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Diagnosis of schistosomiasis mansoni: an evaluation of existing methods and research towards single worm pair detection

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Review Article

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Abstract

The inadequacy of current diagnostics for the detection of low worm burdens in humans means that schistosomiasis mansoni is more widespread than previously acknowledged. With the inception of mass drug treatment programmes aimed at disease elimination and the advent of human vaccine trials, the need for more sensitive diagnostics is evident. In this review, we evaluate the merits and limitations of the principal diagnostic methods, namely detection of eggs in faeces; anti-schistosome antibodies in serum; parasite-derived proteins and glycans in serum or urine; parasite DNA in blood, faeces or urine. Only in the baboon model, where actual worm burden is determined by portal perfusion, have faecal smear and circulating antigen methods been calibrated, and shown to have thresholds of detection of 10–19 worm pairs. There is scope for improvement in all the four methods of detection, e.g. the identification of single targets for host antibodies to improve the specificity of enzyme linked immunosorbent assay. Despite recent advances in the definition of the schistosome secretome, there have been no comprehensive biomarker investigations of parasite products in the urine of infected patients. Certainly, the admirable goal of eliminating schistosomiasis will not be achieved unless individuals with low worm burdens can be diagnosed.

Introduction

Although great strides have been made in the treatment of schistosomiasis mansoni, especially *via* mass treatment programmes, the disease remains widespread, not least because the diagnosis is problematic. The World Health Organization identified it as the second most important human parasitic disease in the world, after malaria. The causative agents are trematode worms (Phylum Platyhelminthes) of the Genus *Schistosoma* and three principal species infect humans: *Schistosoma japonicum*, *Schistosoma mansoni* and *Schistosoma haematobium*, along with other minor species like *Schistosoma intercalatum* and *Schistosoma mekongi*. Adult worms of *S. japonicum* and *S. mansoni* reside in the mesenteric veins and those of *S. haematobium* in the vesicle venous plexus of the bladder. This intravascular location has always made validation of the sensitivity of diagnostic techniques for schistosomiasis difficult (De Vlas and Gryseels, 1992). The current prevalence is hard to gauge but 10–15 years ago it was estimated to affect approximately 207 million people in tropical countries, 20 million of whom had severe illness (Engels *et al.* 2002; Chitsulo *et al.* 2004; Steinmann *et al.* 2006). Since then, while the intensity of infection has undoubtedly declined due to mass treatment programmes (Fenwick, 2017), the overall prevalence may not have, and recent studies indicate that the number of low-intensity cases has been seriously and consistently underestimated (Colley *et al.* 2017; Ortu *et al.* 2017), perhaps by a factor of 3–6-fold (Kittur *et al.* 2016). Egg-induced pathology, the hallmark of the disease, is related to worm burden so low levels of infection may not be clinically important. However, in the context of disease elimination possession of sensitive diagnostic tools, ideally detecting a single worm pair is essential. Based on current widely used diagnostic techniques, 93% of the infected individuals live in sub-Saharan Africa (Steinmann *et al.* 2006; Hotez and Kamath, 2009), with approximately 76% of those in high-transmission areas (Rujeni *et al.* 2017). The disease is also endemic in four countries of the Western Pacific region, with 6.7 million people in the Philippines alone at risk of infection with *S. japonicum* (Magalhães *et al.* 2014). Additionally, schistosomiasis mansoni is a public health concern in Brazil, Venezuela and some Carribean Islands (Barreto *et al.* 2016; Zoni *et al.* 2016).

A range of parasitological, immunological and molecular methods have been used for diagnosis of schistosomiasis mansoni, each having advantages and limitations (comprehensively reviewed by Weerakoon *et al.* 2015). The most widely used has been stool examination for eggs, using the Kato-Katz faecal smear (Rujeni *et al.* 2017). However, its limited sensitivity has been acknowledged in the detection of individuals with a low worm burden, reducing its effectiveness in determining overall prevalence in areas of low endemicity (Feldmeier and Poggensee, 1993; Kongs *et al.* 2001). The detection of antibodies specific to schistosome antigens using enzyme-linked immunosorbent assay (ELISA) is a more sensitive way to find

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light infections and can be used on a large scale (Sorgho *et al.* 2005; Alarcón de Noya *et al.* 2007). Its sensitivity depends on the right choice of coating antigen (Ishida *et al.* 2003; Sorgho *et al.* 2005; Alarcón de Noya *et al.* 2007), but the principal limitation is that it does not discriminate between ongoing and previous infections (Kato-Hayashi *et al.* 2010). A third approach has been to detect parasite DNA in host-derived samples using molecular techniques. (Pontes *et al.* 2003; Sandoval *et al.* 2006). Finally, the detection of *Schistosoma* antigens in the serum or urine has become a widely applied alternative. To date this has been focused on the glycans, Circulating Cathodic Antigen (CCA) and Circulating Anodic Antigen (CAA), released from the parasite gut into the bloodstream (van Dam *et al.* 2004; Corstjens *et al.* 2014; Ortu *et al.* 2017). The poorly defined sensitivity of current diagnostics remains an important impediment in large-scale control programmes, intended ultimately to eliminate the disease.

Our involvement in the topic of schistosome diagnostics emerged from vaccine trials in primates, and by extension, the need to evaluate such trials in humans. One problem encountered was the impossibility of estimating egg output by the Kato-Katz method in the fluid, opaque stools produced during the acute phase of infection between 7 and 12 weeks (Kariuki *et al.* 2006). To circumvent this, we developed a density gradient method to enrich eggs from faeces using Percoll medium (Eberl *et al.* 2002). Its superiority over faecal smears was subsequently demonstrated on human samples from Egypt. In vaccine experiments using the olive baboon (*Papio anubis*) we charted the progress of challenge infections using both faecal smears and circulating antigen assays (Kariuki *et al.* 2004, 2006). Since the animals were ultimately perfused to determine worm burden, it was possible for the first time to determine the absolute sensitivity of these diagnostic tools in a large animal model of intestinal schistosomiasis (Wilson *et al.* 2006). Furthermore, sera from baboon experiments have subsequently been used to determine the sensitivity of improved circulating antigen assays (Corstjens *et al.* 2014). We review here the advantages and limitations of diagnostic methods for *S. mansoni*, which are crucial for monitoring programmes intended to control and eliminate the disease, and for current and future human vaccine trials. We also evaluate the results of proteomic analyses over the last decade that have defined the secretome of adult worms and eggs and may provide new diagnostic targets.

