The double diffusion precipitin test in human fascioliasis

PHILIP H. EVERALL

From the Department of Pathology, Royal Salop Infirmary, Shrewsbury

SYNOPSIS The recent outbreaks of human fascioliasis in Britain provided the opportunity for employing the double diffusion precipitin test in the diagnosis of this disease. In sensitivity and specificity this test appears to equal the complement-fixation test and has the advantages of simplicity and speed in its performance.

The definitive test in human fascioliasis is the demonstration of the ova of *Fasciola hepatica* in the faeces or bile of the patient. However, ova are never found during the first three to four months following infection while the young flukes are migrating to their final position in the bile ducts of their host and attaining sexual maturity. Furthermore, even after the expiry of this latent period the demonstration of liver fluke eggs can only be made in about 70% of patients (Coudert, 1960a). For these reasons serological tests have an important role in the diagnosis of this disease. The one most commonly used in Britain is the complement-fixation test, but passive agglutination tests, precipitin tests (either in their classical liquid phase or by the double diffusion method), and immunoelectrophoretic analysis have all been employed (Coudert, 1960b; Capron, Biguet, Tran Van Ky, and Rosé, 1964; Biguet, Rosé, and Capron, 1965).

The recent outbreak of human fascioliasis in Shropshire (Ashton, Boardman, D'Sa, Everall, and Houghton, 1970) provided the stimulus and some of the material for an investigation of the value of the double diffusion precipitin test in this disease.

Received for publication 6 March 1970.

Materials and Methods

HUMAN SERUM

Sera from 54 persons suspected of being infected were received from Shropshire (16 persons), Gloucestershire (33 persons), Warwickshire (3 persons), and Buckinghamshire (2 persons).

Additionally, 139 sera which had been submitted for serological tests for syphilis and one pool (C-reactive protein + serum) of an unknown number of human sera which had previously been found positive for C-reactive protein were tested. (The C-reactive protein + serum was included as a control to demonstrate that precipitation was not occurring as a result of the interaction of C-reactive protein and C substance. The latter is sometimes present in small quantity in helminth extracts.)

DOUBLE DIFFUSION PRECIPITIN TEST

Antigen

Adult liver flukes obtained from the local abattoir were washed rapidly in four changes of sterile physiological saline and then frozen at -20°C . After thawing, 20 g of flukes was ground

in a pestle and mortar with the aid of a little silver sand. One hundred ml of sterile saline was added to the emulsion thus obtained. The mixture was shaken vigorously and then centrifuged at 3,000 revolutions per minute for 10 minutes. The opalescent supernatant constituted the antigen solution. Sodium azide was then added to a concentration of 0.1% and the antigen stored at 4°C until required for use.

Buffered agar gel (pH 7.4)

This has the composition:

Oxoid agar no. 1	..	..	..	20 g
Sodium chloride	..	..	..	17 g
Sodium azide	..	..	..	2 g
Potassium hydroxide	..	..	..	8 g
Disodium salt of ethylene diamine tetra-acetic acid (EDTA)	..	..	..	0.5 g
Potassium dihydrogen phosphate (KH ₂ PO ₄)	..	..	..	27.5 g
Distilled water	..	..	..	2,000 ml

The agar is dissolved in the water by heating; the remainder of the reagents is then added.

The molten, buffered gel was poured into scratch-free 9 cm Petri dishes (15 ml per dish), and after setting, holes were cut as shown in the figure. A cutter,¹ which was originally designed for quantitative analysis of protein solutions (Soothill, 1962), was employed for this, but is not essential.

METHOD

Into each of the two large cups (A) on the left side of the plate was pipetted 0.04 ml of neat antigen solution, and antigen diluted 1/3 was similarly pipetted into the large cups (B) on the right side of the plates. The smaller peripheral cups were filled with the sera under investigation, each serum being tested against both neat antigen and antigen in 1/3 dilution. In this way, 12 sera were tested on each plate at two different antigen/antibody ratios (Fig. 4).

The plates were incubated at 37°C and read daily for three days for the appearance of precipitin lines. Where present, they were checked for the reaction of identity with known positive sera.

COMPLEMENT-FIXATION TESTS

These were carried out by Dr D. S. Ridley at the Hospital for Tropical Diseases, London.

Results

Of the 54 patients originally suspected of infection with *F. hepatica*, 19 had positive stool examinations for ova and a further 13 (making 32 in all)

were diagnosed as infected when compatible clinical and epidemiological findings existed in the presence of a positive serological test.

In Table I the results of the complement-fixation test and the precipitin test on these 32 patients are compared.

