A Contribution to the Diagnosis of *Capillaria hepatica* Infection by Indirect Immunofluorescence Test

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A highly specific pattern of immunofluorescence was noted when sera from Capillaria hepatica-*infected rats were tested against the homologous worms and eggs present either in paraffin or cryostat sections from mouse liver. The pattern was represented by a combined apple green fluorescence of the internal contents of worms and eggs, which persisted in serum-dilutions of 1:400 up to 1:1600. Unequivocal fluorescent pattern was observed from 15 days up to 3 months following inoculation of rats with embryonated* C. hepatica *eggs and such result was confirmed by the ELISA. After the 4th month of infection, the indirect immunofluorescence test turned negative, probably revealing the extinction of parasitism, however the ELISA was contradictory, disclosing high levels of antibodies in this period . The IIF was also negative when control normal rat sera and sera from rats administered by gavage with immature* C. hepatica *eggs (spurious infection), or for reactions made against* Schistosoma mansoni *eggs, although a weakly positive pattern occurred with* Fasciola hepatica *eggs. The indirect immunofluorescence test may be recommended for use with human sera to detect early* C. hepatica *infection in special clinical instances and in epidemiological surveys, since it is a simple, inexpensive, and reliable test, presenting excellent sensitivity and specificity. Although the diagnosis is positive only during early infection, this is the period when the symptoms are usually more severe and the need for differential diagnosis is greater.*

Key words: *Capillaria hepatica* - immunofluorescence test - rat capillariasis

Capillaria hepatica is a nematode of worldwide distribution, especially frequent in rodents and lagomorphis, but that can also parasitize man (Cochrane et al. 1957, Piazza et al. 1963, Cislaghi & Radice 1970, Berger et al. 1990, Choe et al. 1993). Natural infection of rats in the large cities runs about 60% or more (Luttermoser 1938, Wright 1961, Galvão 1976, Chieffi et al. 1981, Juncker-Voss et al. 2000). Transmission to man may also be frequent where precarious hygiene prevails, especially in children. There are about 40 human cases published in the world literature, the great majority being autopsy reports from instances of extremely severe infections (Cochrane et al. 1957, Piazza et al. 1963). It has been suspected that less severe cases usually pass unnoticed (Galvão 1979, 1981, Bhattacharya et al. 1999, Juncker-Voss et al. 2000). There are plenty of reasons for that. First, the clinicians rarely think of *C. hepatica* as a parasite of man, since the current literature leaves the impression that hepatic capillariasis is an exceptionally rare infection of man. Also, clinical manifestations in milder cases probably share signs and symptoms common to many other trivial conditions. However, the main hindrance to diagnosis is related to the life cycle of the parasite. No eggs appear in the host feces. Eggs are retained in the carcass of infected animals, but still need to rest for some 28-30 days in the environment, exposed to adequate conditions of moisture and temperature, in order to become infecting. Adult *C. hepatica* worms lives a transient life inside the host liver. Soon after egg lying the worms die and disintegrate, causing focal necro-inflammatory lesions. These lesions, containing worm debris and eggs, tend to heal by encapsulation, calcification, and resorption (Luttermoser 1938, Ferreira & Andrade 1993, Gotardo et al. 2000). It can be presumed that during the formation of such hepatic lesions some degree of painful liver enlargement, accompanied by fever, splenomegaly, and peripheral eosinophilia may follow, which are probably undistinguishable from other "tropical eosinophilia" syndromes (Beaver et al. 1952, Beaver 1969, Kaplan et al. 2001). The possibility to detect these cases depends on our awareness about the role *C. hepatica* may play in human pathology, and the availability of a good immunological test. Indirect immunofluorescence test (IIF), made on histological slides containing parasite and eggs, have been tried with good results. Such test have been considered to be practical, reliable and sensible (Galvão 1976, 1981, Juncker-Voss et al. 2000). The present study confirms and extends these previous results by experimentally testing the sensibility and specificity of the method, and by investigating the influence of several factors on its performance, such as time and type of infection, parasite load, cross reactivity, serum dilution, and type of histological material utilized.

