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Comparative evaluation of indigenous ELISAs for detection of anti-cysticercus IgG antibodies in serum from clinically and radiologically suspected cases of neurocysticercosis

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Abstract. Neurocysticercosis (NCC) is an important but neglected tropical infectious disease, which is recently recognized as a global problem due to its potentiality for human-to-human transmission beyond tropics. The laboratory diagnosis of NCC is considered useful to confirm clinical and radiological diagnosis. However there is a lack of indigenous diagnostic method particularly in the tropical developing countries. Present study aimed to develop and evaluate indigenously developed anti-cysticercus IgG-ELISAs for possible diagnosis of NCC among patients presenting with seizures. Three indigenous antibody detection assays were developed employing three different antigenic preparations from *T. solium* metacestode larvae (*viz*., TsM-CF, TsM-CW and TsM-PS). The overall test results showed varying levels of IgG titers in response to the three antigenic preparations as compared with the standard commercially procured antibody-ELISA. Total soluble protein extract of protoscoleces or TsM-PS-Ag employed in the indigenously developed IgG ELISA is recommended to be used as a routine screening test for a confirmatory diagnosis of NCC and other forms of cysticercosis in humans.

INTRODUCTION

Neurocysticercosis (NCC) is a disease of the central nervous system (CNS) that is caused by accidental ingestion of egg(s) of *Taenia solium* (Garcia *et al.*, 2003). The clinical manifestations of NCC are varied and non-specific. Seizures, intracranial hypertension, headache and psychiatric disturbances are the most common manifestations of NCC. Signs and symptoms depend on the number of lesions as well as the developmental stage of the metacestode(s) (Medina & De Giorgio, 2002; Flisser, 1994; Del Brutto *et al.*, 1992; Parija, 2008; Prasad *et al.*, 2008a).

A confirmatory diagnosis of NCC is practically impossible on clinical data alone. A definitive diagnosis is made using a combination of methods including clinical characteristics, neuroimaging, serology (detection of specific antibodies or antigens), and exposure history. Neuroimaging methods by computed tomography (CT) or magnetic resonance imaging (MRI) are helpful to detect the presence and characterization of larval cysts in the brain. However the high costs of neuroimaging or complex immunological assays in endemic areas limit the diagnostic capacity because this disease is frequently associated with poverty (Sarti

et al., 1994; Carpio *et al.*, 1998; WHO., 2002; Bobes *et al.*, 2006). Therefore an efficient method for immunodiagnosis of NCC would be the most practical way to aid in a confirmatory diagnosis of this neglected tropical disease in the developing world that would also enable epidemiological studies with a low-cost indicator of prevalence of infection (Esquivel-Velazquez *et al.*, 2011).

Prevalence of NCC is high in India, it is reported from most of the parts including the state of Andhra Pradesh (Parija & Sahu, 2003; Prasad *et al.*, 2008b; Parija & Raman, 2011; Sahu *et al.*, 2014). However, NCC is often underdiagnosed or underreported in the northern coastal districts of Andhra Pradesh (Pappala *et al.*, 2016).

The laboratory diagnosis of NCC is useful as an adjunct to clinical and radiological diagnosis since there are no definite criteria recommended for its confirmation at present (Dorny *et al.*, 2003). Several immunological tests for detecting specific antibodies in the serum and cerebrospinal fluid (CSF) have been evaluated over last many decades. Among different methods, enzyme linked immunosorbent assay (ELISA) is a sensitive method and easier than enzyme linked immunoelectrotransfer blotting (EITB). It has been applied widely for detecting anti-*T. solium* metacestode antibodies in various specimens of NCC patients (Diaz *et al.*, 1992; Bueno *et al.*, 2000; Pal *et al.*, 2000; Gekeler *et al.*, 2002; Kojic and White, 2003; Villota *et al.*, 2003; Hancock *et al.*, 2004; Sahu *et al.*, 2014; Sahu *et al.*, 2015). In NCC, IgG is the predominant antibody detected; though other antibodies (IgA, IgE and IgM) are observed they are of little value in diagnosis, as they do not correlate with the patient's clinical condition (Carpio *et al.*, 1998; Bueno *et al.*, 2000).

There is a lack of indigenous diagnostics for a laboratory confirmation of cysticercosis in many countries including India where the disease is endemic. Hence the present study aimed to develop and evaluate indigenously developed anti-cysticercus IgG-ELISAs for possible diagnosis of NCC among patients presenting with seizures. Thereby the extent of the problem of NCC among patients presenting with recent onset seizures could be estimated by employing indigenously developed serodiagnostics.

