Urine Antigen Detection for the Diagnosis of Human Neurocysticercosis

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Abstract. Neurocysticercosis (NCC) is a major cause of seizures and epilepsy. Diagnosis is based on brain imaging, supported by immunodiagnosis in serum or cerebrospinal fluid (CSF). Lumbar puncture is invasive and painful. Blood sampling is slightly painful and poorly accepted. Urine antigen detection has been used for other parasites and tried in NCC with suboptimal performance. We used a monoclonal antibody-based ELISA to detect *Taenia solium* antigens in urine from 87 Peruvian neurocysticercosis patients (viable cysts, $N = 34$; subarachnoid cysticercosis, $N = 10$; degenerating parasites, $N = 7$; calcified lesions, $N = 36$) and 32 volunteers from a non-endemic area of Peru. Overall sensitivity of urine antigen detection for viable parasites was 92%, which decreased to 62.5% in patients with a single cyst. Most patients (30/36, 83%) with only calcified cysticercosis were urine antigen negative. Antigen levels in paired serum/urine samples (evaluated in 19 patients) were strongly correlated. Non-invasive urine testing for *T. solium* antigens provides a useful alternative for NCC diagnosis.

INTRODUCTION

Central nervous system infection by the metacestode of the tapeworm *Taenia solium* (neurocysticercosis [NCC]) is a major cause of epilepsy and thus a serious public health problem in many Latin American, Asian, and African countries.[1–3] Diagnosis is supported principally by imaging and serologic methods. Computed tomography (CT) and magnetic resonance imaging (MRI) are the main imaging methods, showing the number, size, and location of NCC-associated lesions, which is crucial for diagnosis and follow-up.[4] Serologic diagnosis has mainly been based on antibody-detecting assays. From these, the enzyme-linked immunoelectrotransfer blot (EITB; Western blot) has 98% sensitivity and 100% specificity for detecting antibodies directed against glycoproteins purified from the parasites.[5]

However, a specific antibody response may result from exposure to the parasite without established infection,[6,7] and antibodies may also persist in circulation for a long time after the parasites have died.[8] It follows that antibody detection provides insufficient information to base decisions on antiparasitic treatment. Antigen detection assays show the presence of viable parasites.[9] Antigen-detection ELISA (Ag-ELISA) using monoclonal antibodies (MoAb) are more specific and reliable than those based on polyclonal antibodies.[7,10–12] These have been used in serum and cerebrospinal fluid (CSF), with sensitivities of ~85% and have also been shown to sharply drop after antiparasitic treatment.[13–16]

For both clinical and epidemiologic purposes, non-invasive urine sampling may be more convenient than blood or CSF sampling. Urine sampling is not invasive, painless, is not culturally inappropriate, and can be obtained immediately in most settings. It would be of great advantage when multiple samples are needed (i.e., clinical follow-up) or for field epidemiology studies. Urine antigen detection has been used for the diagnosis of other parasitic infections,[17–21] and a previous publication used a polyclonal antibody to detect NCC antigen,[22] although this assay did not achieve good sensitivity or specificity. In this study, we prospectively determined the presence of specific antigens in the urine of patients with NCC and in negative controls and assessed the relationships of specific antigens with the viability of brain parasites.

MATERIALS AND METHODS

Study population. Urine samples were collected from September 2003 to February 2005 from NCC patients attending the Cysticercosis Unit at the Instituto de Ciencias Neurológicas, Lima (one sample from each patient). Samples were included in the study if 1) the patient had a diagnosis of NCC based on available brain imaging (either CT or MRI) information and 2) the time between the urine sample collection and the date of the most recent brain image did not exceed 30 days. According to their CT scan or MRI findings, NCC-compatible lesions were classified as having subarachnoid NCC (associated or not to hydrocephalus and/or intraparenchymal cysts), viable intraparenchymal cysts, degenerating intraparenchymal cysts (enhancing lesions or cysticercal granulomas), or calcified cysts. Patients with intraparenchymal lesions in more than one stage of the parasite were assigned to the group of more viable lesions (viable — degenerating — calcified cysts). All patients with suggestive CT or MRI images were included independently of their serologic results.

Negative control urine samples were obtained from volunteers living in Iquitos, a non-endemic zone of the Peruvian jungle.[23] Control urine samples were collected anonymously and assigned a numeric code. No other sampling or testing was done in this volunteer population, so concurrent geohelminth infection cannot be ruled out. Written consent was obtained from the participants. The study protocol and consent forms were approved by the Institutional Review Board of the Universidad Peruana Cayetano Heredia.

Urine sample collection. Urine samples were collected in disposable plastic containers and kept in refrigeration. At the end of each day, samples were archived in 50-mL screw cap polypropylene tubes and stored at −20°C until processed.

