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Evaluation of metacestode of *Taenia solium* antigens for detection of anti-cysticercal IgG among patients with epilepsy in three districts of Northern Uganda

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Neurocysticercosis causes serious public health and socioeconomic problems in developing countries including Uganda. The aim of the study was to evaluate the metacestodes of *Taenia solium (T. solium)* antigens for detection of anticysticercal Immunoglobulin G (IgG) antibodies among patients with epilepsy in three districts of Northern Uganda. Three hundred (300) random samples were screened for anticysticercal IgG using indirect antibody Enzyme Linked Immunosorbent assay (ELISA). Samples positive for anticysticercal IgG were confirmed using western blot. The sensitivity and specificity of the ELISA method was 90 and 95.4% with positive predictive value and negative predictive value of 77.8 and 98.4% respectively.. The kappa value at 95% CI was 0.668 (0.545-0.791). The strength of agreement between ELISA assays and immunoblot was good. The finding indicates the ELISA based method using locally derived antigens can be used to screen most patients with epilepsy for exposure to T. *solium* antigens and the method can be employed in resource limited settings in most developing countries.

Key words: Evaluation, metacestode, Taenia solium antigen, ELISA, Neurocysticercosis, Northern Uganda

INTRODUCTION

Cysticercosis is a human parasitic disease caused by eating pork infected with *Taenia (T) solium* cysticerci that infect humans and free roaming pigs in developing countries. (Carabin et al., 2009; Abdo et al., 2010). Human cysticercosis can also be acquired by ingesting embryonated *T. solium* eggs in food or water contaminated with fecal matter of persons harbouring the adult tapeworm Zoli et al., 2003, Waiswa et al., 2009).

The eggs of the adult tapeworm hatch and release the first larvae onchospheres in the duodenum. These onchospheres penetrate the intestinal mucosal and enter into the blood circulation. Subsequently, the onchospheres invade striated muscles, brain, liver, and other organs where they form cysticerci (Del Brutto and

García, 2013). The most severe manifestation of the disease occurs when the metacestode (larval stage) of the parasite infect the human central nervous system (CNS), causing neurocysticercosis (NCC) (Morales et al., 2008; Winkler et al., 2009). This disease may be asymptomatic or may present with a number of nonspecific indications, such as seizures, headaches, focal neurological deficits, increased intracranial pressure, or cognitive decline (Del Brutto and García, 2014; Suzuki et al., 2007; Alarakol et al., 2018). Globally, approximately 50 million are infected with *T. solium* parasites and 50,000 people die of cysticercosis related diseases annually (Fleury et al 2006; Lescano et al., 2007). In developed countries particularly United States of America, cases of human cysticercosis have been reported among immigrants (Lescano et al., 2007). In Uganda, information about NCC is limited (Alarakol et al., 2017). Neuroimaging techniques such as computer tomography (CT) scans and magnetic resonance imaging (MRI) have been recognized as the gold standard for diagnosing NCC (Suzuki et al., 2007; Winkler et al., 2012). However, these techniques do not provide a definitive diagnosis of NCC due to the complex pathological processes of this disease in the CNS, which often mimic other infectious or non-infectious diseases (Mayta et al., 2008; Del Brutto and García, 2014). While these techniques are essential in the diagnoses of NCC, the costs of the equipment are immense and most health facilities in rural setting in developing countries are unable to bear. More so, serologic findings particularly enzyme linked immuno-sorbent assay (ELISA) based techniques are essential in the screening for NCC, however, their setbacks associated with false negatives and false positives make them unreliable (Marcello et al., 2006.) The Electroimmunotransfer blot (EITB) technique developed by Tsang et al. (1989) which had the sensitivity and specificity of 98 and 100% respectively provides an alternative to this limitation. Despite these efforts, many rural settings are unable to use this technique due to long procedures, sophisticated equipment and cost of reagents required to carry out these procedures. (Alarakol et al., 2017). The aim of this study was to evaluate *T. solium* antigens for serodiagnosis of human cysticercosis using antibody ELISA based model suitable for use in resource limited settings of Northern Uganda.