In the present study three *in-house* antibody detection ELISAs are developed employing three different antigenic preparations from *T. solium* metacestode larvae – *viz.*, cyst fluid (CF), cyst wall (CW) and protoscolex (PS). The overall test results of *in-house* tests are compared with the standard commercially procured antibody-ELISA.

MATERIALS AND METHODS

The present diagnostic evaluation study was conducted in a tertiary care teaching hospital. All the patients included in the study were referred from three north coastal districts of Andhra Pradesh (India).

Study Group

This is a prospective study in which 160 consecutive serum samples were collected from patients with recent onset seizures who were clinically and radiologically (CT and MRI scan) suspected to be NCC. A total of 200 serum samples were also collected from apparently healthy individuals, who did not have any past history of seizures or any known infectious disorders as healthy control group. These samples were subjected to Ab-ELISA using a commercial kit as standard and in-house ELISAs using three different antigenic preparations of the parasite larvae for evaluation.

Each adult study subject as well as legal guardians of pediatric subjects were explained in vernacular and provided with informed consent form to be filled prior to sample collection. A questionnaire regarding epidemiologic, demographic, clinical information and detailed history was collected from each subject. It included name, gender, age, education level, eating habits, consumption of pork, household income, history of seizures, headache, other neurological problems, history of head injury, history of tuberculosis, association with pig rearing and/or slaughtering, proglottid expulsion during defecation and feces disposal.

Inclusion criteria– Seizure patients clinically and/or radiologically suspected to be NCC, patients living in the study area for at least a year and patients above three years of age were included.

Exclusion criteria– Patients not living in the study area at least for an year or short term tourists/visitors, of age range either < 2 years or > 65 years, female patients with pregnancy. Patients having past history of head injury, symptoms of febrile seizures in children, recent history of any other chronic disease were excluded from the present study. Cases with epidemiologically suspected or previously detected cases of hydatid disease were also excluded.

Serum

Three ml of venous blood was collected from each patient and apparently healthy subjects under aseptic precautions, and the whole blood samples were allowed to clot. The serum was then separated, and stored in aliquots at –80°C till further use. For standardization of indigenous diagnostics, the standard positive and negative control sera were obtained from Kalinga Institute of Medical Sciences, Bhubaneswar, India which were confirmed previously by CDC immunoblot testing.

Parasites

T. solium metacestode antigens were prepared from cysticerci are obtained from naturally infected pig muscle, from slaughter local houses located in Madhurawada, Kancharapalem, Narava villages of Visakhapatnam district (Andhra Pradesh, India). *T. solium* metacestode larvae were dissected out free from the surrounding tissue, and collected in sterile phosphate buffer saline (PBS), pH 7.2, containing antibiotics and protease inhibitor phenylmethylsulphonyl fluoride (PMSF) (0.006%) at room temperature. The cysts were then washed in several changes of the same buffer before to antigen preparation. Two different somatic components were prepared from the same larvae denoted as *T. solium* protoscolex (PS) antigen and cyst wall (CW) antigen. The cyst fluid (CF) component from the same larvae was also collected separately. Protein content of each antigenic preparation was estimated by Bradford (1976) method before storing them for future use as mentioned in the following section.

T. solium metacestode CW antigen (TsM-CW Ag) – Cyst wall materials were dissected out free from the protoscolesces and CW antigen was prepared by homogenization and sonication as per the method described for complete somatic antigen preparation (Sreenivasa murthy *et al.*, 1999). Homogenization and sonication were done under cooling conditions. The dissected cyst wall materials were homogenized separately in tissue lyser with PBS (pH 7.2) containing PMSF (0.1 mM). The homogenized tissue suspension was then sonicated 8 times at 12kH with 30 seconds cooling interval. Each cycle of sonication was for 1 minute. The sonicated material was then centrifuged at 4°C for 30 minutes at 14,000 rpm. The supernatant was stored in aliquots at -80°C till use.

T. solium metacestode PS antigen (TsM-PS Ag) – Protoscolesces were dissected out free from the wall materials. The protoscolex antigen was prepared by homogenization followed by sonication of protoscolesces as per the method described for complete somatic antigen preparation (Sreenivasa murthy *et al.*, 1999). The dissected protoscolesces were homogenized separately in tissue lyser with PBS (pH 7.2) containing PMSF (0.1 mM). The TsM-PS Ag was prepared by homogenization, sonication, and centrifugation as described for TsM-CW Ag preparation. The supernatant was stored in aliquots at -80°C till use.

