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Point of care diagnosis of multiple schistosome parasites: Species-specific DNA detection in urine by loop-mediated isothermal amplification (LAMP)

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ABSTRACT

Schistosomes are easily transmitted and high chance of repeat infection, so if control strategies based on targeted mass drug administration (MDA) are to succeed it is essential to have a test that is sensitive, accurate and simple to use. It is known and regularly demonstrated that praziquantel does not always eliminate an infection so in spite of the successes of control programs a residual of the reservoir survives to re-infect snails. The issue of diagnostic sensitivity becomes more critical in the assessment of program effectiveness. While serology, such as antigen capture tests might improve sensitivity, it has been shown that the presence of species-specific DNA fragments will indicate, most effectively, the presence of active parasites. Polymerase chain reaction (PCR) can amplify and detect DNA from urine residue captured on Whatman No. 3 filter paper that is dried after filtration. Previously we have detected *S. mansoni* and *S. haematobium* parasite-specific small repeat DNA fragment from filtered urine on filter paper by PCR. In the current study, we assessed the efficacy of detection of 86 urine samples for either or both schistosome parasites by PCR and loop-mediated isothermal amplification (LAMP) that were collected from a low to moderate transmission area in Ghana. Two different DNA extraction methods, standard extraction kit and field usable LAMP-PURE kit were also evaluated by PCR and LAMP amplification. With *S. haematobium* LAMP amplification for both extractions showed similar sensitivity and specificity when compared with PCR amplification (100%) verified by gel electrophoresis. For *S. mansoni* sensitivity was highest for LAMP amplification (100%) for standard extraction than PCR and LAMP with LAMP-PURE (99% and 94%). The LAMP-PURE extraction produced false negatives, which require further investigation for this field usable extraction kit. Overall high positive and negative predictive values (90% − 100%) for both species demonstrated a highly robust approach. The LAMP approach is close to point of care use and equally sensitive and specific to detection of species-specific DNA by PCR. LAMP can be an effective means to detect low intensity infection due to its simplicity and minimal DNA extraction requirement. This will enhance the effectiveness of surveillance and MDA control programs of schistosomiasis.

1. Introduction

Point of care diagnosis is a priority for strategies that rely on mass drug administration (MDA) for the control of many of the neglected tropical diseases (NTDs). As these strategies are applied and evaluated it is clear that the use of signs such as hematuria for urinary schistosomiasis or examination of stool smears such as the Kato-Katz (KK) test are unsatisfactory as diagnostics because of the lack of sensitivity (Lodh et al., 2013). Currently a point of care test for *Schistosoma mansoni*, the circulating cathodic antigen (CCA) test is in use, but it is similar in sensitivity as the KK smear in detecting *S. mansoni* and quite inadequate to detect *S. haematobium* infection (Stothard et al., 2006). Recently, however, it has been shown at least with schistosomes, that fragments of somatic DNA from both *S. haematobium* and *S. mansoni* are detectable in urine (Ibironke et al., 2011), and can be extracted from filter paper through which a urine specimen was filtered and dried and thus avoids the collection and handling of stool specimens (Lodh et al., 2014). Although collecting and examining specimens has been greatly improved by filtration of urine in the field, and examination in a central laboratory, there still remains the need for thermocycler and electrophoresis

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equipment for the examination. These procedures can be replaced in the field by the loop-mediated isothermal amplification (LAMP) technology (Abbasi et al., 2010).

Detection of parasite species-specific DNA in urine indicates the presence of the actual parasite even when eggs or antigens are not always detectable, and schistosome DNA in urine has been shown to clear from infected people by two weeks after praziquantel treatment (Ibironke et al., 2012). In a carefully controlled study from Zambia, Lodh et al. (2013) showed that detecting *S. mansoni* species-specific DNA fragment in the urine precipitate, there was a 30% disparity between CCA and PCR test. In China, (Xu et al., 2014) showed a six-fold difference between the KK smear (performed nine times per sample) and *Schistosoma japonicum* specific DNA detection from serum. Clearly it is important to add parasite species-specific DNA detection to the diagnostic repertoire and keep the technology as close to the point of care as possible (Shiff, 2014). The LAMP procedure commonly is made up from supplies obtainable from scientific vendors. It is highly specific as it uses four sets of primers to amplify six target regions of the DNA (Notomi et al., 2000). It also amplifies DNA fragments independent of the standard thermocycler and electrophoresis and can easily be adapted for clinical trials. The procedure depends on extraction and purification of the target DNA. This can be done by use of the standard DNA extraction kit (such as,QIAmpDNA Blood mini kit), or by other means of purification. The Eiken Chemical Company, Japan have produced a LAMP-PURE (Procedure for Ultra Rapid Extraction) kit, which incorporates a proprietary method for DNA extraction and purification in order to improve the sensitivity of the amplification process. In this work, we compared PCR amplification of DNA extracted and purified with QIAmpDNA Blood Mini kit against LAMP amplification of DNA extracted and purified with QIAmp and LAMP-PURE to determine whether there was any advantage in diagnostic accuracy from 86 filtered urine specimens collected from a community in southern Ghana. The objective of this study was to compare the efficacy of two methods of extracting DNA from urine deposit on coarse filter paper (Whatman No 3.) dried after filtration and stored at room temperature (Ibironke et al., 2011) and their effect on two different types of amplification.