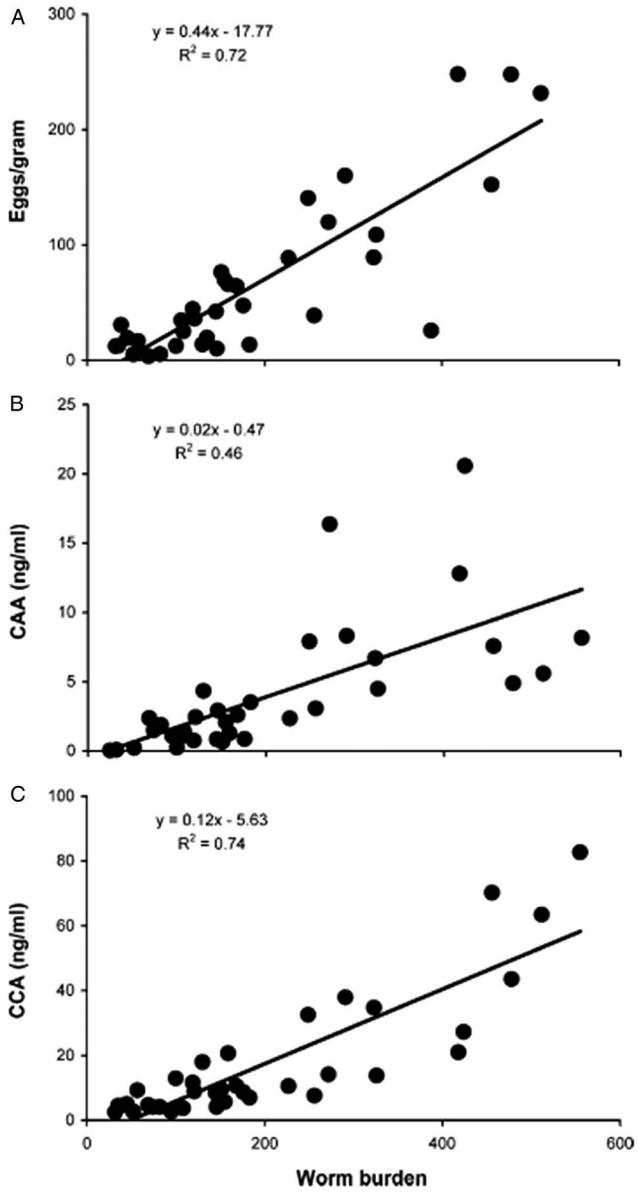

Fig. 1. Linear regression of surrogate values against corresponding worm burdens for (A) faecal eggs, (B) circulating anodic antigen (CAA) and (C) circulating cathodic antigen (CCA). Values are means of nine, three and two replicates, respectively; zero values for eggs per gram (three) and CAA (two) were omitted to avoid biasing the regression calculation. One animal was also omitted from the egg regression due to incomplete data. The regression coefficient R^2 provides a measure of goodness of fit to the data. When $y = 0$, the respective intercepts of the x-axis represent the threshold of detection for each technique. (From Wilson *et al.* 2006).

Parasitological methods

Diagnosis of schistosomiasis infection by the demonstration of eggs in stool or urine specimens is often referred to by clinicians as the 'diagnostic gold standard' in patients from endemic countries. Equally, the disappearance of eggs after drug treatment has been considered as confirmation of its success (Wichmann *et al.* 2009). Parasitological methods or the use of questionnaires for self-reporting of characteristic symptoms (Lengeler *et al.* 2002; Rabarijaona *et al.* 2003) are frequently used for targeting population-based mass drug administration (MDA) in schistosomiasis control campaigns. After MDA, the number of negative stool tests increases and the performance characteristics of standard diagnostic tests decrease (especially in terms of sensitivity and negative predictive value) and test-to-test variability increases (Carabin *et al.* 2005). In addition, it is observed that the sensitivity of parasitological methods diminishes when prevalence and intensity of infection are low, making them less appropriate for low-endemic areas or post-treatment situations. Parasitological methods are also unable to diagnose recent infections where worms have not yet started to produce eggs (the prepatent period) (Shane *et al.* 2011).

The Kato-Katz technique is preferred because it requires relatively simple technology, based on a slide template that can take 50 mg of faecal material, allowing a rapid estimation of infection burden, expressed as eggs per gram (epg) of a stool sample (Katz *et al.* 1972). One drawback is that the sensitivity of Kato-Katz smears is influenced by the consistency of the faecal material (Teesdale *et al.* 1985; Feldmeier and Poggensee, 1993); for instance, moderate to heavy infections may cause diarrhoea and seasonal variations in the availability of fruit can lead to changes in faecal fibre content (Stelma *et al.* 1994), both situations obscuring egg identification. The results may be affected by day to day variability in egg excretion (Barreto *et al.* 1978; Teesdale *et al.* 1985; Kongs *et al.* 2001; Knopp *et al.* 2009). Most importantly, the technique has an automatic detection limit of 20 epg, based on the ~50 mg capacity of the chamber (Katz *et al.* 1972). A

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theoretical calculation illustrates the problem. A single female *S. mansoni* produces ~300 eggs per day not all of which will be excreted in the faeces (Moore and Sandground, 1956; Pellegrino and Coelho, 1978). The median faecal production per day of an adult human in a low-income country is estimated as 250 gm wet weight (Rose *et al.* 2015). Assuming the most favourable condition of total egg excretion, detection of one egg in a single Kato-Katz smear equates to the daily egg output of 17 worm pairs. To have a chance of detecting lower burdens, multiple smears must be made. Put another way, the daily production of one worm pair equates to one egg per 0.8 gm faeces, which would need 16 smears to locate. Compare that with the 2.5×10^5 egg output of a single female *Ascaris lumbricoides* per day that equates to 20 eggs in a single Kato-Katz smear.

