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Evaluation of five immunodiagnostic techniques in echinococcosis patients

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*Double diffusion (DD), indirect haemagglutination (IHA), immunoelectrophoresis (IEP), latex agglutination (LA), and complement fixation (CF) tests were evaluated for sensitivity and specificity in the diagnosis of 141 surgically-proven Turkana echinococcosis patients and 10 controls. The overall sensitivities for the tests were: IHA, 86.7%; LA, 53.3%; CF, 63.3%; DD, 55.0%; IEP, 55.0%. LA and CF tests produced a high number of false positive results; IHA gave a false positive result in 10% of cases; no false positives were obtained with IEP and DD. A combination of the latter three tests would therefore offer the best chance of detecting specific anti-*Echinococcus *antibodies, with an average sensitivity of 62.7%. The possible reasons for the relatively high incidence of false negative values are discussed.*

This paper presents the results of tests on the sensitivity and specificity of diagnostic techniques for human echinococcosis in 90 surgically-confirmed patients from Turkana district, Kenya, which has the highest incidence of the disease in the world (*1*, *2*). Additional studies were carried out on a further 51 patients to evaluate the combined use of three tests. Results were obtained in male and female echinococcosis patients of different ages, and grouped according to the stage of the infection and the condition of the cysts.

MATERIALS AND METHODS

Reference antigen and antisera

A crude lyophilized hydatid cyst fluid antigen (20 mg) and antisera (1 ml)[a] were used as reference standards.

Processing of human hydatid cyst fluid antigen (HHCF)

HHCF was collected from hepatic, mesenteric, and abdominal cysts in echinococcosis patients undergoing surgery, and processed as described elsewhere (*3*). Protein content was estimated using a modification of the Lowry method (*4*). Dialysis was carried out against acetate buffer, and the fluid was lyophilized and used at a concentration of 100 g/litre in barbital buffer solution.

Production of hyperimmune rabbit serum

Adult rabbits (3–4 kg) were inoculated with 0.5 mg of crude HHCF antigen in phosphate-buffered saline (PBS), emulsified with an equal volume of Freund's complete adjuvant. After 6 weeks, they were given the same amount of antigen in Freund's incomplete adjuvant. Blood samples were taken 10 days later and monitored for precipitating antibodies to the HHCF antigens by double diffusion (DD) and immunoelectrophoresis (IEP) tests. Both the antigen and the antisera were standardized using the reference standards.

Serum samples

Serum samples were collected from surgically-proven echinococcis patients in Lodwar and Kakuma hospitals in Turkana district, north-west Kenya. These samples were preserved in 0.015 mol/litre sodium azide and stored at −20 °C until tested. All the patients examined were screened for other parasitic infections. Negative control sera were also collected from the same district.

Immunodiagnosis

A panel of five immunodiagnostic techniques was employed to detect anti-*Echinococcus* antibodies in the patients' sera, as described below.

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Some of the serum samples were examined independently in three other laboratories. This collaborative programme was carried out using a blind test procedure and the results were communicated by the coordinator after receiving all the data.

Immunoelectrophoresis. Conventional IEP was carried out as described elsewhere (*5*), using 12.0 g of agarose per litre of sodium barbital buffer (pH 8.6). Aliquots of 15 μl of HHCF antigen were applied to the wells in the gel, and electrophoresis was carried out for 90 min at a constant current. A serum sample was then added to the troughs. After the precipitation bands had formed, the slides were washed in 0.15 mol/litre NaCl and the plates dried with filter papers. Finally, the slides were stained with a 0.5 g/litre solution of Coomassie blue in 1.75 mol/litre glacial acetic acid and 432 ml/litre ethanol, and then destained using 0.01 mol/litre trisodium citrate in the same concentration of ethanol and acetic acid.

Double diffusion (DD). Agarose gel (12 g/litre) was prepared using PBS (pH 7.2), and antigen and antisera were applied to the wells, as described elsewhere (*6*). The plates were then incubated in a moist chamber and processed as described for the IEP slides.

Indirect haemagglutination test (IHA). The IHA test was based on the tanned-cell technique (*7*) with a few modifications. Sheep red blood cells were coated with 4 g of HHCF antigen per litre, at pH 6.4. All the test sera were diluted 1:256 (the diagnostic titre) and the agglutination reactions recorded.