MATERIALS AND METHODS

Study area

The study was conducted in three rural districts of Northern Uganda between June 2014 to April 2015. This includes; the districts of Gulu (Latitude, 2.77° North, Longitude 32.31° East), Moyo (Latitude 3.37° North, Longitude, 31° 46 East) and Adjumani (latitude, 3.3833°N, longitude, 31.7667° E and 32.7833°N) covering an estimated 6,500 km^2.with an estimated population of 916,000 inhabitants(https://en.wikipedia.org/wiki/Adjumani_DistrictWikipedia,

(http://www.ugandatravelguide.com/guludistrict.html),(https://en.wiki pedia.org/w/index.php?title=Moyo_District&action=info).

Study design

A cross sectional study was conducted on sera samples of patients with epilepsy recruited and referred for further management and care at epilepsy Treatment Centers in Gulu Regional Referral Hospital (GRRH), Moyo Hospital (MH) and Adjumani Hospital (AH), Northern Uganda. Detection of anticysticercal Immunoglobulin G (IgG) was done using indirect antibody ELISA (Ab-ELISA). Positive Sera samples for anticysticercal IgG was confirmed using western blot analysis. The research protocol were approved by Gulu University Research Ethic Committee and Uganda National Council of Science and Technology (Ref: HS987).

Sampling and sample collection

One thousand three hundred eighty three (1383) people suspected of epileptic seizures at households' level were referred for further management and care at epilepsy Treatment Centres in the three hospitals; MH, AH and GRRH. These patients were initially identified from a large community based study conducted in the area as described by Alarakol et al. (2017). Patients presented to the hospitals or health facilities were recorded in the register using the patients` identification number. Simple random sampling was then conducted on the six hundred patients confirmed for epileptic seizures by neurologists. This was done to ensure each patient selected had an equal chance of being included in the study. A total of 300 patients confirmed for epileptic seizures were selected for the study. Blood specimens were collected from selected patients into plain vacuotainer tubes. These were allowed to clot at ambient temperature, later centrifuged and the sera were extracted and stored at -20°C for subsequent use (Alarakol et al., 2017). The sera samples were used in antibody ELISA assays done at Faculty of Medicine, Gulu University Uganda.

Preparation metacestode of *Taenia solium* antigens

Crude antigens for detecting anti-cysticercal IgG in patients with epilepsy was prepared from cysts collected from heavily infected pigs. The antigen was prepared as described by Gottstein et al. (1986). Briefly, 3.0 g of frozen whole cysts thawed in 50 ml falcon tubes on dry ice were added to 7 ml Phosphate Buffered Saline (PBS) containing 0.01% of NaN$_3$ (Invitrogen, Germany). This was homogenized on ice for fifteen minutes. The samples were freeze-thawed twice in liquid nitrogen for further denaturation. These were ultrasonicated in ice bath six times, at intervals of 30 seconds for a total of three minutes, and thereafter centrifuged at 4 degrees Celsius in ultracentrifugation at 13,000g for 45 min. The protein concentration of the supernatant was determined using Biuret's method and the characteristic purity evaluated using sera samples obtained from known NCC positive patients and confirmed with western blot. The antigens were stored at -80°C until use.

Detection of anticysticeral IgG using ELISA

The IgG from patient sera was screened using indirect Ab- ELISA as described by Sloan et al. (1995). Each ELISA plate was coated with antigens. The antigens were prepared in the Department of Infectious Diseases and Tropical Medicine (DITM), University of Munich, Germany. Briefly, 1.0 µg/ml of antigens was coated on to the 96 polystyrene microplate (Nunc® Maxisorp) wells and incubated overnight. The unbound antigens were washed four

