

## Identification of a 17-Kilodalton *Fasciola hepatica* Immunodiagnostic Antigen by the Enzyme-Linked Immuno-electrotransfer Blot Technique

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Sera obtained from human patients, calves, sheep, and rabbits infected with *Fasciola hepatica* were tested by the Falcon assay screening test enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme-linked immuno-electrotransfer blot (EITB) techniques with *Fasciola hepatica* excretory-secretory antigens in order to evaluate their immunodiagnostic potential. The study included sera from 13 patients infected with *F. hepatica* or a history suggesting fascioliasis, 5 patients infected and treated with bithionol or praziquantel (3 were cured with bithionol), 10 patients infected with *Schistosoma mansoni*, 6 infected with *Trichinella spiralis*, and 13 controls and sera from calves, sheep, and rabbits with a primary *F. hepatica* infection. By FAST-ELISA with *F. hepatica* excretory-secretory antigens, the serum samples from fascioliasis patients gave the highest absorbance values, and the schistosomiasis patient sera gave intermediate values compared with a normal human serum control. Also by FAST-ELISA, the values for serum from patients with fascioliasis decreased steadily after cure, reaching normal levels 20 to 47 weeks postcure. In contrast, the serum from two patients who had been treated but were not yet cured had high levels of antibodies for up to 3 years of infection. By EITB, the serum samples from humans, rabbits, cattle, and sheep with fascioliasis recognized two antigenic polypeptides of 17 and 63 kilodaltons (kDa) in the form of sharp bands. For humans, this recognition lasted for at least 3 years of infection. Sera from individuals with schistosomiasis mansoni or trichinosis or from normal controls did not recognize the 17-kDa *F. hepatica* antigenic polypeptide. However, serum from one human with *S. mansoni* and one with *T. spiralis* infection had slight bands in the 63-kDa region, suggesting cross-reactivity. Reactivity to the 17-kDa polypeptide was absent in fascioliasis patients at 1 year postcure. Reactivity to the 63-kDa polypeptide was significantly diminished in fascioliasis patients at 1 year postcure. The sera from rabbits with a primary *F. hepatica* infection also recognized both the 17- and 63-kDa antigenic polypeptides by week 4 of infection. Reactivity to both antigens diminished significantly 6 weeks postcure and disappeared by 8 weeks postcure. The sera from infected cattle and sheep recognized these two antigenic polypeptides by week 8 of infection. These studies suggest that the 17-kDa *F. hepatica* excretory secretory antigen is an excellent candidate for the immunodiagnosis of acute and chronic fascioliasis. Purification of this antigen and its application to quantitative serologic tests will permit further analysis of its predictive value to evaluate cure.

*Fasciola hepatica* worms mature and lay eggs after 8 weeks of infection. However, symptoms often occur much earlier, at which time eggs would not be found in stool examination, making definitive parasitological diagnosis by this means ineffective. In addition, in humans the parasite eggs are often not found even after repeated stool examinations. Thus, serodiagnosis of fascioliasis is a useful alternative to parasitologic diagnosis (4).

Numerous antigens and test systems have been used for the serodiagnosis of fascioliasis, and numerous attempts have been made with conventional chromatographic techniques to isolate and characterize potential specific somatic or excretory-secretory (ES) antigens to define infections (4, 10).

The enzyme-linked immuno-electrotransfer blot (EITB) technique described by Tsang et al. (13, 14) is a precise tool for identifying the molecular weights of potential serodiagnostic antigens. This method was used to identify an *F. hepatica* ES antigen of 17 kilodaltons (kDa) which is recognized by the serum of humans, calves, sheep, and rabbits with fascioliasis, does not cross-react with the sera from humans with schistosomiasis mansoni or trichinosis, and

disappears after successful chemotherapy, making it a potentially immunodiagnostic antigen.