Complement fixation (CF) test. Serum samples from guinea pigs were used as the source of complement. The serum was kept at −70 °C until used. The CF test system was then set up as described elsewhere (*3*, *8*). By a checkerboard titration procedure, the optimal complement activity was found to be 1:1000. After further standardization of the system the optimal antigen and antibody proportions were found to be 4.88 mg/litre (HHCF) and 1:32.

Latex agglutination (LA) test. A commercial preparation of Bacto-latex particles, 0.18 μm diameter,[b] was diluted 1:4 with glycine buffer, pH 8.2. The latex particles were sensitized using 3 g of HHCF antigen per litre. Agglutination tests with positive control samples showed that the diagnostic titre was 1:5. Agglutination reactions for the patients' serum samples were recorded using dilutions of 1:5, 1:10, and undiluted sera.

[b] From Difco Laboratories, Michigan, USA.

RESULTS

Standardization

The strengths of the prepared HHCF antigen and the antisera raised in the rabbits were comparable with those of the reference standards. These were therefore used to check the antigen preparations used in the immunodiagnostic tests.

The protein concentration in each patient's cyst fluid was between 0.23 g/litre and 1.02 g/litre. The concentration of proteins in the HHCF antigen used was 0.5 g/litre.

Sensitivity and specificity

Table 1 shows the results of the five immunodiagnostic tests on samples from 90 surgically-confirmed echinococcosis patients and 10 negative controls. The highest sensitivity obtained was 86.7%, with IHA, and the lowest was 53.3% (LA). In 32 patients all the tests were positive, while in 11 they were all negative. IHA was positive in 17 patients for whom all other tests were negative. There was no clear correlation for the remaining 33.3% of the cases.

Although IHA had the highest sensitivity, the false positive results obtained indicated that other tests are also needed to confirm the diagnosis. LA and CFT were less discriminating in monitoring for human echinococcosis, producing a fairly high number of

Table 1. Comparison of results of immunodiagnostic tests in 90 echinococcosis patients from Turkana district, Kenya

No. of patients	Percentage of patients	Immunodiagnostic test				
		IHA	LA	CFT	DD	IEP
32	35.6	+	+	+	+	+
11	12.2	−	−	−	−	−
5	5.6	+	+	+	−	−
3	3.3	+	−	+	+	+
2	2.2	+	+	−	−	−
5	5.6	+	−	+	−	−
6	6.7	+	+	+	−	+
17	18.9	+	−	−	−	−
5	5.6	+	−	+	+	−
3	3.3	+	+	−	+	+
1	1.0	−	−	+	+	−
No. found positive		78/90	48/90	57/90	44/80	44/80
%		86.7	53.3	63.3	55.0	55.0
Negative control samples No. found positive		1/10	2/10	1/10	0	0
%		10	20	10	0	0

Table 2. Results of IHA, DD, and IEP tests on 51 echinococcosis patients

Clinical status	No. of patients	IHA		DD		IEP		Mean detection rate (%)
		No. positive	%	No. positive	%	No. positive	%	
Primary echinococcosis	40	30	75.0	22	55.0	22	55.0	61.7
Recurrent echinococcosis	11	9	81.8	7	63.6	7	63.6	69.7
Fertile cysts	19	16	84.2	11	57.9	11	57.9	66.7
Sterile cysts	32	17	53.1	13	40.6	13	40.6	44.8
Male patients	18	15	83.3	11	61.1	11	61.1	68.5
Female patients	33	26	78.7	20	60.6	20	60.6	66.6
Age group: 1–18 years	17	13	76.5	10	58.8	10	58.8	64.7
19–45 years	31	23	74.2	18	58.1	17	54.8	62.4
> 45 years	3	2	66.7	1	33.3	1	33.3	44.4

false positive results.

The results of the studies on the additional 51 patients are shown in Table 2. The overall sensitivities in primary and recurrent *Echinococcus* infection were 61.7% and 69.7%, respectively. The breakdown of the results showed that the percentage of positive reactions varied according to the age of the patient, with 64.7% in the 1–18-year age group, 62.4% in the 19–45-year age group, and 44.4% in the group over 45 years old. Different values were also obtained for males and females (68.5% and 66.6%, respectively).

