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Usefulness of environmental DNA for detecting *Schistosoma mansoni* occurrence sites in Madagascar

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ABSTRACT

Objectives: Schistosomiasis is an important disease in Madagascar, and several studies on the disease have focused on the occurrence of the parasite in humans. However, the range of the pathogen in the environment and its impact on human infection is difficult to predict. An environmental DNA (eDNA) detection system for *Schistosoma mansoni* was developed to improve schistosomiasis eco-epidemiology studies.
Methods: Primers and probes were designed and tested in experimental biotopes. The field study was conducted in Maevatanana District of Madagascar. Seven water sources with human use were sampled, with a total of 21 water samples collected. Snails were collected, and patients were examined by ultrasound to determine the occurrence of schistosomiasis in the study area.
Results: One water source with active transmission was identified through the detection of *S. mansoni* eDNA in the water and the intermediate host *Biomphalaria pfeifferi* collected from the same water source. People with clinical schistosomiasis were found in the area, reinforcing the findings.
Conclusions: The application of eDNA in eco-epidemiology enables the determination of hot spots and safe spots in endemic areas, constituting an alternative ecological tool for follow-up and monitoring of control programs for schistosomiasis, and contributing information on water safety for improving the standard of living of the people in endemic areas.

Introduction

Schistosomiasis is a waterborne blood fluke infection, and over 90% of affected people live in the countries of Sub-Saharan Africa, where there is poor access to clean water and sanitary facilities (Chitsulo et al., 2000; Adenowo et al., 2015). It is one of the leading causes of impaired health and socio-economic development in the world, and the control measures currently in use are based on large-scale preventive chemotherapy, as recommended by the World Health Organization (WHO) (WHO, 2012, 2013).

Schistosomiasis is considered endemic in Madagascar and it constitutes a major public health issue in 107 of the 114 districts of the country (Madagascar, 2016a). The north part and the west slope of the island have high transmission of *Schistosoma haematobium* (urogenital form); the east coast, central highland, and south part of Madagascar are endemic for *Schistosoma mansoni* (intestinal form) (Doumenge et al., 1987; Madagascar, 2016a,b). Maevatanana, one of three districts of Betsiboka Region, is particularly endemic for both intestinal and urogenital forms of schistosomiasis (Amat-Roze, 1978). Maevatanana has already benefited from three rounds of large-scale chemotherapy combining praziquantel and mebendazole, targeted at school-age children since 2011. Furthermore, a recent study through baseline prevalence of neglected tropical diseases (NTDs) sentinel sites showed that there is limited access to adequate water, sanitation, and

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hygiene facilities in the schools of western Madagascar (Madagascar, 2016a,b).

The prevention of schistosome infection of individuals can be directly related to the population's awareness of safe water sources (Chitsulo et al., 2000; Adenowo et al., 2015; Fenwick and Jourdan, 2016). However, determining the safety of water is a difficult task. This has been done in ecological surveys by searching for intermediate hosts snails and performing shedding tests for cercaria at each collection spot to determine the risk of infection (Wolmarans et al., 2002; King et al., 2006).

The analysis of environmental DNA (eDNA) as a technique for the detection of specific organisms has been performed previously in studies on the ecology, distribution, and phylogeny of cercozoa using soil samples (Bass and Cavalier-Smith, 2004). Furthermore, to improve surveying methods, eDNA has been used to detect the presence of amphibians and fish through the direct detection of specific DNA in water samples (Ficetola et al., 2008; Minamoto et al., 2012). More recently, the eDNA technique was successfully applied for the detection of the liver fluke *Opisthorchis viverrini* and its intermediate fish hosts in water of endemic areas (Hashizume et al., 2017). With regard to schistosomiasis, studies have been done for the detection of *Schistosoma japonicum* cercaria DNA in water samples (Hung and Remais, 2008; Worrell et al., 2011).

In this study, we developed a real-time PCR detection system by designing species-specific short-amplicon primers and a probe targeting the mitochondrial cytochrome c oxidase subunit I (COI) region of *S. mansoni*. After confirming the specificity and sensitivity of the assay, it was possible to detect *S. mansoni* eDNA in an endemic area of mixed infection in Madagascar, indicating that this system could be used as an alternative detection method in eco-epidemiology studies and surveillance in areas endemic for schistosomiasis.

