

## THE DIAGNOSTIC IMPORTANCE OF SPECIES SPECIFIC AND CROSS-REACTIVE COMPONENTS OF *Taenia solium*, *Echinococcus granulosus*, AND *Hymenolepis nana*.

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### SUMMARY

Sera from patients infected with *Taenia solium*, *Hymenolepis nana* and *Echinococcus granulosus* were tested against homologous and heterologous parasite antigens using an ELISA assay, and a high degree of cross-reactivity was verified. To identify polypeptides responsible for this cross reactivity, the Enzyme Linked Immuno-electro Transfer Blot (EITB) was used.

Sera from infected patients with *T.solium*, *H.nana*, and *E.granulosus* were assessed against crude, ammonium sulphate precipitated (TSASP), and lentil-lectin purified antigens of *T.solium* and crude antigens of *H.nana* and *E.granulosus*.

Several bands, recognized by sera from patients with *T.solium*, *H.nana*, and *E.granulosus* infections, were common to either two or all three cestodes. Unique reactive bands in *H.nana* were noted at 49 and 66 K-Da and in *E.granulosus* at 17-21 K-Da and at 27-32 K-Da. In the crude cysticercosis extract, a specific non glycoprotein band was present at 61-67 K-Da in addition to specific glycoprotein bands of 50, 42, 24, 21, 18, 14, and 13 K-Da.

None of the sera from patients with *H.nana* or *E.granulosus* infection cross reacted with these seven glycoprotein bands considered specific for *T.solium* infection.

**KEYWORDS:** *Taenia solium*; Cysticercus; antigens; *Hymenolepis nana*; *Echinococcus granulosus*; Hydatid Cysts; SDS-PAGE; Electrophoresis; Immunoblotting.

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### INTRODUCTION

Cysticercosis is a disease caused by larva of the cestode *Taenia solium*. The larval form frequently infects the brain causing severe neurologic morbidity and occasionally death as a result of in-

creased intracranial pressure and/or seizures. The disease is common and poses a serious threat to human health in developing countries of Latin America and the world<sup>6,17,20</sup>.

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The diagnosis of cysticercosis is difficult because the neurological symptoms are protean and may mimic those of other diseases including brain tumors, cerebral vascular accidents, and idiopathic epilepsy. Many infections occur at the base of the brain making diagnosis difficult, even through the use of computerized tomography. In some cases the diagnosis is only made following brain surgery<sup>26</sup>.

A variety of immunoassays have been developed to diagnose cysticercosis including: complement fixation<sup>18</sup>, indirect hemagglutination<sup>14</sup> and ELISA<sup>4,6,7,8,10,15</sup>. Sera from patients infected with *Echinococcus granulosus* have been noted to cross-react in such immunoassays<sup>4,8</sup>. The clinical picture of hydatidosis, however, is usually distinct from that of cysticercosis so that cross-reactions do not usually pose a diagnostic problem. Preliminary studies in our laboratory also suggest that patients infected with *Hymenolepis nana* have antibodies that cross-react with *T.solium* antigens. *H.nana*, in contrast to hydatidosis, is frequently encountered in individuals throughout the world<sup>1</sup>. In Lima, Peru, it is the most common intestinal helminth diagnosed by stool examination with a prevalence rate varying from 5% to 50%<sup>24</sup>.

The present study demonstrates that antibodies from patients infected with *H.nana* and *E.granulosus* recognize cysticerci antigens. Antigenic bands unique to each of the cestodes also were identified using the EITB technique.

## MATERIALS AND METHODS

### OVERALL STUDY DESIGN -

In order to identify specific and common cross-reactive antigens of *T.solium*, *H.nana* or *E.granulosus*, sera were obtained from patients confirmed as being infected with each of these parasites and either crude or purified antigen preparations from the same parasites were utilized. Sera were tested for the presence of cross reacting antibodies using both ELISA and EITB assays.

### I. HUMAN SERA -

Sera were obtained from the following patient groups:

Group 1 : Peruvian patients with cysticercosis confirmed by histopathology (n=22).

Group 2 : Peruvian patients with *H.nana* infections presenting positive stool examination (n=59).

Group 3 : Peruvian patients with pulmonary or hepatic *E.granulosus* infection confirmed by biopsy (from cysts excised by surgery) (n=18).

