Diagnosis and post-treatment monitoring of schistosomiasis in endemic and non-endemic settings by quantification of schistosome circulating anodic antigen

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Voor Roeland en onze kinderen,
Micah, Hannah & Sarah

Wat u ook doet, doe het van harte,
alsof het voor de Heer is en niet voor de mensen.

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1.

General introduction

Adapted and extended from:
Context-Specific Procedures for the Diagnosis of Human Schistosomiasis – A Mini Review

Pytsje T. Hoekstra, Govert J. van Dam and Lisette van Lieshout

Frontiers in Tropical Diseases (2021)

Schistosomiasis is a neglected tropical disease that is caused by infection with parasitic blood flukes of the *Schistosoma* genus [1]. Infection occurs in freshwater bodies when humans come in contact with cercariae – released from the intermediate snail host – which penetrate the human skin and migrate into the vascular system. Here, they mature into adult worms, mate and start producing eggs which can be excreted in the environment via urine or stool, depending on the *Schistosoma* species, or retained in the hosts' tissue where they induce inflammatory lesions and immunopathology (Figure 1). The three main species infecting humans are *S. mansoni*, *S. japonicum* (causing intestinal schistosomiasis) and *S. haematobium* (causing urogenital schistosomiasis) [1,2]. *S. mansoni* and *S. haematobium* both occur in Africa and the Middle East, while in the Americas only *S. mansoni* is present. In Asia, mainly the Philippines and China, *S. japonicum* is present. Furthermore, hybrid infections, resulting from interactions between human and animal schistosome species and potentially enhanced by zoonotic transmission, are increasingly being reported and may present a considerable risk of human pathology [3,4]. Worldwide, over 250 million people are infected with *Schistosoma* spp, with the majority residing in Sub-Saharan Africa [2]. Schistosomiasis epidemiology is characterized by its focal distribution (i.e. prevalence and infection intensity can vary substantially within a small region) regulated by the interaction between humans, the intermediate snail host, environmental conditions and human-water contact.

Schistosoma infection can lead to significant morbidity, mainly due to the egg-induced pathology, and even mortality if not treated [1,2]. At population level, clinical symptoms are generally related to infection intensity (i.e. higher worm and egg loads). Schistosome infection can be cured if an accurate and timely diagnosis is made and adequate treatment is given. The most effective and widely used drug is praziquantel (PZQ), which is safe and efficacious against the adult worm stages of all *Schistosoma* spp [5]. It is used for mass drug administration (MDA), also known as preventive chemotherapy, to at-risk populations for control of schistosomiasis in endemic areas, as well as for individual case management.

Diagnosis of schistosomiasis

The use of sensitive and specific diagnostic tests to correctly identify those who are infected is crucial in order to successfully reduce the burden of disease and to eventually move towards elimination of schistosomiasis. This is also clearly recognized by the WHO in the recently published NTD road map for 2021-2030 where the need for field-deployable and sensitive diagnostics to evaluate pre- and post-intervention prevalence is highlighted [6]. Diagnostic laboratory tests for schistosomiasis include conventional microscopy, antibody detection methods, nucleic acid amplification tests (NAATs) and antigen detection methods. Additional clinical diagnostic methods such as clinical markers (e.g. haematuria), physical examinations and *in vivo* imaging techniques, including the recent developments in portable and affordable ultrasound machines, are beyond the scope of this thesis and have been described in detail elsewhere [2,7-10].

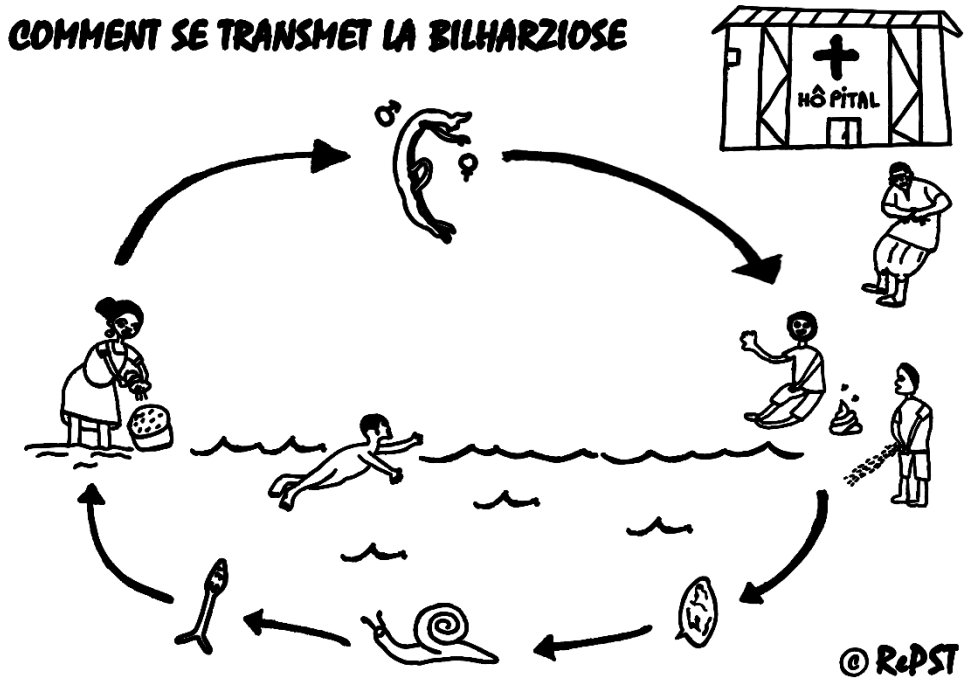

Figure 1. Schistosomiasis life cycle based on drawings of children who participated in a repeated praziquantel treatment trial in Côte d'Ivoire. © RePST

Parasitological methods

The current reference standard for diagnosing schistosomiasis is based on the detection of eggs in stool (for intestinal schistosomiasis) or in urine (for urogenital schistosomiasis) by microscopy. Additionally, information about clinical symptoms and possible exposure history is often taken into account in the final diagnosis [7]. For population based surveys or control programs in endemic settings the microscopy based Kato-Katz technique and the urine filtration or urine sedimentation technique to quantitatively assess the intensity of *S. mansoni* and *S. haematobium* infections respectively, are most commonly used [11-15]. Other available microscopy methods, including flotation techniques, have been described and summarized extensively previously [7]. Even though microscopy techniques are highly specific and can accurately detect infections of moderate-to-high intensity, they are not sensitive enough for low intensity infections and subsequently not suitable for post-treatment monitoring [7]. New developments include optical devices combining digital image recognition with automated data analysis and reporting using artificial intelligence. Although further technical improvement and validation in the field is needed, these smart and simplified optical diagnostic devices have the potential to identify moderate-to-high intensity infections in stool or urine in an automated or semi-automated way at low costs [16,17].

Antibody detection methods

Schistosome-specific antibodies can be demonstrated within a few weeks to months after infection, often before eggs are excreted [2,7]. Various serological methods can be used to detect these antibodies in human plasma or serum [7,13,14,18]. Most of these methods are reasonably specific within the context of travel medicine, i.e. for the diagnosis of imported infections. Although a certain level of cross-reactivity with other helminthic diseases cannot be excluded, this is generally accepted because co-infections are not observed very often in travelers [7,19]. Sensitivity of antibody detection methods varies significantly depending on the test format used and the infecting species as well as the targeted *Schistosoma* antigen(s) which also affect the observed timepoint of seroconversion [19,20]. In general, serology gives neither an indication of the status (past or present) nor the intensity of an infection (21). Still, antibody detection remains useful, especially in the case of travelers who often have not been exposed previously [7,21]. Furthermore, in settings where transmission is assumed to have been interrupted for a longer period of time, highly sensitive antibody detection may play an important role to assess small pockets of ongoing risk of infection [22-26].

Nucleic acid amplification tests

For the detection and quantification of *Schistosoma*-specific DNA, several NAATs have been described for different types of clinical samples, including serum, stool and urine [27-34]. In particular those that apply urine samples are of interest as this type of sample can be easily and non-invasively acquired. Although the described NAATs are mostly in-house assays, some are becoming commercially available [35]. The majority of these tests claim a specificity of 100%, while the sensitivity ranges from equal to significantly higher than standard microscopy [7]. NAATs have also demonstrated to be very useful for detection and strain typing of hybrid schistosome infections, in particular in research settings [3]. To overcome the need for expensive laboratory equipment and highly trained personnel, more field-friendly alternatives have been developed, such as for example loop-mediated isothermal amplification (LAMP) [36,37] and recombinase polymerase amplification (RPA) [9,38], although both need further validation before large scale implementation can be considered.

Antigen detection methods

Living schistosomes release a number of antigens into the hosts' bloodstream and measurement of such schistosome specific antigens allows accurate diagnosis of active infections. Most research has focused on two *Schistosoma* gut-associated glycoconjugates that were already identified in the 1970's at the Leiden University Medical Center (LUMC), the Netherlands [39,40]: circulating cathodic antigen (CCA) and circulating anodic antigen (CAA), named after their migration behavior in an electric field [39]. Both antigens are regularly regurgitated by live *Schistosoma* worms into the hosts' circulation, their presence indicating an active infection. Antigen-levels are generally associated with the number of worms present, in particular with increasing infection intensities [41]. Unique characteristics of both antigens include the clearance from the blood circulation into the urine with little day-to-day variation [42,43] and the decrease

to undetectable levels within days to weeks after PZQ treatment [44-50], making antigen detection well suited for (individual) treatment monitoring. The initial development of monoclonal antibody based sandwich ELISAs has resulted in sensitive and highly specific detection of both antigens [51-55]. Since then, major progress has been made in the field of circulating antigen diagnostics.

For the detection of CCA, a rapid field-applicable test format was developed based on a lateral flow (LF) assay with carbon-labelled anti-CCA monoclonal antibodies [56,57]. Eventually, this has resulted in the point-of-care circulating cathodic antigen (POC-CCA) urine test commercially available via Rapid Medical Diagnostics (Pretoria, South Africa). The POC-CCA test is a non-invasive, user-friendly and field-applicable LF test and has been studied extensively in schistosomiasis endemic settings. It is mainly used for diagnosing intestinal schistosomiasis, in particular in African settings for *S. mansoni* infections [15,58-66], and to a lesser extent for urogenital schistosomiasis [67]. Lower specificity has been observed in pregnant women [68-70], small children [70], as well as in individuals with urinary tract infections or hematuria [71] potentially due to the presence of cross-reactive antigens. These false positive observations are most likely related to the chemical structure of CCA that contains repeating Lewis-x trisaccharide units, which can also be found as part of glycoconjugates on human immune cells [55,72], on several human serum glycoproteins, as well as in several other organisms including some bacteria. Other recent occurring issues are the interpretation of the POC-CCA test in particular in case of faint signals (the so-called 'traces') [73-76] and the problem of batch-to-batch variation [77-79]. Nevertheless, the test has been accepted as a valuable tool and is now being recommended by the WHO as a more user-friendly and more sensitive alternative to Kato-Katz for mapping prevalence of intestinal schistosomiasis as well as for surveillance purposes [6,15,74].

For the detection of CAA, the introduction of a LF test platform in combination with a unique and highly sensitive luminescent reporter label, up-converting reporter particles (UCP), allowed for a significant improvement in sensitivity: the lower limit of detection was improved more than 10-fold compared to previous ELISA assays [80,81]. The UCP-LF CAA test has demonstrated high specificity and sensitivity for the detection of all (human) schistosome species [47,82-87]. The test is applicable to various sample types, including urine [87,88], serum [87], plasma [89], and dried blood spots [90]. To optimize schistosomiasis control efforts, the UCP-LF CAA test was further improved in terms of its sensitivity and available formats: applying larger sample volumes increased the sensitivity of the test, while the development of a dry format allowed storage and worldwide shipment of reagents without the need for a cold chain [87,91]. Even though the most sensitive format still requires some basic laboratory equipment, it is particularly useful for quantifying low worm burdens. Taken together, the UCP-LF CAA test has great potential for accurately determining the presence as well as the intensity of *Schistosoma* infections and to monitor efficacy of PZQ treatment.

Scope and outline of this thesis

The aim of the research described in this thesis is to further evaluate the application of the UCP-LF CAA test for diagnosing schistosomiasis, including post-treatment monitoring, in different settings.

The first part of this thesis focuses on the performance of the UCP-LF CAA test in non-endemic settings, i.e. in the absence of any reinfection. In **chapter 2** the diagnostic value of CAA detection was assessed for early diagnosis and follow-up of acute schistosomiasis in a cluster of Belgian travelers with a *Schistosoma* hybrid infection between *S. haematobium* and *S. mattheei*. In **chapter 3** the UCP-LF CAA test was evaluated and compared to a range of other diagnostic methods in a group of Eritrean migrants with chronic but asymptomatic schistosomiasis, including monitoring of treatment efficacy.

In the second part of this thesis, the UCP-LF CAA test was evaluated in endemic settings. **Chapter 4** describes a clinical trial that was designed to assess the efficacy of repeated PZQ treatment on the clearance of *S. mansoni* infections in school-aged children from Côte d' Ivoire with a range of diagnostic methods. Initial outcomes based on the diagnostics used in the field (i.e. Kato-Katz and POC-CCA) are described in **chapter 5. Chapter 6** focuses on the outcomes based on the more sensitive diagnostic methods that were additionally applied (i.e. real-time PCR and UCP-LF CAA). In **chapter 7** the presence of schistosomiasis was investigated with a panel of non-microscopy diagnostics, including the UCP-LF CAA test, on a set of banked samples from the Democratic Republic of the Congo.

Results are summarized and discussed in **chapter 8** in the broader context of literature, including recommendations for the use of the UCP-LF CAA test as well as regarding potential new lines of diagnostic research needed to move towards elimination of schistosomiasis.

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Part I.

Performance of circulating anodic antigen detection in non-endemic settings

2.

Early diagnosis and follow-up of acute schistosomiasis in a cluster of infected Belgian travellers by detection of antibodies and circulating anodic antigen (CAA): A diagnostic evaluation study

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ABSTRACT

Background: In order to evaluate the diagnostic value of schistosome circulating anodic antigen (CAA) detection, serum and urine CAA-levels were determined in a single cluster of 34 Belgian tourists at three timepoints within a period of 14 weeks following proven *Schistosoma* exposure in South Africa and compared with two in-house antibody assays.

Methods: Samples were collected 4-5 and 7-8 weeks post-exposure and subsequently 5-6 weeks following praziquantel treatment. *Schistosoma* antibodies were detected by an adult worm antigen-immunofluorescence assay (AWA-IFA) and a soluble egg antigen-enzyme-linked immunosorbent assay (SEA-ELISA), while CAA concentrations were determined by the Up-Converting reporter Particle labelled Lateral Flow (UCP-LF) test.

Results: Antibodies were detected in 25/34 (73%) travellers pre-treatment and in 27/34 (79%) post-treatment, with the AWA-IFA showing better performance than the SEA-ELISA. Pre-treatment, CAA was detected in 13/34 (38%) and 33/34 (97%) of the travellers in urine and serum, respectively. Post-treatment, all except one traveller became serum CAA negative. This in contrast to the detected antibodies, as well as the previously reported diagnostic results of this cluster.

Conclusions: The UCP-LF CAA serum assay has been demonstrated as the most sensitive method for the diagnosis of early *Schistosoma* infections and post-treatment monitoring in travellers.

Keywords: Circulating anodic antigen; Diagnostic accuracy; *Schistosoma haematobium* complex; Sensitivity; Treatment efficacy.

INTRODUCTION

Schistosomiasis, a disease caused by parasitic blood flukes of the *Schistosoma* genus, affects more than 230 million people worldwide [1]. Diagnosing acute schistosomiasis in travellers returning from endemic areas is a known challenge: in non-endemic regions the number of infections might be underdiagnosed due to lack of proper diagnostic procedures. Clinical symptoms might appear before egg production has started or before specific antibodies can be demonstrated [2], implying that these methods are not really suitable for confirming acute *Schistosoma* infections. Treatment at this stage of the disease includes some important considerations. The anti-schistosomal drug praziquantel (PZQ) is known to be less effective against juvenile worms and may sometimes even aggravate symptoms, for which additional corticosteroids need to be given either concomitantly or prior to PZQ treatment [1,3].

Classic diagnosis of *Schistosoma* infections is often still based on microscopic detection of parasite eggs in stool or urine, depending on the species [4]. However, this approach lacks sensitivity in low-burden infections [5], which is often the case in infected travellers. *Schistosome* antibody detection is a far more sensitive method for confirming infection in exposed travellers [4,6] and it is therefore the most commonly used method for diagnosing schistosomiasis in non-endemic routine diagnostic laboratories. Antibodies usually develop within a few weeks to months after infection, generally before *Schistosoma* eggs can be detected, but usually after the first clinical symptoms [4]. However, detection of antibodies is not suitable for monitoring treatment efficacy as they remain present after treatment [7,8]. Furthermore, sensitivity of these methods may vary considerably depending on the specific method and on the targeted *Schistosoma* biomarkers [9,10]. Detection of *Schistosoma* DNA in serum is a recent development that allows both for early detection after infection and for species identification [11]. However, individuals remain DNA-positive in serum after treatment, making this method unsuitable to monitor treatment efficacy [11,12].

There clearly is a need for a diagnostic marker which can be accurately detected from the early infection stages onwards and which is cleared soon after treatment. Circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) are two well-studied *Schistosoma* antigens which are regurgitated by living *Schistosoma* worms, and therefore indicative of an active infection [13]. While the field-applicable point-of-care CCA (POC-CCA) test is being used extensively in *S. mansoni* endemic settings, it has demonstrated inconsistent performance in imported cases in non-endemic routine clinical settings [14,15]. Detection of CAA, present within weeks after infection as observed recently in a controlled human schistosomiasis infection model [16], seems to be a promising alternative for diagnosing (acute) schistosomiasis in returning travellers [17,18]. Furthermore, CAA-levels decline rapidly after treatment with PZQ [17,19], allowing monitoring of treatment efficacy. Using an ultrasensitive reporter technology (Up-Converting reporter Particles, UCP) in combination with lateral flow (LF) immunochromatography, CAA can be accurately measured in urine and in serum [20,21]. This UCP-LF CAA assay has demonstrated high sensitivity and specificity in detecting the four major *Schistosoma* species (*S. mansoni*, *S. haematobium*, *S. japonicum* and *S. mekongi*) in endemic areas [21-26] as well as in a non-endemic routine diagnostic setting [17].

In a cluster of Belgian travellers, who all were most likely infected with a *S. mattheei* x *S. haematobium* hybrid in South Africa, the performance of two commercial schistosome antibody assays and a serum schistosome real-time PCR assay was evaluated as described previously [11]. Here, we compare these previously described diagnostics with two in-house antibody assays and the schistosome UCP-LF CAA assay for antigen detection in urine and serum, before and after PZQ treatment.

MATERIALS AND METHODS

Samples were available from a cluster of 34 Belgian travellers (17 males, 17 females) with a PCR-confirmed *Schistosoma* infection, determined shortly after exposure during a holiday in a known *Schistosoma*-endemic region in South Africa. All participated in a prospective study evaluating new diagnostic tests for acute schistosomiasis at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, where ethical clearance had been obtained [11]. Informed consent included storage of samples for future use in studies evaluating new diagnostic tests for schistosomiasis. At LUMC, all samples were tested anonymously.

In total, 16 parents (8 males) aged 39–49 years (median, 43 years) and 18 children (9 males) aged 5–15 years (median, 12 years) were seen at ITM and samples collected at three moments during the course of infection (Fig. 1). Firstly at 4–5 weeks post-exposure, where 32/34 (94%) had developed symptoms of acute schistosomiasis and who were treated with corticosteroids accordingly; secondly at 7–8 weeks post-exposure when symptoms had abated and all received PZQ (40 mg/kg) according to current practice at ITM; and thirdly in a final post-treatment visit 13–14 weeks post-exposure (5–6 weeks after treatment) receiving a second treatment with PZQ.

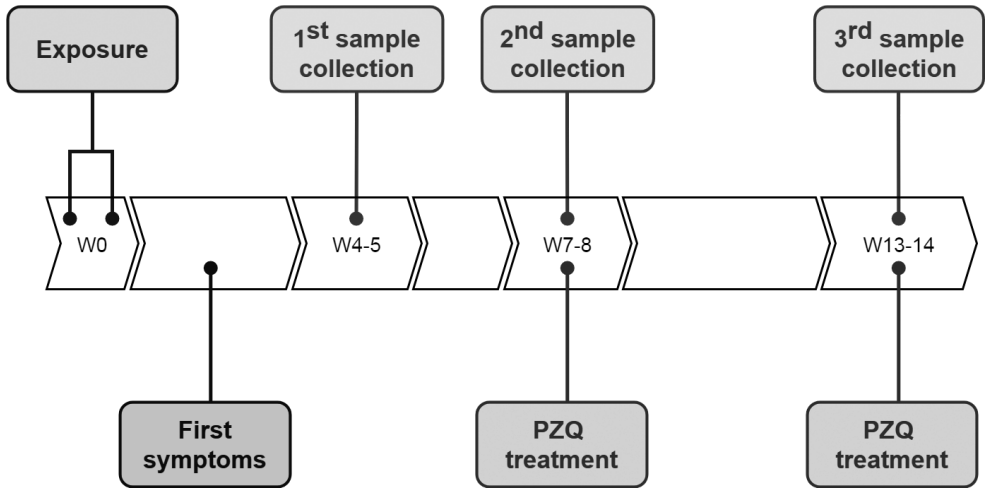

Figure. 1. Timeline of exposure, first appearance of symptoms, sample collection and treatment in a cluster of 34 infected Belgian travellers.

Serum samples were tested at the ITM using two commercially available schistosome antibody assays, an enzyme-linked immunosorbent assay (*S. mansoni* ELISA, Bordier Affinity Products) and an indirect hemagglutination inhibition assay (IHA, ELITechGroup Microbiology), as well as an in-house real-time PCR assay detecting Dra-1, a target which is specific for the *S. haematobium* complex [11,27,28]. Sequencing of schistosome DNA obtained from serum of 3 travellers with very low Ct-values in the serum PCR demonstrated a hybrid infection with *S. mattheei* x *S. haematobium* [11]. No eggs or parasite DNA were detected in any of the collected urine and stool samples [11].

At the Leiden University Medical Center (LUMC), samples from the three visits were subjected to serum antibody testing and urine and serum CAA detection. Serum antibody assays comprised the adult worm antigen-immunofluorescence assay (AWA-IFA) and the soluble egg antigen-enzyme-linked immunosorbent assay (SEA-ELISA) as used in our routine clinical setting. *Schistosoma*-specific IgM antibodies against adult worm gut antigens were determined by AWA-IFA using sections of Rossmann's fixed adult male *S. mansoni* worms and IgG antibodies against *S. mansoni* SEA were detected by SEA-ELISA. Both assays are ISO 15189:2012-certified and have been used for the routine clinical diagnosis of schistosomiasis at the LUMC since the 1990s [17,29,30]. Additional details of these antibody assays can be found elsewhere [16,31].

Detection of CAA in urine and serum samples was done using the laboratory-based, ultra-sensitive and highly specific UCP-LF CAA assay: the UCAA2000 and the SCAA500, respectively, as described previously [18,20,22,32]. Urine samples were subjected to the user friendly dry-format assay, while for serum samples the wet-format assay was used, which includes the additional step of UCP-sonication [32]. Briefly, 500 µl of serum (or 2 ml of urine) was mixed with an equal volume of 4% trichloroacetic acid, incubated and centrifuged. Of the clear supernatant 500 µl of serum (or 2 ml of urine) was concentrated to 20 µL using a 0.5 ml (or 4 ml) centrifugal filter device (Amicon Ultra-4, Millipore). The concentrates were added to microtiter plate wells containing UCP reporter particles labelled with anti-CAA antibodies hydrated in 100 µL LF assay buffer and incubated on a shaker at 37 °C. After 1 h, LF strips were added to the wells and incubated overnight, followed by scanning the strips using a Packard FluoroCount microtiter plate reader [20]. Samples with a known CAA concentration were included as a reference standard to quantify individual CAA-levels and to validate the cut-off of the assay. A predefined cut-off level was used; 0.3 pg/ml for the urine UCAA2000 assay and 1 pg/ml for the serum SCAA500 assay [20]. Samples with a CAA concentration above these cut-offs were considered positive, whereas samples below these cut-offs were considered negative. Data were entered into a Microsoft Excel spreadsheet and analysed using GraphPad Prism 8.1.1 (GraphPad Software Inc.; California, USA) and SPSS version 25 (IBM Corp.; Armonk, USA). Statistical analysis was performed using descriptive statistics.

RESULTS

In Table 1 an overview is given of the outcomes of the diagnostic tests that were performed in the current study. At the first post-exposure visit, schistosome antibodies were detected in 13 travellers (39%), all positive by AWA-IFA with two (6%) by both AWA-IFA and SEA-ELISA. Positivity increased substantially at the second post-exposure (pre-treatment) visit where 68% of

travellers tested positive by AWA-IFA compared to 32% by SEA-ELISA. After treatment, the number of AWA-IFA-positive travellers further increased to 74%, while the number of SEA-ELISA-positive travellers decreased to 12%. For comparison, the outcomes of the diagnostic tests that were previously performed at ITM (11) have also been included in the table. Results of the SEA-ELISA at LUMC were comparable to results with the commercial ELISA at ITM.

Table 1. Number of positive cases at the three time points according to the different diagnostic tests in a cluster of 34 infected Belgian travellers.

	Pre-treatment			Post-treatment
	4-5 weeks post-exposure n (%)	7-8 weeks post-exposure n (%)	Cumulative positive pre- treatment n (%)	13-14 weeks after exposure n (%)
Diagnostic tests performed at ITM[a]				
ELISA	0/33	12/34 (35%)	12/34 (35%)	11/34 (32%)
IHA	3/33 (9%)	0/34[b]	3/34 (9%)	1/34 (3%)[c]
Serum PCR	24/33 (73%)	30/34 (88%)	31/34 (91%)	24/34 (71%)
Diagnostic tests performed at LUMC				
AWA-IFA	13/33 (39%)	23/34 (68%)	23/34 (68%)	25/34 (74%)
SEA-ELISA	2/33 (6%)	11/34 (32%)	11/34 (32%)	4/34 (12%)
Urine CAA	8/30 (27%)	9/32 (28%)	13/33 (39%)	0/30
Serum CAA	30/33 (91%)	30/34 (88%)	33/34 (97%)	1/34 (3%)

Abbreviations: AWA-IFA; adult worm antigen-immunofluorescence assay, CAA; circulating anodic antigen, IHA; indirect hemagglutination inhibition assay, ITM; Institute for Tropical Medicine, Antwerp, Belgium, LUMC; Leiden University Medical Center, PCR; polymerase chain reaction, PZQ; praziquantel, SEA-ELISA; soluble egg antigen-enzyme-linked immunosorbent assay.
a. Data derived from Cnops L et al., Clin Infect Dis, 2020.
b. Uninterpretable in 13 cases
c. Uninterpretable in 2 cases

CAA was detected in urine of 8 (27%) and 9 (28%) travellers 4–5 weeks and 7–8 weeks post-exposure, respectively, with a total of 13 (39%) travellers being positive on at least one of these time points. All travellers became urine CAA negative after PZQ treatment. In serum, CAA was detected in samples from 30 travellers of 4–5 weeks (91%) and 7–8 weeks (88%) post-exposure. Cumulatively, 33 (97%) travellers were serum CAA positive pre-treatment, with all but one turning negative after treatment. When compared to the previously published serum PCR results, the serum CAA showed a higher number of positives at the first post-exposure visit. One the other hand, all 34 travellers had detectable Dra-1 by PCR in at least one of the serum samples, while 33 tested positive for CAA. The traveller who tested negative for CAA also remained negative in all antibody detecting tests performed. No association was observed between CAA-levels and gender or age of the host.

Supplementary Table 1 gives an overview of all collected data per traveller, indicating that among the diagnostic tests performed, only the UCP-LF CAA assay demonstrated a significant decline following praziquantel treatment. Individual CAA-levels over time are also shown in Fig. 2. Between the first and second post-exposure visit, thus before treatment, CAA-levels increased in urine of 6 travellers (20%) and in serum of 11 travellers (33%). In addition, a spontaneous reduction in CAA-levels was observed during the same time period in urine of 6 travellers (19%), four of them even becoming negative, and in serum of 20 travellers (59%), with three of them becoming negative. From two travellers, who were urine CAA positive before

treatment, a post-treatment urine sample was missing. However, no CAA was detected in the post-treatment serum sample of these two individuals. Overall, CAA-levels in urine were lower compared to CAA-levels in serum. All urine CAA-positive cases were also serum CAA positive.

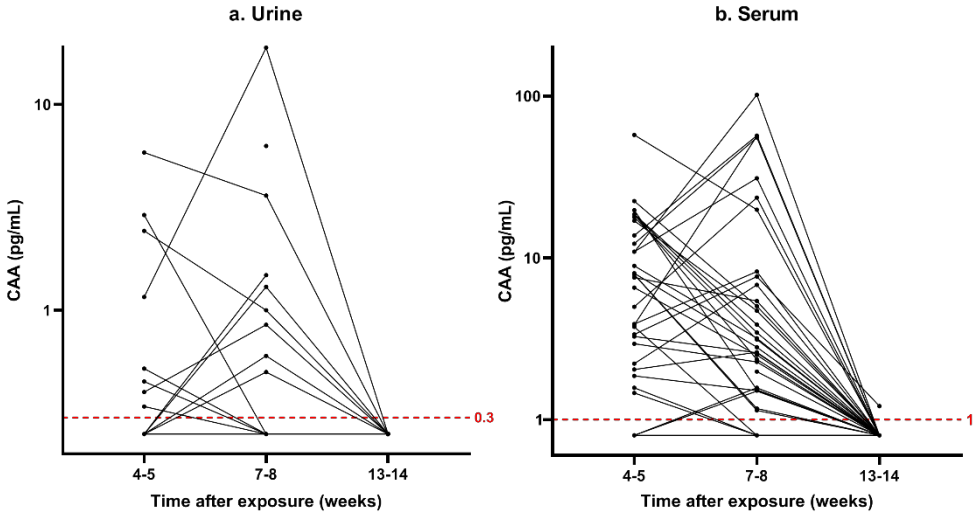

Figure. 2. Individual circulating anodic antigen (CAA) concentrations in urine (a) and serum (b) in a cluster of 34 infected Belgian travellers at three different time points.

DISCUSSION

Accurate diagnosis of acute schistosomiasis in travellers is challenging as in this early stage of the infection egg detection methods are often not reliable. Antibody detection assays are commonly applied in non-endemic routine diagnostic laboratories, especially for those travellers who have been exposed for a relatively short period. This, despite the fact that these assays have several known limitations, some of which are confirmed again in the current study. In this cluster of acutely infected travellers, the serum CAA assay was the most sensitive test to confirm active *Schistosoma* infection as well as to assess cure. The serum CAA assay also showed an overall better performance compared to a real-time serum PCR assay which has been considered by some authors as the most accurate diagnostic reference test for this specific target population [12].

Even though almost all travellers showed symptoms of acute schistosomiasis, no eggs were detected in stool or urine at any of the pre-treatment visits [11]. It was hypothesized that the infective species in this cluster, a *S. mattheei* x *S. haematobium* hybrid as demonstrated by schistosome DNA sequences extracted from serum of three travellers, is sterile [10]. In such a setting, microscopic examination of stool or urine, even after further sample concentration or repeated sample collection, will never be reliable for accurately detection of schistosome infections. Alternative explanations for the absence of eggs in stool or urine could be a mono-sexual infection, immaturity of the worms which do not yet produce eggs or a very low worm burden as often found in travellers [33]. The finding that around 32% of the travellers had detectable antibodies against egg antigen (e.g. positive SEA-ELISA) does not prove any

deposition of eggs, as antibodies against the schistosome egg antigen have also been detected in volunteers following infection with male parasites only [16].

Serum antibody tests for schistosomiasis come in many assay formats and use a variety of schistosome antigen extracts: from adult worm, eggs or even cercariae. ELISA is the most commonly used format in commercial serological tests, and therefore considered as a benchmark test in established schistosome infection. The rationale for including our in-house antibody test in the current diagnostic test evaluation rests on our overall positive experiences with these tests. In particular the AWA-IFA assay, detecting schistosome IgM, has been found to be highly sensitive in early detection of schistosomiasis in returning travellers, as well as in a controlled human *S. mansoni* infection model, showing 100% sero-conversion within 4–6 weeks after exposure [16,17,31]. In the current study, however, the performance of the AWA-IFA, although much better than the SEA-ELISA, remained inferior to serum CAA and serum PCR detection, possibly because of the *S. haematobium* hybrid nature of the present infection. Our in-house SEA-ELISA format, based on soluble egg antigen, did not perform better than a commercial ELISA using a mixture of egg and adult worm antigen (11). Antibody tests are particularly useful for detecting infections in asymptomatic travellers, and they are often used for screening purposes [4,31,34]. However, for diagnosing acute schistosomiasis within a limited number of weeks after exposure, antibody tests seem to be less suitable. In addition, our results confirmed that antibody tests are not suitable to monitor the effect of PZQ treatment, as the majority of individuals remained or even became positive after successful treatment.

No correlation was observed between the level of parasite DNA and CAA, while CAA in serum and urine became negative after treatment in almost all individuals, the majority remained PCR positive. These findings suggest that the output of this specific PCR assay is not a reflection of the actual worm burden, but a measure of the overall amount of parasite DNA present, probably released from surrounding tissues, including trapped eggs. Notable is the single traveller who showed a discrepancy between PCR and serum CAA results. Based on previously described data from this cluster, it was assumed that all individuals of this cluster had been infected with *Schistosoma* [11]. However, the single individual showing no serum CAA did not present any clinical signs of acute schistosome infection, no eosinophilia, nor any positive serological test result at any of the three time points. The PCR test of this CAA-negative individual showed a high Ct-value of 43.8, which corresponds to a very low level of parasite DNA, suggesting that the PCR result might be false positive and that most likely this individual was not infected.

Overall, in this cluster of travellers, the serum CAA assay performed better than the urine CAA assay. CAA-levels in serum seem to be higher and more stable, as has been observed in some previous studies [13,35-37]. All detected urine CAA-levels were above but very close to the assay cut-off, indicating that testing with even larger urine volumes would have been more beneficial. Although urine CAA-levels were low, all urine CAA positive individuals became negative after treatment, indicating that the infection has been cleared. In some individuals, urine CAA-levels already decreased to undetectable levels even before treatment. A similar trend was observed in serum: in some individuals serum CAA-levels decreased at the second follow-up visit before treatment while in others serum CAA-levels still increased. These fluctuations indicate that the course of infection as well as intensities of infection vary from individual to

individual in the first weeks after exposure. The growth/maturation of (young) worms will cause an increase in CAA-levels over time, while the decrease in CAA-levels indicate that non-matured worms die before treatment is given. After treatment, CAA-levels in serum decreased significantly to below the cut-off in all but one traveller, indicating that they cleared the infection. Even though in this cluster the infecting species turned out to be a *S. mattheei* x *S. haematobium* hybrid [11], the serum CAA assay was able to detect all infected individuals, confirming again that CAA is a *Schistosoma*-genus specific antigen [21,38].

The current study confirms the usefulness of CAA detection for diagnosis of schistosomiasis in travellers and has several advantages over existing methods. First and foremost, the presence of CAA indicates an active *Schistosoma* infection [35,39]. Furthermore, CAA is excreted by all schistosome species, making the UCP-LF CAA assay generally applicable in all *Schistosoma* endemic areas [20], and as shown in this study also for detection of hybrid infection. Serum CAA has shown to be an excellent marker for demonstrating active *Schistosoma* infections at a very early stage, as the majority of travellers had detectable CAA-levels in serum already 4–5 weeks after exposure. Clearance of CAA from the host circulation appears to be relatively fast, within 5–6 weeks after treatment, as also previously observed [40]. Unlike PCR and serological tests, CAA is the only reliable marker to assess treatment efficacy soon after administration of PZQ. Furthermore, the better sensitivity of detecting CAA in serum compared to urine fits nicely with clinical routine diagnostic settings where, in contrast to endemic countries, the use of serum is often preferred over urine.

A limitation of the UCP-LF CAA assay is its inability to differentiate between *Schistosoma* species, as CAA is excreted by all *Schistosoma* species. However, if needed, known geographical distribution of *Schistosoma* could be of use to indicate the suspected species, especially to decide whether and how individuals should be treated. Another limitation is the availability of the UCP-LF CAA assay. Efforts are ongoing to implement the serum CAA assay in our clinical routine diagnostic laboratories as well as to make a CAA detection test generally available.

CONCLUSIONS

This study confirms the accuracy and usefulness of the UCP-LF CAA serum assay for diagnosing active *Schistosoma* infections in (recently) exposed travellers and its ability to assess efficacy of treatment. The UCP-LF CAA serum assay is the only test that fulfils the criteria for correctly determining cure.