MATERIALS AND METHODS

Infection and experimental groups - Thirty-five Wistar adult rats, males and females, kept on a balanced commercial diet and water ad libitum, were infected by the administration of embrioned *C. hepatica* eggs, by means of a gastric tube. The eggs had been collected from the livers

of rats or mice by the 40-50 days after experimental infection, as described elsewhere (Gotardo et al. 2000, Souza et al. 2001). The animals were divided into four groups, as follows:

Group 1 - Ten rats were infected with 600 embryonated eggs, representing a *true* infection with high worm load. The inoculum was chosen in order to produce massive infection, according to Oliveira and Andrade´s data (2001).

Group 2 - Ten rats infected with 30 embryonated eggs, representing a *true* infection with low worm load.

Group 3 - Ten rats were administered with 600 immature eggs, representing a so-called *spurious* infection (Nascimento & Sadigursky 1986).

Group 4 - Five rats were administered with saline by a gastric tube, representing normal non-infected controls.

Sera tested - The sera used for the IIF and ELISA were collected from the tail vein of Wistar rats from the four groups. About 0.5 ml of blood was drawn at a time. After coagulation, the blood was centrifuged at 14,000 rpm/3min, and the serum collected and stored at –20ºC until the moment of use. The sera were collected at the following experimental points: 0, 9, 15, 18, and 40 days, and 4, 7, and 12 months following infection of the animals.

IIF - All the tests were made on histological sections taken from the livers of CBA mice on the 3rd-4th week after infection with 150 embryonated *C. hepatica* eggs. This is a heavy infection, well tolerated by mice. Fragments from these livers were either fixed in neutral 10% formalin and embedded in paraffin or mounted in Tissuetek (Sakura, Tokyo, Japan) and immediately frozen in liquid nitrogen and cut in a cryostat at –20ºC. Indirect immunofluorescence test was used to detect the presence of *C. hepatica* antigens in the sections of mouse liver. Sections were either used with previous treatment for antigen recovering or not. In the first case the parafinized sections were place in pH 6 citrate buffer and subjected to three cycles of micro-wave treatment. Sections were covered with sera (primary antibodies), diluted in phosphate buffered saline, pH 7.2, during 30 min at 37ºC. Sera dilutions were 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600. These same sera were tested against sections of mouse liver containing either *S. mansoni* or *F. hepatica* eggs. PBS replaced the sera in negative control slides. After a series of washing with iced buffered saline, sections were treated with anti-rat IgG conjugated to fluorescein-isothiocyanate (Sigma, Saint Louis, MO) under a 1:640 dilution. Slides were mounted in carbonate buffer and examined with a Zeiss microscope under UV light, with appropriate barrier and exciter filters.

ELISA - Sera were submitted to an ELISA test for detection of total antibodies, by using a goat anti-rat IgG conjugated to peroxidase at 1:1,000.(Sigma). The plates were sensitized with 10 µg/ml/per well of *C. hepatica*-egg and worm antigen, diluted in carbonate buffer, pH 9.9. Sera were diluted 1:1000 in PBS with 3% skimmed milk and 0.05% Sigma tween-20. All tests were used in duplicates, including the blank. Lecture was made on a microplate reader "Molecular devices-Thermomax" spectrophotometer (Sunyvaley, CA) under 450 nm wave-length, connected to a computer with MDS-Soft Max.

RESULTS

The sections taken from the livers of mice experimentally infected with *C. hepatica* contained well-preserved worms, their eggs and disintegrating worms. The details about the fluorescence staining were centered on the sections of well-preserved worms and eggs. When treated with saline and examined under UV light, only a yellowish auto-fluorescence was observed in the egg shell. Sera from normal intact rats and from those with spurious infection gave essentially negative results. However, sera with 1:50 up to 1:200 dilutions exhibited non-specific fluorescence (Fig. 1). This appeared at the worm cuticle in the egg-shell. The yellowish auto-fluorescence of the eggshell then acquired a bright whitish hue, while its inner contents appeared uniformly black.

