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Brief Communication

Comparisons between scolex and membrane antigens of *Cysticercus fasciolaris* and *Cysticercus cellulosae* larvae for immunodiagnosis of neurocysticercosis

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The antigen source for enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunotransfer blot for neurocysticercosis is generally *Taenia solium*. A comparison of the membrane and scolex extracts of *Cysticercus cellulosae* and *Cysticercus fasciolaris* (larval stage of *Taenia taeniaeformis*) for the immunodiagnosis of neurocysticercosis has been performed. *C. fasciolaris* cysts were produced experimentally in rat liver. *C. cellulosae* was obtained from muscle of infected pigs. The antigen extracts of membrane and scolex were compared using ELISA in 50 patients and 50 control participants to detect immunoglobulin (Ig) G or IgM antibodies. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were immunoprobed using pooled and individual sera. The gold standard for diagnosis was visualization of scolex in ring lesions by magnetic resonance imaging or computed tomography scans. ELISA for IgG antibodies using *C. fasciolaris* membrane had the highest sensitivity of 94%. Specificity ranged from 78% to 90%. Immunoreactive bands common to all 4 antigens were seen between 60 and 70 kDa and 40 and 45 kDa. The presence of comparative antigenic bands between human and rat pathogens provides convincing evidence for use of *C. fasciolaris* antigens for immunodiagnostic procedures. The antigen can be produced in small animals in standardized laboratory conditions within 60 days.

Key words: *Cysticercus*; Enzyme-linked immunosorbent assay; Immunoblotting; Neurocysticercosis

Introduction

Taenia solium cysticerci are pathogenic to humans and cause cysticercosis by accidental infestation. Cysts in the central nervous system present with high morbidity, including epilepsy, meningitis, hydrocephalus, and other manifestations. Radiological studies form the mainstay of diagnosis. However, diagnostic specificity is not high and immunoassays are frequently used as adjuncts to diagnosis. The antigen in immunoassays is frequently derived from *T. solium*, although other species of cysticerci, including *Taenia crassiceps* [1-3], *Taenia hydatigena* [4], and *Taenia saginata* (adult worm) [5], have been tested in immunological assays such as enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunotransfer blot (EITB) with good results. These authors have previously reported the use of membrane antigen of *Cysticercus fasciolaris*, the larvae of *Taenia taeniaeformis*, for the detection of immunoglobulin (Ig) G and IgM antibodies in serum of patients with neurocysticercosis using ELISA [6]. The study described here was performed to compare the use of membrane and scolex antigens of *C. fasciolaris* (the rat pathogen) with those of *Cysticercus cellulosae* (the human pathogen) to detect IgG and IgM antibodies by ELISA in the serum of patients with neurocysticercosis and healthy and infected control participants. Comparison of the antigenic bands of the membrane and scolex antigens of the 2 pathogens was done by immunoblot assay.

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Methods

Study design

This was a case-control study for diagnostic test evaluation performed at a tertiary hospital, King George's Medical University, Lucknow, India. Fifty patients with neurocysticercosis, aged 5 to 49 years (mean ± standard deviation, 8.8 ± 11.4 years). The male-to-female ratio was 35:15. Neurocysticercosis was diagnosed by the absolute criterion of presence of ring-enhancing lesions by magnetic resonance imaging (MRI) and computed tomography scan with visualization of scolex as an eccentric nodule [7]. Forty one patients had single parenchymal ring-enhancing lesions and 9 had multiple ring-enhancing lesions. The 50 control participants included 36 patients with tuberculoma diagnosed by MRI, magnetic resonance spectroscopy, and response to therapy, 4 with hydatid disease of the liver, 2 with intestinal taeniasis, and 8 healthy participants.

Preparation of antigens

The gravid segments of *T. taeniaeformis*, collected from the stool of infested Indian cats, were teased in normal saline to release eggs. Mature and viable eggs were counted and the volume of the stock suspension was then adjusted using normal saline so that each mL contained 1000 eggs. Adult male *Rattus rattus* were given 0.2 mL of egg suspension through a feeding needle attached to a tuberculin syringe. After 60 days, the eggs developed into mature cysticerci inside the rat liver. The rats were anesthetized, the liver was removed, and the cysts were dissected on ice and washed with cold phosphate-buffered saline (PBS). The membranes and scolices were separated, washed with PBS, homogenized, and sonicated to yield *C. fasciolaris* antigens. The suspension was cold centrifuged at 20,000 g for 30 min at 4°C.