Abbreviations

AWA, (Adult Worm Antigen); CAA, (Circulating Anodic Antigen); ELISA, (Enzyme-Linked ImmunoSorbent Assay); IFA, (ImmunoFluoresence Assay); IHA, (Indirect Hemagglutination inhibition Assay); ITM, (Institute for Tropical Medicine, Antwerp, Belgium); LUMC, (Leiden University Medical Center, Leiden, The Netherlands); PZQ, (Praziquantel); SCAA, (Serum Circulating Anodic Antigen); SEA, (Soluble Egg Antigen); UCAA, (Urine Circulating Anodic Antigen); UCP-LF, (Up-Converting reporter Particle Lateral Flow).

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Declaration of competing interest
All authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. Overview of all diagnostic outcomes in a cluster of Belgian travelers at three different time points: (1) early symptomatic visit (4-5 weeks post-exposure), (2) treatment visit (7-8 weeks post-exposure), and (3) post-treatment visit (13-14 weeks post-exposure and 5-6 weeks post-treatment)

	ELISA ITM[a,b]			IHA ITM[a,c]			Serum PCR[a,d]			AWA-IFA LUMC[d]			SEA-ELISA LUMC[e]			Urine CAA[f]			Serum CAA[g]		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	-	+	+	-	-	-	34,3	34,2	34,2	-	-	-	-	-	64	-	-	-	2,2	6,8	-
2	-	-	-	-	0,5[h]	-	-	36,3	38,6	16	128	64	-	-	-	n/a	-	-	1,9	1,5	-
3	-	-	+	-	0,5[h]	-	-	35,1	33,2	16	64	32	-	-	-	-	0,5	-	57,5	19,8	-
4	-	-	-	-	-	-	29,8	32,2	31,9	-	-	-	-	-	-	0,3	-	-	1,7	4,7	-
5	-	-	-	-	-	-	30,4	32,6	35,2	16	64	64	-	-	-	-	-	-	3,7	-	-
6	-	-	-	-	-	-	30,3	37,5	-	16	32	64	-	-	-	-	-	-	1,6	-	-
7	-	-	-	-	-	-	29	33,4	33	-	32	32	-	-	-	5,8	3,6	-	13,8	57	-
8	-	+	-	-	0,5[h]	-	31	-	-	-	32	16	-	32	-	n/a	-	-	19,7	0,2	-
9	-	+	-	-	-	-	34,5	36	-	16	128	64	-	-	-	-	-	n/a	2	2,6	-
10	-	-	-	-	-	-	-	34,2	32,5	-	-	32	-	64	-	1,2	18,9	-	3,9	56,6	-
11	-	-	-	-	-	-	32,7	36,1	38,5	-	16	-	-	-	-	2,9	-	n/a	7,9	1,1	-
12	-	-	-	-	0,5[h]	-	29,4	35	-	-	32	-	-	-	-	-	-	-	1,5	-	-
13	-	-	-	-	0,5[h]	-	32,5	34	33,1	32	16	16	-	64	-	0,5	-	-	22,4	5	-
14	-	-	-	-	-	-	32,4	32,9	32,7	-	64	128	-	-	-	-	1,3	-	12,2	55,5	-
15	-	-	-	-	-	-	28,1	32,4	35,3	-	32	16	-	-	-	-	-	-	-	1,6	-
16	-	+	-	-	0,5[h]	-	31,6	33,4	32,5	-	256	64	-	64	-	n/a	n/a	-	10,9	31	-
17	-	+	+	-	0,5[h]	160	-	32	35	-	32	256	-	-	-	-	1,4	n/a	8,9	3,2	-
18	-	+	+	-	-	-	30,4	29,9	32,1	-	-	-	-	32	32	-	n/a	-	10,9	101,7	-
19	-	+	+	-	0,5[h]	-	25,2	30,9	-	-	32	64	-	64	-	-	-	-	7,5	5,4	-
20	-	-	-	-	-	-	-	37,8	-	-	-	16	-	-	-	-	-	-	2,9	2,3	-
21	-	-	-	-	-	-	-	-	43,8	-	-	-	-	-	-	-	-	-	-	-	-
22	-	+	+	-	0,5[h]	0,5[h]	33,2	30,7	33,8	32	256	128	-	32	-	-	-	-	6,6	2,5	-
23	-	+	+	-	0,5[h]	-	32	39,3	-	32	64	64	-	32	-	0,5	-	-	8	2,8	-
24	-	+	-	-	0,5[h]	-	27,9	34,8	33,8	128	64	64	32	64	-	-	-	-	18,6	2,4	-
25	-	-	+	-	-	-	-	33,8	-	-	-	-	-	-	-	0,4	0,8	-	3,9	8,2	-
26	-	-	-	-	-	-	27	-	42,2	-	-	64	-	-	-	-	-	-	-	1,5	-
27	-	-	-	-	-	-	-	32,1	-	-	-	-	-	-	-	-	-	-	3,3	2,6	-
28	-	+	+	-	-	-	28,7	29,9	33	-	64	64	-	-	-	-	-	-	17,9	3,1	-
29	-	-	-	1/640	0,5[h]	0,5[h]	32,7	30,9	32,6	32	64	128	-	-	-	-	-	-	7,9	1,2	-
30	-	+	+	-	-	-	30,2	30,8	34,1	32	64	64	-	32	32	-	0,6	-	3,4	7,7	1,2
31	-	-	-	-	-	-	28,1	33,7	-	32	32	16	-	-	-	-	-	-	18,5	3,5	-
32	-	-	-	-	-	-	-	-	35,2	-	-	-	-	-	-	-	-	-	5	23,6	-
33	-	-	-	1/320	0,5[h]	-	30,2	36	36	64	64	64	32	32	16	2,4	1	-	18,6	3,9	-
34	n/a	-	+	n/a	-	-	n/a	32,5	33,2	n/a	-	32	n/a	-	-	n/a	6,3	n/a	n/a	2	-

a. ELISA ITM: Cut-off ratio of 1. Results obtained from Cnops et al (1).
b. IHA ITM: Cut-off titer of 160. Results obtained from Cnops et al (1).
c. PCR ITM: Results given as Ct value. Results obtained from Cnops et al (1).
d. AWA-IFA LUMC: Results displayed as titer; cut-off 1:16
e. SEA-ELISA LUMC: Results displayed as titer; cut-off 1:32
f. Urine CAA: Results given in pg/ml, cut-off 0.3 pg/ml
g. Serum CAA: Results given in pg/ml, cut-off 1 pg/ml
h. IHA ITM, NI = results not interpretable
n/a: no sample

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3.

Sensitive Diagnosis and Post-Treatment Follow-Up of *Schistosoma mansoni* Infections in Asymptomatic Eritrean Refugees by Circulating Anodic Antigen Detection and Polymerase Chain Reaction

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ABSTRACT

The increasing number of refugees coming from or passing through *Schistosoma*-endemic areas and arriving in Europe highlights the importance of screening for schistosomiasis on arrival, and focuses attention on the choice of diagnostic test. We evaluate the diagnostic performance of circulating anodic antigen (CAA) detection in 92 asymptomatic refugees from Eritrea. Results were compared with already-available stool microscopy, serology, and urine point-of-care circulating cathodic antigen (POC-CCA) data. For a full diagnostic comparison, real-time polymerase chain reaction (PCR) and the POC-CCA were included. All outcomes were compared against a composite reference standard. Urine and serum samples were subjected to the ultra-sensitive and highly specific up-converting particle lateral flow CAA test, *Schistosoma* spp. real-time PCR was performed on urine and stool, and the POC-CCA was used on urine using the G-score method. CAA was detected in 43% of urine and in 40% of serum samples. Urine PCR was negative in all 92 individuals, whereas 25% showed *Schistosoma* DNA in stool. POC-CCA was positive in 30% of individuals. The CAA test confirmed all microscopy positives, except for two cases that were also negative by all other diagnostic procedures. Post-treatment, a significant reduction in the number of positives and infection intensity was observed, in particular regarding CAA levels. Our findings confirm that microscopy, serology, and POC-CCA lack the sensitivity to detect all active *Schistosoma* infections. Accuracy of stool PCR was similar to microscopy, indicating that this method also lacks sensitivity. The CAA test appeared to be the most accurate method for screening active *Schistosoma* infections and for monitoring treatment efficacy.

INTRODUCTION

Schistosomiasis affects more than 230 million individuals worldwide and is mainly endemic in sub-Saharan Africa [1]. The increasing number of African refugees and migrants traveling to Europe from or passing through *Schistosoma*-endemic regions stresses the importance of screening for *Schistosoma* infections [2], but at the same time raises the question of which diagnostic test is most suitable for this specific population. Although the commercially available point-of-care (POC) test detecting circulating cathodic antigen (CCA) is being used more often for diagnosing *S. mansoni* infection in non-endemic study settings [3-7], the detection of *Schistosoma*-specific antibodies remains the most widely used and recommended first-line test for screening [8,9], whereas the detection of eggs in urine or stool is still often used as a reference standard to confirm infection as well as to determine the species [10].

These diagnostic methods, however, lack the sensitivity to detect infections of low intensity. In particular, in the case of refugees and migrants, diagnosing schistosomiasis is challenging because microscopy is often negative when performed on a single stool sample, and antibody detection is unable to differentiate between active and past infection. An alternative and more sensitive method for the diagnosis of imported *Schistosoma* infections in routine laboratory settings is the detection of *Schistosoma*-specific DNA. For example, *Schistosoma* internal transcriber-spacer-2 (ITS2) real-time polymerase chain reaction (PCR) has been shown to detect egg-negative cases and demonstrate that individual DNA loads decrease after treatment [11], indicating its suitability to monitor efficacy of treatment. In addition, a significant number of microscopy-negative cases have detectable levels of circulating anodic antigen (CAA), a unique gut-associated antigen that is excreted continuously by adult worms and is therefore detectable within weeks of infection as well as in chronic infections [12-14]. CAA is detected by highly sensitive and specific monoclonal antibodies [15], making the up-converting particle (UCP) lateral flow (LF) CAA test highly specific. The presence of CAA in urine or serum demonstrates the presence of living worms [13], indicating active infection. CAA levels decline rapidly (within weeks) after treatment with praziquantel [16,17], making this test suitable for both individual and community screening as well as for follow-up after praziquantel treatment. The UCP-LF CAA test is a genus-specific test able to identify active *Schistosoma* infections of all known species [13]. This test has shown high accuracy in detecting the four major *Schistosoma* species in different endemic [13,18-21] as well as non-endemic routine diagnostic settings [16,22-24].

We evaluated the clinical diagnostic value of the UCP-LF CAA test for diagnosing *Schistosoma* infections among asymptomatic East African refugees and migrants within 2 years after their arrival in Switzerland, and compared this to currently used routine diagnostics consisting of microscopic detection of eggs in stool and urine and antibody detection [25, 26]. For a full diagnostic comparison, DNA detection by real-time PCR in stool and urine samples and the POC-CCA were included, and outcomes were compared against a composite reference standard. In addition, previous studies have shown that *Schistosoma* circulating antigens decrease after treatment, but at the same time suggest that the standard dose of praziquantel may not kill all worms present [12]. Therefore, this diagnostic comparison study includes not only the detection of infected cases, but also evaluates the UCP-LF CAA test for monitoring the efficacy of praziquantel treatment.

MATERIALS AND METHODS

This was a nested study within the original study, which focused on assessing infectious and non-communicable health conditions among East African refugees at arrival and post-integration in Switzerland [7,25,26]. Ethical approval was obtained from the institutional research commission of the Swiss Tropical and Public Health Institute (Swiss TPH; ref. no. FK 120) and the ethics committee of Northwest and Central Switzerland (ref. no. EKNZ 2015-353). For the nested study, a complete baseline sample set—consisting of stool, urine, and serum samples—was available from 92 asymptomatic Eritrean refugees (87 men; median age, 26 years; age range, 18–63 years). At the Swiss TPH, stool samples were subjected to microscopy using a large-volume sedimentation technique to maximize the yield of detectable eggs. Serology consisted of three in-house assays that are used routinely at the Swiss TPH diagnostic center, the details of which have been described previously [25]. In addition, urine samples were tested using the POC-CCA according to manufacturer's instructions; visible lines were documented according to their intensity as weak positive or clearly positive. Individuals who were found positive by any of these methods were treated with two doses of praziquantel (60 mg/kg body weight/dose) and monitored after approximately 12 to 18 months, when the same diagnostic procedures were repeated [26]. A complete post-treatment sample set—consisting of stool, urine, and serum samples—was available from 23 individuals (22 men; median age, 27 years; age range, 18–44 years).

At the Leiden University Medical Center (LUMC), samples were subjected to CAA detection (urine and serum), as well as DNA detection (urine and stool) and CCA detection (urine). Detection of CAA in urine and serum samples was performed using the laboratory-based, and most sensitive, concentration format of the UCP-LF CAA test, as described previously [13,18]. For urine samples the dry format of the test was used; serum samples were subjected to the wet format, which includes an additional UCP sonication step [13]. A reference standard of samples with a known CAA concentration was included to quantify individual CAA levels and to validate the cutoff (0.2 pg/mL for the urine test (UCAAhT3333) and 1 pg/mL for the serum test (SCAA500) as described in Corstjens et al. [13]). Samples were considered positive if the CAA concentration exceeded this cutoff; samples below the cutoff were considered negative.

The *Schistosoma* genus-specific ITS2 real-time PCR was used to detect *Schistosoma* DNA in urine and stool samples, as described previously [27,28]. Since its implementation in 2019, we scored 100% for the *Schistosoma* PCR at the annual international helminths external quality assessment scheme provided by the Dutch Foundation for Quality Assessment in Medical Laboratories based on the distribution of genuine clinical samples [29]. The stool PCR output consisted of a cycle threshold value, which represented the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence and thereby indicated the presence of parasite-specific DNA in the sample that was tested.

To determine the reproducibility of the POC-CCA test and to evaluate a more standardized scoring method, the test was repeated at LUMC using the same batch that also had been used at the Swiss TPH [25,26] (batch no. 50182; Rapid Medical Diagnostics, Pretoria, South Africa), which was still within the expiration date. The test was performed according to the

manufacturer's instructions, but now using the G-score scoring method for a more standardized interpretation and quantification of the test outcome [30]. POC-CCA G-scores were transformed into the more frequently used visual scores of trace (G2–3), 1+ (G4–5), 2+ (G6–7), and 3+ (G8–10) [29]. POC-CCA traces were considered negative for the analysis [31].

Data were entered into a Microsoft Office 365 Excel spreadsheet (Microsoft Corp., Redmond, WA) and analyzed using GraphPad Prism 8.4.2 (GraphPad Software Inc., San Diego, CA) and SPSS version 25 (IBM Corp., Armonk, NY). Statistical analysis was performed using descriptive statistics. The agreement between the pre-treatment outcomes of the different diagnostic tests was determined by κ statistics. McNemar's χ^2 test was used to compare sensitivity and specificity between diagnostic methods. In the absence of a single suitable reference standard, the sensitivity and specificity of the diagnostic tests were determined against a composite reference standard. To obtain the highest specificity, the diagnostic tests included in the composite reference standard were stool microscopy, urine CAA, serum CAA, and stool PCR. In this study, an individual was considered to be infected if either microscopy was positive or if at least two of the other diagnostic tests (i.e., urine/serum CAA, stool PCR) were positive. Spearman's ρ was used to assess the relationship between CAA levels in urine and serum, as well as the relationship between CAA levels (urine/serum) and POC-CCA G-scores and PCR cycle threshold values.

RESULTS

Pre-treatment (N=92). Figure 1 presents an overview of the percentage of positive results of the different diagnostic tests performed. The greatest number of positives was observed with the UCP-LF CAA test; 43.5% of individuals had detectable CAA levels in urine and 40.2% of individuals had detectable CAA levels in serum. At the Swiss TPH, in 23.9% of individuals, *S. mansoni* eggs were observed in stool samples, whereas no eggs were found in urine samples and, based on antibody detection, 42.4% of individuals were positive. In addition, at the LUMC, DNA was detected in the stool samples of 25.0% of individuals, whereas no DNA was detected in urine samples and POC-CCA was positive in 30.4% of individuals.

The agreement between urine and serum CAA and stool microscopy, serology, and stool PCR and POC-CCA is shown in Figure 2. In total, 48 individuals (52.2%) were positive by at least one of these five diagnostic tests. The majority of stool microscopy positives was detected by urine and/or serum UCP-LF CAA, stool PCR, or POC-CCA. Almost half of the CAA positive cases were not detected by stool microscopy nor by stool PCR, whereas the POC-CCA positives showed more overlap with the CAA positives. The degree of agreement as well as the discordance between the UCP-LF CAA test, stool PCR, and POC-CCA are shown in Table 1. Additional analyses of the degree of agreement and the discordance compared with the Swiss TPH data of stool microscopy and serology are included in Supplemental Table 1. The correlation between the UCP-LF CAA test, stool PCR, and POC-CCA is shown in Supplemental Figure 1 and Supplemental Table 2.

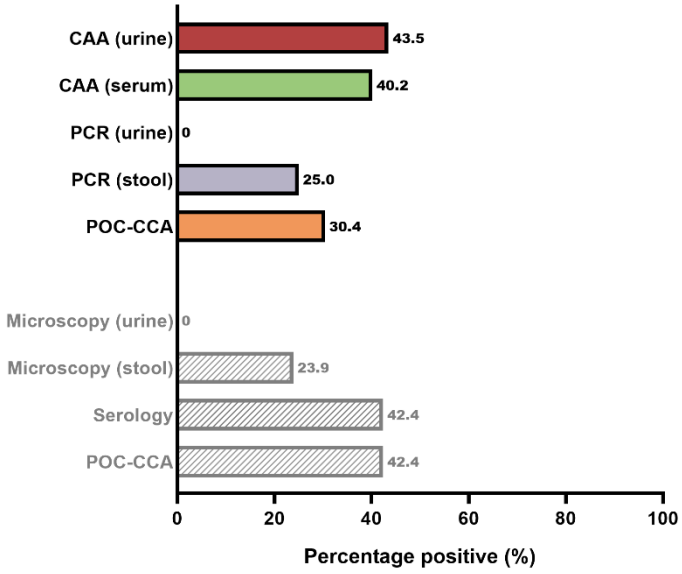

Figure 1. Percentage positive by urine and serum circulating anodic antigen (CAA), stool polymerase chain reaction (PCR), and point-of-care circulating cathodic antigen (POC-CCA) (in color) compared with percentage positive by stool sedimentation microscopy, serology, and POC-CCA (Swiss Tropical and Public Health Institute, in gray) in a group of 92 asymptomatic Eritrean refugees.

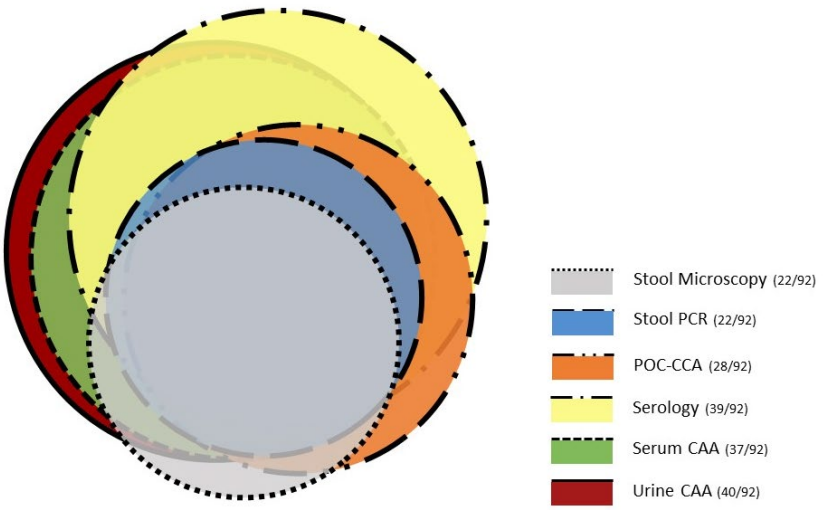

Figure 2. Proportional Venn diagram of urine and serum circulating anodic antigen (CAA) positives compared with stool sedimentation microscopy, serology, stool polymerase chain reaction (PCR), and point-of-care circulating cathodic antigen (POC-CCA) positives in a group of 92 asymptomatic Eritrean refugees.

Post-treatment (N=23). The total number of positive participants decreased after treatment, as detected by urine CAA (21.7%), serum CAA (17.4%), stool PCR (8.7%), and POC-CCA (8.7%). A significant decrease in average CAA levels in urine and serum was observed after treatment, with intensity reduction rates of 95.0% and 93.6% respectively. Individual pre- and post-treatment outcomes of urine and serum CAA, stool PCR, and POC-CCA are shown in Figure 3. The majority of individuals became negative after treatment. One individual remained positive by all four diagnostic methods (indicated by the green shapes in Figure 3), whereas another individual remained positive by serum CAA and stool PCR only (indicated by the red shapes in Figure 3B and 3C).

The sensitivity and specificity of all diagnostic tests compared with the composite reference standard are shown in Table 2. In total, 39 individuals (42.4%) were positive by the composite reference standard. The UCP-LF CAA test in serum showed the highest sensitivity (95%), followed by the UCP-LF CAA test in urine (90%). The POC-CCA test showed a lower sensitivity (62%), comparable to stool PCR (56%) and stool microscopy (56%).

Table 1. The level of agreement between circulating anodic antigen (CAA), stool polymerase chain reaction (PCR), and point-of-care circulating cathodic antigen (POC-CCA) by Cohen's $\varkappa$ coefficient and McNemar's χ^2-test in a group of 92 asymptomatic Eritrean refugees.

Diagnostic test	Reference test *n*		Cohen's kappa			McNemar's *P* value
			$\varkappa$ value	Interpretation*	*P* value	
	CAA (urine)					
CAA (serum)	Positive	Negative				
Positive	36	1	0.89	Almost perfect	<0.001	0.375
Negative	4	51				
	PCR (stool)					
CAA (urine)	Positive	Negative				
Positive	22	18	0.56	Moderate	<0.001	<0.001
Negative	1	51				
	PCR (stool)					
CAA (serum)	Positive	Negative				
Positive	22	15	0.61	Substantial	<0.001	0.001
Negative	1	54				
	POC-CCA					
CAA (urine)	Positive	Negative				
Positive	23	17	0.50	Moderate	<0.001	0.017
Negative	5	47				
	POC-CCA					
CAA (serum)	Positive	Negative				
Positive	24	13	0.60	Moderate	<0.001	0.049
Negative	4	51				
	PCR (stool)					
POC-CCA	Positive	Negative				
Positive	18	10	0.60	Moderate	<0.001	0.302
Negative	5	59				

CAA = circulating anodic antigen; PCR = polymerase chain reaction; POC-CCA = point-of-care circulating cathodic antigen.
*Interpretation of $\varkappa$ coefficient: ≤0, chance; 0.01 to 0.20, slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; 0.81 to 0.99, almost perfect.

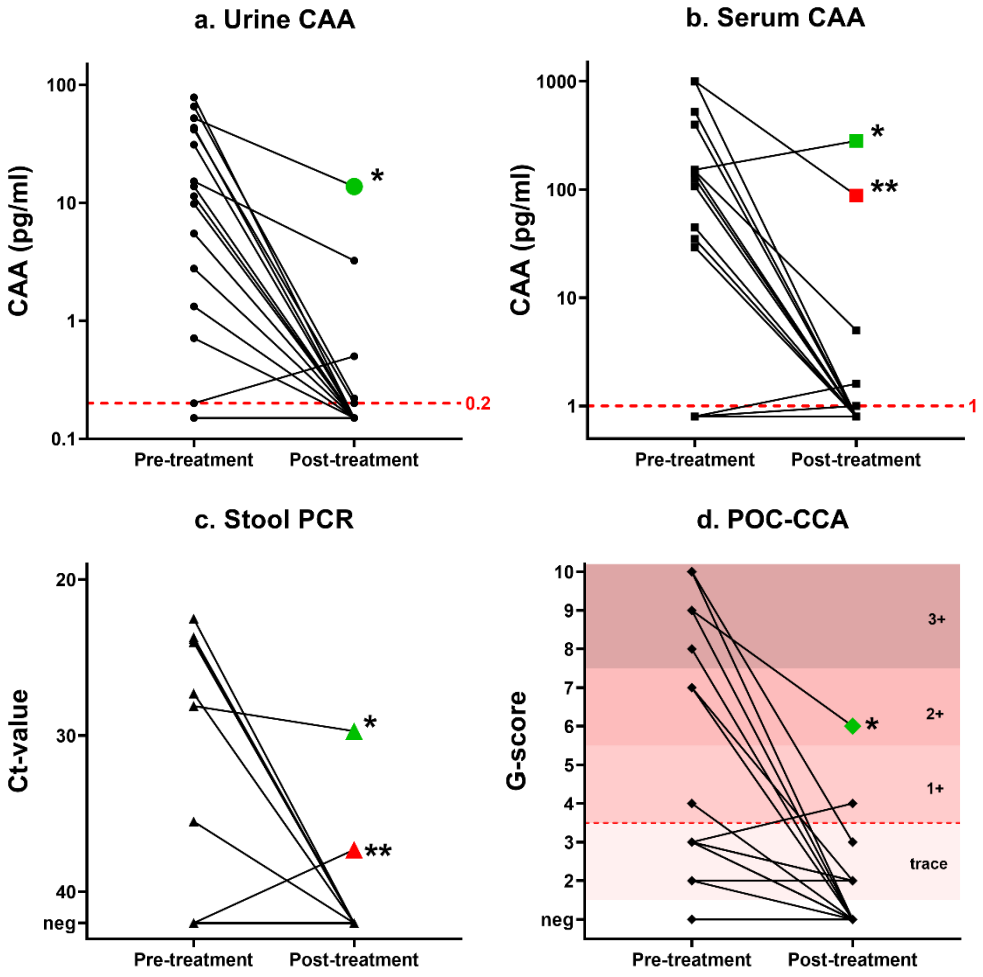

Figure 3. Effect of treatment on individual test outcomes: (A) urine circulating anodic antigen (CAA), (B) serum CAA, (C) stool polymerase chain reaction (PCR) and (D) point-of-care circulating cathodic antigen (POC-CCA) in a group of 23 asymptomatic Eritrean refugees. Ct-value = cycle threshold value.
* Individual who tested positive in all four tests after treatment.
** Individual who tested positive by serum CAA and stool PCR after treatment.

Table 2. Accuracy of the different diagnostic tests compared against a composite reference standard in a group of 92 asymptomatic Eritrean refugees.

Diagnostic test and location	Outcome	CRS*, *n*		Diagnostic accuracy, %		Cohen's κ			
		Positive	Negative	Sensitivity	Specificity	κ	Interpretation†	*P* value	McNemar's *P* value
LUMC, Leiden, the Netherlands									
CAA (urine)	Positive	36	3	90%	94%	0.845	Almost perfect	< 0.001	1.000
	Negative	4	49						
CAA (serum)	Positive	37	0	95%	100%	0.955	Almost perfect	< 0.001	0.500
	Negative	2	53						
PCR (stool)‡	Positive	22	1	56%	98%	0.576	Moderate	< 0.001	< 0.001
	Negative	17	52						
POC-CCA§	Positive	24	4	62%	92%	0.561	Moderate	< 0.001	0.019
	Negative	15	49						
Swiss TPH, Basel, Switzerland||									
Microscopy (stool)¶	Positive	22	0	56%	100%	0.599	Moderate	< 0.001	<0.001
	Negative	17	53						
Serology	Positive	31	8	79%	85%	0.644	Substantial	< 0.001	1.000
	Negative	8	45						
POC-CCA§	Positive	29	10	74%	81%	0.555	Moderate	< 0.001	1.000

CAA = circulating anodic antigen; CRS = composite reference standard; LUMC = Leiden University Medical Center; PCR = polymerase chain reaction; POC-CCA = point-of-care circulating cathodic antigen; Swiss TPH = Swiss Tropical and Public Health Institute

* CRS was based on the detection of eggs in stool and/or DNA in stool and/or CAA in urine/serum: an individual was considered positive if either microscopy was positive or at least two out of other diagnostic tests were positive.

† Interpretation of κ coefficient: ≤0, chance; 0.01-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; 0.81-0.99, almost perfect.

‡ All urine samples were negative by urine PCR.

§ The same POC-CCA batch was used at LUMC and Swiss TPH (#50182), but a different scoring approach, see Materials and Methods.

|| Data available from Swiss TPH.

¶ All urine samples were negative by urine microscopy.

DISCUSSION

The high prevalence of *S. mansoni* infections in this group of asymptomatic Eritrean refugees, as demonstrated previously by stool microscopy, serology, and POC-CCA, was confirmed in our study by the highly sensitive and specific UCP-LF CAA test, stressing the importance of timely and efficacious screening of refugees and migrants for *Schistosoma* infections.

Previous results from the main study suggested that a combination of POC-CCA and serology would be the ideal screening method for *S. mansoni* infections in asymptomatic refugees [25]. In our study, the UCP-LF CAA test was evaluated as a potential new tool for screening and post-treatment monitoring of *Schistosoma* infections. Our results demonstrate that a single urine or serum UCP-LF CAA test detected an even greater number of *Schistosoma* positives compared with stool microscopy and serology. The UCP-LF CAA test was able to confirm all microscopy positives, except two cases that were positive only by microscopy and negative by all other diagnostic tests. In addition, 21 positives were observed by the UCP-LF CAA test that were negative by microscopy, confirming the added value of CAA detection in this specific population. Especially in the case of low worm burden the detection of eggs can be very difficult [32,33], while living worms continue to release CAA which is detected by the UCP-LF CAA test. In our study, observed CAA levels were relatively high, in particular given the fact that none of the individuals showed any schistosomiasis-related signs or symptoms [25]. Because CAA is excreted by all *Schistosoma* species, even in the case of hybrid infections [24], the UCP-LF CAA

test is applicable to migrants originating from all *Schistosoma*-endemic areas. Extensive parasitological diagnosis has been performed in this cohort and there was no evidence of other helminth infections [7], which is in line with the high specificity of the UCP-LF CAA test.

In the absence of a single suitable reference standard, the diagnostic methods were compared against a composite reference standard [34,35], which is an approach that has also been used previously [17,19]. It can serve as an alternative to, for example, latent class analysis, as criteria for this type of analysis are often difficult to meet, especially in the case of schistosomiasis diagnostic evaluation studies (e.g., conditional dependence among tests, small sample size, the limited number of tests included in the analysis) [36]. The urine UCP-LF CAA test showed a sensitivity of 90% compared with the composite reference standard, which is similar to findings from previous studies, even though they were performed in endemic settings and some of them concerned other *Schistosoma* species [19,20,37]. Compared with the composite reference standard, the UCP-LF CAA test in serum demonstrated the highest sensitivity of 95%, thereby signifying its potential use for screening purposes for accurate detection and monitoring of *Schistosoma* infections in refugees and migrants. Although the performance of stool PCR was expected to be better than microscopy, as also observed in previous studies, our results show a similar sensitivity. An explanation for this could be that a large-volume sedimentation technique with at least 10 g of stool was used to detect the eggs, resulting in a substantially higher sensitivity than a single Kato-Katz thick smear, as often used in endemic settings [38]. Although the absolute number of positive cases was similar by either CAA or serology, the diagnostic accuracy of serology was substantially less compared with that of the UCP-LF CAA test. This seems to be a combination of 1) serology missing some cases with an active infection based on the presence of CAA and 2) persisting positive serology when no active infection could be demonstrated. This inability of serology to differentiate between active and passive infection is well known [39].

CAA levels in urine and serum decreased significantly after treatment, indicating clearance of infection and thereby again confirming the specificity of the UCP-LF CAA test. Although the praziquantel treatment regimen (two doses of 60 mg/kg body weight) was aimed at parasite eradication rather than parasite reduction (standard single dose of 40 mg/kg recommended for mass drug administration in endemic regions by the WHO), in some individuals, urine and serum CAA levels remained detectable 12 to 18 months after treatment. Reinfection can be ruled out as individuals remained in Switzerland during the study period [26]. Because CAA levels decreased in most cases after treatment, and because treatment compliance was not controlled (no directly observed treatment), these individuals might not have complied fully with the treatment schedule of two doses of praziquantel, thereby resulting in an uncleared infection. Because CAA levels decrease (within weeks) after treatment [12,16,17], short-term follow-up is possible. This would be a major advantage especially for refugees or migrants, because, in general, they are a very mobile population often resulting in lower compliance and greater lost-to-follow-up rates.

When looking at the reproducibility and performance of the POC-CCA test, a comparable percentage of positives was observed at both the Swiss TPH and LUMC, demonstrating the consistent performance of this specific POC-CCA batch and confirming that testing after shipping and storing the urine samples frozen for 1 year does not influence the

outcome of the test when using the same batch. Unfortunately, although the same batch was used, it was difficult to compare the individual results because of the difference in the scoring method; the POC-CCA test at the Swiss TPH was only scored (weak) positive or negative [25]. A moderate $\varkappa$ agreement and a strong correlation was found between the POC-CCA test performed at the LUMC and the other diagnostic tests (stool microscopy, stool PCR, serum/urine CAA), whereas only a slight to moderate $\varkappa$ agreement between these diagnostic tests and the POC-CCA test performed at the Swiss TPH was found. A clear effect of treatment was observed based on G-scores, whereas at the Swiss TPH, several inconclusive and inconsistent results were observed at follow-up [26]. Overall, this comparison stresses the necessity of using a standardized method when scoring POC-CCA test outcomes pre-treatment as well as post-treatment.

Because the UCP-LF CAA test is a genus-specific test [13], the results of our study cannot confirm whether the CAA test only detected *S. mansoni* or also other *Schistosoma* infections. However, based on microscopy, only *S. mansoni* eggs were detected in this study population, which is confirmed by the presence of *Schistosoma* DNA in stool samples and the absence of any *Schistosoma* DNA in urine samples. Furthermore, considering their area of origin (Eritrea) and migration route, only *S. mansoni* infections were assumed in this group [25].

It was not possible to determine the correlation between intensity of infection based on stool microscopy and diagnostic tests performed at the LUMC because of the absence of fecal egg counts, but such a correlation has been described in previous studies [25,40-42].

Unfortunately, a complete post-treatment follow-up data set was only available from a relatively small number of individuals. In addition, only those who tested positive at baseline (pre-treatment) were monitored after treatment. For better comparison, follow-up could have taken place in all those who participated in the initial study and, preferably, also earlier than 12 to 18 months after treatment.

The UCP-LF CAA test is not commercially available yet. At the moment, the test is used for research purposes and in collaborative projects. However, recently the UCP-LF CAA test has been implemented into the routine diagnostic laboratory of the LUMC and is now available for individual case detection. In addition, initiatives have been taken to develop a more easy-to-use and visually scored POC-CAA test based on a finger-prick blood sample (https://www.finddx.org/ntd/schisto-rdts/).

CONCLUSION

This study confirms the importance of screening (asymptomatic) refugees and migrants for schistosomiasis. The accuracy of stool PCR was similar to extensive microscopy, indicating that this method also lacks sensitivity in this specific population. The UCP-LF CAA assay appears to be the most sensitive method for screening *Schistosoma* infections. In addition, although tested in only a small number of individuals, our results also confirm CAA to be a suitable, genus-specific marker for monitoring praziquantel treatment efficacy.

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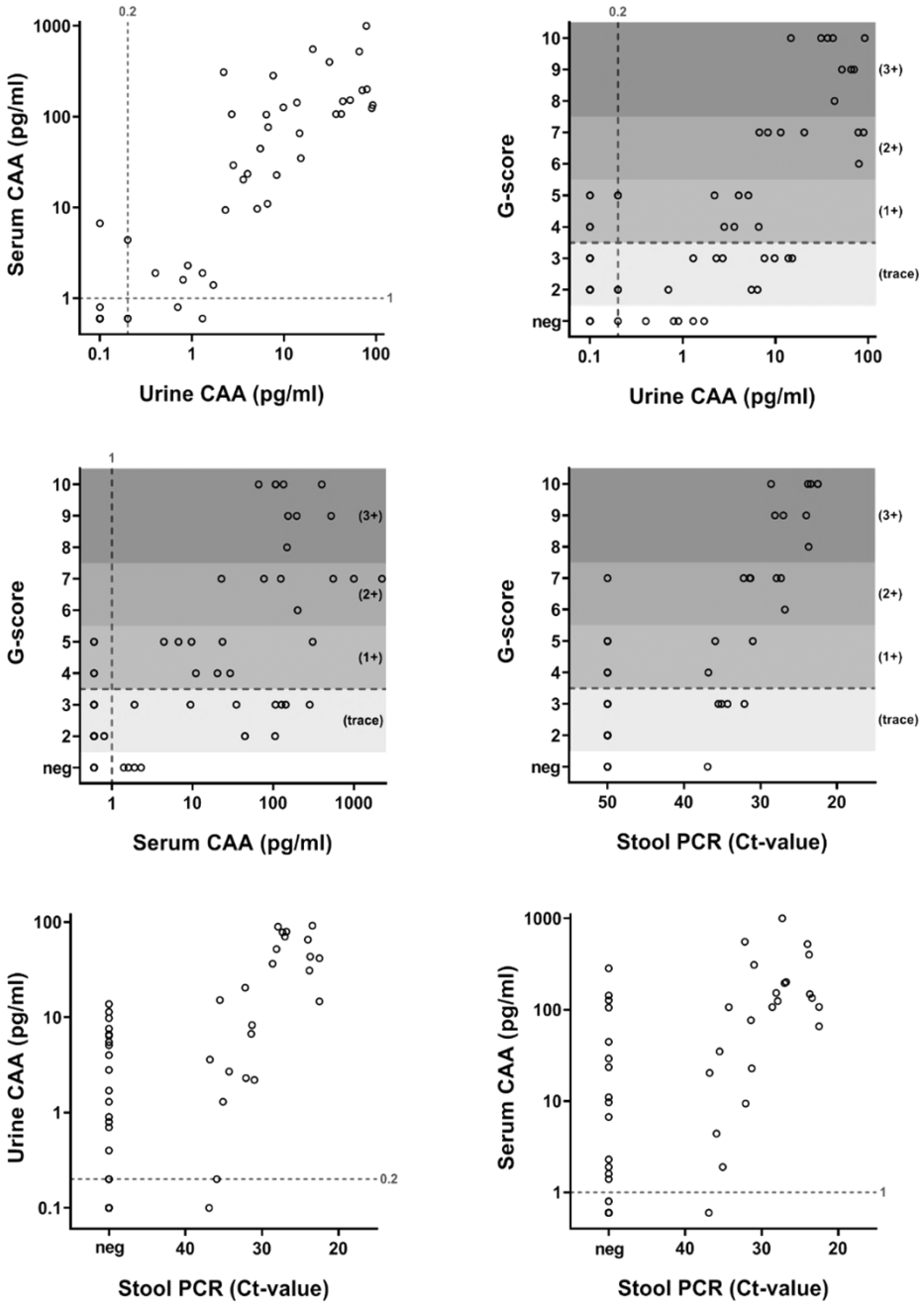
SUPPLEMENTARY MATERIALS

Supplementary Table 1. The level of agreement between CAA, stool PCR and POC-CCA and routine diagnostic tests performed at Swiss TPH (stool sedimentation microscopy and serology) by Cohen's kappa coefficient and McNemar's χ^2-test in a group of 92 asymptomatic Eritrean refugees.