Group 4 : Negative control comprised children and adult from Bangladesh, a Muslim country where cysticercosis is not endemic (n=50); and from upper class adult Peruvians (10-40 years of age) living in Lima, who had low rates of exposure to parasitic disease, and showing negative results in immunoblot technique for cysticercosis. Controls for *H.nana* infections were adult outpatients (6-30 years of age) (low to low middle socioeconomic class) with negative stool in two examinations for *H.nana* (n=30). Controls for *E.granulosus* infections included adult patients with a negative chest x-ray and no hydatidosis verified during the abdominal surgery (n=47).

The sera from group 4 were also employed as the negative control to determine the ELISA cut-off point for each of the studied antigens.

### II. ANTIGENS -

#### a) Crude *T.solium* Larva -

Whole *T.solium* cysticerci were excised from the skeletal muscle of naturally infected pigs. Larvae (10 g) were washed in 30 ml Phosphate Buffered Saline, pH 7.4 (PBS), at 4°C containing protease inhibitors (0.25 M phenylmethylsulfonylfluoride (PMSF) and were homogenized using a glass tissue homogenizer. The mixture was sonicated on ice for 3 min at 20 Khz. This material was centrifuged (11,720 x g, 90 min, at 4°C) and the supernatant was used as a

crude antigen preparation in which the protein concentration<sup>2,9</sup>, varied from 1-9 mg/ml.

- b) Ammonium sulphate precipitated antigen (TSASP) - Twenty ml of the crude antigen preparation was precipitated overnight (4°C) with an equal volume of a 20% saturated solution of ammonium sulphate (final concentration of 10%) and then centrifuged (20,840 x g, 15 min, 4°C). The precipitate was dissolved in 1 ml of PBS and used immediately<sup>11</sup>.
- c) Glycoprotein antigens from *T.solium* larvae - This antigen was prepared using lentil-lectin affinity-purification<sup>23</sup>.
- d) Crude *H.nana* extract - Adult worms were removed (30 days post-infection) from the intestine of infected mice<sup>1</sup>. The worms were homogenized in PBS at 4°C, sonicated for 5 min at 21 Khz, centrifuged (20,000 x g, 20 min).
- e) Crude *E.granulosus* cyst fluid - Cyst fluid from ovine hydatid cysts were aspirated and centrifuged at 1,000 x g for 30 min. The supernatant was then dialyzed against PBS, pH 7.4, and lyophilized<sup>25</sup>.

All antigens were aliquoted into vials<sup>13</sup> and stored at -70°C, after the protein content of antigen preparations was determined using the Bradford protein assay<sup>2</sup>.

### III. IMMUNOENZYMATIC ASSAY (ELISA) -

The conditions influencing ELISA were optimized by checkerboard titration (antigen concentration, incubation period, serum dilution, and peroxidase conjugate concentration). Each microplate well (Immulon I, Dynatech Laboratories, Chantilly, VA.) was coated with 100 µl (1 µg/ml) of either *E.granulosus*, *H.nana*, or *T.solium* crude antigen preparation in carbonate buffer, pH 9.6, overnight at 4°C.

The excess antigen was removed by washing the wells with PBS containing 0.05% Tween 20.

Free binding sites were blocked with 1% skimmed bovine milk diluted in PBS-Tween for 1 hr at 37°C.

The microplate wells were washed, duplicate serum dilutions in PBS-Tween were added before incubating for 1 hr at 37°C. After washing, goat anti-human IgG-peroxidase conjugate was added (1/10000, Kirkegaard & Perry Laboratories, Gaithersburg, MD.) and incubated for 1 hr at 37°C. The microplates were washed and 100 µl of substrate (10 µl H<sub>2</sub>O<sub>2</sub> and 4 mg o-phenylenediamine) per 10 ml citrate buffer was added. The plates were incubated for 10-15 min at room temperature. The reaction was stopped by adding 25 µl 1 N H<sub>2</sub>SO<sub>4</sub> and read at 490 nm in a spectrophotometer (DIWAN et. al. 1982., HARRIS, 1973). A sample was considered positive if the specific O.D. value was greater than the mean values of the negative sera plus two standard deviations, corresponding to the stipulated cut-off value for each dilution. Sensitivity and specificity of each test was calculated (ESPINOZA et. al., 1986., ESPINOZA et. al., 1982). The sera dilution which provided the highest sensitivity and specificity for each antigen were: 1/2500 for crude cysticercosis extract (Cut-off 0.060), 1/500 for TSASP (Cut-off 0.094), 1/100 for *H.nana* crude antigen (Cut-off 0.386) and 1/2500 for hydatid cyst fluid (Cut-off 0.072).