### MATERIALS AND METHODS

**Antigens.** *F. hepatica* ES antigens were isolated from live, adult worms essentially as described in Santiago and Hillyer (11). Live, intact adult *F. hepatica* worms were obtained from bovine livers at a local abattoir and washed three or four times at room temperature during a 1-h period with 0.01 M phosphate-buffered saline, pH 7.2. The key element in the wash process is to remove all traces of blood and bile prior to the 3-h incubation in 0.01 M phosphate-buffered saline, pH 7.1, at 37°C. After incubation, the medium was collected and concentrated by vacuum dialysis. The antigen preparation was centrifuged at 5,000 × *g* at 4°C, and the supernatant was collected. Protein concentration was determined by the Bradford method (1), and the antigens were stored at -80°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ES obtained in this manner showed that it was composed primarily of a large cluster of proteins of 19 to 26 kDa and isolated bands of 17 and ca. 63 kDa (11).

**Sera.** The following human sera were used in this study. (i) Sera from 13 patients infected with *F. hepatica* (5 defined by coprology, 8 by serology [4]). Of these patients, three were

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treated with bithionol (50 mg/kg) and two with praziquantel (25 mg/kg) and followed parasitologically by the modified Ritchie formol-ether concentration method (9) for up to 3 years.

(ii) Sera were obtained from six individuals infected with *Schistosoma mansoni*, as determined by the presence of parasite eggs in their stools (9), and four individuals positive serologically by the circumoval precipitin (COP) reaction (7).

(iii) Sera from six individuals with confirmed trichinosis were kindly donated by Shirley Maddison, Centers for Disease Control, Atlanta, Ga.

Normal human serum samples were obtained from 13 individuals with no history of eating watercress and were negative by enzyme-linked immunosorbent assay (ELISA) against *S. mansoni* soluble egg antigen (SEA) and *F. hepatica* ES antigens.

The following sera from animals experimentally infected with *F. hepatica* were used in this study. (i) A 7-week-old New Zealand White rabbit was prebled, infected with 30 *F. hepatica* metacercariae, bled periodically for 20 weeks, treated with a curative dose of rafoxanide (Merck) (6), and bled for an additional 30 weeks. (ii) Five 6-month-old calves were prebled, infected with 500 *F. hepatica* metacercariae, and bled at 2-week intervals for 10 weeks. Each of the five 2-week serum samples obtained from each bleeding was pooled for use in this study. (iii) Five sheep were prebled, infected orally with 400 *F. hepatica* metacercariae, and bled at 2-week intervals for 10 weeks. Each of the five 2-week serum samples obtained from each bleeding was pooled for use in this study.

**Immunologic tests.** The Falcon assay screening test ELISA (FAST-ELISA) was done as described by Hancock and Tsang (3). The antigen concentration for the assay was 4 µg/ml. All sera were tested at twofold dilutions from 1:32 to 1:256. Goat anti-human immunoglobulin G (IgG) plus IgM plus IgA (heavy- and light-chain specific [H+L]) (Kirkegaard & Perry Laboratories, Inc. [KPL], Gaithersburg, Md.) affinity-purified peroxidase-labeled conjugates were used at a 1:300 dilution in 0.3% phosphate-buffered saline-Tween. The reactions were read on a Bio-Rad Laboratories 2550 ELISA reader at 600 nm.

The EITB was done as described by Tsang et al. (13, 14) and Santiago and Hillyer (11). All sera were tested at a 1:200 dilution. The following affinity-purified peroxidase-labeled conjugates were used (at a 1:2,000 dilution): goat anti-human IgG+IgM+IgA (H+L) (KPL), goat anti-bovine IgG (H+L) (KPL), goat anti-rabbit IgG (H+L) (Bio-Rad), and rabbit anti-sheep IgG (H+L) (KPL).

