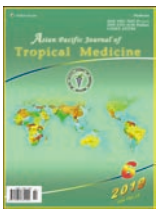

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Laboratory diagnosis of schistosomiasis mansoni: Current status and future trends

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ABSTRACT

Schistosomiasis is a neglected tropical disease that affects about 290 million patients worldwide. Children aged between 5 and 14 years represent 45.8% of the affected patients, in addition, schistosomiasis has been reported in *Schistosoma*-free areas, mostly because of tourism and immigration from endemic countries. Intestinal schistosomiasis caused by *Schistosoma mansoni* is mainly diagnosed *via* direct stool examination for egg detection. Immunological methods are favoured for disease monitoring and preliminary checking for communities in areas with low infection rates, and for patients with light and chronic infections where parasitological tests are negative. PCR-based diagnostic techniques are more sensitive, but expensive. Tegument proteins and miRNAs are promising markers for diagnosis of schistosomiasis. Here we review the diagnostic methods for schistosomiasis mansoni aiming to reach a standardized technique for diagnosis of early infection to help better control of the disease.

1. Introduction

Schistosomiasis caused by *Schistosoma* species is an important neglected tropical disease that attracts more attention due to its chronic nature, association with water resources, and lack of a protective vaccine. It is dominant in rural poor communities among agriculture and fishing workers, moreover, contact with infected water as in car and clothes washing, as well as recreational activities increases the risk of infections. Out of the Six *Schistosoma* species affecting humans, *Schistosoma(S.) mansoni* is responsible for intestinal schistosomiasis in African countries. Nearly, 54 million of infected cases are present in sub-Saharan Africa, while 393 million are at risk[1]. The emergence of schistosomiasis in low endemic or even non-endemic areas can be facilitated by movement of population to urban areas, international tourism, increase in migration, international cooperation programs and environmental changes[2]. Therefore, physicians should consider the likelihood for this parasitic infection in new parts of the globe.

Early diagnosis of schistosomiasis in endemic areas is crucial for patient management, evaluation of treatment efficiency and monitoring of control programs. In non-endemic parts, schistosomiasis diagnosis at the individual level by using more accurate diagnostic test is very essential not only for a curative treatment but also for preventing expansion of *Schistosoma* in new areas. Additionally, as there is no affordable sensitive and rapid standard test for schistosomiasis, there is an eminent need for a gold standard technique with high sensitivity to which newly developed diagnostic tests can be compared[3].

Herein, we review the current diagnostic techniques for *S. mansoni* as it accounts for the majority of intestinal schistosomiasis in Africa with emphasis on recently developed techniques and new diagnostic markers.

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2. Direct parasitological diagnosis

Examination of stool samples for egg detection is a common utilized method for species identification and recording infection intensity, but it is not suitable for diagnosis of acute disease since egg deposition starts 4-6 weeks post infection(PI). The WHO recommended the Kato-Katz (KK) thick stool smear technique for diagnosis at community level. This technique is highly specific and simple; by microscopic examination of fresh stool sample (41.7 mg) cases with a minimum of 20 eggs/g can be detected. The KK is inexpensive (US$0.1–0.3 for a KK kit) and easily used in field studies[4] to assess the impact of control programs by measuring the changes in the prevalence and infection intensity in post-treatment surveys where mass drug administration using praziquantel is employed. However its poor sensitivity for detecting light intensity infections causes substantial variation in results and in turn alternative techniques are being tested[5,6]. The variability in the test results are due to the daily fluctuation in egg excretion, uneven distribution of eggs in the stool sample and the level of experience of technicians. The sensitivity of KK can be improved by using replicate samples on different days, besides, several concentration techniques were developed to improve the microscopic examination of stool such as formol-ether sedimentation, salt flotation and centrifugation, and the interaction of magnetic microspheres with eggs[7].

FLOTAC is a flotation technique with higher sensitivity than KK[8] but it needs specific equipment, specific flotation solution (Zinc sulphate for *S. mansoni* eggs) and suitable preservative medium, which can affect the test[9].

Also, a Mini-FLOTAC (Prof. Giuseppe Cringoli, University of Naples Federico II, Naples, Italy) was developed as a quick and low cost method, which can be used in developing countries[8]. The new Parasep feces centrifuge tube method showed better results than the KK and Mini-FLOTAC due to large stool sample (0.5 g) and using ethyl ether, which removes fat and organic compounds from the sample leading to a clearer field[10].