N=92			Cohen's kappa $\varkappa$	Interpretation[1]	*p*-value	McNemar *p*-value
	Microscopy (stool)					
CAA (urine)	Positive	Negative				
Positive	19	21	0.440	Moderate	$p<0.001$	$p<0.001$
Negative	3	49				
	Microscopy (stool)					
CAA (serum)	Positive	Negative				
Positive	20	17	0.540	Moderate	$p<0.001$	$p=0.001$
Negative	2	53				
	Microscopy (stool)					
PCR (stool)	Positive	Negative				
Positive	18	5	0.735	Substantial	$p<0.001$	$p=1.000$
Negative	4	65				
	Microscopy (stool)					
POC-CCA	Positive	Negative				
Positive	18	10	0.618	Substantial	$p<0.001$	$p=0.180$
Negative	4	60				
	Serology					
CAA (urine)	Positive	Negative				
Positive	30	10	0.579	Moderate	$p<0.001$	$p=1.000$
Negative	9	43				
	Serology					
CAA (serum)	Positive	Negative				
Positive	30	7	0.642	Substantial	$p<0.001$	$p=0.804$
Negative	9	46				
	Serology					
PCR (stool)	Positive	Negative				
Positive	21	2	0.529	Moderate	$p<0.001$	$p<0.001$
Negative	18	51				
	Serology					
POC-CCA	Positive	Negative				
Positive	23	5	0.515	Moderate	$p<0.001$	$p=0.027$
Negative	16	48				

1. Interpretation of $\varkappa$ coefficient: ≤0, chance; 0.01-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; 0.81-0.99, almost perfect.
Abbreviations: CAA, circulating anodic antigen; PCR, polymerase chain reaction; POC-CCA, point-of-care circulating cathodic antigen.



Supplementary Figure 1. Pre-treatment correlation between CAA, stool PCR and POC-CCA in a group of 92 asymptomatic Eritrean refugees.
Abbreviations: CAA, circulating anodic antigen; Ct-value, cycle threshold value; PCR, polymerase chain reaction; POC-CCA, point-of-care circulating cathodic antigen.

Supplementary Table 2. Pre-treatment correlation between CAA, stool PCR and POC-CCA in a group of 92 asymptomatic Eritrean refugees.

Diagnostic comparison	Spearman's rho[1]	*p*-value	Interpretation
Urine CAA vs Serum CAA	0.874	$p<0.01$	Very strong
Urine CAA vs Stool PCR	-0.659	$p<0.01$	Strong
Serum CAA vs Stool PCR	-0.636	$p<0.01$	Strong
Urine CAA vs POC-CCA	0.611	$p<0.01$	Strong
Serum CAA vs POC-CCA	0.683	$p<0.01$	Strong
POC-CCA vs Stool PCR	-0.676	$p<0.01$	Strong

1. Interpretation of Spearman's rho: ≤0, chance; 0.00 -0.20, negligible; 0.21-0.40, weak; 0.41-0.60, moderate; 0.61-0.80, strong; 0.81-1.00, very strong.
Abbreviations: CAA, circulating anodic antigen; PCR, polymerase chain reaction; POC-CCA, point-of-care circulating cathodic antigen.

Part II.

Performance of circulating anodic antigen detection in endemic settings

4.

Repeated doses of Praziquantel in Schistosomiasis Treatment (RePST) – single versus multiple praziquantel treatments in school-aged children in Côte d'Ivoire: a study protocol for an open-label, randomised controlled trial

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ABSTRACT

Background: Large scale administration of the anthelminthic drug praziquantel (PZQ) to at-risk populations is the cornerstone of schistosomiasis control, although persisting high prevalence of infections in some areas and growing concerns of PZQ resistance have revealed the limitations of this strategy. Most studies assessing PZQ efficacy have used relatively insensitive parasitological diagnostics, such as the Kato-Katz (KK) and urine-filtration methods, thereby overestimating cure rates (CRs). This study aims to determine the efficacy of repeated PZQ treatments against *Schistosoma mansoni* infection in school-aged children in Côte d'Ivoire using the traditional KK technique, as well as more sensitive antigen- and DNA-detection methods.

Methods: An open-label, randomised controlled trial will be conducted in school-aged children (5 to 18 years) from the region of Taabo, Côte d'Ivoire, an area endemic for *S. mansoni*. This 8-week trial includes four two-weekly standard doses of PZQ in the "intense treatment" intervention group and one standard dose of PZQ in the "standard treatment" control group. The efficacy of PZQ will be evaluated in stool samples using the KK technique and real-time PCR as well as in urine using the point-of-care circulating cathodic antigen test and the up-converting phosphor, lateral flow, circulating anodic antigen assay. The primary outcome of the study will be the difference in CR of intense versus standard treatment with PZQ on individuals with a confirmed *S. mansoni* infection measured by KK. Secondary outcomes include the difference in CR and intensity reduction rate between the intense and standard treatment groups as measured by the other diagnostic tests, as well as the accuracy of the different diagnostic tests, and the safety of PZQ.

Discussion: This study will provide data on the efficacy of repeated PZQ treatment on the clearance of *S. mansoni* as measured by several diagnostic techniques. These findings will inform future mass drug administration policy and shed light on position of novel diagnostic tools to evaluate schistosomiasis control strategies.

Trial registration: The study is registered at EudraCT (2016–003017-10, date of registration: 22 July 2016) and (NCT02868385, date of registration: 16 August 2016).

Keywords: Schistosomiasis, *Schistosoma mansoni*, Praziquantel, Treatment efficacy, Diagnostic test, Kato-Katz, CAA, CCA, PCR.

BACKGROUND

Schistosomiasis is still a major public health problem in many tropical countries, particularly in Africa where more than 90% of the global burden of schistosomiasis occurs [1]. Large-scale administration of the anthelminthic drug praziquantel (PZQ) to at-risk populations has become the cornerstone of schistosomiasis control [2,3]. This strategy – known as preventive chemotherapy – has been successful in reducing infection intensities, and hence morbidity [4,5]. As morbidity is a result of cumulative exposure to a high number of schistosomes, school-aged children bear the largest burden of disease because they carry the highest intensity infections [6]. Therefore, mass drug administration (MDA) of preventive chemotherapy targets school-aged children primarily [6,7]. These children are intermittently treated with a single oral dose of 40 mg/kg PZQ, the frequency of which depends on the prevalence of the infection in the community [8]. Target populations are divided into high-, moderate- and low-risk communities in which school-aged children are treated with PZQ once a year, once every 2 years or twice during their primary schooling age, respectively [9]. PZQ is the drug of choice for the treatment of all forms of schistosomiasis due to its high efficacy and excellent safety profile [10].

Observed cure rates (CRs) after a single dose of PZQ treatment (40 mg/kg) range between 42 and 79% for *Schistosoma mansoni* and between 37 and 93% for *S. haematobium* in school-aged children [11]. A second dose of PZQ at a later time point can increase the CR up to 93% for *S. mansoni* [11-13] and up to 99% for *S. haematobium* [11]. However, the estimated efficacy of PZQ is highly dependent on the diagnostic tool used to measure CRs. Most studies have used traditional parasitological methods, such as the Kato-Katz (KK) and urine-filtration (UF) methods based on microscopy and determining the presence/excretion of eggs. These methods lack sensitivity for diagnosing low level infections and as such overestimate CRs [14,15]. More sensitive diagnostic tools for schistosomiasis which are currently available and can be implemented in the field, are much more suited to evaluate the efficacy of PZQ and alternative dose regimens. For example, the commercially available point-of-care circulating cathodic antigen (POC-CCA) test, which indicates active worm infection by detection of parasite CCA in urine, has shown a high diagnostic accuracy for *S. mansoni* with a sensitivity ranging between 78 and 92% and specificity approaching 100% [15-17]. Over the past 10 years, this test has been widely evaluated in sub-Saharan Africa and is now recommended as the first line diagnostic to map schistosome prevalence and facilitate preventive chemotherapy strategic decision-making [7,16,18]. In addition, there is a pressing need for ultra-sensitive diagnostic tools for areas where prevalence and infection intensity have been reduced to very low levels. Such diagnostic tools are needed to confirm interruption of transmission and possibly elimination of schistosomiasis. The circulating anodic antigen (CAA) detection assay fulfils these requirements. This assay measures parasite antigen both in urine and serum using an ultrasensitive reporter technology (up-converting phosphor particles, UCP) in combination with common immune-chromatography, lateral flow (LF). This UCP-LF CAA assay has shown high sensitivity and specificity for all four main schistosome species (*S. haematobium*, *S. japonicum*, *S. mansoni* and *S. mekongi*) [19-22]. In addition to the UCP-LF CAA assay, highly specific and sensitive molecular polymerase chain reaction (PCR) techniques that detect parasite-specific DNA in stool and urine have also become available [16]. The combination of worm-derived antigens and egg-derived

nucleic acids, are envisaged to further increase the sensitivity and specificity of the diagnostic toolbox and allow for a comprehensive assessment of PZQ efficacy, with respect to both parasite worm dynamics and fecundity.

Rationale

There is a need for a re-evaluation of previously established PZQ CRs to provide evidence for continuing mass distribution of PZQ in high risk communities. Previously, CRs may have been overestimated due to insensitive diagnostic tools, whereas continuing reinfections and the fact that PZQ has little activity on immature worms, might have led to an underestimation of the therapeutic effect [23-26]. Repeated treatment with PZQ at short intervals (e.g. 2–8 weeks) in areas with ongoing transmission will more effectively target non-susceptible schistosomula as they will have matured into drug susceptible worms during this period [11,27], thereby increasing the drug effectiveness. As the short metabolic half-life of PZQ may also limit its effectiveness by suboptimal plasma levels, repeated dosing will increase the chance that all worms are affected [28]. Whether this will be reflected in a significant decrease in schistosome prevalence and the potential to interrupt transmission, remains to be investigated [11,15,29]. In this study, we will evaluate the effect of multiple doses of PZQ on parasite clearance and tolerance in individuals infected with *S. mansoni*.

The primary objective of this study is to determine the efficacy of PZQ treatment for clearing *S. mansoni* infections in a multiple dose regimen (standard dose, four times, two-week intervals) using the KK technique. Secondary objectives include determining the efficacy of PZQ for clearing *S. mansoni* infections in a multiple dose regimen using DNA- and antigen-detection techniques, evaluationg the safety of PZQ and determining the accuracy of the different diagnostic tests used in this study. Exploratory objectives include modelling the effect of multiple PZQ treatments on the transmission of schistosomiasis as well as modelling the biological effects of multiple PZQ treatments on individual worm burden, egg load and re-infection rates.

METHODS

Study design

To evaluate the repeated doses of PZQ in schistosomiasis treatment (RePST), an open-label, randomised controlled trial will be conducted, with the primary aim to compare the efficacy of one versus four doses of PZQ in *S. mansoni* infected school-aged children in Côte d'Ivoire, using the traditional KK thick smear technique as well as with more sensitive antigen- and DNA-detection methods (Fig. 1). After screening for eligibility, participants are randomised into two groups in a 1:1 ratio. Individuals assigned to the standard treatment group will receive a single dose of PZQ (40 mg/kg) at baseline (week 0) and will receive no further treatment until the final visit. Individuals assigned to the intense treatment group will receive four doses of PZQ at baseline (week 0) and at three other time points with 2-week intervals. Follow-up and sample collection will take place every week for a period of eight weeks. At the end of the study, all children from selected communities (including study participants) will be offered a standard dose of PZQ (single oral dose of 40 mg/kg) as well as albendazole (single oral dose of 400 mg)

according to international guidelines of the World Health Organization (WHO), in coordination with the National Control Programme of Côte d'Ivoire (Programme National de Lutte contre les Maladies Tropicales Négligées à Chimiothérapie Préventive, PNLMTN-CP), for the treatment of schistosomiasis and soil-transmitted helminth infections, respectively.

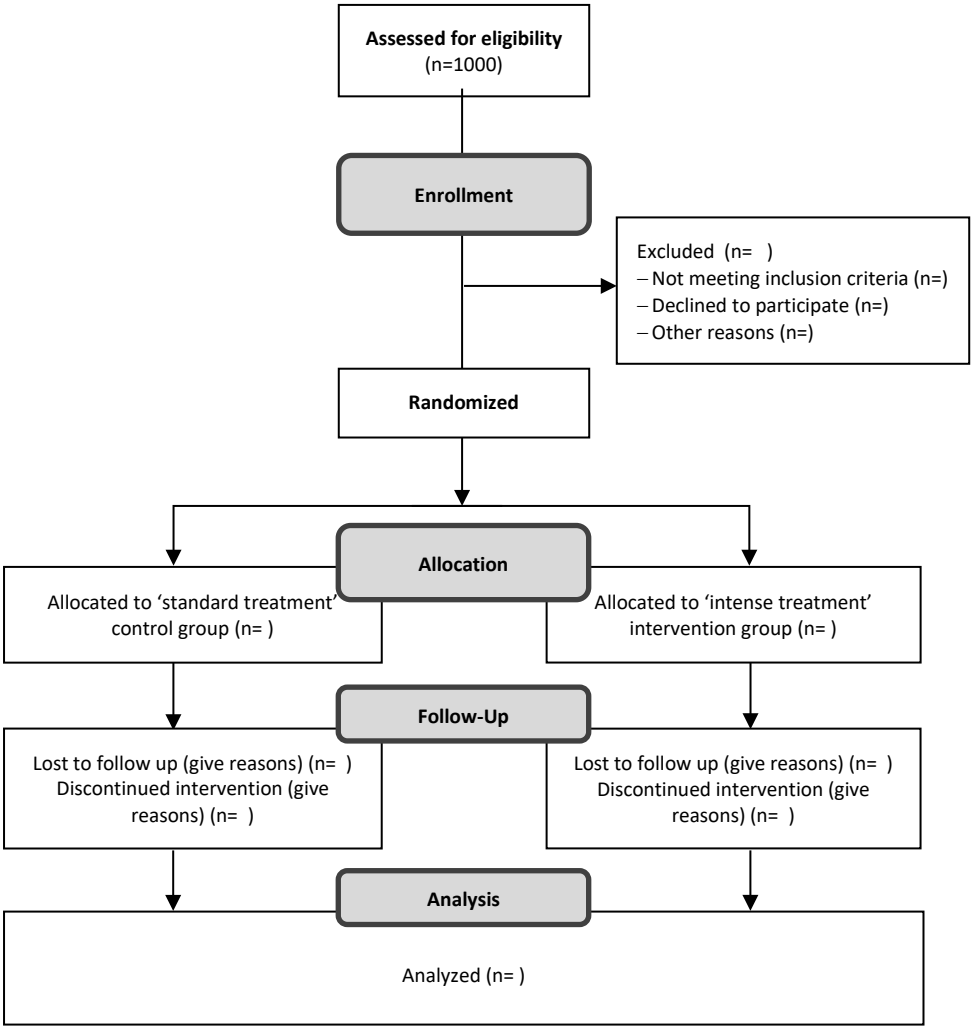

Figure 1. Flow-diagram.

Study area and population

The study population will consist of school-aged children (5 to 18 years) from selected villages of Singrobo, Ahouaty and N'Denou in the Taabo health district, south-central Côte d'Ivoire. This district is part of the Taabo health and demographic surveillance system (HDSS) and is located approximately 150 km north of Abidjan and has been described in more detail previously

[30,31]. It comprises a small town, 13 villages and over 100 hamlets, with a total population of 42.480 inhabitants in 2013. The Taabo HDSS focuses, among other things, on neglected tropical diseases such as schistosomiasis and soil-transmitted helminths. Repeated cross-sectional epidemiological surveys on approximately 5–7% of the population and specific, layered-on haematological, parasitological and questionnaire surveys have been regularly conducted every year since 2009 within the Taabo HDSS [30].

Inclusion criteria

In order to be eligible to participate in this study, an individual must meet the following criteria:

- provide oral and signed assent as well as written informed consent signed by parents/legal guardian(s)
- have a confirmed *S. mansoni* infection (i.e. positive test result for POC-CCA and at least one positive KK thick smear);
- be between 5 and 18 years of age;
- have a good medical condition, as determined by the study physician based on biochemical, physical and clinical indicators (i.e. absence of acute or severe chronic disease);
- have received no PZQ treatment in the past 3 months; and be able and willing to provide multiple blood, stool and urine samples during the study.

Exclusion criteria

A potential participant who does not meet the inclusion criteria or who meets any of the following criteria will be excluded from participation in this study:

- have a confirmed *S. haematobium* infection (as determined by UF);
- have a known allergy to study medication (i.e. PZQ and albendazole); and
- is pregnant (confirmed by pregnancy HCG test) or lactating.

Procedures

Screening and informed consent

Individuals will be assessed for eligibility during a 2-week baseline screening. This will include public meetings of awareness and health education, to allow all members of the communities to be well informed on the project. During these meetings, particular emphasis will be placed on modes of transmission, associated pathologies and risk factors related to schistosomiasis in particular and intestinal worms in general. After explaining the purpose of the study in the local language, potential participants will be asked to participate. After obtaining oral and written assent from the children and signed informed consent from their parents/legal guardians, standard demographic and other characteristics (e.g. age and sex) will be collected. Participants will be asked to provide a urine sample which will be tested on site immediately for *Schistosoma* infection by the POC-CCA urine test as well as with UF. The initial screening by POC-CCA will increase the likelihood of finding participants with a positive KK. Those with a positive POC-CCA test result (scoring 1+ or higher) as well as a negative UF result, will be asked to provide one stool sample which will be examined for *S. mansoni* eggs by triplicate KK. If at least one out

of three smears is positive, the participant will be asked to undergo a biological, clinical and physical examination to determine whether he/she is in good health. For the biological exam, a blood sample will be obtained and tested for haematological indicators, liver function and renal function parameters. A study physician will perform the physical and clinical examinations, which will consist of checking the participant's physical condition as well as determining if the participant has any chronic disease(s). All participants who meet the inclusion criteria will be enrolled and randomised.

Follow-up, sampling procedure and storage of samples

After randomisation, follow-up will take place on the selected groups every week. Participants will be asked to provide one urine sample every week and one stool sample every two weeks. A more detailed and schematic representation is given in Fig. 2. Each urine sample will be tested with the POC-CCA test and KK examination will be performed on each stool sample. An additional amount of sieved stool (300–400 µL of volume) will be mixed with 1 mL of 96% ethanol and stored for real-time PCR [32]. After every visit, all collected samples will be taken to the laboratory of the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire (CSRS)-Fairmed in Taabo for temporary storage. Urine samples will be stored at − 20 °C and stool samples stored in ethanol. Once sample collection is completed, all samples will be transported from the CSRS-Fairmed laboratory in Taabo to CSRS in Abidjan.

From all samples, one aliquot will be stored at CSRS in Abidjan for long-term storage and one aliquot will be sent to the Leiden University Medical Center (LUMC), the Netherlands, for additional testing with the UCP-LF CAA assay on urine samples and real-time PCR on stool samples.

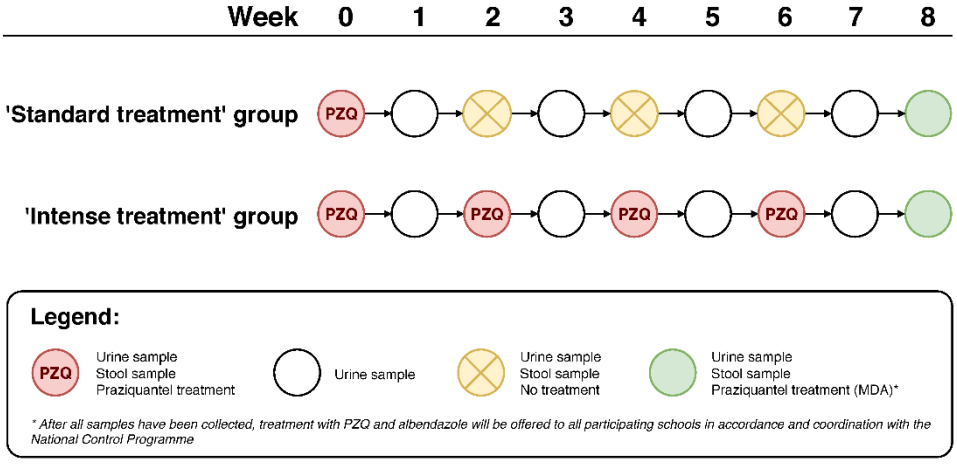

Figure 2. Schematic representation of follow-up, treatment and sampling procedures in the standard and intense treatment group.

Diagnostics

As part of the inclusion-exclusion process, several tests will be executed to determine eligibility for the study. Absence of pregnancy, if appropriate, will be confirmed with locally available pregnancy (HCG) tests. Infection with S. haematobium, will be determined by filtration of 10 mL of urine and microscopical examination, as described previously [33]. Finally, to verify a potential participant's good health, a blood sample will be tested for basic biochemical and haematological parameters (e.g. urea, creatinine, liver enzymes and haemoglobin) in a qualified local laboratory. All urine samples will be tested at the CSRS-Fairmed laboratory in Taabo using the POC-CCA assay (batch no.: 170522062, Rapid Medical Diagnostics, Pretoria, South Africa) as previously described [7,17,34]. At 20 min, valid tests will be scored as negative or positive according to a set of 10 novel artificial cassettes with inkjet-printed test lines representing negative and positive results depending on the intensity of the test line (artificial score 1–10 0). These novel artificial scores will be transformed into stratified positive scores of trace, 1+, 2+ or 3+. All tests will be read independently by two trained laboratory technicians. In case of discordant results, a third independent investigator will be consulted and results will be discussed until agreement is reached within 25 min. UF will be performed on urine samples collected at the final time point (week 8). Additionally, all urine samples will be examined at the LUMC using the UCP-LF CAA assay: a maximum of 2 mL urine will be analysed with a cut-off threshold of 0.1 pg/ml, as previously described [19,35]. CAA results will be reported quantitatively in pg/ml.

From each stool sample triplicate KK thick smears will be prepared at the laboratory of CSRS-Fairmed in Taabo, using 41.7 mg templates, following standard protocols [36]. Briefly, three KK thick smears (A, B and C) will be prepared on microscope slides and examined quantitatively under a microscope by two independent, experienced laboratory technicians one (A), two (B) and three (C) days after preparation. Results will be reported as eggs per slide and converted to eggs per gram of faeces (EPG).

To detect the presence of *Schistosoma* DNA, real-time PCR analysis will be performed on the ethanol-preserved stool samples at the LUMC. Sample handling, DNA isolation and PCR analysis will be performed as described previously [37,38]. The PCR output consists of a cycle-threshold (Ct) value, which represented the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence, thereby indicating the amount of parasite-specific DNA in the sample that was tested.

Treatment and adverse events

Treatment of participants with PZQ will be administered by the study physician. The standard treatment group will receive a standard dose of PZQ (single oral dose of 40 mg/kg) at week 0, while the intense treatment group will receive PZQ at week 0, week 2, week 4 and week 6. PZQ treatment will be given after a light meal, as recommended by WHO [2], to minimize potential adverse events. The tablets will be administered under supervision of the study physician. If a participant vomits within 1.5 h following PZQ administration, another standard dose of PZQ will be given.

Participants will be monitored for adverse events at 3 h and 24 h after PZQ administration. An adverse event is defined as any undesirable experience occurring to a

participant during the study, whether or not considered related to PZQ treatment. All adverse event intensities will be assessed by the study physician, following local guidelines and will be graded as mild, moderate or severe. Additionally, to monitor the functioning of the vital organs in the intense treatment group, participants will be asked to provide blood samples at weeks 3 and 7, after the second and fourth treatment, respectively, to be tested for haematological indicators, liver function and renal function parameters.

At the end of the study all school-aged children in the selected communities, including those who participated in the study and those who participated in the baseline screening but were not eligible to participate in the study based on inclusion/exclusion criteria as well as those who were not invited for baseline screening, will be offered PZQ and albendazole treatment according to and supplied by the PNLMTN-CP in Côte d'Ivoire.

Withdrawal

Participation is voluntary and participants can decide not to continue their participation in the study at any time for any reason if they wish to do so, without any consequences. The principal investigator can also decide to withdraw a participant from the study for any (urgent) medical reasons.

Outcomes

The primary outcome for the study is:

- Difference in CR of one versus four standard doses of PZQ (given two weeks apart) in participants infected with *S. mansoni*, measured by KK at baseline compared to week 8.

Secondary outcomes are:

- Difference in CR of one versus four standard doses of PZQ in participants infected with *S. mansoni* as measured by the different diagnostic tests at baseline compared to week 8.
- Difference in CR of one versus two or three standard doses of PZQ as measured by the different diagnostic tests at baseline compared to weeks 4 or 6, respectively.
- Difference in intensity reduction rate (IRR) of one versus four standard doses of PZQ (given two weeks apart) on participants infected with *S. mansoni* measured by the different diagnostic tests at baseline compared to week 8.
- Difference in IRR between intervention and control group of one versus two or three standard doses of PZQ as measured by the different diagnostic tests at baseline compared to weeks 4 or 6, respectively.
- Sensitivity and specificity of the different diagnostic tests at different time points.
- Safety of repeated standard doses of PZQ.

Outcome measures

The CR is defined as the proportion of participants who were *S. mansoni* egg positive at baseline and who became *S. mansoni* egg negative at week 8, as determined by KK. For the primary outcome, the CR in the standard treatment group will be compared to the CR in the intense treatment group. For the other diagnostic tests, the CR will be the proportion of participants

who were positive by urine POC-CCA, urine UCP-LF CAA or stool PCR at baseline and who became negative at week 8. Differences between the standard and intense treatment group will be compared. The overall IRR in the intense and standard treatment groups will be calculated as the intensity of infection at week 8 compared to the intensity of infection at baseline, as determined by the different diagnostic tests (intensity referring to egg counts for KK, artificial score for POC-CCA, CAA level in pg/ml for UCP-LF CAA, Ct-value for real-time PCR). Additionally, the CR and IRR will be determined at intermediate time points after one versus two and one versus three standard doses of PZQ. All adverse events occurring within 24 h after PZQ treatment will be recorded to evaluate the safety and tolerability of repeated PZQ treatment.

Randomisation and blinding

All participants will be randomised at baseline in a 1:1 ratio, by an independent statistician. Local nurses and physicians will not be blinded to treatment. Study personnel, laboratory technicians and investigators will be blinded. Data analysis will be performed blinded to the intervention.

Sample size calculation

The sample size estimation is based on the difference in CR of one versus four repeated standard doses of PZQ measured with KK 8 weeks after treatment. Based on previous, data we assume a CR of 66% after one standard dose of PZQ [11] and aim for an increased CR of 98.7% after four repeated doses of PZQ, leading to a sample size of 30 participants per group, with a power of 90% and a level of significance of 5% (2-tail) [39]. Because we anticipate a considerable loss to follow-up, we aim to include approximately 100 participants in each group, hence 200 participants in total. Assuming a *S. mansoni* prevalence of approximately 25% by KK (unpublished data, Taabo HDSS survey February 2016) it is estimated that at least 1000 children will have to be screened to obtain a minimum of 200 KK positives in the selected region.

Data management and statistical analysis

Each participant will be given a unique study identification number. Data will be collected using paper based case report forms (CRFs) and will be double entered and managed by well-trained data entry personnel using the REDCap electronic data capture tools hosted at the Leiden University Medical Center, the Netherlands, via Emory University, Atlanta, GA, USA [40].

All analyses will be conducted blinded to the treatment allocations and will be performed using STATA version 12 (StataCorp; College Station, TX, USA) or IBM Statistical Package for Social Sciences version 23 (SPSS Inc., Chicago, Illinois, USA) or R language (R Foundation for Statistical Computing; Vienna, Austria). Response and dropout rates will be assessed and reported. Demographics and outcome parameters will be summarised using descriptive summary measures, expressed as mean (standard deviation) or median (interquartile range) for continuous variables depending on whether the data are normally distributed and frequencies (percentage) for categorical variables. The proportion of participants positive for intestinal schistosomiasis will be calculated as the proportion of participants who tested positive at various time points (e.g. baseline, week 1, week 2, etc.). These percentages positive for

schistosomiasis will be reported separately for each diagnostic test. Baseline characteristics between the control and intervention groups will be compared using the X2 test for categorical variables and t-test or Mann-Whitney U for continuous variables depending on the distribution of the data.

For the primary and secondary outcomes, a repeated measurements analysis approach will be used [41]. With this approach, the correlation between the measurements collected from the same participant over time will be modelled using properly chosen correlation matrices. Based on this analysis the proportions cured at different time points will be estimated and differences between the intense and standard treatment group will be tested for statistical significance. Similarly, for the IRR the progression of the reduction in intensity will be estimated and differences between the two groups at different time points will be tested for statistical significance. This model-based approach is becoming a more popular method to properly analyse longitudinal data on treatment efficacy of PZQ among individuals as well as among groups of individuals [42-44]. Transformation of data will be applied if needed. The model will be adjusted for covariates such as age and sex. Although the assumption will be made that missing data will be missing at random, reasons for individuals missing treatment at each time-point will be recorded.

In the absence of a true 'gold' standard, the sensitivity and specificity for KK, POC-CCA, UCP-LF CAA and PCR will be estimated by using an imperfect 'gold' standard (based on assumptions of 100% specificity for KK, CAA and PCR, similarly to Knopp et al., 2015 [45]) as well as by using latent class analysis (LCA). LCA uses all available data to estimate the proportion of true positives that test positive for each test (i.e. the sensitivity of each test), the proportion of true negatives that test negative for each test (i.e. the specificity of each test), and the proportion of individuals truly positive in the study population (i.e. the infection prevalence within the study population), as described previously [46,47]. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of all diagnostic tests will be calculated with 95% confidence intervals (CIs). For all analysis, a p value of < 0.05 will be taken as the level for statistical significance.

Data safety and monitoring board

An independent data safety and monitoring board (DSMB) has been established which will review safety data from the first and third treatment during the study and provide recommendations to the sponsor concerning the continuation of the study.

Ethical considerations

The study is registered at ClinicalTrials.gov (reference number NCT02868385) as well as at the EU Clinical Trials Register (EudraCT, reference number 2016–003017-10) and will be conducted in accordance with the latest version of the Helsinki Declaration. The study has been approved by the National Ethics Committee of the Ministry of Health in Côte d'Ivoire (CNESVS, registration number 091–18/MSHP/ CNESVS-km, 27 June 2018) as well as by the Direction de la Pharmacie, du Médicament et des Laboratoires de Côte d'Ivoire (DPML, registration number 99433-/MSHP/DGS/ DPML/DAR and clinical trial number ECCI00618, 22 October

2018) and has been reviewed by the Ethics Committee of the Leiden University Medical Center in the Netherlands without any objections (CME, registration number P16.254, 11 January 2017). This study protocol, including the statistical analysis section, has been written before start of the trial. The data analysis of the main publication will follow this plan. The SPIRIT protocol checklist is given in Additional file 1.

DISCUSSION

The RePST trial is the first and currently the only clinical trial that will investigate the efficacy of four repeated standard doses of PZQ on the clearance of *S. mansoni* in a randomised trial design. Efficacy will be assessed by using several diagnostic techniques, including real-time PCR and the ultra-sensitive UCP-LF CAA assay. By using an extensive panel of diagnostics in a frequent post-treatment sampling schedule, this study is the first of its kind to comprehensively assess the efficacy of single as well as repeated doses of PZQ.

This study is a proof of concept study to determine the efficacy of repeated PZQ treatment in an endemic setting. The context is to provide evidence and tools for evaluating current schistosomiasis control approaches, as well as input for developing new strategies for individual cure and transmission interruption rather than population-wide morbidity control. Clearly this study is not aiming to design repeated PZQ treatment schedules for implementation in large-scale control programmes. Results will also prove to be highly relevant in individual test-and-treat approaches using non-invasive POC diagnostics, even in non-endemic settings. The in-depth analysis and validation of different diagnostic tools (before and) after treatment is essential to determine the effect of PZQ on different parasite-related parameters providing additional information on CRs, re-emergence of infections, and even on transmission potential. Accurate diagnosis of light intensity infections (after intensive chemotherapy) will also be crucial in the light of elimination of schistosomiasis now being a target in several endemic countries [48,49] and clearly advocated by WHO [9].

Trial status

This open-label, randomised controlled trial has started recruitment in October 2018. We envisage the sample collection period to be finished by the end of 2018, and sample processing and testing at the LUMC to start early 2019.

Abbreviations

CAA: Circulating anodic antigen; CCA: Circulating cathodic antigen; CI: Confidence interval; CR: Cure rate; CRF: Case report form; CSRS: Centre Suisse de Recherches Scientifiques; Ct: Cycle threshold value; DNA: Deoxyribonucleic acid; DSMB: Data safety and monitoring board; EPG: Eggs per gram of faeces; HDSS: Health and demographic surveillance system; IRR: Intensity reduction rate; KK: Kato-Katz technique; LCA: Latent class analysis; LF: Lateral flow; LUMC: Leiden University Medical Center; MDA: Mass drug administration; NPV: Negative predictive value; PCR: Polymerase chain reaction; PNLMTN-CP: Programme National de Lutte contre les Maladies Tropicales Négligées à Chimiothérapie Préventive; POC: Point-of-care; PPV: Positive predictive value; PZQ: Praziquantel; RePST: Repeated doses of praziquantel in schistosomiasis treatment; UCP: Up-converting phosphor particles; UF: Urine-filtration technique; WHO: World Health Organization

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Authors' contributions
GJvD, JTC, SK and JU conceptualized and designed the study. PTH, MCP, ASA, RKA, KDS, JTC and GJvD drafted the protocol. LvL, PLAMC, ST and MR provided valuable advice and expertise. PTH, ASA and GJvD drafted the manuscript. All authors read and approved the final version of the manuscript.

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Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
Ethical approval for this study has been obtained from the National Ethics Committee of the Ministry of Health in Côte d'Ivoire (CNESVS, registration number 091–18/MSHP/CNESVS-km, 27 June 2018) as well as from the Direction de la Pharmacie, du Médicament et des Laboratoires de Côte d'Ivoire (DPML, registration number 99433-/MSHP/DGS/DPML/DAR and clinical trial number ECCI00618, 22 October 2018). Oral and signed assent and signed informed consent will be obtained from their legally appointed representative, usually a parent or guardian, responsible for making decisions on their behalf, prior to study enrolment.

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SUPPLEMENTARY MATERIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	ItemNo	Description	Addressed on
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	4
	2b	All items from the World Health Organization Trial Registration Data Set	Additional file 1
Protocol version	3	Date and version identifier	See manuscript
Funding	4	Sources and types of financial, material, and other support	20
Roles and responsib	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	Additional file 1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	20
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N.A.
Introduction			
Background and ratic	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	6
	6b	Explanation for choice of comparators	N/A
Objectives	7	Specific objectives or hypotheses	6, 7
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	7
		Methods: Participants, interventions, and outcomes	
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7, 8
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	8, 9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9-dec
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	12
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	12
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	12
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	13, 14
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	10, Figure 2
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	15
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	15
		Methods: Assignment of interventions (for controlled trials)	
Allocation:			
Sequence generatior	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	14
Allocation concealme	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	14
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	14
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	14
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A

		Methods: Data collection, management, and analysis	
Data collection meth	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	15-17
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	15-17
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	15-17
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	15-17
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	15-17
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	15-17
		Methods: Monitoring	
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	17
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	17
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	14
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
		Ethics and dissemination	
Research ethics app	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	17, 19
Protocol amendment	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	N/A
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	9
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	9, 10
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	15
Declaration of interes	28	Financial and other competing interests for principal investigators for the overall trial and each study site	20
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	20
Ancillary and post-tria	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	17, 18
	31b	Authorship eligibility guidelines and any intended use of professional writers	N/A
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A
Appendices			
Informed consent ma	32	Model consent form and other related documentation given to participants and authorised surrogates	Available on request
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	N/A

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the

5.