C. cellulosae antigen was prepared from cysticerci of *T. solium* collected from freshly slaughtered heavily infected pigs, obtained from the butchery, fresh on ice. The cysts were dissected without any adherent host tissue, and kept on ice. Dissected cyst membrane and scolices were washed thoroughly in cold PBS. Antigen was prepared by homogenization, sonication, and centrifugation as described above for *C. fasciolaris*. The supernatants at a final concentration of 150 mg/dL of protein were used as concentrated antigen. Four antigens — *C. fasciolaris* membrane (CFM), *C. fasciolaris* scolex (CFS), *C. cellulosae* membrane (CCM), and *C. cellulosae* scolex (CCS) — were obtained, aliquoted, and stored at –80°C.

All animal studies were done after prior ethical approval was obtained, and the principles of the Declaration of Helsinki were followed.

Enzyme-linked immunosorbent assay

Indirect antibody detection by ELISA was performed. Checkerboard titration was done to optimize the dilution of antigen, sample, and secondary antibody. Polystyrene-coated flat-bottomed microtiter plates were coupled with 100 μL of antigen, CFM, CFS, CCM, and CCS in bicarbonate buffer at a dilution of 1:1000, and stored overnight at 37°C. Protein coated in each well was 150 μg. Plates were washed 10 times with PBS with 0.1% Tween 20 (PBST) [Bangalore Genei, Bangalore, India], blocked with 2% bovine serum albumin (BSA) in PBS for 1 h, washed 5 times in PBST, incubated with 100 μL of serum diluted 1:1000 in PBS for 2 h, washed 5 times with PBST, incubated with secondary antibody-horse radish peroxidase tagged anti-human IgG or IgM (Dakopatts, Copenhagen, Denmark) diluted 1:500 for 1 h, washed 5 times with PBST, and color developed with tetramethylbenzidine at 1:20 dilution for 15 min. Optical densities (ODs) were measured at 450 nm after stopping the reaction with 5 N sulfuric acid. Eight sets of ELISA were run using 4 different antigens for the detection of anticysticercus human IgG and IgM antibodies. ELISA was performed blindly for all samples from patients and control participants. Negative, low, and high positive control samples were run in each batch. All samples were run in triplicate and the test was repeated on 2 different days to check for reproducibility of results.

Sodium dodecyl sulfate electrophoresis

The membrane and scolex antigens (15 μg/lane) of both parasites were run on 15% acrylamide gel in a mini vertical gel chamber with sodium dodecyl sulfate buffer along with prestained molecular weight standards (Bio-rad, Hercules, CA, USA). Protein samples were denatured in Laemmli sample buffer with beta-mercaptoethanol by heating at 100°C for 5 min. Separation was checked with silver staining.

Silver staining

The gel was fixed for 1 h in a fixing solution (50 mL methanol, 1 mL acetic acid, 38.8 mL water, and 100 μL formaldehyde), followed by washing and the addition of silver nitrate in the dark. Developer (3.0 gm sodium carbonate, 49 mL water, and 50 μL formaldehyde) was added until the protein bands became visible.

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When the bands were half visible, the reaction was terminated by adding 5 mL of 2.3 M citric solution.

Immunoblot assay

Bands were transferred to nitrocellulose membrane in a transfer apparatus (Bio-rad) using Tris glycine buffer at 100 V for 1 h. The nitrocellulose membranes, cut into sections each with 4 protein lanes and standards, were blocked with 2% BSA in Tris buffer overnight. The membranes were incubated with individual case/control or pooled case/control sera at 1:500 dilution for 2 h, washed 3 times with 1% Tween 20 in Tris, and incubated with antihuman IgG/IgM (Dakopatts) at 1:1000 dilution (1 h). Bands were developed with chromogen diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA). The sera from 10 case samples and 5 control samples were tested individually and pooled test and control sera were also tested.

Statistical analysis

Sensitivity, specificity, and positive and negative predictive values of the ELISA tests were assessed. Significance of difference was assessed by Student *t* test. A *p* value of <0.05 was considered significant.

Results

Enzyme-linked immunosorbent assay

The mean ODs of the case and control samples obtained by ELISA are shown in Fig. 1. The results of ELISA using CCM, CCS, CFM, and CFS antigens for the detection of IgG and IgM antibodies in the serum of 50 patients and 50 control participants are shown in Table 1. The sensitivity of ELISA for detection of antibodies in serum ranged from 76% to 94% and the specificity ranged from 78% to 90%. The highest sensitivity of 94% was obtained for CFM ELISA for detection of IgG antibodies while 90% specificity was obtained for CFS and CCM in IgM ELISA and CCS in IgG ELISA. No significant differences were observed between ELISA results using different species or for membranes versus scolices (p=0.8).The distribution of ODs among patient and control samples was well separated.

