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Diagnostic urinary cfDNA detected in human cystic echinococcosis

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Abstract

Cystic echinococcosis (CE) is a major neglected tropical zoonotic disease caused by the tissued-welling larval stage of cestode parasite *Echinococcus granulosus.* For individuals suspected of CE, the diagnostic standard is imaging using ultrasonography, X rays, or computed tomography. These resource-demanding and expensive procedures are rarely available in endemic rural areas where CE is most prevalent. There is a critical need for a new approach to identify CE patients so that they can be managed early in the course of their infection. This study reports on the results of a diagnostic approach that identifies *E. granulosus-derived* cell-free DNA (cfDNA) in the urine of CE patients. Utilizing PCR to amplify a fragment of a major tandem repeat element found in *E. granulosus* nuclear DNA, urine samples from all seven imaging-confirmed CE patients who harbored active liver cysts were positive. In addition, the urine samples from 2/4 patients who presented with non-viable/calcified liver cysts were also PCR positive for the repeat fragment. To

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Authors contributions:

L.T. completed the experimental investigation, she collected urine, filtered in the field, performed all extractions, amplification, did the electrophoresis and interpreted the results. She also wrote the initial draft. S.S. covered the field work, identified and examined the volunteers and interpreted the scan results. He prepared the Table. R.E., C.S. and P.B. were involved with the human volunteers, results and interpreted the scans. H.G. coordinated the entire work, field and laboratory in Peru. He is PI of the Cysticercosis Working Group in Peru. C.J.S. developed the concept of using filtered urine to trap diagnostic DNA. he supervised LT. when doing the initial work in Baltimore. He and AS finished the final document.

Conflicts of interest:

None

our knowledge, this is the first report of using parasite cfDNA from urine to diagnose CE. This approach provides an easy to implement and cost-effective method to survey for the prevalence of *E. granulosus* in humans populations.

Keywords

Diagnosis of hydatid cysts; cell-free DNA; hydatidosis; urinary DNA; *Echinococcus granulosus* ; Peru

1) Introduction

Cystic echinococcosis (CE) is a neglected tropical zoonosis caused by the cestode *Echinococcus granulosus*. Several herbivorous and omnivorous species function as intermediate hosts of *E. granulosus* when they ingest parasite eggs from contaminated food and water. The parasites develop into transmissible larvae in the viscera. Canidae (dogs, dingoes, wolves and coyotes), the definitive hosts, obtain the parasite when they consume larvae-containing viscera. The adult tapeworm, which resides in the intestine of the definitive host, broadcasts embryonated eggs into the environment in the feces. Humans are accidental intermediate hosts when these eggs are inadvertently consumed. The eggs hatch and the larvae cross the intestinal mucosae and encyst principally in the liver or lungs [1, 2].

CE impacts up to a million people per year, and it is considered a serious public health problem in farming regions around the world due its detrimental impact on human health and on the livestock industry resulting in ~$2 billion dollars in loss annually [3]. In rural regions of South America the prevalence of CE can be as high as 5%-10% [1, 3, 4], with an estimated incidence of 3,000+ cases per year in Peru alone [5]. The principal risk factors for CE are the use of dogs to herd sheep, feeding dogs with raw sheep viscera, and absence of an effective sanitary infrastructure [6, 7].

Diagnosis of CE is challenging, particularly in the early stages when the infection is largely asymptomatic. Definitive diagnosis of CE is based on imaging methods using ultrasonography for liver CE and chest X-Ray for lung CE, followed by validation with computed tomography (CT) and/or magnetic resonance imaging (MRI). These imaging techniques are expensive and largely unavailable to the rural populations that are most impacted by this disease. Serological tests have been developed to detect host antibodies or parasite antigens, but these tests have limited sensitivity and lack sufficient specificity. There is a need for diagnostic approaches appropriate for community-based screening to assess the prevalence of CE in children and adults [8-10]. Although there are several reports of using parasite genomic and mitochondrial DNA to differentiate *Echinococcus* species and haplotypes from humans and animals [3, 8, 11-14], the use of cfDNA for the diagnosis of echinococcosis has been limited.