Efficacy of single versus four repeated doses of praziquantel against *Schistosoma mansoni* infection in school-aged children from Côte d'Ivoire based on Kato-Katz and POC-CCA: An open-label, randomised controlled trial (RePST)

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PLoS Neglected Tropical Diseases (2020)

ABSTRACT

Background

Preventive chemotherapy with praziquantel (PZQ) is the cornerstone of schistosomiasis control. However, a single dose of PZQ (40 mg/kg) does not cure all infections. Repeated doses of PZQ at short intervals might increase efficacy in terms of cure rate (CR) and intensity reduction rate (IRR). Here, we determined the efficacy of a single versus four repeated treatments with PZQ on *Schistosoma mansoni* infection in school-aged children from Côte d'Ivoire, using two different diagnostic tests.

Methods

An open-label, randomized controlled trial was conducted from October 2018 to January 2019. School-aged children with a confirmed *S. mansoni* infection based on Kato-Katz (KK) and point-of-care circulating cathodic antigen (POC-CCA) urine cassette test were randomly assigned to receive either a single or four repeated doses of PZQ, administered at two-week intervals. The primary outcome was the difference in CR between the two treatment arms, measured by triplicate KK thick smears 10 weeks after the first treatment. Secondary outcomes included CR estimated by POC-CCA, IRR by KK and POC-CCA, and safety of repeated PZQ administration.

Principal findings

During baseline screening, 1,022 children were assessed for eligibility of whom 153 (15%) had a detectable *S. mansoni* infection, and hence, were randomized to the standard treatment group (N = 70) and the intense treatment group (N = 83). Based on KK, the CR was 42% (95% confidence interval (CI) 31–52%) in the standard treatment group and 86% (95% CI 75–92%) in the intense treatment group. Observed IRR was 72% (95% CI 55–83%) in the standard treatment group and 95% (95% CI 85–98%) in the intense treatment group. The CR estimated by POC-CCA was 18% (95% CI 11–27%) and 36% (95% CI 26–46%) in the standard and intense treatment group, respectively. Repeated PZQ treatment did not result in a higher number of adverse events.

Conclusion/significance

The observed CR using KK was significantly higher after four repeated treatments compared to a single treatment, without an increase in adverse events. Using POC-CCA, the observed CR was significantly lower than measured by KK, indicating that PZQ may be considerably less efficacious as concluded by KK. Our findings highlight the need for reliable and more accurate diagnostic tools, which are essential for monitoring treatment efficacy, identifying changes in transmission, and accurately quantifying the intensity of infection in distinct populations. In addition, the higher CR in the intense treatment group suggests that more focused and intense PZQ treatment can help to advance schistosomiasis control.

Trial registration

www.clinicaltrials.gov NCT02868385.

AUTHOR SUMMARY

The previously established efficacy of the widely used drug praziquantel (PZQ) against schistosomiasis might have been overestimated due to the use of inaccurate diagnostic methods. Repeated PZQ treatment at short intervals in areas with ongoing transmission could more effectively target non-susceptible schistosomula as they will have matured into drug susceptible worms within a few weeks. In the current study, we aimed to determine the cure rate (CR) of repeated PZQ, measured by the Kato-Katz (KK) technique and the point-of-care circulating cathodic antigen (POC-CCA) test, respectively. An open label, randomized controlled trial was conducted assigning 153 school-aged children with a confirmed *Schistosoma mansoni* infection to two groups, one receiving a single PZQ treatment, while the second group received four repeated PZQ treatments, given at two week intervals. Based on the KK test, the CR was significantly higher after four repeated treatments compared to a single treatment. When using POC-CCA, a diagnostic method that has not been utilized before in studies assessing the efficacy of four repeated PZQ treatments, the CR was much lower, even after four repeated PZQ treatments. Our results indicate that worms are still present after multiple PZQ treatments and that PZQ might be less efficacious than previously published.

INTRODUCTION

Schistosomiasis remains a public health problem in different parts of the world with an estimated 779 million people at risk of infection and more than 250 million people infected (1,2). The disease is caused by parasitic blood flukes of the genus *Schistosoma*. The three most important species are *S. japonicum* and *S. mansoni* (causing intestinal schistosomiasis) and *S. haematobium* (causing urogenital schistosomiasis) (3,4). To control schistosomiasis, health authorities rely on preventive chemotherapy, that is the large-scale administration of the anthelmintic drug praziquantel (PZQ) to at risk populations without prior diagnosis (5). This strategy has been successful in reducing the prevalence and, most importantly, the intensity of infection, and thereby controlling morbidity (6,7). The burden of schistosomiasis is greatest in school-aged children, generally presenting the highest prevalence and intensity of infection (8). School-aged children are therefore the main target for preventive chemotherapy, consisting of a single 40 mg/kg oral dose of PZQ, as recommended by the World Health Organization (WHO) (5, 9, 10). PZQ is the drug of choice because it is safe and efficacious against the adult stages of all *Schistosoma* species (11). The efficacy of PZQ is typically expressed as a cure rate (CR) and often also as an intensity reduction rate (IRR), both based on pre- and post-treatment data. Reported CRs in school-aged children range between 42% and 79% for *S. mansoni* and between 37% and 93% for *S. haematobium* after a single 40 mg/kg oral dose of PZQ (12–14). Following a closely spaced second dose of PZQ, considerably higher CRs are reported; 91% for *S. mansoni* (12,15,16) and 99% for *S. haematobium* (12).

Most studies reporting on the efficacy of PZQ have used microscopy-based methods, such as urine filtration for *S. haematobium* and the stool-based Kato-Katz (KK) technique for S. *japonicum* and *S. mansoni*. However, these methods lack sensitivity, especially for detection of low-intensity infections (17,18). Hence, reported CRs based on these parasitological methods are

likely an overestimation (19,20). From a public health perspective, the absence or a significant reduction in the number of *Schistosoma* eggs is essential as they are causing morbidity and keep transmission ongoing. However, from an individual health care perspective, worm absence (cure) is more important. It is known that PZQ targets adult worms, therefore a direct determination of PZQ efficacy would be to measure the number of worms instead of eggs (which are usually used as a proxy for worm burden) with a highly accurate diagnostic tool. The field-applicable and commercially available point-of-care circulating cathodic antigen (POC-CCA) urine test, which identifies active worm infections by detection of schistosome CCA in urine, has shown a higher sensitivity for detecting *S. mansoni* infections than the KK technique (20–22). It is now being recommended for surveillance and mapping of prevalence of intestinal schistosomiasis (10,18,21,23).

In addition to the possible overestimation of CRs due to insensitive diagnostic tools, the limited activity of PZQ on immature worms as well as continuing reinfection might have led to an underestimation of the efficacy (24–27). Furthermore, the short metabolic half-life of PZQ might also limit its effectivity (28). In areas with ongoing transmission, where repeated infections and hence the presence of schistosomula in the human body is likely, repeating PZQ treatment a few weeks after the first dose might increase its overall effectiveness for parasitological cure (12,29).

In the current study, we assessed the effect of multiple doses of PZQ on parasite clearance and tolerance in school-aged children from Côte d'Ivoire with a confirmed *S. mansoni* infection. As primary outcome we determined the difference in CR of a single versus four repeated doses of PZQ, measured by the KK technique in stool samples 10 weeks after the first treatment. Secondary outcomes included CR measured by the POC-CCA test, IRR by KK and POC-CCA, and safety of repeated PZQ treatments. Given the paucity of highly effective control measures for schistosomiasis, the results of our study are essential to assess the most optimal PZQ strategy from a public health control and best-care perspective.

METHODS

Ethics statement

Ethics approval was obtained from the Comité National d'Éthique des Sciences de la Vie et de la Santé de Côte d'Ivoire (CNESVS; reference no. 091-18/MSHP/CNESVS-km, date of approval 27 June 2018), the Direction de la Pharmacie, du Médicament et des Laboratoires de Côte d'Ivoire (DPML; reference no. 99433/MSPH/DGS/DPML/DAR and clinical trial number ECCI00618, date of approval 22 October 2018), and the Ethics Committee of the Leiden University Medical Center in the Netherlands (P16.254, date of approval 11 January 2017). Communities and health authorities were informed on the purpose and procedures of the study. Participating children were informed about the objectives, procedures, and potential risks and benefits of the study using lay terms. Written informed consent was obtained from children's parents or guardians, while children provided oral assent. The trial is registered at ClinicalTrials.gov (registration no. NCT02868385).

Study design and participants

We conducted an open-label, randomized controlled trial with two arms in which children aged 5–17 years from three villages located in the Taabo health district in south-central Côte d'Ivoire (30) were included.

The trial was conducted from October 1, 2018 to January 14, 2019. In the first month of the study (October 2018), children were assessed for eligibility during a baseline screening. Children who were found positive for *S. mansoni* by KK and POC-CCA and egg-negative for *S. haematobium* by urine filtration were eligible. A detailed description of the inclusion and exclusion criteria is provided in the study protocol published elsewhere (31). Eligible children were randomized into the 'standard treatment' group, receiving a single PZQ treatment at baseline, or the 'intense treatment' group, receiving PZQ treatment at baseline and again at two, four, and six weeks after the initial dose, totalling four treatments with two-week intervals in the intense treatment group. Due to logistic reasons and school holidays, final sample collection had to be postponed from 8 weeks (as described in the study protocol (31)) to 10 weeks after baseline treatment (see S1 Fig).

Randomization and masking

Eligible school-aged children were randomly assigned to the standard or intense treatment group, as described elsewhere (31). Participants as well as nurses and the study physician were not blinded to the treatment assignments, while laboratory technicians and investigators were blinded to the treatment assignments.

Outcomes

The primary outcome was the difference in CR of a single versus four repeated PZQ treatments, based on KK in the intention-to-treat population. CR was defined as the proportion of children being *S. mansoni* egg-positive at baseline who became egg-negative 10 weeks after the first treatment. Secondary outcomes included the *S. mansoni* infection percentage positivity and intensity over time based on KK and POC-CCA, the CR based on POC-CCA, the IRR (defined as the percentage reduction in the median intensity, either expressed by eggs per gram of stool (EPG) or by visual score of the POC-CCA, of the positive individuals, 10 weeks after the first treatment, based on KK and POC-CCA), and safety of a single or multiple doses of PZQ.

Procedures

Detailed descriptions of field and laboratory procedures are provided in the published study protocol (31). In brief, during the baseline survey, single urine and single stool samples were collected from each participating child. Urine samples were subjected to POC-CCA (batch #170522062; Rapid Medical Diagnostics, Pretoria, South Africa) using the semi-quantitative scoring method called 'G-scores' (32). With this POC-CCA batch, the provided quality control (QC) standard-series S0, S1, S2, and S3 resulted in a G1, G4, G8, and G10, respectively (see standard operating procedure (SOP), provided as an appendix in Casacuberta-Partal et al. (32)). To exclude the most abundant *S. haematobium* infections, urine filtration was performed on single

baseline urine samples. Stool samples were processed using the KK technique with triplicate 41.7 mg thick smears prepared from each sample, as described previously (31,33).

To assess treatment efficacy, additional urine and stool samples were collected from each participating child weekly and two-weekly, respectively, at eight time points over a period of 10 weeks. At each time point, all urine and stool samples were subjected to POC-CCA and KK, respectively, as described above.

Treatment and monitoring of adverse events

At baseline, all included children were given PZQ (600 mg tablets; Biltricide, Bayer, Abidjan, Côte d'Ivoire), according to the calculated dose per kg of bodyweight (40 mg/kg, weight measured by a Seca 877 digital scale). Prior to treatment, breakfast was provided to each child. After sample collection, directly observed treatment was applied by the study physician and was accompanied with water and lunch, provided by the research team. Children allocated to the intense treatment group were re-treated with PZQ at 2, 4, and 6 weeks after the first treatment.

After treatment, children remained under medical supervision for at least 3 hours and adverse events were recorded. If needed, symptomatic treatment for adverse events was provided by the study physician. In case vomiting occurred within 1.5 hours, children were readministered a dose of PZQ. Twenty-four hours post-treatment, children were interviewed about the occurrence of adverse events. An adverse event was defined as any undesirable sign, symptom, or disease occurring to a participant during the study, whether or not related to PZQ treatment. Intensity of adverse events was graded by the study physician as mild, moderate, or severe, following guidelines by the European Medicine Agency.

Statistical analysis

A detailed description of the sample size calculation is given elsewhere (31). In brief, to detect an increase in CR from 66% after a single PZQ treatment (12) to 99% after four repeated PZQ treatments with a two-sided 5% significance level and a power of 90%, a minimum sample size of 30 children per group was required (31, 34). To account for follow-up losses, expected due to the intense weekly follow-up, the sample size was increased to 100 children in each group, hence 200 children in total. Assuming a *S. mansoni* infection prevalence of approximately 25% based on KK (Taabo health and demographic surveillance site survey carried out in February 2016), at least 1,000 children needed to be screened in order to obtain a minimum of 200 KK-positive children in the Taabo region.

Data were double entered by two well-trained data entry clerks and managed using a REDCap electronic data capture tool hosted at Leiden University Medical Center (Leiden, the Netherlands), via Emory University (Atlanta, United States of America) (35,36). Descriptive statistics were performed using IBM Statistical Package for Social Sciences version 25 (SPSS Inc., Chicago, United States of America).

Infection intensity, as expressed by EPG, was calculated by multiplying the sum of egg counts from triplicate KK thick smears by a factor of 8. Intensity of infection was classified according to WHO guidelines into light (1–99 EPG), moderate (100–399 EPG), and heavy (≥400 EPG) (5). POC-CCA G-scores were classified into negative (G1), trace (G2-3,

conservatively considered as negative in the analysis presented here), 1+ (G4-5), 2+ (G6-7), or 3+ (G8- 10) (10,22,32,37).

To determine the prevalence over time as well as CRs (based on KK and POC-CCA) and IRRs (based on KK), mixed effects models were employed to take into account the correlation between the different measurements from the same individual (38–40). For the prevalence, we used mixed effects logistic regression where prevalence was modeled as a function of time, treatment group and their interaction. In the case of KK, the time variable was taken as categorical, while for POC-CCA we modeled progression over time using natural cubic splines with four knots. For the KK-based IRR, we used a zero-inflated negative binomial mixed model where the logarithm of the mean egg counts is modeled as a function of time (using natural cubic splines), treatment group and their interaction. For POC-CCA, the IRRs could not be obtained from the mixed effects model, as the output (G-score) is not a continuous variable. Therefore, the IRR based on POC-CCA was calculated according to WHO guidelines (1 − (arithmetic mean after treatment/arithmetic mean before treatment)) × 100 (41). In all models, the within subject correlation was modeled using a random intercepts term. The models used provide results under the missing at random assumption for the missing data which is valid in this study. All analyses were done in R (version 3.43) using the GLMMadaptive package. CR and IRR estimated from the model are given with their corresponding 95% pointwise confidence intervals (CIs). P-values <0.05 were considered to indicate statistical significance.

RESULTS

Fig 1 shows the study flow. At baseline, 1,022 children aged 5–17 years were assessed for eligibility. Of these, 153 had a detectable *S. mansoni* infection and met the inclusion criteria. They were randomly assigned to one of the two study arms; 70 were assigned to the standard treatment group, and 83 were assigned to the intense treatment group. Regular randomization was performed instead of block randomization, and hence, the size of the two groups differed. At baseline, all 153 children (100%) received treatment. Three children (one in the standard treatment group and two in the intense treatment group) were lost to follow-up from week 6 onwards because they moved out of the study region during the follow-up period (see S1 Table). In the intense treatment group, compliance to each following treatment was unexpectedly high, from 100% in week 2 (second treatment) to 98% in week 6 (fourth and final treatment). All 153 children were included in the intention-to-treat analysis.

The demographic and parasitological baseline data for the participating children are summarized in Table 1. The median age and sex of children were balanced among the two groups. In both groups, most of the children had a light to moderate *S. mansoni* infection, while heavy infection intensities were observed in 18 (26%) children in the standard treatment group compared to 12 (14%) children in the intense treatment group. At pre-treatment, the median fecal egg count was 172 EPG and the median G-score in urine was 6 in the standard treatment group, and 128 EPG and G-score 7 in the intense treatment group, respectively.

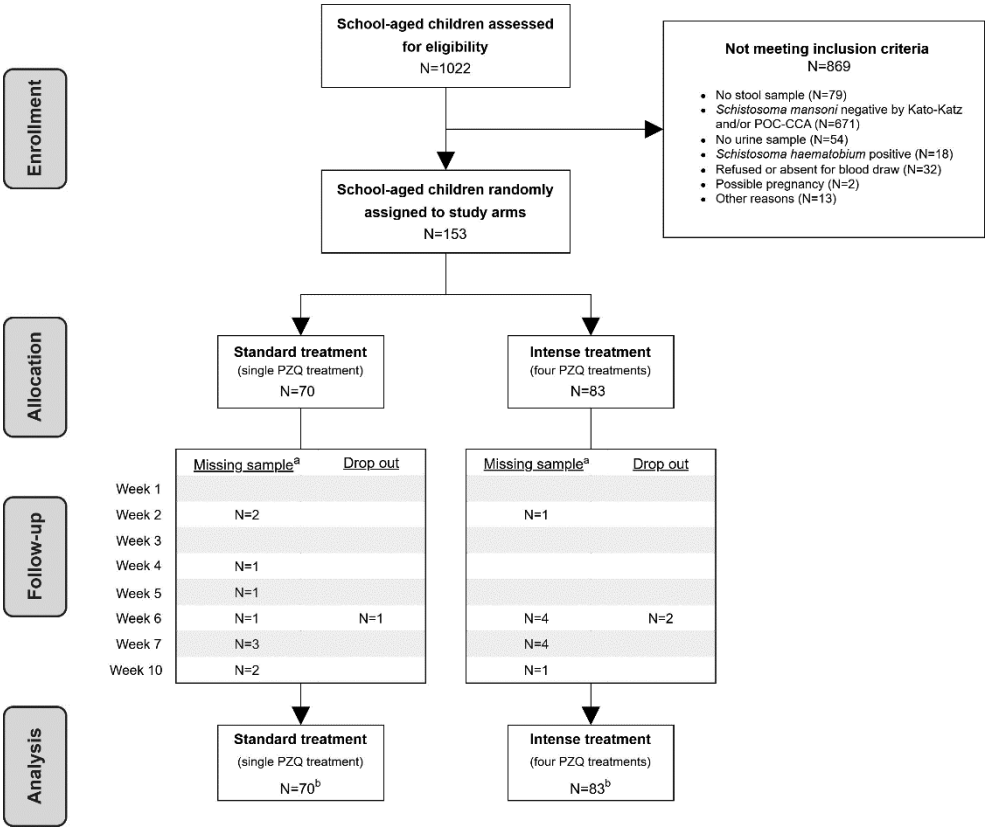

Figure 1. Trial profile.

a. Sample (urine and/or stool) not provided.
b. Intention-to-treat analysis.

Prevalence over time

Figure 2 illustrates the percentage of *S. mansoni* positives over time based on KK (Fig 2A) and on POC-CCA (Fig 2B). In the standard treatment group, the overall *S. mansoni* prevalence based on triplicate KK thick smears was decreased from 100% to 58% (95% CI 48–68%), measured 10 weeks after treatment. In the intense treatment group, who received a total of four doses of PZQ, the prevalence decreased to 14% (95% CI 8–23%), measured 10 weeks after the first treatment. Based on POC-CCA, measured at the final follow-up time point, 82% (95% CI 72–89%) and 64% (95% CI 54–74%) POC-CCA positives were observed in the standard and intense treatment group, respectively.

Table 1. Baseline characteristics of the standard treatment group and the intense treatment group in a randomized trial. The trial was conducted in late 2018 among school-aged children in south-central Côte d'Ivoire and compared single versus four repeated PZQ treatments against *S. mansoni*.

	Standard treatment group (1x PZQ)	**Intense treatment group** (4x PZQ)
	N=70	**N=83**
Age, years	10.5 (9-12)	10.0 (9-12)
Weight, kg	32.2 (27.3-38.4)	32 (26.5-38.0)
Height, cm	137 (130-145)	140 (128-146)
Haemoglobin (g/dl)	11.3 (10.8-11.8)	11.3 (10.8-12.0)
Sex		
Boys	43 (61.4%)	51 (61.4%)
Girls	27 (38.6%)	32 (38.6%)
Village		
Ahouaty	35 (50.0%)	41 (49.4%)
N'Denou	27 (38.6%)	33 (39.8%)
Singrobo	8 (11.4%)	9 (10.8%)
Infection intensity		
Kato-Katz		
Light (1-99 EPG)	24 (34.3%)	35 (42.2%)
Moderate (110-399 EPG)	28 (40.0%)	36 (43.4%)
Heavy (≥400 EPG)	18 (25.7%)	12 (14.4%)
POC-CCA[a]		
1+	16 (22.9%)	22 (26.5%)
2+	38 (54.2%)	50 (60.2%)
3+	16 (22.9%)	11 (13.3%)

Data are median (IQR) or n (%). Abbreviations: EPG, eggs per gram of stool; IQR, interquartile range; POC-CCA, point-of-care circulating cathodic antigen.

[a] POC-CCA positive G-scores were classified into 1+ (G4-5), 2+ (G6-7) or 3+ (G8-10).

Intensity of infection over time

The intensity of infection over time based on KK and POC-CCA is shown in Fig 3 (see also S2 Fig and S3 Fig). Based on the KK technique (Fig 3A and 3B), most of the remaining infections after the first treatment were of low intensity. In the standard treatment group, the proportion of low, moderate and, to a smaller extent, heavy intensity infections showed an increase 10 weeks after treatment. In the intense treatment group, a small proportion of infections of low intensity was observed at the final time point. Based on POC-CCA (Fig 3C and 3D), the overall prevalence did not change dramatically and the proportion of 3+ scores (indicating high infection level) remained similar over time in the standard treatment group as well as in the intense treatment group. The proportion of POC-CCA negatives (including traces) increased over time, particularly in the intense treatment group. In both groups, most children remained POC-CCA positive at the final time point. A positive correlation was observed between fecal egg counts and POC-CCA visual scores before treatment (Spearman's rho = 0.44, P<0.01) (S4 Fig).

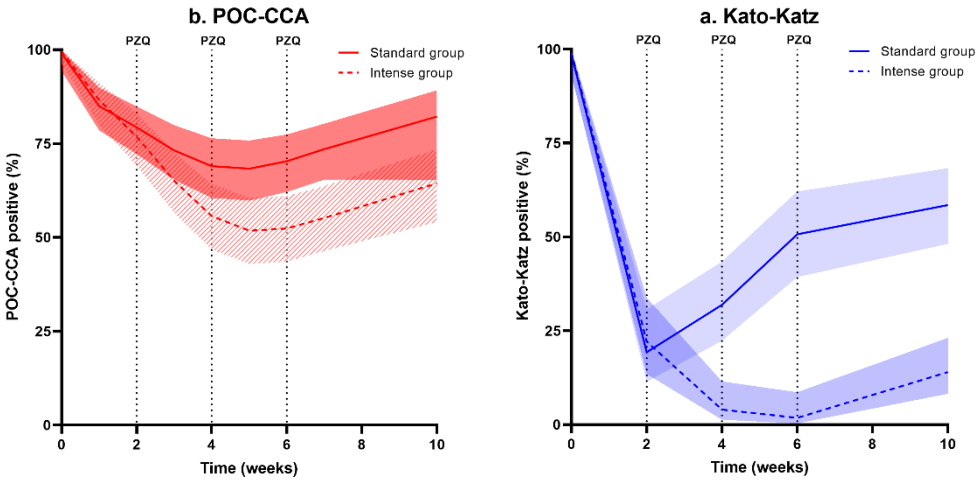

Figure 2. Prevalence over time (with corresponding pointwise 95% confidence intervals) estimated from the mixed effects logistic regression model. Data pertain to (a) triplicate Kato-Katz (KK) thick smears from a single stool sample and (b) single point-of-care circulating cathodic antigen (POC-CCA) urine test in the standard treatment group (single dose of PZQ, solid line) and the intense treatment group (four doses of PZQ at W0, W2, W4, and W6, dashed line).

Cure rate

In the standard treatment group, CR based on triplicate KK thick smears was 42% (95% CI 31–53%) (Table 2; see also S2 Table). A significantly higher CR (86%, 95% CI 75–92%) was observed in the intense treatment group, (P<0.001; primary outcome, both CRs measured 10 weeks after first treatment). When using the same time interval post-treatment to compare CRs between the two groups, i.e., four weeks after the first treatment for the standard treatment group and four weeks after the fourth treatment for the intense treatment group, the observed CR in the standard treatment group was 68% (95% 57–78%) compared to 86% (95% CI 75–92%) in the intense treatment group (P<0.01).

POC-CCA-based CRs were much lower compared to CRs based on the KK technique; only 18% (95% CI 11–27%) in the standard treatment group and 36% (95% CI 26–46%) in the intense treatment group (P<0.01). Using the 4-week post-treatment time points, CRs were similar in both groups; 31% (95% CI 23–40%) in the standard treatment group and 36% (95% CI 26–46%) in the intense treatment group (P=0.23).

Intensity reduction rate

Based on the KK technique, the IRR in the standard treatment group was 72% (95% CI 55–83%), compared to 95% (95% CI 85–98%) in the intense treatment group (P<0.01). When using the same time interval post-treatment to compare IRRs between the two groups (4 weeks), the observed IRR in the standard treatment group was 83% (95% CI 69–91%) versus 95% (95% CI 85–98%) in the intense treatment group (P<0.01). The decrease in the mean POC-CCA G-score was larger in the intense treatment group compared to the standard treatment group, resulting

in an IRR of 27% and 9%, respectively (measured 10 weeks after first treatment). When using the same time interval post-treatment for the standard treatment group, the IRR was 20%.

Safety of PZQ

Observed and reported (within 3 hours) adverse events are summarized in Table 3, stratified by treatment group and follow-up time point. After the first treatment, stomach ache was the most common adverse event (overall 38%), followed by headache (overall 5%) and vomiting (overall 3%). Most of the adverse events were mild and all of them resolved 24 hours after treatment. Adverse events decreased with subsequent treatments in the intense treatment group.

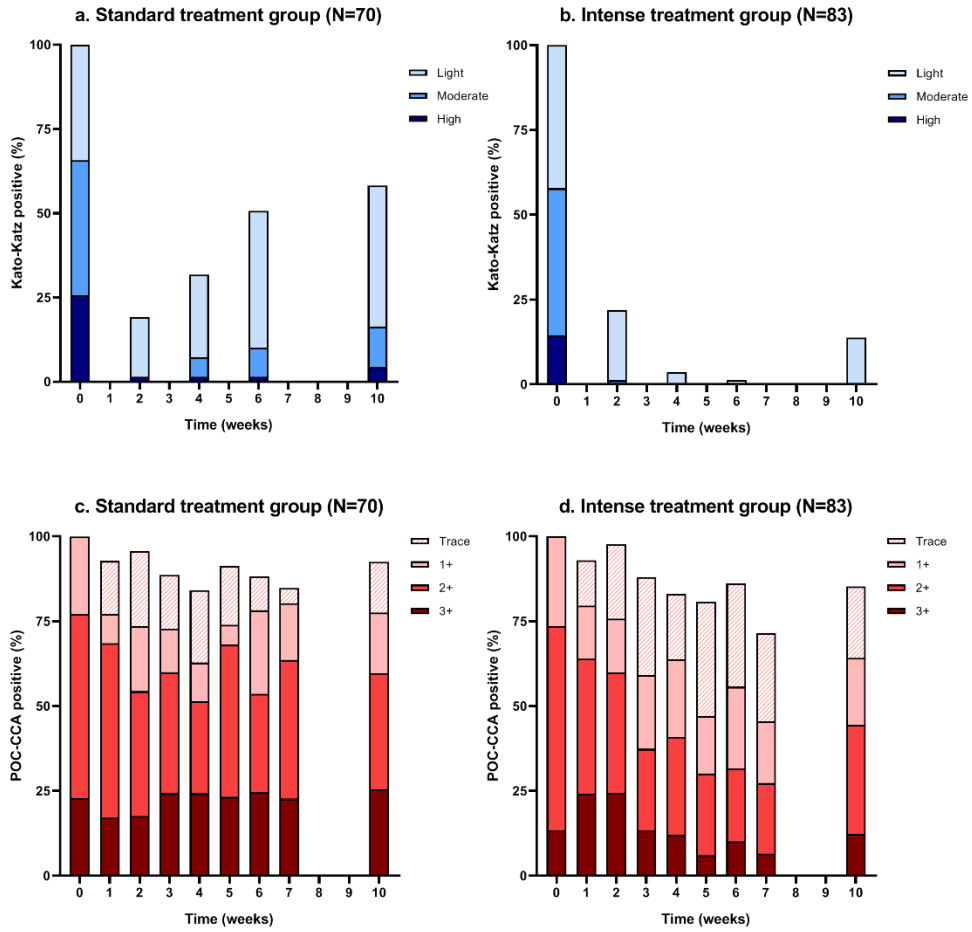

Figure 3. Intensity of infection. Data are based on triplicate Kato-Katz (KK) thick smears from a single stool sample (a, b) and single point-of-care circulating cathodic antigen (POC-CCA) urine test (c, d) in the standard treatment group (single dose of PZQ) and the intense treatment group (four doses of PZQ at W0, W2, W4, and W6).

Table 2. Cure rate (CR) and intensity reduction rate (IRR) of a single (standard treatment group) and four (intense treatment group) repeated PZQ treatments in school-aged children infected with *S. mansoni*. Data are based on triplicate Kato-Katz thick smears from a single stool sample and single point-of-care circulating cathodic antigen (POC-CCA) urine test.

	Standard treatment group (1x PZQ) N=70	**Intense treatment group** (4x PZQ) N=83
Kato-Katz		
Infected children before treatment	70	83
Cured children 10 weeks after first treatment	28	69
CR[a,b]	41.6% (95% CI 31.1-52.9)	86.0% (95% CI 75.4-92.4)
Cured children 4 weeks post-treatment[c]	47	69
CR[b]	68.2% (95% CI 57.1-77.6)	86.0% (95% CI 75.4-92.4)
Median EPG[d]		
Before treatment	172	128
10 weeks after first treatment	64	8
4 weeks post-treatment[c]	36	8
Arithmetic mean EPG		
Before treatment	298.2	242.7
10 weeks after first treatment	97.7	3.2
IRR[e]	72.3% (95% CI 54.6-83.1)	95.1% (95% CI 85.1-98.4)
4 weeks post-treatment[c]	45.8	3.2
IRR[e]	83.3% (95% CI 68.9-91.2)	95.1% (95% CI 85.1-98.4)
POC-CCA		
Infected children before treatment	70	83
Cured children 10 weeks after first treatment	15	29
CR[b]	17.9% (95% CI 11.3-27.2)	35.7% (95% CI 26.4-46.1)
Cured children 4 weeks post-treatment[c]	26	29
CR[b]	31.2% (95% CI 23.4-40.2)	35.7% (95% CI 26.4-46.1)
Median G-score[d]		
Before treatment	6	7
10 weeks after first treatment	7	6
4 weeks post-treatment[c]	6	6
Arithmetic mean G-score		
Before treatment	6.4	6.3
10 weeks after baseline treatment	5.8	4.6
IRR[f]	9.3%	27.0%
4 weeks post-treatment[c]	5.1	4.6
IRR[f]	20.3%	27.0%

Abbreviations: CR, cure rate; EPG. eggs per gram of stool; IRR, intensity reduction rate; POC-CCA, point-of-care circulating cathodic antigen test

[a] Primary outcome

[b] CR as calculated from the model

[c] Measured four weeks after first treatment for the standard treatment group, and four weeks after the fourth treatment for the intense treatment group

[d] Median of the positives

[e] IRR based on the reduction in mean EPG as calculated from the model

[f] IRR based on the reduction in mean POC-CCA G-score as calculated manually

Table 3. Main type of adverse events observed and reported 3 hours after PZQ administration in *S. mansoni*-infected children in the standard treatment group and the intense treatment group.

	Standard treatment group		Intense treatment group							
	First treatment W0		First treatment W0		Second treatment W2		Third treatment W4		Fourth treatment W6	
	N=70		N=83		N=82		N=82		N=78	
Adverse events										
Stomach ache	25	36%	33	40%	25	30%	22	27%	10	13%
Headache	3	4%	5	6%	14	17%	4	5%	2	3%
Vomiting	3	4%	2	2%	2	2%	4	5%	2	3%
Dizziness	2	3%	2	2%	3	4%	9	11%	6	8%
Diarrhoea	2	3%	1	1%	0		0		0	
Nausea	0		0		0		1	1%	1	1%

DISCUSSION

Based on stool microscopy, we observed a significantly higher CR after four closely spaced PZQ treatments compared to a single dose, measured 10 weeks after the first treatment, without any difference in the frequency and severity of adverse events. Employing the POC-CCA test, the observed CRs were considerably lower compared to KK, even after four repeated treatments, indicating that worms are still present and that PZQ might be less efficacious than previously published.

Our aim of administering PZQ four times at 2-week intervals was to achieve a high CR, as this approach not only targets adult schistosomes, but also the immature forms, which were not yet drug-susceptible during the first treatment (12,29,42). Indeed, 10 weeks after the baseline survey, four repeated treatments resulted in a significantly higher CR than a single treatment based on the KK technique, but failed to cure all infections. The primary outcome according to the study protocol, i.e., the difference in CR of a single versus four repeated PZQ treatments, was calculated by comparing the prevalence of infection at baseline and 10 weeks after the first treatment (31). This implied that the time interval after treatment was not the same for both groups; 10 weeks after the single treatment in the standard treatment group (allowing for a 10-week period of possible worm maturation, worm recovery, as well as renewed parasite exposure and re-infection) versus four weeks after the fourth treatment in the intense treatment group. To render the comparison between the groups more representative, CRs were also determined using the same time interval for both groups, i.e., for the standard treatment group taking four weeks after the first treatment as the final time point. While the CR in the standard treatment group was significantly higher four weeks after treatment compared to the CR obtained 10 weeks after treatment, there was no significant difference in the CRs between the standard treatment group and the intense treatment group four weeks after the last treatment, measured with both KK and POC-CCA. Hence, following this evaluation approach, there is no indication that four repeated PZQ treatments outperform a single treatment in curing schistosomiasis.

Moreover, although four repeated PZQ treatments resulted in a statistically significantly higher CR when utilizing the KK technique compared to POC-CCA, the estimated

CR was considerably lower than what we had expected (31), and the proportion of KK-positives increased again from 1% before the fourth treatment to 14% four weeks after the fourth treatment. This might indicate continued parasite exposure, ongoing re-infection, and worm maturation after treatment over the course of the study, not excluding possible PZQ resistance.

Compliance to treatment was very high at each treatment, most likely due to the relatively mild and short-lived adverse events in combination with the commitment of the field and laboratory team, and enthusiastic participation of children. At each treatment time point, directly observed treatment was applied by the study physician. We therefore conclude that the observed increase in proportion of KK-positives at the final time point in the intense treatment group cannot be explained by the fact that some children might not have taken the (repeatedly) administered drugs, but points to parasite survival in the host or rapid reinfection.

To more accurately assess the CR, the POC-CCA test was employed in addition to KK. CRs based on POC-CCA were significantly lower in both groups compared to CRs based on the KK technique. Even though the number of POC-CCA negatives increased after four treatments, still more than half of the participants remained positive (traces conservatively considered as negative), indicating that active *Schistosoma* infections were still present in our cohort or new infections occurred within short periods.

Previous studies have demonstrated false positive POC-CCA results in people with urinary tract infections (43) as well as potentially in pregnant women (44) and new born babies. In our study, no urinary tract infections were noted, two female participants were excluded because of possible pregnancy, and only children aged 5 years and above were included. To further minimize the inclusion of false positives, traces were considered as negative in our analysis. Recent studies have shown that prevalence estimates of *S. mansoni* below 20% according to the KK technique might correspond to POC-CCA prevalence estimates that are 3- to 4-fold higher (18,5). Furthermore, studies applying latent class analysis to determine the performance of diagnostic assays have shown that the POC-CCA test has a considerably greater sensitivity and a comparable specificity than the KK technique, especially when traces are considered negative (20,46). In contrast, other studies have shown that POC-CCA might be of limited use to diagnose *S. mansoni* infection, especially in low endemic areas (47). More accurate diagnostic methods, such as PCR (48,49) or the upconverting phosphor lateral flow (UCP-LF) assay detecting circulating anodic antigen (50), both being well established laboratory-based assays, could be used to determine prevalence more accurately.

