Vaccines and diagnostics for zoonotic schistosomiasis japonica

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Running title: Development of vaccine and diagnostics for *Schistosoma japonicum*

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SUMMARY

Schistosomiasis is one of the most prevalent, insidious and serious of the tropical parasitic diseases. Although the effective anthelmintic drug, praziquantel, is widely available and cheap, it does not protect against reinfection, drug-resistant schistosome may evolve and mass drug administration programs based around praziquantel are probably unsustainable long term. Whereas protective anti-schistosome vaccines are not yet available, the zoonotic nature of *Schistosoma japonicum* provides a novel approach for developing a transmission-blocking veterinary vaccine in domestic animals, especially bovines, which are major reservoir hosts, being responsible for up to 90% of environmental egg contamination in China and the Philippines. However, a greater knowledge of schistosome immunology is required to understand the processes associated with anti-schistosome protective immunity and to reinforce the rationale for vaccine development against schistosomiasis japonica. Importantly as well, improved diagnostic tests, with high specificity and sensitivity, which are simple, rapid, and able to diagnose light *S. japonicum* infections, are required to determine the extent of transmission interruption and the complete elimination of schistosomiasis following control efforts. This article discusses aspects of the host immune response in schistosomiasis, the current status of vaccine development against *S. japonicum* and reviews approaches for diagnosing and detecting schistosome infections in mammalian hosts.

Key words: *Schistosoma japonicum*, vaccine, diagnostics

INTRODUCTION

Some two hundred million people are infected with schistosomes in 74 countries; 120 million of these are symptomatic, with 20 million suffering severe schistosomiasis disease (Ross et al., 2002). The disease burden in 2010 calculated for schistosomiasis was 3,309,000 disability-adjusted life years (DALYs) (Murray et al., 2012). A meta-analysis assigned 2-15% disability weight to schistosomasis (King et al., 2005). Despite the availability of the effective drug praziquantel (PZQ), its relative inactivity against migratory juveniles and developing worms (Gonnert & Andrews, 1977), its inability to prevent reinfection, and the possibility of resistant schistosome parasites emerging, due to years of mass administration of the drug, are important shortcomings. Schistosomiasis remains one of the most devastating tropical parasitic diseases (Colley et al., 2014) and imposes a high socioeconomic burden on many developing countries where schistosomiasis is endemic due to their impact on human health, particularly as these bloodflukes are commonly found as co-infections with human immunodeficiency virus/AIDS (HIV/AIDS) (Kallestrup et al., 2006), malaria (Diallo et al., 2004) and tuberculosis (Elias et al., 2005). Accordingly, the Bill and Melinda Gates Foundation and other agencies, such as the World Health Organization, have targeted schistosomiasis, along with a number of other neglected infectious diseases, for elimination through investment in strategy evaluation, product development, and operational research.

Five schistosome species, *Schistosoma mansoni*, *S. japonicum*, *S. mekongi*, *S. intercalatum*, and *S. haematobium* infect humans. This article focuses mainly on *S. japonicum*, which is endemic in China, the Philippines and Indonesia and which, in addition to man, infects a range of reservoir mammalian hosts including water buffaloes, cattle, rodents, dogs, sheep and pigs. Human infection is normally acquired through activities such as fishing, bathing, farming, washing clothes and swimming as schistosomes are transmitted via freshwater containing infectious cercariae. After shedding their bifurcated tails, cercariae transform into schistosomula which locate and enter a blood vessel (more rarely a lymphatic vessel) and are carried by the flow of blood to the lungs via the pulmonary artery. After several days, the worms arrive in the hepatic portal system and further develop to adult worms (schistosomes are dioecious). The males and females pair up, mature, migrate downstream, and the female worms of *S. japonicum* begin egg production when the paired worms reach mucosal branches of the inferior mesenteric and superior hemorrhoidal veins. Many eggs are mainly entrapped in liver and intestines whereas others traverse the intestinal wall and are ejected in the faeces. Then the eggs hatch, if they contact with fresh water, to release miracidia, which can infect amphibious *Oncomelania hupensis* snails. A miracidium forms a sporocyst which asexually produces daughter sporocysts that migrate to the snail hepatopancreas and, following another phase of asexual reproduction, release larval cercariae into fresh water.