times with Phosphate buffered Saline (PBS)/TWEEN (PH = 7.2) and this was followed by blocking with PBS/bovine serum albumen (BSA)/TWEEN for 1 h at 37°C. 10 μl of antisera were then added and incubated for 1 h at 37°C. The unbound sera components were washed and rabbit anti human horseradish-peroxidase polyclonal IgG (Invitrogen, Germany) specific for the first antibody in the sera was added to form antigen-antibody complex. The unbound antibody enzyme conjugate was washed and a substrate o-phenlydiamine (OPD) (Invitrogen) was added. This was followed by dark incubation of the samples for 30 min. The reaction was stopped by adding 0.5 M H_2SO_4 (BDH UK). This conjugate catalysed formation of colored substance which was measured photometrically at 492 nm using ELISA reader (Tecan Austria GmbH, Sunrise). Known positive and negative control sera were included for validation of the test results. The cut-off points were calculated as the mean of the optical density (OD) values obtained with 8 negative serum samples plus two standard deviations (SD). A sample was considered positive if the OD value was greater than the estimated cut-off point.

Positive control sera

The control positive sera were obtained from 15 patients with confirmed neucysticercosis using clinical, CT scan and/MRI and immunodiagnosis. The patients were referred to the hospital after presenting with convulsions which had not been previously investigated and none of them were on treatment program.

Negative control sera

The negative control samples were from 15 healthy individuals no history of seizures and were screened for other helminthes namely; *Taenia saginata*, *Echinococcus granulosus*, *T. hydatigena,* askarids, strongyloides. Additionally, they also underwent serodiagnosis with western blot

Polyacrylamide gel electrophoresis

The sera samples which were positive in Ab-ELISA were run on western blot for confirmation. This was preceded by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Preparation of the immunoblot strips for serodiagnosis was done as previously described by Parija and Raman (2011) but with some modifications of the procedures. These were required in order to shorten the time of separation of the glycoprotein in Tris –Trisine buffers using high voltage and current. Briefly, 750 ug/ml of crude antigens were run on a SDS-PAGE in a 16.4% resolving and 4.0% stacking gel (in Tis-Tricine buffers) at 100V 150 mA 10W for 5 h. The antigens were separated under non reducing conditions by SDS-PAGE in a 10 × 8 cm Mini gel (Austria). The gels were stained with bromophenol blue and the molecular weight marker (ladder) (Invitrogen, Germany) placed at both ends.

Electroimmunotransfer blotting (Western blot)

The separated antigenic proteins on the gels were electronically transferred in wet 0.22 um pores size nitrocellulose membranes (USA, Billerica, MA) in a mini Transblot cells (BIORAD) at 150 V 100 mA 30 W for 1 h as described by Parija and Raman (2011). The two small portions on the nitrocellulose membrane branded with the molecular weight markers were cut and air dried. The larger portion of the nitrocellulose membrane containing separated antigenic proteins was placed on 5% BSA for 30 min to block nonspecific binding sites and thereafter air dried. The immunoblot strips of 4 mm were cut from the larger portion of the dried nitrocellulose membrane and stored at 4°C in fulcon tubes for serodiagnosis.

Electroimmunotransfer blot analysis

Western blot analysis was conducted as previously described by Parija and Raman (2011) with some modifications. Briefly, serodiagnosis with the stored immunoblot strips was done on all patients samples which were positive with antibody ELISA. The immunoblot strips were placed in Mini incubator Trays (Hindenerg, Germany) and incubated on 5% BSA for 30 min. This was followed by washing with Phosphate Buffered Saline (PBS)//TWEEN (PH= 7.2) five times with ELISA washer (Tecan Austria GmH, Hydrofolex). All subsequent steps were washed five times. The patient sera were added and incubated on a shaker (Hindenberg, Austria) for 70 min. This was followed by addition of a secondary enzyme alkaline phophatase linked Poly Rabbit anti-human IgG antibody (Invitrogen, Germany) and incubation on a shaker for 75 min. The last incubation and washing was done for 5 and 10 min, thereafter, the substrate diaminobenzidine (DAB) (Invitrogen, Germany) added. The strips developed the bands within 10 min on a shaker (Heinsburg, Germany). Thereafter, the strips were removed from incubator tray and placed in water to stop the reaction. These were then removed and air dried on glass plate. The presence of the antibody in the sera were confirmed when at least two specific bands of 8, 10, kDa were observed on the diagnostic region. The molecular weight of *T. solium* characteristic antigenic peptides were determined by comparing the bands in diagnostic region with the standard molecular weight markers placed alongside the strips. Major antigenic peptides were; 8, 10, 18, 32, 40, 50, 76 and 100 kDa. The bands outside the diagnostic regions were not considered as specific for *T.* solium because these were shared by other helminthes (Alarakol et al., 2017). The proportions of patients positive for *T. solium* antibody in Ab-ELISA and those in immunoblot were determined.