**SDS-PAGE** - Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of parasite antigen preparations was carried out using thin minislab 12% gels. The samples were heated for 15 min at 65°C and each lane was loaded with 0.2 µg/µl/mm. Electrophoresis was carried out at 200 volts for one to two hrs at 4°C. Pre-stained low and heavy molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD.) also were run<sup>22</sup>.

**IMMUNOBLOTTING** - Electrophoretic transfer of proteins to nitrocellulose was carried out at 1 Amp. for 1 hr. The nitrocellulose strips were quenched overnight in 5% skimmed milk in PBS, pH 7.2<sup>22</sup>. Pooled antibodies from patients infected with either *T.solium*, *H.nana*, or *E.granulosus*, and from rabbits immunized with one of the antigen preparations was diluted 1/50 and incubated with the strips for 1 hr at room temperature. After ex-

tensive washing in 0.4% Tween 20 in PBS, horse-radish-peroxidase (HRP) - conjugated to goat anti-polyvalent human Ig (1/2500, CDC) was incubated with strips for 1 hr at room temperature. The strips were washed and then visualized using 3,3-diaminobenzidine (DAB) as substrate.

## RESULTS

**CROSS-REACTIVITY OF ANTIBODIES FROM INFECTED PATIENTS BY ELISA ASSAY** - A high degree of cross reactivity was observed between *T.solium*, *H.nana*, and *E.granulosus* crude antigens in ELISA. The highest degree of cross reactivity was observed between the antigens of *T.solium* and *H.nana* (Table 1). There was however a significant decrease in cross reactivity (7%) when the sera from *H.nana* infected patients were tested against the TSASP antigen in comparison to the crude antigen.

Antigens were tested against sera from *E.granulosus* infected patients. No reduction in the cross-reactivity rate occurred when TSASP antigen was used instead of crude *T.solium*.

Sera from *T.solium* infected patients provided very high rates of cross-reactivity with *E.granulosus* (75%) and *H.nana* (88%) antigens (Table 1).

**CHARACTERIZATION OF IMMUNOREACTIVE PROTEINS BY EITB** - Seven patient sera positive only for *H.nana* infection were pooled and tested against the three cysticercosis antigens and also against the crude antigen preparation of *E.granulosus*. These sera cross-reacted with the crude extract of cysticercosis (bands 48, 23-21 k-Da), with the TSASP antigen (bands 27, 14-13 K-Da), and with *E.granulosus* antigen (bands 64, 38 K-Da). (Figure 1 and Table 2). One of 59 sera from *H.nana* infected patients gave a reaction to glycoprotein bands (50, 42-39, 24 K-Da) typical of patients infected with *T.solium* (TSANG et al., 1989).

A large number of cross-reacting bands appeared when *T.solium* crude antigens were reacted with pooled sera from *E.granulosus* patients. The bands were identified at 120, 105, 62, 54, 40, 38, 17, 13, 12 K-Da whereas with the TSASP antigen bands of 105, 92, 62, 40, 17, 13, 12 K-Da were identified. (Figure 2 and Table 2).

TABLE 1

Positivity and cross reactivity among the sera from patients with *Hymenolepis nana*, *Echinococcus granulosus* and *Taenia solium* infections in homologous and heterologous antigenic systems.

INFECTION (SERUM)	TECHNIQUE				
	ELISA				IMMUNOBLOTTING
	Crude	Extract	Antigen	TSASP <sup>a</sup>	Glycoprotein Antigen
	<i>H.nana</i>	<i>E.granulosus</i>	<i>T.solium</i>	<i>T.solium</i>	<i>T.solium</i>
<i>H.nana</i>	100% <sup>b</sup> (59/59)	17% (10/59)	20% (12/59)	7% (4/59)	2% (1/59)
<i>E.granulo-</i> <i>sus</i>	50% (9/18)	94% (17/18)	11% (2/18)	22% (4/18)	0% (0/18)
<i>T.solium</i>	88% (14/16)	75% (15/20)	82% (18/22)	73% (16/22)	91% (20/22)

<sup>a</sup>TSASP: *Taenia solium* ammonium sulphate precipitated antigen. b: Percentage of seropositivity is referred to as: Number of positive sera / Total Number of tested sera.