Important biological features of *S. japonicum* are its zoonotic nature and the fact female worms can produce thousands of eggs per day, ten times more in number than *S. mansoni* and *S. haematobium*. It is the schistosome eggs that are responsible for transmission and pathology, the latter due to the granulomas which

form around the eggs trapped in the liver and other organs. Unlike the other human schistosome species, the zoonotic transmission of schistosomiasis japonica provides a novel feature that can be utilised for the development of a transmission-blocking veterinary vaccine in domestic animals, especially bovines, to help prevent human *S. japonicum* infection and resultant disease. Bovines (cattle and water buffaloes [*Bubalus bubalis*]) are the major reservoirs, contributing about 90% of the *S. japonicum* infection source in China (Chen & Lin, 2004). A study of bovines in Samar province in the Philippines in 2010 indicated a similar picture to that found in China with more than 90% of bovines infected with *S. japonicum* (Gordon et al., 2012). It is logical to target bovines for treatment/vaccination in schistosomiasis japonica control programs because these animals produce considerably more faeces on a daily basis than do humans. The first published mathematical model of *S. japonicum* transmission dynamics predicted that, in the lakes and marshlands of the Yangtze River basin in China, a bovine vaccine with 45% efficacy (the level of many current prototype vaccines) would reduce the endemic prevalence, but would not result in elimination [10]. However, if accompanied by an initial period of human treatment and by improvements in human sanitation or a reduction in contaminated water contact by humans, elimination would be possible (Williams et al., 2002). Due to the fact that water buffaloes are responsible for much of the schistosomiasis transmission in the marshland areas of China (Guo et al., 2006), a vaccine with 48-52% efficacy targeting these bovines, used in conjunction with PZQ treatment, could lead to a significant reduction in transmission with the predicted equilibrium prevalence reduced to zero after 5 years (Da'dara et al., 2008).Despite a number of research publications, knowledge of schistosome immunology in mammalian hosts is still limited, but this is critical to further understand the mechanism of pathogenesis in schistosomiasis and the processes associated with protective immunity in order to reinforce the rationale for successful vaccine development. Improved diagnostic tests for schistosomiasis are also required that can build on a better understanding of the immune response to schistosomes so that light infections can be identified with high specificity and sensitivity so as to determine the extent of the interruption of transmission and the elimination of schistosomiasis in a particular area. Here we review prospects for the development of vaccines and new diagnostics for zoonotic schistosomiasis.

THE HOST IMMUNE RESPONSE TO SCHISTOSOMES

The immunobiology of schistosomiasis includes the nature of the host innate and adaptive response to the schistosome parasites, knowledge of which is built on infection and immunization (with schistosome extracts or defined antigens) studies mainly in mice (including wild type and gene knockouts) but also in nonhuman primates and other mammalian hosts.

As reviewed by Mo *et al*. (Mo et al., 2014), the immune response towards schistosomes comprises two separate components: 1) Immunopathogenesis and/or immunoregulation, which results from released antigens from eggs trapped in tissues. This leads to fibrosis and granuloma formation, collagen deposition

and, in the case of *S. japonicum* and *S. mansoni*, severe hepatic periportal fibrosis, morbidity chronic inflammation and anemia; and 2) Age-dependent concomitant immunity against reinfection resulting over time from repeated natural adult worm death leading to the development of partially protective natural immunity in areas endemic for schistosomiasis. The protective effect of PZQ is thought, in part, to be due to the immunity induced against the drug-killed adult schistosomes (Mo et al., 2014). This partial protection has been associated with increased eosinophilia, $CD23^+$ B cells, IL-5 and anti-adult worm antigen IgE antibodies together with low levels of IgG4 antibodies against these worm components (Mo et al., 2014).

A better understanding of innate immunity to schistosomiasis is necessary in developing strategies to protect hosts from infection or restrict immunopathology. Schistosomiasis results in a range of morbidities, most of which are not caused by the adult worms. Instead they are associated with the T-cell-dependent immune response of the mammalian host induced by schistosome eggs trapped in tissues and granuloma formation and related pathologies in target organs - mainly the liver and intestine with *S. japonicum* infection. The main immunopathology of schistosomiasis is induced by molecules secreted by the eggs, resulting in a marked $CD4^+$ T-cell mediated granulomatous inflammation involving monocytes, eosinophils, and lymphocytes, likened to a form of delayed type hypersensitivity (McManus & Loukas, 2008).