Most of the patients examined had abdominal cysts, and 63.4% (26/41) gave positive results in the immunodiagnostic tests. The condition of the cyst also appeared to influence the detection rate of anti-*Echinococcus* antibodies in the serum. Of the patients with sterile cysts, 44.8% were positive, while of those with fertile cysts, 66.7% gave positive reactions.

Table 3. Comparison of results of tests carried out in Switzerland and Kenya, on serum samples from patients and controls from Turkana district, Kenya

Patient no.	Switzerland		Kenya		Surgery	Clinical status
	IIF[a]	IHA	IEP	DD		
1	–	–	–	–		Negative control
2	–	–	–	–		Negative control
3	±	–	–	–	+	Primary echinococcosis
4	+	±	+	+	+	Primary echinococcosis
5	±	±	+	+	+	Primary echinococcosis
6	+	+	+	+	+	Primary echinococcosis
7	+	–	–	–		Negative control
8	–	–	–	–	+	Primary echinococcosis
9	–	–	–	–		Negative control
10	+	+	+	+	+	Primary echinococcosis
11	–	–	–	–		Negative control
12	–	–	–	–	+	Primary echinococcosis
13	±	–	–	–	+	Primary echinococcosis
14	–	–	–	–	+	Primary echinococcosis
15	–	–	–	–		Negative control
16	+	+	+	+	+	Primary echinococcosis
17	–	–	–	–	+	Primary echinococcosis

[a] IIF = Indirect immunofluorescent test

Table 4. Comparison of results of tests carried out in Argentina, France, and Kenya on serum samples from patients and controls from Turkana district, Kenya

Patient no.	France		Argentina	Kenya		Surgery	Clinical status
	IEP	IHA	DD	IEP	DD		
15	-	-	-	-	-		Control
57	-	-	-	-	-		Control
63	-	-	-	-	-		Control
92	-	+	-	-	-		Control
77	-	-	-	-	-		Uterine fibroids
33	+	+	+	+	+	+	Primary echinococcosis
42	+	+	+	+	+	+	Primary echinococcosis
72	+	+	+	+	+	+	Primary echinococcosis
95	+	+	+	+	+	+	Primary echinococcosis
29	-	-	-	-	-	+	Primary echinococcosis
56	-	-	-	-	-	+	Primary echinococcosis
48	-	+	-	-	-	+	Primary echinococcosis
53	-	+	-	-	-	+	Primary echinococcosis
14	+	+	+	+	+	+	Recurrent
81	+	+	+	+	+	+	Recurrent
89	±	+	-	-	-	+	Recurrent
17	+	+	+	+	+	+	Recurrent

Collaborative studies

Table 3 shows the results of a collaborative study on 17 Turkana patients. The percentage sensitivities obtained in the two laboratories were: IIF, 45.4%; IHA, 27.3%; IEP, 45.4%; and DD, 45.4%. Only the indirect immunofluorescence test (IIF) was positive in one negative control (patient no. 7).

Table 4 shows data from three laboratories where sera from 17 Turkana patients were tested. No false positive reactions were obtained with the IEP and DD tests. IHA gave one false positive result (patient no. 92) at a titre of 1/80. The overall sensitivity of both IEP and DD was 58.3% (7/12); IHA was very sensitive, since in patients no. 48, 53, and 89 it gave highly significant titres of 1/640 when the IEP and DD tests both gave negative results. Only 2 patients (no. 29 and 56) had a low titre of 1/80, which was below the diagnostic titre, giving an overall percentage sensitivity for IHA of 83.3% (10/12). Thus there was complete agreement in the results of the four world laboratories on echinococcosis patients' examination.

DISCUSSION

Results have been presented on the performance of several immunodiagnostic tests for echinococcosis in patients from Turkana district. There is very little *Echinococcus* infection in other areas of Kenya.

The average sensitivity of the five tests was 62.7%, so that the diagnostic procedures employed would fail to detect anti-*Echinococcus* antibodies in 37.3% of surgically-proven cases. The IHA test had an overall sensitivity of 86.7%, but the average sensitivity of the other tests was rather low. Results obtained by the independent collaborating laboratories confirmed these low values, whereas results from Caucasian communities have produced false negative values of 10–20% (*9–11*).