## RESULTS

The first question was whether *F. hepatica* ES antigens could identify antibodies in the serum of patients with confirmed fascioliasis ( $n = 3$ ) or suspected of having fascioliasis due to history ( $n = 7$ ) by FAST-ELISA, and this was indeed the case (Fig. 1). The second question was whether *F. hepatica* ES antigens cross-reacted with the serum of individuals infected with *S. mansoni* (confirmed by stool examination,  $n = 6$ ; confirmed by positive COP reaction,  $n = 4$ ). As can also be seen in Fig. 1, the serum samples from individuals infected with *F. hepatica* had the highest absorbance values against *F. hepatica* ES antigens, and the sera from the individuals infected with *S. mansoni* had intermediate but clearly reactive levels compared with a normal control pool.

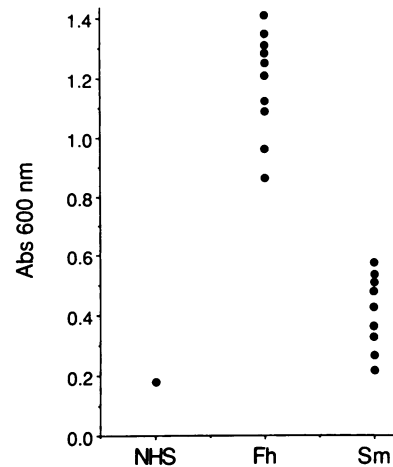


FIG. 1. FAST-ELISA with *F. hepatica* ES antigens reacted against normal human serum (NHS) or the serum of humans infected with *F. hepatica* (Fh) of *S. mansoni* (Sm). Abs, Absorbance.

The third question was whether FAST-ELISA with *F. hepatica* ES antigens could be used to monitor the success of chemotherapy in patients with fascioliasis. Figure 2 shows the absorbance values for samples from three patients treated and cured with bithionol and followed up for up to 50 weeks. Before treatment, all three patients with fascioliasis had serum absorbance values similar to the positive control serum. In one patient, antibody levels increased slightly 2 weeks posttreatment (Fig. 2a) and then decreased by 5 weeks posttreatment, steadily decreasing thereafter toward normal values through the 23 weeks posttreatment tested. In the second patient, antibody levels decreased within 3 weeks posttreatment, steadily decreasing thereafter toward normal values through the 47 weeks posttreatment tested. In the third patient, antibody levels dropped near to normal values by 16 weeks posttreatment, remaining at these low levels through 50 weeks tested.

The antibody absorbance patterns of samples from two patients treated with praziquantel and not cured of fascioliasis were followed for 83 and 147 weeks posttreatment. As seen in Fig. 3, the antibody levels of these two infected patients remained high during the follow-up period, which in one case was almost 3 years.

In order to best define the *F. hepatica* ES antigenic polypeptides seen in infected patients, the EITB was performed. Sera from the five patients infected with *F. hepatica* as defined parasitologically all reacted in EITB, producing multiple bands. An apparently nonspecific cluster of 2+ bands was seen at ca. 29 kDa (Fig. 4). One remarkable observation was that the serum from all *F. hepatica*-infected individuals had antibodies which reacted with the 17- and 63-kDa bands. Moreover, all eight serum samples from patients having antibodies to *F. hepatica* ES antigens also had antibodies which reacted with the 17- and 63-kDa bands. In addition, the three patients who received curative doses of bithionol had their antibody levels to the 17-kDa antigenic polypeptide diminish rapidly (12 to 16 weeks postcure). In the same patients, antibody levels to the 63-kDa antigenic polypeptide diminished more slowly (16 to 26 weeks), requiring almost 1 year for total disappearance. Representative EITBs showing these patterns are shown in Fig. 4 and 5. The

