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Evaluation of IgG4 and Total IgG Antibodies against Cysticerci and Peptide Antigens for the Diagnosis of Human Neurocysticercosis by ELISA

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SUMMARY To support the clinical diagnosis of human neurocysticercosis (NCC), we evaluated two peptides, HP6-3 and Ts45W-1, as well as crude saline extract (SE) of *Tenia solium* cysticerci as antigens for the detection of specific IgG4 subclass and total IgG antibodies by an enzyme-linked immunosorbent assay (ELISA). The sera of definitive diagnosed NCC patients, patients infected with other parasitoses and healthy controls were examined. The diagnostic sensitivity for IgG4 and total IgG detection of the ELISA against SE antigen was 100% and 64.3% with a high amount of cross-reactions to taeniasis saginata at 88.9% (8/9) and 100% (9/9), respectively. The SE-based IgG4-ELISA showed the highest specificity (80.9%). Both peptide-based IgG4-ELISAs provided a superior sensitivity (78.6%) to the total IgG tests whereas their specificity was 66.7% for HP6-3 and 69.8% for Ts45W-1 only. The SE-based ELISA for the detection of specific IgG4 antibody can be used for the diagnosis of neurocysticercosis as well as for serological surveys of NCC endemic areas. The peptide-based IgG4 ELISAs potentially provide a reliable and cost effective alternative method independent from live parasite supply.

Human neurocysticercosis (NCC) caused by the larval cyst (cysticercus) of *Taenia solium*, is considered the most common helminthic disease of the brain worldwide especially in the developing countries of Latin America, Africa and Asia.[1] It has recently been recognized as an emerging parasitic disease in developed countries because of the influx of immigrants from endemic areas.[2-4] In Thailand, the disease also seems to have become more prevalent.[5] Humans acquire NCC by accidental ingestion of infective eggs in food contaminated by human feces. The actual incubation period in human is still unknown. The initial phase of the infection is often clinically silent except if the location or size of the pathology causes signs and symptoms. NCC presents with various clinical features according to the location, number, size as well as viability or stage of the degenerative cysts and the host's reaction.[4] Clinical presentations are therefore highly diverse, ranging from asymptomatic to severe. The diagnosis of NCC typically relies on pathognomonic clinical manifestations, neuroimaging, epidemiological data and confirmatory serological tests.[6] Computed tomo-

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graphy (CT) and/or magnetic resonance imaging (MRI) can be regarded as the 'gold standards' for the diagnosis of NCC in endemic areas[7], however, these methods are expensive and not accessible in many hospitals. CT and MRI can categorically identify cysts in the brain and can be conducted to determine whether cysticerci are viable, colloidal or calcified. MRI may be better than CT at detecting brainstem, or intraventricular lesions.[8] Notwithstanding technological precision, the diagnosis of NCC should be based on the proper interpretation of clinical findings, neuroimaging and antibody assays for anticysticerci antibodies.[6]

Neuroimaging results without knowledge of the underlying disease can be difficult to interpret. Serology helps to confirm the neuroimaging results by offering an infectious pathogenesis. Hospitals where MRIs are unavailable may use serological methods despite their inability to indicate the stage of infection or specify the site of the pathologic feature in the central nervous system. Immunoblot assays using cysticercus glycoprotein antigens are widely accepted for their specificity and sensitivity, approaching 100%.[9,10] The antigens, however, require a complicated technique for preparing the samples and usually rely on the availability of cysticerci collected from naturally infected swine. Recently, *Taenia saginata* derived synthetic peptide-based ELISAs were used for the confirmation of the diagnosis of cysticercosis.[7,11,12] The two peptides HP6-3 (RFVKTPSKKKTKSR), derived from a sequence of the 18 kDa *T. saginata* surface/secreted oncospheral adhesion antigen (GenBank accession no. X95983) identified by HP6 monoclonal antibody, and Ts45W-1 (AYEQPIERTVVGHQTLRDIFVWG), derived from the sequence of the *T. saginata* homologue of the *T. ovis* 45W protective gene family (GenBank accession no. AJ430567), were previously used as antigens for the serodiagnosis of NCC with a high sensitivity and specificity.[7,12] However, these results were obtained with Mexican and South American i.e. Brazilian and Venezuelan samples. Additional worldwide data are necessary to provide more insight into the reliability of these antigens for the diagnosis of NCC. Furthermore, assays detecting IgG4 subclass antibodies using crude extract and/or antigen B of *T. solium* cysticerci have been successful in diagnosing cysticercosis.[13,14] However, specific IgG4 subclass antibody detection by an ELISA based on peptides has not been reported. In the present study, we evaluated the diagnostic potential of the two peptides, i.e. HP6-3 and Ts45W-1, as antigens for the detection of specific IgG4 subclass and total IgG antibodies in NCC patient sera from Thailand. The results of the assay using the peptide antigens were compared to those using the antigen derived from crude saline extract (SE) of *T. solium* cysticerci.