T. solium metacestode CF antigen (TsM-CF Ag) – Approximately, 200 cysts were washed thrice in PBS, pH 7.2 to remove extraneous matter and then ruptured aseptically to remove the cyst fluid. The cyst fluid was then collected with a sterile syringe followed by filtration through sterile membrane filter of 0.22 µm pore size (*Millipore*, *USA*). The fluid was stored in aliquots at –80°C till use.

***In-house* ELISA**

ELISA was performed as per the standard method of Crowther with few modifications (Crowther, 1995). One µg protein per 100 µL concentration of the antigen was prepared in antigen coating buffer (PBS pH 7.2) used for coating plates (*NUNC*) followed by blocking non-specific binding sites by 2% BSA in PBS (pH 7.2). Optimum dilution (1:50) of test serum sample and known *T. solium* larval stage antibody-positive serum samples were prepared in sample dilution buffer, added in duplicate in the wells, and goat anti-human-IgG-HRP conjugated secondary antibody (*Bangalore Genei*, *India*) was added to each well for detection. Substrate solution prepared freshly by adding one mg of 2, 2'-Azino-di (3-ethylbenzthiazoline-6-sulfonate) in 10 mL of citrate buffer pH 4 and 10 µL of H_2O_2 (30%) was added just before use. The reaction was stopped by adding 100 µL of 1% sodium dodecyl sulfate (SDS) in each well to avoid over reaction and development of optimum color. The absorbance was taken at 405nm in an ELISA reader (BIORAD). Various controls such as antigen blank, antibody blank, negative control serum, and known positive control serum were used in parallel for validity of the assay. Samples with an OD_{405} value more than cut-off (cut-off OD_{405}= mean OD_{405} of 200 negative serum samples + 2 SD) was considered as positive (cut-off values are shown under results section).

All the above three antigenic preparations (TsM-CW Ag, TsM-PS Ag, TsM-CF Ag) were used separately to coat wells and all the wells were blocked with bovine serum albumin, then detection of parasite specific IgG in serum was performed and processed following standard ELISA protocol. Known positive and negative serum samples were assayed for test validity. The ELISA parameters were standardized by Checker Board Titration Curve analysis. Anti-Cysticercus antibodies were detected against the above three antigenic preparations.

Commercial ELISA

A commercially procured ELISA kit (*NovaTec Diagnostics*, *Germany*) was employed for detection of anti-Cysticercus IgG antibodies in sera. All collected serum samples were tested using the same ELISA kit following the manufacturer's instructions as described previously (Sahu *et al.*, 2015). Antigen coated wells incubated with 1:10 diluted patient serum (dilution fluid provided in kit). A negative control serum, low positive control serum, and high positive control serum (all control sera were provided in the kit) for validation of the test. Absorbance was measured at 450 nm. The sensitivity and specificity were previously estimated to be 85% and 94% respectively (Pappala *et al.*, 2016).

RESULTS

In the present study the cut-off value was estimated to be 1.044 for the ELISA detecting IgG antibodies against TsM-CW Ag. The cut-off OD values for the ELISAs using TsM-PS Ag and TsM-CF Ag were estimated to be 1.164 and 0.920 respectively (Table 1).

Sensitivity and specificity of the three different in-house ELISAs were estimated by comparing results based on the test using sera from confirmed positive NCC cases and normal healthy subjects. Table 2 shows the test quality parameters for the three in-house ELISAs for detection of anti-*T. solium* metacestode antibodies in serum for diagnosis of NCC.

The overall results of the three different in-house ELISA assays are compared and presented in Table 3. Highest cases of NCC were diagnosed when TsM-PS Ag(30.6%) based ELISA was used followed by TsM-CF Ag(30%), TsM-CW Ag(28.1%) and the commercial ELISA (27.5%).

The results of the different assays were compared and analyzed considering patient information parameters, clinical profile, and radio-imaging characteristics of the cases presenting with seizures in this study.