Our work with the olive baboon confirmed the low sensitivity of the Kato-Katz smear. This host is highly permissive for *S. mansoni* infection and provides a realistic laboratory model with which to assess and compare the efficacy of diagnostic techniques (Kariuki and Farah, 2005). Indeed, we argue that it is the only such model regularly available and its principal attribute is that surrogate estimates of worm burden, such as epg of faeces, can be compared directly with actual worm burdens obtained by portal perfusion of infected animals. Our studies, based on data from 37 baboons, used simple linear regression of mean epg from nine replicate Kato-Katz smears on worm burden and revealed a detection threshold of 40 worms (Wilson *et al.* 2006) (Fig. 1). Note also that eggs were not detected in nine replicate smears from three animals with a measurable worm burden (25 or more worms; 8%) which would have been false negatives by the Kato-Katz smear technique. On the positive side, the R^2 of 0.72 shows what is normally assumed, namely that faecal egg counts are good quantitative surrogate estimates of actual worm burden. Adjusted for sex-ratio (Kariuki *et al.* 2006), the threshold is equivalent to approximately 16 worm pairs. The estimate is remarkably similar to the theoretical calculation for the baboon, based on a faecal production of 200 gm per day (Wasser *et al.* 1994), of 13 worm pairs.

One way of circumventing the low frequency of eggs in a faecal mass, their random distribution and the small sample taken for analysis, is to enrich eggs prior to counting using a larger amount of faces. Flotation methods, such as zinc sulphate solutions (SG 1.2–1.35), used for many nematode eggs in faecal samples, are intrinsically less suitable for schistosome eggs because these lack an impermeable vitelline membrane. The high osmotic pressure of such solutions means they suck all the water out of the eggs and crush them. Nevertheless, both ether concentration (Marti and Escher, 1990) and the FLOTAC method (Cringoli *et al.* 2010) have been evaluated against the Kato-Katz smear technique (Glinz *et al.* 2010). One advantage is that the methods can be used on fixed and stored faecal samples, with a much larger amount of starting material (~0.5 to 1 g). Both estimated a higher % prevalence of *S. mansoni* infections in human faecal samples, compared to triplicate Kato-Katz smears. However, the FLOTAC method, in particular, produced lower quantitative epg counts, suggesting that many eggs were not recovered by the flotation. The fragility of the schistosome egg in the FLOTAC zinc sulphate solution was confirmed by the images displayed by (Glinz *et al.* 2010).

We previously addressed the problem of egg fragility using a biocompatible 60% Percoll solution in 0.9% saline, as a separation medium (Eberl *et al.* 2002). Percoll is a colloidal suspension of silica particles coated with polyvinylpyrrolidone that exerts a very low osmotic pressure. The method works on the opposite principle to FLOTAC, being of slightly lower density than viable eggs (SG 1.224). It was used with 250 mg faeces to float off the colloidal material that would obscure eggs in a smear (especially at the acute stage of infection), leaving the eggs in the pellet for counting. For both rhesus macaques and chimpanzees the method was 10–20 times more effective than the Kato-Katz smear. It proved similarly superior when employed with human faecal samples from Egypt. However, the Percoll method requires viable eggs, a centrifuge and an expensive reagent, and so seems best suited to research applications, rather than mass treatment programmes.

Another approach, based on the physical properties of eggs, termed Helmintex, has been developed in Brasil (Teixeira *et al.* 2007). It starts with 30 gm of faecal material, processed by passage through a series of sieves, excluding larger debris but allowing colloidal particles to travel in the flow-through. The eggs are then stained and concentrated further using paramagnetic beads, before counting (Favero *et al.* 2017). The method has been calibrated by spiking of faeces with known numbers of eggs and shown to give a mean recovery rate of 27%. The method proved significantly superior to both the Kato-Katz smear and a saline gradient method in diagnosing individuals with a light infection (Pinheiro *et al.* 2012). Its main drawback is the time taken to process each sample. However, using the values given above for egg output per worm (300) and daily human faecal production (250 gm), with a 30 gm faecal sample and a 27% recovery rate, the method should be capable of detecting an infection comprising one worm pair.

It is hard to avoid the conclusion that, although widely used, the Kato-Katz smear has serious limitations. In areas of high transmission, it can be used with confidence to control morbidity by identifying individuals with high worm burdens, whose health and prospects will be markedly improved by chemotherapy. However, where worm burdens are low, its insensitivity gives the impression that prevalence is much lower than the reality, perhaps even causing complacency about the status of control measures.

Antibody-based tests

In order to overcome some of the drawbacks of the Kato-Katz smear and other faecal sampling techniques, attempts have been made to develop more sensitive diagnostic tests based on the detection of antibodies in serum reactive with schistosome antigens, particularly *in situ*ations of light infections (Noya *et al.* 2002; Doenhoff *et al.* 2004; Bottieau *et al.* 2006). Such tests, usually in ELISA format, are most valuable in patients with infrequent exposure to schistosome cercariae, such as tourists visiting an endemic area (Coltart *et al.* 2015). They are generally more sensitive than examination of stool samples, particularly in low transmission areas (Cesari *et al.* 2005). Antibody assays may utilize crude antigen extracts such as schistosome egg antigen, soluble adult worm antigen preparation (SWAP), or can be constructed to detect purified or recombinant antigens (Oliveira *et al.* 2008). In patients with Katayama syndrome – an acute inflammatory response driven by the body's immune response to schistosomula migration and egg deposition, often characterized with nocturnal fever, cough, myalgia, headache and abdominal tenderness (Ross *et al.* 2007), – a positive antibody test is usually the earliest diagnostic laboratory result. Still, a large fraction of patients will initially test negative (Hamilton *et al.* 1998; Zhu, 2005). Such false negative tests prevent timely treatment of schistosomiasis in travellers who present with fever of unknown origin. A potential solution to the early diagnosis of acute schistosomiasis using ELISA was provided by the identification of a glycan epitope shared between keyhole limpet haemocyanin and the schistosome surface (Grzych *et al.* 1987). In recently exposed individuals in both Egypt (Mansour *et al.* 1989) and Brasil (Alves-Brito *et al.* 1992) strong antibody responses to the glycan were detected, even before eggs were present in faecal samples

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and the method readily distinguished between patients with acute *vs* chronic disease in almost all instances.