Most of the sera positive in the precipitin test gave one strong line but some sera gave one or two weaker lines in addition. A complement-fixation test positive at 1/5 to 1/10 is thought to be probably significant and positive results at 1/20 or higher are considered definite positives (Ridley, 1969).

The individual laboratory findings on those five patients whose sera gave negative results with

Fig. Double diffusion precipitin test

Cups A and B (7.5 mm in diameter) contain *fasciola* antigen, neat in A and diluted 1/3 in B. Small cups, 1-12 (diameter 5 mm), are filled with patients' sera, each serum being tested against the two antigen concentrations. The distance between the large and small cups is 3 mm. Precipitin lines with a reaction of identity are given by sera 1, 2, 3, and 4, and 10 and 11.

¹Peter Brasshouse Ltd, Spring Hill, Birmingham 18.

	CFT+ ¹ Precipitin+	CFT+ Precipitin—	CFT— Precipitin+	CFT— Precipitin—
Ova present in stools (19 cases)	12	2	3	2
Ova not demon- strated (13 cases)	10	1	2	—
Totals	22	3	5	2

Table I Results of complement-fixation and precipitin tests.

¹Indicates a titre of 1/5 or greater.

Patient	Eosinophilia	Ova	CFT (highest titre recorded)
M.L.	+	+	Negative
P.P.	—	+	1/5
G.H.	—	+	Negative
J.H.	—	+	1/10
R.P.	+	—	1/5

Table II Negative results with precipitin tests in five patients.

the precipitin test when other tests were positive are summarized in Table II.

Included in the 22 patients in whom a diagnosis of fascioliasis was not made was one patient (M.B.) whose serum gave a positive complement-fixation test result at 1/5 and a negative precipitin test, and a second patient (J.B.) whose serum, whilst giving a negative complement-fixation test, produced two weak lines in the precipitin test, neither of which gave a reaction of identity with lines from other positive sera. On clinical findings alone, it was considered unlikely that either of these two patients were, in fact, suffering from fascioliasis.

No precipitin lines were developed against the *F. hepatica* antigen by any of the 139 sera submitted for serological tests for syphilis or with the C-reactive protein + serum.

Discussion

The antigen used in the precipitin tests was a crude saline extract of adult *F. hepatica*, no attempt having been made either to remove lipid or to fractionate it in any other way. Such an antigen may give precipitin lines with the serum of patients infected with helminths other than *F. hepatica*. Thus, Biguet, Capron, and Tran Van Ky (1962), demonstrating 15 antigens in saline extracts of adult *F. hepatica* using a hyperimmune rabbit serum, showed by the use of extracts prepared from other helminths that only five of the

15 were specific to *F. hepatica*. However, the five specific antigens included one strongly antigenic fraction, the antibody to which is the first to appear in experimental immunization of the rabbit and in the naturally acquired disease in humans. In this country, where helminthic infection with associated brisk antibody response is not common, the risk of false reactions must be slight. It is possible that the positive precipitin test given by the serum of patient J.B., who was not thought to be suffering from fascioliasis, is an example of a false reaction due to common helminth antigens. (This patient's faeces were not examined at any time during this investigation.)

Capron *et al* (1964) recommended the technique of immunoelectrophoresis for the diagnosis of human fascioliasis. This allows antigenic components reacting with the antibodies in the patient's serum to be distinguished by their electrophoretic mobility. However, the technique requires the preliminary two-fold concentration of the patient's serum, is time consuming, and does not permit of the easy identification of 'reaction of identity' as does the ordinary double diffusion technique. Apart from being simple and quick to perform, the merit of the double diffusion precipitin test is this ability to demonstrate 'reaction of identity', a more delicate test of chemical relationship than electrophoretic mobility.

Capron *et al* (1964) also drew attention to the dangers of false positives arising from the interaction of C-reactive protein in the serum of patients with the C substance which they found to be present in small amounts in their *F. hepatica* antigen. This can, of course, be avoided by the choice of buffer for the gel used in the diffusion tests since calcium ions are essential for the reaction between C-reactive protein and C substance. The EDTA incorporated in the gel used in the series of tests reported here appeared effectively to inhibit this reaction since no reaction was obtained between C-reactive protein + serum and the crude antigen.

The specificity and sensitivity of the complement-fixation test and the double diffusion precipitin test appear to be very similar when applied to residents of the United Kingdom. Of the 32 patients diagnosed as having fascioliasis, both serological tests were positive in 22, the complement-fixation test was positive alone in three, and the precipitin test positive alone in five. The sensitivity of the precipitin test appeared to be even greater as compared with the complement-fixation test if the results on contemporary serum specimens submitted to both tests were compared.

While the performance of a complement-fixation test for fascioliasis demands the skills of a laboratory specializing in such work, the relative simplicity of the double diffusion precipitin test makes it a useful screening procedure capable of being performed in most routine medical laboratories.