Schistosoma blood flukes depend on signals from host $CD4^+$ T cells for their growth and maturation in the mammalian host by inducing Th2-biased inflammatory granulomas (Riner et al., 2013). While B cells suppress granulomatous pathology in schistosomiasis, it remains unclear whether these cells effect schistosome maturation, reproduction and granuloma development without the aid of CD4(+) T lymphocytes (Tang et al., 2013). However, it has been shown that T and B cells play a crucial role in both generating protection and exacerbating disease outcomes by orchestrating the immune response during *S. japonicum* infection in rodent models showing resistance to the parasite (Hu et al., 2012). Following cercarial infection, the early immune response is predominantly Th1, targeted at the adult worm. After egg deposition in tissues, the Th2 response becomes prominent, suggesting that egg antigens may directly inhibit the Th1 response (Liang et al., 2012). In general, recent investigations have demonstrated that T-cell-mediated immunity is necessary to promote acquired resistance to schistosomes in the murine model, a process mediated by activated macrophages. Furthermore, cytokine studies also suggest that a schistosome vaccine that can induce activated macrophages to produce Th1 cytokines [gamma interferon (IFN-ɣ) and IL-2] may be useful in preventing disease (McManus & Loukas, 2008). It has been shown that IFN-γ and IgG2 antibodies, characteristic of Th1 responses and cytotoxicity, correlate with the high level of protection induced by an irradiated *S. japonicum* cercarial vaccine in pigs (Tian et al., 2010).

The CD4+ Th2 cellular response against schistosome egg antigens coordinates the development of granulomas which comprise cells (eosinophils, CD4+ T cells, macrophages) and collagen fibres around individual eggs (Pearce & MacDonald, 2002) (Fig.1). Dendritic cells (DCs) can activate naive CD4+ T cells

during their migration to lymphoid tissues, and acquire egg antigens, thereby inducing a Th2 response. Toll-like receptor 2 (TLR2), present at the DC cell surface, influences their maturation by inducing IL-10-secreting regulatory T cells (Kane et al., 2004) (Fig.1). However, exposure of egg antigens to DCs does not stimulate them to synthesise interleukin-12 (IL-12) or co-stimulatory molecules such as CD80, CD86 or CD40. Generation of the Th2 response depends on IL-4 from an alternative source to the DC. IL-4 limits the Th1-response and acts as a growth factor to increase the Th2 response. IL-10 produced by B cells and DCs induces regulatory T-cell activity, with the potential to suppress the Th1 cell response to helminth worm-derived antigens, thereby ensuring Th2 cell polarization (McKee & Pearce, 2004). IL-10 may have a role in suppressing IL-12 production and minimizing the progression of the Th1 response.

The roles of TLR2-MHC class II, CD40–CD154 and OX40L–OX40 are important in the generation of Th2 responses to schistosome antigens (de Jong et al., 2002). An important feature of this Th2 immunity is the induction of alternatively activated macrophage (AAM) populations, which is crucial in regulating the pathology and worm expulsion that is essential for the host surviving schistosomiasis (Horsnell & Brombacher, 2010). Interleukin-13 (IL-13) is the main Th2 cytokine that is responsible for fibrosis (Fallon et al., 2000) and a series of recent studies have elucidated that IL-13 is able to promote fibrogenesis (Hesse et al., 2000; Hesse et al., 2001; Modolell et al., 1995). IL-13 and its receptor complex have been identified as important regulators in controlling the progression of schistosomiasis (Mentink-Kane & Wynn, 2004) suggesting the possibility of IL-13-blocking therapies for the disease. A schistosome egg glycoprotein is able to induce the release from basophils of IL-4 and IL-13 by non-specifically binding and cross-linking cell-surface IgE (Schramm et al., 2003). The fibrogenic role of IL-13, together with IL-4, is essential for upregulating the expression of arginase in macrophages (Hesse et al., 2001). Arginase metabolises L-arginine to produce proline which is necessary for the formation of collagen and fibrosis development. Th1 response mediators [NO, TNF, interferon-γ (IFN-γ), IL-12] can inhibit the Th2-response and induce macrophages to express, rather than arginase, inducible nitric oxide synthase (iNOS) which uses arginine for the production of citrulline and nitric oxide (NO). During this process, L-hydroxyarginine is produced which inhibits arginase and reduces the level of expressed proline, thereby reducing collagen synthesis. The Th2 response stimulates massive blood and bone eosinophilia, increased levels of serum IL-5, and the egg-induced granuloma formation which results in collagen being deposited, tissue fibrosis, and the manifestations associated with schistosomiasis (Wilson et al., 2007). The resolution of any helminth infection is generally correlated with a Th2 immune response by the mammalian host. Recent research has shown that ~~discovered that expect eggs, -~~ schistosome worms also stimulate~~s~~ functional type 2 responses~~,~~; for example, a parasite cysteine protease is an inducer of Th2 responses at the early stage of schistosome infection (de Oliveira Fraga et al., 2010).