A major limitation of antibody-based tests is that while the methods tend to be more sensitive than Kato-Katz smears, parasite-specific antibodies remain detectable in the circulation for years after the infection has been cleared. As a result, measuring anti-schistosome antibody titres in serum may not distinguish between current and previous infections. In addition, antibody levels in serum do not necessarily correlate with the intensity of the schistosome infection as determined by mean epg of faeces (Shane *et al.* 2011). This limits the clinical value of antibody detection for confirmation of the success of chemotherapy (Rabello *et al.* 1997) since specific antibodies continue to be present long after the worms have disappeared. The occurrence of cross-reactivity with other helminthic infections due to shared antigenic epitopes constitutes another limitation of the immunodiagnostic approach. One way to circumvent is to select specific antigens for coating ELISA plates (Valli *et al.* 1997; Oliveira *et al.* 2003). Use of peptides from schistosome proteins has an even higher potential of eliminating cross-reactivity while improving on specificity (Oliveira *et al.* 2008). Sufficient information is now known about the secreted proteins from the gut and tegument of adult worms, and from mature eggs (Wilson, 2012), for researchers to evaluate their potential in such a diagnostic assay (see below). The recent review by Hinz *et al.* (Hinz *et al.* 2017) has revealed how little progress has been made away from use of crude extracts towards single specific targets as diagnostics.

Circulating glycan-based antigens

One solution to the problems associated with testing for reactive antibodies in infected individuals is to detect parasite-derived antigens in the circulation or urine (Pontes *et al.* 2002). Several assays have been described in different laboratories (de Jonge *et al.* 1991; Barsoum *et al.* 1992; Hassan *et al.* 1992) but the major player in their development has been the group of Deelder and colleagues in Leiden, The Netherlands. The current assays depend on adult worms vomiting CAA and CCA into the host bloodstream (Nash, 1978; Deelder *et al.* 1994) and the detection of their immunologically reactive O-linked glycan residues. The repeated epitope in CAA is unique to the worm (Bergwerff *et al.* 1994) but the multiple Lewis X epitopes of CCA (van Dam *et al.* 1994) are also present in the host, and this can result in high backgrounds for the assay (Polman *et al.* 2000). A prerequisite for these assays was the generation of monoclonal antibodies specific for the glycan epitopes (van Lieshout *et al.* 1994; Al-Sherbiny *et al.* 1999). A feature of the ELISA format is that the assays are performed on serum samples after precipitation of proteins by trichloroacetic acid, releasing the glycans from immune complexes and leaving them in the supernatant (de Jonge *et al.* 1987).

Detection of CAA and CCA by ELISA (Deelder *et al.* 1989; de Jonge *et al.* 1990; van Lieshout *et al.* 2000) demonstrates only active infections, it is suitable for assessing the effect of treatment on worm burden, and has a high specificity. Early in their development, the tests were extended to detection in urine (Deelder *et al.* 1994) opening up the possibility that invasive methods of sample collection could be eliminated. CCA detection in urine was found to be as sensitive as a single Kato-Katz test in areas that have a high intensity of infection (Polman *et al.* 1995). However, although the detection of circulating antigens by ELISA is highly specific (Hamilton *et al.* 1998; Attallah *et al.* 1999), in areas of low endemicity it was not thought to be more sensitive than the detection of eggs by the Kato-Katz faecal smear (de Jonge *et al.* 1988; Van Lieshout *et al.* 1995; Hamilton *et al.* 1998).

In our vaccine studies with the olive baboon, we were able to compare the detection limits of circulating antigen assays *vs* faecal smears (Fig. 1) (Wilson *et al.* 2006). When surrogate estimates of worm burden provided by CAA and CCA assays on serum from 37 animals (three and two replicates, respectively) were compared by linear regression with the number of worms recovered by portal perfusion, thresholds of detection of 24 and 47 worms respectively, were revealed. The respective R^2 values of 0.74 and 0.46, respectively, indicate that CAA was a better predictor of worm burden than CCA. Extrapolated to human populations as with the Kato-Katz smear, these assays were just as likely to miss individuals with low worm burdens. Furthermore, two animals with 25 or more worms were false negatives after three replicate CAA tests.

Given the diagnostic potential of circulating antigens, considerable efforts were made to simplify their application in endemic areas. Two urine CCA assays were developed and commercialized (Shane *et al.* 2011). The first, using a colloidal carbon conjugate of a monoclonal antibody specific for CCA, was designed for use in the laboratory (van Dam *et al.* 2004). A version of this assay was used in studies of children less than 3 years of age (Odogwu *et al.* 2006) and in school-aged children with sensitivities and specificities in the low 80th percentiles when compared with stool egg data (Odogwu *et al.* 2006), but is no longer available. The second assay was a gold-conjugated, lateral flow cassette-based assay, which was designed to be a point of care CCA assay (POC-CCA) (Shane *et al.* 2011), and its introduction has changed the schistosome diagnostics landscape.

Shane *et al.* (2011) showed that the SWAP ELISA and the cassette urine CCA assay were the most sensitive assays, albeit also the least specific when analysed by latent class analysis. The authors also observed that the CCA urine assays were more sensitive than examination of three stools by Kato-Katz and were as sensitive as the adult worm-specific antibody tests. The urine CCA assays are also easy to use and less time consuming than the other methods currently employed for *S. mansoni* screening. CCA assays may also have the potential to assess cure, as CCA levels declined significantly from week 1 after treatment of individuals with *S. mansoni* (van Lieshout *et al.* 1993). The manufacturer's technical brochure suggests that false positive CCA results may occur if the individual being tested has haematuria or pio-uria due to urinary tract infection. Shane *et al.* (2011) found a strong correlation between stool egg concentration and intensity of the CCA test band and concluded that the CCA urine assays are an effective screening tool for *S. mansoni* infections in areas of high prevalence. However, a complication of the POC-CCA assay is the subjective visual assessment of the test strip result, and the way in which a 'trace' reading should be interpreted (Colley *et al.* 2017). Nevertheless, the POC-CCA has now been widely used in mass treatment programmes in several African countries, where it has outperformed the faecal smear as a diagnostic tool (Kittur *et al.* 2016; Colley *et al.* 2017; Ortu *et al.* 2017). One conclusion of all these studies could be that in so-called 'egg negative' individuals, adult worms are still present, and the prevalence of schistosomiasis mansoni has been seriously underestimated (Colley *et al.* 2017). Indeed, the diagnostic deficit may apply to all the species of *Schistosoma* infecting humans (Colley *et al.* 2017). It is unfortunate that the POC-CCA cassette assay was not available when we performed our baboon vaccine experiments so no data are available on how its sensitivity relates to actual worm burdens.