Silver staining

The silver-stained gel showed multiple fine and prominent bands ranging from 201 kDa to lesser molecular weight bands of less than 31.8 kDa in all 4 antigens (Fig. 2A). The maximum number of bands was observed for CCM. Several protein bands were common in membrane and scolex antigens of both species.

Immunoblot analysis

Multiple thick and fine bands were visualized for all 4 antigens, varying in size from 18 to 200 kDa (Fig. 2B). *C. fasciolaris* showed 6 antigenic bands in membrane and 8 in scolex. *C. cellulosae* showed 8 antigenic bands in membrane and 7 in scolex. Prominent bands between 60 to 70 kDa and 40 to 45 kDa were common to membranes and scolices. These bands were recognized by both IgG and IgM antibodies in all patient sera and were more intensely stained than other bands. Six of 10

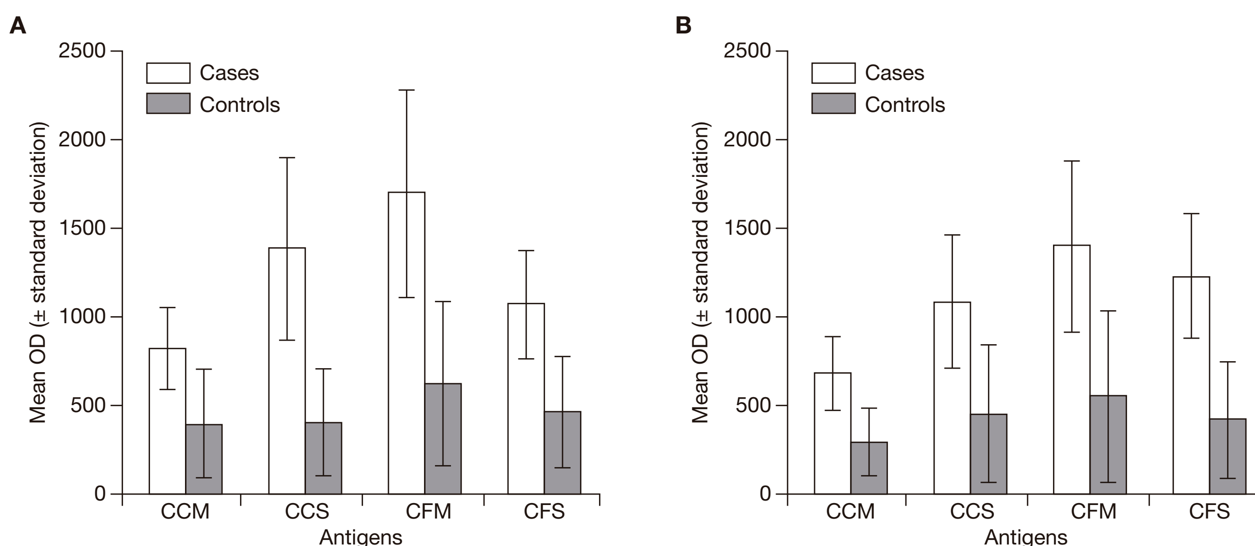

Fig. 1. Distribution of mean optical density (OD) in patient and control samples by enzyme-linked immunosorbent assay (ELISA) using 4 different antigens. (A) Immunoglobulin (Ig) G ELISA; and (B) IgM ELISA. CCM = *Cysticercus cellulosae* membrane; CCS = *C. cellulosae* scolex; CFM = *Cysticercus fasciolaris* membrane; CFS = *C. fasciolaris* scolex.

Table 1. Enzyme-linked immunosorbent assay (ELISA) for the detection of anticysticercus immunoglobulin (Ig) G and IgM antibodies

	Antigens							
	IgG ELISA				IgM ELISA			
	CFM	CFS	CCM	CCS	CFM	CFS	CCM	CCS
Patients								
Positive	47	44	41	44	40	46	38	41
Negative	3	6	9	6	10	4	12	9
Controls								
Positive	8	10	11	5	9	5	5	10
Negative	42	40	39	45	41	45	45	40
Sensitivity	94	88	82	88	80	92	76	82
Specificity	84	80	78	90	82	90	90	80
PPV	85.4	81.4	78.8	89.7	81.6	90.1	88.3	80.8
NPV	83.3	86.9	81.2	88.2	80.3	91.8	80.8	81.6