Natural killer T (NKT) cells are activated to proliferate by glycolipids present in both schistosome eggs and worms (Zaccone et al., 2003). NKT cells may also play roles in modulating the classical T cell response,

accompanied by the up-regulation of CD4 and down-regulation of CD94 expression in infected mesenteric lymph node (MLN) natural killer (NK) cells and enhanced expression of IL-4 and IL-17 in both the NK and NKT cells of infected mice (Luo et al., 2013). NKT cells, mast cells and eosinophils are all potential sources of IL-4 (Sabin et al., 1996). A large amount of IL-17 induced by *S. japonicum* infection in mouse pulmonary lymphocytes contributes to granulomatous inflammatory and fibrosing reactions in the liver (Chen et al., 2013a). However, IL-17 is a signature cytokine of Th17 cells and has been implicated in the induction of chronic inflammatory diseases (Zhang et al., 2012b). Severe hepatic granulomatous inflammation is associated with high levels of IL-17 (Zhang et al., 2012b) and lower IL-17 levels may result in favourable host protective responses (Wen et al., 2011). Th17 cells are able to express more IL-4 and IL-5 than IFN-γ, but not IL-10 (Chen et al., 2013b; Luo et al., 2012). It has been suggested that activated NK cells in the liver can down regulate egg-induced liver fibrosis by producing IFN-γ and killing activated hepatic stellate cells (Hou et al., 2012). It is well established that IFN-γ is essential for the development of acquired resistance against murine schistosomiasis although recent evidence suggests IFN-γ is not always a positive regulator of immune responses. In IFN-γ knockout mice, the disruption of IFN-γ signaling may up-regulate the cytotoxic T-cell-mediated immune responses to *S. japonicum* infection (Du et al., 2011). However, studies on cytokine-deficient and B-cell-deficient mice demonstrated that successful anti-schistosome vaccination required the induction of strong Th1 and Th2 responses (McManus et al., 2010). Further research has also showned that a balance between Th1, Th17 and Th2 cytokines is required for effective schistosome larval elimination in the mouse model (El Ridi et al., 2010; Tallima et al., 2009)

It should be emphasized that ~~Meanwhile,~~ the mechanisms of immune responses to ~~of~~ schistosome infections in the ~~mouse model~~ mice can not be completely ~~be~~ generalized to humans or other natural hosts~~.~~ (Lebens et al., 2004). Clinical v~~V~~accine development against schistosome infection has been hampered by a limited understanding of the mechanisms of protective immunity in humans. As reviewed by Siddiqui et al. (2011), ~~(Siddiqui et al., 2011)~~, factors predictive of resistance in humans from ~~in multiple~~ a number of immune-epidemiological studies include a high concentration of serum parasite-specific IgE, increased circulating CD23+ B cells~~-~~, eosinophilia and secretion of IL-5 in response to c~~ur~~ude worm extracts. However, whether any of these immune responses is directly involved in worm killing has not been elucidated. As arguably the most relevant nonhuman primate model for human clinical trials, the baboon has similar immune responses, ontogeny, reproductive physiology as a human and develops a human-like schistosomiasis acute syndrome and ~~the~~ chronic disease after exposure to schistosome ~~the~~ cercariae ~~of schistosome~~. The b~~B~~aboon has been used as a ”protection” model to determine the efficacy of schistosome vaccine candidates, including *S. mansoni* 28GST(Boulanger et al., 1991)~~-~~, *S. mansoni* calpain (Sm-p80) (Karmakar et al., 2014; Siddiqui et al., 2005), *S. mansoni* heat shock protein 70 (Kanamura et al., 2002) and attenuated schistosome~~s parasites~~ including attenuated cercariae (Kariuki et al., 2006) and schistosomula (Reid et al., 1995). Siddiqui and his colleagues~~,~~ showed recently that the~~s~~ Sm-p80 vaccine generates intricately balanced proinflammatory (Th17 and Th1) responses and, to a much smaller extent, antiinflammatory (Th2) types of immune responses ~~studies showed that Th1 and Th17 type immune responses are important for a vaccine-mediated protection~~

against *S. mansoni* challenge in the baboon model resulting in both prophylactic and therapeutic efficacies, while a complicated balance in Th1, Th17 and Th2 responses is required for generating the protection (Karmakar et al., 2014). While, high cost and statistical calculation associated with nonhuman primate model are major concerns with the use of nonhuman primate studies.