2. Materials and methods

2.1. Study design and population

The study location and population had been described in detail in previous publication (Lodh et al., 2014). Briefly, the study was carried out at Tomefa, a district of the greater Accra region in Ghana. Colleagues of the Noguchi Memorial Institute for Medical Research (NMIMR) chose this area because of the presence of both *S. mansoni* and *S. haematobium* and an ongoing study that focused on intestinal schistosomiasis for NMIMR. Stool specimens for *S. mansoni* were examined using the KK method, but there was no urine examination for *S. haematobium*. For this study, urine samples were collected from 86 participants between 5 and 23 years of age specifically for DNA detection. Approval for the diagnostic research was provided by NMIMR (IRB: 043/12-13). Written study consent was collected from parents or guardians in case of minors and from individuals in case of 18 years or older. Participants found infected parasitologically were treated by praziquantel by NMIMR and local health centers.

Urine samples (approximately 40–50 mL) were collected between 10:00 am and 2:00 pm and were evaluated for color, pH, and specific gravity and for presence of protein, glucose bilirubin and hematuria with Hemastix (Bayer, Elkhart, IN). After physiological assessment, approximately 50 mL whole urine was filtered through Whatman No. 3 filter paper (Whatman International, Maidstone, England), then dried under a fly-proof net and individually packed in Ziploc bags with desiccant. Filter papers without any personal information and only with number associated with specimen along with age, sex, physiological and diagnostic information were brought back to Johns Hopkins School of Public Health, Baltimore, Maryland for molecular diagnosis. DNA was extracted from urine sediment captured on filter paper by QIAmpDNA Blood Mini Kit (Qiagen, MD) and LAMP PURE kit (Eiken Chemical Co., Ltd, Japan). DNA extracted by Qiagen kit was evaluated by both PCR and LAMP. LAMP PURE kit extracted DNA was evaluated only by LAMP.

2.2. DNA extraction from urine by Qiagen and LAMP PURE kit

Each filter paper was folded to form a cone to drain the urine. DNA was extracted from marked inner quadrant at the base of filter paper by QIAmpDNA Blood Mini Kit (Qiagen, MD, USA). From each quadrant 15 pieces of ~1 mm diameter paper disc was punched off by a regular paper punch. After every use, the paper punch and scissor was cleaned by 10% bleach solution and distilled water to avoid any contamination. All 15 paper discs for each sample were placed in 1.5 mL Eppendorf tube with 600 μL nuclease-free water and heated at 95 °C for 10 min and then kept on a rotor at room temperature (22–25 °C) for 16–18 h. The next day water solution was transferred to a Qiagen QIAmp 2 mL column tube and DNA was extracted by following manufacturer's protocol.

The adjacent quadrant was used to extract DNA by LAMP-PURE kit. The PURE kit was consisted of a series of extraction and purifying steps. Step 1: An aliquot of sample (water extract as described above) was heated at 90 °C for 10 min using a water bath to lyse any cellular material. This was added to the manufacturers DNA extraction solution, mixed by inversion and incubated. Step 2: The sample was then treated with adsorbent ingredient to remove inhibitory materials contained in the sample and to neutralize the solution without any loss of the target DNA.

DNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Qiagen-kit-extracted DNA concentration was ranged from 1 to 10 ng/μL. PURE-kit-extracted DNA concentration was ranged from 50 to 1200 ng/μL. Therefore, PURE-kit-extracted DNA was diluted at 1:10 ratio as DNA concentration was too high for amplification.

2.3. PCR amplification for S. mansoni and S. haematobium

PCR amplification was carried out in 10 μL volume with positive and negative controls. For *S. mansoni*, KK positives were used as positive control and for *S. haematobium*, hematuria and urine filtration positives were used as positive control. Water was used as negative control for both parasite species. For PCR amplification, the reaction volume consisted of 5 μL of PCR Master Mix, 2X (Promega, Madison, WI, USA), 0.5 μL (10 μM) of each amplification primers, 1.5–2 μL of 25 mM $MgCl_2$, 2 μL of DNA (concentration: 4–6 ng/μL) and rest nuclease-free-water. The amplification profile for *S. mansoni* was initial denaturation at 95 °C for 10 min and 35 cycles at 95 °C for 30 s, 60 °C for 90 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. For *S. haematobium* the denaturing step was at 95 °C for 10 min, followed by 33 cycles of 95 °C for 30 s, 53 °C for 90 s, and 72 °C for 1 min, followed by a final extension step at 60 °C for 5 min. To confirm amplification and correct amplicon size, PCR products were visualized in 2% agarose gel stained with ethidium bromide (10 mg/μL) with 50 bp DNA marker (New England BioLabs Inc., Ipswich, MA, USA).

Eighty-six urine samples were investigated by PCR for both schistosome species DNA obtained from Qiagen miniprep. For *S. mansoni*, PCR was carried out by amplifying 110 bp fragment from a highly repeated 121 bp region of *S. mansoni* described by Hamburger (Hamburger et al., 1991). Primers for PCR amplification were described by Pontes et al. (Pontes et al., 2002). For *S. haematobium* 121 bp *Dra I* repeat fragment was amplified by primers previously designed by Hamburger et al. (Hamburger et al., 2001). In case of both schistosome species the repeat fragments comprise of 12–16% of each parasite genome (~600,000 copies per cell) occur in different region of