Materials and methods

Obtaining eDNA from water samples

Purification of eDNA was performed using the DNeasy Blood and Tissue Kit (Qiagen), as described previously (Uchii et al., 2016), with some modifications. Briefly, water samples were filtered through glass fiber filter papers (GF/F, 0.7 μm; Whatman, UK) and fixed in 70% ethanol (Minamoto et al., 2016). Filter papers containing the filtered material were placed in Salivette tubes (Sarstedt, Germany), and 200 μl of Buffer AL and 20 μl of proteinase K were added. The tubes were incubated for 30 min at 56 °C and centrifuged at 5000 *g* for 3 min. One rinse step was done, adding 200 μl of Tris-ethylenediaminetetraacetic acid (TE) buffer directly onto the filter papers. After 1 min at room temperature, the tubes were centrifuged at 5000 *g* for 3 min, after which 300 μl of ethanol was added to the filtrated solution. The binding step was done by transferring 650 μl to a DNeasy column, followed by centrifugation for 1 min at 6000 *g*. The binding step was repeated for the entire filtrated solution. The subsequent steps were performed as recommended in the manufacturer's protocol, with an elution volume of 110 μl of Buffer AE. The purified eDNA was stored in a freezer at −25 °C until further use.

Primers, probes, and real-time PCR

The mitochondrial COI DNA sequences of 22 *Schistosoma* species were obtained from NCBI GenBank and a primer set and probe were designed for species-specific eDNA detection of *S. mansoni* using Primer Express Software 3.0 (Applied Biosystems, USA) with default settings. The forward primer, Sma-COI-F (5′-CAGGGGTTTCAAGTCTAATTGGAT-3′), and the reverse primer, Sma-COI-R (5′-CAAATAATAACATCGTTATTCCTCTGG-3′), were designed for partial amplification of the *S. mansoni* COI with a predicted designed amplicon size of 162 bp. The TaqMan MGB probe (Thermo Fisher Scientific, Waltham, MA, USA) was designed as Sma-COI-P (5′-FAM-TTCAAATGTTCGATAATA-NFQ-MGB-3′). The specificity of the primers/probe was then tested in silico in comparison with other organisms using BLAST.

To determine specificity, the newly designed real-time PCR assay (Sma-COI) was conducted to specifically distinguish *S. mansoni* from *S. japonicum* and *Schistosoma mekongi* using DNA obtained from adult worms. Real-time PCR was performed with a StepOnePlus thermocycler (Thermo Fisher Scientific). The reaction was performed in a 20-μl final volume containing 10 μl of 2 × Taqman Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.1 μl of AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific), 2 μl of DNA template (including 100 pg of total DNA derived from worms), 900 nM each of the Sma-COI-F/R primers, and 125 nM of the Sma-COI-P probe. The PCR conditions were 2 min at 50 °C and 10 min at 95 °C as initial steps, followed by 55 cycles of 15 s at 95 °C and 60 s at 60 °C. All reactions were conducted with three replications.

PCR and sequencing

PCR reactions were done as described previously in 20-μl volume reactions with SMA-COI-F and SMA-COI-R primers and using 2 μl of sample DNA as template. One positive control (tissue-derived DNA) and a negative control were included in each batch of tests. The PCR was performed in a StepOnePlus thermocycler (Thermo Fisher Scientific) with the following conditions: 50 °C for 2 min, followed by 95 °C for 10 min and 55 cycles of 95 °C for 15 s, 60 °C for 1 min. PCR for other species of *Schistosoma* was performed as described by Kato-Hayashi et al. (2010). After PCR, electrophoresis was performed at 100 V for 60 min using 2% agarose gel. The PCR products were purified and directly sequenced using an ABI3730XL sequencer (Applied Biosystems, USA) at Macrogen Inc. (Korea) and at Dokkyo Medical University (Japan).

Detection of eDNA in S. mansoni *experimental infection water samples*

Water was collected from aquariums (30 cm length × 20 cm width × 20 cm height) containing *Biomphalaria glabrata* snails infected and not infected with *S. mansoni*. Briefly, *S. mansoni* eggs were recovered from experimentally infected mice and hatched as described by Standen (1951). Snails were placed in 24-well microplates and each snail was infected overnight at 25 °C with five miracidia collected by pipetting under stereomicroscope observation. Non-infected snails were from the same breeding batch as the snails used for *S. mansoni* infection.