MATERIALS AND METHODS

Peptides and *T. solium* crude saline extract

The two selected peptides, HP6-3 (acetyl-RFVKTPSKKKTKSR-NH2) and Ts45W-1 (acetyl-AYEQPIERTVVGHQTLRDIFVWG-NH2),[12] were synthesized as an amide at the C-terminus and an acetyl at the N-terminus by Mimotopes Pty Ltd (Clayton Victoria, Australia). The SE of the *T. solium* cysts was prepared as previously described[15] with some modifications. Briefly, the larval cysts were homogenized with a tissue grinder in a small volume of 0.85% NaCl in distilled water containing proteinase inhibitors, 0.1 mM phenyl-methyl sulfonyl fluoride, 0.1 mM *p*-tosyl-L-phenylalanine-chloromethyl ketone, and 1 μM trans-3-carboxy-oxiran-2-carbonyl-L-leucylagmatine. The preparation was then sonicated with an ultrasonic disintegrator and centrifuged at 10,000 × *g* at 4°C for 1 hour. The protein concentration of the supernatant was determined.[16] The supernatant was aliquoted and stored at -70°C before being used as source of the antigen.

Human sera

The NCC sera were obtained from 14 patients (1 woman and 13 men) with an age range of 4 to 59 years (median age of 36) who were admitted at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. Their serum samples were obtained immediately after admission. These 14 definitive diagnosed NCC patients were categorized as previously described[17] into 3 groups as follows. Group 1 (n = 1)- presence of one absolute criterion, *i.e.*, pathologic demonstration of living cysticerci from muscle nodules and presence of cystic lesions showing scolex on neuroimaging (Fig. 1). Group 2 (n = 1)- presence of two major criteria, *i.e.*, lesion highly suggestive of NCC on neuroimaging

(Fig. 2) and the presence of plain X-ray films showing multiple cigar shaped calcifications in the muscles. Group 3 (n = 12)- presence of one major criterion, *i.e.* evidence of neuroimaging lesions suggestive of NCC, plus two minor criteria, *i.e.* clinical manifestations suggestive of NCC and the disappearance of intracranial lesions after treatment with albendazole, plus the epidemiologic criterion of living in an endemic area of NCC. The medical records were examined for clinical manifestations of the minor criteria suggestive for NCC, *i.e.* seizures, focal neurological deficit, intracranial hypertension (headache, vomiting, drowsiness, etc.) and sometimes syncopes, accompanied by a disappearance of the intracranial lesions after anticysticercus therapy. Major criteria were met by the evidence of lesions suggestive of NCC in neuroimaging studies such as a small thin-walled cystic ring lesion without scolex (Fig. 2) and/or abnormally enhanced lesions or calcifications or a hydrocephalus referring to inactive lesions. Twenty serum samples were obtained from apparently healthy adults whose stool examination at the time of the blood collection gave no evidence of any intestinal parasitic infection and whose history was without evidence of convulsions or seizures, or eating uninspected meat. These sera were used as controls and provided the baseline cut-off for antibody levels of infected subjects. Positive and negative reference sera were prepared from NCC or healthy sera, respectively, and were used for observation of day-to-day variations in the antibody detection. Nine taeniasis saginata sera were obtained from subjects whose stools contained *T. saginata* gravid proglottids. Other parasitosis sera (angiostrongyliasis, intestinal capillariasis, strongyloidiasis, fascioliasis, trichinellosis opisthorchiasis, and gnathostomiasis) were obtained from parasitologically confirmed cases.