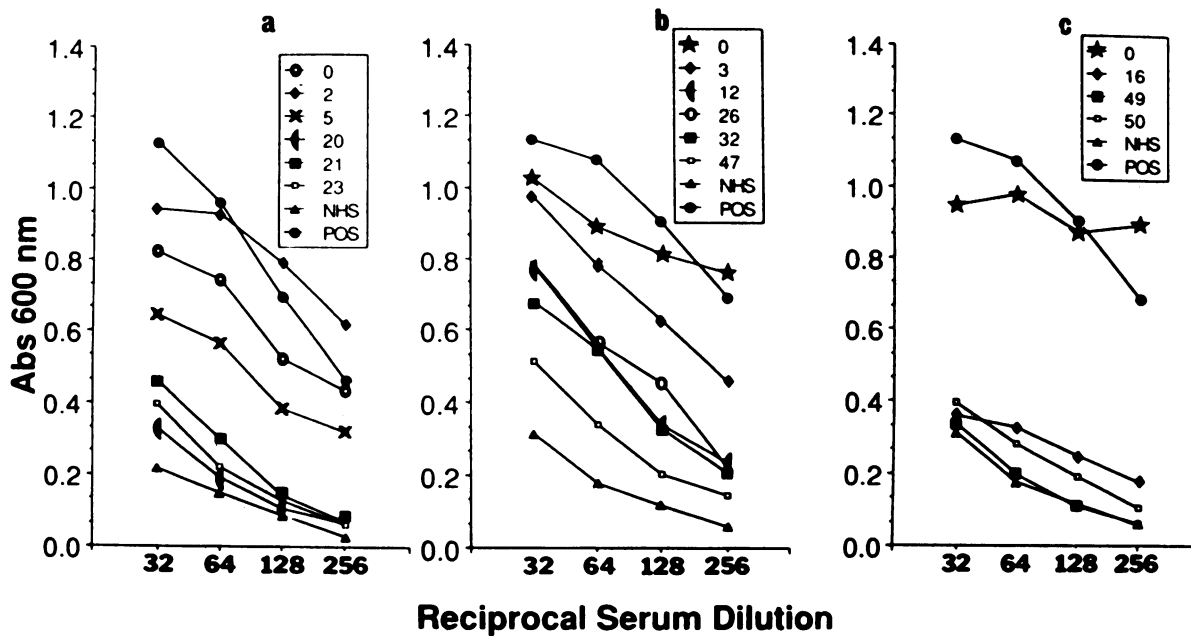


FIG. 2. FAST-ELISA of serum from three fascioliasis patients reacted with *F. hepatica* ES antigens. These patients were treated with bithionol and cured of infection as determined by multiple parasitological examinations (formol-ether concentration method). Abs, Absorbance; NHS, normal human serum; POS, positive control serum. Numbers beside symbols indicate weeks posttreatment.

serum from patients treated with praziquantel but not cured did not have diminished antibody to the 17- and 63-kDa antigenic polypeptides up to 147 or more weeks posttreatment (and postinfection) (Fig. 6).

As can be seen in Fig. 4 and 6, the serum from humans infected with either *S. mansoni* or *Trichinella spiralis* reacted with *F. hepatica* ES antigens, primarily in the 20- to 30-kDa region, as did normal human serum, with one or two

bands at ca. 29 kDa. Additional bands cross-reactive with the serum of *S. mansoni*-infected individuals were seen in the 15- and 40-kDa regions. In Fig. 6, one of the two human *S. mansoni* serum samples (but not the two in Fig. 4) and one of the three *T. spiralis* sera also appeared to react with the 63-kDa antigenic polypeptide. Moreover, the serum from *T. spiralis*-infected individuals also recognized several antigenic polypeptides of less than 14 kDa.

