Am. J. Trop. Med. Hyg., 100(2), 2019, pp. 327–329
doi:10.4269/ajtmh.18-0706

Detection of *Taenia solium* DNA in the Urine of Neurocysticercosis Patients

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Abstract. Neurocysticercosis (NCC), caused by *Taenia solium* larvae that reside in the central nervous system, results in serious public health and medical issues in many regions of the world. Current diagnosis of NCC is complex requiring both serology and costly neuroimaging of parasitic cysts in the brain. This diagnostic pipeline can be problematic in resource-constrained settings. There is an unmet need for a highly sensitive and clinically informative diagnostic test to complement the present diagnostic approaches. Here, we report that *T. solium*–derived cell-free DNA is readily detectable in the urine of patients with the subarachnoid and parenchymal forms of NCC, and discuss the potential utility of this approach in enhancing and refining *T. solium* diagnostics.

INTRODUCTION

Taenia solium is endemic in regions of Latin America, sub-Saharan Africa, and Asia where pigs are raised under semi-feral conditions. Although the adult form of this parasitic cestode resides in the intestine, the tissue migrating *T. solium* larvae can invade the brain resulting in neurocysticercosis (NCC), a life threatening and debilitating form of the infection.[1] Whereas detection of the intestinal infection with adult tape worms is straight forward, definitive diagnosis of the central nervous system (CNS) form of the disease is more difficult as it requires neuroimaging of parasitic cysts in the brain.[1] The number of parasite cysts, their position in the brain, and other factors are used to devise patient-specific management plans, and an important step in this process is the efficient, often field-based, identification of those individuals who are candidates for costly neuroimaging.[1] Presently, this is carried out by the detection of antibodies in blood or cerebro-spinal fluid or the identification of circulating *T. solium* antigens.[1–4] Although these diagnostic approaches have proven useful, they suffer from a number of immunological and logistical issues and suboptimal combinations of sensitivity and specificity.[1] There is an unmet need for a highly sensitive and clinically informative diagnostic test for NCC to complement or replace current diagnostic approaches.

In the fields of cancer and infectious diseases, interest is growing in the use of cell-free DNA (cfDNA) found in serum, saliva, urine, and other body fluids for diagnosis of disease and monitoring the efficacy of interventions.[5–7] The detection of cfDNA has been demonstrated to be useful for the diagnosis of parasitic diseases including malaria, trypanosomiasis, leishmaniasis, schistosomiasis, strongyloidiasis, and filariasis (reviewed in ref. 6). Here, we report that *T. solium*–derived cfDNA is readily detectable in the urine of NCC patients and discuss the potential utility of this approach in enhancing and refining *T. solium* diagnostics and assessing intervention efforts.

MATERIALS AND METHODS

Sample collection. Following informed consent, urine and blood samples were collected from 23 neuroimaging-confirmed Peruvian NCC patients in accordance with ethical approval certificate R-033-03-17 issued by the main Institution Review Board of the Universidad Peruana Cayetano Heredia. In addition, five blood and urine samples from non-infected volunteers were also obtained. Fresh urine (40 mL, processed within 3 hours) was filtered through a filter paper cone (12.5 cm Whatman No. 3), dried overnight, and stored in a plastic bag with desiccant at 4°C. An additional 3 mL of urine from each patient was frozen at –20°C for subsequent magnetic bead–based extraction of cfDNA using MagMAX© Cell-Free DNA Isolation Kit (Life Technologies, Austin, TX).

Serology. Blood samples were assessed for the presence of anti–*T. solium* antibodies using the enzyme-linked immune-electro-transfer blot (EITB) assay[4] and circulating *T. solium* antigen using a monoclonal antibody-based ELISA[3] at the Universidad Peruana Cayetano Heredia and the Cysticercosis Unit in the Instituto de Ciencias Neurológicas, both in Lima, Peru. Each sample was tested in duplicate and was considered positive if the value was ≥ 3 standard deviations greater than the mean of the negative controls.