Table 4 depicts the gender-wise distribution of cases with a positive detection of *T. solium* metacestode larval antibodies detected in serum where different in-house ELISAs are compared with the commercial ELISA. In the present study there were 60%

Table 1. Estimation of cut-off titers for the in-house ELISAs

Antigen	OD Range		Mean average	Standard deviation	Cut-off
	Maximum	Minimum			
TsM-CW Ag	0.885	0.227	0.496865	0.274026	1.044916
TsM-PS Ag	0.917	0.267	0.56951	0.297261	1.164031
TsM-CF Ag	0.908	0.215	0.508873	0.205606	0.920084

Cut-off was calculated based on the formula written in methodology section. *i.e.*, Cut-off = Mean average + 2SD.

Table 2. Estimation of test quality parameters of the in-house ELISAs

Study groups	No. of subjects	n(%) of cases positive by in-house IgGELISA employed using various antigens of the parasite		
		TsM-CW Ag	TsM-PS Ag	TsM-CF Ag
Conformed positive cases	30	24 (80%)	26 (86.6%)	25 (83.3%)
Conformed negative cases	60	57 (95%)	59 (98.3%)	57 (95%)
Sensitivity	80%	86.6%	83.3%	
Specificity	95%	98.3%	95%	
PPV	88.8%	96.2%	89.2%	
NPV	90.47%	93.6%	91.9%	
Efficiency	90%	94.4%	91.1%	

PPV: positive predictive value, NPV: negative predictive value, %: percentage. (Microsoft Office Excel – windows 10 used for calculate the P value).

Table 3. Overall results of the commercial anti cysticercus IgG ELISA and in-house ELISAs using different antigen preparations of *T. solium* metacestode larvae

Method	Total No. of patients	% (n) of sera Positives	% (n) of sera Negatives
Anti cysticercus IgG ELISA commercial by Kit	160	27.5 (44)	72.5 (116)
In-house ELISA by using TsM-CW Ag		28.1 (45)	71.9 (115)
In-house ELISA by using TsM-PS Ag		30.6 (49)	69.4 (111)
In-house ELISA by using TsM-CF Ag		30 (48)	70 (112)

Statistical analysis of the results showing P value=0.916, that there was no significant difference (P>0.05) between different methods of ELISAs for detection of anti-Cysticrccus IgG antibodies. (Microsoft Office Excel – windows 10 used for calculate the P value).

Table 4. Gender wise distribution of clinically and radiological suspected cases of NCC and the ELISA results for anti-Cysticercus IgG antibodies in serum

Method	Total No. of patients	% (n) subjects tested either positive or negative for anti-Cysticercus IgG antibodies in serum			
		Males (n=96)		Females (n=64)	
		Positive	Negative	Positive	Negative
Anti-Cysticercus IgG ELISA by Kit	160	22.9 (22)	77. 1 (74)	34.3 (22)	65.6 (42)
ELISA using TsM-CW Ag		31.2 (30)	68.8 (66)	23.4 (15)	76.6 (49)
ELISA using TsM-PS Ag		31.2 (30)	68.8 (66)	29.6 (19)	70.3 (45)
ELISA using TsM-CF Ag		33.3 (32)	66.7 (64)	25 (16)	75 (48)

Statistical analysis of the results showing P value = 0.4 in males & P value = 0.509 in females, there was no significant difference (P>0.05) between different methods of ELISAs for detection of anti cysticrccus antibodies. (Microsoft Office Excel – windows 10 used for calculate the P value).

males and 40% females. In case of males highest percentage of positives were observed when diagnosed using TsM-CF Ag(33.3%) in the in-house ELISA followed by TsM-PS Ag(31.2%),TsM-CW Ag(31.2%). In females highest percentage of positives observed when diagnosed by TsM-PS Ag(29.6%) based ELISA followed by TsM-CFAg(25%), TsM-CW Ag(23.4%).

Age wise distributions of positively diagnosed cases detected by different in-house methods are presented in the following section. In this study the age range of these cases the predominant age group was between 13 to 50. In case of >12 years aged subjects highest number of positives diagnosed when TsM-PS Ag(30.2%) based ELISA was used followed by TsM-CF Ag(29.4%), TsM-CW Ag(28.6%) and lastly with the commercial ELISA (Table 5). In case of children (3–12 years) highest numbers of positive cases were diagnosed by anti-TsM-PS (32.4%) and anti-TsM-CF (32.4%) IgG ELISAs.

The distribution of percentages of cases diagnosed positive by different in-house ELISAs methods are presented with respect to their different associated symptoms (Table 6). In patients with generalized seizures the highest number of positive cases were diagnosed by anti-TsM-CF IgG ELISA (35.2%) followed by anti-TsM-PS, and anti-TsM-CW IgG ELISAs (31.8% and 30.6% respectively). However, only 26.1% cases with generalized seizures were diagnosed positive by the commercial ELISA.