Abbreviations: CFM = *Cysticercus fasciolaris* membrane; CFS = *C. fasciolaris* scolex; CCM = *Cysticercus cellulosae* membrane; CCS = *C. cellulosae* scolex; PPV = positive predictive value; NPV = negative predictive value

patient sera recognized low molecular weight immunoreactive bands between 18 to 25 kDa for all 4 antigens. Multiple fine bands of molecular weight more than 100 kDa and less than 18 kDa were variably recognized in membrane and scolex antigens in pooled sera (Fig. 2B). There was considerable overlap in the immunoreactivity of corresponding bands of different antigens using individual and pooled patient sera. Cross-reactivity with control sera was not observed with the prominent bands in the regions described above. A few finer bands in the high molecular weight region showed immunoreactivity (Fig. 2C).

Discussion

For ELISA, the cut-off OD was calculated from the receiver operating characteristic curves. Samples were run in duplicate and the mean OD was taken as the OD of the sample. A variation of >10% in the sample OD was not acceptable and such samples were tested again. The tests were repeated on 2 different occasions and reproducibility of the results was 100%. ELISA has been widely employed as a useful tool for the diagnosis of neurocysticercosis using serum and cerebrospinal fluid [8-10] because of its technical simplicity. The sensitivity of ELISA for detection of antibodies in serum has varied from 65% [11] to 93% [12]. In this study, sensitivity between 76% and 94% and specificity between 78% and 90% were observed (Table 1).

The results of the immunoblot analysis suggest that antigenic bands of *C. fasciolaris* and *C. cellulosae* in the range of 18 to 25 kDa, 40 to 45 kDa and 60 to 70 kDa are excellent candidates for the development of an optimal test for serological diagnosis of cysticercosis. These immunoreactive bands have also been observed in other publications using *C. cellulosae* antigen [13-20]. Katti and Chandramukhi analyzed various antigenic fractions of porcine pathogen and observed an immunodominant antigen with molecular weights between 64 and 68 kDa and 24 and 28 kDa [13]. Grogl et al have also observed polypeptides and molecular weights of 64 kDa, 53 kDa, and 30 to 52 kDa was present in all antigenic fractions [14]. Grill et al have shown a high incidence of serological markers for cysticercosis among patients with epilepsy, and observed significant bands in western blot analysis with molecular weights of 13 kDa, 14 kDa, and 18 kDa in the sera of children with epilepsy [15]. Bragazza et al [16] and Pardini et al [17] have observed strong immunoreactivity at 18 kDa and 14 kDa using *T. crassiceps* antigen. Aguilar-Rebolledo et al have reported 24 kDa, 39 to 42 kDa, and 50 kDa as the most frequently recognized proteins in enzyme-linked immunoelectroblot [18]. Proaño-Narvaez et al reported 39 to 42 kDa, 24 kDa, and 13 kDa as immunodiagnostic bands [19]. In a seroprevalence study of patients with epilepsy by Bucardo et al, the bands most frequently recognized were 39 to 42 kDa, 24 kDa, and 14 kDa [20].

It appears that *C. fasciolaris* is antigenically related to the porcine and human pathogen, and is a practical alternative as an antigen source for ELISA and EITB assay for human cysticercosis. However, cysticercosis can be produced in rat liver within a shorter time of