I am grateful to all those who kindly provided serum specimens from patients suspected of fascioliasis, and to Dr G. Grant for his advice and encouragement.

References

- Ashton, G., Boardman, P., D'Sa, C. J., Overall, P. H., and Houghton, A. W. J. (1970). Human fascioliasis in Shropshire. *Brit med. J.*, 3, 500-502.
- Biguet, J., Capron, A., and Tran Van Ky, P. (1962). Les antigènes de *Fasciola hepatica*. Étude électrophorétique et immunoelectrophorétique. Identification des fractions et comparaison avec les antigènes correspondant à sept autres helminthes. *Ann. Parasit. hum. comp.*, 37, 221-231.
- Biguet, J., Rosé, G., and Capron, A. (1965). Le diagnostic de la distomatose a *Fasciola hepatica* par la réaction d'hémagglutination. Comparaison avec les résultats de l'immunoélectrophorèse et de la réaction d'hémolyse. *Bull. Soc. Path. exot.*, 58, 866-878.
- Capron, A., Biguet, J., Tran Van Ky, P., and Rosé, G. (1964). Possibilités nouvelles dans le diagnostic immunologique de la distomatose humaine a '*Fasciola hepatica*'. *Presse med.*, 72, 3103-3107.
- Coudert, J. (1960a). Valeur comparée des éléments de diagnostic de laboratoire dans le dépistage et la conduite de traitement de la distomatose hépatique Européenne. *Path. et Biol.*, 8, 145-150.
- Coudert, J. (1960b). Mise en évidence des anticorps spécifiques dans quelques helminthiases tissulaires. *Path. et Biol.*, 8, 139-144.
- Ridley, D. G. (1969). Personal communication.
- Soothill, J. F. (1962). Estimation of eight serum proteins by gel diffusion precipitin techniques. *J. Lab. clin. Med.*, 59, 859-870.

Reports and Bulletins prepared by the Association of Clinical Biochemists

The following reports and bulletins are published by the Association of Clinical Biochemists. They may be obtained from The Administrative Office, Association of Clinical Biochemists, 7 Warwick Court, Holborn, London, WC1R 5DP. The prices include postage, but airmail will be charged extra. Overseas readers should remit by British Postal or Money Order. If this is not possible the equivalent of 10s. is the minimum amount that can be accepted.

SCIENTIFIC REPORTS

- 3 Automatic Dispensing Pipettes.** An assessment of 35 commercial instruments 1967 P. M. G. BROUGHTON, A. H. GOWENLOCK, G. M. WIDDOWSON, and K. A. AHLQUIST 17s (\$2)
- 4 An Evaluation of 5 Commercial Flame Photometers suitable for the Simultaneous Determination of Sodium and Potassium** March 1970 P. M. G. BROUGHTON and J. B. DAWSON 17s (\$2)

TECHNICAL BULLETINS

- 9 Determination of Urea by Auto-Analyzer** November 1966 RUTH M. HASLAM 8s 6d (\$1)
- 10 Filter Fluorimeters.** A comparative list of 14 instruments March 1967 HANNELORE BRAUNSBURG (Re-issued in response to demand. Text still valuable, list now out of date) 8s 6d (\$1)

- 11 Determination of Serum Albumin by AutoAnalyzer using Bromocresol Green** October 1967 B. E. NORTHAM and G. M. WIDDOWSON 8s 6d (\$1)

- 13 An Assessment of the Technicon Type II Sampler Unit** March 1968 B. C. GRAY and G. K. MCGOWAN 8s 6d (\$1)

- 14 Atomic Absorption Spectroscopy.** An outline of its principles and a guide to the selection of instruments May 1968 J. B. DAWSON and P. M. G. BROUGHTON 8s 6d (\$1)

- 15 A Guide to Automatic Pipettes** (2nd edition) June 1968 P. M. G. BROUGHTON 8s 6d (\$1)

- 16 A Guide to Automation in Clinical Chemistry** May 1969 P. M. G. BROUGHTON 12s 6d (\$1.50)

- 17 Flame Photometers** (2nd edition) 1969 P. WILDING 12s 6d (\$1.50)

- 18 Control Solutions for Clinical Biochemistry** (4th edition) March 1970 P. M. G. BROUGHTON 12s 6d (\$1.50)

- 19 Spectrophotometers.** A comparative list of low-priced instruments readily available in Britain May 1970 C. E. WILDE and P. SEWELL 12s 6d (\$1.50)

- 20 Quantities and Units in Clinical Biochemistry** June 1970 P. M. G. BROUGHTON 12s 6d (\$1.50)
More than 30 copies in units of 10 at 4s