Extraction of cfDNA from filter paper. Fifteen 1.0-mm discs were punched from the central portion of the filter, covered with 600 µL of water, incubated at 95°C for 10 minutes, and then gently agitated overnight at room temperature. The tubes were centrifuged at 4,000 rpm for 5 minutes and the cfDNA in the supernatant was extracted using a QIamp Mini Kit (Qiagen, Hilden, Germany) followed by quantitation using a

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NanoDrop (NanoDrop Technologies LLC, Wilmington, DE) and storage at –20°C.

Detection of cfDNA. We selected the best performing primer set from nine different primer sets tested. The primer set designated P1 (P1F-5′-CACGTGGCAGGGTGTGAC-3′; P1R-5′-AGGAGGCCAGTTGCCTAGC-3′) amplified a 116-bp fragment from the pTsol-9 gene described by Chapman et al.[8] The amplicon was sequence-verified and matched 100% with pTsol-9. Amplification was performed at a 15 µL final volume that contained 7.5 µL of Taq 2× Master mix (New England Biolabs, Ipswich, MA), 0.75 µL of each primer (10 µM), 1–2 µL of $MgCl_2$, 1.5 µL of water, and 2 µL of cfDNA (8–20 ng/µL). The polymerase chain reaction (PCR) conditions were: 95°C for 10 minutes, 30–35 cycles at 95°C for 1 minute, 57–59°C for 1.5 minutes, 72°C for 1 minute, and a final extension step of 72°C for 5 minutes. The amplicon was assessed on a 2% agarose gel. Each sample was assayed in duplicate and samples with noncongruent results were reanalyzed to establish their status. Only samples that were PCR positive in both replicates were considered to be positive for *T. solium* cfDNA. Positivity was confirmed by sequencing the amplicons.

RESULTS

Based on neuroimaging and neurological interpretation, the 23 patients were classified as harbouring subarachnoid, viable parenchymal, or calcified parenchymal NCC (Table 1). Patients with subarachnoid NCC had moderate to severe disease and were part of a clinical trial to determine the efficacy of drug treatment. The two parenchymal NCC patients each carried a single viable brain cyst, one 1.0 cm and the other 1.7 cm in diameter. Calcified NCC patients were part of a cohort study of resolved, nonviable NCC and presented with between one and 11 parenchymal brain calcifications. Whereas the EITB results for the presence of anti–*T. solium* antibodies were positive for all subarachnoid and viable parenchymal patients, most, but not all, of the patients were positive for circulating parasite antigen. A lower proportion of the five patients harboring calcified cysts were antibody and circulating antigen–positive.

TABLE 1

Results of the EITB, monoclonal antibody-based ELISA for detection of circulating *Taenia solium* antigen (Ag-ELISA), and the PCR-based detection of *T. solium* cfDNA from urine on serum and urine samples obtained from neuroimaging-confirmed NCC patients and controls (SCAN-NEG)

NCC type	EITB	Ag-ELISA	cfDNA
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	–	–
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	+
SUBARACHNOID	+	–	–
SUBARACHNOID	+	+	+
SUBARACHNOID	+	+	–
SUBARACHNOID	+	+	+
PARENCHYMAL	+	+	+
PARENCHYMAL	+	+	+
CALCIFIED	+	–	+
CALCIFIED	+	+	–
CALCIFIED	–	–	–
CALCIFIED	+	–	+
CALCIFIED	–	–	–
SCAN-NEG	–	–	–
SCAN-NEG	–	–	–
SCAN–NEG	–	–	–
SCAN-NEG	–	–	–
SCAN-NEG	–	–	–

cfDNA = cell-free DNA; EITB = enzyme-linked immune-electro-transfer blot; NCC = neurocysticercosis.
+ and – indicate that the outcome of the assay was positive or negative, respectively.

