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A new surveillance and response tool: Risk map of infected *Oncomelania hupensis* detected by Loop-mediated isothermal amplification (LAMP) from pooled samples

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ABSTRACT

Although schistosomiasis remains a serious health problem worldwide, significant achievements in schistosomiasis control has been made in the People's Republic of China. The disease has been eliminated in five out of 12 endemic provinces, and the prevalence in remaining endemic areas is very low and is heading toward elimination. A rapid and sensitive method for monitoring the distribution of infected *Oncomelania hupensis* is urgently required. We applied a loop-mediated isothermal amplification (LAMP) assay targeting 28S rDNA for the rapid and effective detection of *Schistosoma japonicum* DNA in infected and prepatent infected *O. hupensis* snails. The detection limit of the LAMP method was 100 fg of *S. japonicum* genomic DNA. To promote the application of the approach in the field, the LAMP assay was used to detect infection in pooled samples of field-collected snails. In the pooled sample detection, snails were collected from 28 endemic areas, and 50 snails from each area were pooled based on the maximum pool size estimation, crushed together and DNA was extracted from each pooled sample as template for the LAMP assay. Based on the formula for detection from pooled samples, the proportion of positive pooled samples and the positive proportion of *O. hupensis* detected by LAMP of Xima village reached 66.67% and 1.33%, while those of Heini, Hongjia, Yangjiang and Huangshan villages were 33.33% and 0.67%, and those of Tuanzhou and Suliao villages were 16.67% and 0.33%, respectively. The remaining 21 monitoring field sites gave negative results. A risk map for the transmission of schistosomiasis was constructed using ArcMap, based on the positive proportion of *O. hupensis* infected with *S. japonicum*, as detected by the LAMP assay, which will form a guide for surveillance and response strategies in high risk areas.

Introduction

Schistosomiasis is a neglected tropical disease and remains a serious health problem, affecting more than 200 million people worldwide (Steinmann et al., 2006). The World Health Organization (WHO) identified schistosomiasis as the second most important human parasitic disease in the world, after malaria (Croft et al., 2003). The causative organism, *Schistosoma japonica*, is a zoonotic parasite prevalent in Asia, including the People's Republic of China (PR China), Indonesia and Philippines. In PR China, *S. japonicum*, and its intermediate snail host, *Oncomelania hupensis*, are distributed along the middle and lower reaches of the Yangtze River, especially around southern tributaries, lakes and marshlands, and in some hilly and mountainous regions of Southwestern China (Liang et al., 2006; Li et al., 1999). Control efforts over the last 60 years have effectively controlled most infections in endemic areas; for instance, the disease has been eliminated in five out of 12 endemic provinces, including Fujian, Guangdong, Guangxi, Shanghai and Zhejiang provinces. The remaining endemic areas, located in lake regions of Hunan, Hubei, Jiangxi, Anhui, Jiangsu provinces and in mountainous regions of Sichuan, Yunnan provinces, have shown very low prevalences during last 5 years (Collins et al., 2012). For example, three provinces, Sichuan, Yunnan and Jiangsu, achieved

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the national criteria of transmission control by the end of 2010, which indicated that prevalence of schistosomiasis in all three provinces was less than 1% (Li et al., 2009; Hao et al., 2009, 2010; Lei et al., 2011). By the end of 2010, 32854 *S. japonica* infections had been reported, representing a reduction of 10.92% compared with those in 2009. Among these, only 43 acute cases were reported, representing a decrease of 44.16% over the number of acute cases in 2009 (Lei et al., 2011). However, schistosomiasis control is still vital, especially in regions with lakes and marshlands, where a large area of schistosomiasis transmission risk remains present. To achieve further reduction of the risk of human infection in this background of low prevalence, real-time surveillance and response systems are required to monitor and predict the risk areas of schistosomiasis transmission (Bergquist and Whittaker, 2012; Zhou et al., 2013). These surveillance and response systems require an innovative tool to rapidly locate infected snails so that they can be controlled using molluscicides in these areas, using a multidisciplinary approach, to reduce the risk of human infection through water contact (Zhou, 2012). The innovative tool should be affordable (require less expensive, simple equipment), sensitive, specific, user-friendly, rapid and deliverable to be used in the diseases control program in the field (Mabey et al., 2004; So and Ruiz-Esparza, 2012).

Molecular techniques, such as conventional polymerase chain reaction (PCR), are capable of detecting schistosomal DNA in samples obtained from snails, feces and vaginal lavage (Standley et al., 2010; Oliveira et al., 2010; Kumagai et al., 2010; Gomes et al., 2010; Kjetland et al., 2009). Real-time PCR and nested-PCR have also been established to detect *S. japonicum* infections (Lier et al., 2008; Tong et al., 2009). Although PCR-based assays provide excellent sensitivity and specificity, their dependence on expensive apparatus, professional skill and long reaction times restrict their widespread application for clinical diagnosis. Therefore, cost-effective, simple, and rapid detection methods need to be developed for the diagnosis of schistosomiasis.