Data analyses

The data are presented as mean, standard deviation, frequency and variance. The data were analysed using Pearson's chi square to test for statistical level of significance between means of proportions (dependent and independent variables). The dependable variables included optical densities of the IgG and study locations while the independent variables included age and sex of the patients. The sensitivity was calculated by dividing the proportion of samples of people with NCC who have a positive antibody ELISA results. While the specificity was calculated by dividing the proportion of samples of people without the NCC who have a negative antibody ELISA results. Student-t test was used to test for differences between the means of proportions (optical density) for levels of significance. MacNemar chi-square ($χ2$) with Yates correction continuity factor was used to compare the differences between the proportions for independence. The probability value of $p<0.05$ were considered to be statistically significant. Data was also analysed to check the levels of agreement between tests using Kappa values.

RESULTS

Demographic characteristics

Out of 300, epileptic patients who presented to the health

Table 1. Demographic characteristics of study population.

Variable	Frequency	Percentage
Sex		
Male	170	56.7
Female	130	43.3
Age group		
10-19***	132	44.2
20-29**	78	26.2
30-39*	40	13.4
40-49	20	6.7
≤50	30	10.0

**; Age group 10-19 were more predominant in the present study, follows by ** ; 20-29 and *; 30-39; Number of males were significantly higher than female counterpart, *P*=0.00.
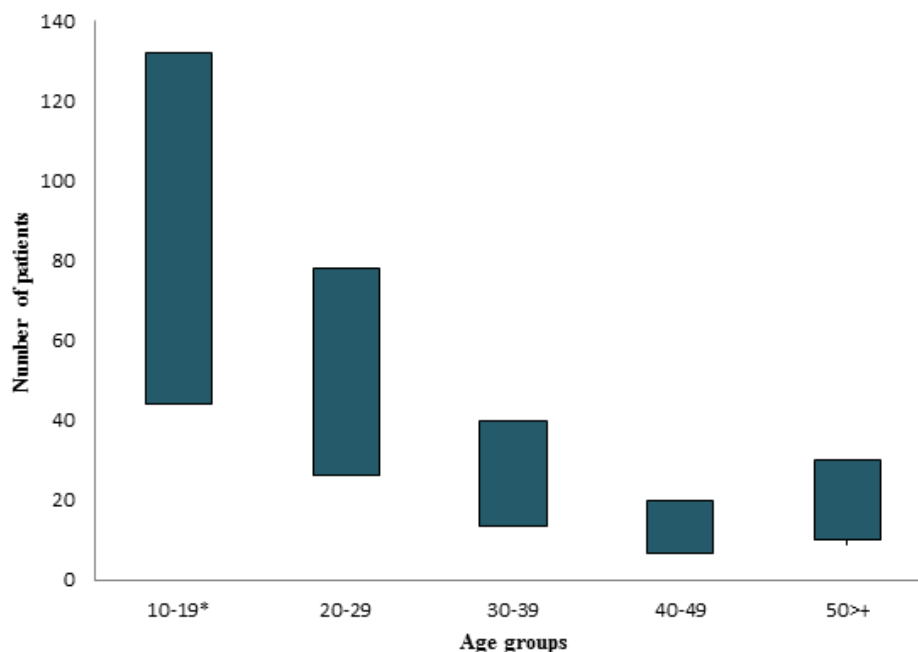