The analysis of cell-free DNA (cfDNA) has found wide used in the clinic setting for non-invasive prenatal testing [15, 16], cancer diagnosis and monitoring [17, 18], and detecting pathogens [19]. Parasite-derived cfDNA in body fluids has been demonstrated to be useful for the diagnosis of malaria, trypanosomiasis, leishmaniasis, schistosomiasis,

strongyloidiasis, filariasis [20-22], and echinococcosis [7, 23-26]. *Echinococcus*-derived nuclear cfDNA was detectible in the circulation of experimentally infected animals [23] and patients [23, 26] with polycystic echinococcosis. Studies have employed deep sequencing approaches to define the complexity and nature of the parasite cfDNA circulating in the blood of patients with various clinical forms of echinococcosis [25, 26]. Ji et al. [25] showed that *E. granulosus* cfDNA in the circulation of patients ranged from ~100 bp to over 350 bp and mapped uniformly across the nuclear and mitochondrial genomes.

Our group has developed PCR-based analyses to detect helminth-derived cfDNA in the urine of patients with schistosomiasis (*Schistosoma mansoni, S. haematobium*), stongyloidiasis (*Strongyloides stercoralis*) and neurocysticercosis (*Taenia solium*) with high sensitivity and specificity [27-30]. This non-invasive sampling method is well-suited for population-based surveys to assess the prevalence of the infection, something that heretofore has not been feasible. The goal of this study was to provide proof-of-principle evidence that *E. granulosus*-derived cfDNA can be detected in the urine from imaging-confirmed CE patients and to demonstrate the potential of urine-based diagnostics as a first-line test for detection of hydatid cysts in a rural setting in Peru.

2) Materials and Methods

a) Study population

Urine samples were collected from 12 patients who were confirmed as harboring liver or lung CE cysts by ultrasound or thorax/abdominal CT scans. The patients resided in Corpacancha-Junín, a highly endemic village in the Central Peruvian Highlands, with altitude of 4,141 m above sea level. A brief survey was applied to register their epidemiological information and to obtain written consent to participate in an ultrasound survey for CE and to provide urine samples. Subjects between the ages of 3 and 18 years required parental consent to participate.

Additionally, urine samples were collected from 25 healthy volunteers from Cayetano Heredia research laboratories to serve as controls. These volunteers were selected based on self-reporting that they had no history of exposure to the *E. granulosus*. The study protocol and consent forms were reviewed and approved by the main IRB of the Universidad Peruana Cayetano Heredia (approval code 102270).

b) Sample collection

A total of 40 ml of fresh urine was collected in a disposable plastic receptacle and filtered into a 50 ml Falcon tube through a filter paper cone fitted with Whatman #3 paper (GE Healthcare, Illinois, USA) [27]. The urine was filter processed within 3 hours of collection. The filter paper specimens were dried overnight at room temperature, labeled, placed individually in a sealed plastic bag with desiccant, stored at room temperature and transported to the facilities of Cayetano Heredia University in Lima for extraction and PCR analysis.

c) DNA extraction

Fifteen 1.0 mm diameter discs were punched from the central area of the filter paper and the discs were placed in 500 μL nuclease-free water in a 1.5 ml microcentrifuge tube. The tubes were incubated at 95°C for 10 minutes in dry bath and then were left at room temperature with gentle agitation for at least 12 hours. The next day, the tubes were centrifuged at 8,000 rpm for 5 minutes and the supernatant was transferred to a Qiagen spin column. Cell-free DNA was recovered from the column following the manufacturer's instructions (QIamp Mini Kit -Qiagen, Hilden, Germany). DNA quantification was carried out with a Nanodrop system (NanoDrop Technologies LLC, Wilmington, DE) and the extracted cfDNA was stored at −20°C.