Table 5. Age wise distribution of clinically and radiological suspected cases of NCC and the ELISA results for anti-Cysticercus IgG antibodies in serum

Method	Total No. of patients	Pediatrics (n=34)		Adults (n=126)	
		% (No) of sera Positive	% (No) of sera Negative	% (No) of sera Positive	% (No) of sera Negative
Anti-Cysticercus IgG ELISA (Commercial)	160	29.4 (10)	70.6 (24)	27 (34)	73 (92)
Anti-TsM-CW IgG ELISA		26.5 (09)	73.5 (25)	28.6 (36)	71.4 (90)
Anti-TsM-PS IgG ELISA		32.4 (11)	67.6 (23)	30.2 (38)	69.8 (88)
Anti-TsM-CF IgG ELISA		32.4 (11)	67.6 (23)	29.4 (37)	70.6 (89)

Statistical analysis of the results showing age wise distribution with P value = 0.944 in pediatrics and P value = 0.953 in adults, there was no significant difference (P>0.05) between different methods of ELISAs for detection of anti cysticrccus antibodies. (Microsoft Office Excel – windows 10 used for calculate the P value).

Table 6. Seizure patterns and associated symptoms of suspected NCC cases, and results of various ELISAs for detection of anti-Cysticercus IgG antibodies in serum

Seizure patterns	No. of patients	% (n) cases tested positive for anti-Cysticercus IgG antibodies in serum by commercial and in-house ELISAs			
		Commercial ELISA	In-house ELISAs detecting		
			Anti-TsM-CW IgG	Anti-TsM-PS IgG	Anti-TsM-CF IgG
Generalized	88	26.1 (23)	30.6 (27)	31.8 (28)	35.2 (31)
Simple Partial	50	32 (16)	28 (14)	34 (17)	28 (14)
Complex Partial	22	22.7 (5)	18.1 (4)	18.1 (4)	13.6 (3)
Other associated symptoms					
Headache	88	30.7 (27)	30.7 (27)	31.8 (28)	30.7 (27)
Vomiting	20	40 (8)	40 (8)	35 (7)	30 (6)
Hemiparesis	11	36.4 (4)	36.4 (4)	45.4 (5)	36.4 (4)
Muscle weakness	17	23.5 (4)	29.4 (5)	35.2 (6)	29.4 (5)
Unconscious ness	9	33.3 (3)	33.3 (3)	33.3 (3)	22.2 (2)
Altered sensorium	7	28.6 (2)	28.6% (2)	28.6 (2)	28.6 (2)
Total	160	27.5 (44)	28.1 (45)	30.6 (49)	30 (48)

In patients with simple partial seizures the highest number of positive cases were diagnosed by anti-TsM-PS IgG ELISA (34%) followed by the commercial ELISA (32%); 14 were diagnosed by anti-TsM-CW and anti-TsM-CF IgG ELISAs (either one 28%). In patients with complex partial seizures the highest number of positive cases were diagnosed by commercial ELISA kit (22.7%) followed by anti-TsM-CW (18.1%), anti-TsM-PS (18.1%) and the least by anti-TsM-CF (13.6%) IgG ELISAs.

In patients with headache (31.8%), hemiparesis (45.4%), muscle weakness (35.2%) the highest number of positive cases were diagnosed by anti-TsM-PS IgG ELISA compared to other in-house ELISAs. In patients with history of vomiting the highest number of positive cases were diagnosed by anti-TsM-CW IgG ELISA (40%) and commercial ELISA (40%) followed by anti-TsM-PS IgG ELISA (35%) and anti-TsM-CF IgG ELISA (30%).

Table 7 depicts the comparison of ELISA results using different antigenic preparations with respect to the imaging features. In patients with single lesion (29.4%) and multiple lesions (32%) more positive results were diagnosed by *in-house* anti-TsM-PS IgG ELISA comparative to other methods.

The comparative results of different diagnostic methods with respect to location of lesions based on imaging are presented in Table 8. In patients with lesions in parietal lobe, more number of positive results were diagnosed by Anti-TsM-CF IgG and commercial ELISA (27.9%) compared to other in-house method but in patients with lesions in other areas of brain, in-house methods gave more positive results.