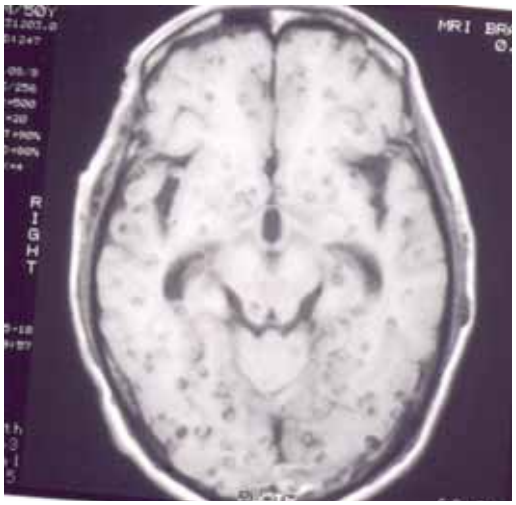

Fig. 1 Magnetic resonance image showing multiple small active cystic lesions with scolex (MRI axial T1W image).

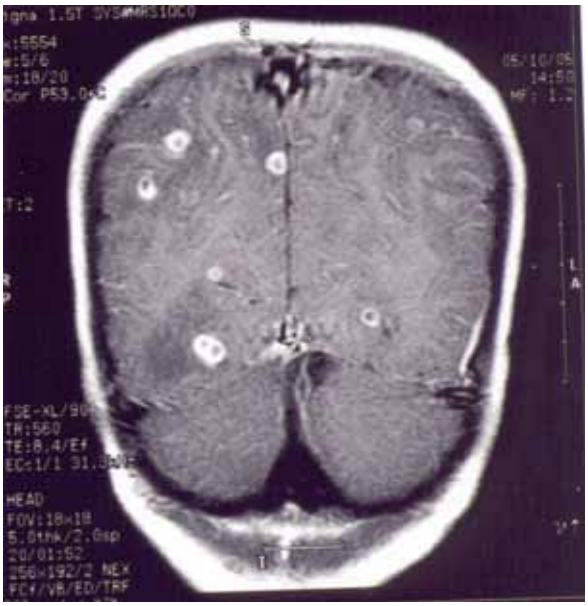

Fig. 2 Magnetic resonance image showing multiple small thin-walled cystic rings enhanced lesions without scolex (MRI coronal T1W post contrast image).

This study was approved by the Human Ethics Committee of Khon Kaen University (No: HE500214).

Enzyme-linked immunosorbent assay (ELISA)

The method was performed as previously described[18] with some modifications. The optimum assay conditions were established by titration. For the determination of specific IgG4 antibody, each well of the ELISA plate was sensitized with 0.5 µg of each peptide in 0.1 ml of 10 mM phosphate buffered saline (PBS, pH 7.5) or with 0.5 µg of SE in a carbonate buffer, pH 9.6 at 4°C overnight. The wells were washed three times with 10 mM PBS (pH 7.4) containing 0.05% Tween-20 (PBS/T) and blocked with 3% bovine serum albumin (BSA) in PBS/T for

1 hour at 26°C. After washing with PBS/T, the wells were incubated at 37°C for 1 hour with 0.1 ml of human sera diluted 1:50 with 1% BSA in PBS/T. After another washing step with PBS/T, a peroxidase-conjugated anti-human IgG4 subclass (Zymed Laboratories, Inc., South San Francisco, CA) diluted 1:500 with 1% BSA in PBS/T was used as secondary antibody. The wells were then washed with PBS/T and incubated with 0.1 ml orthophenylene diamine as substrate for 1 hour. The reaction was stopped with 0.05 ml of 8 N H_2SO_4 and the optical density (OD) was measured at 492 nm using a microplate ELISA reader (Tecan, Salzburg, Austria).

For the determination of specific total IgG, the ELISA was performed with the following modifications: after incubation of the serum samples (diluted 1:400 for the peptide-based ELISA and 1:50 for the SE-based ELISA), peroxidase conjugated monoclonal anti-human total IgG antibody (Zymed Laboratories, Inc.), diluted 1:80,000 in PBS/T containing 1% BSA was added. After 1 hour incubation at 37°C, the assay was continued as described above.

Data analysis

The OD differences between the cases and controls were analyzed by Student's *t* test using the statistical software of Sigma Stat (San Rafael, CA). The cut-off value for positivity was defined as the mean OD plus 2 SD of the healthy sera group. The diagnostic sensitivity, specificity, accuracy and predictive values were calculated and expressed using the method of Galen (1980).[19] The coefficients of variations showed that all antigen-based ELISAs had statistically acceptable day to day variations.