Unique bands - In the crude extract of *H.nana* there were 2 antigenic bands at 66 and 49 K-Da that did not react with antisera of either *T.solium* or *E.granulosus* infected patients. There were also two non-cross reacting bands at 17-21 KDa and 27-32 K-Da in *E.granulosus* crude extract when tested with sera of *H.nana* and *T.solium* infected patients. the crude cysticercosis extract also had one broad reacting band at 61-67 K-Da (in addition to the 7 glycoprotein bands described by TSANG et. al., 1989) that did not cross react with antisera to the other parasites (Figure 3 and Table 2).

### DISCUSSION

Serum antibodies from humans infected with either *E.granulosus* or *H.nana* cross react with antigens of the cysticercus stage of *T.solium*. The existence of conserved proteins among cestodes has been described in hydatidosis<sup>21</sup>, but not in *H.nana* infections.

Sensitivity and specificity of ELISA were similar to those found in both Mexico<sup>8</sup> and South

TABLE 2

Cross Reacting And Specific Protein bands found in Antigen Preparations of *Taenia solium*, *Hymenolepis nana*, *Echinococcus granulosus* with homologous and heterologous sera from infected patients.

ANTIGEN	HUMAN SERA INFECTED WITH		
	<i>T.solium</i>	<i>H.nana</i>	<i>E.granulosus</i>
Crude <i>T.solium</i> larva	61,50,42, 18,14,13.	48,23,21.	120,105,62, 40,17,13,12.
Ammonium sulphate precipitated antigen (TSASP)	61,50,42, 18,14,13.	27,14-13.	105,92,62, 40,17,13,12.
Glycoproteins antigen from <i>T.solium</i>	50,42,24, 21,18,14, 13	-	62,40.
Crude extract from <i>H.nana</i>	54,13.5.	66-64,49-48, 38,27.	88.
Crude <i>E.granulosus</i> cyst fluid	54,38.	64,38.	62,27,17,13.

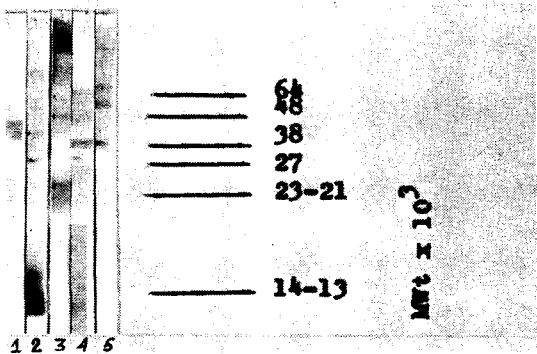


Fig.1. Antibodies from patients infected with *Hymenolepis nana* reacting with components of the three parasites: Lane (1). Glycoprotein antigen from *Taenia solium*. Lane (2). Ammonium sulphate precipitated antigen from *Taenia solium* (TSASP). Lane (3). Crude antigen from *T.solium*. Lane (4). Crude extract from *Hymenolepis nana*. Lane (5). Crude cyst fluid from *Echinococcus*

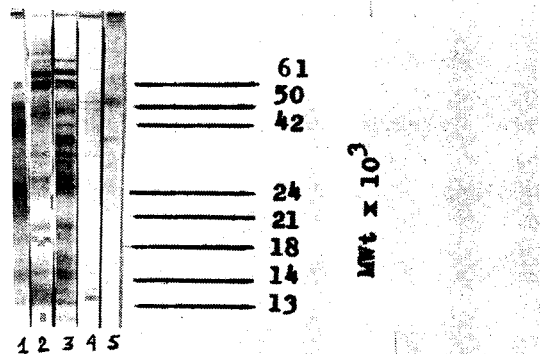


Fig.2. Antibodies from patients infected with *Echinococcus granulosus* reacting with components of these cestodes. Lane (1). Glycoprotein antigen from *T.solium*. Lane (2). Ammonium sulphate precipitated antigen from *T.solium* (TSASP). Lane (3). Crude antigen from *T.solium*. Lane (4). Crude extract from *H.nana*. Lane (5). Crude cyst fluid from *E.granulosus*.

Africa<sup>19</sup>. The specificity of crude extracts *T.solium* and TSASP, nevertheless, was low; many bands showed cross reactivity with *H.nana* and *E.granulosus*. TSASP antigens had less cross-reactivity for *H.nana* sera when compared with the crude *T.solium* antigen.

The existence of a 38 K-Da band cross-reacting with other cestode, trematode, and nematode parasites<sup>21</sup> was confirmed by our study. In addition, the 62 and 48 K-Da cross-reacting bands of our study appear to be similar to the 62 and 49 K-Da antigenic bands which react with normal human sera which have been previously noted<sup>12</sup>.