Table 7. Results of In-house anti-Cysticercus IgG ELISAs in patients with respect to CT and MRI features

Number of lesions in brain	No. of patients	% (n) cases tested positive for anti-Cysticercus IgG antibodies in serum by commercial and in-house ELISAs			
		Commercial ELISA	In-house ELISAs detecting		
			Anti-TsM-CW IgG	Anti-TsM-PS IgG	Anti-TsM-CF IgG
Single lesion	85	24.7 (21)	27 (23)	29.4 (25)	29.4 (25)
Multiple lesions	75	30.6 (23)	29.3 (22)	32 (24)	30.6 (23)
Total	160	27.5 (44)	28.1 (45)	30.6 (49)	30 (48)

Statistical analysis of the results showing P value = 0.993 that there was no significant difference (P>0.05) in relation to the commercial kit and in-house ELISA. (Microsoft Office Excel – windows 10 used for calculate the P value).

Table 8. Results of in-house anti-Cysticercus IgG ELISAs in suspected NCC patients with respect to location of lesions in brain

Location of lesions in brain	No. of patients	% (n) cases tested positive for anti-Cysticercus IgG antibodies in serum by commercial and in-house ELISAs			
		Commercial ELISA	In-house ELISA		
			Anti-TsM-CW IgG	Anti-TsM-PS IgG	Anti-TsM-CF IgG
Parietal lobes	61	27.9 (17)	21.5 (15)	24.5 (15)	27.9 (17)
Frontal and fronto-parietal	32	28.1 (9)	25 (8)	31.2 (10)	25 (8)
Occipital and parieto-occipital	20	15 (3)	30 (6)	35 (7)	40 (8)
Temporal lobes	5	20 (1)	0 (0)	(1)	0 (0)
Parieto-temporal	3	33.3 (1)	0 (0)	0 (0)	0 (0)
Basal ganglia	2	0 (0)	0 (0)	0 (0)	50 (1)
Ventricles	1	0 (0)	100 (1)	0 (0)	0 (0)
Cerebellum	1	100 (1)	100 (1)	100 (1)	100 (1)
All regions	35	34.3 (12)	40 (14)	42.85 (15)	37.1 (13)
Total	160	44	45	49	48

Table 9. Lesion characteristics based on neuroimaging in clinically and radiologically suspected cases of NCC, and the ELISA results for anti-Cysticercus IgG antibodies in serum

Lesion characteristics	% (n) cases tested positive for anti-Cysticercus IgG antibodies in serum by commercial and in-house ELISAs			
	Commercial ELISA	In-house ELISAs		
		Anti-TsM-CW IgG	Anti-TsM-PS IgG	Anti-TsM-CF IgG
Total no of cases with single lesion	50 (22)	51.1(23)	51 (25)	52.1 (25)
Total no of cases with multiple lesions	50 (22)	48.9 (22)	49 (24)	47.9 (23)
Total no of cases with vesicular stage (either one or more)	6.8 (3)	6.6 (3)	6.1 (3)	12.5 (6)
Total no of cases with granular-nodular stage (with or w/o inflammation) (either one or more)	54.5 (24)	51.1 (23)	53.1 (26)	43.75 (21)
Total no of cases with calcified stage (either one or more)	16 (7)	17.8 (8)	16.3 (8)	18.75 (9)
Total no of cases with all stages (multiple cysts)	22.7 (10)	24.5 (11)	24.5 (12)	25 (12)
Total no. of positive cases	44	45	49	48

Comparison of results of different diagnostic methods with respect to the stage of the parasite basing on imaging features, are presented in Table 9. The percentages of diagnosis of NCC with single lesion and with multiple lesions were nearly same when we use both commercial kit ELISA and in house ELISA *i.e*, 22–25%. But when observed individually, in cases of NCC with vesicular stage and calcified stage the percentage of diagnosis was found to be more with usage of TsM-CF-Ag *i.e*, 12.5% and 18.75% respectively, and in cases of NCC with granular-nodular stage, the percentage of diagnosis was found to be more with usage of TsM-PS-Ag(53.1%).

DISCUSSION

NCC has been reported to be an existing underlying problem of seizures in various districts of Andhra Pradesh (Yashodhara & Elizabeth, 2015; Sahu *et al.*, 2014; Murthy *et al.*, 2004; Avvaru *et al.*, 2012). The only hospital based screening study conducted recently revealed that the three northern coastal districts of this state are not free from this zoonotic public health problem (Pappala *et al.*, 2016). At present, no serological confirmation is performed in clinically suspected cases of NCC, in most of the hospitals located in the present area as well as in other parts of India particularly which are previously unexplored for cysticercosis (Sahu *et al.*, 2015).