RESULTS

The abnormal brain lesions of the 14 NCC patients were classified into parenchymal, ventricular, and subarachnoid types and the number of cysts was determined. Multiple abnormal lesions were demonstrated in eight patients whereas the remaining six patients showed a single cyst each. In one patient, living cysticerci could be demonstrated from a muscle biopsy as well as multiple active cystic lesions with scolex in the MRI (Fig. 1). This patient's serum was used as positive control throughout the study. The other 13 patients demonstrated either degenerative cysts in the brain parenchyma (8 cases), or the parenchyma and ventricles (2 cases), or racemose forms in the parenchyma and subarachnoid space (2 cases) or only the subarachnoid space with meningitis (1 case).

The absorbance values of the ELISAs for specific IgG4 and total IgG against the two synthetic peptides (HP6-3 and Ts45W-1) and the SE of *T. solium* cysticerci for all serum groups are shown in Table 1. It was found that the percent positivity in the neurocysticercosis group ranged from 21.4% for the total IgG ELISA with HP6-3 antigen to 100% for the IgG4 ELISA with SE. Cross reactivity among helminthiasis sera was 0% to 100% depending upon the type of ELISA and antigen used. The highest cross reaction rate was found for fascioliasis (75-100%), opisthorchiasis (0-100%) and taeniasis saginata (11.1-100%). The comparative ELISA values for the sera of NCC, healthy control and taeniasis saginata cases are shown in Fig. 3. The mean OD values against each antigenic type were significantly different between the NCC group and the healthy controls ($p < 0.001$) except for the total IgG against HP6-3 antigen where no significant difference was detected ($p > 0.05$). To demonstrate the usefulness of this ELISA for the diagnosis of neurocysticercosis, the predictive values of the ELISA using different antigens were determined and are summarized in Table 2.

The highest positive predictive values were achieved by the IgG4-ELISA using either SE or HP6-3 antigens and the total IgG-ELISA with HP6-3 or Ts45W-1 antigens. The positive predictive values were found to be reduced considerably to 12.5-52.8% when other parasitosis sera were included in the calculation (Table 2). These tests had negative predictive values ranging from 79.2 to 100%.

DISCUSSION

The sensitivity and specificity of diagnostic tests for various parasitic infections can be improved by the detection of specific IgG subclass antibodies such as those for hookworm infection,[20] fascioliasis,[21,22] gnathostomiasis,[23,24] angiostrongyliasis[25] and paragonimiasis.[26] Similarly, the detection of specific IgG4 subclass antibody increases the diagnostic values for the diagnosis of neurocysticercosis

Table 1 ELISA absorbance values against three antigenic types in various serum samples

Type of sera	Mean ± SD (no. positive/% positivity)					
	SE		HP6-3		Ts45W-1	
	TIgG[a]	IgG4	TIgG	IgG4	TIgG	IgG4
Neurocysticercosis	1.07 ± 0.91	1.91 ± 1.26	0.10 ± 0.08	0.15 ± 0.11	0.18 ± 0.08	0.20 ± 0.18
n =14	(9/64.3%)	(14/100%)	(3/21.4%)	(11/78.6%)	(8/57.1%)	(11/78.6%)
Healthy controls	0.23 ± 0.13	0.03 ± 0.03	0.07 ± 0.02	0.04 ± 0.02	0.09 ± 0.03	0.04 ± 0.02
n = 20	(1/5%)	(0/0%)	(0/0%)	(0/0%)	(0/0%)	(2/10%)
Taeniasis saginata	1.04 ± 0.25	1.04 ± 0.89	0.10 ± 0.07	0.06 ± 0.03	0.10 ± 0.05	0.05 ± 0.02
n = 9	(9/100%)	(8/88.9%)	(1/11.1%)	(2/22.2%)	(1/11.1%)	(2/22.2%)
Angiostrongyliasis	0.383 ± 0.125	0.007 ± 0.018	0.092 ±0.028	0.053 ± 0.028	0.112 ± 0.033	0.024 ± 0.007
n = 5	(1/20%)	(0/0%)	(4/80%)	(1/20%)	(0/0%)	(0/0%)
Fascioliasis	0.592 ± 0.090	0.221 ± 0.105	0.158 ± 0.026	0.271 ± 0.049	0.171 ± 0.059	0.276 ± 0.062
n = 5	(4/100%)	(3/75%)	(4/100%)	(4/100%)	(3/75%)	(4/100%)
Trichinosis	0.370 ± 0.090	0.021 ± 0.009	0.108 ± 0.016	0.082 ± 0.018	0.131 ± 0.024	0.064 ± 0.023
n = 5	(1/20%)	(0/0%)	(3/60%)	(2/40%)	(1/20%)	(1/20%)
Capillariasis	0.326 ± 0.078	0.028 ± 0.019	0.094 ± 0.002	0.154 ± 0.094	0.061 ± 0.025	0.125 ± 0.120
n = 5	(0/0%)	(0/0%)	(1/20%)	(4/80%)	(0/0%)	(4/80%)
Strongyloidiasis	0.475 ± 0.120	0.007 ± 0.018	0.103 ± 0.018	0.061 ± 0.046	0.107 ± 0.025	0.039 ± 0.041
n = 5	(2/40%)	(0/0%)	(1/20%)	(2/40%)	(1/20%)	(0/0%)
Opisthorchiasis	0.428 ± 0.148	0.017 ± 0.027	0.156 ± 0.107	0.125 ± 0.024	0.232 ± 0.201	0.120 ± 0.042
n = 5	(2/40%)	(0/0%)	(3/60%)	(3/60%)	(5/100%)	(3/60%)
Gnathostomiasis	0.468 ± 0.218	0.049 ± 0.053	0.131 ± 0.018	0.181 ± 0.021	0.167 ± 0.016	0.160 ± 0.018
n = 5	(1/20%)	(1/20%)	(4/80%)	(3/60%)	(4/80%)	(3/60%)