d) Detection of cfDNA

Primers were synthesized to amplify a 133 bp fragment of the *E. granulosus* EgG1 *Hae* III repeat as reported by Abbasi et al. [31]. The repeat is estimated to be found in the *E. granulosus* genome at ~7,000 copies that are arranged in tandem in groups of 2-6 repeats [31]. The primer sequences were: forward Eg1121a GAATGCAAGCAGCAGATG and Eg1122a reverse GAGATGAGTGAGAAGGAGTG. The PCR amplification mix contained 15 μL of final volume containing 7.5 μL of Taq 2× Master mix (New England Biolabs, Ipswich, MA), 0.75 μL of each primer (10 μM), 1.5 μL of $MgCl_2$ (25 mM), 2.5 μL of water, and 2 μL of cfDNA. The PCR conditions were: 95°C for 10 minutes, 35 cycles of 95°C for 1 minute, 57°C for 90 seconds and 72°C for 1 minute; and a final extension step of 72°C for 5 minutes. A positive control was obtained by pooling the cfDNA from 10 confirmed, image-positive patients who reside in the same community as the test patients. The PCR product was visualized on a 2% agarose gel. The samples were assayed in duplicate and triplicate for noncongruent results. All of the amplicons produced in this study were sequence-verified as the *E. granulosus* EgG1*Hae* III repeat fragment.

3) Results

a) Patients and Imaging findings:

Of the 12 CE cases that tested positive using either ultrasound or CT scans, four were male and eight were female with ages ranging from 7 to 98 years (Table 1). The control group contained seven men and 18 women with ages ranging from 22 to 63 years. Of the 11 CE patients that harbored liver cysts, five had cysts in other organs: three in the lungs (patients 2, 3, and 11), one in the spleen (patient 1), and one in the uterus (patient 6). Two patients had multiple liver cysts (patient 3 had three and patient 9 had two). According to the ultrasound classification developed by the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) for CE in the liver, of the 11 patients with liver cysts, five CE patients harbored eight active cysts (CE1 in classification), two CE patients had two transitional cysts (CE3 in classification), and four CE patients had four inactive cysts (one CE4 and three CE5 in classification) (Table 1). For the three patients who harbored cysts in both the liver and the lungs, all of the lung cysts were active. For the single patient that harbored cysts only in the lungs (patient 12), there was a mix of active and inactive cysts.

b) PCR results:

The Eg1121a-Eg1122a primer set generated a major PCR product at 133 bp fragment from the 269 pb EgG1 *Hae III* tandem repeat with as little as ~1 fg of *E. granulosus* template DNA [31] (data not shown). The PCR product generated when the urine-derived cfDNA was used as a template was 133 bp and when sequenced the PCR products had 92% to 95% identity to the reported EgG1 *Hae III* sequence (GenBank Accession DQ157697.1; data not shown).

Between 2.96 to 9.42 ng/μl of total cfDNA was extracted from the test and control urine filter samples. The amount of total cfDNA recovered from the urine did not correlate with the number of cysts carried by the image-positive patients (R^2=0.185).

Overall, the *E. granulosus* EgG1*Hae* III repeat-derived cfDNA was PCR-amplified from the urine samples of 9/12 (75%) of the imaging-confirmed CE cases (Table 1). Of note, all seven of the urine samples from patients with presumptively viable (CE1) or transitional (CE3) liver cysts were EgG1*Hae* III PCR-positive. Of the urine samples obtained from patients with calcified and degenerating liver cysts (CE4 and CE5) two were EgG1*Hae* III PCR-positive and two were EgG1*Hae* III PCR-negative. The third PCR-negative urine sample was derived from the single patient who was ultrasound positive only in the lungs with a mix of active and inactive cysts.

It is important to report that one out of the 25 urine samples collected from control volunteers, one tested positive. Interestingly, upon further questioning, it was revealed that the person who provided the positive sample originated from an area endemic for *E. granulosus*, thus raising the possibility that the volunteer was cryptically infected with the parasite.