Laboratory diagnosis is considered to be useful in confirming the presumptive diagnosis of NCC based on clinical presentations and imaging features. Therefore, in NCC where parasitological proof of infection is difficult to obtain, there is a need of a sensitive and specific serological test as recommended elsewhere (Proano-Narvaez *et al.*, 2002). Also it is important to develop cost-effective tools. In the present study total soluble protein preparations from different components of the metacestode are employed for antibody detection specific to these antigenic preparations, and the findings are discussed in the following sections. Besides that a commercially available ELISA method was also employed in parallel and results are compared with that of the indigenously developed ELISAs.

Results from the present study showed that the commercial ELISA to be less sensitive than the *in-house* ELISAs based on the total soluble protein antigens of the locally collected *T. solium* larval cysts. This indicates the usefulness of the in-house ELISAs for detection of specific antibodies in diagnosis of cysticercosis in human subjects. There may be many reasons, the main reason being nondisclosure of the source/origin of the parasite material, by the manufacturer. In reality no information is available for the commercial diagnostic kit particularly about which antigen of the parasite is coated onto the wells in this ELISA. The coated antigen might have been prepared from a different strain of the parasite which is not well recognized by the anti-Cysticercus antibodies in patient serum, these being actually raised against the local strain of the parasite, upon natural infection (Barcelos *et al.*, 2012).

In present study overall percentages of sero-positivity for cysticercosis in females was predominant over males when commercial ELISA was used. Earlier study also reported female predominance over males (Foyaca-Sibat *et al.*, 2009). Some studies reported predominance of males over females (Sahu *et al.*, 2015; Blocher *et al.*, 2011). A pattern of male predominance was observed when in-house ELISAs were used in the present study. Among the in-house ELISAs this study, detected more percentage of sera positive for anti-*T. solium* metacestode antibodies among males, and the highest percentage was obtained when diagnosed by anti-TsM-CF IgG ELISA. Whereas among the females, majority detected to be positive by the anti-TsM-PS IgG ELISA even though it was lesser, compared to the percentage of positive results out of the commercial ELISA.

In present study the >12 years aged subjects the highest number of positive results was obtained when TsM-PS Ag based ELISA was used; whereas among the children of 3–12 years age, the highest positive results was obtained when anti-TsM-PS and/or anti-TsM-CF IgG ELISAs were done. The age wise distribution with respect to serologically positive diagnosis of cysticercosis in man, varies among studies. Previous studies observed that the predominant age group for infection varies in between 8-65 years (Parija & Raman, 2011; Sutisna *et al.*, 1999; Kotokey *et al.*, 2006; Rottbeck *et al.*, 2013; Ellsiott *et al.*, 2013).

In present study the percentage of serologically positive subjects among the cases with generalised seizures were 35.2%, cases with simple partial seizures were 34%, cases with complex partial seizures were 22.7%, cases with headache were 31.8%, and cases with muscle weakness were 35.2%. In another Indian study, diagnosis of NCC among cases with generalised seizures, simple partial seizures, and complex partial seizures were 22.6%, 32.3%, and 45.2% respectively (Yashodhara & Elizabeth, 2015). In a previous study by this author in Andhra Pradesh it was found 52.17% cases with generalised seizures, 26.08% with simple partial seizures, and 21.73% with complex partial seizures; cases with headache were 73.91% and cases with muscle weakness were 13.04% (Sahu *et al.*, 2014). In another study by some authors of present group done in the neighboring state Odisha, the percentage of subjects diagnosed to be positive for NCC among cases with generalised seizures were 35.17%, whereas cases with simple partial seizures were 25%, and those with complex partial seizures were 18.75% (Sahu *et al.*, 2015).

The in-house ELISAs were found to be more sensitive in confirming the diagnosis of NCC with generalized seizures and simple partial seizures though their sensitivities were less in confirming NCC with complex partial seizures compared to the commercial ELISA. Among the patients with generalised seizures the highest number of positive test was detected when diagnosed by anti-TsM-CF IgG ELISA. Similarly among the patients with simple partial seizures the highest number of positive test was detected when diagnosed by anti-TsM-PS IgG ELISA. Among patients presenting with headache, hemiparesis and muscle weakness the highest number of positive cases were diagnosed by anti-TsM-PS IgG ELISA. Whereas, among patients with vomiting the highest number of positives cases

were diagnosed by anti-TsM-CW IgG ELISA as well as the commercial ELISA. However no statistical significance in these differences could be established. Among patients with lesions in parietal lobe more number of positive cases were diagnosed by commercial ELISA in comparison to the in-house methods but among patients with lesions in other areas of brain, in-house ELISAs detected more number of positives.