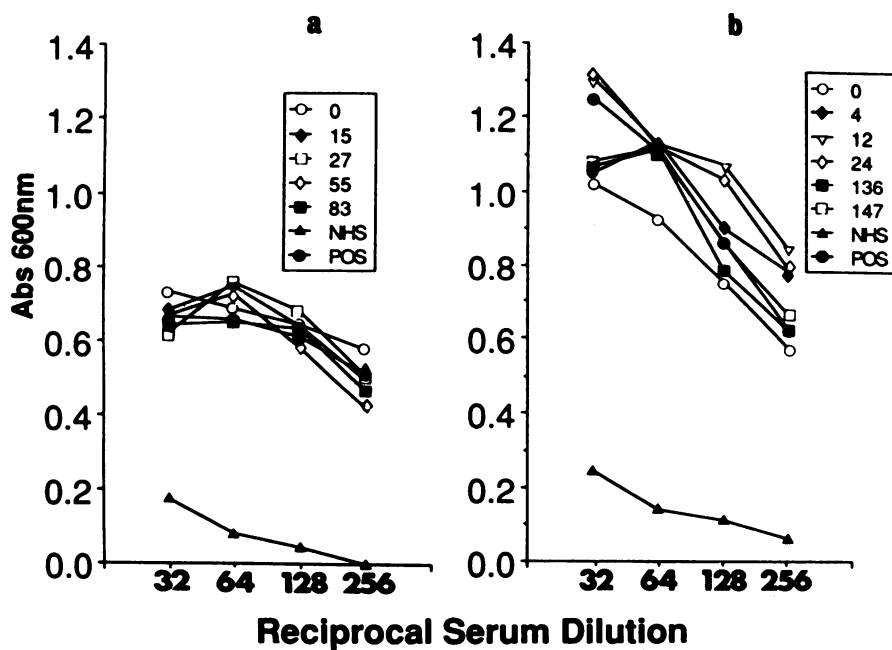


FIG. 3. FAST-ELISA of the serum from two fascioliasis patients reacted with *F. hepatica* ES antigens. These patients were treated with praziquantel and not cured of infection as determined by multiple parasitological examinations (formol-ether concentration method). Abs, Absorbance; NHS, normal human serum; POS, positive control serum. Numbers beside symbols indicate weeks posttreatment.

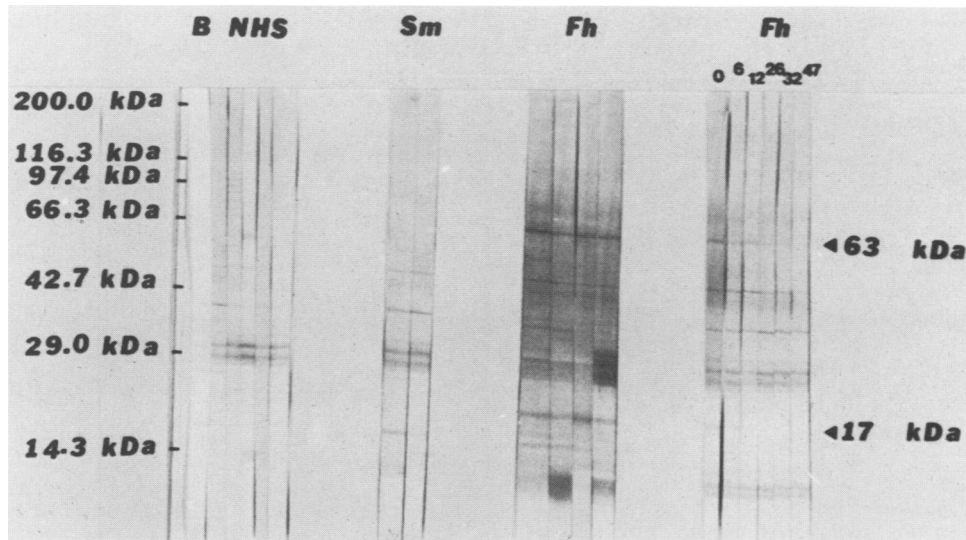


FIG. 4. Immunoblot (EITB) of five normal human sera (NHS), and the serum samples from two patients with schistosomiasis mansoni (Sm), four with fascioliasis (Fh), and one fascioliasis patient who received a curative dose of bithionol. All samples from patients with fascioliasis recognized the 17-kDa antigenic polypeptide; the other sera did not. The serum of the cured fascioliasis patient did not react to the 17-kDa antigenic polypeptide by 12 weeks postcure. Antibody levels to the 63-kDa antigenic polypeptide diminished more slowly and virtually disappeared 1 year (47 weeks) postcure (numbers above lanes).