Two aquariums containing 10 liters of water and 20 infected snails each were used to determine the capability of detection of *S. mansoni* eDNA in water samples. Water samples were also collected from two aquariums containing non-infected snails under the same conditions. The water in all aquariums was changed weekly, and the collection for this study was done 24 h after a water change, 60 days after infection of the snails.

A total of 250 ml of water was collected from each aquarium, to which 250 μl (1:1000 v/v) of 10% benzalkonium chloride solution (w/v) was added to each sample to prevent DNA degradation (Yamanaka et al. 2017). All samples were filtered using a glass fiber filter (GF/F, 0.7 μm; Whatman, UK). After water filtration, 10 ml of 70% ethanol was passed for fixation, and the filter papers were dried, wrapped in aluminum foil, and stored in a freezer at −25 °C. DNA trapped on the filters was extracted using the method described above.

Field trial of Schistosoma eDNA detection in Madagascar

Location of the study

This study was performed in Maevatanana District (Figure 1). With a totalarea of 10 752.2 km^2, Maevatanana District is composed of 17 communes, which range in area from 33.1 km^2 to 1670.5 km^2. Communes are at an average altitude of 282.1 m, with a lower average altitude of 30 m in Ambalajia and a highest average altitude of 918.9 m in Mahatsinjo. The average temperature in Maevatanana District is 35.6 °C during the day and 20.7 °C at night, with a minimum average reading of 16.0 °C. The average precipitation in this district is 1215 mm per year, varying from 1065 mm to 1337 mm (IUNC/UNEP/WWF, 1987; WMO, 1996; IRI, 2006). According to the latest data, the precipitation trend has remained constant despite minor fluctuations from year to year (IRI, 2006).

Considering Maevatanana District as a mixed infection endemic area with reported cases of *S. mansoni* and *S. haematobium* (Amat-Roze, 1978; Madagascar, 2016a,b), this study focused on the two different communes of Ambalanjanakomby and Ampisavankaratra, mixed endemic areas for *S. haematobium* and *S. mansoni*, respectively, according to local health center data.

Environmental sampling in Madagascar

Water samples were collected from seven different water sources in Ambalanjanakomby and Ampisavankaratra communes in June 2016. The water collection spots are indicated in Figure 1 and are located at the following coordinates: 16°41′33.91″S 47°3′57.19″E, 16°41′30.77″S 47° 3′0.15″E, and 16°41′31.90″S 47° 3′23.58″E (collection spots 1, 2, and 3, respectively, from three water sources in Ambalanjanakomby village, with a total of nine samples), and 17°27′24.99″S 46°58′28.67″E, 17°27′26.78″S 46°58′27.22″E, 17°27′10.54″S 46°57′47.08″E, and 17°27′11.10″S 46°57′46.59″E (collection spots 4, 5, 6, and 7, respectively, from four water sources in Ampisavankaratra village, with a total of 12 water samples). The sampling locations were determined using a wireless Holux M-241 GPS logger (Holux, Taiwan) and an eTrex Vista HCx GPS receiver (Garmin, USA). Water temperature and pH were estimated using a portable LAQUAact D-74 multimeter (Horiba, Japan). For each sample, 500 ml of water was collected from at least three different points of the water source. All samples were filtered using a glass fiber filter (GF/F, 0.7 μm; Whatman, UK), and 10 ml of 70% ethanol was passed for fixation after filtration.

A snail survey was performed at the same points as the water collection, using the fractional technique, for a limited time; snail scoops were used and the sampling time was fixed at 30 min. The collected snails were identified in the Helminthology Unit of Institut Pasteur de Madagascar.