The results were nearly same with commercial kit ELISA and in-house ELISAs for diagnosis of NCC with single or multiple cysts CNS. But when we observed individually the percentage of diagnosis of NCC is more when we used anti TsM-PS IgG and TsM-CF IgG ELISAs. The seropositivity was more in multiple cysts when compared to single lesion.

Many studies shown that TsM-PS Ag and TsM-CF Ag aremost specific for differential serodiagnosis of NCC. The TsM-PS Ag and TsM-CF Ag used in present study are similar to them. The sensitivity of the in-house ELISAs using different antigens *i.e* TsM-CW, TsM-PS, TsM-CF were similar to the studies of Diwan and Sloan (Diwan *et al.*, 1982, Sloan *et al.*, 1995) but appear to be much greater than that of Mittal & Samiakirmani (Mittal *et al.*, 2001; SamiaKirmani *et al.*, 2014). The specificity values of present study tests were comparable to that of Sloan, SamiaKirmani, Proano-Narvaez (Proano-Narvaez *et al.*, 2002; Sloan *et al.*, 1995; SamiaKirmani *et al.*, 2014). The percentage of sero-positivity by ELISA of present study 30.6% was nearly same as that of Sahu (Sahu *et al.*, 2014) study which was 37.7%. Although the results of ELISA showed that both TsM-PS Ag and TsM-CF Ag can be used to diagnosis the NCC but the use of TsM-PS Ag has more advantages in terms of interpretation of results due to its constitution.

Nevertheless there were few limitations in the present study which are stated in the following section. Present sample size was 160 which were mainly based on the previous study where the major objective was to estimate the prevalence of NCC among the cases presenting with recent onset seizures originated from the northern coastal districts of Andhra Pradesh in India (Pappala *et al.*, 2016). Present study aimed at evaluating the in-house developed ELISAs as discussed before. However the relative frequencies of detection of IgG antibodies specific to different antigen preparations of the parasite might be altered if extrapolation of the sample size is done for testing a larger patient population. Moreover the evaluations of the in-house ELISAs are done exclusively on symptomatic patients those who attended hospital. There is probably a need to evaluate the diagnostic efficacy of asymptomatic cases as required to screen high-risk populations in epidemiological studies.

T. solium is now divided into two genotypes: the Asian type, and the Africa-American type (Ito *et al.*, 2002). Hence, genetic polymorphisms among *T. solium* from different areas across an endemic country might be influencing antibody detection results in patients with NCC as suggested elsewhere (Barcelos *et al.*, 2012). While comparing the results of the present in-house ELISAs with the commercial ELISA, the manufacturing location of the diagnostic kit and also the source of the parasite material coated onto the wells in the commercial kit should be considered as there may be minor difference in antigenic epitopes in the parasites isolated from different geographic regions (Barcelos *et al.*, 2012). So the relative higher or lower efficiency of the tests might be analyzed in this backdrop. Scope of the present study, which limits a follow up, is also a matter of concern. Probably a follow up study is required to validate the utility of our in-house tests in post treatment monitoring.

CONCLUSION

The present study indicates that NCC is an underlying cause of recent onset of seizures in north coastal districts of Andhra Pradesh. Since there is a continuous demand for simple and cost effective in-house diagnostics in endemic countries including India, the presently evaluated tests employing three antigenic preparations for *T. solium*

metacestode larva (TsM- CW, TsM-PS, TsM-CF) where found to be diagnostic with varying degrees of sensitivity and specificity. Moreover the test qualities of these in-house ELISAs were found to be comparable or even better than the commercial ELISA used in present study. The antibody detection ELISA using TsM-PS Ag showed the highest specificity and sensitivity. Therefore TsM-PS Ag based IgG detection ELISA is highly recommended due to the ease in preparing the antigen, and availability of large quantity of the antigenic material, and its high sensitivity as well as specificity. These tests can be used as a substitute for traditionally used techniques for antibody detection in sera for the diagnosis of NCC.

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Conflict of Interest
None.

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