These results demonstrate that the detection of parasite-derived cfDNA from a urine sample has potential utility as a first-line diagnostic approach for the identification of CE patients harboring active and transitional *E. granulosus* cysts in the liver. Although the numbers were limited, findings also indicated that the use of the EgG1*Hae* III PCR-based detection in urine samples from patients who have inactive cysts or active cysts outside the liver may be less sensitive.

4) Discussion

The absence of obvious symptoms during the early stages of CE presents a challenge to identify infected individuals. Although attempts have been made over the years to develop antibody-based and antigen-based serological diagnostic approaches to screen populations, CE remains under diagnosed and typically identified only when complications arise or by chance. Currently there is no practical way of defining community-based prevalence of this infection. The tests designed to detect anti-parasite antibodies or parasite-derived antigens have proven to lack the sensitivity and specificity required to diagnose CE in the clinic or in the field [32]. The WHO standard for diagnosis is ultrasonography imaging of parasite cysts associated with the internal organs followed by validation with CT and/or MRI scans. This resource intensive approach likely identifies only a fraction of the infected individuals.

There is a clear need to develop diagnostic approaches with sufficient sensitivity and specificity to be a useful tool to screen asymptomatic children and adults for infection with *E. granulosus*. Here we report on the potential effectiveness of an approach that detects *E. granulosus* cfDNA amplified from the urine of image-confirmed CE patients.

In this study, we adapted a simple, low-tech field processing protocol that has been used previously in urine-based diagnostic tests for other helminth parasites [27-30]. A key feature of this processing protocol is a step where ~40 ml of freshly collected urine is passed through a cellulose membrane to capture, concentrate and stabilize cfDNA and then filter is dried for easy transport of the sample to the lab for analysis and/or long-term storage. In our hands, parasite cfDNA can be extracted from properly stored filters for over one year [27]. While such factors as the mechanism of binding of the cfDNA to the filter, the influence of urine pH, protein and lipid content on cfDNA binding, the efficiency of cfDNA capture on and elution from the cellulose membrane are not known, the procedure has proven a reliable, practical, and cost-effective for the collection pathogen-derived transrenal cfDNA in a field setting [27, 29, 30].

The proportion of *E. granulosus*-derived cfDNA contained in the total cfDNA isolated from the filter-processed urine samples is not known. Ji et al. used a non-biased sequencing approach to identify the cfDNAs isolated from serum samples obtained from CE patients and reported that on average parasite-derived sequences were found at the level of 0.433 sequences per million read-pairs [25]. Wan et al., using a similar sequencing approach, also concluded that *Echinococcus* cfDNA in plasma is a very low proportion of the total cfDNA [26]. This is in stark contrast to the levels of fetal cfDNA in the mother's blood which ranges from ~3% during early gestation to ~6% during the later stages of pregnancy [33]. Since the parasite cfDNA in the serum is the presumed proximal source of the cfDNA found in the urine [34, 35], it is likely that only a small proportion of the cfDNA isolated from the urine of patients is parasite-derived.

Based on our extensive experience targeting repetitive genomic DNA to devise urine-based cfDNA diagnostics for other helminth parasites [27-30], the EgG1*Hae* III repeat was selected for the development of the PCR assay. The rationale for this selection is based on the presumption that, if the cfDNA was generated from the parasite's nuclear DNA at random, this repeat, which is present at ~7,000 tandem copies arranged in 2-6 clusters [31], would likely be one of the most highly represented nucleic acid species in the parasite cfDNA. These assumptions are supported by findings that demonstrate that the genomic release points for parasite cfDNAs isolated from blood map evenly across the known *Echinococcus* nuclear genome [25] and that a majority of *Echinococcus* cfDNA mapped to non-coding and low complexity regions of the parasite's genome [26]. Further, Wan et al. demonstrated that a number of different repeats present in the *Echinococcus* genome are readily detected amongst the cfDNAs isolated from patient's blood [26]. In future work, it will be of interest to know if any of these additional repeat fragments found in the blood are also found in the urine of patients and can be incorporated into a multiplex assay that will increase the sensitivity of urine-based diagnosis of CE.