Figure 1. Graph showing variation of number of patients within different age groups. Majority of patients were in the age group 10-19** follows by 20-29 and 30-39. Males were more among age groups 10-19, 75(56.9%), 20-29, 37 (47.4%), 30-39, 24 (60%), 40-49, 13(65%) and 50>, 21(70%). While the females were more among aged groups 20-29, 37(47.4%).

facilities for medical care, 170 (56.7%) were males and 130 (43.3%) were females with the mean age of 25±13. The proportion of patients were more among age groups 10-19, 132(44.2%), 20-29, 78 (26.2%), 30-39, 40 (13.2%), 40-49, 20(6.2%) and 50>,30(10.0%) (Table 1). Males were more among age groups 10-19, 75(56.9%), 20-29, 37 (47.4%), 30-39, 24 (60%), 40-49, 13(65%) and ≤50, 21(70%) (Figure 1). While the females were more among aged groups 20-29, 37(47.4%).

Detection of anticysticercal IgG antibody using antibody ELISA

Out of 300 serum samples Gulu, 17 had representing 15.3% of patient samples positive for anticysticercal IgG. Adjumani and Moyo had 18(19.6%) and 10 (10.3%) of patients samples positive for anticysticercal IgG respectively. There was statistical significance difference observed between the positivity of patients' sera of Gulu

Table 2. Seropositivity of anticysticercal IgG antibody using antibody ELISA (N=300).

District	Number of patients screened	Antibody ELISA positive samples (n)	Percent (%)
Gulu	111	17	15.3
Adjumani	92	18	19.6
Moyo	97	10	10.3
Total	**300**	**45**	**15.0**

n = represents number of samples positive for anticysticercal IgG antibody, N = represents total number of patients screened in the study area. The positivity to anticysticercal IgG differed significantly between Gulu and Adjumani (*P*=0.00), Gulu and Moyo (*P*=0.26) when screened with ELISA.

Table 3. Seropositivity of IgG antibody using immunoblot assays.

District	Antibody ELISA positive samples[a]	8and 10 kDa positive samples[b]	Percent (%)[c]
Gulu	13	12	30.8
Adjumani	18	16	41.0
Moyo	15	11	28.2
Total**	**45**	**39**	**86.7****

[a], samples positive for anticysticercal IgG on Ab-ELISA; [b], total of samples positive for immunoblot 8 and 10 kDa, [c]: percentage of patients samples positive for immunoblot 8 and 10 kDa bands, ** Total number of antibody positive patients samples positive for 8 and 10 kDa.

and Adjumani (*P*=0.00). While no significant difference in the level of positivity was observed between those of Gulu, Adjumani and Moyo (*P*=0.26).Table 2.

Detection of anticysticercal antibody IgG using immunoblot assays

Table 3 shows forty five antibody positive sera analyzed by Immunoblot for specific *T. solium* glycoproteins. Of the 45 samples analyzed, 86.7% were positive on the diagnostic bands 8 and 10 kDa proteins. Adjumani had significantly (*P*=0.00), highest patients tested positive for 8 and 10 kDa proteins than Gulu 12 (30.8%) and Moyo 11(28.2%) respectively (Table 3). Eighteen, 18(40.0%) patients sera strongly reacted with higher molecular weight of 14 kDa, 21 kDa, 24 kDa, 38-42 and 50kDa proteins (Figure 2). Fourteen 14(31.1%) patients sera showed reactions below the diagnostic bands (below 8 - 10 kDa) (Figure 2).