Perhaps as a tacit acknowledgment of still inadequate performance, attempts to improve the sensitivity of circulating antigen assays have been made using a combination of various quantitative lateral flow (LF)-based assays utilizing up-converting phosphor (UCP) reporters (Deelder *et al.* 2012; Colley *et al.* 2013;

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Corstjens *et al.* 2014). Of note, the UCP-LF assay for CAA detection has been adapted to a dry reagent format that is stable at ambient temperature and worldwide shipping without the need for a cold chain (van Dam *et al.* 2013). The stability of the target antigen has a direct effect on the assay sensitivity and the CAA glycan fraction is a stable component in urine and blood, detectable after storage of clinical samples for prolonged periods of time (Corstjens *et al.* 2014). Corstjens and colleagues showed that the quantitative UCP-LF assay had the capacity to determine active infections of various *Schistosoma* species with excellent clinical sensitivity and specificity (Corstjens *et al.* 2014). Nevertheless, to achieve maximum sensitivity they needed to concentrate the CAA in the serum or urine sample using centrifugal filters with a 10 kDa cut-off. With baboon sera from vaccination experiments and graded infections, they obtained an improved resolution of the relationship between CAA levels and worms, in the low burden range. Used in conjunction with a concentration step, the UCP-LF assay takes us some way towards the goal of detecting a single worm pair.

Molecular approaches

Schistosomes do not multiply within the definitive host implying that there would be a little turnover of DNA from the adult worms (apart perhaps from shedding of spermatozoa by excess unmated males). However, a salient feature of *S. mansoni* infections is the deposition of ~300 eggs per female in the tissues each day. Those which die in the tissues release parasite DNA, which must reach the bloodstream and urine to serve as the basis for diagnosis. Several groups have developed polymerase chain reaction (PCR) methods to improve the direct detection of schistosome infections. The tests can be done on urine, stool, or organ biopsy samples and involve the recovery of parasite-derived DNA prior to PCR amplification (Pontes *et al.* 2003; Sandoval *et al.* 2006). Unfortunately, only a small volume of starting material – faeces, blood or urine – can be processed for DNA extraction. In the case of faecal sampling, successful PCR amplification depends on whether the processed sample contains eggs or not; it has the same limitations as optical microscopy and does not provide a significant clinical benefit.

Nonetheless, cloning and characterization of a 121-bp, tandemly repeated DNA sequence (Sm1–7), which comprises 12% of the genomes of both male and female *S. mansoni* (Hamburger *et al.* 1991) opened the opportunity for using PCR technique to diagnose schistosomiasis mansoni (Pontes *et al.* 2003; Wichmann *et al.* 2009). The small, repeat sequences are suitable probes because their copy number (600 000 per schistosome cell) should make for high detection sensitivity, even if only a few DNA fragments reach the blood stream. Secondly, such sequences are most likely to be non-coding and therefore evolve more rapidly than the rest of the genome, making for high specificity. A second DNA sequence, the ITS2 spacer situated between the small and large subunits of the ribosomal RNA genes and present as thousands of copies, has been independently developed for PCR-based diagnosis (Obeng *et al.* 2008).

The utility of PCR was revealed when a single survey, using primers designed from Sm1–7 repeat sequence, detected more cases of infection with *S. mansoni* than three Kato-Katz stool examinations (Pontes *et al.* 2003). The discrepancy confirmed the greater sensitivity of PCR when eggs were detected by further faecal sampling from the same patients. PCR has the ability to achieve amplification with as little as 1fg of *S. mansoni* egg template DNA (Pontes *et al.* 2002) and the specificity of the test was shown by the absence of amplification when DNA from four other helminths commonly found in the same endemic areas as *S. mansoni* was used as templates. The ITS2 spacer was initially detected in urine samples from patients with a *S. haematobium* infection where it compared favourably with a CCA strip test. Subsequently this PCR technique has been extended to the detection of *S. mansoni* DNA (i.e. eggs) in stool samples from human populations in areas of high (Senegal) and low transmission (Kenya), where it proved more sensitive than one or more faecal smears; it was especially useful in areas with low-level infections (Meurs *et al.* 2015). It should be noted that the PCR method applied to stool samples suffers the same constraints as direct faecal sampling for eggs by microscopy. At low worm burdens, if the amount of stool sampled is small, it may not contain any eggs. In the Sm1–7 study (Pontes *et al.* 2003) 0.5 gm was processed from each patient so it was very likely to miss some infections. With the ITS2 study (Meurs *et al.* 2015) 0.7 mls of faeces was sieved and diluted in ethanol. Again this was probably not an adequate amount of starting material to detect the eggs released by single worm pair. If technically feasible, DNA extraction from a larger faecal sample appears essential, to increase sensitivity.

When stool and serum/plasma were compared as the source of DNA for PCR amplification (Pontes *et al.* 2002; Wichmann *et al.* 2009), serum DNA bands were not as strong as those seen with faecal samples, suggesting limited quantities of free-circulating DNA (Pontes *et al.* 2002). Nevertheless, Pontes *et al.* (2002) concluded that PCR is probably more sensitive than the Kato-Katz technique *in situ*ations of low worm burden. Wichmann *et al.* (2009) also detected cell-free parasite DNA (CFPD) in human plasma, which in contrast to eggs in stool or urine, should be equally distributed throughout the plasma volume of the patient, resolving the issue of random sampling that confounds clinical sensitivity of classical detection methods. The disappearance of CFPD from the bloodstream might also be a way to confirm the elimination of schistosomes after treatment (Wichmann *et al.* 2009). Additionally, CFPD can be very useful in early diagnosis of Katayama syndrome caused by acute *Schistosoma* infection which is a major differential diagnosis in returning travellers presenting with fever of unknown origin (Ross *et al.* 2007; Wichmann *et al.* 2009).