Clinical and ultrasound examination

To confirm active schistosomiasis cases occurring in the sampling area, a total of six patients from Ambalanjanakomby, four from Maevatanana, and seven from Ampisavankaratra presented voluntarily at each corresponding health center and

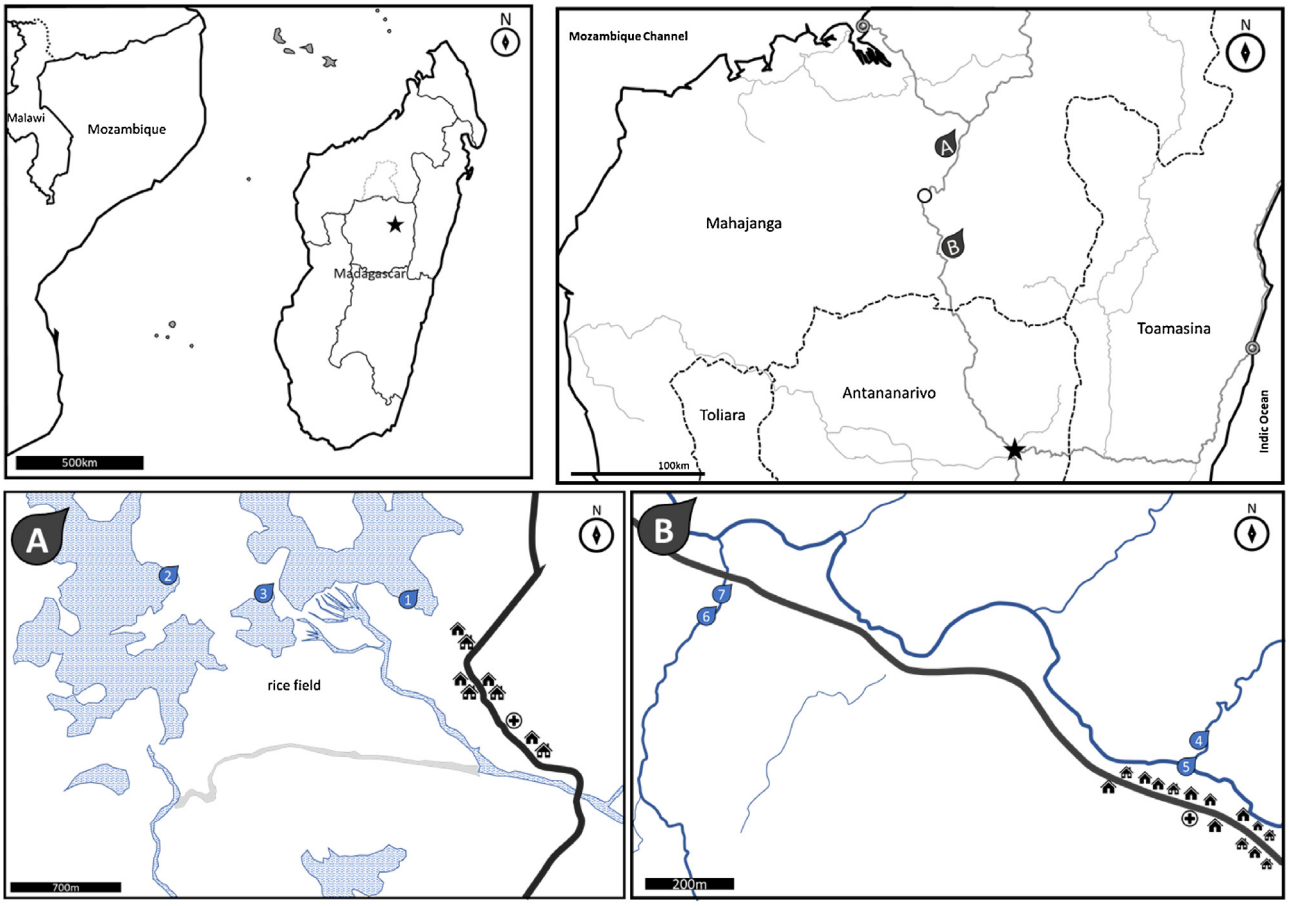

Figure 1. Madagascar and a detailed map of the water sampling spots. The upper left map shows Madagascar, divided politically into six provinces (thin black lines). The area surrounded by the dotted gray line represents Maevatanana District, where this study was performed. Antananarivo, the capital city, is represented as a black star in the upper left and right maps. In the upper right map, the gray lines correspond to the main road network, and the city of Maevatanana is represented as a closed white circle. The dotted lines are the province limits, with the capital cities Toamasina and Mahajanga represented with donut marks in the respective provinces. Lower left and right maps (drop A and B) show the water bodies, the villages (houses) with the health centers (represented as a cross in a circle), and the points of collection in this study, numbered from 1 to 7.