[a]Total IgG

Table 2 Diagnostic ELISA values (%) against three antigenic types

Antigen	Antibody types	Sensitivity	Specificity	Accuracy	PPV1[a]	PPV2[b]	NPV[c]
SE	TIgG[d]	64.3	66.7	66.2	90	30	89.4
	IgG4	100	80.9	84.4	100	53.8	100
HP6-3	TIgG	21.4	66.7	58.4	100	12.5	79.2
	IgG4	78.6	66.7	68.8	100	34.4	93.3
Ts45W-1	TIgG	57	76.2	72.7	100	34.8	88.9
	IgG4	78.6	69.8	71.4	84.6	36.7	93.6

[a]Positive predictive value 1 was calculated based on samples consisting of NCC and healthy controls.
[b]Positive predictive value 2 was calculated based on samples consisting of NCC, healthy controls and other parasitosis cases.
[c]Negative predictive value.
[d]Total IgG.

using crude soluble extract and/or antigen B of *T. solium* cysticerci.[13,14] The disadvantage of using these rather complex antigens is the requirement of *T. solium* cysticerci from naturally infected pigs as source for the antigen. Experimental infection of pigs is impractical and antigen preparation is complicated and not cost-effective. Recombinant antigens and synthetic peptides potentially provide a reliable, reproducible and cost effective alternative, as well as independence from live parasite supply. Many such synthetic peptides with high antigenic indices have already been used for the serodiagnosis of NCC.[7,12]