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Urine concentration methods. Urine concentration was used as an attempt to improving assay performance in terms of increased sensitivity without losing specificity. First, using a dilution curve obtained from a known positive urine sample diluted in a pool of negative urine samples, four concentration methods were tested in comparison to non-concentrated urine: ethanol precipitation,[22] lyophilization,[24] polyethylene glycol,[25] and ammonium sulphate.[26] Each sample was concentrated to a 15 times smaller volume. Urine samples from NCC patients and uninfected controls with enough remaining volume were tested by the two best concentration methods to compare their performances.

Serum samples. The Cysticercosis Unit archives were examined for blood samples of the same patients taken within 6 months before or after the urine sample collection date. Archived aliquots of these samples were also tested by Ag-ELISA, and their results were compared with the corresponding urine sample to assess their degree of correlation.

Ag-ELISA. In 1992, Brandt and others[10] developed a IgM monoclonal antibody (MoAb)-based Ag-ELISA directed to a secretory-excretory antigen from *Taenia saginata*, later modified by the same research group. The best diagnostic performance was obtained with B158C11 and B60H8 IgG MoAbs of murine origin.[27] An ELISA protocol was described for use on serum samples.[28,29] A modified protocol was developed for antigen detection in urine samples (Brandt J, Rodriguez-Hidalgo R, Van Hul A, unpublished data): plates were sensitized with 100 µL of trapping MoAb (B158C11) in bicarbonate buffer at 5 µg/mL, shaken for 30 minutes at 37°C, and washed with PBS-Tween 20 at 0.05%. Blocking solution (150 µL of PBS Tween 20–newborn calf serum [NBCS] 1%) was added to all the wells and incubated for 15 minutes. Simultaneously, urine samples, homogenized and diluted 1:2 in PBS-Tween 20–NBCS 1%, were incubated together with biotinylated MoAb (B60H8) for 1 hour at 37°C (a total of 250 µL). Pre-treated urine (100 µL/well) was left for 15 minutes at 37°C in the wells under gentle shaking, and the contents were discarded to proceed to incubate overnight at 4°C with a new 100-µL volume of pre-treated urine sample. Then, after washing the plate five times, 100 µL streptavidin diluted 1:10^4 in PBS-Tween 20–NBCS 1% was added and incubated for 15 minutes at 37°C under gentle shaking. Plates were washed again for five times, and 100 µL of ortho-phenylenediamine diluted in citrate buffer was added as substrate/chromogen and incubated in the dark for 15 minutes, and the reaction was stopped with 50 µL of 2N H_2SO_4. Plates were read at 492/650 nm. A sample was considered positive if the OD was higher than the mean of eight known negative samples plus 3 SD. Results were also analyzed as a continuous variable, expressed as percent positivity (PP), obtained from dividing the sample OD (×100) by the OD of a positive standard pool from known NCC patients. Antigen detection in serum samples was done using a volume of 100 µL in a dilution of 1:2 and adding a step of immune-complex break with trichloroacetic acid, as described before.[29]

Analysis. Sensitivity of the assay was calculated by dividing the numbers of Ag-ELISA–positive patients with viable parasites (either viable intraparenchymal cysts or subarachnoid NCC) by the total number of the respective strata. Specificity was calculated as the number of negative Ag-ELISA patients in the control population (excluding the eight samples used to define the cut-off point). Confidence intervals (95% CIs) were calculated for each proportion. Associations between discrete variables were assessed using the Fisher exact test. Correlations between numbers of lesions and Ag-ELISA PP

values, or between serum and urine Ag-ELISA PP values, were analyzed using the Spearman correlation test.

RESULTS

Study population. Urine samples from 87 NCC patients met the inclusion criteria and were considered for analysis. According to their CT/MRI findings, NCC patients were classified as having subarachnoid NCC ($N = 10$), viable cysts ($N = 34$), single enhancing lesions ($N = 7$), or calcifications only ($N = 36$) (Table 1). NCC patients were 47 (54%) males and 40 (46%) females, with a mean age of 35.3 years (range, 4–75 years; SD = 12.98 years). Control samples came from 32 volunteers from a non-endemic region in the jungle of Peru. Control samples were made completely anonymous.

Urine concentration. From the four methods used, lyophilization and ethanol precipitation yielded higher values on antigen levels compared with concentration by ammonium sulphate or polyethylene glycol (Figure 1). Ninety-one urine samples were tested in triplicate (non-concentrated urine, concentrated by ethanol precipitation, and lyophilized). Samples included 41 samples from patients with NCC (11 from patients with subarachnoid NCC and 30 from patients with intraparenchymal NCC) and 50 apparently uninfected controls from a non-endemic region. Compared with non-concentrated urine, the area under the receiver operating curve (ROC) curve was slightly wider for lyophilized urine (0.956–0.934), and smaller for samples concentrated by ethanol precipitation (0.907). This did not reflect in increased performance: six of the positive samples were missed by all methods and one, two, and five samples from the negative group were positive in non-concentrated, lyophilized, and ethanol-precipitated urine, respectively.