When treated with sera from *C. hepatica*-infected rats, the structures of worms and eggs presented a strong apple-green fluorescence, mainly marked at the internal genitalia (uterus) and the hypodermis (especially at the so-called "bacillary bands") (Fig. 2). The eggs were strongly marked at the shell, and their interior appeared empty most of times. However, when the contents of the eggs were preserved in the sections, they were strongly fluorescent. This staining of eggs and worms represented a highly specific feature, present in positive cases even at the highest dilutions, and entirely absent from controls. Actually, nine days after inoculation the results were still negative, but turned weakly positive by the 15th day (Table) even at a 1:1600 dilution. However, the 15-day positive result was only observed in slides submitted to antigen retrieval. From then on, a strong positive reaction was regularly observed during the first three months following infection, with or without the retrieval of antigen. The positive fluorescence and its patterns occurred in infected rats regardless of parasite load. However, with serum dilutions of 1:400 or higher, it did not persist when *C. hepatica* infection had lasted for 4 months or over. Sera from rats with 4, 7 or 12 month-old infection revealed consistent negative results by IIF, although anti-*C. hepatica* antibodies continued to be detected by ELISA (Fig. 3). When the IIF was made using rat anti-*C. hepatica* serum, against tissue containing *Schistosoma mansoni* eggs, results were negative. The same test using *Fasciola hepatica* eggs resulted in a weakly positive reaction even when dilutions of 1:400 and over were used.

DISCUSSION

Examining 500 children from low income families in Salvador, BA, Brazil, Galvão (1979) found that 44.2% of them exhibited several patterns of positive fluorescence for *C. hepatica* in the sera eluted from a drop of blood collected in filter paper. In nine cases fluorescence was particularly strong, disclosing a peculiar pattern even under high dilutions. This pattern was characterized by the fluorescence of the inner contents of the eggs and of the internal structures of the worms, particularly at the gonads. The present study confirms these results and adds a further detail, the focal staining of "bacillary bands", which are present just below the worm cuticle. These combined fluorescent sites formed a highly characteristic pat-
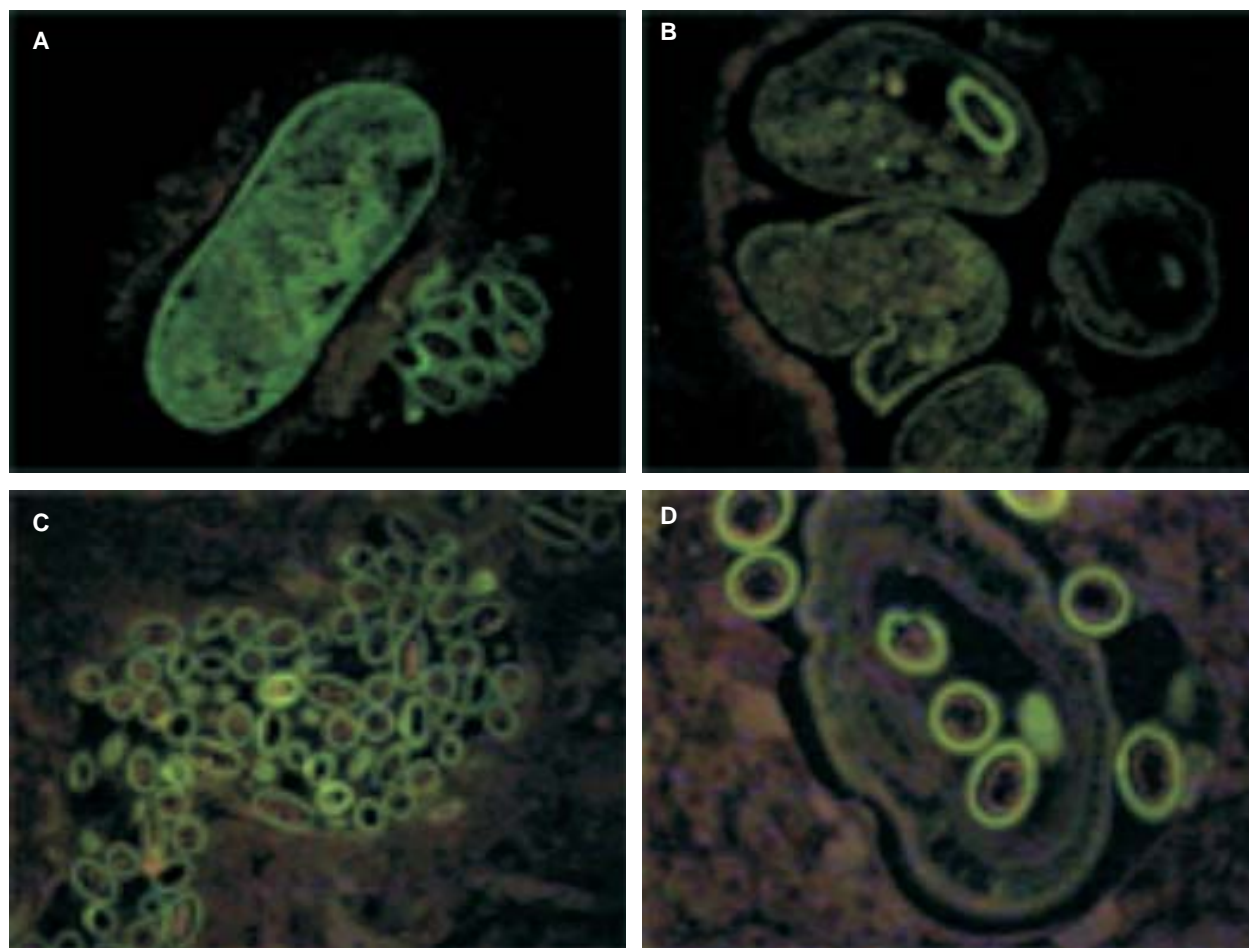
Fig.1: sections of mouse liver, containing *Capillaria hepatica* worms and eggs, treated with control serum from normal rats. A: diffuse fluorescence revealing the internal structures of the worms, especially the digestive tube and also the cuticle. The egg shells exhibit an apple green fluorescence. Intensity of fluorescence progressively diminishes with increasing in dilutions, as can be noted by comparing the picture at A (1:50) with that at B (1:100); C: collections of eggs treated with 1:400 serum dilution. The internal contents of the eggs lack fluorescence. The worm seen at D is also unstained. Magnification 200X, except D, 400X.