The results for cfDNA analysis from the filter paper and the magnetic bead protocols were largely congruent suggesting that both of these DNA-capture approaches are viable alternatives for sample collection. The data presented in Table 1 represent a combination of the two capture methods. For the subarachnoid and viable parenchymal NCC patients, 13/16 (81%) and 2/2 (100%), respectively, had detectable levels of *T. solium* cfDNA in their urine. Of interest, 2/5 (40%) patients with calcified cysts in the brain had detectable cfDNA in their urine, suggesting that these calcified deposits are releasing parasite material or these two patients harboured viable cysts in their somatic tissues. Although the overall sensitivity of cfDNA detection in the urine was 74% (17/23), for the patients harboring demonstrably viable parasites in the brain (subarachnoid and parenchymal), the sensitivity of the cfDNA detection was 83% (15/18). Negative controls were from anonymous donors never exposed to *T. solium* who tested negative for antibody, antigen, and cfDNA.

DISCUSSION

The standard for the diagnosis of NCC is neuroimaging using computed tomography and magnetic resonance imaging. This is typically confirmed by serology using either the EITB which detects antibodies directed against parasite-derived glycan residues.[4] Enzyme-linked immune-electrotransfer blot has high sensitivity for patients who harbor two or more live cysts in their CNS, but sensitivity drops significantly for lower parasite loads.[1] Like all antibody-based diagnostic approaches, the EITB is an indirect measure that cannot distinguish between CNS and somatic infection or between current infections and past exposure. Thus, EITB serology is usually supported with a second test based on detection of circulating parasite antigen in the blood. The most widely used assay is a monoclonal antibody-based ELISA that detects a circulating parasite antigen, however this test is limited by its modest sensitivity in patients with a low number of CNS cysts.[1] Thus, there is a need for an additional diagnostic approach that can facilitate the reliable diagnosis of *T. solium* CNS infection in the clinic and in the field.

The presence of parasite-derived cfDNA in the body fluids of patients has been demonstrated for a number of parasitic infections[6] and detection of cfDNA in the urine has been shown to be a sensitive and specific diagnostic approach for several helminth infections.[9–12] Although *T. solium* cfDNA had been detected in cerebrospinal fluid of NCC patients,[13] our work is the first to report that *T. solium*–derived cfDNA can be detected in an easily obtained body fluid such as urine and serve as a sensitive measure of infection. It is notable that 3/16 patients harboring subarachnoid cysts containing viable parasites were negative for cfDNA. Although it is possible that

these represent a subset of patients that do not pass parasite cfDNA in the urine, it is also possible that these patient's samples have cfDNA that is derived from other regions of the parasite's genome. Incorporating additional *T. solium* cfDNA targets in a multiplex version of the assay could expand the sensitivity of this diagnostic approach.

As with most infections, it is important to know if a *T. solium*–infected patient is cured after treatment. Estimates for the half-life of cfDNA in the blood of humans range between 4 minutes and 12 hours.[14] Assuming that the immediate upstream source of cfDNA in the urine is the cfDNA from the blood, this short half-life strongly suggests that detection of parasite-derived cfDNA in the urine reflects the presence of parasites and its absence could be used as a sensitive measure of the effectiveness of chemotherapy efforts. Indeed, cfDNA-positive *Schistosoma haematobium* patients tested negative 2 weeks after receiving praziquantel.[9]

There is a need for new diagnostic methods to better identify patients who harbor *T. solium* in the CNS so they can be assessed by neuroimaging and their disease properly managed. Although serology- and urine/cfDNA-based assays have utility in the identification of infected individuals, the current forms of these assays are not capable of discriminating somatic from CNS involvement. Thus, additional refinements to these assays will be necessary to confirm that patients harbor parasites that have localized to the CNS. Recent advances in the identification of biomarkers for CNS inflammation in the context of a spectrum of brain diseases have demonstrated that these markers are detectable in the urine of patients.[15–17] Combining cfDNA analysis with identification of biomarkers of CNS inflammation holds the potential for a sensitive and specific approach for the diagnose NCC from a single urine sample.

Received August 27, 2018. Accepted for publication October 22, 2018.

Published online December 10, 2018.

Financial support: This study was partially supported by grants U19AI129909 from the National Institute of Allergy and Infectious Diseases (Peru TMRC Program, NIAID/NIH), R01NS054805 from the National Institute of Neurological Disorders and Stroke (NINDS/NIH), and D43TW001140 training grant from the Fogarty International Center (FIC/NIH).

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