Sometimes, PCR can report false negative results due to many factors such as inhibition of the amplification reaction by faecal compounds and/or DNA degradation during transportation from the field (Engels *et al.* 1996).

A recent development has been the successful application of PCR for detection of the Sm1–7 DNA fragment (Hamburger *et al.* 1991) in urine samples from potentially infected individuals (Lodh *et al.* 2013), in comparison with the CCA dipstick assay and Kato-Katz faecal smear. The PCR detection of parasite DNA in patient urine consistently outperformed the other two assays. The major drawback is the need for a PCR instrument and a well-equipped laboratory so at this time it cannot be described as a point-of-care assay. Nevertheless, it would be worth calibrating the PCR technique against worm burden in a realistic host like the baboon, using both the Sm1–7 and ITS2 sequences as DNA targets to see how they compare in sensitivity.

Loop-mediated isothermal amplification (LAMP), a novel molecular alternative to PCR, has also been developed and tested. It differs from PCR in that four or six primers are used for the amplification of a single target gene at a single temperature step from 63 to 65 °C, maintained at 65 °C for 60 min. Many amplicons with various structural conformations are produced in LAMP reactions which can be detected by simple turbidity or fluorescence (Zhang *et al.* 2011). LAMP, initially described in 2000 (Notomi *et al.* 2000), has rapidly gained acceptance for detection of a variety of infectious agents including *Plasmodium falciparum* and *S. japonicum* (Poon *et al.* 2006; Xu *et al.* 2010). It does not require complex equipment for DNA amplification

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or for amplicon detection (Mori *et al.* 2001) and is potentially suitable for molecular monitoring in basic laboratory facilities (Boehme *et al.* 2007). Given these features, LAMP is potentially useful for work in the field and has already been used in rural laboratories in developing areas for the diagnosis of tuberculosis (Boehme *et al.* 2007). However, some points still need to be addressed in considering LAMP implementation in the field. First, a simple and inexpensive DNA preparation method is required. For achieving field applicability, the expensive DNA extraction kits should be replaced by user-friendly and affordable DNA preparation tools, particularly where large numbers of samples are to be examined. Also, to be applicable in field laboratories, LAMP reaction mixtures will need to be premixed, ready for use, and storable under field-laboratory conditions (Abbasi *et al.* 2010). Reaction mixtures having these features are now available for PCR (Sohni *et al.* 2008) and can likely be developed for LAMP. The diagnosis of schistosomiasis mansoni infection in humans using Sm1–7 sequence amplification for copro-PCR (Pontes *et al.* 2003) or plasma-PCR (Wichmann *et al.* 2009) has already been accomplished and it can detect low-grade infections with high sensitivity. It is feasible that LAMP assays would add another order of magnitude of detection sensitivity, namely 0.1 fg worm DNA compared with 1 fg detection sensitivity of the corresponding PCR assays (Hamburger *et al.* 1991, 2001).

Diagnostic sensitivity and human vaccine testing

The search for a schistosome vaccine has a long history and at least three candidates, TSP-2, Sm-14 and Smp80 calpain (Siddiqui and Siddiqui, 2017), are at Phase I testing in humans, whilst one (ShGST) has reached Phase III, but with unknown outcome (summarised by Merrifield *et al.* 2016). How will the efficacy of these vaccines be tested at Phase II and Phase III in human populations in areas endemic of schistosomiasis? Our experience with trials of the radiation-attenuated cercarial vaccine in baboons has highlighted the problems caused by the diagnostic deficit (Kariuki *et al.* 2006). In five separate experiments protection was evaluated ten weeks after challenge when surrogate estimates of worm burden in test and control animals were at steady state. Compared with actual worm burden, epg faeces overestimated protection by >5% on all occasions, four times by >20% CAA overestimated protection on 3/5 and CCA on 4/5 occasions. The existence of thresholds of detection is clearly a major contributory factor in this overestimation (Wilson *et al.* 2006). Human vaccine trials are likely to be undertaken in individuals given chemotherapy to 'clear' their worms. These individuals will then gradually accrue worm burdens that will only become reliably detectable as they rise above the thresholds. The threshold distortion diminishes over time with accumulating worm burden but protection will always be overestimated in the test relative to the placebo group. The outcome would be a misleading impression that the vaccine is performing (much) better than the reality, especially if the evaluation took place early after patency.

Secreted schistosome proteins as potential new diagnostics

Large-scale sequencing of schistosome cDNAs to define the transcriptome (Verjovski *et al.* 2003) was followed by the sequencing, assembly and annotation of the *S. mansoni* genome (Berriman *et al.* 2009). Over the same period advances in mass spectrometry made it possible to determine the precise protein composition of parasite fractions (Wilson *et al.* 2007). An intense period of investigation followed in which the proteins secreted by invading cercariae (Curwen *et al.* 2006), migrating schistosomula were characterized (DeMarco *et al.* 2010). An inventory of the proteins exposed at, or released from the adult tegument surface (Braschi *et al.* 2006*a*; Braschi and Wilson, 2006) and those vomited into the blood stream from the blind-ending gut (Hall *et al.* 2011) was also compiled. Lastly, the products of the live mature egg, released into the tissues to aid its passage through host tissues, were defined (Cass *et al.* 2007; Mathieson and Wilson, 2010). It should be noted that there are wide discrepancies in the results of these compositional studies, and we take here a conservative view that only proteins with a signal peptide are components of the secretome, unless there is a very good reason to believe otherwise. The potential for the secretions of adult worms and eggs to act as new diagnostic targets was reviewed in 2012 (Wilson, 2012) and a Table incorporating recent findings is presented here (Table 1). This information could be used to improve the specificity of antibody detection and to find new markers of active infection in blood or urine. It should assist future searches for schistosome-derived biomarkers in those fluids.