It is important to note that the two diagnostic methods employed in our study detect different *Schistosoma* life-cycle stages, namely eggs in case of KK thick smears examined under a microscope and antigens derived from adult worms in case of the POC-CCA urine test. Finding a high proportion of individuals positive by POC-CCA after treatment, while no eggs are detected by KK, indicates that the infection is not fully cured. Mature worms could have been affected by PZQ but not killed, resulting in the (temporary) reduction or cessation of fecal egg excretion (13), while still excreting CCA detectable by POC-CCA. Furthermore, individuals can harbor living (single sex) worms with only sporadically excreting eggs in stool or with no detectable eggs at all (51–54). CCA might also originate from new infections or immature worms (55) or perhaps from dead worms that were killed by PZQ. Lastly, PZQ treatment could have resulted in a reduction of fecundity, indicating that egg-based diagnostic methods will

overestimate the reduction in worm burden (56,57). However, even with the presence of low worm numbers, which excrete relatively few eggs, there is a continued risk of pathology (58,59), as eggs could retain in the host tissue, where they induce inflammatory responses resulting in 'subtle morbidity' (60).

Little is known about the transmission of schistosomes in the study setting of south-central Côte d'Ivoire; transmission is suspected to be ongoing, given the number of observed cases and the frequent surface water contact of the inhabitants with the man-made Lake Taabo (27,30). This trial focused on simply measuring the efficacy of PZQ and did not include sanitation and behavioral interventions, which in this specific setting would have had an additional impact on the number and intensity of *Schistosoma* infections. Moreover, since this study focused on a subset of school-aged children, other children in the same age range, as well as preschool-aged children or adolescents/adults from the community would still serve as a reservoir of infection and therefore contribute to ongoing transmission.

The CR and IRR as determined by the KK technique were higher after four treatments compared to a single treatment, both at four and 10 weeks after treatment. This observation suggests that repeated treatment has an added value on reducing the number of infections and *Schistosoma*-related infection intensity and thus morbidity in areas where people are likely infected with different developmental stages of the parasite and rapid re-infection is obvious. However, since microscopy lacks sensitivity, especially in infections of low intensity and posttreatment settings, not being able to detect eggs does not necessarily mean that the infection is cured and that all worms have been killed. In contrast to the high IRR based on the KK technique, only a minor reduction in POC-CCA-based infection intensity was observed, which did not increase significantly after four repeated PZQ treatments. This contradicts previous studies that indicated a decrease in POC-CCA intensity score rapidly after treatment (20,47,61). More accurate diagnostic methods, such as the UCP-LF CAA test or quantitative PCR could be applied to more accurately determine the reduction in intensity.

Previous studies indicate that the frequency and severity of adverse events is related to *Schistosoma* infection intensity, with more events reported in infections with a heavy intensity (62,63). Over the course of the trial, mostly mild and short-lived adverse events were observed, which presumably can be attributed to the relatively low intensity of infection in our study population. Overall, repeated PZQ treatment was well tolerated, indicating that repeated PZQ treatment can be considered as safe. Repeated PZQ treatment might help to enhance the control of schistosomiasis. This should not preclude the notion that treatment, whether single or repeated, should always be combined with other control measures, such as behavior change, sanitation, safe water, and snail control interventions in order to bolster the effect of PZQ and to move toward interruption of transmission.

CONCLUSION

Based on stool microscopy using the KK technique, four repeated doses of 40 mg/kg PZQ at 2-week intervals resulted in a CR against *S. mansoni* infection of 86%, as determined 10 weeks after the initial treatment. When using the more sensitive POC-CCA test, the observed CR was significantly lower (27%), indicating that PZQ might not be as efficacious as previously reported.

The same trend is shown when efficacy is expressed as IRR, which highlights the relevance of accurate diagnostic methods in monitoring treatment efficacy as well as other control approaches (e.g. vaccine development). This study signifies that the development and field implementation of reliable and more accurate diagnostic tools are essential to systematically map transmission intensity and measure efficacy of control strategies, ultimately providing rational guidance on the path toward elimination of schistosomiasis.

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Data Availability Statement
All relevant data are available within the manuscript and its supporting information file.

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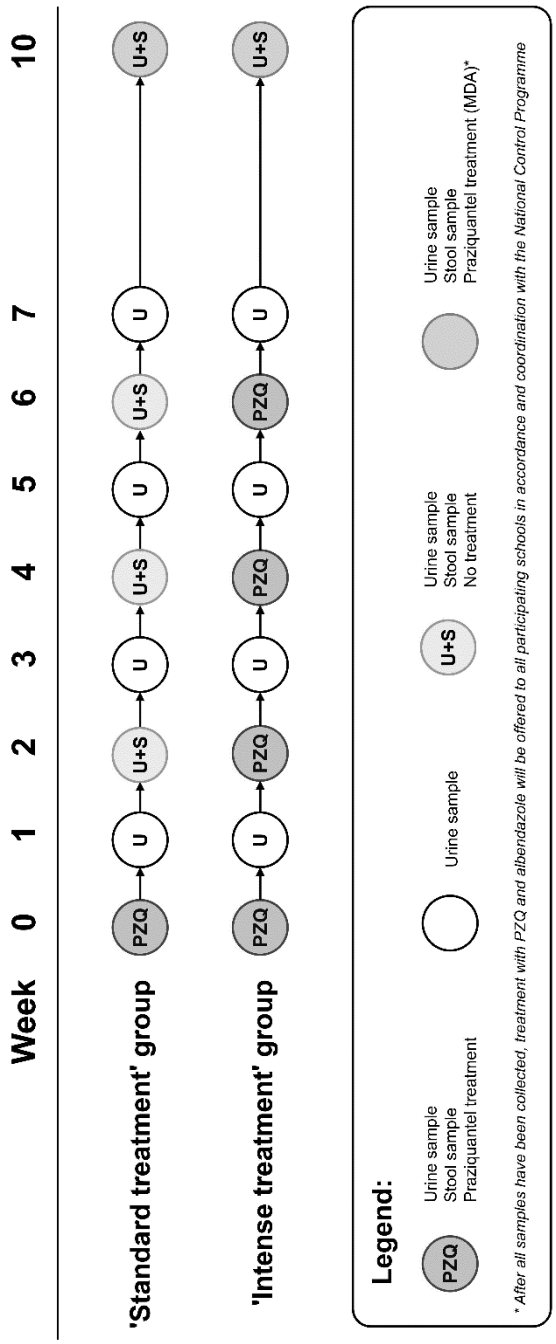
SUPPLEMENTARY MATERIALS

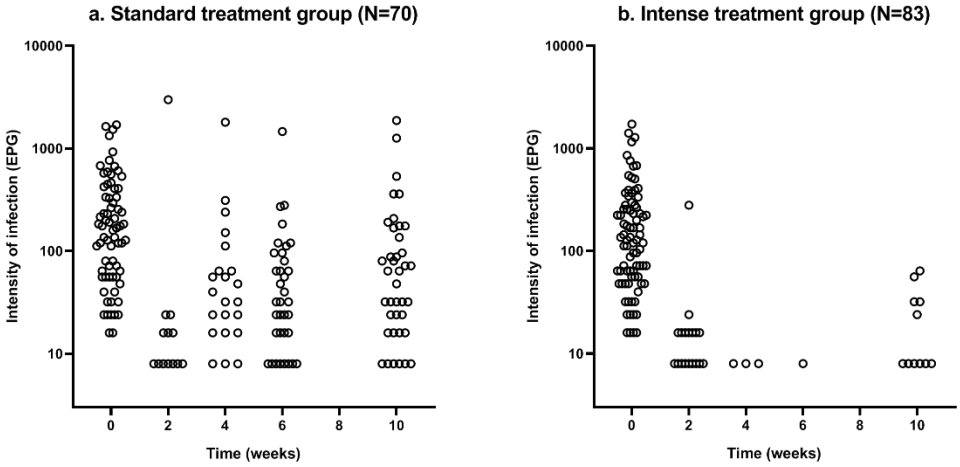
CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Title
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	Abstract
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	Introduction
	2b	Specific objectives or hypotheses	Introduction, paragraph 4
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	Methods, paragraph 2-4
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	Methods, paragraph 3
	4b	Settings and locations where the data were collected	Methods, paragraph 2
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Methods, paragraph 4, 9, 10
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	Methods, paragraph 6
	6b	Any changes to trial outcomes after the trial commenced, with reasons	Methods, paragraph 4
Sample size	7a	How sample size was determined	Methods, paragraph 11
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Methods, paragraph 5
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Methods, paragraph 5
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Methods, paragraph 5
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Methods, paragraph 5
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Methods, paragraph 5
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	Methods, paragraph 14
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Methods, paragraph 14
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Results, paragraph 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	Results, paragraph 1 and S1 Table
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Methods, paragraph 3
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Results, paragraph 2 and Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Results, paragraph 1 and Figure 1
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Results, paragraph 3-6 and Table 2
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Table 2
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	NA
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Results, paragraph 7 and Table 3
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Discussion, paragraph 4, 5
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Discussion, paragraph 1, 2, 7 and Conclusion
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Discussion, paragraph 7 and Conclusion
Other information			
Registration	23	Registration number and name of trial registry	Abstract and Methods, paragraph 1
Protocol	24	Where the full trial protocol can be accessed, if available	Published (see https://www.ncbi.nlm.nih.gov/pubmed/30547750)
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	End of manuscript

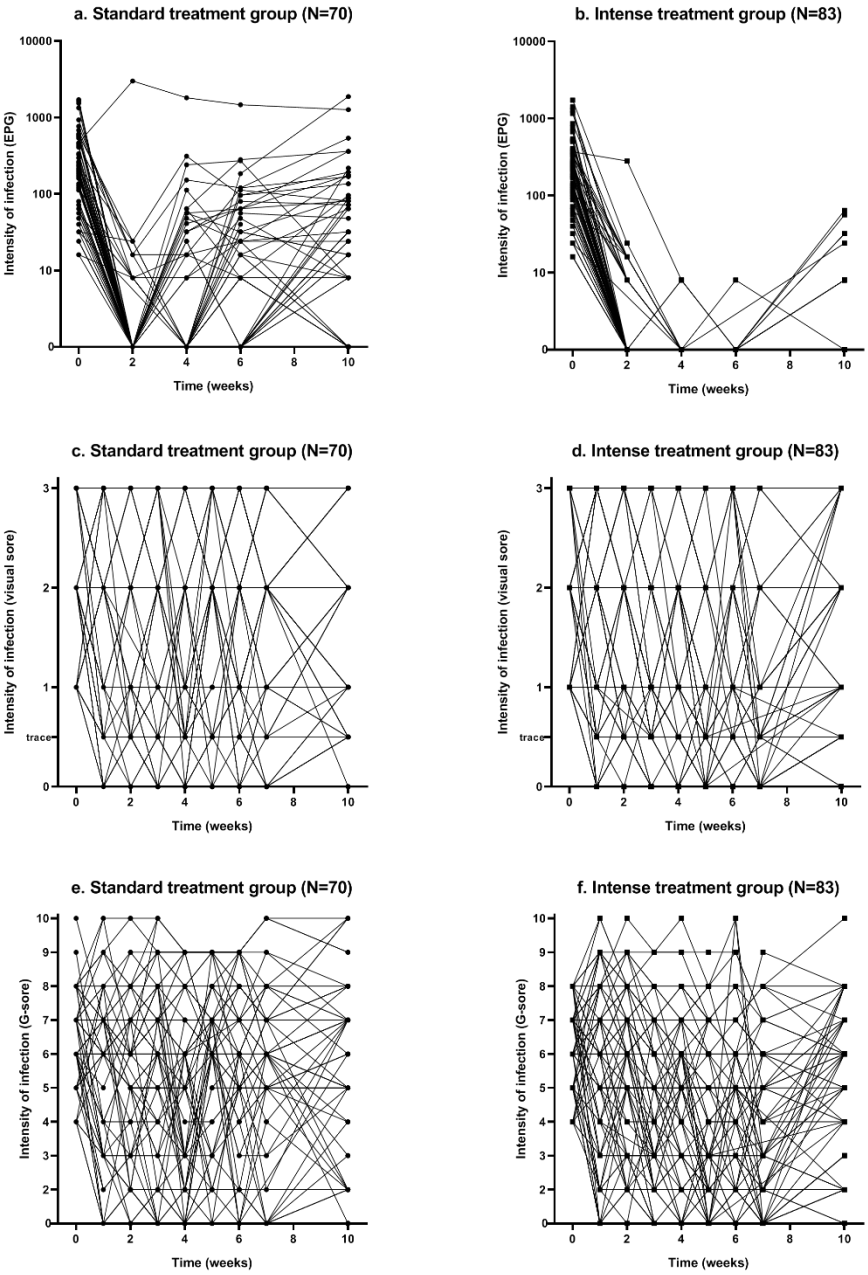
*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.



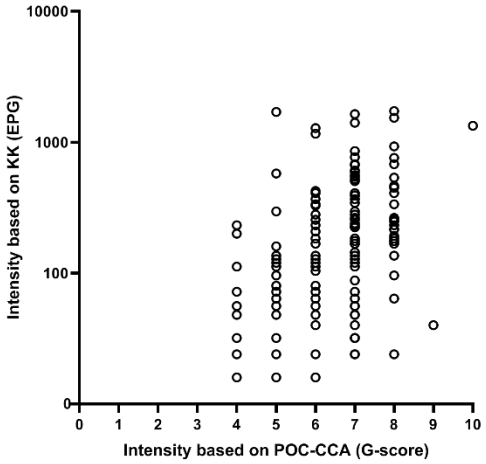
S1 Figure. Schematic representation of follow-up, treatment and sampling procedures in the standard and intense treatment group. Adapted from the published study protocol.



S2 Figure. Intensity of infection of KK-positives over time based on triplicate thick smears from a single stool sample in the standard treatment group (single PZQ treatment) (a) and the intense treatment group (four repeated PZQ treatments at W0, W2, W4, and W6) (b).



S3 Figure. Individual intensity of infection over time based on triplicate Kato-Katz (KK) thick smears from a single stool sample (a, b) and single point-of-care circulating cathodic antigen (POC-CCA) urine test using visual scores (c, d) or G-scores (e, f) in the standard treatment group (single PZQ treatment) and the intense treatment group (four repeated PZQ treatments at W0, W2, W4, and W6).



S4 Figure. Correlation between EPG (based on KK) and G-scores (based on POC-CCA) before treatment.

S1 Table. Detailed overview of missing samples and drop-outs during follow-up in the standard treatment group and the intense treatment group.

	Standard treatment group (1x PZQ) N=70			**Intense treatment group** (4x PZQ) N=83		
	Total	Missing	Participant ID	Total	Missing	Participant ID
W0						
Received PZQ treatment	N=70	N=0		N=83	N=0	
Provided stool sample	N=70	N=0		N=83	N=0	
Provided urine sample	N=70	N=0		N=83	N=0	
W1						
Provided urine sample	N=70	N=0		N=83	N=0	
W2						
Received PZQ treatment	NA	NA		N=82	N=1	2023
Provided stool sample	N=68	N=2	1155 2453	N=82	N=1	2023
Provided urine sample	N=68	N=2	1155 2453	N=82	N=1	
W3						
Provided urine sample	N=70	N=0		N=83	N=0	
W4						
Received PZQ treatment	NA	NA		N=82	N=1	2457
Provided stool sample	N=69	N=1	1105	N=83	N=0	
Provided urine sample	N=70	N=0		N=83	N=0	
W5						
Provided urine sample	N=69	N=1	1535	N=83	N=0	
W6						
Received PZQ treatment	NA	NA		N=78	N=5	2050[a] 2149 2260 2282 2457[a]
Provided stool sample	N=68	N=2	1105[a] 2449	N=77	N=6	1425 2050[a] 2149 2282 2371 2457[a]
Provided urine sample	N=69	N=1	1105[a]	N=79	N=4	2050[a] 2149 2282 2457[a]
W7						
Provided urine sample	N=66	N=4	1105[a] 1416 1549 2336	N=77	N=6	1243 2050[a] 2146 2149 2282 2457[a]
W10						
Provided stool sample	N=67	N=3	1034 1105[a] 1155	N=80	N=3	1512 2050[a] 2457[a]
Provided urine sample	N=67	N=3	1034 1105[a] 1155	N=81	N=2	2050[a] 2457[a]

NA; not applicable.

[a] Lost to follow-up.

S2 Table. Cure and intensity reduction rates after one, two, three and four treatments with PZQ at two-week intervals in school-aged children infected with *S. mansoni* based on triplicate Kato-Katz thick smears from a single stool sample and single point-of-care circulating cathodic antigen (POC-CCA) urine cassette test.

	Standard treatment group	Intense treatment group			
	First treatment (W0)	First treatment (W0)	Second treatment (W2)	Third treatment (W4)	Fourth treatment (W6)
Kato-Katz	N=70	N=83	N=82	N=82	N=78
Infected children before treatment[a]	70	83	83	83	83
Cured children after treatment[b]	55	64	80	76	69
CR[c]	80.8% (95% CI 69.6-88.6)	80.0% (95% CI 68.8-85.0)	96.0% (95% CI 89.2-98.6)	98.2% (95% CI 91.4-99.7)	86.0% (95% CI 75.4-92.4)
Median EPG[d]					
Before treatment	172	128	128	128	128
After treatment[b]	8 *(out of 13 positive)*	12 *(out of 18 positive)*	8 *(out of 3 positive)*	8 *(one positive)*	8 *(out of 11 positive)*
Arithmetic mean EPG					
Before treatment	298.2	242.7	242.7	242.7	242.7
After treatment[b]	46.2	6.0	0.3	0.1	3.2
IRR[e]	95.6% (95% CI 90.4-98.0)	97.1% (95% CI 94.1-98.6)	99.9% (95% CI 99.7-100.0)	100.0% (95% CI 99.9-100.0)	95.1% (95% CI 85.1-98.4)
POC-CCA (traces considered negative)					
Infected children before treatment[a]	70	83	83	83	83
Cured children after treatment[b]	18	20	30	29	29
CR[c]	20.9% (95% CI15.0-28.4)	23.2% (95% CI 16.6-31.3)	44.4% (95% CI 35.5-53.7)	47.7% (95% CI 38.8-56.7)	35.7% (95% CI 26.4-46.1)
Median G-score[d]					
Before treatment	6	7	7	7	7
After treatment[b]	6	6	5	5	6
Arithmetic mean G-score					
Before treatment	6.4	6.3	6.3	6.3	6.3
After treatment[b]	5.4	5.7	4.7	4.2	4.6
IRR[f]	15.6%	9.5%	25.4%	33.3%	27.0%

[a] Number of infected children at baseline
[b] Measured 2 weeks post-treatment for one, two and three treatments and measured 4 weeks post-treatment for four treatments
[c] CR as calculated from the model based on the probability of being cured
[d] Median of the positives
[e] IRR based on the reduction in mean EPG as calculated from the model
[f] IRR based on the reduction in mean POC-CCA G-score as calculated manually
Abbreviations: CR, cure rate; EPG, eggs per gram of stool; IRR, intensity reduction rate; POC-CCA, point-of-care circulating cathodic antigen test.

6.

Limited efficacy of repeated praziquantel treatment in *Schistosoma mansoni* infections as revealed by highly accurate diagnostics, PCR and UCP-LF CAA (RePST trial)

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PLoS Neglected Tropical Diseases (2022)

ABSTRACT

Background: Most studies assessing praziquantel (PZQ) efficacy have used relatively insensitive diagnostic methods, thereby overestimating cure rate (CR) and intensity reduction rate (IRR). To determine accurately PZQ efficacy, we employed more sensitive DNA and circulating antigen detection methods.

Methodology: A sub-analysis was performed based on a previously published trial conducted in children from Côte d'Ivoire with a confirmed *Schistosoma mansoni* infection, who were randomly assigned to a standard (single dose of PZQ) or intense treatment group (4 repeated doses of PZQ at 2-week intervals). CR and IRR were estimated based on PCR detecting DNA in a single stool sample and the up-converting particle lateral flow (UCP-LF) test detecting circulating anodic antigen (CAA) in a single urine sample, and compared with traditional Kato-Katz (KK) and point-of-care circulating cathodic antigen (POC-CCA).

Principal findings: Individuals positive by all diagnostic methods (i.e., KK, POC-CCA, PCR, and UCP-LF CAA) at baseline were included in the statistical analysis (n = 125). PCR showed a CR of 45% (95% confidence interval (CI) 32-59%) in the standard and 78% (95% CI 66-87%) in the intense treatment group, which is lower compared to the KK results (64%, 95% CI 52-75%) and 88%, 95% CI 78-93%). UCP-LF CAA showed a significantly lower CR in both groups, 16% (95% CI 11-24%) and 18% (95% CI 12-26%), even lower than observed by POC-CCA (31%, 95% CI 17-35% and 36%, 95% CI 26-47%). A substantial reduction in DNA and CAA-levels was observed after the first treatment, with no further decrease after additional treatment and no significant difference in IRR between treatment groups.

Conclusion/significance: The efficacy of (repeated) PZQ treatment was overestimated when using egg-based diagnostics (i.e. KK and PCR). Quantitative worm-based diagnostics (i.e. POC-CCA and UCP-LF CAA) revealed that active *Schistosoma* infections are still present despite multiple treatments. These results stress the need for using accurate diagnostic tools to monitor different PZQ treatment strategies, in particular when moving toward elimination of schistosomiasis.

Clinical trial registration: www.clinicaltrials.gov, NCT02868385.

AUTHOR SUMMARY

Efficacy of praziquantel (PZQ) for the treatment of schistosomiasis is usually assessed by classical microscopic detection of parasite eggs in stool or urine. Due to low sensitivity, especially in case of low-intensity infections, the prevalence of infection is underestimated leading to an overestimated cure rate (CR) when using these methods. In a repeated treatment trial, the efficacy of one versus four repeated PZQ treatments, given at 2-week intervals, was investigated in school-aged children from Côte d'Ivoire by applying a range of diagnostic methods, including traditional microscopy as well as more sensitive DNA and circulating antigen detection methods. Our results demonstrate that PZQ efficacy measurements vary based on the diagnostic method used: while egg-based diagnostics (stool microscopy and DNA detection methods) show an improved CR after repeated treatment, the CR determined by worm-based diagnostics (urine circulating antigen detection methods) remained poor over time. Although all four diagnostic methods showed a significant reduction in intensity of infection already after a single treatment, more accurate antigen diagnostics revealed that, in most cases, worms remain present even after multiple treatments. Hence, using accurate diagnostic tools is essential to determine the true infection status and to monitor and evaluate treatment programs.

INTRODUCTION

Schistosomiasis remains a major public health problem in many sub-Saharan African countries. The cornerstone of schistosomiasis control programs is large-scale administration of the anthelmintic drug praziquantel (PZQ) [1]. Even though the World Health Organization (WHO) recommends using the intensity reduction rate (IRR) to measure the efficacy of PZQ treatment, the efficacy is still often expressed as cure rate (CR) [2]. Observed CRs in school-aged children range from 42% to 79% for *Schistosoma mansoni* and between 37% and 93% for *S. haematobium* (3). Repeating PZQ treatment can increase the CR up to 99% [3-5]. However, the majority of studies assessing the efficacy of PZQ treatment have used classical parasitologic methods, including Kato-Katz (KK) to determine the CR [6]. It is widely acknowledged that these methods lack sensitivity, especially in case of low intensity infections – as often observed after treatment [7-9] – thereby underestimating the prevalence of infection, and hence, overestimating the CR [10-11].

To determine the efficacy of PZQ more accurately, diagnostic methods with a higher sensitivity should be used. Highly specific and sensitive molecular PCR techniques detecting *Schistosoma*-specific DNA in stool and urine are available and could be suitable for monitoring PZQ efficacy [7,12]. Alternatively, as PZQ affects the adult worm, treatment efficacy could also be evaluated by measuring the worm burden. Schistosome worms regurgitate various antigens into the blood circulation, which are then excreted via the urine, for example the antigens 'circulating cathodic antigen' (CCA) and 'circulating anodic antigen' (CAA); two antigens that have been described and studied extensively [13,14]. Detection of CCA is done via the commercially available point-of-care (POC) CCA urine cassette test, which is currently being recommended by WHO as an alternative for KK for diagnosing intestinal schistosomiasis [15-18]. CAA is detected quantitatively using an ultra-sensitive reporter technology (up-converting

particle, UCP), combined with common immunochromatography, lateral flow (LF) [19]. This UCP-LF CAA test has demonstrated high sensitivity and specificity for the main human schistosome species (*S. haematobium*, *S. mansoni*, *S. japonicum*, and *S. mekongi*) [19-22]. Combining egg-derived nucleic acid and worm-derived antigen detection methods is expected to provide a more accurate determination of the efficacy of PZQ, both in terms of parasite worm dynamics as well as fecundity.

Previous results from the Repeated Praziquantel for Schistosomiasis Treatment (RePST) study–an open-label randomized controlled trial evaluating the efficacy of repeated PZQ on parasite clearance in school-aged children with a confirmed *S. mansoni* infection–showed a significantly higher CR after four PZQ treatments compared to a single PZQ based on the traditional KK technique [23]. However, the CR based on POC-CCA was substantially lower than the CR based on KK, even after four repeated PZQ treatments, indicating that worms are most likely still present after repeated treatment. Here, we further evaluate the efficacy of PZQ by using two additional sensitive and highly specific diagnostic methods, i.e., stool PCR and the UCP-LF CAA urine test, to determine the CR as well as the IRR after single and four repeated treatments. The current study uniquely combines all diagnostic methods, and provides new insights into the post-treatment dynamics of *S. mansoni* infections.

METHODS

Ethics statement

Ethical approval was granted in Côte d'Ivoire from both the Comité National d'Éthique des Sciences de la Vie et de la Santé de Côte d'Ivoire (no. 091-18/MSHP/CNESVS-km) and the Direction de la Pharmacie, du Médicament et des Laboratoires de Côte d'Ivoire (no. 99433/MSPH/DGS/DPML/DAR and ECCI00618), and in The Netherlands from the Ethics Committee of the Leiden University Medical Center (P16.254). Oral assent from school-aged children as well as signed informed consent from children's parents or guardians was obtained before data and sample collection.

Study design and participants

Previously, an open-label, randomized controlled trial was conducted between October 2018 and January 2019 in the Taabo health district in south-central Côte d'Ivoire, results of which have been published elsewhere [23,24]. Briefly, after clinical and parasitologic assessment for eligibility, school-aged children (5–17 years) with a confirmed *S. mansoni* infection (i.e., positive by KK (>8 eggs per gram of stool, EPG) and POC-CCA (traces excluded)), were randomized into the 'standard treatment' or into the 'intense treatment' group [23]. At baseline, all participating children were given PZQ (40 mg/kg). Subsequently, children assigned to the standard group did not receive any further treatment during the trial period, while children assigned to the intense treatment group received additional PZQ treatment at 2, 4, and 6 weeks after the initial treatment [23].

Diagnostic procedures

A detailed description of all field and laboratory procedures can be found in the previously published study protocol [24]. In brief, urine and stool samples were collected from each participating child weekly and two-weekly, respectively, over a period of 10 weeks in total. In the field, urine samples were subjected to the POC-CCA test using the G-score method [25], while stool samples were processed and examined using the KK technique [23,24]. Aliquots of all available urine and stool (mixed with ethanol for storage and transport purposes [26]) samples were stored at -20°C. After the trial was completed, all available urine and stool aliquots were shipped to the Leiden University Medical Center (Leiden, The Netherlands) and stored at -20°C pending further testing.

The *Schistosoma* genus specific (ITS2) real-time PCR was used for the detection of *Schistosoma* DNA in stool samples, as described previously [27,28]. Besides negative and positive control samples included in each amplification run, phocin herpes virus-1 (PhHV-1) was added to the lysis buffer in each sample as an internal positive control. Virus-specific primers and detecting probe were included in each reaction mixture. Fifty PCR amplification cycles were run per sample, using the amplification cycle in which the level of fluorescent signal exceeded the background as the cycle-threshold (Ct)-value PCR output. Since its implementation, the LUMC-team scored 100% in sensitivity and specificity of their *Schistosoma* PCR at the annual international Helminths External Molecular Assessment Scheme (HEMQAS) provided by the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) [29].

Urine samples were subjected to the UCP-LF CAA test [19]. A set of reference standards with a known CAA concentration was included to quantify CAA-levels as well as to validate the cut-off (0.6 pg/ml for the urine UCAAhT417 test [19]). Samples were considered positive if the CAA concentration exceeded the cut-off, while samples below the cut-off were considered negative.

Statistical analysis

Only individuals with a positive baseline outcome in all diagnostic methods (i.e., KK, POC-CCA, PCR, and UCP-LF CAA) were included in the analysis. As the diagnostics used typically focus on direct detection of the presence of worms (by their metabolic excretion products, CCA and CAA) or indirectly by showing the presence of the eggs (KK and PCR), data were analyzed to compare both approaches. Descriptive statistics were performed using IBM Statistical Package for Social Sciences version 25 (SPSS Inc., Chicago, United States of America). Prevalence over time as well as CR and IRR based on PCR and UCP-LF CAA were determined using a mixed effects model taking into account the correlation between the different measurements from the same individual [30-32]. CR and IRR were determined by comparing baseline data to outcomes at 4 weeks after the last PZQ treatment, i.e., considering week 4 as the final time point for the standard treatment group and week 10 as the final time point for the intense treatment group. Ct-values were transformed into arbitrary units (AU) of copy numbers of *Schistosoma* DNA, as described before [33]. In short, to each low positive sample, i.e., a Ct-value between 35 and 50, an arbitrary value of 1 AU was assigned. Starting from Ct-value 35, for each PCR cycle reduction a duplication of AU was computed, assuming 100% efficacy of the DNA multiplication process.

To determine the prevalence over time as well as the CR and IRR (based on all diagnostic methods) mixed effects models were used, as described previously [23,31]. This model framework takes into account the zero inflation, the correlation between repeated measurements from the same individual and gives valid results under the missing at random assumption for the missing data, which is valid in this study. For the prevalence and CR, we used mixed effects logistic regression where prevalence was modeled as a function of time, treatment group, and their interaction. In the case of KK and PCR, the time variable was taken as categorical, while for POC-CCA and UCP-LF CAA we modeled progression over time using natural cubic splines with knots. For the IRR, the mean was modeled using the main effect of time (taken as categorical), the main effect of treatment and their interaction. KK data were analyzed in time using a zero-inflated mixed effects negative binomial model, while PCR and UCP-LF CAA data were analyzed in time using a two-part linear mixed model. The correlation between the repeated measurements of each individual is modeled using random effects (i.e., random intercepts). All analyses have been done in R (version 3.43) using the GLMMadaptive package. CR and IRR estimated from the model are given with their corresponding 95% pointwise confidence intervals (CIs).

In the absence of a true 'gold' standard, the sensitivity of the different diagnostic methods was determined by comparison against a composite reference standard (CRS) [34-36], which was based on a positive result by KK and/or PCR and/or UCP-LF CAA, all considered highly specific methods. Consequently, an individual was considered true positive if either KK, PCR, or UCP-LF CAA was positive.

RESULTS

From the 153 school-aged children who participated in the original trial, in 19 cases no aliquots from baseline urine and/or stool samples were available for additional testing. Another nine school-aged children were omitted as they had a negative UCP-LF CAA (n = 7) or a negative PCR (n = 2) at baseline. In total, 125 school-aged children were included in the final analysis, 56 and 69 in the standard and intense treatment group, respectively (S1 Fig). The demographic and parasitologic baseline data for the school-aged children included in the current analysis are summarized in S1 Table.

Prevalence over time

In Fig 1, the percentage of *S. mansoni* positive samples over time based on PCR (Fig 1A) and UCP-LF CAA (Fig 1B) is shown, including previously published results based on KK and POC-CCA [23] (Fig 1C and 1D, respectively). Based on PCR, the total percentage of positives decreased to 55% (95% CI 41-68%) in the standard treatment group and to 22% (95% CI 13-34%) in the intense treatment group, both measured 4 weeks post-treatment. The corresponding CR for the standard and intense treatment group were 45% (95% CI 32-59%) and 78% (95% CI 66-87%), respectively (Tables 1 and S2). Based on UCP-LF CAA, the total percentage of positives decreased to 84% (95% CI 76-90%) in the standard treatment group and to 81% (95% CI 74-89%) in the intense treatment group, both measured 4 weeks post-treatment. The

corresponding CR for the standard and intense treatment group were 16% (95% CI 11-24%) and 18% (95% CI 12-26%), respectively (Tables 1 and S2).

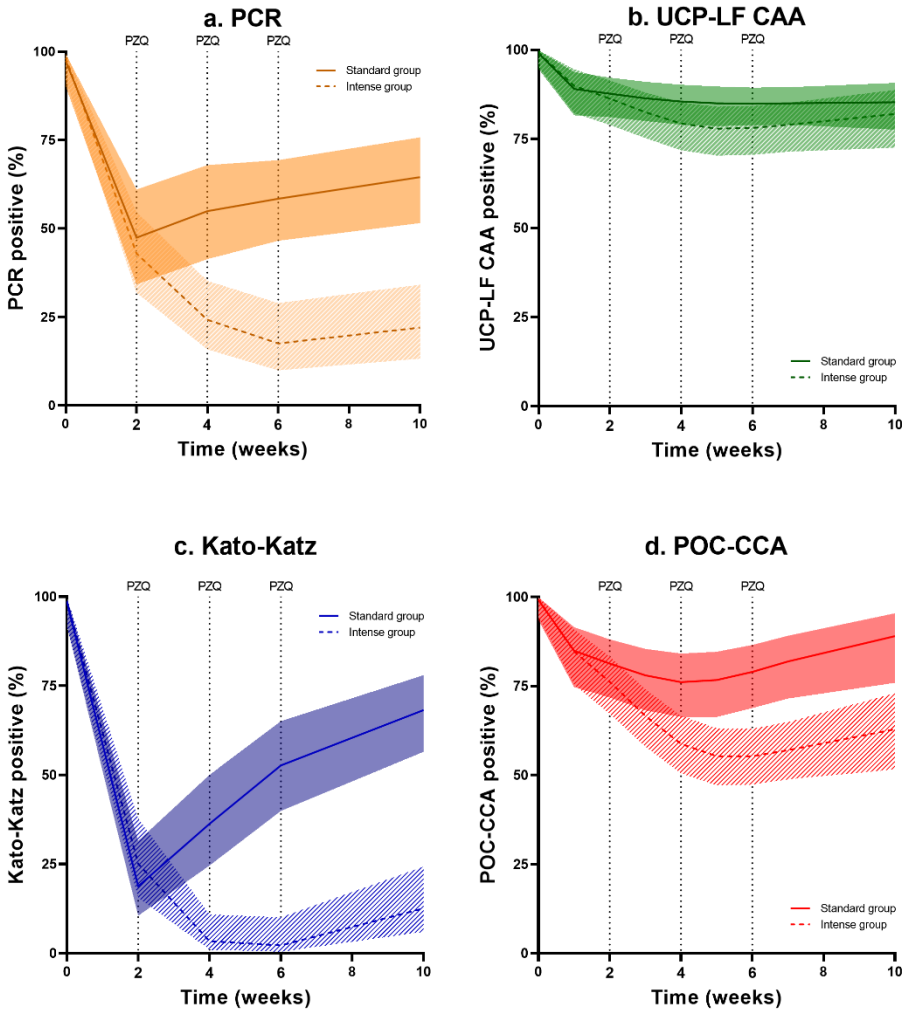

Figure 1. Percentage positive over time with corresponding 95% confidence intervals estimated from the mixed effects logistic regression model. Data based on (a) polymerase chain reaction (PCR), (b) up-converting particle circulating anodic antigen (UCP-LF CAA), (c) Kato-Katz (KK), and (d) point-of-care circulating cathodic antigen (POC-CCA), in the standard treatment group (n = 56, single dose of PZQ at week 0, solid line) and intense treatment group (n = 69, four doses of PZQ at weeks 0, 2, 4, and 6, dashed line).

Table 1. Cure rate (CR) and intensity reduction rate (IRR) of a single (standard treatment group) and four (intense treatment group) repeated PZQ treatments in 125 school-aged children with a confirmed *S. mansoni* infection. Data based on polymerase chain reaction (PCR) and up-converting particle circulating anodic antigen (UCP-LF CAA).