The serum of a rabbit infected with *F. hepatica* developed antibodies to both the 17- and 63-kDa antigenic polypeptides by week 4 of infection and remained strong through week 20 of infection, at which time the rabbit received a curative dose of rafoxanide. Six weeks postcure, the antibodies to both the 17- and 63-kDa antigenic polypeptides virtually disappeared, being more clear-cut with the 17-kDa polypeptide (Fig. 7).

Finally, pooled serum samples of sheep or calves with fascioliasis were reacted with *F. hepatica* ES antigens. Although the earliest and largest number of reactive bands were in the high 20s to low 30s range, these infected animals

also developed antibodies to the 17- and 63-kDa bands by 8 weeks of infection (Fig. 8).

DISCUSSION

Serodiagnosis of human fascioliasis is important because infected individuals often have clinical symptoms well before parasitologic diagnosis by finding eggs in the feces is possible. In the absence of commercially available purified antigens, the first step in immunodiagnostic testing for fascioliasis often involves crude antigens (4). In our experience, crude *F. hepatica* adult worm extracts yield different

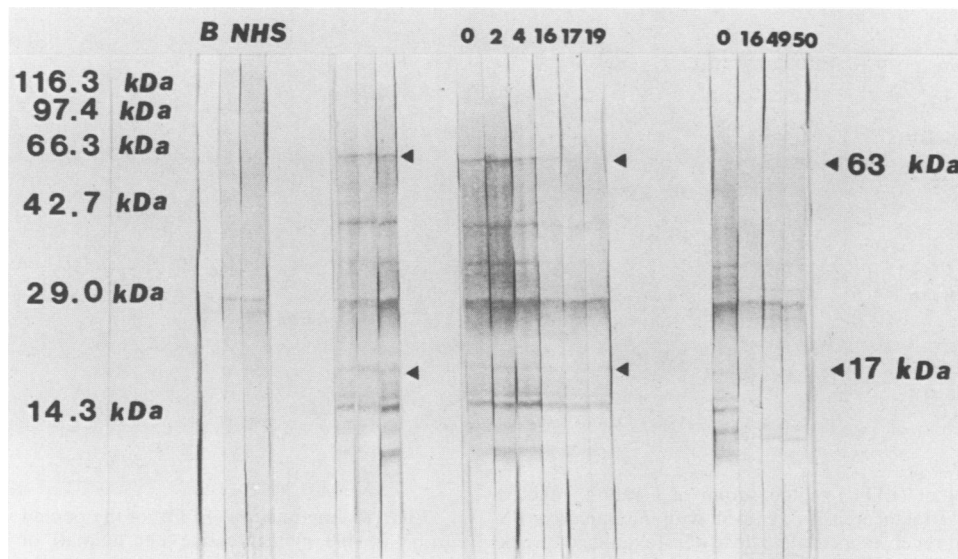


FIG. 5. Immunoblot (EITB) of two normal human sera (NHS) and samples from three patients with fascioliasis (Fh) and two other fascioliasis patients who received curative doses of bithionol. All samples from fascioliasis patients recognized the 17- and 63-kDa antigenic polypeptides. The sera of the cured patients were virtually negative to the 17- and 63-kDa antigenic polypeptides by 16 or 20 weeks postcure (numbers above lanes).