Sensitivity and specificity of Ab-ELISA Vs. immunoblot

Ab ELISA findings were analyzed using immunoblot as the gold standard, the sensitivity (Se) and specificity (Sp) of local Ab- ELISA was 89.7 and 95.4% with PPV and NPV of 77.8 and 98.4% respectively (Table 4). The McNemar χ2 square test with Yates continuity Correction for small samples sizes revealed that the percentage of

samples positive with local antibody ELISA differed significantly from local immunoblot, $\chi2$ (1, n= 300) = 0.02, P < 0.05, the odd ratios with associated 95% confidence limits was 1.7 (1.1-2.5); (P<0.01). When analyzed for kappa values, the numbers of observed and expected agreements were 92.0% (n=300) and 75.1% (n=300) of the observations respectively. The kappa value at 95% CI was 0.668 (0.545-0.791). Therefore, the strength of agreement between local Ab ELISA assays and immunoblot was good (Table 4).

DISCUSSION

Diagnosis of NCC is a challenging construct that requires technical knowledge, skills, and robust equipment in its execution. However, these are mostly possible in fairly developed facilities in urban and peri-urban centres. Solving these inadequacies require development of a robust, simple and cheap technique that can be used in the screening of NCC in rural settings. The present study evaluated the diagnostic potential of the metacestodes of *T. solium* antigens for use in ELISA based format. Our findings indicate that Ab-ELISA format with locally derived antigens had sensitivity and specificity of 90 and 95.4%, respectively. This indicates that the ELISA based method can detect majority (90%) of the patients associated with NCC as positive for anti-cysticeral IgG, and with a specificity at 95.4%. These are important attributes, particularly when the ELISAs are used for screening purposes to identify previous exposures to T. *solium*
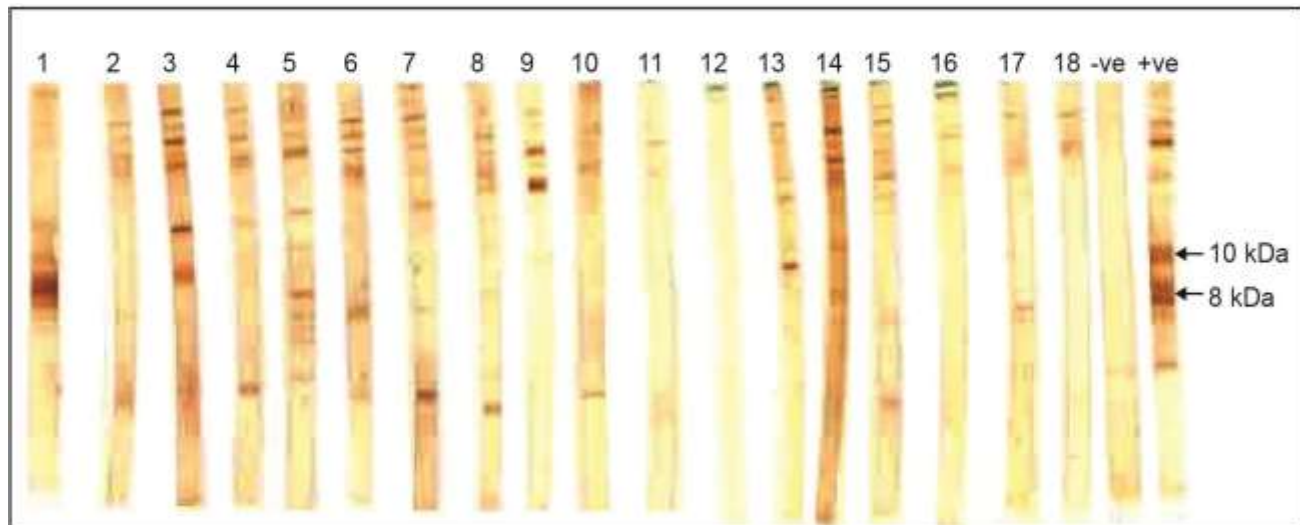
Figure 2. Immunoblot results for patients positive for 8 and 10kDa glycoproteins of *Taenia Solium*. Lane (+ve) (right); immunoblot containing known NCC sera (positive control). Lane (-ve); negative control; Lane 1, 3, 5, 6, 7, 14 and 17 are positive for 8 and 10 kDa glycoproteins

Table 4. Sensitivity and specificity of Ab- ELISA assays Vs. local immunoblot.

