

EVALUATION OF *CAPILLARIA PHILIPPINENSIS* COPROANTIGEN IN THE DIAGNOSIS OF INFECTION

By

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Abstract

In order to overcome the false negative diagnosis of infection with *C. philippinensis* at time of absence of eggs in stool, coproantigen prepared from stools of infected patients was evaluated serologically. This antigen was able to detect anti-*Capillaria* antibodies in the sera of infected cases at the same OD level produced with *Capillaria* crude worm antigen using indirect ELISA technique. *C. philippinensis* coproantigen did not cross-react with sera from patients with schistosomiasis mansoni, fascioliasis or strongyloidiasis at 1:00 serum dilution. Laboratory-prepared hyperimmune sera versus crude worm antigen of *C. philippinensis* succeeded in capturing *Capillaria* antigen prepared from the stools of infected patients and did not cross react with coproantigens prepared from stool samples of cases infected with *S. mansoni* or *Fasciola* using sandwich ELISA technique.

Introduction

Intestinal capillariasis is a recently identified zoonotic parasitic disease of humans. It occurs as a result of infection with *Capillaria philippinensis*; a tiny nematode parasite of

migratory fish-eating birds. Human infection occurs accidentally and leads to the development of serious manifestations that may end fatally if untreated (Cross and Bhaibulaya, 1983). The first human case was reported in the Philippines in 1963 (Chitwood *et al.*, 1964) then more and more cases were detected in the Philippines and Thailand (Bhaibulaya, 1975 and Cross and Bhaibulaya, 1983). Few sporadic cases were detected in other countries in the Far East and Middle East, However, it is believed that the geographical distribution of the disease is much more prevalent than what has already been reported. It is noteworthy to mention that the disease has taken a serious situation in Egypt with continuous detection of new indigenous cases and it became the country with the highest number of reported cases outside the endemic area (El-Dib and Doss, 2002). Diagnosis of infection is usually easy especially in endemic areas. Physicians may largely depend on the typical clinical picture and the stages of the parasite usually appear in stool and /or duodenal aspirate (Cross and Bhaibulaya, 1983; El-Dib *et al.*, 1992). However, it has been observed that egg-shedding shows a considerable decrease with changes in the reproductive activity of the worms (Banzon *et al.*, 1975; Cross and Bhaibulaya, 1983). Usually most of the suspected cases were subjected to repeated stool analysis to reach an accurate diagnosis which is time consuming (El-Dib *et al.*, 1999; El-Dib and Doss, 2002). That is why it was necessary to develop a more sensitive diagnostic technique to help in identifying infection with *C. philippinensis* for clinical and epidemiological purposes in suspected cases without eggs or parasite stages in their stools. Only very few serological tests have been published which were on experimental basis as those carried out by Banzon *et al.* (1975) and Cross and Chi. (1978). *C. philippinensis* worms are very tiny and difficult to isolate from human feces therefore antigen preparation from worms would not be very practical.

The present study was designed to prepare *C. philippinensis* coproantigen from the stools of infected patients and to determine its diagnostic value using ELISA technique.

Material and methods

Twenty cases have been included as follows: Five cases with intestinal capillariasis, five cases with fascioliasis, five cases with schistosomiasis *mansoni* and five cases with strongyloidiasis. The healthy control group includes five parasite-free individuals. All cases were chosen from the Cairo University Hospital. All cases were diagnosed by stool analysis and serum samples were collected from them and stored at 70C till used.

Preparation *C. philippinensis* crude worm antigen: Adult worms were isolated from freshly voided stool samples of infected cases particularly in the 2nd day after the administration of albendazole. A drop of liquid stool was spread on a slide and examined by the light microscope using 4x objective lens and 10 x eye lens. A fine needle was used to remove the tiny worms from the slide and transfer them to a small Eppendorf tube half filled with PBS (PH 7.4) per tube. A number of 20-30 worms were collected in each tube. Worms were washed several times in PBS, homogenized in a suitable amount of buffer and centrifuged at 6000 rpm for 3 minutes. The supernatant was aspirated and its protein content was estimated according to Lowry *et al.* (1951) before it was aliquoted and stored at -70 C till used.

C. philippinensis coproantigen: This was prepared from stool samples of infected cases as was described by El-Bahy *et al.* (1992) with some modification. The collected stool samples were mixed with equal amount of PBS (PH.7.4), sieved through a double layer of gauze, homogenized for 3 minutes in a homogenizer, sonicated for 5 minutes in an ice bath then centrifuged at 10.000 rpm for ten minutes. The supernatant was then aspirated & its protein content was estimated as mentioned before. The antigen was aliquoted & stored at -70C till used. Other antigens used for the detection of cross-reactivity with patients sera were: *Fasciola gigantica* E.S antigen prepared according to River - Marrero *et al.* (1988), hydatid cyst antigen prepared according to Kaddak *et al.* (1992) *Sarcocystis fusiformis* cystozoites antigen, prepared according to Morsy *et al.* (1994) and the crude larval antigen of *Toxocara canis*,

prepared according to Kagan *et al.* (1958) were kindly supplied by Dr. M. El-Bahi, Prof. of Parasitology, Cairo University. *Schistosoma mansoni* soluble egg antigen was obtained from Theodore Bilharze Institute, Imbaba, Cairo, Egypt.