were examined. A full explanation of the objectives and methodology of this study and the use of information, including the opt-out possibility at any stage of the study, was given in the Malagasy language for a complete understanding. The ultrasonography examination was performed using a portable Vscan device (GE Healthcare, USA). The history and grade of the disease were recorded and paired with the observed imaging data. The image diagnosis was performed according to the clinical morbidity: acute, chronic, urinary, intestinal, hepatosplenic, and ectopic schistosomiasis, following the latest standardization and reviewed classification (Richter et al., 2000; Sah et al., 2015).

Data analysis

All data collected on paper forms were tabulated and analyzed in Excel 2016 (Microsoft) and EpiInfo version 7.1.5.0 (CDC, Atlanta, USA). GPS data were collected and analyzed using Holux logger utility software and Google Earth Pro version 7.1.5.1557.

Results

Schistosome eDNA detection in experimentally infected snail aquarium water and the ecological survey in Madagascar

Each designed primer/probe set (Sma-COI-F, Sma-COI-R, Sma-COI-P) was tested in PCR using *S. mansoni* genomic DNA samples. DNA amplification was performed in three repetitions. The expected amplicon of 162 bp was successfully obtained. Confirmation certifying species-specific amplification was done using non-target *Schistosoma spp* DNAs as templates or negative controls with no amplification, as shown in Figure 2A. eDNA was obtained from snail aquarium water samples, and the detection of *S. mansoni* was tested for each aquarium. Real-time PCR could correctly discriminate the aquariums with snails infected with *S. mansoni* from the aquariums containing non-infected snails, which presented no signal, as shown in Figure 2B.

Water collection in Madagascar was performed at seven points (Figure 1) for a total of 21 water samples. All collection spots were confirmed to be water sources with human use. The pH and temperature of the collected water were 7.7/24.8 °C, 9.8/25.9 °C, and 9.0/26 °C at points 1, 2, and 3, respectively, of Ambalanjanakomby, and 8.5/19.7 °C, 8.25/19.4 °C, 8.28/19.6 °C, and 8.43/19.9 °C at points 4–7 in Ampisavankaratra.

One out of 14 environmental water samples was positive in the Sma-COI real-time PCR assay. The positive sample was from sampling site number 5 (Figure 1), a small river behind some community houses. This water source was used as a place for washing and cleaning domestic utensils and clothes, and for leisure. The amplicon was sequenced and confirmed to be *S. mansoni*.

Schistosomiasis and the snail hosts in the study area

All 17 patients examined were residents of Maevatanana District and described a history compatible with schistosomiasis, confirming the information provided by the local health agents of the occurrence of the disease in the area. The ultrasonography examinations revealed typical lesions of chronic schistosomiasis infection in the cases examined. It was seen that advanced stages of schistosomiasis are present in Maevatanana District.

The snail survey was performed at the water sources used by the community for washing, cooking, and drinking. The presence of *Biomphalaria pfeifferi* snails was detected at sample spot number 5 in Ampisavankaratra (Figure 1B). There were no snails at any of the sample spots of Ambalanjanakomby Commune water sources (Figure 1A).

Discussion

Usefulness of eDNA in schistosomiasis surveillance

The provision of a safe water supply and education of people on behavioral changes are the key factors in schistosomiasis control (Rollinson et al., 2013; Adenowo et al., 2015). Additionally, ecological surveys are necessary to determine the safety (or risk) of water sources. Ecological surveys of trematodes in the environment are based on the collection of intermediate hosts and performance of shedding tests with snails to determine locations with transmission of the disease (Phongsasakulchoti et al., 2005; King et al., 2006; Kiatsopit et al., 2012). In this study, the same characteristic of host dependence/disease was observed using the eDNA detection survey. The positive sample was collected at the same place where *B. pfeifferi*, the local intermediate host for *S. mansoni*, was found.