Helmintex is another method developed for improving sensitivity of egg detection. This technique promotes the isolation of *S. mansoni* eggs by using magnetic microspheres which form conjugates between the parasitic eggs and magnetic microspheres since iron is generally present in the eggshell pores[11] but other factors as electrostatic forces between the magnetic particles and the surface ornamentation of the eggs may play a role. Helmintex allows examination of a larger sample (30 g), thus increases the diagnostic sensitivity[11]. Although the recent modification for Helmintex significantly decreased the examination time[12], and the overall cost, it is still expensive and not suitable for field studies, yet can be used for evaluation of other tests[13].

The viability of the detected eggs can be assessed by a hatching test. A miracidia hatching apparatus was developed based on the potent positive phototropic behaviour of the miracidia[14]. Although the test is simple, cheap, useful for diagnosis and improving the diagnostic sensitivity[14], hatching can be affected by temperature and quality of water and is not suitable for routine, large-scale screening or in people with light infections[15].

3. Immunological diagnosis

Immunological methods are relatively simple, more sensitive and essential during early stages of infection, in cases with fibrosis, or decreased infection prevalence and intensity as after praziquantel administration, and for initial screening at point-of-care facilities[16].

3.1. Antibody detection

Detection of antibodies against different *Schistosoma* stages in different human samples is important for diagnosis of infection in low endemic areas, in children with initial active infection, and in travellers returning from endemic areas. Antibody detection tests remain positive for years after successful treatment, so combination of serological tests and/or using different antigens enable differentiation between active and chronic disease[17]. Also, antibody detection is not suitable for reporting infection intensity, and usually there is cross-reactivity with other helminths as the antigen used is a crude extract of schistosomes leading to decreased test specificity[18]. Available antibody-based assays include the circumoval precipitin test that depends on the development of precipitate after incubation of the serum of a patient suffering from schistosomiasis with lyophilized Schistosoma eggs[18]. The test is positive if there is more than 9% of precipitation around the mature eggs[16]. Circumoval precipitin test is highly sensitive and specific and has been used with copro-microscopy to increase the diagnostic accuracy, but it is laborious, complex with long reaction time for sero-conversion post-treatment[16].

Cercarien Hüllen reaction is another antibody detection test, where live cercariae are incubated with the serum of an infected patient, then a precipitate is formed and cercariae become immobilized. Currently, it is not used because of several disadvantages. The test is complex, time consuming and remains positive several years after successful treatment leading to misdiagnosis of cleared infection[19].

The indirect hemagglutination test is commonly used for routine diagnosis of schistosomiasis. It detects reactivity between antibodies and red blood cells (human O-type) coated with schistosomal antigen. The test is simple, highly sensitive, used in large-scale surveys, and as a monitoring method in schistosomes-endemic areas, but it has the same disadvantages of antibody detection tests[20].

The indirect immunofluorescence assay detects the reaction between parasite antigens and anti-schistosomal antibodies in patient serum or other body fluids. The test is highly sensitive, especially in low-prevalence areas. Paraffin sections of adult worms can be used to detect antibodies against gut-associated antigens, eggs and cercariae in acute and chronic infections[21]. Immunofluorescence assay can be used with other diagnostic methods to improve its diagnostic value, however, the need for costly reagents, microscopes and technical expertise limits its use in community surveys[19,22].

Enzyme-linked immunosorbent assay (ELISA) is widely used, yet crude antigens used have low sensitivity and may exhibit cross-reactivity, so purified and recombinant antigens are preferred[23].