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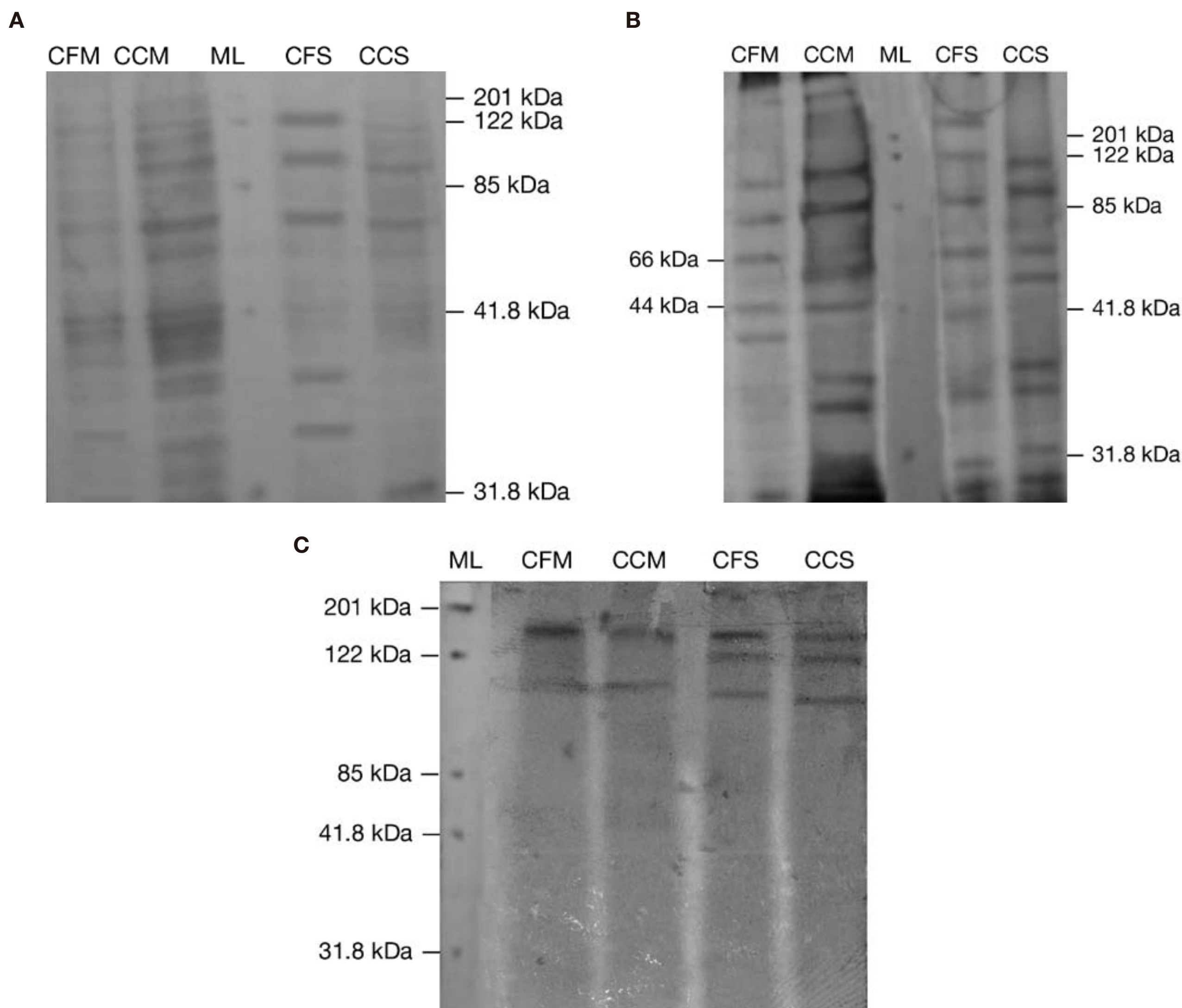

Fig. 2. (A) Silver-stained sodium dodecyl sulfate polyacrylamide gel showing separated protein bands; (B) immunoblot analysis with pooled patient sera and antihuman immunoglobulin G secondary antibody; and (C) immunoblot analysis with pooled negative control sera. CFM = *Cysticercus fasciolaris* membrane; CCM = *Cysticercus cellulosae* membrane; ML = marker lane; CFS = *C. fasciolaris* scolex; CCS = *C. cellulosae* scolex.

60 days compared with a minimum of 6 months for the human-pig cycle [21], rats are easier to breed in laboratory conditions, and are lower in cost.

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References

1. Garcia E, Ordoñez G, Sotelo J. Antigens from *Taenia crassiceps* cysticerci used in complement fixation, enzyme-linked immunosorbent assay, and western blot (immunoblot) for diagnosis of neurocysticercosis. J Clin Microbiol. 1995;33:3324-5.
2. Bueno EC, Vaz AJ, Machado LD, Livramento JA, Mielle SR. Specific *Taenia crassiceps* and *Taenia solium* antigenic peptides for neurocysticercosis immunodiagnosis using serum samples. J Clin Microbiol. 2000;38:146-51.
3. Pinto PS, Vaz AJ, Germano PM, Nakamura PM. Performance of the ELISA test for swine cysticercosis using antigens of *Taenia solium* and *Taenia crassiceps* cysticerci. Vet Parasitol. 2000;88:127-30.
4. Cheng RW, Ko RC. Purification of larval *Taenia solium* antigens by gel filtration. Vet Parasitol. 1992;43:65-73.
5. Morakote N, Nawacharoen W, Sukonthasun K, Thammasonthi W, Khamboonruang C. Comparison of cysticercus extract,