Climate change, urbanization, and the shift in land use (extractivism, to cropping, to pasture, and so on) have a direct impact on the habitat of the snails – the intermediate hosts of schistosomiasis (Tanaka and Tsuji, 1997). These changes are happening faster and it is important to determine how this is interfering in the patterns of diseases like schistosomiasis. Thus,

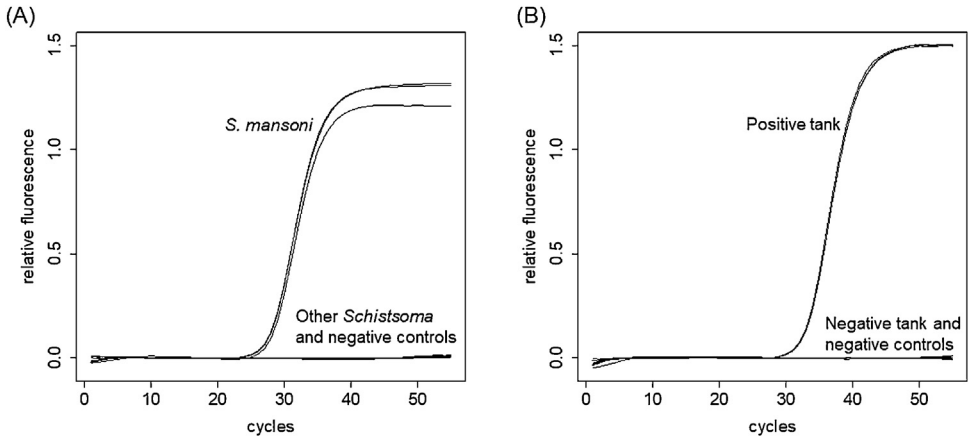

Figure 2. The determination of specificity was done using *Schistosoma mansoni* and other *Schistosoma* species, using the designed primers/probe on the left side (A) where a specific signal was observed. Tests of aquarium water by qPCR, on the right side (B), show the capability of the Sma-COI real-time PCR system for use on eDNA from water samples, with a specific signal successfully detected in the infected snail aquarium (positive tank).

rapid and low-cost survey methods are required to provide timely information in endemic areas for an integrated disease prevention system (Hashizume et al., 2017; Sato et al., 2018). As such, eDNA analysis could be an interesting alternative. This method can be applied for ecological surveys of different organisms and has been reported to be a useful ecological tool for detecting target pathogen DNA, reducing time and costs (Rees et al., 2014; Hashizume et al., 2017; Minamoto et al., 2009, 2012, 2016; Pitula et al., 2012; Hyman and Collins, 2012; Huver et al., 2015; Hall et al., 2015). The use of this method for the detection of cercaria DNA in water samples for schistosomiasis studies has also been reported (Hung and Remais, 2008; Worrell et al., 2011). The detection of eDNA constitutes a feasible technique in endemic areas to determine the safety of water sources for human use.

The primers for the detection of *Schistosoma spp* in environmental samples developed in this study target the COI gene of mitochondrial DNA. This gene is often used for gene barcoding and with diagnostic objectives (Kato-Hayashi et al., 2010, 2015), with products from 254 bp to 614 bp in PCRs. For eDNA samples, because of the degradation occurring under environmental conditions, primers are generally designed to yield short amplicons targeting high copy number genes, enhancing the detection level of the assays (Huver et al., 2015; Strickler et al., 2015). The primer set developed in this study can be considered highly versatile for application on different sources of material, including non-environmental samples. The system was able to detect species of schistosomes using genomic DNA and field environmental water samples, demonstrating its practical value, and it was successful in detecting *S. mansoni* from eDNA of water sources in an endemic area of Madagascar (Figure 1B). It is important to note that positive samples were detected only at the spot where *B. pfeifferi* snails were found, whereas schistosomiasis patients were confirmed in both Ambalanjanakomby and Ampisavankaratra communes. Interestingly, despite the presence of patients with an ultrasound diagnosis of *S. haematobium*, no *Bulinus spp* snails were found in the field. Also, the PCR tests could not detect *S. haematobium* eDNA in the water samples.

This study is the first field trial using eDNA detection for schistosomiasis. The technique presented promising results, and it could be used for accurate discrimination of dangerous waters and moreover to determine safe water for human use. It could also be used as a tool to follow up the impact of all eventual efforts at community behavior change. However, to ensure that its application is effective, the system needs to be tested in further field studies, in wider areas, and compared with the current conventional methods.