Soluble egg antigen (SEA/ELISA) was widely used and anti-SEA IgG4 was used as biomarker for detecting schistosomiasis in endemic areas. The purified cationic egg fraction 6 proved more sensitive and useful in detecting praziquantel efficacy especially in children but with low specificity[24]. When fractionated SEA was used in ELISA (FA-ELISA), it was able to detect current infection with low cross reactivity with other parasites[25]. The soluble cercarial antigen of *S. mansoni* was detected in serum from early infection using ELISA[26]. Cercarial antigen was also used in rapid card diagnostic tests, which were reported as sensitive as duplicate KK, and can be an alternative for urine circulating cathodic antigen (CCA) in diagnosis of schistosomiasis in endemic areas[27]. When used with ELISA, schistosomula tegument was better in diagnosis of acute infection than adult worm antigen particularly in non-endemic areas[28]. Yet, using *S. mansoni* microsomal antigen (MAMA) was better. As modification of conventional ELISA, the modified Falcon assay screening test used adult worm antigen gave similar specificity and sensitivity as conventional tests but in short time[29].

The keyhole limpet hemocyanin (KHL) is a protein extracted from the marine mollusc *Megathura crenulata*. KHL can be used to differentiate between acute and chronic schistosomiasis using ELISA[30], as a shared carbohydrate epitope between this protein and tegumental antigens from *S. mansoni* schistosomula has been reported[30]. Although KHL represents an attractive cheap, stable and commercially available antigen for large-scale screening, yet conflicting results have been reported from field studies[31].

Lateral flow-based assays and up-converting phosphor reporters is a technique recently introduced for rapid detection of anti-schistosomal antibodies in blood, urine and even saliva. By using a single strip carrying multiple antigens or targets (up to 20), antibodies specific to certain infection can be detected simultaneously in a format suitable for field application[32].

Three antibody detection tests showed high sensitivity have been developed recently but only suitable for *S. japonicum*. The dot immunogold filtration assay depends on using a nitrocellulose membrane as a carrier for japonicum egg proteins and gold colloid as a marker for qualitative detection of antibodies[33], the dipstick dye immuno-assay is composed of colloidal dye-labeled protein from cercaria or egg used as a probe. The assay is suitable for screening large number of people[34] and the Electrochemical immunosensor array test, which uses either SEA or recombinant antigen (SjE16; a calcium-binding protein) from *S. japonicum* or both (8 parts SjE16: 1 part SEA). Sensitivity when using both antigens was 100% with minimum cross reactivity[35]. This promising test is rapid, sensitive, specific, fairly inexpensive and suitable for epidemiological screen of schistosomiasis on large scale since the equipments used are simple and portable.

3.2. Antigen detection

Circulating anodic antigen (CAA) and CCA originating from the worm gut, are indicators of active infection since they are detected in blood three weeks post-infection, also they are useful for follow up. When used in ELISA, detection of circulating anodic antigen in serum and CCA in urine gives similar sensitivities, and both can be used in low endemic areas. CCA detection in urine was developed as a rapid lateral flow cassette assay to diagnose *S. mansoni* infection, then the technique was changed to dipstick format, as point-of-care urine CCA assay used in epidemiological surveys[36]. The test is easy and more sensitive than the KK, yet expensive, but detection of CCA in urine is a rapid way for identification of active infection by using a non-invasive technique. A newly tested method utilizes paramagnetic microspheres coated with anti-CCA monoclonal antibody for specific detection of CCA epitopes in low endemic area. This method was effective, but it needs specially equipped laboratories[36].

4. Nucleic acid-based amplification techniques

Nucleic acid detection using several techniques is an accurate method for diagnosis, particularly in field settings where collection and storage conditions are difficult to control.

In the DNA-based diagnosis, extraction of DNA is the key procedure since the yield and quality of DNA directly affects the outcome of the amplification procedure. Traditional extraction method of DNA is based on extraction with phenol-chloroform followed by precipitation using ethanol or isopropanol, but these techniques are time consuming and the exposure to these hazardous chemicals may cause human adverse effects[37]. The appearance of commercial DNA extraction kits reduces time and effort and improves the quality and quantity of DNA[38].

In conventional polymerase chain reaction (PCR), DNA extracted from blood or stool samples of schistosomiasis patient is the template for amplification using a probe targeting specific fragments of *Schistosoma* genome. A set of primers used to amplify a highly repeated 121-basepair sequence of *S. mansoni* were reported by Hamburger *et al.*[39], and a PCR assay was established to diagnose schistosomiasis using serum and stool samples. The detection limit was 10 times lower than the KK. Also, mitochondrial gene fragment has been tested[40] and identified the *Schistosoma* species. Other techniques as ELISA and restriction fragment length polymorphism can be used with conventional PCR to improve its sensitivity[41].