		Immunoblot**		Total
		Positive	**Negative**	
Ab-ELISA	Positive	35	10	45
	Negative	4	251	255
Total		39	263	300

** Immunoblot was the gold standard, sensitivity (Sn) and specificity (Sp); sn; 90.%, sp; 95.4%, PPV; 77.6, NPV; 98.4%, χ^2 =0.02, P<0.05.

infections. The ELISA based methods for detecting the presence of IgG and circulating antigens have long been described in previous studies (Decker et al., 2010). These serologic findings, together with neuroimaging techniques have been used in the diagnosis of NCC (Garcia et al., 2005). The latter have been used for confirmation of the NCC among suspected patients. The sensitivity was defined as the proportion of people with NCC who have a positive antibody ELISA results. While the specificity was defined as the proportion of people without the NCC who have a negative antibody ELISA results. Although, the present study used crude antigens of *T. solium* metacestodes, the sensitivity and specificity obtained from this ELISA method is comparable to the previous studies. Previous studies have reported on Ab-ELISAs with varying results. Mittal et al. (2001) reported the sensitivity and specificity of 10.4 and 70% (Sloan et al., 1995), 93 and 95% (Parija and Raman 2011), 91 and 96% for crude *T. solium* metacestodes. Minozzo et al. (2008) reported a sensitivity and specificity of ELISA at 96 and 90% using serum samples. Ito et al. (2006) on the

other hand observed that the sensitivity of ELISA in confirming active NCC case with serology was 94%, of which 93% was from native antigens. Whereas Ito et al. (2006) reported high sensitivity and specificity with native antigens and chimeric recombinant antigens in both ELISA and immunoblot; in this study, the low sensitivity (90%) of locally derived antigens in Ab-ELISA format may be due to cross reactions with other helminthes in the patients' sera which interact with the antigenic epitope, hence blocking the true antibody the accessibility (Alarakol et al., 2017). Fleury et al. (2001) reported that un-purified antigens have moderate sensitivities and relatively poor specificities. While the research on the antigenic properties of cyst fluid and surface associated glycoprotein, and improved protein purification have resulted into much more reliable serological tools (Dorny et al., 2003); none of these have eliminated the issue of cross reactions. Previous studies have reported that most tests that employ crude *T. solium* metacestodes antigens lack sensitivity and specificity (Chung et al., 1999. In the present study, the locally derived antigens used in Ab-

ELISA might have had problems with cross reacting helminthes. This is evident from the results of the Se = 90% and Sp = 95.4%), (P>0.05). Purified antigens from crude metacestodes of *T. solium* have yielded good results in most studies especially the use of chimeric recombinant and the synthetic proteins in Ab- ELISA. It is not clear whether the purity of the antigens used in these Ab-ELISA formats significantly contributed to the low sensitivity and specificity in the ELISA formats. Therefore, more study is necessary to investigate other potential cross reacting parasites in the endemic areas. Further studies need to explore the use of purified antigens in the diagnosis of *T. solium* cysticercosis. This will widen the scope of searching for better antigens for use in the Ab-ELISA formats. The present study had some limitations which include the use of small sample size for evaluation of this ELISA based technique. However, larger sample size is required for better understanding of the performance of this technique for the screening of NCC in a rural setting. More so, there is need to include more healthy control samples before this can be introduce for routine screening of NCC in the rural communities.

In conclusion, This study has evaluated the ELISA based model using locally derived antigens of metacestodes of *T. solium*. The finding indicates that the ELISA based method using locally derived antigens can be used to screen most patients with epilepsy for exposure to *T. solium* antigens and the method can be employed in resource limited setting in most developing countries. Finally, more highly purified antigens need to be used in the Ab-ELISA format for better detection of exposure to *T. solium* antigens among patients with Neurocysticercosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest

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