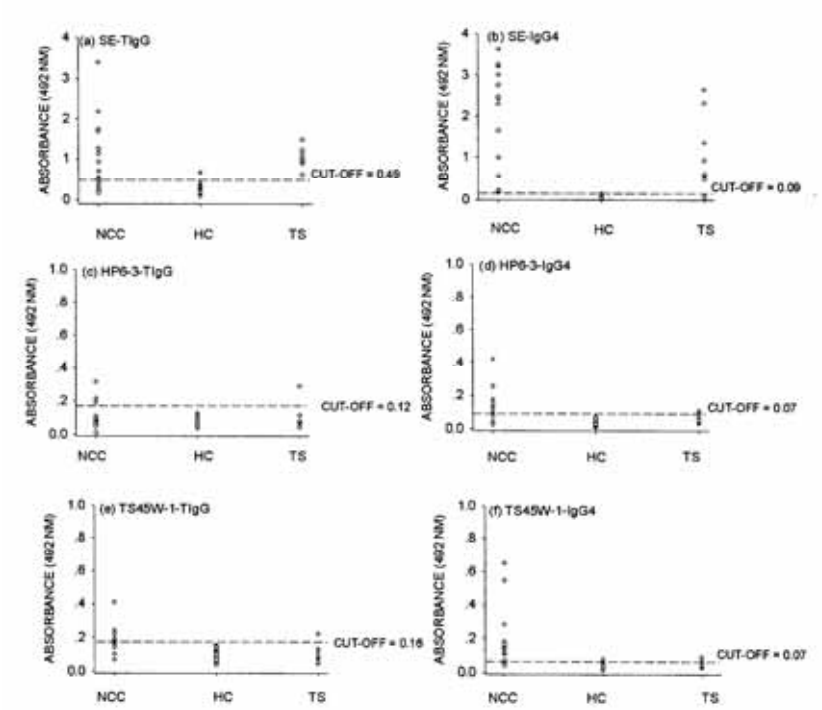

Fig. 3 Absorbance values (at 492 nm) of the ELISA using saline extract of *Taenia solium* cysticerci (SE, a and b) and peptides HP6-3 (c and d) and Ts45W-1 (e and f) as antigens for the detection of specific total IgG (TIgG) (a, c and e) and IgG4 subclass antibody (b, d and f) in sera from cases with neurocysticercosis (NCC), healthy controls (HC) and taeniasis saginata (TS).

Here we evaluated IgG4 and total IgG antibodies to support the diagnosis of NCC by a peptide-based ELISA and the results were compared to an SE-based ELISA. Our findings showed that, in terms of ELISA sensitivity, IgG4/SE, IgG4/HP6-3, and IgG4/Ts45w-1 had acceptable values of 100, 78.6 and 78.6%, respectively. These results, however, did not take into consideration cross-reactivity with *T. saginata*, an infection which is well known to give serological cross reactions with serodiagnostic tests for cysticercosis.

As expected, a high positivity rate was attained with taeniasis sera, notably up to 100% with SE (Table 1). This finding impedes the use of this ELISA in Thailand and other Asian countries where taeniasis is prevalent.[5] However, owing to its 100% sensitivity, the SE-based IgG4-ELISA can be used for supporting the diagnosis of patients with clinical manifestations and neuroimaging lesions suggestive of NCC if their stool examination was negative for proglottids and/or eggs of taeniasis saginata. Additionally the ELISA may be useful for verifying whether antihelminthic treatment has successfully removed the worm by turning negative. Cross reactions of the SE-based IgG4-ELISA with fascioliasis and gnathostomiasis were also documented (Table 1). These cross reactions do not pose a real problem in a clinical setting because infections with these parasites normally present with different neuroimaging features. Furthermore, specific serological tests are available for both diseases.[21-24, 27, 28]

Concerning the peptide-based ELISA, the sensitivity for IgG4 detection was higher than for total IgG antibody. This is probably due to an increase in IgG4 subclass antibody in chronic helminthic infections.[29] On the other hand, the NCC serum samples from group 3 were negative for both IgG4-peptide ELISAs. These negative results may possibly have been caused by either a variation in the antibody response among infected subjects or by low antibody levels because of inactive NCC disease or because antibody responses differ in different stages of the degenerated cysts.

Although *T. solium* has been classified into two genotypes, the Asian type and the American/African type,[30] a variation in their ability to induce antibody responses has not been established. In the present study, the two antigenic peptides (HP6-3 and Ts45W-1) derived from the amino acid sequences of a tapeworm that reacted with the sera from NCC patients from Mexico and South America could also react with Thai NCC patients, demonstrating that the *T. solium* in Thailand share antigenic epitopes with the tapeworms from Mexico and South America.

Although the IgG4-ELISA using peptide antigens reached only 78.6% sensitivity, it can be useful where cysticerci can not be obtained from naturally infected pigs and crude saline extract is not available. The cross reactions with other parasitoses could be differentiated by other available serodiagnostic tests. [21-28,31-36]

In the present study we present a specific and sensitive SE-based immunoassay detecting specific IgG4 antibody for the diagnosis of NCC patients. In patients with inconclusive imaging results and a confirmed absence of taeniasis saginata and other parasitoses, the SE and peptide-based ELISAs can give a positive predictive value of 84.6% or more. This value decreased when the calculation included taeniasis saginata and other parasitoses suggesting that the use in serological surveys still requires careful consideration, especially in areas where several helminthiases are prevalent.[5,7] For laboratory units that lack cysticerci antigen supply, ELISAs using synthetic peptides as antigens can be useful in terms of availability and standardization, resulting in good reproducibility, in contrast to live cysticerci products which tend to vary from batch to batch and highly cross-react with taeniasis saginata sera.

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