The purified cysticercosis glycoprotein antigens used in our study yielded superior specificity when compared to either the crude or the TSASP antigens. The purified antigens were also highly specific; none of the sera from 18 patients with hydatidosis demonstrated cross-reacting antibody to any of the seven specific bands. There was, however, one patient infected with *H.nana* whose serum gave a strong positive result with the purified *T.solium* glycoprotein antigen. The pattern of the three bands that reacted in this patient were the same as those characteristically found in patients with cysticercosis. These bands were not the bands normally found in sera from patients with *H.nana* when tested against homologous antigen. In neurologically intact patients living in highly endemic regions, seropositivity ranged between 2-8% when the purified glycoprotein antigen and EITB technique were used<sup>5</sup>. It is likely, therefore, that this patient was coinfecting with cysticercosis, although the possibility of a rare cross-reacting antibody cannot be ruled out.

The ELISA assay, which utilizes crude extracts of *T.solium*, has two serious defects: 1) it is not highly sensitive and 2) it gives a high rate of false positive reactions, particularly in zones where *H.nana* infection is endemic<sup>3,16</sup>. In the present study, 20% of *H.nana* patients had a positive reaction in the cysticercosis ELISA which used crude *T.solium* antigen. When this ELISA test is used it may become imperative to screen all patients for *H.nana* by stool examination.

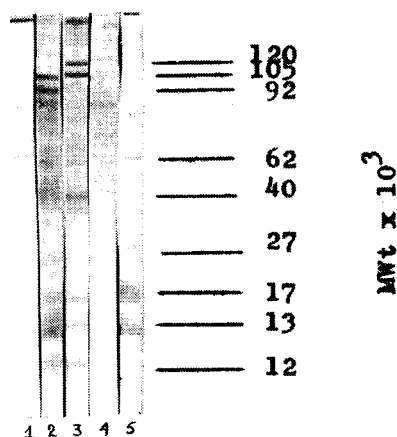


Fig. 3. Antibodies from patients infected with *Taenia solium* reactive with the components of the different cestodes: Lane (1). Glycoprotein antigen from *T.solium*. Lane (2). Ammonium sulphate precipitated antigen from *T.solium* (TSASP). Lane (3). Crude antigen from *S. solium*. Lane (4). Crude extract from *H. nana*. Lane (5). Crude cyst fluid from *E.granulosus*.

The seroepidemiology of cysticercosis has been hampered by the lack of a sensitive and specific test. The use of purified antigen in immunoassays from which most cross-reacting bands have been removed will provide higher sensitivity and specificity than crude antigen. At present, the immunoblot assay, utilizing purified glycoprotein antigens, would appear to be a suitable test for both clinical and epidemiological studies of *T.solium* infection.

## RESUMO

**Importância diagnóstica da reação cruzada espécie-específica de componentes da *Taenia solium*, *Echinococcus granulosus* e *Hymenolepis nana*.**

Soros de pacientes infectados com *Taenia solium*, *Hymenolepis nana* e *Echinococcus granulosus* foram testados contra antígenos parasitários homogêneos e heterogêneos usando o teste de ELISA e foi verificado alto grau de reatividade cruzada. Para identificar os

polipetídeos responsáveis por esta reatividade cruzada foi utilizado o teste "Enzyme Linked Immuno-electro Transfer Blot (EITB)".

Soros de pacientes infectados por *T.solium*, *H.nana*, e *E.granulosus* foram colocados em contato com precipitado de sulfato de amônia e antígenos não purificados de *T.solium* e os de *H.nana* e *E.granulosus*.

Várias bandas reconhecidas pelos soros de pacientes com infecção por *T.solium*, *H.nana* e *E.granulosus* foram comuns a dois ou três destes cestódeos. Uma única banda foi notada em *H.nana* a 49 e 66K-Da e no *E.granulosus* a 17-21 K-Da e 27-32 K-Da. No extrato não purificado de cisticercose uma banda específica não glicoproteica estava presente a 61-67 K-Da além das bandas de glicoproteínas específicas de 50, 42, 24, 21, 18, 14 e 13 K-Da. Nenhum destes soros de pacientes com infecção por *H.nana* ou *E.granulosus* reagiu de forma cruzada com estas sete bandas de glicoproteína consideradas específicas à infecção por *T.solium*.

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