Preparation of hyperimmune sera: Mice and rabbits hyperimmune sera were prepared against the crude worm antigen of *C. philippinensis* according to Langley and Hillyer (1989) with modification. A primary dose of the crude worm antigen, adjusted to contain 1.2mg protein and mixed in an equal volume of Freund's complete adjuvant was injected subcutaneously into a rabbit. One tenth of this dose was used for a mouse. Another 3 consecutive doses of the antigen, each contains one third of the initial protein content mixed with an equal volume of complete Freud's adjuvant were injected deeply intramuscular at weekly intervals. The blood of the immunized animals was collected 10-14 days after the last injection and the serum was separated and stored at -70°C.

Detection of reactivity of coproantigen using indirect ELISA: Sera from all cases of the study were tested against *C. philippinensis* coproantigen and crude worm antigen as well as antigens of *F. gigantica*, *S. mansoni*, hydatid cyst, *Sarcocystis* and *T. canis*. Serum samples were used in 2 dilutions 1:50 and 1:100. The test was conducted (Espino *et al.*, 1978). Each plate was coated with the tested antigen at the optimal dilution (4ug/well) in coating buffer, adjusted after checkerboard titration. Horseradish peroxidase conjugated rabbit antihuman IgG (Sigma) was used at 1:1000 dilution. The test was applied in duplicate, with both serum dilutions. The results of the reaction were read at 499 nm using Titertech Multiscan ELISA reader. Positive results were accepted in samples with mean OD values equal to double the values of negative control readings.

Sandwich ELISA: *C. philippinensis* coproantigen and coproantigens prepared from stool of cases infected with *S. mansoni*, cases with *Fasciola* and parasite free individuals (as positive and negative controls) were tested against two different laboratory prepared anti-*C. philippinensis* hyperimmune sera of rabbit and mouse according to Espino and Finlay (1994). The plates were coated with 100ul/well of mouse anti-*Capillaria* hyperimmune serum at 1:20 dilution overnight (the required

concentration of IgG was obtained after checkerboard titration). After washing, 100 ul/well from each antigen (diluted 1:1 in PBS-Tween 20) was added in 2 separate rows and incubated at room temperature for 2 hours. The plates were washed before the addition of 100ul/well of rabbit anti-*Capillaria* hyperimmune serum at 1:20 dilution, then they were incubated at room temperature for 2 hours. After another wash, horseradish peroxidase conjugated goat anti-rabbit Ig G (sigma) diluted 1:100 in PBS was added in 100ul/well and the plates were incubated again for 1 hour then washed before the addition of 100ul of the phenylene - diamine substrate to each well. The reaction was allowed to proceed for 15 minutes at room temperature in dark then stopped by addition of 1M Sulphuric acid in 100ul /well. Positive results were evaluated as before.

Results and Discussion

In the present study, two different ELISA techniques were used to determine the value of *C. philippinensis* coproantigen for accurate diagnosis of *C. philippinensis* infection in suspected cases with negative stool samples. The results revealed that *C. philippinensis* coproantigen has the same diagnostic value as *C. philippinensis* crude worm antigen in detection of anti-*Capillaria* antibodies in the sera of infected patients using indirect ELISA technique (table:1&2).

Coproantigens prepared from stool samples of cases infected with *C. philippinensis* reacted with sera of patients infected with *C. philippinensis* in indirect ELISA with a mean OD reading value of (0.650). This reading was lower than that obtained in the reaction between the crude worm antigen and the same serum samples (0.860). However the coproantigen has the advantage of being more specific as no cross reactions occurred with sera of patients with other parasitic infections (table 2).

El Bahy *et al.* (1992) reported that the specificity of coproantigens may be due to the nature of these antigens which lack one or more of the cross reacting epitopes, which usually lead to high ELISA OD values and occurrence of cross reactions with other related antibodies.