	Standard treatment group (n=56)	**Intense treatment group** (n=69)
PCR		
Cured children 4 weeks post-treatment	25	52
CR (95% CI)[a]	45.3% (32.3-58.8%)	78.1% (66.4-86.6%)
Median AU[b]		
Before treatment	32,768	16,384
4 weeks post-treatment	2,048	24
Arithmetic mean AU[a]		
Before treatment (95% CI)	5.6×10^5 (2.3×10^5-1.2×10^6)	2.0×10^5 (9.5×10^4-4.2×10^5)
4 weeks post-treatment (95% CI)	1.0×10^4 (3.4×10^3-2.8×10^4)	9.9×10^2 (2.2×10^2-4.3×10^3)
IRR (95% CI) [a]	99.6% (98.6-99.9%)	99.5% (97.2-99.9%)
UCP-LF CAA		
Cured children 4 weeks post-treatment	9	13
CR (95% CI)[a]	16.1% (10.5-24.0%)	17.8% (11.5-26.3%)
Median urine CAA-level (pg/ml)[b]		
Before treatment	286	270
4 weeks post-treatment	61	14
Arithmetic mean urine CAA-level (pg/ml)[a]		
Before treatment (95% CI)	145.6 (93.3-217.8)	153.4 (102.5-219.3)
4 weeks post-treatment (95% CI)	40.6 (25.2-62.2)	15.2 (9.3-23.3)
IRR (95% CI)[a]	72.1% (62.4-79.4%)	90.1% (86.9-92.5%)

Abbreviations: AU, arbitrary unit (see Methods for definition); CAA, circulating anodic antigen; CR, cure rate; IRR, intensity reduction rate; PCR, polymerase chain reaction; PZQ, praziquantel; UCP-LF, up-converting particle lateral flow.

a. Calculated from the model

b. Median of the positives

Intensity of infection over time

Fig 2 illustrates the infection intensity over time based on PCR (Fig 2A) and UCP-LF CAA (Fig 2B), including KK and POC-CCA results (Fig 2C and 2D, respectively). Based on PCR, most remaining infections after the first PZQ treatment were of low intensity. In the standard treatment group, the average PCR outcome (AU) decreased from 56×10^4 (95% CI 23×10^4-12×10^5) to 10×10^3 (95% CI 34×10^2-28×10^3), and in the intense treatment group from 20×10^4 (95% CI 95×10^3-42×10^4) to 99×10^1 (95% CI 22×10^1-43×10^2), both measured 4 weeks post-treatment. The corresponding IRR for the standard and intense treatment group were 99.6% (95% CI 98.6-99.9%) and 99.5% (95% CI 97.2-99.9%), respectively (Tables 1 and S2). Based on UCP-LF CAA, infection levels decreased from 146 pg/ml (95% CI 93-218) to 41 pg/ml (95% CI 25-62) in the standard treatment group, and from 153 pg/ml (95% CI 103-219) to 15 pg/ml (95% CI 9-23) in intense treatment group. The corresponding IRRs for the standard and the intense treatment group were 72.1% (95% CI 62.4-79.4%) and 90.1% (95% 86.9-92.5%), respectively (Tables 1 and S2).

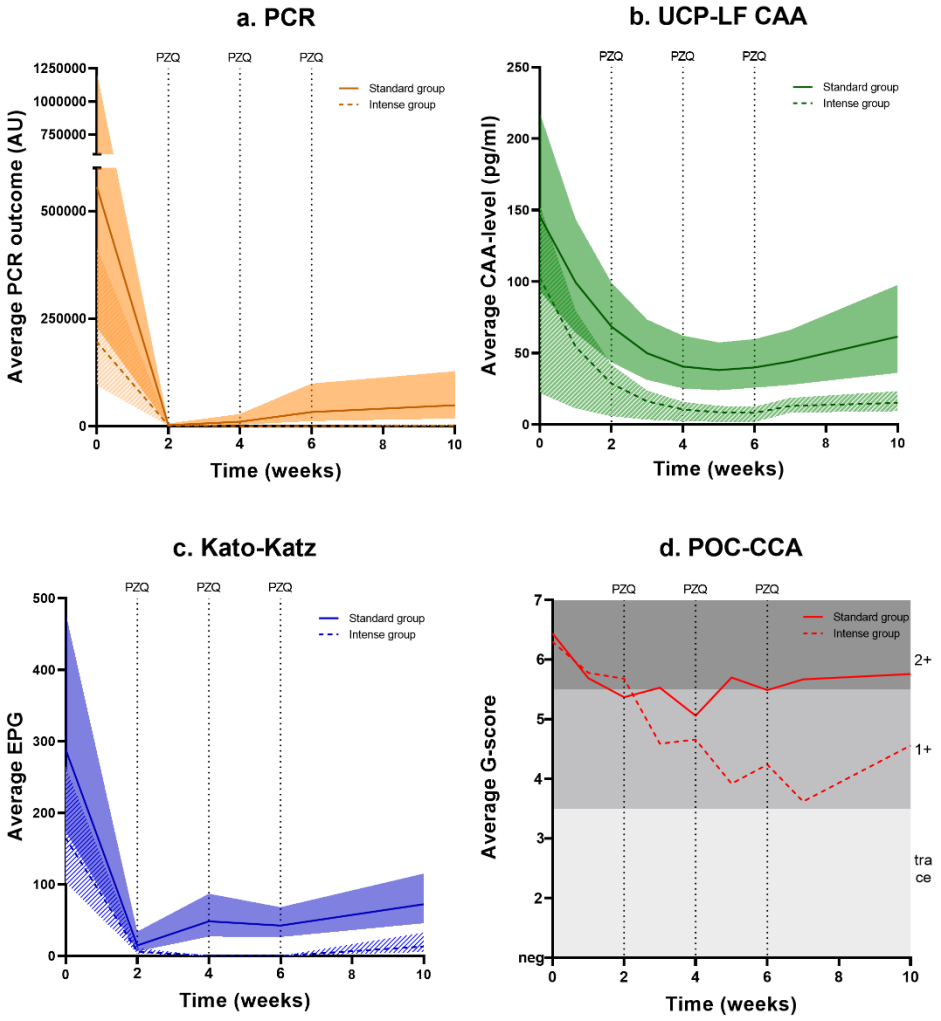

Figure 2. Average infection levels over time with corresponding 95% confidence intervals* estimated from the mixed effects logistic regression model. Data shown for (a) polymerase chain reaction (PCR), (b) up-converting particle circulating anodic antigen (UCP-LF CAA), (c) Kato-Katz (KK), and (d) point-of-care circulating cathodic antigen (POC-CCA), in the standard treatment group (n = 56, single dose of PZQ at week 0, solid line) and intense treatment group (n = 69, four doses of PZQ at weeks 0, 2, 4, and 6, dashed line). **for POC-CCA no confidence intervals available.*

Correlation between tests

In Fig 3, the baseline correlation between egg-based detection methods (Fig 3A) and worm-based detection methods (Fig 3B) is shown. The correlation between egg-based methods PCR (in AU) and KK (in EPG) at baseline was higher (Spearman's rho 0.64, $p<0.01$) compared to the correlation between worm-based detection by UCP-LF CAA and POC-CCA (Spearman's rho 0.37, $p<0.01$). The majority of individuals with ≥400 EPG (86%) had a POC-CCA G-score of 6 or higher (corresponding to a visual score of 2+ or higher) and a urine CAA-level of ≥100 pg/ml (S2 and S3 Figs).

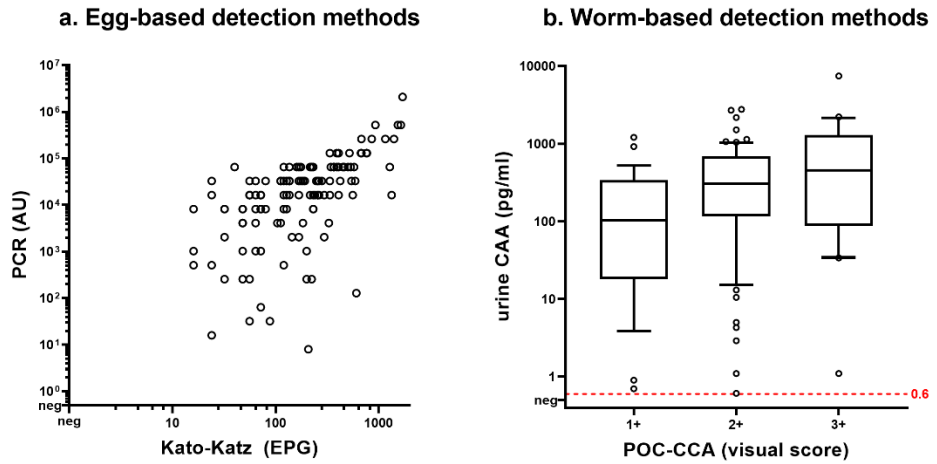

Figure 3. Baseline correlation between (a) egg-detection methods and (b) worm-based detection methods. Data shown for (a) polymerase chain reaction (PCR) versus Kato-Katz (KK) and (b) point-of-care circulating cathodic antigen (POC-CCA) versus up-converting particle circulating anodic antigen (UCP-LF CAA) at baseline (n = 125).

Fig 4 shows the post-treatment correlation between egg-based detection methods (Fig 4A) and worm-based detection methods (Fig 4B). A similar correlation was observed between PCR and KK (Spearman's rho 0.51, $p<0.01$) and between UCP-LF CAA and POC-CCA (Spearman's rho 0.59, $p<0.01$) 4 weeks after treatment. The majority of egg- and PCR-positive individuals were observed in the standard treatment group, while UCP-LF CAA and POC-CCA positives were observed in both groups (S4 Fig).

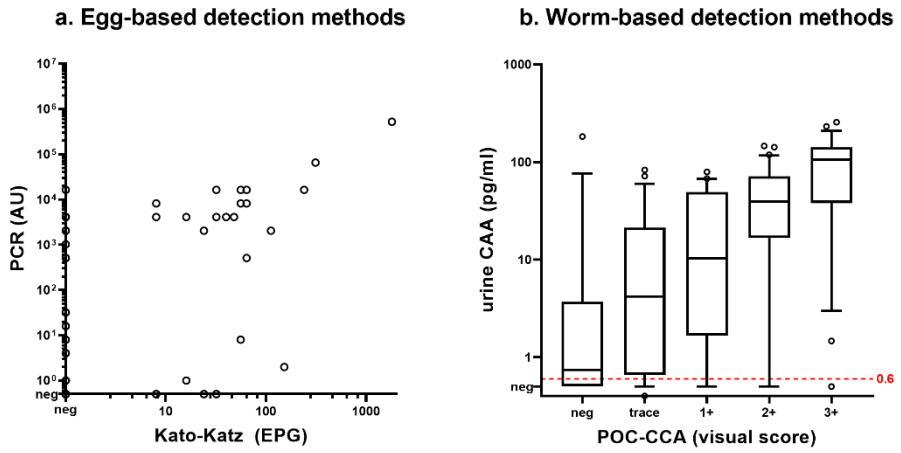

Fig 4. Post-treatment correlation between (a) egg-detection methods and (b) worm-based detection methods. Data based on (a) polymerase chain reaction (PCR) versus Kato-Katz (KK) and (b) point-of-care circulating cathodic antigen (POC-CCA) versus up-converting particle circulating anodic antigen (UCP-LF CAA) 4 weeks after treatment (n = 125).

Accuracy of tests

Sensitivity of egg-based diagnostic methods decreased over time after treatment; a higher reduction in sensitivity was observed in the intense treatment group compared to the standard treatment group (Table 2). While in both groups the sensitivity of POC-CCA fluctuated over time, the sensitivity of UCP-LF CAA remained high and stable, regardless of additional treatment.

Table 2. Sensitivity of polymerase chain reaction (PCR), Kato-Katz (KK), up-converting particle circulating anodic antigen (UCP-LF CAA), and point-of-care circulating cathodic antigen (POC-CCA) compared to a composite reference standard (CRS) over time. Data shown for the standard group (n = 56) who received a single PZQ treatment at week 0 and the intense treatment group (n = 69) who received four repeated PZQ treatments at weeks 0, 2, 4, and 6. The composite reference standard (CRS) was based on KK, PCR, and UCP-LF CAA: an individual was considered positive if at least one of these tests was positive (i.e., test specificity assumed to be 100%).

			Number of individuals positive per diagnostic test and corresponding sensitivity (%) compared to the composite reference standard (CRS)								
		N	**CRS**	**Stool PCR**		**Kato-Katz**		**UCP-LF CAA**		**POC-CCA**	
Standard group	Baseline	56	56	56	100%	56	100%	56	100%	56	100%
	Week 2	54	48	25	52.0%	10	20.8%	46	95.8%	42	87.5%
	Week 4	55	50	30	60.0%	20	40.0%	46	92.0%	38	76.0%
	Week 6	55	49	32	65.3%	29	59.2%	46	93.9%	43	87.8%
	Week 10	53	51	34	66.7%	36	70.6%	44	86.3%	45	88.2%
Intense group	Baseline	69	69	69	100%	69	100%	69	100%	69	100%
	Week 2	68	61	29	47.5%	17	27.9%	58	95.1%	51	83.6%
	Week 4	69	57	16	28.1%	2	3.5%	55	96.5%	42	73.7%
	Week 6	63	49	11	22.4%	1	2.0%	46	93.9%	34	69.4%
	Week 10	66	55	14	25.5%	8	14.5%	54	98.2%	40	72.7%

DISCUSSION

In this study, performing additional diagnostic analysis on samples previously collected at a multiple treatment trial, comparing single versus 4 treatments with PZQ in school-aged children from Côte d'Ivoire, we confirmed our prior conclusion that highly accurate diagnostic methods are important for determining PZQ efficacy [23].

Due to its high sensitivity compared to KK, while remaining intrinsically specific, the *Schistosoma* genus-specific ITS2 PCR detecting *Schistosoma* DNA in stool samples revealed more positive cases over time. Yet, the overall post-treatment dynamics were similar to KK results [23]. This supports the notion that both tests reflect the number of *Schistosoma* eggs in stool (as adult *Schistosoma* worms are located in the mesenteric blood vessels), although the ITS2 PCR target is considered to be present in all life-stages of the parasite. The good correlation between KK and PCR is consistent with previous studies [12,27,28,37,38] indicating the capability of the *Schistosoma* ITS2 PCR to determine infection intensity. PCR also showed a significant reduction in DNA levels after treatment, as observed previously [12,38], confirming its usefulness to monitor PZQ treatment efficacy. The latter is in contrast to other studies using a serum-based PCR that remained positive for months after treatment [39,40].

Turning to worm-based diagnostics, the highly accurate UCP-LF CAA test confirmed the already low CR observed previously by POC-CCA [23]. In 285 out of 483 (59%) post-treatment samples, CAA was detected in urine, while no eggs were observed in the stool. To a lesser extent, this was already shown by previously published POC-CCA positive but egg-negative individuals [23,41,42]. As both CAA and CCA are worm-derived antigens, the presence of either antigen indicates that worms are still metabolically active and that the infection is hence not fully cleared, indicating that the CR is even lower than generally assumed [3,6,43]. The opposite, egg-positive with no CAA in urine, was observed in only a few cases (15 out of 483, 3%). This apparent incongruence of absence of CAA despite presence of eggs could be due to variation in biological excretion, but administrative errors such as sample processing and/or labelling cannot be excluded.

Egg-based diagnostic methods (i.e., PCR and KK), showed an increased CR after repeated treatment, as also observed in other studies [3-5]. Contrastingly, the more sensitive worm-based methods, UCP-LF CAA and POC-CCA, demonstrated a poor CR, which did not significantly improve with additional treatments. Observed CRs should therefore be interpreted with care, keeping in mind that they depend on the type of diagnostic method used. In addition, the time point after treatment chosen for measurement of CRs also influences its value, e.g., for KK it is highest 2 weeks after treatment, and subsequently decreases in the next weeks if no additional treatment is given (S5A Fig). Using different diagnostics, our results confirm that the CR has limited value in assessing PZQ efficacy. Diagnostic sensitivity has a higher impact on the CR than on the IRR, as the detection of an egg, DNA, or circulating antigen makes the difference between cured and non-cured (S5 and S6 Figs).

A significant decrease in urine CAA-levels was observed already after a single treatment and no substantial differences were observed whether data were calculated as arithmetic or geometric mean or median (S7 Fig). By repeating the treatments at relatively short intervals, it was anticipated that non-PZQ-susceptible schistosomula would be targeted more effectively as

within a few weeks they would have matured into PZQ-susceptible worms. Also, an additional effect on mature worms was expected, as the short metabolic half-life of PZQ might limit its effectiveness when given as single treatment. Our results indicate that even though a further reduction in egg output occurred with multiple treatments, worm numbers reflected by urine CAA-levels continued to decrease slightly and remained solidly low from 3 weeks onwards, with only very few cases becoming negative. The rapid rise in egg numbers after treatment would indicate that in this setting transmission continued which, for CAA, resulted in continuous low levels originating probably from young worms (2-4 weeks old) [14,19]. Alternatively or additionally, worms could reside in capillaries not reached by a single dose of PZQ, or worms could revitalize after the damage occurred by PZQ and the host immune attack, thus surviving the treatment. This would suggest that repeating treatment at 2-week intervals is not sufficient and that shorter intervals or perhaps a higher dose could be considered. Although generally rapidly cleared, it cannot be excluded that also a slow release of antigens, restrained in various tissues, contributed to the low CAA-levels after treatment. To further elucidate these mechanisms and to differentiate between surviving adult worms and establishing new infections (reinfection), research in endemic settings with various (seasonal) transmission patterns, in particular the absence of transmission is needed. Alternatively, experimental infections in animals or controlled human infection models would be helpful in unravelling the exact metabolic aspects of CAA excretion and immune-mediated clearance patterns. Due to limited sensitivity of KK for light intensity infections after treatment, eggs may also continue to be excreted but remain below the detection limit of microscopy. Eggs that may be trapped in the host tissue, and therefore not detected, could still result in a continued risk of pathology [44]. Further research is needed to determine the impact of the continued presence of worms without the detection of eggs and to what extent the egg production and excretion is reconstituted over time.

CONCLUSION

A single PZQ treatment had a major impact on the egg output as well as on the worm burden measured by CAA-levels, but it appears difficult to get rid of the remaining worms. Even though additional treatment resulted in a further decrease in the number of eggs, the effect on the number of worms remaining in the host was limited, which indicates that–in this setting–(up to) four repeated treatments at a relatively short interval were not sufficient to clear all *S. mansoni* infections. In particular when moving toward interruption of transmission or even elimination of schistosomiasis, it is important to focus on complete clearance of infection, i.e., not only the absence of eggs, but more importantly the absence of worms. To determine the ideal schistosomiasis treatment schedule, still considering other important factors influencing post-treatment status such as transmission season, environmental conditions, and water contact behavior, the use of accurate egg- and worm-based diagnostic tools is essential.

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Competing interests

The authors have declared that no competing interests exist.

Data Availability Statement

All relevant data are available within the manuscript and its supporting information file.

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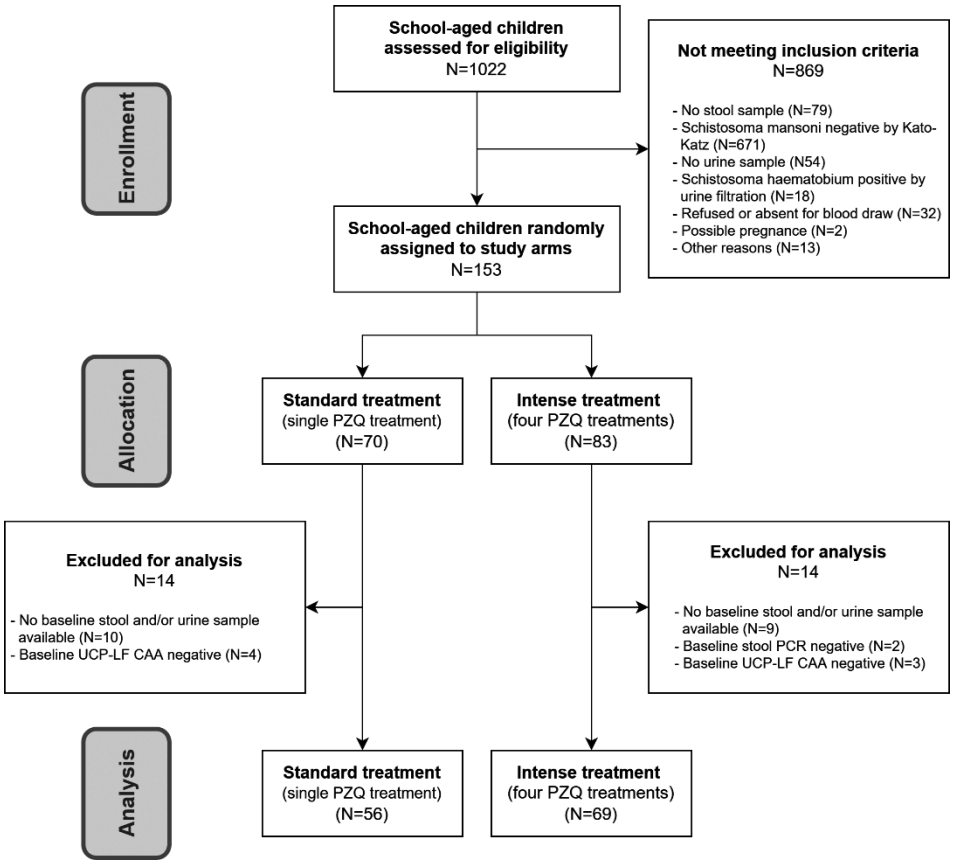
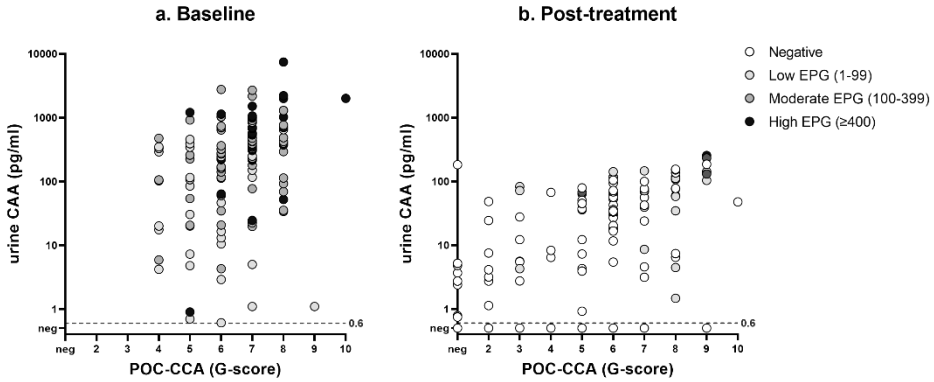
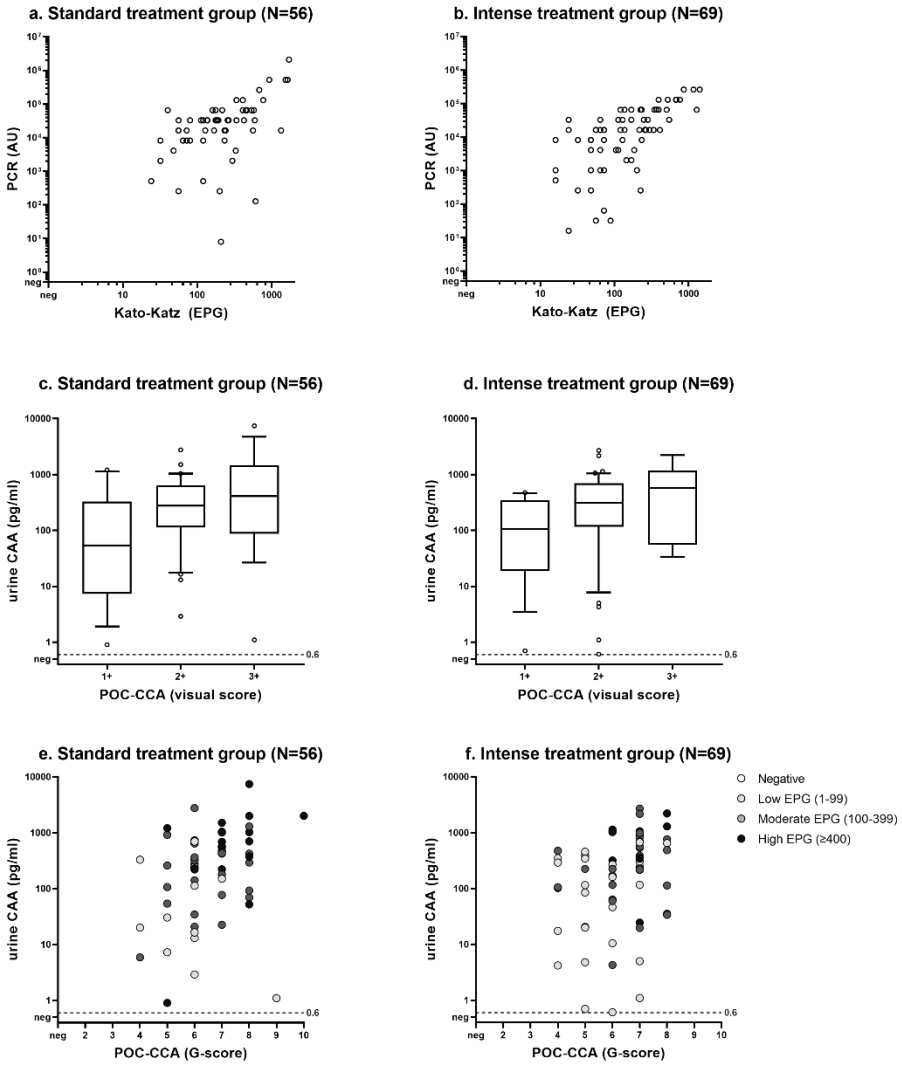
SUPPLEMENTARY MATERIALS



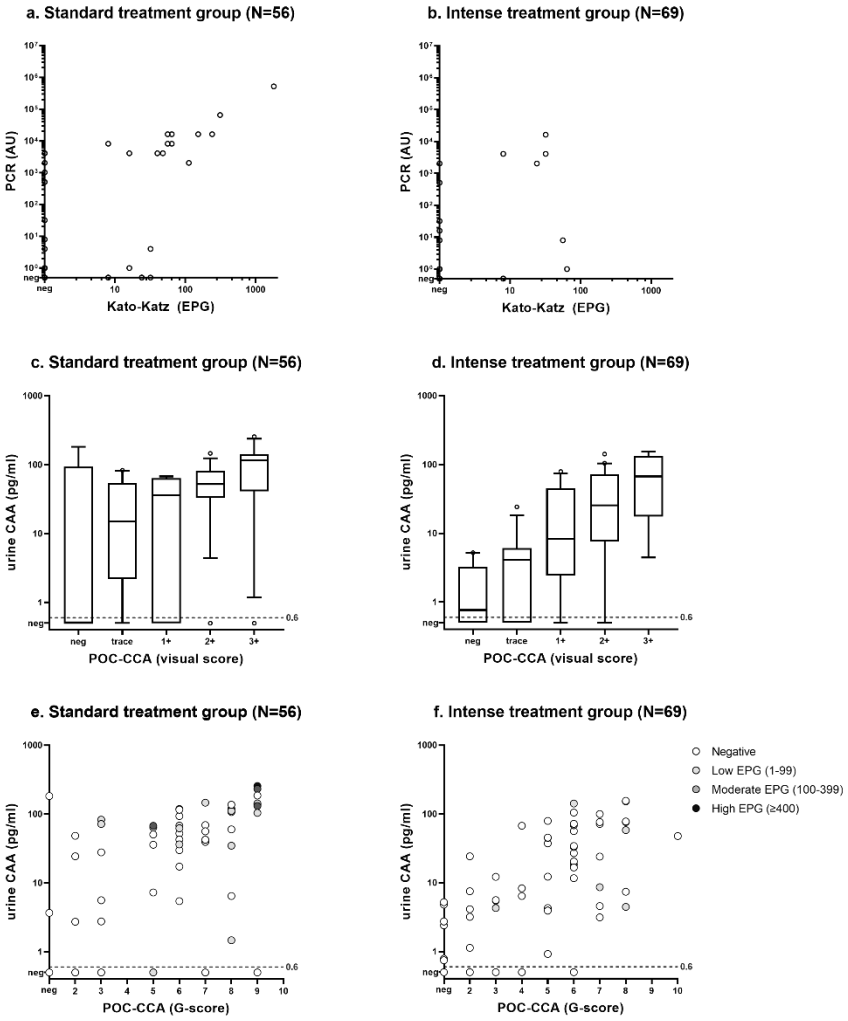
S1 Figure. Trial profile.



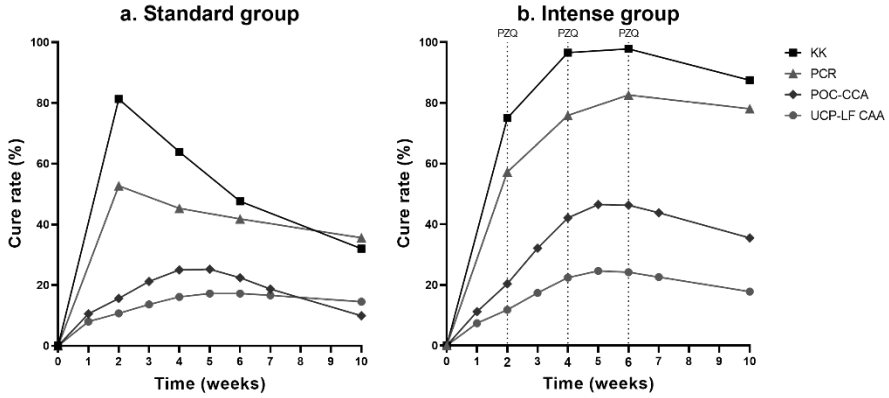
S2 Figure. Correlation between worm-based detection methods including intensity categories based on Kato-Katz (eggs per gram of feces, EPG) at (a) baseline and (b) 4 weeks after (the last) treatment. Data based on point-of-care circulating cathodic antigen (POC-CCA) and up-converting particle circulating anodic antigen (UCP-LF CAA) in combination with Kato-Katz (KK), with colors indicating the EPG intensity (n = 125).



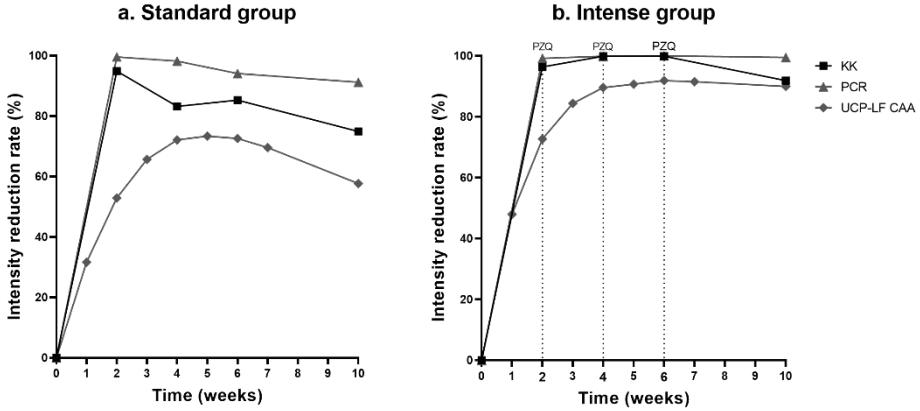
S3 Figure. Baseline correlation between egg-detection methods and worm-based detection methods. Data based on stool polymerase chain reaction (PCR) versus Kato-Katz (KK) (a-b) and point-of-care circulating cathodic antigen (POC-CCA) versus up-converting particle circulating anodic antigen (UCP-LF CAA) (c-f) at baseline (n = 125).



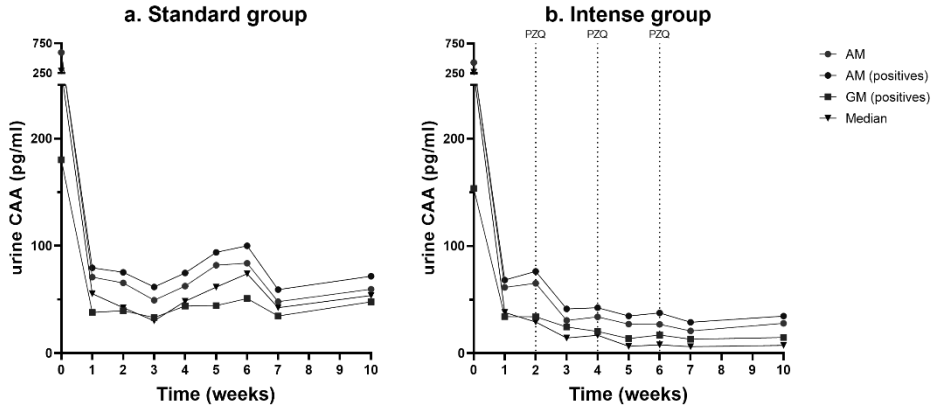
S4 Figure. Post-treatment correlation between egg-detection methods and worm-based detection methods. Data based on stool polymerase chain reaction (PCR) versus Kato-Katz (KK) (a-b) and point-of-care circulating cathodic antigen (POC-CCA) versus up-converting particle circulating anodic antigen (UCP-LF CAA) (c-f) 4 weeks post-treatment (n = 125).



S5 Figure. Cure rate (CR) over time determined by the different diagnostics in (a) the standard treatment group, who received a single dose of PZQ at week 0, and (b) the intense treatment group, who received four doses of PZQ at weeks 0, 2, 4, and 6. Data based on stool polymerase chain reaction (PCR), Kato-Katz (KK), urine up-converting particle circulating anodic antigen (UCP-LF CAA), and point-of-care circulating cathodic antigen (POC-CCA) (n = 125).



S6 Figure. Intensity reduction rate (IRR) over time determined by the different diagnostics in (a) the standard group, who received a single dose of PZQ at week 0, and (b) the intense group, who received four doses of PZQ at weeks 0, 2, 4, and 6. Data based on stool polymerase chain reaction (PCR), Kato-Katz (KK), and urine up-converting particle circulating anodic antigen (UCP-LF CAA) (n = 125).



S7 Figure. Circulating anodic antigen (CAA) levels over time in (a) the standard group, who received a single dose of PZQ at week 0, and (b) the intense group, who received four doses of PZQ at weeks 0, 2, 4, and 6. Data shown as arithmetic mean (AM), arithmetic mean of the positives, geometric mean (GM) of the positives, and median.

S1 Table. Baseline characteristics of the standard treatment group and the intense treatment group. Adapted from (1), for the current study based on data from 125 school-aged children.

	Standard treatment group (N=56)	**Intense treatment group** (N=69)
Age, years	10.5 (9-12)	10 (9-12)
Sex		
Boys	36 (64%)	45 (65%)
Girls	20 (36%)	24 (35%)
Village		
Ahouaty	30 (53%)	39 (57%)
N'Denou	21 (38%)	25 (36%)
Singrobo	5 (9%)	5 (7%)
Infection intensity		
Kato-Katz		
Light (1-99 EPG)	14 (25%)	26 (38%)
Moderate (110-399 EPG)	25 (45%)	32 (46%)
Heavy (≥400 EPG)	17 (30%)	11 (16%)
POC-CCA[a]		
1+	11 (20%)	17 (25%)
2+	31 (55%)	44 (64%)
3+	14 (25%)	8 (11%)
PCR		
Low (35 ≤ Ct < 50)	0	0
Moderate (30 ≤ Ct < 35)	1 (2%)	3 (4%)
High (Ct < 30)	55 (98%)	66 (96%)
UCP-LF CAA		
Low (0.6-9 pg/ml)	5 (9%)	7 (10%)
Moderate (10-99 pg/ml)	12 (21%)	12 (17%)
High (>100 pg/ml)	39 (70%)	50 (72%)

Data are median (IQR) or n (%). Abbreviations: Ct, cycle threshold; EPG, eggs per gram of stool; IQR, interquartile range; PCR, polymerase chain reaction; POC-CCA, point-of-care circulating cathodic antigen; UCP-LF CAA, up-converting particle lateral flow circulating anodic antigen.

[a] POC-CCA positive G-scores were classified into 1+ (G4-5), 2+ (G6-7) or 3+ (G8-10).

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S2 Table. Cure rate (CR) and intensity reduction rate (IRR) of a single (standard treatment group) and four (intense treatment group) repeated PZQ treatments in 125 school-aged children with a confirmed *S. mansoni* infection. Data based on polymerase chain reaction (PCR), Kato-Katz (KK), up-converting particle lateral flow circulating anodic antigen (UCP-LF CAA), and point-of-care circulating cathodic antigen (POC-CCA).