The immunological aspects of the host–parasite relationship in patients from the Turkana district appear to differ from those seen in other areas. Work done on isoenzyme characterization and *in vitro* culture (C. Macpherson, personal communication, 1979) suggests the existence of a different strain of *E. granulosus* in Kenya. Infection of the Turkana people with this strain may change their immune responses and affect the diagnostic results.

The high number of false negative results may also be a result of several other factors. The presence of inhibitors in the serum samples and the role of immune complexes ought to be considered. Although Richard-Lenoble et al. (*11*) found only 2 of 13 echinococcosis patients with positive sera to have immune complexes, our preliminary data indicate a much higher proportion with circulating immune complexes. Unlike the cysts in Caucasian populations, those found in Turkana patients were often very large, containing up to 4 litres of cyst fluid. This could mean

that massive release of *Echinococcus* antigens occurs and immune complexes are formed readily in these patients. The effect of these circulating immune complexes on diagnostic techniques remains to be investigated.

It is therefore important to interpret the immunodiagnostic results in Turkana patients carefully, taking into consideration the immune status of the patient, possible strain differences in *E. granulosus*, and the presence of immune complexes and other inhibitors in the serum samples. No single test provides 100% sensitivity and specificity for echinococcosis and a combination of IHA, DD, and IEP appears to give the most reliable results.

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RÉSUMÉ

EVALUATION DE CINQ TECHNIQUES IMMUNODIAGNOSTIQUES CHEZ DES MALADES ATTEINTS D'ECHINOCOCCOSE

Dans les régions où l'échinococcose humaine sévit de façon endémique, il est urgent de pouvoir disposer d'épreuves immunodiagnostiques tant pour procéder à des études épidémiologiques que pour assurer le suivi des malades traités chirurgicalement. L'article présente les résultats d'études sur la sensibilité et la spécificité de plusieurs techniques utilisées pour l'examen d'échantillons de sérum prélevés chez des cas chirurgicalement confirmés provenant du district de Turkana, au Kenya, où l'incidence de l'échinococcose est plus élevée que partout ailleurs dans le monde.

Dans l'ensemble, la sensibilité des épreuves a été la suivante: hémagglutination indirecte 86,7%; agglutination de particules de latex 53,3%; fixation du complément 63,3%; double diffusion 55%; et immunoélectrophorèse 55%. La sensibilité moyenne était de 62,7%, ce qui veut dire que ces techniques diagnostiques n'ont pas décelé les anticorps anti-*Echinococcus* chez 37,3% des malades positifs. La double diffusion et l'immunoélectrophorèse n'ont donné aucun résultat faussement positif tandis que l'hémagglutination indirecte, la fixation du complément et l'agglutination du latex en ont donné respectivement 10%, 10% et 20%. C'est l'hémagglutination indirecte détectant les anticorps agglutinants anti-*Echinococcus*, qui a présenté la sensibilité la plus élevée, tandis que les sensibilités moyennes des autres techniques, fondées sur la détection des anticorps précipitants ou des anticorps activant le complément, étaient plutôt faibles. Ces résultats ont été confirmés par l'examen indépendant de certains des échantillons de sérum dans des laboratoires en Suisse, en Argentine et en France.

Il convient, toutefois, d'interpréter avec circonspection ces résultats. Il est important d'étudier l'état immunitaire des malades, en particulier leurs réponses immunitaires cellulaires. Il est aussi avéré qu'il existe une souche locale différente de *E. granulosus*, dont il faudrait explorer la capacité de donner des antigènes ayant une valeur diagnostique et son effet sur les aspects immunologiques de la relation hôte–parasite. En outre, la présence d'immuncomplexes et d'autres inhibiteurs sériques décelés chez certains des patients doit être étudiée et leur importance pour l'immunodiagnostic démontrée.

A Turkana, par conséquent, aucune épreuve n'a à elle seule eu une sensibilité et une spécificité de 100% et l'utilisation combinée de l'hémagglutination indirecte, de la double diffusion et de l'immunoélectrophorèse semble donner les résultats les plus fiables. Il faut, avant tout, continuer à étudier la réponse immunitaire des malades atteints de *E. granulosus*.

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