Table :1 Mean ELISA OD values of serum samples of the study group versus different antigens (serum dilution at 1:50)

Antigen	Mean ELISA OD values of Tested serum of study cases infected with				
	<i>Capillaria</i> (n=5)	<i>Fasciola</i> (n=5)	<i>S.mansoni</i> (n=5)	<i>Strongyloides</i> (n=5)	-Ve (n=5)
<i>C.philip.</i> crude Ag	0.764(+)	0.494(c.r.)	0.323(-)	0.270(-)	0.283(-)
<i>C. Philip.</i> Copro.	0.695(+)	0.465(c.r.)	0.286(-)	0.250(-)	0.267(-)
<i>F.gigantica</i> ES Ag.	0.649(c.r.)	0.735(+)	0.237(-)	0.229(-)	0.285(-)
<i>S.mansoni</i> egg Ag.	0.503(c.r.)	0.310 (-)	0.264(-)	0.264(-)	0.216(-)
Hydatid cyst Ag.	0.631(c.r.)	0.402(c.r.)	0.225(-)	0.241(-)	0.245(-)
<i>Toxocara canis</i> Ag.	0.615(c.r.)	0.488(c.r.)	0.323(-)	0.276(-)	0.290(-)
<i>Sarcocystis</i> Ag.	0.617(c.r.)	0.421(c.r.)	0.248(-)	0.254(-)	0.248(-)

(+)= Positive samples, (-) = negative samples, (cr) = cross reacted value

Table (2); Mean ELISA OD value
in the study group serum samples versus different antigens
(Serum dilution at 1:00)

Tested antigen	Mean ELISA OD values of tested sera of study cases infected				
	<i>Capillaria</i> (n=5)	<i>Fasciola</i> (n=5)	<i>S.mansoni</i> (n=5)	<i>Strongyloides</i> (n=5)	-Ve (n=5)
<i>C.philipin.</i> crude Ag	0.860 (+)	0.467(*)	0.321(-)	0.277(-)	0.232(-)
<i>C.philipin.</i> copro Ag	0.650(+)	0.369(-)	0.250(-)	0.189(-)	0.182(-)
<i>F.gigantica</i> ES Ag	0.428(*)	0.851(+)	0.407(*)	0.205(-)	0.236(-)
<i>S.mansoni</i> egg Ag	0.158(-)	0.398(*)	0.854(+)	0.175(-)	0.196(-)
Hydatid cyst Ag	0.194(-)	0.264(-)	0.244(-)	0.184(-)	0.210(-)
<i>Toxocara canis</i> Ag.	0.139(-)	0.273(-)	0.246(-)	0.244(-)	0.250(-)
<i>Sarcocystis</i> Ag	0.146(-)	0.243 (-)	0.186 (-)	0.199 (-)	0.179 (-)

(+)= positive results, (-)= negative results, (*)= Presence of cross reacted antibodies.

Table (3): Detection of reactivity of different coproantigens versus anti-*Capillaria philippinensis* hyperimmune sera using sandwich ELISA

Coproantigens from stool of	No.	Mean ELISA OD values	Results
<i>Capillaria</i> cases	5	0.655 - 0.750	+ve
<i>Fasciola</i> cases	5	0.250 - 0.285	-ve
<i>Schistosoma mansoni</i> cases	5	0.220 - 0.280	-ve
Control negative people	5	0.220 - 0.270	-ve

Cross reactions were recorded between the antigens prepared from *Fasciola*, *S. mansoni*, hydatid cyst, *Toxocara canis* and *Sarcocystis* and the sera of the study group at 1:50 serum dilution (table 1). The cross reactions almost disappeared at further dilution of the serum samples to 1:100, and the test became more specific (table 2). Serum samples from cases infected with *C. philippinensis* showed a high degree of specificity versus the other tested antigens as there was no cross reaction between them at 1:100 serum dilution, where as sera from patients with other parasitic diseases showed positive ELISA OD values versus their target antigens.

Banzon *et al.* (1975) reported no cross reactions between sera of cases with *C. philippinensis* and *S. japonicum* antigens by double diffusion and indirect haemagglutination (IHA) test meanwhile they reported cross reaction between sera of *C. philippinensis* cases with antigens of *T. spiralis* and *Trichuris*. The authors added that the level of antibodies in the sera of infected patients, were apparently not related to the clinical severity of the disease.

Juncker Voss *et al.* (2000) using indirect immunofluorescence assay (IFT) described cross reaction between sera of cases with *Capillaria hepatica* and antigens of other parasites, which disappeared at higher serum dilution.

In the present work, low level of cross reaction was observed between sera of cases with intestinal *capillariasis* and *F. gigantica* ES antigen at 1/100 serum dilution. A similar result was previously recorded by Lin *et al.* (1990), who reported cross reactions in ELISA between sera of patients with *C. philippinensis* and antigens prepared from the liver fluke *Clonorchis sinensis* as well as from *Sparganum proliferum* and *T. canis*.

Concerning the detection of reactivity of *C. philippinensis* coproantigen against anti-*Capillaria* antibodies in the hyperimmune sera, there was marked specificity in capturing these antibodies with OD reading value of 0.655-0.750 nm, (table 3). This reaction was not detected with other coproantigens used (from stools of *S. mansoni* and *Fasciola* infected persons).

This study is a step forward in the immunological diagnosis of infection with *C. philippinensis* depending on detection of a specific antigen in the stools of patients. Stool samples are more abundant than serum, easily obtainable in a non-invasive way. The presence of the antigen is related to active infection unlike antibodies which may remain in the serum of patients for variable durations even after treatment.

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