Ag-ELISA. The entire study set was tested by Ag-ELISA without using any concentration method. A cut-off OD of 0.028 (corresponding to a PP of 2.70) was calculated using the mean and SD of the initial eight consecutive control samples (Figure 2). The proportions of patients with positive Ag-ELISA results in urine per group are shown in Table 2.

The calculated sensitivity of the Ag-ELISA in urine samples of individuals with viable NCC was 90.9% (40/44; 95% CI, 0.82–0.99), and its specificity was 100%. Four samples of patients with viable intraparenchymal brain parasites were negative on Ag-ELISA. Negative results were more frequent in patients with a single viable cyst (3/8 versus 1/26, $P = 0.033$, one-sided Fisher exact test), giving a sensitivity of 62.5% in this subgroup. The number of viable brain cysts was significantly correlated with the levels of antigen in urine (Spearman $\rho = 0.461$, $P = 0.006$).
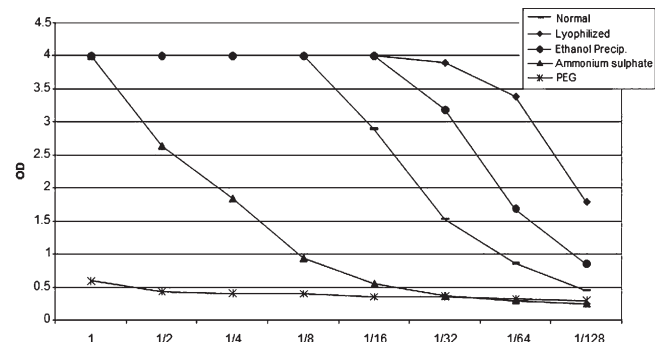

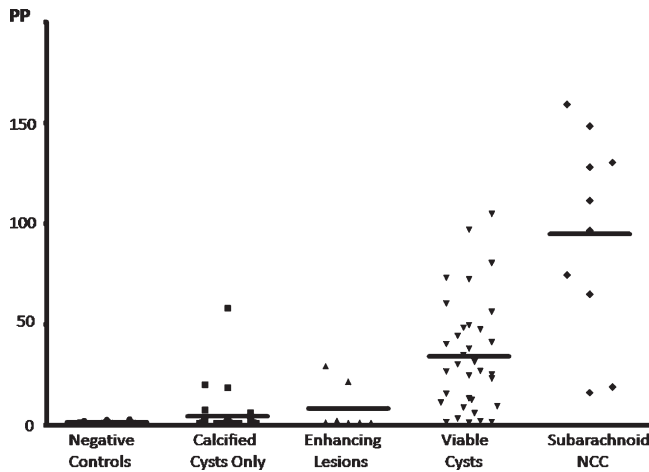FIGURE 1.   ELISA ODs of a dilution curve of positive urine using different concentration methods.

FIGURE 2. Ag-ELISA results of individual samples by type of NCC.

TABLE 2
Results of Ag-ELISA on urine samples from NCC patients and uninfected controls

Type of NCC	Positive	Ag-Elisa PP (mean, SD)
Subarachnoid NCC	100.0% (10/10; 95% CI, 0.69–1.00)	94.89 ± 27.77
Viable intraparenchymal cysts	88.2% (30/34; 95% CI, 0.73–0.97)	34.28 ± 50.42
Degenerating (enhancing lesions)	28.6% (2/7; 95% CI, 0.04–0.71)	8.47 ± 11.95
Calcified only	16.7% (6/36; 95% CI, 0.06–0.33)	4.55 ± 10.17
Uninfected controls*	0.0% (0/24; 95% CI, 0.00–0.14)	1.67 ± 0.47

*A second set of 50 negative controls run later to compare urine concentration methods showed one positive sample of 50 (1/50, specificity 98%).

Positive reactions were present in 2 of 7 patients with degenerating brain parasites and 6 of 36 patients with calcified lesions only. There was no significant correlation between Ag-ELISA PP and number of degenerated or calcified brain parasites (1/9 versus 5/27, $P = 0.525$, one-sided Fisher exact test).

Comparison of antigen levels in urine and serum of the same patients. From 19 of the 87 participant patients, an archive serum sample from the examined period was available. Ag-ELISA results in serum and urine of the same patient were strongly correlated (Spearman $\rho = 0.767$, $P < 0.001$; Figure 3).