	Standard treatment group (N=56)	Intense treatment group (N=69)
PCR		
Cured children 4 weeks post-treatment	25	52
CR (95% CI)[a]	45.3% (32.3 – 58.8%)	78.1% (66.4 – 86.6%)
Median AU[b]		
Before treatment	32768	16384
4 weeks post-treatment	2048	24
Arithmetic mean AU[a]		
Before treatment (95% CI)	$5.6x10^5$ ($2.3 x10^5$ – $1.2x10^6$)	$2.0x10^5$ ($9.5x10^4$ – $4.2x10^5$)
4 weeks post-treatment (95% CI)	$1.0x10^4$ ($3.4x10^3$ – $2.8x10^4$)	$9.9x10^2$ ($2.2x10^2$ – $4.3x10^3$)
IRR[a]	99.6% (98.6 – 99.9%)	99.5% (97.2 – 99.9%)
Kato-Katz		
Cured children 4 weeks post-treatment	35	58
CR (95% CI)[a]	63.9% (51.7 – 74.5%)	87.5% (77.9 – 93.2%)
Median EPG[b]		
Before treatment	204	136
4 weeks post-treatment	0	0
Arithmetic mean EPG[a]		
Before treatment (95% CI)	288 (174 – 478)	164 (102 – 264)
4 weeks post-treatment (95% CI)	48 (27 – 87)	13 (5 – 33)
IRR (95% CI)[a]	83.2% (70.5 – 90.4%)	91.9% (77.8 – 97.1%)
UCP-LF CAA		
Cured children 4 weeks post-treatment	9	13
CR (95% CI)[a]	16.1% (10.5 – 24.0%)	17.8% (11.5 – 26.3%)
Median urine CAA-level (pg/ml)[b]		
Before treatment	286	270
4 weeks post-treatment	61	14
Arithmetic mean urine CAA-level (pg/ml)[a]		
Before treatment (95% CI)	145.6 (93.3 – 217.8)	153.4 (102.5 – 219.3)
4 weeks post-treatment (95% CI)	40.6 (25.2 – 62.2)	15.2 (9.3 – 23.3)
IRR (95% CI)[a]	72.1% (62.4 – 79.4%)	90.1% (86.9 – 92.5%)
POC-CCA		
Cured children 4 weeks post-treatment	16	24
CR (95% CI)[a]	25.0% (17.2 – 34.9%)	35.5% (25.5 – 46.9%)
Median G-score[b]		
Before treatment	6.5	7.0
4 weeks post-treatment	6.0	5.0
Arithmetic mean G-score[a]		
Before treatment	6.6	6.3
4 weeks post-treatment	5.6	4.6
IRR[c]	15.2%	27.0%

Abbreviations: AU, arbitrary unit (see Materials and Methods for definition); CAA, circulating anodic antigen; CR, cure rate; IRR, intensity reduction rate; PCR, polymerase chain reaction; POC-CCA, point-of-care circulating cathodic antigen; PZQ, praziquantel; UCP-LF, up-converting particle lateral flow.

a. Calculated from the model
b. Median of the positives
c. Calculated manually

7.

Diagnosis of Schistosomiasis without a Microscope: Evaluating Circulating Antigen (CCA, CAA) and DNA Detection Methods on Banked Samples of a Community-Based Survey from DR Congo

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ABSTRACT

Detection of *Schistosoma* eggs in stool or urine is known for its low sensitivity in diagnosing light infections. Alternative diagnostics with better sensitivity while remaining highly specific, such as real-time PCR and circulating antigen detection, are progressively used as complementary diagnostic procedures but have not yet replaced microscopy. This study evaluates these alternative methods for the detection of *Schistosoma* infections in the absence of microscopy. Schistosomiasis presence was determined retrospectively in 314 banked stool and urine samples, available from a previous survey on the prevalence of taeniasis in a community in the Democratic Republic of the Congo, using real-time PCR, the point-of-care circulating cathodic antigen (POC-CCA) test, as well as the up-converting particle lateral flow circulating anodic antigen (UCP-LF CAA) test. *Schistosoma* DNA was present in urine (3%) and stool (28%) samples, while CCA (28%) and CAA (69%) were detected in urine. Further analysis of the generated data indicated stool-based PCR and the POC-CCA test to be suitable diagnostics for screening of *S. mansoni* infections, even in the absence of microscopy. A substantial proportion (60%) of the 215 CAA-positive cases showed low antigen concentrations, suggesting that even PCR and POC-CCA underestimated the "true" number of schistosome positives.

INTRODUCTION

Schistosomiasis is a neglected tropical disease affecting over 250 million people worldwide [1], with an estimated 779 million people at risk of the disease [2]. Traditionally, schistosomiasis is diagnosed through microscopical examination of urine (for *Schistosoma haematobium*) or stool (for *S. mansoni*) [3,4]. Although this method is highly specific, it is also known for its low sensitivity – especially in low-intensity infections – leading to underestimation of the prevalence of infection [1]. Alternative and more sensitive diagnostic methods, such as real-time PCR and circulating antigen detection, are progressively used as complementary diagnostics, but these methods do not yet completely replace microscopy. Detecting *Schistosoma* DNA in stool or urine by real-time PCR is proven to be highly specific and more sensitive than microscopy [5,6]. Circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) are two genus-specific carbohydrate antigens that are continuously regurgitated by live *Schistosoma* worms into the bloodstream of the host [7,8], from where they are excreted in the urine [8,9,10] with limited day-to-day variations [11,12]. These characteristics make them excellent markers for detecting active *Schistosoma* infections as well as a proxy for worm burden [13]. A point-of-care test is commercially available for the detection of CCA in urine and is particularly useful for diagnosing intestinal schistosomiasis [14,15,16,17,18,19,20,21] and, to a lesser extent, also for urogenital schistosomiasis [22]. This test has been studied extensively and is currently recommended by the WHO as an alternative to microscopy for diagnosing intestinal schistosomiasis [23,24,25,26]. CAA has a chemically unique structure and is detected using highly sensitive luminescent up-converting reporter particle (UCP) technology in combination with lateral flow (LF) [27]. This laboratory-based UCP-LF CAA test is quantitative and specific for the main human schistosome species (*S. haematobium*, *S. mansoni*, *S. japonicum*, and *S. mekongi*) [27,28,29,30]. The aim of the current study was to investigate the application of a panel of non-microscopy diagnostic methods to determine the presence of *Schistosoma* infections in the absence of microscopy, in order to provide better insight into the performance of these alternative methods as well as to determine whether an accurate diagnosis of schistosomiasis can be made without traditional microscopy.

MATERIALS AND METHODS

Study Design and Data Collection

The current study was performed on banked urine and stool samples which were available from a previous study on the prevalence and risk factors of *Taenia solium* cysticercosis conducted in 2009 in Malanga, Bas-Congo, the Democratic Republic of the Congo [31,32]. After obtaining informed consent, participants were asked to provide a stool and urine sample. Due to a lack of time and staff, no extended parasitological examination was performed at the time of sample collection. Samples were stored at 4 °C until transport to the local hospital laboratory, where urine samples were stored at −20 °C. Of each collected stool sample, an aliquot of approximately 1 g was mixed with 2 mL of 70% ethanol and stored at −20 °C. All samples were transported under frozen conditions to the Institute of Tropical Medicine, Antwerp, Belgium, and

subsequently transferred to the Leiden University Medical Center (LUMC), Leiden, The Netherlands, and stored at −20 °C until use.

Laboratory Analysis

Real-Time PCR

After DNA extraction, the *Schistosoma* genus-specific real-time PCR was executed as described previously, using a 200 µL sample volume [5,6,32]. *Schistosoma*-specific primers (Ssp48F and Ssp124R) and the double-labeled probe Ssp78T were used to amplify a 77-bp fragment of the internal transcribed spacer-2 (ITS-2) region. An internal control (Phocin Herpes Virus-1) was included for the detection of potential inhibition of amplification. A CFX real-time detection system (Bio-Rad Laboratories, USA) was used for amplification, detection and analysis. The PCR output consisted of a cycle-threshold (Ct)-value, representing the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence and thereby indicating the presence of parasite-specific DNA. Since its implementation, the LUMC-team scored 100% in sensitivity and specificity of their *Schistosoma* PCR at the annual international Helminths External Molecular Assessment Scheme (HEMQAS) provided by the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) [33]. Intensity of infection was classified arbitrarily as either negative (Ct = 50), low intensity (35 ≤ Ct < 50), medium intensity (30 ≤ Ct < 35), high intensity (25 ≤ Ct < 30), or very high intensity (Ct < 25), based on previous studies [5,34,35].

POC-CCA

The commercially available POC-CCA test (batch no. 50174; Rapid Medical Diagnostics, Pretoria, South Africa) was performed for the detection of CCA, according to the manufacturer's instructions. In brief, one drop of urine was added to the well of the cassette, followed by one drop of buffer (provided with the test kit). Results were read after 20 min. In case the control line did not develop, the test was considered invalid and the sample was retested. Each POC-CCA cassette was scored as negative, trace (weak line), or positive (1+, 2+, or 3+) by three independent readers, after which the average was taken as the final score. POC-CCA traces were considered negative for the analysis [36].

UCP-LF CAA

The UCP-LF CAA test was performed for the detection of CAA, as described previously [27,37,38]. All urine samples were tested via the UCAA10 format using 10 µL of urine, and subsequently, also with the most sensitive concentration format using 2 mL of urine (UCAA2000). In brief, each urine sample was mixed with an equal volume of 4% trichloroacetic acid, incubated and centrifuged. In the case of the UCAA2000 format, the clear supernatant was concentrated to 20 µL using a 4 mL centrifugal device (Amicon Ultra-4, Millipore, Merck Chemicals B.V., Amsterdam, The Netherlands). The resulting 20 µL concentrate was subsequently used in the assay. Samples with known CAA-levels were included as a reference standard to quantify CAA concentrations as well as to validate the cut-off of the assay. A CAA concentration below 0.1 pg/mL was considered negative [27].

Statistical Analysis

Participants with a complete dataset (i.e., all diagnostic tests performed) were included in the final analysis. Statistical analyses were performed using SPSS version 25 (IBM). Data were summarized using descriptive statistics. The agreement between the diagnostic methods was determined by Kappa ($\varkappa$) statistics. The nonparametric Spearman's rank correlation was applied to measure the relationship between PCR Ct-values, POC-CCA scores and CAA-levels. To compare the sensitivity and specificity of the different diagnostic methods, McNemar's χ^2 test was used. In the absence of a suitable reference standard, diagnostic accuracy was compared to a composite reference standard (CRS) assuming 100% specificity for PCR as well as for UCP-LF CAA, meaning that an individual was considered positive if PCR and/or UCP-LF CAA was positive.

Ethics Approval and Consent to Participate

Ethical permission for this study was obtained from the Ethical Committee of the University of Kinshasa, DRC, as well as the Institutional Review Board of the Institute of Tropical Medicine in Antwerp, Belgium (No. 650/09) and the Ethical Committee of the University of Antwerp, Belgium (No. 9/11/47). All participants provided written informed consent before the start of the study.

RESULTS

A complete set of urine and stool samples was available from 314 individuals (46% male, median age 18 years, range 1–80 years). Figure 1 presents an overview of the percentage of positive results of the different diagnostic methods. The highest number of positives was found with the UCP-LF CAA test; in 215 out of 314 (69%) individuals, CAA was detected in urine. The POC-CCA test was positive in 86 (27%) individuals, while 44 (14%) individuals showed a trace. DNA was detected in stool samples of 87 (28%) individuals, while in 10 (3%) individuals, DNA was detected in urine.

Intensity of Infection

The intensity of infection is shown in Table 1. Of the 87 individuals positive by PCR in stool, the majority of infections were of high intensity (58%). Most urine PCR positives were of low to moderate intensity (70%). With the POC-CCA, mainly low-intensity infections were found (56%). The majority of urine CAA positives were of very low to low intensity (61%). Of those individuals positive by UCP-LF CAA only, the median CAA-level was 1.1 pg/mL (range 0.1–298 pg/mL). In Figure 2, the intensity of infection per age group is demonstrated for each diagnostic method. With increasing age, the number of *Schistosoma* DNA positives first increased, peaking in the age group of 15–19 years, and subsequently decreased with increasing age. CCA was detectable in all age groups, but overall a lower prevalence was observed in the oldest age group. Overall, the number of CAA positives was similar in all age groups, except in the youngest

(≤5 years) where fewer positives were found. More high-intensity infections were observed in children aged 10–19 years compared to the other age groups.

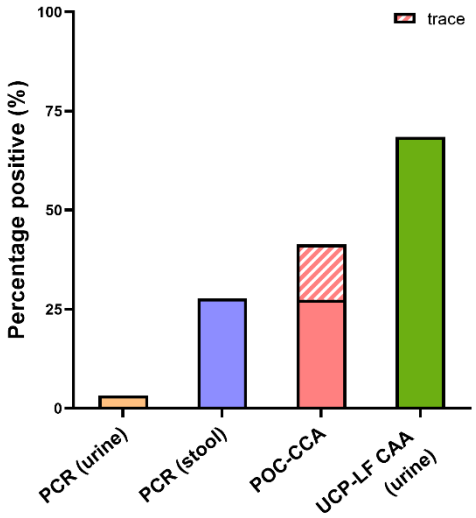

Figure 1. Percentage positive by polymerase chain reaction (PCR) in urine and stool, point-of-care circulating cathodic antigen (POC-CCA), and the up-converting lateral flow circulating anodic antigen (UCP-LF CAA) test in 314 individuals. Shaded area represents POC-CCA trace results.

Table 1. Intensity of infection based on polymerase chain reaction (PCR) in urine and stool, point-of-care circulating cathodic antigen (POC-CCA), and the up-converting particle, lateral flow circulating anodic antigen (UCP-LF CAA) urine test in 314 individuals.

Diagnostic Method	N (%)
PCR (urine)	
35 ≤ Ct < 50 (low)	2 (0.6%)
30 ≤ Ct < 35 (medium)	4 (1.3%)
25 ≤ Ct < 30 (high)	3 (1.0%)
Ct < 25 (very high)	1 (0.3%)
PCR (stool)	
35 ≤ Ct < 50 (low)	15 (4.8%)
30 ≤ Ct < 35 (medium)	4 (1.3%)
25 ≤ Ct < 30 (high)	18 (5.7%)
Ct < 25 (very high)	50 (15.9%)
POC-CCA	
Trace	44 (14.0%)
1+ (low)	48 (15.3%)
2+ (moderate)	28 (8.9%)
3+ (high)	10 (3.2%)
UCP-LF CAA (urine)	
0.1–1 pg/mL (very low)	64 (20.4%)
1–10 pg/mL (low)	66 (21.0%)
10–100 pg/mL (moderate)	58 (18.5%)
>100 pg/mL (high)	27 (8.6%)

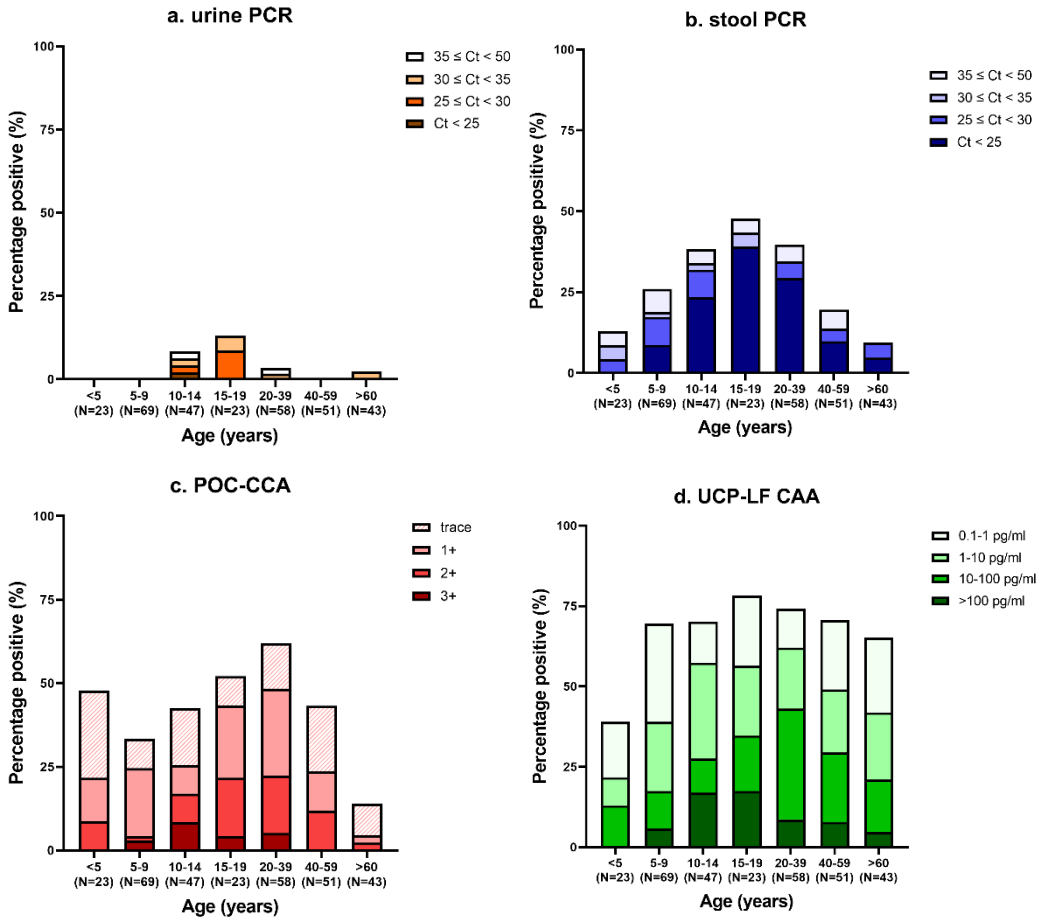

Figure 2. Intensity of infection per age group. Data based on (a) PCR in urine; (b) PCR in stool; (c) Point-of-care circulating cathodic antigen (POC-CCA); and (d) up-converting particle lateral flow circulating anodic antigen (UCP-LF CAA).

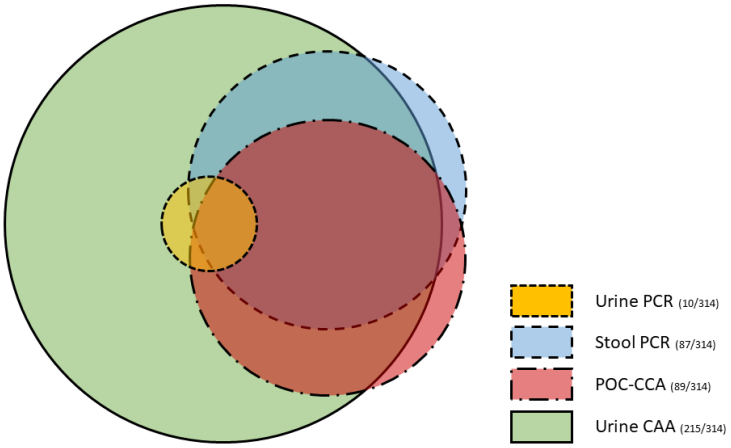

Figure 3. Proportional Venn diagram of polymerase chain reaction (PCR) in urine and stool compared to the point-of-care circulating cathodic antigen (POC-CCA) and the up-converting particle lateral flow circulating anodic antigen (UCP-LF CAA) urine test in 314 individuals.

Table 2. The level of agreement between polymerase chain reaction (PCR) in urine and stool, point-of-care circulating cathodic antigen (POC-CCA), and the up-converting particle, lateral flow circulating anodic antigen (UCP-LF CAA) urine test by Cohen's κ coefficient and McNemar's $\chi 2$ test in 314 individuals.

Diagnostic test	Reference test		K value	Interpretation [1]	*p* Value	McNemar's *p* Value
PCR (stool)	Positive	Negative				
Positive	8	79	0.114	Slight	<0.001	<0.001
Negative	2	225				
	POC-CCA					
PCR (stool)	Positive	Negative				
Positive	60	27	0.577	Moderate	<0.001	1
Negative	26	201				
	UCP-LF CAA					
PCR (stool)	Positive	Negative				
Positive	80	7	0.223	Fair	<0.001	<0.001
Negative	135	92				
	UCP-LF CAA					
POC-CCA	Positive	Negative				
Positive	79	7	0.220	Fair	<0.001	<0.001
Negative	136	92				
	PCR (urine)					
POC-CCA	Positive	Negative				
Positive	8	78	0.116	Slight	<0.001	<0.001
Negative	2	226				
	PCR (urine)					
UCP-LF CAA	Positive	Negative				
Positive	10	205	0.030	Slight	0.029	<0.001
Negative	0	99				

[1] Interpretation of k coefficient: 0, chance; 0.01 to 0.20, slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; 0.81 to 0.99, almost perfect.

The correlation between DNA-levels in stool and POC-CCA visual scores was strong (Spearman's rho −0.62, $p < 0.001$); see Figure 4a. A moderate but still significant correlation was observed between the DNA levels in stool and CAA-levels in urine, as well as between CAA-levels in urine and POC-CCA visual scores (Spearman's rho −0.55, $p < 0.001$ and 0.56, $p < 0.001$, respectively); see Figure 4.

Table 3. Sensitivity and specificity of polymerase chain reaction (PCR) in urine and stool, point-of-care circulating cathodic antigen (POC-CCA), and the up-converting particle circulating anodic antigen (UCP-LF CAA) urine test compared to a composite reference standard (CRS).

		CRS (PCR & UCP-LF CAA) [1]		Diagnostic Accuracy	
		Positive	Negative	Sensitivity	Specificity
PCR (urine)	Positive	10	0	4.5%	100% [2]
	Negative	212	92		
PCR (stool)	Positive	87	0	39.2%	100% [2]
	Negative	135	92		
POC-CCA	Positive	80	6	36.0%	93.5%
	Negative	142	86		
UCP-LF CAA	Positive	215	0	96.8%	100% [2]
	Negative	7	92		

[1] Composite reference standard (CRS) was based on PCR (urine/stool) and UCP-LF CAA: an individual was considered positive if at least one of these tests was positive. [2] Specificity is 100% by definition.

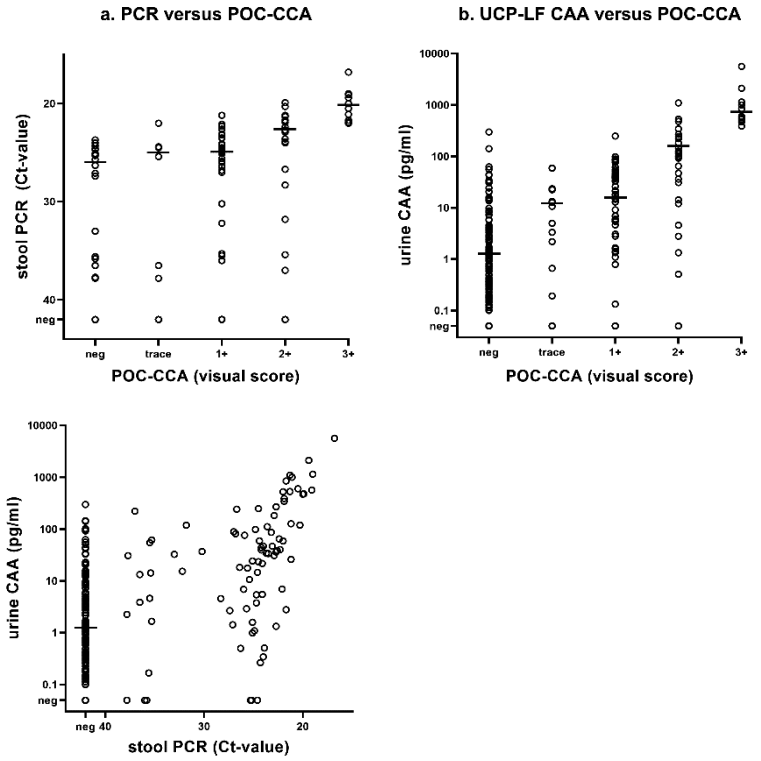

Figure 4. Correlation between the different tests: (a) PCR in stool versus POC-CCA, (b) UCP-LF CAA versus POC-CCA, (c) UCP-LF CAA versus PCR. Horizontal lines indicate the median Ct-value (a) or the median CAA concentration (b, c) of the positive tested samples.

DISCUSSION

There is a need for a sensitive, specific, rapid and easy to perform diagnostic test for the diagnosis of schistosomiasis. This study is the first to describe the presence of schistosomiasis based on a combination of PCR and circulating antigen detection in the absence of traditional microscopy. It was designed to evaluate different diagnostic tests on banked stool and urine samples in order to provide better insight into the performance of the different tests as well as to determine whether an accurate estimate of the presence of schistosomiasis can be made without traditional microscopy. While a range of studies have evaluated the comparison between microscopy and PCR, or microscopy and circulating antigens, no studies have compared the outcome of real-time PCR on both stool and urine with urine CCA and CAA levels in the same study population.

Our results showed that both the field-applicable POC-CCA as well as the PCR in stool are equally suitable for a first screening of the schistosomiasis prevalence in an endemic region. A fair to moderate agreement was found between the diagnostic methods. Intensity categories of each diagnostic method were either pragmatic (POC-CCA, UCP-LF CAA) or arbitrary (PCR) and therefore difficult to compare, but when looking at an individual level, a positive correlation was observed between the increasing intensity of the POC-CCA visual score and CAA-levels in urine, which corresponds to previous findings [39,40]. Also, a strong correlation was observed between POC-CCA visual scores and Ct values. The majority of cases that were positive by POC-CCA and PCR (in stool) also had detectable CAA-levels in urine, confirming the ability of both tests to detect active *Schistosoma* infections. Still, numerous additional cases, mainly of low intensity (i.e., <10 pg/mL), were detected by UCP-LF CAA, suggesting that the percentage of schistosomiasis positives is much higher than assumed by POC-CCA and PCR alone. Indeed, the UCP-LF CAA test has proven to be an ultra-sensitive test for the detection of active *Schistosoma* infections [40,41].

The high number of cases detected by the diagnostic methods applied in the current study indicates substantial schistosomiasis transmission levels in this study population. The majority of infections is assumed to be caused by *S. mansoni*, based on the higher frequency of DNA present in stool samples compared to urine samples. Although the PCR assay used in this study was not species specific, *Schistosoma* spp. DNA detected in stool samples most likely indicates an infection with *S. mansoni* [5]. This was confirmed by the results of the POC-CCA, which is known to detect mainly *S. mansoni* infections. In this study, very few individuals were urine PCR positive, pointing towards a possible *S. haematobium* infection [6,35]. While these were all confirmed as *Schistosoma* spp positive by the UCP-LF CAA test, 8 out of 10 were also positive by POC-CCA and PCR in stool. This suggests a possible co-infection of *S. haematobium* with *S. mansoni*, although these urine samples might also have been contaminated with stool and, therefore, could represent a *S. mansoni* infection only. Alternatively, ectopic egg elimination cannot be excluded, as *S. mansoni* eggs have been occasionally observed in urine as well [42,43,44]. In such cases, microscopy could have provided additional information concerning the *Schistosoma* species, provided that the infection intensity was sufficiently high.

Urine CAA results showed a similar age-related distribution of *Schistosoma* infection compared to circulating antigen results as well as egg counts from previous studies [45,46]. The prevalence of infection increased with age, but did not decrease in adults, while the intensity of infection decreased in individuals of 20 years and older. Although a high number of positives

was observed in school-aged children, still numerous cases were found in children <5 years of age as well as in adults, stressing the importance of improving treatment uptake in these age groups [47]. Furthermore, with increasing age, the presence of CCA and CAA indicates that worms are still present without a relationship with the number of eggs, as indicated by the decreasing number of *Schistosoma* egg DNA positives with increasing age.

While *Schistosoma* species could have been determined with microscopy, we believe it would not have added any extra value here since the detection of DNA in stool as well as CCA in urine (POC-CCA) both point towards the presence of *S. mansoni* infections. Furthermore, the diagnostic methods applied in this study have proven to be more sensitive compared to traditional microscopy, so it is likely that microscopy would have missed several cases due to its limited sensitivity, in particular in detecting low-intensity infections [14,48,49]. Moreover, microscopy is labor-intensive, and the costs are often higher than for example the POC-CCA [50]. Based on consumables only, the costs of real-time PCR and the UCP-LF CAA assay are roughly 10 times higher than POC-CCA. Therefore, in view of the recent recommendation from the WHO, the POC-CCA is considered to be a good alternative for microscopy. However, when more resources are available, real-time PCR and UCP-LF CAA could be considered to obtain a more accurate estimate of the presence of schistosomiasis.

CONCLUSION

A moderate to high percentage of *Schistosoma* infections was observed in this study population based on non-microscopy diagnostic methods. The results of this study indicate that the POC-CCA and PCR on stool are suitable screening tools for *S. mansoni* infections when microscopy is unavailable. However, both methods may still significantly underestimate the "true" number of *Schistosoma* infections since a large number of additional, mainly low positive, cases were found by the ultrasensitive and highly specific UCP-LF CAA test. In conclusion, even without microscopy, sufficient alternative diagnostic methods are available to accurately determine the presence as well as the intensity of schistosome infections in an endemic area.

Author Contributions:
Conceptualization, J.M., K.P., L.v.L. and P.L.; methodology, J.M., K.P., L.v.L. and P.L.; formal analysis, G.J.v.D., L.v.L., P.L.A.M.C., P.T.H. and R.v.G.; investigation, E.A.T.B. and R.v.G.; resources, K.P., J.M. and P.L.; data curation, R.v.G. and P.T.H.; writing—original draft preparation, P.T.H.; writing—review and editing, E.A.T.B., G.J.v.D., L.v.L., J.M., K.P., P.L.A.M.C., P.L., P.T.H. and R.v.G.; visualization, P.T.H.; supervision, K.P. and L.v.L.; project administration, L.v.L., P.T.H. and R.v.G.; funding acquisition, L.v.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data Availability Statement
Data is contained within the article.

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Informed Consent Statement
Informed consent was obtained from all subjects involved in the study. All participants provided written informed consent before the start of the study.

Institutional Review Board Statement
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Kinshasa, DRC, the Institutional Review Board of the Institute of Tropical Medicine in Antwerp, Belgium, as well as the Ethics Committee of the University of Antwerp, Belgium.

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Part III.

Reflections on circulating anodic antigen detection for context-specific diagnosis of schistosomiasis

8.

Summarizing discussion

Adapted and extended from:
Context-Specific Procedures for the Diagnosis of Human Schistosomiasis – A Mini Review

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The use of sensitive and specific diagnostic tests for schistosomiasis, to correctly identify those who are infected, is crucial to successfully reduce the burden of disease and to eventually move towards elimination of schistosomiasis. However, the diagnostic need differs between settings with each setting requiring its own distinctive approach. In addition to accurately diagnosing schistosomiasis, monitoring the efficacy of praziquantel (PZQ) treatment also requires highly accurate diagnostic methods. It is generally acknowledged that traditional microscopy lacks sensitivity in detecting low intensity infections, as is often the case after treatment. Since PZQ is known to target adult schistosome worms, accurately measuring the worm burden would be a more direct way to determine efficacy of treatment, compared to determining number of eggs (often used as a proxy for worm burden). In this thesis, we evaluated the performance of the UCP-LF CAA test in the context of endemic and non-endemic settings for diagnosing schistosomiasis and monitoring efficacy of PZQ treatment. The UCP-LF CAA test is a lateral flow (LF) test for quantitative detection – using luminescent high sensitivity up-converting reporter particles (UCP) – of circulating anodic antigen (CAA) regurgitated by live schistosome worms in the human circulation. As CAA is assumed to be excreted by all schistosomes, the UCP-LF CAA test is expected to be suitable for detection of infections with all *Schistosoma* species, including veterinary ones and hybrid infections (chapter 2). The presence of CAA, measured in urine and/or serum, reflects an active *Schistosoma* infection: CAA-levels decrease rapidly to low or undetectable levels after treatment thereby confirming the efficacy of PZQ (chapter 2, 3 & 6). In non-endemic settings (i.e. the absence of reinfection), undetectable CAA-levels indicate clearance of infection (chapter 2 and 3). This in contrast to endemic settings where – with continuous exposure and ongoing transmission – CAA-levels significantly decrease but often remain detectable (chapter 6). Similarly to egg microscopy, but with relevantly higher sensitivity, the UCP-LF CAA test not only indicates active infection but also allows better perception on the intensity (worm burden) of *Schistosoma* infections (chapter 7).

Using a composite reference standard in diagnostic evaluation studies

In all studies, the UCP-LF CAA test showed a significantly higher number of positive cases compared to other diagnostic methods, regardless of the setting. The most important indication for the UCP-LF CAA test being more accurate is the fact that CAA-levels significantly decrease after PZQ treatment, confirming that CAA is excreted by live worms and indicating the effect of treatment on active *Schistosoma* infections (chapter 2, 3 & 6). Still, it remains important to have an accurate estimation of the sensitivity and specificity of the UCP-LF CAA test. In order to obtain this, the UCP-LF CAA test was compared against a composite reference standard (CRS) in three different studies described in this thesis (chapter 3, 6 & 7). This is a commonly applied approach when there is no single suitable reference standard available [1,2] and has already been used in several schistosomiasis diagnostic evaluation studies [3-5]. It is an alternative to, for example, latent class analysis, since the conditions for this kind of statistical analyses are often difficult to meet in particular in studies evaluating diagnostic methods for schistosomiasis [6]. For example, there is conditional dependence among diagnostic tests, the sample size is often too small, and the number of different tests available for the analysis is insufficient.

In all studies (chapter 3, 6 & 7), the CRS consisted of a set of diagnostic tests that were assumed to have or approach a specificity of 100%. Depending on the type of diagnostic tests included in the CRS, an individual was classified as true positive based on the OR rule (i.e. individuals with at least one positive test were classified as disease positive and those with all negative tests were classified as disease negative, chapter 6 & 7) or the K rule (i.e. disease positive if at least K tests were positive, chapter 3). Even though the UCP-LF CAA test was being investigated, it was decided to include the test in the CRS based on the fact that in previous studies the detection of CAA has proven to be highly specific [3-5,7-11], likely due to the chemically unique structure of CAA which has not been observed in organisms other than schistosomes [12]. Nevertheless, it is important to keep in mind that this could potentially have led to an overestimation of the sensitivity of the UCP-LF CAA test. It was difficult to compare the performance of the UCP-LF CAA test between different studies as the reported accuracy of the test also depends on the disease prevalence which varied between the studies described in this thesis. However, despite these differences in prevalence and setting, the sensitivity of the UCP-LF CAA in the studies described in this thesis was consistently high (>90%), similar to previous findings [3,8,13,14].

Performance of circulating anodic antigen detection in non-endemic settings

Distinctive populations can be identified within non-endemic settings, primarily short-term travelers (including tourists and expatriates) and migrants (including refugees). In general, travelers have not been exposed previously and are therefore considered to be immune-naïve, whereas migrants, when originating from *Schistosoma* endemic areas, have often been exposed since childhood and are more likely to present with chronic infections. In exceptional cases, migrants originating from non-endemic schistosomiasis areas may have acquired an acute *Schistosoma* infection when passing through a schistosomiasis endemic area. In areas not endemic for schistosomiasis, diagnosis is usually focused on test-and-treat of the individual patient, i.e. early detection of infection and confirmation of cure following intervention. The general aim within clinical settings is complete eradication of infection in each individual patient as there is no risk of reinfection.

Travelers

Only 30-50% of schistosome infected travelers present with clinical symptoms [15,16], but if they develop acute schistosomiasis, the so-called Katayama syndrome, it is generally seen several weeks before eggs can be detected. In combination with the low worm burden that travelers often harbor, this makes diagnosis with conventional microscopy challenging [9,16-18]. Since seroconversion usually occurs within 4 to 8 weeks after exposure, detection of schistosome-specific antibodies is the most commonly used alternative diagnostic approach for diagnosing schistosomiasis in previously naïve individuals. However, a major disadvantage of antibody detection methods is that they cannot distinguish between current and past infection nor provide any information regarding the intensity of infection. Alternatively, detection of *Schistosoma* DNA in blood has shown to be highly sensitive and specific for early diagnosis of acute schistosomiasis, but also this PCR outcome remains positive for many months after treatment [19,20]. In chapter 2 the diagnostic value of CAA detection was evaluated against a panel of

antibody and DNA detection methods for early diagnosis of schistosomiasis and monitoring PZQ treatment efficacy in a cluster of exposed Belgian travelers with a confirmed hybrid infection between *S. mattheei* x *S. haematobium* infection [20]. The UCP-LF CAA test in serum was the most sensitive test to confirm active *Schistosoma* infection as well as to assess cure: CAA-levels were detectable 4 weeks after exposure and decreased to undetectable levels following PZQ treatment, as also observed in previous studies [9,10,21]. Even though in this study it appeared to be a hybrid infection, CAA was detected in serum of all infected individuals, confirming that these *Schistosoma* species also excrete CAA [22-24]. The UCP-LF CAA test is not only suitable for detecting active infection in (recently) exposed travelers, but interestingly it can also be used to demonstrate the absence of an active infection in travelers who for example show a positive serology, but have no additional indications of harboring viable worms.