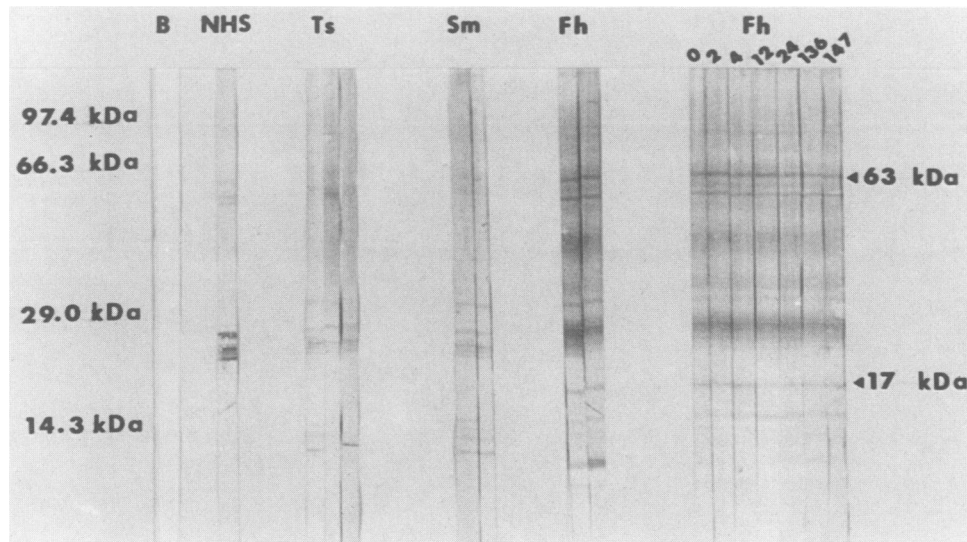


FIG. 6. Immunoblot of one normal human serum (NHS) and sera from two patients with schistosomiasis mansoni (Sm), three with trichinosis (Ts), two with fascioliasis (Fh), and a third with fascioliasis treated repeatedly with different doses of praziquantel and not cured. All fascioliasis patient sera recognized the 17-kDa antigenic polypeptide. The others did not, suggesting improved specificity. The serum of the treated but not cured patient still reacted with the 17- and 63-kDa antigenic polypeptides 147 weeks (almost 3 years) postinfection (and postinfection) (numbers above lanes).

banding patterns in EITB when different animal models are used. Moreover, crude *F. hepatica* adult worm extracts, even in the presence of protease inhibitors, deteriorate rather quickly when stored at 4°C (data not shown). For these reasons, we decided to use a “cleaner” starting material and selected ES products for their ease of processing. Recently, a FAST-ELISA has been described in which the immunodiagnostic test can be completed in 15 min, assuming a prior antigen incubation of 2 h (3). Thus, we first adapted the *F. hepatica* ES products to a FAST-ELISA. Because of the possible presence of cross-reactive antigens in the *F. hepatica* ES antigenic preparation (4), we decided

to test in ELISA the serum of humans infected with either *S. mansoni* or *F. hepatica*. The sera from the humans with *S. mansoni* infection showed intermediate but clearly reactive antibody levels compared with the normal human serum pool and the serum from humans infected with *F. hepatica*. The demonstration that the serum from patients with schistosomiasis mansoni cross-reacted with *F. hepatica* ES antigens at a level intermediate between the normal human serum pool and the serum from patients with fascioliasis suggests that there are a limited number of cross-reactive epitopes. However, this cross-reactivity must be taken into consideration when performing serologic tests with serum

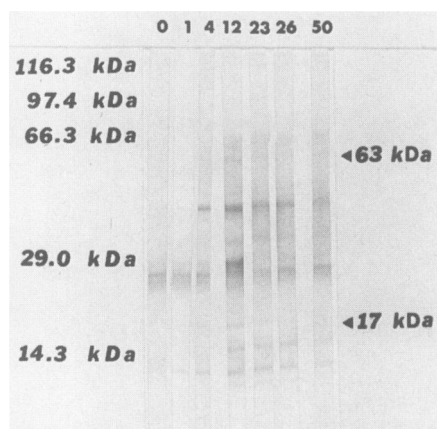


FIG. 7. Immunoblot (EITB) of the serum of a rabbit infected with 30 *F. hepatica* metacercariae reacted with *F. hepatica* ES antigens. This rabbit received a curative dose of rafoxanide on week 20 of infection. Antibodies to both the 17- and 63-kDa antigenic polypeptides appeared by week 4 of infection (numbers above lanes). Antibodies to the 17- and 63-kDa antigenic polypeptides virtually disappeared within 6 weeks postcure (week 26), although it was more clear-cut with the 17-kDa antigenic polypeptide.