DISCUSSION

NCC is endemic in most of the world and is considered a major public health problem.[1–3] Diagnosis is based on expensive neuroimaging tests including CT and MRI, which may not be available in many endemic areas. Antiparasitic treatment improves the prognosis of NCC-associated seizures in patients with live parasites.[30] Although neuroimaging is the key for diagnostic purposes and to guide an appropriate management of NCC, immunologic discrimination of individuals with live parasites may be useful to select subgroups of patients requiring specialized radiology or referral to centers with appropriate facilities for neuroimaging or management of antiparasitic therapy.

Cysticercosis antigen detection is an interesting option. Monoclonal-based Ag detection ELISAs for cysticercosis have been reported since 1989.[12,31] In 1992, Brandt and others[10] developed a sandwich ELISA using two monoclonal antibodies against a repetitive glycoprotein epitope found in the membrane and in excretory secretory products of *Taenia saginata* cysticerci. This assay has been recently shown to have 85% sensitivity and 97% specificity for NCC with more than one viable lesion (Dorny P, unpublished data). In this study, we showed that cysticercal antigens are excreted in the urine

of infected patients, thus representing a promising alternative for the diagnosis of human cysticercosis.

Non-invasive sampling using saliva[32,33] or urine[17–21] provides a series of logistic advantages and should increase compliance. Urine sampling has been used for serologic diagnosis of many infections including tissue parasitic diseases like hydatid disease,[18] *Schistosomiasis*,[19] Chagas' disease,[20] or filariasis,[21] among others. Another group has previously reported the use of a polyclonal antibody–based ELISA for cysticercus antigen detection in urine samples,[22] with 62.5% sensitivity and 91% specificity. The overall sensitivity of the MAb-based assay used in this study was 88% for intraparenchymal NCC, higher in patients with subarachnoid NCC or those with multiple intraparenchymal cysts, and lower in patients with a single intraparenchymal brain cyst. Interestingly, we failed to significantly increase the assay's sensitivity by concentrating urine samples. Most patients with only degenerating or calcified parasites tested negative on Ag-ELISA, thus suggesting that urine antigen detection could orientate the attending neurologists in regard to the viability of NCC. Our data strongly suggest that urine is a good alternative to serum sampling for cysticercosis antigen detection. Serum and urine antigen levels were strongly correlated. Moreover, urine antigen levels were also correlated to the number of viable brain cysts. A correlation between antigen level in serum and number of cysts was previously shown in pigs.[32]

TABLE 1
Urine cysticercus antigen detection: type of NCC and number of patients in each group

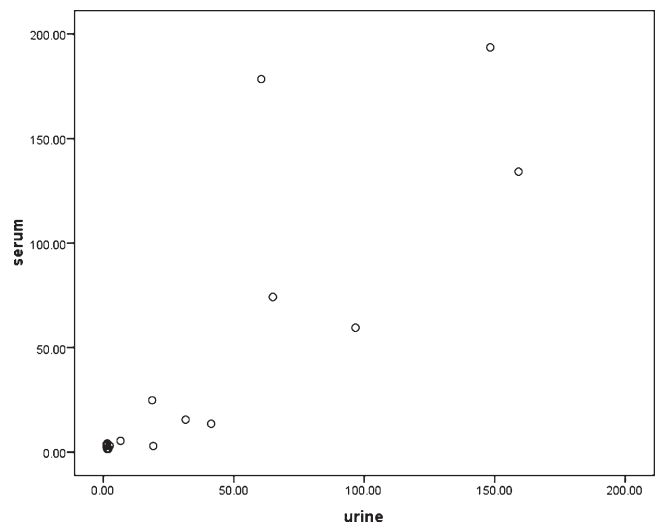| Type of NCC	No.
Subarachnoid NCC	10
Viable intraparenchymal cysts	34 (single 8, multiple 26)
Degenerating (enhancing lesions)	7 (single 3, multiple 4)
Calcified only	36 (single 9, multiple 27)

FIGURE 3. Scatterplot of serum versus urine antigens of 19 paired samples from Peruvian NCC patients.

Antigen levels in patients with subarachnoid NCC are markedly higher than those of individuals with intraparenchymal cysts. As previously reported for serum samples,[13,14] non-invasive urine sample collection may serve to monitor the evolution of circulating antigen levels and decrease the need for expensive and scarce neuroimaging in patients clinically well controlled.

It is unclear to us why some patients with calcified NCC had a positive Ag-detection ELISA result. This finding could be interpreted as that, even in late scarring stage of degeneration, membrane antigens of parasite cells may still be cleared. Alternatively, there could have been other parasitic cysts that went unnoticed on brain imaging or anywhere else in their bodies.

Urine antigen detection using the B158C11-B60H8 MoAb-based Ag-ELISA has good diagnostic performance. Its use could be of great value in the clinical field, particularly for screening in endemic regions where image studies are not available or for diagnostic confirmation in cases where CT/MRI images are inconclusive.

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