Migrants

The increasing number of migrants, coming from or passing through *Schistosoma* endemic regions and arriving in Europe, stresses the importance of timely and effective screening for *Schistosoma* infections [25-27]. Detecting *Schistosoma*-specific antibodies remains the recommended and most used first-line test for screening migrants [28,29]. Despite being the most commonly used diagnostic method for imported infections, antibody detection procedures still have their limitations both in sensitivity and specificity for suspected infections. It is common practice in many settings to use the detection of eggs in urine or stool as a confirmation test to prove an active infection, despite the fact that it is generally acknowledged that microscopy lacks sensitivity [30]. A recent study in migrants showed that the point-of-care circulating cathodic antigen (POC-CCA) test – a commercially available and field-applicable rapid test particularly suitable for diagnosing intestinal schistosomiasis – can, in combination with serology, be an efficient screening tool for *S. mansoni* infections when used in a standardized manner [31-33]. Additionally, the detection of *Schistosoma* DNA by the *Schistosoma* genus-specific ITS2 real-time PCR in stool and urine sample has demonstrated to be of clinical value when monitoring schistosomiasis in migrants after their arrival in Europe [34]. In contrast to the real-time PCR in blood which – despite being highly sensitive and specific for diagnosing schistosomiasis – remains positive after treatment [19,20], there are strong indications that the clearance of *Schistosoma* DNA in stool or urine occurs within weeks to months following PZQ treatment [35]. This was confirmed in chapter 3 where a panel of diagnostics, including real-time PCR and circulating antigen (POC-CCA and UCP-LF CAA) diagnostics, were applied to samples from asymptomatic migrants. The majority of stool PCR-positive individuals became negative after treatment. However, the UCP-LF CAA test on serum seems more suitable for migrants originating from different schistosomiasis endemic regions as CAA is excreted by all *Schistosoma* species including hybrids (shown in chapter 2), is detectable even in low intensity infections [10,34] and simply because serum is the preferred sample type from a routine diagnostic procedure perspective. In chapter 3 the UCP-LF CAA test was further investigated and confirmed the previously observed high microscopy-based prevalence of *S. mansoni* infections in asymptomatic migrants. CAA results showed a good agreement with microscopy as well as with serology, real-time PCR and POC-CCA results. However, almost half of the CAA-positive cases could not be confirmed with microscopy nor real-time PCR, and even

though a slightly better overlap was observed with POC-CCA results, this still implied that a significant amount of *Schistosoma* infections had been missed. Also here a significant decline to very low or undetectable CAA-levels was observed after treatment, indicating that an active infection was present which was cured after treatment, similar to previous findings [9,34,36]. Compared to diagnostic methods currently used in routine diagnostic settings (in particular antibody detection and microscopy), the UCP-LF CAA test is more suitable for diagnosing *Schistosoma* infections as well as to determine individual cure shortly after treatment. Especially in the migrant population, a short follow-up period is preferable. Newly arrived migrants tend to be very mobile during the first months to years after their arrival, so this population would benefit most from a diagnostic test that is able to determine efficacy of treatment (i.e. clearance of infection) within weeks, allowing additional treatment if needed.

Performance of circulating anodic antigen detection in endemic settings

In schistosomiasis endemic regions more attention is generally given to diagnosis for public health purposes than to the identification of individual cases. In such settings, determination of an infected individual is often based on clinical symptoms only, sometimes combined with the detection of eggs in stool or urine. From a public health perspective, the absence or a significant reduction in the number of *Schistosoma* eggs is crucial as eggs are the cause of morbidity and ongoing transmission. The cornerstone of schistosomiasis control in endemic settings mainly relies on large-scale administration of PZQ at regular intervals to at-risk populations aiming to decrease and keep the overall worm burden low within the population [37-40]. The efficacy of mass drug administration (MDA) with PZQ is commonly evaluated by detection of parasite eggs in urine or stool [41-43]. Due to the reduced sensitivity of microscopy, in particular in case of low infection intensity, previously reported efficacy of MDA has been overestimated [44-47]. Repeating treatment at short intervals in regions with ongoing transmission could potentially increase the efficacy of PZQ, as non-susceptible schistosomula [48] – who within a few weeks will have matured into drug susceptible worms – are targeted as well. This approach of repeating PZQ treatment was investigated in a group of school-aged children with a confirmed *S. mansoni* infection. To this end, an open-label, randomized controlled clinical trial called 'Repeated doses of Praziquantel in Schistosomiasis Treatment' (RePST) was conducted in Côte d'Ivoire (chapter 4). Over the course of a 10-week period, children received either a single PZQ treatment or four repeated PZQ treatments at 2-week intervals. A wide range of diagnostic methods was applied in this study, classified as egg-based diagnostics (i.e. Kato-Katz and real-time PCR) and worm-based diagnostics (i.e. circulating antigen detection methods: POC-CCA and UCP-LF CAA). Chapter 5 subsequently describes the initial results from the diagnostic methods used in the field, i.e. Kato-Katz and POC-CCA. After a single treatment, the cure rate as well as the intensity reduction rate based on Kato-Katz were found to be similar compared to previous findings [41]. However, the majority of children remained positive by POC-CCA even after repeated treatment, resulting in very poor cure rates. Therefore, more accurate methods (i.e. real-time PCR and UCP-LF CAA) were applied to investigate to what extent POC-CCA positives reflected true infections (chapter 6). Urine CAA-levels correlated well with egg counts in feces, in particular in increasing infection intensity, confirming previous observations [49-55]. Nonetheless, discrepancies between the different diagnostic methods occurred, a phenomenon

that is inherent to diagnostic evaluation studies. Of particular interest were egg-positive cases with no detectable CAA in urine or serum. This disagreement of the absence of CAA while eggs have been observed could be due to passing of remaining eggs while the worms already died or variation in biological excretion of CAA [56,57], but administrative errors such as sample processing and or labeling cannot be excluded either. In contrast, the opposite – CAA-positive but no eggs in stool or urine – was observed more often. Since CAA is a worm-derived antigen, its presence is indicative of an active *Schistosoma* infection. Nevertheless, it is important to note that different schistosome life stages are being measured, namely the detection of eggs in stool or urine by microscopy versus the detection of worm-derived CAA in serum or urine by the UCP-LF CAA test. The absence of eggs while CAA is being detected can largely be attributed to the low sensitivity of microscopy, but could for example also be due to individuals harboring living (single sex) worms with only sporadically excreting eggs or with no detectable eggs at all [47,58-60] or a reduced fecundity due to PZQ treatment [61]. Overall, in chapter 6 we showed that PZQ efficacy measurements vary based on the diagnostic method that is used: while the cure rate determined by egg-based diagnostics (Kato-Katz, real-time PCR) significantly increased after repeated treatment, the cure rate determined by worm-based diagnostics (POC-CCA, UCP-LF CAA) remained poor over time. Even though all four diagnostic methods demonstrated a significant reduction in infection intensity already after a single treatment, the circulating worm-derived antigen diagnostics indicated the presence of active *Schistosoma* infections despite multiple treatments. CAA results confirmed the number of infected children to be abundant, even after repeated treatment, albeit with relatively low CAA-levels. Another recent study investigating the dynamics of parasite clearance and re-infection by Kato-Katz and POC-CCA also concluded that timing of post-treatment sampling is important as well as the diagnostic test used to determine cure rate and re-infection [62,63]. To better understand and optimize treatment strategies, highly sensitive methods such as real-time PCR and UCP-LF CAA should be used in conjunction to provide adequate insight into the host-parasite interaction and post-treatment dynamics of schistosome circulating antigens.

In the final study, a panel of non-microscopy diagnostics was applied to a set of banked samples from a schistosomiasis endemic area in the Democratic Republic of the Congo in order to determine whether an accurate estimate of the presence of schistosomiasis could be made when traditional microscopy is unavailable (chapter 7). Both the POC-CCA test as well as real-time PCR on stool showed to be equally suitable for a first screening of the presence of *Schistosoma* infections, whereas the UCP-LF CAA test detected many additional cases, mainly of low intensity. These results confirm that – even in the absence of microscopy – sufficient alternative diagnostic methods are available to accurately determine the presence as well as the intensity of schistosome infections in an endemic area. The POC-CCA findings, confirmed by real-time PCR, consolidate the WHO recommendation to use the POC-CCA as an alternative for microscopy in African intestinal schistosomiasis [37,64]. When more resources are available, the use of real-time PCR or the UCP-LF CAA test – provided that the UCP-LF CAA test becomes widely available – could be considered to obtain a more accurate estimation of the presence of schistosomiasis.

Reflections on the use of circulating anodic antigen detection in context-specific settings

A comparison of CAA-levels between the studies described in this thesis

The work presented in this thesis confirmed that the UCP-LF CAA test is suitable for application in both endemic and non-endemic settings. The test can be applied on non-invasive urine samples (often preferred in endemic settings) and on serum samples (more suitable for non-endemic settings). The sensitivity of the test is dependent on the test format used: by applying larger sample volumes (i.e. concentration of a pre-treated sample) a lower limit of detection can be reached (i.e. lower concentrations of CAA can be detected) [11]. Which test format is most suitable depends on the setting (low/moderate/high endemicity), the type and volume of sample available for analysis, but also on the availability of the required equipment (i.e. centrifuge) to execute the UCP-LF CAA test.

Figure 1 combines the CAA-levels as described in the different chapters of this thesis, with relevant additional details included in Table 1. In the studies where the UCP-LF CAA test was applied on both urine and serum samples (chapter 2 and 3), CAA-levels in serum tended to be higher than in urine. An observation which has also been made in previous studies [8,10,56,65-69]. CAA-levels in migrants resembled CAA-levels in individuals from endemic settings (chapter 3, 6 and 7), most likely due to repeated exposure over a longer time period in migrants. Different CAA-levels were observed in endemic settings (chapter 6 and 7), most likely depending on the level of endemicity but also on the population being measured: school-aged children in Côte d'Ivoire (chapter 6) compared to individuals of all ages in the Democratic Republic of the Congo (chapter 7). Generally, school-aged children tend to have a higher infection intensity compared to adults [70]. CAA-levels in travelers appear to be lower than those in migrants or individuals from endemic areas [9,17,21]. However, in a group of Belgian travelers relatively high CAA-levels were observed (chapter 2), suggestive of a high worm burden. This in contrast to a recent prospective study in travelers where very few individuals were positive by UCP-LF CAA while antibodies were detected in 21% of individuals [17], indicating that these travelers had a low worm burden or did not even establish an active infection.

Recommendations for using circulating antigen diagnostics in context-specific settings

In Table 2 an overview is given of diagnostic requirements and recommendations for context-specific settings. Generally, the focus in endemic settings is on ease-of-use and field applicability and costs are often crucial, whereas in non-endemic settings more advanced diagnostic methods are applied and costs usually have low priority. Early 2022, the routine diagnostics microbiology laboratory of the LUMC implemented the UCP-LF CAA test [71], making the test available for individual case detection in order to only treat those who really need it. In endemic settings, the UCP-LF CAA test could also be an ideal candidate for field use and integration into national programs, provided that the test becomes available on a large scale. The test meets the need of a highly sensitive and specific diagnostic tool – applicable to all *Schistosoma* species – for precisely mapping schistosomiasis prevalence and infection intensity at high spatial resolution to guide treatment and identify transmission hotspots.

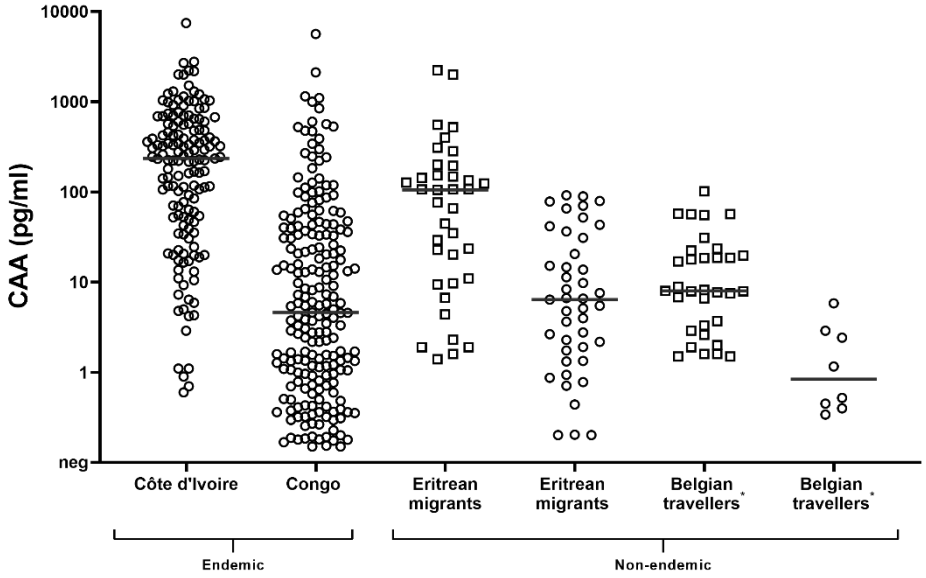

Figure 1. Overview of CAA-levels as observed in the different chapters of this thesis. Côte d'Ivoire (**chapter 6**) and Congo (**chapter 7**) are both *Schistosoma* endemic areas while the Eritrean migrants (**chapter 3**) and Belgian travelers (**chapter 2**) are representatives of non-endemic settings. CAA was measured in urine (circles) and / or serum (squares) samples. Median CAA-level (of the CAA-positives) is indicated with a horizontal red line. For more specific details see Table 1 below.
* highest observed CAA-level before PZQ treatment was used.

Table 1. Overview of additional details from the studies described in this thesis

Study (chapter)	Côte d'Ivoire (chapter 6)	Congo (chapter 7)	Eritrean migrants (chapter 3)	Eritrean migrants (chapter 3)	Belgian travellers (chapter 2)	Belgian travellers (chapter 2)
Age range study population	5-17 years	1-80 years	18-63 years	18-63 years	5-49 years	5-49 years
Species	*S. mansoni*	*S. mansoni*	*S. mansoni*	*S. mansoni*	*S. mattheei* x *S. haematobium*	S. *mattheei* x S. *haematobium*
UCP-LF CAA test format	UCAA*bT*417 wet	UCAA2000 wet	SCAA500 wet	UCAA*bT*3333 dry	SCAA500 wet	UCAA2000 dry
Sample type	urine	urine	serum	urine	serum	urine
Limit of detection	0,6 pg/ml	0,1 pg/ml	1 pg/ml	0,2 pg/ml	1 pg/ml	0,3 pg/ml
N tested	147	314	92	92	34	34
N positive	147	205	43	37	33	8
Median CAA-level of the positives	221 pg/ml	5 pg/ml	107 pg/ml	7 pg/ml	8 pg/ml	0.9 pg/ml

Table 2. Overview of recommended diagnostic methods for each context-specific setting.

	Essential characteristics				Proposed diagnostic method(s)[1]	
	Accuracy	Sample type	Ease of use	Cost of test	First choice	Optional / Alternative
ENDEMIC SETTINGS						
Control programs / Monitoring & Evaluation	Moderate	Urine, Finger prick blood	Field applicable, rapid	High priority	POC-CCA	CAA-RDT[2] Hematuria dipsticks[3]
Elimination as a public health problem	Moderate	Urine	Field applicable, rapid	High priority	POC-CCA RPA[2]	
	High	Urine	Minimal labor (central laboratory facility)	Low to moderate priority	UCP-LF CAA	Real-time PCR[4]
Interruption of transmission	High	Urine, Serum	Minimal labor (central laboratory facility)	Low to moderate priority	UCP-LF CAA	Real-time PCR[4] Antibody detection[5]
NON-ENDEMIC SETTINGS						
Travelers	High	Serum	Suitable for routine diagnosis	Low priority	UCP-LF CAA Antibody detection[5] Real-time PCR[4]	
Migrants	High	Urine, Serum	Suitable for routine diagnosis	Low priority	UCP-LF CAA Real-time PCR[4]	POC-CCA Antibody detection[5]
RESEARCH-SPECIFIC SETTINGS						
Controlled Human Infection Model	High	Urine, Serum	Highly standardized	Low priority	UCP-LF CAA Antibody detection[5,7]	
Clinical studies[6]	Moderate	Urine, Finger prick blood	Field applicable	Moderate priority	POC-CCA	CAA-RDT[2]
	High	Urine	Minimal labor (central laboratory facility)	Moderate priority	UCP-LF CAA	Real-time PCR[4]

1. Antibody detection (all *Schistosoma* spp.), PCR (all *Schistosoma* spp.), POC-CCA (*S. mansoni*), RPA (*S. haematobium*), UCP-LF CAA (all *Schistosoma* spp), CAA-RDT (all *Schistosoma* spp), Hematuria dipsticks (morbidity marker for *S. haematobium*).
2. Needs further validation.
3. Hematuria dipsticks only provide information with regard to morbidity most likely related to *S. haematobium* infection.
4. Depending on the DNA target and test format used, in addition might need collection of stool and/or urine samples.
5. Depending on the antigen target, antibody type and test format used.
6. Depending on the endemic situation, availability of resources and research question.
7. Assuming infection of schistosomiasis naïve individuals.

The high focality of schistosomiasis transmission and its dependence on a complex interplay between socio-economic, sociological (behavior of people, social situation), geographical (distance to water bodies/transmission sites) and ecological (vegetation, rainfall, occurrence of intermediate snail host) factors often result in a large variation of prevalence and intensity of infection within an area. The high accuracy and quantitative outcome of the UCP-LF CAA test, in combination with its applicability on easy and non-invasively obtained urine samples, make it amendable to pooled sample testing strategies through which information from whole communities can be obtained in a presumably more cost-effective way [72]. Compared to exhaustive individual sampling and testing approaches, appropriate pooling strategies can significantly reduce logistical and laboratory costs, with minimal loss of sensitivity and specificity [72-74]. Preliminary exercises have shown that average CAA-levels from pooled urines in defined low and high prevalence and infection intensity easily can be detected with the UCP-LF CAA test [72].

Control programs (endemic setting)

Schistosomiasis control in endemic countries relies mainly on transmission intervention measures combined with large-scale administration of PZQ without prior diagnosis, which has been successful in reducing infection intensity, and hence morbidity [38-40,75]. Programs are based on pilot surveys often performed on a limited number of school-aged children. For monitoring and evaluation of these programs and to determine whether MDA schemes should be adapted or even stopped, more sensitive non-microscopy diagnostic procedures are needed [76]. An example is the POC-CCA urine strip test, recommended as an alternative tool to Kato-Katz for mapping prevalence of African intestinal schistosomiasis as well as for surveillance purposes [37,64,77,78]. As there is no direct rapid diagnostic test for diagnosing *S. haematobium* infections commercially available, an optional method for obtaining an indication of urogenital schistosomiasis prevalence would be hematuria dipsticks that test for *S. haematobium* related microhematuria, since this strongly correlates with urogenital schistosomiasis [79]. However, although hematuria dipsticks are not expensive and relatively easy to use, they only provide information on morbidity and do not provide a confirmed schistosome-specific diagnosis of infection. A visually scored finger prick blood-based CAA rapid diagnostic test (currently under development, see below and [80-82]) could potentially be a suitable candidate for easy, quick and more accurate screening of the presence of infections with all *Schistosoma* spp.

Elimination of schistosomiasis as a public health problem (endemic setting)

In areas where morbidity has been significantly reduced, the next aim is elimination of schistosomiasis as a public health problem. This has been defined by the WHO as <1% of school-aged children with schistosomiasis being categorized as heavily infected [83]. For intestinal schistosomiasis, this means >400 eggs per gram of feces and for *S. haematobium* >50 eggs per 10ml of urine [84], detected by Kato-Katz or urine filtration, respectively. However, a recent viewpoint from Wiegand et al (2022) suggests there is not enough evidence supporting this definition and highlights the need for more accurate measurements to develop an evidence-based framework focusing on the use of overall prevalence of infection rather than the prevalence of heavy-intensity infections [85]. As the POC-CCA test has been shown to be a more sensitive and cost-effective alternative for determining *S. mansoni* prevalence, attempts

have been made to estimate equivalent measures of prevalence between POC-CCA and Kato-Katz (11,78,86-88). In addition, the diagnostic position of the potentially user-friendly recombinase polymerase amplification (RPA) assays, in particular as an alternative for the POC-CCA test in case of *S. haematobium* infections, should be further explored [89]. A visually scored finger prick blood-based CAA rapid diagnostic test (currently under development, see below and [80-82]) would also be a good alternative for accurately screening prevalence and intensity of infection with all *Schistosoma* spp.

Interruption of transmission (endemic setting)

Accurate diagnosis of schistosomiasis is also crucial for determining interruption of transmission and eventual elimination, especially in regions where extensive control measures have reduced the prevalence and intensity of infection to very low levels [3,8,68,90-92]. This is clearly recognized by the WHO and international stakeholders in the NTD Roadmap 2030 where they highlight the need for field-deployable, intelligent diagnostics and sampling strategies to evaluate pre-and post-intervention prevalence, especially for low endemic and near elimination areas. The UCP-LF CAA test has demonstrated to be suitable for determining the presence of *Schistosoma* infections in low-endemic settings [3,90,92]. Recently, a strategy for the sustained, local interruption of transmission of schistosomiasis was presented in a viewpoint paper stressing the need for highly sensitive diagnostics (e.g. the UCP-LF CAA test) and intelligent testing procedures such as pooled sampling [74]. Additionally, identifying, treating, and following-up positive cases, including those with very light intensity infections, has the added advantage of avoiding reappearance of infection that is sometimes seen in such settings due to limited cure rates and the fact that immature schistosomes are refractory to praziquantel [93]. To what extent DNA detection methods might be suitable for defining interruption of transmission, needs more research [94-96]. Eventually, in settings where transmission appears to have been interrupted, detection of specific antibodies may also play an important role, for example in assessing exposure in young children [47,97-102].

Research

The UCP-LF CAA test has been investigated, evaluated and applied in numerous studies in Africa, Asia and South America, as well as in projects in Europe and the USA, including the studies described in this thesis. Even though implementation of the UCP-LF CAA test requires a basic equipped laboratory, the current format of the test has proven to be well-developed and robust enough to be implemented in low-resource settings. This has already been realized in two clinical trials in Madagascar (*S. mansoni*) and Gabon (*S. haematobium*) within the freeBILy project, where the UCP-LF CAA test, together with the POC-CCA, is being evaluated for diagnosing and monitoring treatment of *Schistosoma* infections in the still often neglected group of pregnant women and their new born children [103-105]. Moreover, the UCP-LF CAA test has been implemented in Tanzania for HIV-*Schistosoma* coinfection and PZQ treatment studies [106-116], and more recently also in Texas (USA) and Uganda for accurate assessment of vaccine efficacy in upcoming *S. mansoni* vaccine trials [117,118].

The UCP-LF CAA test has also great potential for use in controlled human schistosome infection trials. The UCP-LF CAA test appeared to be highly suitable for monitoring cure, as demonstrated in a recent experimental human *S. mansoni* infection model, where healthy volunteers were intentionally infected with male-only or female-only parasites [10,119]. This model provided insight into the development of (acute) schistosomiasis in terms of symptoms, the related immune responses, and the performance of diagnostic tests over time. Following experimental infection, all previously schistosomiasis-naïve volunteers showed detectable antibodies against adult worm gut antigen within 4 to 6 weeks, while the UCP-LF CAA test was most suitable to determine presence as well as cure of infection after treatment. In general, post-exposure CAA-levels appeared to be relatively low (with serum concentrations ranging between 1-10 pg/ml), but after treatment decreased to below the detection limit of 1 pg/ml.

While the UCP-LF CAA test has demonstrated excellent performance, with the most sensitive concentration format in serum showing a lower limit of detection corresponding to a single worm pair in a non-human primates model [11,120], it currently does not meet the current target product profile (TPP) requirements [121]. In particular in terms of the ease-of-use and throughput, which are currently not met due to the sample pre-treatment and concentration step, improvement is needed. Furthermore, a dedicated strip reader is needed to visualize and interpret the outcome of the UCP-LF CAA test. Especially when moving to field settings, requirements for such a reader include the ease-of-use, affordability and suitability for remote settings. Efforts to make CAA detection generally available are ongoing, with a recent initiative focusing on the development of a more easy-to-use, accurate, affordable and visually scored CAA-RDT [80-82]. This CAA-RDT will be able to detect all *Schistosoma* spp in a single finger prick blood sample without the need for sample preparation or a reader for detection in order to support national schistosomiasis control and elimination programs.

Concluding remarks

This thesis has provided further evidence on the suitability of CAA detection for diagnosing *Schistosoma* infections and monitoring treatment efficacy by evaluating the UCP-LF CAA test in the context of various endemic and non-endemic settings. CAA seems to be the only diagnostic marker that is accurately detectable from the early infection stages onwards as well as cleared soon after treatment. Compared to a range of other diagnostics, the UCP-LF CAA test was confirmed to be highly sensitive and specific. Although CAA detection seems the most favorable choice overall, alternative procedures such as antibody and DNA detection methods will remain crucial for specific purposes depending on the setting. As there is no 'one size fits all', diagnostic tests for schistosomiasis need to be carefully selected based on the data they provide in order to respond adequately to a specific situation. This is the first and foremost important step after which further choices can be guided by for example practicability, test availability and costs.

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Appendices.

List of abbreviations
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List of abbreviations

CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CRS	Composite reference standard
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoassay
LAMP	Loop-mediated isothermal amplification
LF	Lateral flow
LUMC	Leiden University Medical Center, The Netherlands
MDA	Mass drug administration
NAATs	Nucleic acid amplification tests
NTD	Neglected tropical disease
PCR	Polymerase chain reaction
pg/ml	picogram per milliliter
POC-CCA	Point-of-care circulating cathodic antigen urine test
PZQ	Praziquantel
RDT	Rapid diagnostic test
RePST	Repeated doses of praziquantel in schistosomiasis treatment
RPA	Recombinase polymerase amplification
TPP	Target product profile
UCP	Up-converting reporter particles
WHO	World Health Organization

Nederlandse samenvatting

Schistosomiasis is een ziekte die wordt veroorzaakt door parasitaire wormen, genaamd *Schistosoma*. Wereldwijd zijn er meer dan 250 miljoen mensen geïnfecteerd, van wie de meerderheid in Afrikaanse landen ten zuiden van de Sahara woont. De ziekte kan worden opgelopen door contact met zoet water in tropische gebieden waarin zich geïnfecteerde zoetwaterslakken – de tussengastheer voor de parasiet – bevinden. Infectie vindt plaats wanneer larven (cercariën), afkomstig van de slak, de intacte huid van een mens penetreren. Deze larven ontwikkelen zich vervolgens in de lever tot volwassen wormen en migreren naar de bloedvaten van verschillende organen, afhankelijk van de *Schistosoma* soort. Vrouwelijke wormen leggen eieren die een immuunreactie en schade aan organen kunnen veroorzaken en kunnen worden uitgescheiden via de urine of feces. Wanneer deze eieren in het water terechtkomen, komen er larven (miracidia) vrij die op hun beurt weer een slak kunnen infecteren, waarmee de cirkel vervolgens rond is.

Schistosomiasis wordt gekarakteriseerd door een focale distributie, dit houdt in dat de prevalentie en intensiteit van infectie aanzienlijk kan variëren binnen een klein gebied. Dit wordt beïnvloed door onder andere de aanwezigheid van de tussengastheer (slak), het menselijke watercontact en leefomstandigheden, in het bijzonder goede sanitaire voorzieningen. Als je voor het eerst geïnfecteerd raakt, veelvoorkomend bij reizigers die nog niet eerder zijn blootgesteld aan de parasiet, kan de ziekte zich presenteren met acute symptomen. Zonder behandeling ontwikkelt de infectie zich tot een chronisch stadium waarbij de gevolgen afhankelijk zijn van onder andere de *Schistosoma* soort, de duur en intensiteit van infectie en gastheer-gerelateerde factoren. Chronische infecties komen veel voor in endemische gebieden en de gevolgen hiervan op populatieniveau zijn mede afhankelijk van de prevalentie. Er zijn verschillende diagnostische methoden beschikbaar om een *Schistosoma* infectie vast te stellen: het detecteren van eieren in urine of feces (microscopie), de detectie van antilichamen, moleculaire methoden (waaronder PCR) en de detectie van antigenen.

Om de ziektelast van schistosomiasis te verminderen is het van essentieel belang dat diagnostische methoden heel gevoelig en specifiek zijn, om bij iedereen die geïnfecteerd is de parasiet aan te kunnen tonen. Naast het correct diagnosticeren van schistosomiasis is ook voor het bepalen van de effectiviteit van behandeling met praziquantel (PZQ) accurate diagnostiek nodig. Het is algemeen bekend dat microscopie niet gevoelig genoeg is, met name in het detecteren van overblijvende infecties met een lage intensiteit zoals vaak wordt waargenomen na behandeling. Omdat PZQ voornamelijk effect heeft op volwassen wormen en de ei productie soms slechts tijdelijk onderdrukt, zou directe aantoning van de aanwezigheid van wormen een betere manier zijn om het effect van behandeling vast te stellen in vergelijking tot een indirecte bepaling d.m.v. van de hoeveelheid gedetecteerde eieren (welke vaak gebruikt wordt als een indicatie voor de wormlast).

Veel onderzoek is gedaan naar twee *Schistosoma* darm-gerelateerde glycoconjugaten, genaamd circulerend kathodisch antigeen (CCA) en circulerend anodisch antigeen (CAA). De aanwezigheid van deze *Schistosoma* specifieke antigenen geeft aan dat er een actieve infectie is

aangezien alleen levende wormen antigenen afscheiden die detecteerbaar zijn in de bloedcirculatie van de gastheer. Vanuit de bloedcirculatie worden deze antigenen uiteindelijk uitgescheiden in de urine, wat vanuit een diagnostisch perspectief interessante opties biedt. Met name het detecteren van CAA door middel van de UCP-LF CAA test heeft grote potentie voor het accuraat bepalen van de aanwezigheid alsook de intensiteit van *Schistosoma* infecties en het effect van behandeling met PZQ te volgen. De UCP-LF CAA test is een laterale flow (LF) test die gebruik maakt van een specifiek infrarood geïnduceerd fluorescent label UCP ('up-converting reporter particles'). De test kan op een kwantitatieve manier CAA detecteren in bloed maar ook in urine van de gastheer. Dit proefschrift onderzoekt de toepassing van de UCP-LF CAA test in endemische en niet-endemische gebieden voor de diagnose van schistosomiasis en het monitoren van de effectiviteit van PZQ. **Hoofdstuk 1** bevat een algemene inleiding over schistosomiasis, waarbij de focus vooral ligt op de diagnostiek van schistosomiasis. Een compact overzicht van de meest gebruikelijke methoden voor het diagnosticeren van schistosomiasis wordt gegeven in dit hoofdstuk.

Het eerste deel van het experimentele werk is gericht op de toepassing en evaluatie van de UCP-LF CAA test in gebieden die niet-endemisch zijn voor schistosomiasis, met andere woorden de *Schistosoma* parasiet komt hier niet voor waardoor er dus geen risico op her-infectie is. In **hoofdstuk 2** wordt de diagnostische waarde van de UCP-LF CAA test onderzocht voor een vroege diagnose van acute schistosomiasis en het vervolg van de behandeling ervan in een groep van Belgische reizigers die slechts enkele weken daarvoor een infectie hadden opgelopen. De UCP-LF CAA test detecteerde alle infecties en bevestigde dat ook bij hybride infecties – in dit geval een hybride infectie tussen *S. mattheei* en *S. haematobium* – CAA detecteerbaar is. **Hoofdstuk 3** beschrijft de toepassing van de UCP-LF CAA als screeningstest in een groep van Eritrese migranten zonder schistosomiasis gerelateerde symptomen. Ook hier bleek de UCP-LF CAA test het meest gevoelig voor het detecteren van actieve *Schistosoma* infecties ten opzichte van andere diagnostische methoden. In zowel **hoofdstuk 2** als **3** werd bevestigd dat de aanwezigheid van CAA in urine en of bloed een actieve infectie reflecteert, aangezien de CAA concentraties significant daalden en afnamen tot nul na behandeling, wat tegelijkertijd ook de effectiviteit van PZQ bevestigt in een populatie waar geen kans is op een nieuwe blootstelling.

Het tweede deel van het experimentele werk omvat twee studies waarbij de UCP-LF CAA test is toegepast in schistosomiasis endemische gebieden waar er, in tegenstelling tot niet-endemische gebieden, sprake is van continue blootstelling en transmissie van schistosomiasis. **Hoofdstuk 4** beschrijft het studie protocol van een klinische studie – genaamd 'Repeated doses of PZQ in Schistosomiasis Treatment' (RePST) – opgezet om het effect van herhaaldelijke behandeling met PZQ van *S. mansoni* infecties te onderzoeken door middel van verschillende diagnostische methoden. De uitkomsten gebaseerd op de diagnostische methoden uitgevoerd in het veld in Ivoorkust, Kato-Katz voor de detectie van eieren in feces en de commercieel beschikbare point-of-care CCA urine test voor het aantonen van CCA in urine, zijn beschreven in **hoofdstuk 5**. Op basis van Kato-Katz daalde zowel de prevalentie als de intensiteit van infectie aanzienlijk na herhaaldelijke behandeling met PZQ. Dit, terwijl POC-CCA resultaten lieten zien dat er nog steeds wormen aanwezig zijn ondanks herhaaldelijk behandelen. Dit suggereert dat PZQ mogelijk minder effectief is in hoog endemische gebieden dan altijd werd aangenomen. In

hoofdstuk 6 is dit verder onderzocht door het toepassen van meer gevoelige diagnostische methoden, namelijk PCR en de UCP-LF CAA test. Hieruit bleek dat het effect van (herhaaldelijke) behandeling wordt overschat wanneer ei-gerelateerde diagnostische methoden worden gebruikt (Kato-Katz en PCR op feces voor de detectie van – hoogstwaarschijnlijk ei-gerelateerd – *Schistosoma* DNA). Worm-gerelateerde diagnostische methoden (POC-CCA en UCP-LF CAA) lieten namelijk zien dat actieve infecties nog steeds aanwezig zijn ondanks herhaaldelijke behandeling, alhoewel in de meeste gevallen de intensiteit van infectie (op basis van CAA concentratie) wel aanzienlijk daalde. De resultaten van de RePST studie benadrukken het belang van het gebruik van accurate diagnostische methoden voor een juiste bepaling van de aanwezigheid van schistosomiasis en de behandeleffectiviteit. In **hoofdstuk 7** wordt een panel van verschillende niet-microscopische diagnostische methoden uitgevoerd op een set van opgeslagen monsters, oorspronkelijk afkomstig uit de Democratische Republiek Congo, om te onderzoeken of een accurate schatting van de aanwezigheid van schistosomiasis gemaakt kan worden wanneer microscopie niet beschikbaar is. In vergelijking met microscopie detecteerde de UCP-LF CAA test meer actieve infecties en gaf ook een beter inzicht van de intensiteit (wormlast) van *Schistosoma* infecties.

Een samenvattende discussie in **hoofdstuk 8** bespreekt de geschiktheid van de UCP-LF CAA test in de context van verschillende endemische en niet-endemische gebieden. CAA lijkt de enige geschikte diagnostische marker te zijn die zowel nauwkeurig gemeten kan worden vanaf de vroege infectiestadia en ook snel verdwijnt na behandeling. Vergeleken met andere diagnostische methoden is de UCP-LF CAA test het meest gevoelig. Over het algemeen lijkt de detectie van CAA de beste keuze, maar alternatieve methoden zoals de detectie van antilichamen en DNA detectie methoden kunnen een belangrijke rol spelen voor specifieke doeleinden afhankelijk van de setting, bijvoorbeeld in het geval van reizigers of post-eliminatie gebieden. Aangezien er geen 'one size fits all' bestaat, moeten diagnostische methoden zorgvuldig worden gekozen. Niet alleen op basis van de data die ze opleveren, maar ook rekening houdend met de gevoeligheid, monstertype, gebruikersgemak en kosten om zodoende adequaat te kunnen reageren op specifieke situaties. Dit is de eerste en belangrijkste stap waarna verdere keuzes gemaakt kunnen worden op basis van bijvoorbeeld uitvoerbaarheid, beschikbaarheid en kosten.

Dank je wel – Thank you – Merci beaucoup – Tige tank

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Curriculum vitae

Pytsje Hoekstra – Mevius is geboren op 26 April 1988 in Heerenveen. Na het behalen van haar VWO diploma in 2006 aan het Bornego College te Heerenveen is ze gestart met de bachelor Farmacie aan de Rijksuniversiteit Groningen. Na het behalen van haar bachelor in 2010 besloot ze een master in Biomedische wetenschappen te doen aan de Vrije Universiteit van Amsterdam. Tijdens haar master heeft ze stage gelopen bij de afdeling Parasitologie van het LUMC waar ze zich richtte op de evaluatie van een real-time PCR assay voor de diagnose van schistosomiasis en vervolgens deze assay heeft geïmplementeerd in een diagnostisch laboratorium in Durban, Zuid-Afrika. Haar laatste onderzoeksstage heeft ze uitgevoerd in Mwanza, Tanzania, waar ze onderzoek heeft gedaan naar seksueel overdraagbare aandoeningen onder zwangere meisjes. Na het behalen van haar master diploma in 2012 is ze gaan werken als data monitor bij de Stichting HIV Monitoring in Amsterdam. Echter, vanwege haar sterke interesse in onderzoek – in het bijzonder de combinatie met de tropen – keerde ze in 2016 terug naar de afdeling Parasitologie van het LUMC waar ze aan de slag ging met de toepassing van circulerende antigeen detectie voor de diagnose van schistosomiasis. Tijdens haar promotieonderzoek is ze naar verschillende Afrikaanse landen gereisd om de UCP-LF CAA test te implementeren in lokale laboratoria. Daarnaast maakte ze van 2018 tot 2023 deel uit van het project management team voor het EDCTP-gefinancierde freeBILy project waarbij de nauwkeurigheid antigeen testen voor het diagnosticeren van *Schistosoma* infecties werd geëvalueerd in vrouwen en jonge kinderen in Gabon en Madagascar. Op dit moment zet ze haar onderzoek naar het gebruik van circulerende antigenen voor de diagnose van schistosomiasis voort als postdoctoraal onderzoeker bij de afdeling Parasitologie van het LUMC.

List of publications

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for from Him and through Him and to Him are all things.
To Him be the glory forever.
Amen