cyst fluid and *Taenia saginata* extract for use in ELISA for serodiagnosis of neurocysticercosis. Southeast Asian J Trop Med Public Health. 1992;23:77-81.
6. Husain N, Jyotsna, Bagchi M, Husain M, Mishra MK, Gupta S. Evaluation of *Cysticercus fasciolaris* antigen for the immunodiagnosis of neurocysticercosis. Neurol India. 2001;49:375-9.
7. Del Brutto OH, Rajshekhar V, White AC Jr, Tsang VC, Nash TE, Takayanagui OM, et al. Proposed diagnostic criteria for neurocysticercosis. Neurology. 2001;57:177-83.
8. Costa JM, Ferreira AW, Makino MM, Camargo ME. Spinal fluid immunoenzymatic assay (ELISA) for neurocysticercosis. Rev Inst Med Trop Sao Paulo. 1982;24:337-41.
9. White AC Jr. Neurocysticercosis: updates on epidemiology, pathogenesis, diagnosis, and management. Annu Rev Med. 2000;51:187-206.
10. Hawk MW, Shahlaie K, Kim KD, Theis JH. Neurocysticercosis: a review. Surg Neurol. 2005;63:123-32; discussion 132.
11. Diaz JF, Verastegui M, Gilman RH, Tsang VC, Pilcher JB, Gallo C, et al. Immunodiagnosis of human cysticercosis (*Taenia solium*): a field comparison of an antibody-enzyme-linked immunosorbent assay (ELISA), and antigen-ELISA, and an enzyme-linked immunoelectrotransfer blot (EITB) assay in Peru. The Cysticercosis Working Group in Peru (CWG). Am J Trop Med Hyg. 1992;46:610-5.
12. Sloan L, Schneider S, Rosenblatt J. Evaluation of enzyme-linked immunoassay for serological diagnosis of cysticercosis. J Clin Microbiol. 1995;33:3124-8.
13. Katti MK, Chandramukhi A. Comparative evaluation of cysticercal antigens and immunoassays in the diagnosis of neurocysticercosis. Ann Trop Med Parasitol. 1991;85:605-15.
14. Grogl M, Estrada JJ, MacDonald G, Kuhn RE. Antigen-antibody analyses in neurocysticercosis. J Parasitol. 1985;71:433-42.
15. Grill J, Pillet P, Rakotomalala W, Andriantsimahavandy A, Esterre P, Boisier P, et al. Neurocysticercosis: pediatric aspects. Arch Pediatr. 1996;3:360-8. [Article in French].
16. Bragazza LM, Vaz AJ, Passos AD, Takayanagui OM, Nakamura PM, Espindola NM, et al. Frequency of serum anti-cysticercus antibodies in the population of a rural Brazilian community (Cássia dos coqueiros, SP) determined by ELISA and immunoblotting using *Taenia crassiceps* antigens. Rev Inst Med Trop Sao Paulo. 2002;44:7-12.
17. Pardini AX, Peralta RH, Vaz AJ, Machado LR, Peralta JM. Use of *Taenia crassiceps* cysticercus antigen preparations for detection of antibodies in cerebrospinal fluid samples from patients with neurocysticercosis (*Taenia solium*). Clin Diag Lab Immunol. 2002;9:190-3.
18. Aguilar-Rebolledo F, Meza-Lucas A, Torres J, Cedillo-Rivera R, Enciso A, Garcia RC, et al. Evaluation of the enzyme-linked immunoelectrotransfer blot assay for diagnosis of neurocysticercosis in children. J Child Neurol. 2002;17:416-20.
19. Proaño-Narvaez JV, Meza-Lucas A, Mata-Ruiz O, García-Jerónimo RC, Correa D. Laboratory diagnosis of human neurocysticercosis: double-blind comparison of enzyme-linked immunosorbent assay and electroimmunotransfer blot assay. J Clin Microbiol. 2002;40:2115-8.
20. Bucardo F, Meza-Lucas A, Espinoza F, García-Jerónimo RC, García-Rodea R, Correa D. The seroprevalence of *Taenia solium* cysticercosis among epileptic patients in León, Nicaragua, as evaluated by ELISA and western blotting. Ann Trop Med Parasitol. 2005;99:41-5.
21. Gonzalez AE, Gilman R, Garcia HH, McDonald J, Kacena K, Tsang VC, et al. Use of sentinel pigs to monitor environmental *Taenia solium* contamination. The Cysticercosis Working Group in Peru (CWG). Am J Trop Med Hyg. 1994;51:847-50.

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