tern disclosed by the positive sera (Fig. 1). The internal structures of the worms appeared marked by the serum taken at the 15th day of infection, but the internal contents of the eggs appeared a little later, around the 18th day. From then on, the typical pattern persisted unchanged throughout the first three months of infection. Therefore, the positive fluorescent pattern appeared early in the course of *C. hepatica* infection, was not influenced by the worm load, by was so by the presence of viable eggs within the host tissue. By the 4th month after inoculation, fibrosis decreased and the eggs probably became metabolically inactive. Luttermoser (1938) observed a gradual reduction of the viability of the eggs with the increasing age of the infections. A trial to embryonate eggs after 12 months of infection resulted unsuccessful (data not shown). Apparently the negative finding observed in old infection was not related to the level of ELISA antibodies, but to their quality. As a matter of fact, sera taken 15 days after infection revealed a definite pattern of positive fluorescence, while presenting ELISA-levels of antibodies

lower than those from animals with old infections (four months or more). Therefore, results from anti-*C.hepatica* antibodies searched by the ELISA method in infected rat sera were concordant with those revealed by the IIF only during the first three months after infection. From then on a diverging result was noted. Besides, positive fluorescence was never observed in control sections or in cases of *C. hepatica* spurius infection (animals subjected to ingestion of non-infective immature eggs). Spurious infection has been claimed to sensitize the host on the basis of the ELISA test (Nascimento & Sadigursky 1986), a finding not confirmed in the present investigation, especially when the IIF was applied. Fluorescence was then weak and non-specific, disappearing when dilutions of 1:400 or over were employed.