For PCR-ELISA technique, it employs ELISA to quantify a specific amplified DNA sequence from samples as stool using a microplate, so the technique is more sensitive in diagnosing light infection[41]. Copro-PCR-based techniques detect parasite DNA in fecal samples[42], but polymerase inactivation by inhibitors in stool is a problem. The common method for detection of PCR amplified products is running the product on gel through gel electrophoresis, but the carcinogenic ethidium bromide should

be added for the amplified product to be seen under ultra-violet trans-illumination. Oligochromatographic dipstick is a simple and quick alternative method for visualization of the amplified product via its hybridization with a gold-conjugated probe[43]. It allows both identification and differentiation between *S. mansoni* and *S. haematobium* by naked eye.

In multiplex PCR, using different sets of primers leads to amplification of multiple nucleic acid fragments in the same reaction, thus saving reagents. A species-specific primer sets targeting *cox1*, a mitochondrial gene encoding cytochrome C oxidase subunit 1 was used to differentiate between different human *Schistosoma* species as the resultant amplified products were species-specific[44].

In quantitative real-time PCR (qRT-PCR), amplification and quantification of the target gene are monitored in real-time by using a fluorescent reporter, and the signal strength is directly proportional to the number of the amplified DNA molecules. The detection method of qRT-PCR uses a sequence-specific probe (TaqMan probe, molecular beacon) or a non-sequence specific DNA-binding dye as SYBR green. The qRT-PCR was tested on fecal and serum samples from *S. mansoni*-infected hamsters and was found to detect Schistosoma DNA from 14 up to 56 days PI[45]. Serum and stool samples from infected individuals were also used to amplify SM1-7 (GenBank accession number M61098), a 121 bp tandem repeats representing nearly 12% of the genome of *S. mansoni* male and female[39].

High-resolution melting analysis is a relatively new post-PCR analysis for characterization of DNA amplicons. By amplifying a conserved region on the *cox1* gene, it was simple and inexpensive to genotype and rapidly differentiate between *S. haematobium* and *S. mansoni*[46]. The high-resolution melting assay can identify and distinguish between *Schistosoma* species infecting human when targeting a portion of the nuclear 18S rDNA, so it can be used as an accurate, precise alternative tool to other probe-based genotyping assays in the epidemiological surveys and diagnostic laboratories detecting as little as 10^{-5} ng genomic DNA from *Schistosoma* species[46].

Droplet digital PCR (ddPCR) is a technique used for quantification of nucleic acid. It is based on the distribution of the individual molecules into many reactions at limiting dilution to amplify the individual template molecules separately, hence resulting in zero or one molecule in each reaction. After reaction termination, the concentration of template can be determined by Poisson statistical analysis of the number of positive and negative reactions. The ddPCR is sensitive and gives absolute quantification without external references. The reactions can be partitioned into nanoliter-sized, aqueous uniform droplets in oil rather than multi-well plates[47], leading to analysis of thousands of droplets per sample and makes ddPCR practical for routine use. Recently, ddPCR was used for detection of *S. japonicum* DNA in human stool, serum, urine, and saliva with high sensitivity compared with the KK[48].

To address high costs associated with DNA amplification techniques, the loop-mediated isothermal amplification (LAMP) has been developed as a one step amplification method using 6 primers; 2 internal, 2 outer, and 2 loop primers to amplify the target DNA sequence under isothermal condition (60-65 ℃) within one hour. LAMP is based on self-recurring strand-displacement synthesis done by reaction primers and assisted by using a special DNA polymerase with strand displacement properties, commonly Bst (Bacillus stearothermophilus) polymerase[49]. Amplification reaction can be inspected because of the turbidity introduced by the presence of magnesium pyrophosphate white precipitate and simultaneous measurement of the turbidity of the samples in real-time by using turbidimetry. Other indicators can be used as SYBR Green I and hydroxy naphthol blue[50]. In experimental *S. mansoni* infection, a mitochondrial minisatellite DNA region [GenBank accession no: L27240] was selected as LAMP target (SmMIT-LAMP assay). The 620 pb was successfully amplified from mice feces one week PI, whereas the KK technique can detect eggs six weeks PI[51]. LAMP is a simple and fast technique with higher amplification capacity, suitable for low-resource countries, but the amplification mechanism can lead to carryover contamination and false-positive results.