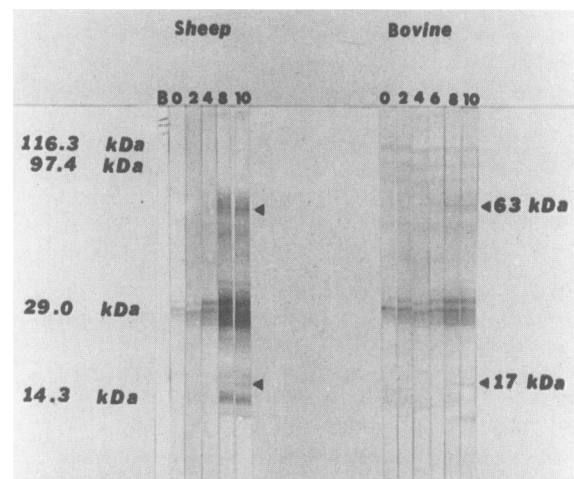


FIG. 8. Immunoblot (EITB) of the pooled serum of either sheep ( $n = 5$ ; 400 metacercariae per animal) or calves ( $n = 5$ ; 500 metacercariae per animal) infected with *F. hepatica* for up to 10 weeks. Both sheep and cattle developed antibodies to the 17-kDa antigenic polypeptide by 8 weeks of infection (numbers above lanes). Antibodies to the 63-kDa antigenic polypeptide appeared earlier in the serum of sheep (2 weeks) than of cattle (8 weeks).

from patients who have been exposed to schistosomes. Recently, Espino et al. (2) did not find any antigenic cross-reactivity when testing serum from humans with a wide variety of parasitic infections, including schistosomiasis (although the species was not defined, it was presumably *S. mansoni* or *S. haematobium* or both). However, this may be due to differences in assay conditions or in the preparation of antigen. Espino et al. (2) obtained their *F. hepatica* ES antigens after a 24-h incubation; on the other hand, ours were obtained after a 3-h incubation.

The decrease in antibody levels seen by FAST-ELISA in the cured fascioliasis patients confirms a previous study of ours reporting antibody changes after chemotherapy in one cured patient in which crude *F. hepatica* worm antigen was used in the ELISA (5).

Irving and Howell (8) isolated *F. hepatica* ES antigens by incubating live worms obtained from infected mice for 5 to 7 days in serum-free medium. Three major antigenic polypeptides incorporated radiolabeled amino acids of 23, 24, and 26 kDa. We have also found this to be one region of major antigenic polypeptide activity in rabbits with fascioliasis (12). In the present report, we found that this region also contained some antigens cross-reactive with *S. mansoni* and *T. spiralis* and nonspecific cross-reactivity with normal human serum.

Our observation herein that humans, rabbits, cattle, and sheep infected with *F. hepatica* all develop antibodies to the 17-kDa antigenic polypeptide seen by EITB suggests that this may be a useful marker to detect infections by this method.

The uniformity of finding the 17-kDa band when the sera of patients with *F. hepatica* were tested by EITB with *F. hepatica* ES antigen preparations, and not finding these reactions with an antigenically closely related trematode, such as *S. mansoni*, or one with cross-reacting antibodies, such as *T. spiralis*, suggests that the 17-kDa band may be specific for fascioliasis. The observation that it disappeared in treated and cured patients but not in treated but not cured patients suggests that it may also serve as a marker to define the success of chemotherapy. This is reinforced by the study on the rabbit that was infected and then treated and cured, in which antibodies to the 17-kDa antigenic polypeptide appeared by week 4 of infection and virtually disappeared within 6 weeks postcure. The observation that infected humans still have similarly high levels of antibodies as at pretreatment 3 years later suggests that this fluke is long-lived in its human host.

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