Although the tests made to investigate cross reactivity with other helmints were very preliminary in the present study, revealing a weakly positive result with *F. hepatica* eggs and totally negative results with *S. mansoni* eggs, there are suggestions in the literature that such cross
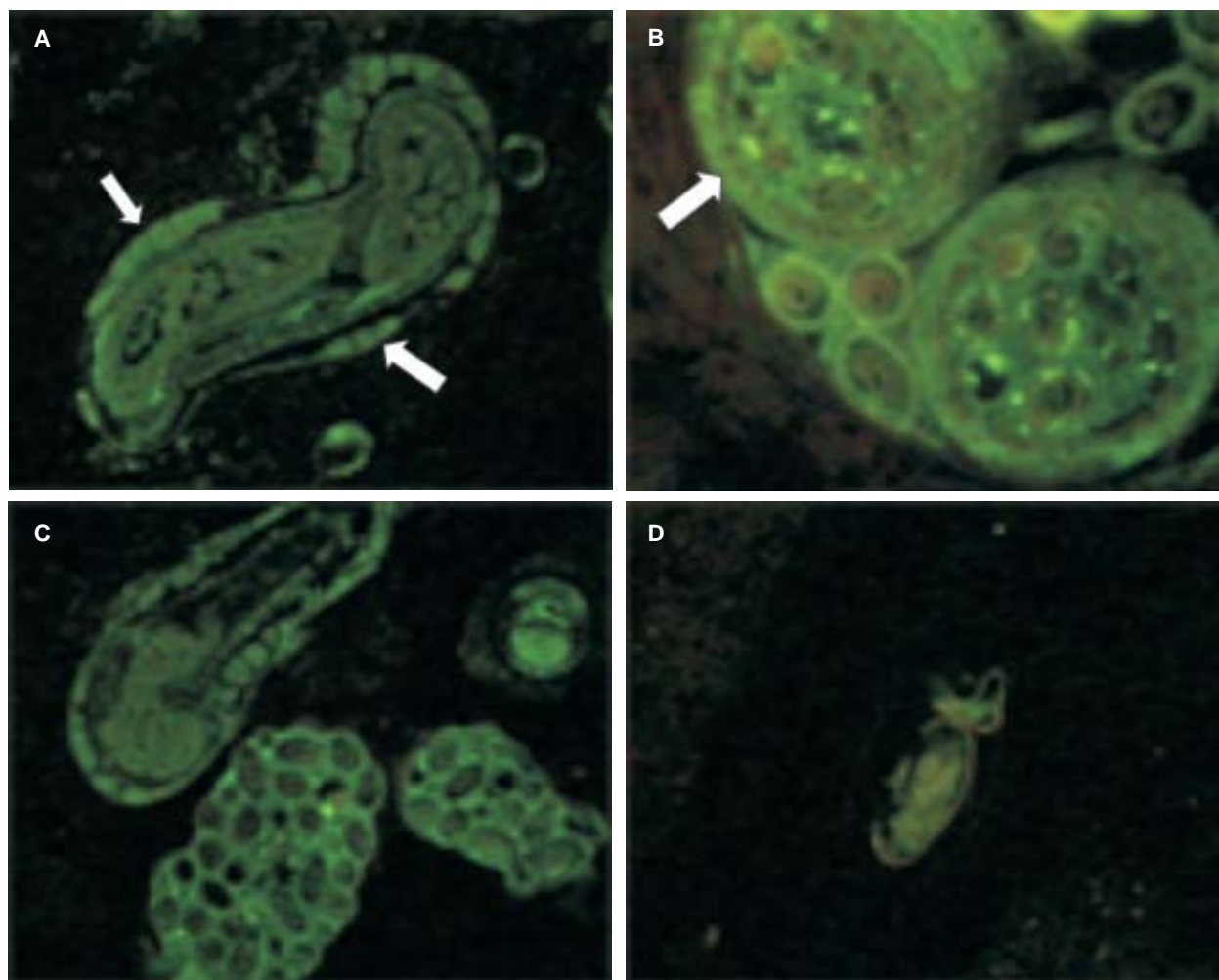
Fig. 2: sections of mouse liver, containing *Capillaria hepatica* worms and eggs, treated with sera from rats with 18-day old *C. hepatica* infection. Bacillary bands (A, arrows) appear fluorescent, as well as the uterus (B, arrow) and the internal contents of the eggs (C). Serum dilutions: A 1:1,600 and B 1:800; C 1:2,600. Picture at the D represents fluorescence of *Fasciola hepatica* egg after treatment with *C. hepatica* rat serum, dilueted at 1:800. Magnifications: B 400X; all others 200X.