Our results demonstrated that the *E. granulosus*-specific DNA repeat fragment was most efficiently detected in the urine of patients with liver cysts classified as active. The urine from patients harboring calcified liver cysts (CE4 and CE5) gave a mixed signal for the *E. granulosus* EgG1*Hae* III repeat DNA (Table 1). These variable results from patients with calcified cysts are likely a function of the age of the calcified cyst and the level of inflammation in the cyst-proximal microenvironment. It is of interest to note that patients harboring calcified brain cysts caused by *Taiena solium* were also shown to be positive for parasite cfDNA in the blood [36] and in the urine [30].

While not a point of care test, the approach outlined here has the potential to be a cost-effective method to identify both asymptomatic and symptomatic individuals harboring active liver cysts and who require confirmatory diagnosis and disease management. The protocol used here outlines a low-tech pipeline where the urine specimen is filtered, dried and packaged in the field in preparation for transport and analysis in the laboratory. In addition to its prospective utility as a scalable diagnostic tool, detection of parasite-derived cfDNA in urine might be used to monitor the efficacy of therapeutic treatments for the elimination of the parasite [27]. Additionally, this approach could prove to be an easy to implement, non-invasive approach for early case detection and to monitor the prevalence of CE in rural communities.

While limited in scope, to our knowledge, this study is the first to report the potential for using a urine-based approach to detect *E. granulosus* cfDNA as a diagnostic test for CE. While more extensive studies are required to validate the findings, this urine-based approach has the potential to be useful as an adjunct screening tool that is a component in a more efficient and cost-effective pipeline to identify the individuals most in need of imaging-based diagnosis for CE.

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Highlights:

- An in house-PCR was designed to specifically amplify a tandem repeat fragment from *Echinococcus granulosus* DNA.
- We established the presence of the *E. granulosus* DNA repeat fragment as part of the cell-free (cf)DNA from the urine of cystic echinococcosis patients, principally in patients with viable liver cysts (9/11, 81.8%).
- Urine-based diagnosis of *E. granulosus* has potential as a non-invasive, cost-effective, practical method to identify individuals with cystic echinococcosis in field conditions.

Table 1.

PCR results for the detection of a 133 bp fragment of the *Echinococcus granulosus* EgG1*Hae* III repeat DNA in the urine of imaging-confirmed patients harboring cysts in the liver and lungs.

CE Patient No.	Sex/Age (years)	ultrasound	Abdominal CT	Tissue	volume (mm3)	WHO IGWE	EgG1 *Hae III* PCR
1*	F/14	positive	NE	liver	32.7	CE1	positive
2	F/10	positive	negative	liver	385	CE1	positive
				lung	7.6	active	
				lung	8.5	active	
				lung	2.25	active	
3	F/46	positive	NE	liver	56.5	CE1	positive
				lung	5.23	active	
4	F/7	positive	negative	liver	4.1	CE1	positive
5	M/10	positive	NE	liver	797	CE1	positive
				liver	23.3	CE1	
				liver	795	CE1	
6**	F/25	positive	NE	liver	2240	CE3	positive
7	F/12	positive	NE	liver	110	CE3	positive
8	F/32	positive	NE	liver	22.4	CE4	negative
9	M/29	negative	positive	liver	8.75	CE5	positive
				liver	2.15	CE5	
10	F/98	negative	positive	liver	82.32	CE5	negative
11	F/8	negative	positive	liver	4.06	CE5	positive
				lung	97.66	active	
12	M/59	positive	NE	lung	1.4	inactive	negative
				lung	21.2	inactive	
				lung	4.6	active	
				lung	66.6	active	

* harbored a cyst in the spleen

** harbored a cyst in the uterus

NE = not evaluated