PCR- based techniques can also detect cell-free parasite DNA in patient serum from the first week of infection. The cell-free parasite DNA is originated from tegument shedding of worms, dead schistosomula and inactive eggs. It is uniformly distributed in serum/plasma and excreted in urine, saliva or other body fluids as CSF. The cell-free parasite DNA is useful in diagnosis of early infection and extra-intestinal disease as neuroschistosomiasis, besides, its amount declines with treatment; so it can be used for monitoring drug efficacy[52].

The ribosomal DNA (rDNA)-based assay has been developed as a sensitive, species-specific technique, since amplicons from different human *Schistosoma* species could be amplified[53]. Moreover, DNA isolation and PCR-based identification of *S. mansoni* were performed by using sediments of urine from infected patients[54].

5. New markers for diagnosis of schistosomiasis

5.1. Extracellular vesicles (EV) protein and micro-RNAs

Parasite EV are membrane-bound vesicles classified based on their sizes, specific proteins and RNA contents into exosomes and microvesicles. Exosomes develop within a cell by inward budding of multi-vesicular endosomes and thus contain components of the parental cell, such as RNAs or proteins[55]. Recently, *S. mansoni* exosome-like EV from adult and schistosomula have been characterized[56], and several proteins were tested as vaccine candidates.

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression in eukaryotes, they are present in EV and play a role in host-parasite interplay[55]. Experimentally, elevation of miRNAs sma-miR-277, sma-miR-3479-3p, and bantam were reported in a murine model of schistosomiasis 8 weeks PI[57]. Furthermore, miR-277, miR-3479-3p and bantam were tested in serum from low and high infection sites in Zimbabwe and Uganda and miRNAs were

able to detect infected individuals in these areas with high sensitivity and specificity[57].

A study has detected miRNAs within EV fractions from infected travellers[58], and highlighted the possibility of using miRNA for evaluating the efficacy of treatment, as miRNA level decreased as early as 11 weeks after treatment. However, tests used for detection of schistosomal miRNA are still expensive and need more validation at the field level.

5.2. Schistosoma tegument proteins

Parasite surface proteins, particularly those expressed in the adult tegument, are considered as valuable vaccine candidates and markers for diagnosis. The identification of certain tegument proteins as potential diagnostic markers can help in improving diagnostic sensitivity of *Schistosoma* infection when used instead of crude extract from adult. Examples of those proteins are as follows: Aquaporins is an integral membrane protein involved in selective transportation of water and solutes through the plasma membrane of mammals, plants and lower organisms. Aquaporin is the most abundant transmembrane protein in *S. mansoni* tegument; expressed in all developmental stages especially intravascular stages. It plays a vital role in parasite survival through osmoregulation and nutrient transport[59].

Dysferlin is a member of the ferlin family involved in plasma membrane repair. In *S. japonicum*, Dysferlin was reported as a tegumental protein upregulated in adult worms, particularly females and was involved in the repair process in the tegument and muscle[60].

The rSm200 is recombinant 200-kDa tegumental protein used in ELISA for diagnosis of schistosomiasis. When used in experimental studies, there were significant differences between infected (acute and chronic) and non-infected animals, the test can also differentiate between healthy persons from non-endemic areas and infected patients, but cannot diagnose patients from low endemic areas[61].

6. Conclusion

Despite control programs, schistosomiasis is still a public health problem. Early and accurate diagnosis of schistosomiasis lead to early treatment, cessation of transmission cycle and prevention of chronic complications. Many diagnostic tests for schistosomiasis are available but have unsatisfactory performance. Although the application of molecular techniques to improve diagnostics for *Schistosoma* infection has resulted in some technical advances, but still with the lack of reliable diagnostic tools, underestimation of disease prevalence will lead to the misuse of protective chemotherapy and ultimately to an increase in costs and efforts. It is important to work on improving the current diagnostic methods for better results, and simultaneously look for new diagnostic markers. Surface proteins such as Aquaporin, Dysferlin and rSm200 are attractive biomarkers, which could be used for development of rapid diagnostic tests and contribute to increase the accuracy of diagnosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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