The secretions of the gastrodermis that lines the gut are a major source of worm proteins entering the host bloodstream when the worm regurgitates the residual contents of a blood meal (Hall *et al.* 2011). They comprise, in approximately equal measure enzymes, largely but not entirely proteases, and a selection of carrier proteins that are probably involved in uptake of inorganic ions (Ferritins, Calumenin) and lipids (Saposins, NPC2) into the worm tissues. The alimentary tract products have recently been augmented by the identification of around 40 esophageal gland proteins (Wilson *et al.* 2015), only the most abundant of which are listed in Table 1. Identification of tegument surface constituents released into the bloodstream has been bedevilled by the extreme fragility of this syncytial layer *in vitro*. Only the products released after cationised ferritin-stimulated sloughing of the membranocalyx (Braschi *et al.* 2006*b*; Wilson, 2012) and those GPI-anchored proteins that can be released from live worms by enzymatic shaving with phosphatidyl-inositol phospholipase C (Sauma *et al.* 1991; Castro-Borges *et al.* 2011), are listed in Table 1. Lastly, the live mature eggs are a major source of secretions (Mathieson and Wilson, 2010), and proteomics has revealed that together IPSE and Omega-1 constitute the vast bulk. When these are depleted from eggs secretions, groups of MEG-2 and MEG-3 family proteins are revealed (DeMarco *et al.* 2010). The contribution of tegument proteins to the total secretome is likely to be slight – this syncytium is primarily concerned with immune evasion. The relative contributions of alimentary tract *vs* eggs secretions are hard to judge. The female gut, in particular, is very active, consuming eight times more blood than the male and presumably, therefore, producing eight times more vomitus. Although only 300 eggs are produced per day, when mature, they secrete their 'escape proteins' for days to weeks so that a single worm pair will be responsible for the secretions of some thousands of viable tissue eggs at any one time.

The search for biomarkers is not a new idea: evidence of secreted schistosome proteins was first reported in 1967 (Berggren and Weller, 1967), who demonstrated the presence of adult worm antigens in the serum and urine of mice and hamsters infected with *S. mansoni*. However, it has received remarkably little attention from researchers. Most reports have been concerned with the detection of host markers in the blood stream as an indicator of tissue damage caused by eggs (e.g. Pereira *et al.* 2016). The CAA and CCA glycoproteins were the first secretome products identified and then developed for diagnostic purposes. We can pose a series of questions about other possibilities:

- Do some other adult worms or egg products have properties that surpass CAA or CCA?
- What is the half-life of worm or egg secreted proteins in the bloodstream?

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Table 1. The secretome of adult worms and eggs characterized by proteomics

ENZYMES IN VOMITUS		MW	MISCELLANEOUS IN VOMITUS		MW
Asp endopeptidase	Smp_075800	46.9	a 2 macroglobulin	Smp_089670	245.5
Cathepsin S	Smp_139240	38.1	LAMP	Smp_167770	43.1
Cathepsin B	Smp_067060	36.8			
Cathepsin B	Smp_103610	36.8	TEGUMENT		
Aspartyl protease	Smp_013040	45.5	Sloughed membranocalyx		
Cathepsin B	Smp_141610	36.5	Sm200 surface glycoprotein	Smp_017730	187.1
Cathepsin L	Smp_139160	36.2	Sm29	Smp_072190	18.1
Cathepsin L	Smp_187140	37.9	Tetraspanin TSP-2	AAN17276[a]	26.2
Carboxypeptidase	Smp_071610	51.1	Sm13	Smp_195190	9.7
Carboxypeptidase	Smp_002600	54.6	Sm8·7 LMWP	Smp_194860	8.8
DNAse II	Smp_008610	41.5	Annexin	Smp_077720	39.4
beta D xylosidase	Smp_043390	91.0			
CARRIER PROTEINS IN VOMITUS			Readily detached by enzymatic shaving		
Saposin	Smp_194910	18.7	Sm200 surface glycoprotein	Smp_017730	187.1
Saposin	Smp_130100	12.6	Alkaline phosphatase	Smp_155890	42.1
Saposin	Smp_105420	20.3	ADP-ribosyl cyclase,	Smp_025830	32.6
Saposin	Smp_105450	12.9	Carbonic anhydrase,	Smp_168730	34.9
Saposin	Smp_016490	20.3	CD59	Smp_019350	11.0
Saposin	Smp_014570	22.2	CD59	Smp_105220	12.2
Niemann Pick C2	Smp_194840	14.4	Sm29	Smp_072190	18.1
Ferritin 2	Smp_047650	19.7			
Ferritin 2	Smp_047660	20.9			
Ferritin 2	Smp_047680	19.9	EGG SECRETIONS		
Apoferritin	Smp_063530	20.0	IPSE	Smp_112110	13.2
Calumenin	Smp_047760	35.7	Omega 1	ABB73002[a]	24.3
ESOPHAGEAL GLANDS			MEG-3.1	Smp_138080	14.2
MEG-4.2	Smp_085840	10.7	MEG-3.2	Smp_138070	13.3
MEG-15	Smp_010550	16.9	MEG-3.3	Smp_138060	14.1
MEG-8.2	Smp_172180	13.8	MEG-2.1/ESP15	Smp_181510	6.8
MEG-4.1	Smp_163630	26.8 #	MEG-2.2	Smp_159810	11.0
MEG-8.1	Smp_171190	19.1	MEG-2.5	Smp_180320	8.7
MEG-12	Smp_152630	5.0	MEG-2.6	Smp_180310	6.1
MEG-9	Smp_125320	5.7	MEG-2.8	Smp_180340	4.9

MW of mature secreted protein minus signal peptide.
[a]GenBank annotation. # actual MW = 100 kDa due to extensive O glycosylation.
Data from: Vomitus, Hall *et al.* 2011; Neves & Castro-Borges unpublished.
Esophageal glands, Wilson *et al.* 2016; Tegument, Wilson, 2012; Egg secretions, DeMarco *et al.* 2010.

- Are they immunogenic and form immune complexes with immunoglobulins leading to their rapid clearance?
- Do they persist in the circulation at concentrations proportional to worm burden?
- Do some of them pass easily into the urine and can be detected there?