Loop-mediated isothermal amplification (LAMP) is a rapid, simple and sensitive technique developed by Notomi et al. (2000), which does not require expensive equipment. This method enables the amplification of a few copies of DNA to 10^9 copies in less than 1 h under isothermal conditions. The amplification products can be observed by the naked eye. The LAMP reaction requires only a single enzyme, *Bst* DNA polymerase, which synthesizes a new strand of DNA while simultaneously displacing the former complementary strand, thereby enabling DNA amplification at a single temperature. Four primers are required for the LAMP reaction, namely, FIP (Forward Inner Primer), BIP (Backward Inner Primer), F3 (Forward outer primer 3), and B3 (Backward outer primer 3).

LAMP assays have been developed to detect parasites, including *Plasmodium* spp. (Han et al., 2007; Poon et al., 2006), *Trypanosoma* spp. (Thekisoe et al., 2007; Njiru et al., 2010), *Leishmania* spp. (Adams et al., 2010; Takagi et al., 2009), *Taenia* spp. (Nkouawa et al., 2009, 2010), *Dirofilaria immitis* (Aonuma et al., 2009), *Toxoplasma gondii* (Zhang et al., 2009a,b; Kong et al., 2012), and *Angiostrongylus cantonensis* (Chen et al., 2011). For *Schistosoma* spp., LAMP assays have been used to detect *S. mansoni*, *S. haematobium* and *S. japonicum* DNA by targeting Sm1-7, DraI and 28S rDNA, respectively. These LAMP assays are sensitive enough to detect infection from the first day after exposure to miracidia, which permits monitoring of prepatent snails (Abbasi et al., 2010). These LAMP assays could be adapted for application in field laboratories (Hamburger et al., 2013). LAMP assays targeting the SjR2 and 28S rDNA to detect *S. japonicum* DNA have been reported by Xu et al. (2010) and Kumagai et al. (2010). In addition to the above-mentioned applications, LAMP assays have also for detection from pooled samples, for example, to detect *Wuchereria bancrofti* and influenza viruses. LAMP detected *W. bancrofti* DNA in a mimic pooled sample of 60 *Culex* spp. mosquitoes containing 100 μL human blood and one microfilaria (Mf) (Takagi et al., 2011). Using pooled RNA samples extracted from influenza viruses corresponding to all 15 HA subtypes (in addition to other avian pathogenic viruses), the RT-LAMP system only amplified H7 AIV RNA (Bao et al., 2012).

Technological developments in geographical information systems (GIS) have provided scientists with the opportunity to develop risk maps of parasitic diseases, including malaria, schistosomiasis, trypanosomiasis and leishmaniasis, resulting in better designed and refined surveillance-impact strategies and early warning systems (De la Rocque et al., 2001; Bavia et al., 2005; Noor et al., 2012. Scholte et al., 2012; Yang et al., 2012; Omumbo et al., 2013; Tsegaw et al., 2013).

Based on a previously published LAMP assay (Kumagai et al., 2010) that targeted 28S rDNA, the present study aimed to apply LAMP assay to pooled samples in the field to provide a rapid and effective method to detect *S. japonicum* DNA in field-collected *O. hupensis*. This new assay will aid the establishment of a surveillance and response system toward schistosomiasis elimination in PR China. The risk map presented here will be useful for the spatial targeting of schistosomiasis control interventions and future efforts focusing on schistosomiasis elimination.

Materials and methods

Parasites

Clonorchis sinensis, *Paragonimus westermani*, *Schisotosoma mansoni* and *Angiostrongylus cantonensis* were prepared in the Zhejiang Academy of Medical Sciences, PR China. Three female New Zealand rabbits, weighing approximately 3 kg, were infected with 1000 *S. japonicum* cercariae for 45 days, anesthetized with Pelltobarbitalum Natricum. Adult worms were collected from the rabbits by perfusing their portal veins with physiological saline. The Institutional Animal Care and Use Committee (IACUC) approved the use of the animals.

Snails and pooled samples

From April to August 2010, *O. hupensis* snails were collected from 28 field sites in five endemic provinces, including Anhui, Hubei, Hunan, Sichuan, and Yunnan, in PR China. Two groups, e.g. individual and pooled samples, were prepared before DNA detection. For individual detection, the collected snails were crushed individually with tweezers classified as infected ones if cercariaes of the parasite were identified under a light microscope. For pooled sample detection, the maximum pool size (MPS) was calculated based on the following pooled sample formula (Gu, 1998):

$$1 - (1 - r)^n - nr(1 - r)^{n-1} < \alpha.$$

where r is the estimated infection rate of the snails, n is the MPS and α is the significant level of detection. Our previous data showed that r is approximately 0.26% (Dang et al., 2006), and α is 0.01; thus, n is about 50. Therefore, 50 snails were pooled and crushed together as one group for each site.

DNA extraction

Genomic DNA of *S. japonicum* from adult worms was extracted using a QIAmp DNA mini kit (QIAGEN, Dusseldorf, Germany), according to the manufacturer's instructions, and the concentration of DNA was measured by NanoDrop spectrometry (Thermo Fisher Scientific, MA, USA). DNA derived from *P. westermani*, *C. sinensis*, *S. mansoni* and *A. cantonensis* was used to confirm the specificity of the LAMP assay. To detect schistosomal DNA from snails, the

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