TABLE

Results of the indirect immunofluorescence test according to intensity and patterns and in relation to variations in serum dilutions

Serum dilution	Normal controls	Heavy true infection [a]	Mild true infection [a]	Spurius infection
1:50	+	+++	+++	+
1:100	+	+++	+++	+
1:200	+	+++	+++	+
1:400	-	+++	+++	-
1:800	-	+++	+++	-
1:1600	-	++	++	-

a: sera taken 15-40 days after infection; -: negative; +: mild and non-specific; ++: moderate and specific; +++: strong and specific

reactivity is not to be expected, at least when high serum dilutions are used. Galvão (1979) observed the *C. hepatica* IIF to be negative in several instances when the sera of children presenting eggs of intestinal helminths in stools were examined. Junker-Voss et al (2000) obtained negative results with sera from cases of *Echinococcus granulosus*, *E. multilocularis*, *F. hepatica*. *S. mansoni*, *Toxocara canis*, *Trichuris trichiura,* and *Trichinella spiralis* infections. Of course the possibility of cross reactivity has not been ruled out and should be better explored in further studies. But, one should consider that a good *C. hepatica* immunological test is to be useful for especial clinical situations and epidemiological surveys as strong presumptive evidence, not necessarily as an absolute di-
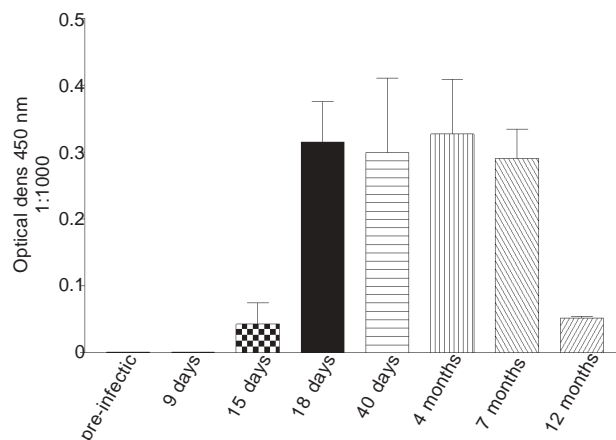
Fig. 3: levels of anti-*Capillaria hepatica* antibodies in the sera of infected rats in relation to the days following infection (ELISA).

agnosis. In special cases, complementary data should be sought through clinical investigation, sometimes including such procedures as ultrasound imaging and liver biopsy.

Examinations made on paraffin sections allow the same results as when cryostat sections were used. As a matter of fact, paraffin sections yielded better localization and definition of fluorescent areas. The practical advantages of paraffin over cryostat sections are obvious. It is easier to keep paraffin blocks and unstained sections for future investigations, and at lower cost, than frozen tissue and sections. Micro-wave antigen enhancement yields brighter and crisp positive fluorescence, but its absence will not interfere with any other aspects of the test.

Considering the high prevalence of *C. hepatica* infection of rats in large cities and the poor hygienic conditions sometimes prevailing in the poor human populations at their outskirts, it seems logical to suspect that human infections with this helminth can occur in such environment. A good serological test for the diagnosis of *C. hepatica* infection should be included for the differential diagnosis when children or even adults present hepato-splenomegaly, unexplained fever and eosinophilia. It is hoped that the availability of a reliable and practical diagnostic tool will facilitate future investigations to determine the real role *C. hepatica* infection plays in human pathology.

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