Factors such as molecular weight, charge and shape will determine whether worm proteins pass through the glomerular membrane filters of the kidney or are retained in the bloodstream (Hall, 2016). Macromolecules like inulin (MW 5.5 kDa) are completely filterable, while 75% of myoglobin (MW 17kD) but only 0.5% of albumin (MW 69 kDa) enter the kidney tubules. Many of the adult worm and egg secreted products listed in Table 1 have relatively low MW so might be expected to enter the urine unless reabsorbed by the tubule cells. Importantly, since 99% of the water solvent filtered through the glomerular membranes is reabsorbed by the tubules, the smaller schistosome proteins entering the filtrate should be concentrated by a factor of 100 in urine, over the blood stream. Remarkably, no-one yet seems to have carried out a systematic biomarker analysis of urine (or plasma) from infected patients or laboratory animals to search for these released products.

There is one report of an oligosaccharide target of a monoclonal antibody being identified by mass spectrometry in the urine of infected individuals, which confirms the existence of such

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biomarkers (Robijn *et al.* 2008). The tegument surface glycoprotein Sm200 has been detected in circulating lipoprotein particles from the blood of schistosome-infected humans (Sprong *et al.* 2006). Lastly, the gastrodermal Cathepsin B protease was previously identified as a suitable target for immunodiagnosis by antibody ELISA (Li *et al.* 1996; El-Sayed *et al.* 1998; Sulbarán *et al.* 2010) and an assay has now been developed to capture the enzyme in the circulation of infected patients using a specific polyclonal rabbit antibody (Gonzalez *et al.* 2016). The activity of the captured enzyme was then measured using a solid phase assay and the test was able to detect low-level infections in Venezuelan patients with 100% sensitivity and 100% specificity. This last report indicates that evaluation of other gastrodermal enzyme components in the circulation of patients is worthwhile, bearing in mind the questions posed above.

Proteins in the worm and egg secretomes can also be used as specific targets to improve antibody detection but research in this area has been sporadic. Before the advent of proteomics, the gut cathepsin B was employed in the diagnosis of schistosomiasis using antibody-detecting ELISA assays in mice and human subjects (Ruppel *et al.* 1991; El-Sayed *et al.* 1998). Doenhoff *et al.* (2004) suggested the use of the major egg secretion IPSE as a potential immunodiagnostic to detect antibody responses after infection. More recently, two gut saposins likely to be prominent in *S. japonicum* vomitus (SjSAPLP4 and SjSAPLP5) have been used in ELISA format to detect schistosome infections in laboratory animals and human patients in China (Liu *et al.* 2016) and the Philippines (Cai *et al.* 2017). Their orthologs are also prominent in proteomic analyses of *S. mansoni* vomitus. Definition of the worm and egg secretome has provided a large number of potential new diagnostic targets.

Concluding remarks

Parasitic helminths differ in one key aspect from other infectious agents as the lack of multiplication means that primary worm burden is the key factor in disease morbidity. For schistosome infections in humans that burden cannot be measured directly so all diagnostics must serve as surrogates for the parameter. As argued above, the baboon is the only realistic laboratory host in which the absolute sensitivity of surrogate estimates can be evaluated against worm burden. If we aim for detection of a single worm pair, what are the merits of each surrogate?

Faecal sampling for eggs. The main attraction has to be the quantitative relationship between egg output and worm burden. However, the low egg output per *S. mansoni* female relative to the daily faecal output is the major obstacle. Enrichment of eggs in faeces (or removal of the contaminants that obscure eggs) offers the only hope for increasing sensitivity. A flotation medium with low osmolality or a sieving method and at least 1 gm of faecal starting material are prerequisites.

Circulating antibodies by ELISA. The sensitivity of antibody detection may be high but the problem remains the difficulty of distinguishing between current and previous infections.The specificity of such assays can be increased by using single secreted targets, and there are now plenty of such targets to choose from. It is also unclear in current infections whether antibody titre relates to worm burden. Ideally, an antibody ELISA test would use a single and specific schistosome protein as the target, recognized by the immune response of all infected humans. The antibody response to that antigen, approximately proportional to the worm burden, would rapidly fall towards zero when the stimulus provided by infection was eliminated by chemotherapy. IgM responses might have better diagnostic features than IgG responses.

Circulating antigens. CAA and CCA ELISAs are as sensitive as the Kato-Katz faecal smear and proportional to worm burden; the POC-CCA dipstick is apparently more sensitive. However, the dipstick has not been calibrated against worm burden in a host like the baboon. The CAA lateral flow test on serum samples has been validated using baboon sera and may be the nearest approach yet to the goal of single worm pair detection. However, this was only achieved by spin-concentrating the target glycan from ~1 ml of serum. There is a need for biomarker studies on blood and especially urine of infected humans and laboratory host like the baboon to identify those markers of the worm and egg secretome that might develop as new diagnostic targets. Given the schistosome specificity of many secreted products, this could overcome the problem that the CCA epitope in the current dipstick is shared with the host.

Detection of parasite DNA. This is the most enigmatic of the diagnostic possibilities because it is unclear whence the schistosome DNA detectable in blood and urine derives. The most likely source seems to be the death and degradation of tissue eggs, a feature that might have an extended kinetic profile of weeks to months. In that respect detection of DNA could prove little better than antibody titre as an indicator of current infection. Set against this, the specificity and abundance of the Sm 1–7 and ITS2 DNA markers in the *S. mansoni* genome are very desirable characteristics. It is entirely possible that use of PCR or LAMP amplification could reach the requisite sensitivity of single worm pair detection, but that needs calibration in the baboon host.

Is the diagnostic ideal of detecting one worm pair *via* a finger-prick blood sample or a urine sample achievable, without the need for enrichment of the parasite stage (egg) or product (protein, DNA)? That goal is still distant and the development of diagnostic techniques for schistosomiasis with greater sensitivity appears to us essential if disease control and eradication are to be attained. When mass chemotherapy is used to reduce infection in school children, low worm burdens become undetectable with current diagnostics. There is still a need to identify such individuals as they may subsequently develop severe pathology and they also serve as reservoirs of infection. The early intervention with effective chemotherapeutic agents is most efficacious when sensitive and specific methods for detecting infection are available.

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