Schistosomiasis in Madagascar and the situation in Maevatanana District

In Madagascar, the Ministry of Health established the National Program against Bilharzia in the mid-1980s, with the aim of reducing the prevalence to less than 20% in each hyper-endemic village, prevent the occurrence of severe complications, and to reduce reinfection (Madagascar, 2002). However, a recent study showed persisting hyper-endemic areas for *S. mansoni* and *S. haematobium* (Madagascar, 2016b). Starting in 2012, free mass drug treatment (praziquantel and mebendazole) of school-age children was started in Maevatanana with the worldwide initiative on an integrated approach to NTDs; however, there is a lack of information on the results of each session of mass drug administration (Dr Rafalimanantsoa, personal communication). We observed chronic and acute schistosomiasis cases in the patients in this study, showing that the problem persists under the current adopted control strategies.

Ultrasonography is an important imaging tool in the diagnosis of schistosomiasis, providing information on the lesions in the target tissues and their pattern, and delineating the possible prognosis after treatment; moreover it is a low-cost, non-invasive, and portable system (Sah et al., 2015; Abdel-Wahab et al., 1992). With a portable ultrasound, it was possible to examine patients in areas without electricity, constituting a great support for diagnosis. All of the patients examined presented typical lesions of chronic schistosomiasis infection, showing advanced stages of complicated cases of schistosomiasis in Maevatanana District, despite years of mass drug administration.

In Maevatanana District, monitoring of freshwater snails (intermediate hosts) and their environment has not yet been conducted. There is no information on the seasonality, transmission, or habitats of Schistosoma transmission in this specific region. This initial study represents a recommencement of eco-epidemiology in endemic areas in Madagascar using a newly developed eDNA technique. The eDNA results can help to determine safe/unsafe water sources, as demonstrated in previous food/waterborne pathogen studies (Hashizume et al., 2017; Jones et al., 2018). Considering that Betsiboka is a farming region and that more than 80% of men and women in Maevatanana work in agriculture, the imminent risk of infection exists in their daily life. The prevention of food/waterborne diseases requires education and sanitation (Tomokawa et al., 2018; Takeuchi et al., 2013; UNICEF, 2009). However, according to EDSMD 2008–2009 EDSMD, 2010EDSMD 2008–2009, 23.6% of people in Betsiboka region have no access to a school education and 58% cannot finish primary school. In rural areas of Maevatanana region, only 1% of households are equipped with a sanitary toilet and 48% have no kind of toilet (EDSMD 2008–2009EDSMD, 2010EDSMD 2008–2009), indicating that environmental contamination by schistosomes or other NTDs occurs continuously. A scale-up field survey should be conducted to map areas of occurrence of the parasite, make people aware of the infection, and generate positive information to improve the health of the people, not only those living in the endemic areas, but also tourists, workers, and people passing through these areas (Sato et al., 2003, 2006; Hashizume et al., 2017).

Over 50% of the population of Madagascar is infected with intestinal or urinary schistosomiasis (Rollinson et al., 2013). The disease slowly debilitates infected persons, who are frequently children, resulting in lower productivity and reduced learning in school-age children. This situation leads to a vicious cycle, perpetuated by several environmental, psychological, and social factors. A multifaceted approach, involving mass treatment, biological control, environmental control, education, and disease surveillance, could lead to schistosomiasis control and elimination in Madagascar. Pathogen-specific eDNA detection, as shown in this study, could contribute as an accurate surveillance tool to be applied to NTDs in the field, helping to establish intelligent control programs for NTDs and contributing to food and water safety for those people living in endemic areas.

Conclusions

In this study, we succeeded in developing specific and shorter amplification length primers targeting the COI region of mitochondrial DNA of *S. mansoni*. The detection of *S. mansoni* DNA in environmental water by applying an environmental DNA analysis was performed successfully. Thus, a new method for eco-epidemiology studies is presented, which could be used as an additional tool in control programs for schistosomiasis in endemic areas.

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Ethical approval

This study was approved by the Ministry of Public Health Madagascar (Official Referenced Letter No. 220 MSANP/SG/DGS – 2016). The animal experiments in this study were approved by the Ethics Committee of Dokkyo Medical University (Document Number 0006/2001).

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Conflict of interest

No competing interest declared.

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