such a result could have been anticipated, since it is well-known that antigenic sharing exists between this cestode and the hydatidosis agent, *Echinococcus granulosus*, both of which belong to the Taeniidae family.

Only two of the three patients infected with *Fasciola hepatica* yielded a positive ELISA titer, and these (both 1:10) were below the diagnostic titer of 1:20.

The Diagnostic Titer

Determining a titer of diagnostic significance tends to increase the specificity of any quantitative serologic method (3). In essence, such a titer can be determined using either of two basic criteria (9); that is, the diagnostic titer should be (a) the lowest at which no response is observed with nonhydatid sera, or (b) the lowest at which a minimum number of cross-reactions is observed.

We chose to adopt the latter criterion in this study for several reasons. In the first place, cross-reactivity occurred, in the case of sera from teniasis patients where it was logical to find it, when whole hydatid fluid was used as the antigen. Second, when the latter criterion was used, the observed rate of false positive responses (2.1 per cent) was very low. Third, it must be kept in mind that the specificity of any given method will be affected by the cross-reactivity of the control sera used to determine the diagnostic titer (3, 9). And finally, because the purified "fraction 5" antigen described by Capron et al. (4) was not used, there is no

reason why a false positive titer higher than any given diagnostic titer could not be attained with some other more cross-reactive sera from teniasis cases. It is for this latter reason that, because of its specificity, the IEP test based on observation of diagnostic arc 5 (Capron et al.—4) continues to be the reference test for diagnosis of human infection with *Echinococcus larvae* (3, 9-12).

Conclusions

Although the ELISA test calls for high-quality reagents and meticulous procedure, and although it may initially seem complicated, once a minimum amount of practice has been acquired it is no more difficult than the indirect hemagglutination test. Furthermore, it has an advantage over the latter in that it does not require red blood cells, which can be difficult for some laboratories to obtain.

The ELISA method described is also rapid (a definitive result is available in 5 or 6 hours), reasonably inexpensive, and repeatable. (Regarding the latter point, at our laboratory we have on several occasions repeated tests with the same sera, and with each serum have obtained the same positive titer or a titer difference no greater than one dilution.)

In view of the frequent difficulties involved in obtaining hydatid fluid and producing standardized antigens by means of immunoelectrophoretic analysis (3), immunologic laboratories find tests that are adequately sensitive and specific and that use only small amounts of antigens to be very desirable. In this regard, it should be noted that the ELISA test uses minimal amounts of antigen. Moreover, the test described here uses whole hydatid fluid instead of the purified fraction 5 that is harder to obtain.

Use of the micromethod employed in this study also makes it possible to test a large number of sera on the same day, because once the antigen has adhered to the microtiter plates, the material keeps well at 4°C for at least a week in all cases.

Since the LA test is a method recommended for diagnosing clinical cases of hydatidosis, as well as for seroepidemiologic surveys, we felt it would be worthwhile to compare ELISA and LA results. As Table 4 shows, the ELISA test detected 12 hydatidosis cases that yielded negative results. (Overall, the LA test detected 80.2 per cent of the hydatidosis cases, while the ELISA test detected 96 per cent.) On the other hand, corroborating the results of earlier studies (7,9,10), the rate of false positive LA responses (1.04 per cent) was very low.

To sum up, a test used in seroepidemiologic studies should meet the following criteria: It should be (a) simple, (b) rapid, (c) inexpensive, (d) repeatable, (e) sensitive and specific, (f) sparing in its use of antigen, and (g) capable of processing many sera simultaneously. On the basis of our results, we believe that evaluating the ELISA method described in seroepidemiologic studies, by using it in surveys assessing large numbers of sera, would yield findings of considerable interest.

Our own results also suggest that the method would be very useful for diagnosing hospital cases of hydatidosis because of its high sen-

sitivity, as indicated by detection of 73 of the 76 cases studied (error probability of false positive results being $p < 0.025$).

Also, it is well-known that the usual serologic tests for human hydatidosis are less sensitive to pulmonary cysts than to cysts at other places in the body, a fact corroborated in our study by the LA and IEP test results (see Table 3). In contrast, the ELISA method used demonstrated great sensitivity (91.3 per cent) to sera from patients with pulmonary cysts, a sensitivity similar to that for sera from patients with hepatic cysts (see Table 2). The ELISA test should therefore make a very valuable contribution to detection of pulmonary hydatidosis, which frequently yields seronegative results in other diagnostic tests.

It should, of course, be remembered that diagnosis of hydatidosis in man is based on a combination of clinical, serologic, radiologic, and scintillographic data. Therefore, although the rate of false positive responses obtained with the ELISA method used was very low, the possibility of such responses should be kept in mind in diagnosing cases where the ELISA test is positive but the IEP test yields negative results.

SUMMARY

The purpose of the study reported here was to evaluate the contribution an indirect enzyme-linked immunosorbent assay (ELISA) method could make in diagnosing cases of human hydatidosis. Accordingly, 76 human sera from surgically confirmed hydatidosis cases and 96 control sera were subjected to simultaneous latex agglutination (LA), immunoelectrophoresis (IEP), and ELISA tests.

The ELISA method chosen employs anti-human globulins conjugated with peroxidase and uses a solution of 5-aminosalicylic acid and H_2O_2 as a substrate. On the basis of positive titers obtained with the control sera, a diagnostic titer of 1:20 was established. Overall, the sensitivity of the ELISA test at titers of diagnostic significance was 96 per cent. No major differences were found between sensitivity to pulmonary as compared to hepatic and other types of hydatidosis cases.

Only two of the 96 nonhydatid (control) sera yielded positive titers higher than 1:10, and both of

these sera came from patients with teniasis caused by *Taenia saginata*. The overall rate of false positive ELISA responses was thus 2.08 per cent.

By comparison, the overall sensitivity of the LA test was 82 per cent, the percentage of cases detected ranging from 56.5 per cent of the pulmonary cases to 90 per cent of the hepatic cases. The overall rate of false positive LA responses was 1.04 per cent. Similarly, the overall sensitivity of the IEP test was 68.4 per cent, the percentage of cases detected ranging from 47.8 per cent of the pulmonary cases to 75.6 per cent of the hepatic cases. There were no false positive IEP results.

Twelve sera from hydatidosis cases that were found negative by the LA test yielded a positive response to the ELISA test. In general, the results obtained suggest that the ELISA method employed could prove useful for seroepidemiologic studies and that it should make a very useful contribution to detection of cases of pulmonary hydatidosis.

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