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Based on the current approach status for in the control of *S. japonicum* in China, endemic areas, studies of protective immunity in bovine schistosomiasis japonica are more important when considering the development of a transmission-blocking veterinary vaccine to assist in integrated control efforts of *S. japonicum* infection (McManus & Dalton, 2006). However, in contrast to murine schistosomiasis, our understanding of the immunology of schistosome infections in water buffaloes and cattle is also very limited. Recently, it has been reported that, following *S. japonicum* infection, worm burdens drop over time in water buffaloes. This self-cure phenomenon appears due to parasite clearance by both immune and non-immune factors, with evidence suggesting that most experimental animals susceptible to schistosomes develop some level of acquired immunity following a primary infection (Li et al., 2014a). However, studies of PZQ treatment and re-infection in bovines infected with *S. japonicum* in China have suggested that age-related resistance likely occurs in water buffaloes but not in cattle (Wang et al., 2006a). Furthermore, it was shown that worm length, worm recovery rate and the number of paired worms were significantly increased in yellow cattle, another natural host for *S. japonicum* in China, compared with water buffaloes, in which more serious pathological damage occurs. Immunological analysis suggested that the number of $CD4^+$ T cells, which might constitute an integral component of the immune response, may correlate with worm development in yellow cattle. A shift from Th1 to Th2 type polarized immunity was identified in yellow cattle, but not in water buffaloes infected with schistosomes (Yang et al., 2012). Based on the fact that water buffaloes are major reservoirs involved in the transmission of *S. japonicum*, further studies on the immunology of those bovines are necessary to select effective *S. japonicum* transmission vaccine candidates (such as the immune response analysis to migrating larvae, which are the likely targets of an anti-schistosome vaccine) and to determine the desirable route of immunization.

CURRENT STATUS OF VACCINE DEVELOPMENT FOR *S. JAPONICUM*

Highly effective vaccines have been developed against several tapeworm species (Vercruysse et al., 2007) indicating it is possible to generate a reliable, high level of protection against complex metazoan parasites, using defined recombinant antigens. A schistosomiasis vaccine is not yet available and substantial development efforts will be required to produce a viable product. Nevertheless, there is evidence indicating that at least partial natural human immunity can develop in schistosomiasis-endemic areas, and that part of this protective effect can be attributed to the immunity that is generated after adult schistosome worms are killed by PZQ. Furthermore, irradiated schistosome cercariae or schistosomula can confer considerable levels of protection against infection in experimental animal challenge models. For example, high levels of

protective efficacy (77.62%, 88.8% and 99.78% reduction in worm burden, liver eggs and faecal eggs, respectively) against *S. japonicum* challenge were obtained with a UV-attenuated cercarial vaccine in pigs, with vaccination evoking an effective IFN-γ response and a strong antibody-mediated response, especially in increased levels of IgG2 antibodies (Lin et al., 2011a). Earlier research in the 1990s showed that water buffaloes vaccinated with irradiation-attenuated *S. japonicum* cercariae gained weight after unattenuated cercarial challenge and developed 89% resistance to *S. japonicum* re-infection (Shi et al., 1990). In addition, Chinese bovines (including cattle and buffaloes) immunized with 36 kR gamma-irradiated schistosomula reduced the worm burden by 65-76% following a normal cercarial re-challenge (Hsu et al., 1984). A major challenge is our limited knowledge of the immunology of schistosome infections in cattle and, especially water buffaloes – due in part to the scarcity of immunological reagents for studying immune responses (McManus & Loukas, 2008). PZQ-treatment and reinfection studies of bovines infected with *S. japonicum* in China have indicated that age-related resistance may occur in water buffaloes but not cattle but whether this self-cure phenomenon has an immunological basis has yet to be determined (Li et al., 2014b). Additional studies on the immunology of buffaloes and cattle represent an important research area and these will be vital to aid in the process of selecting *S. japonicum* vaccine antigens and in defining optimum routes of immunization. In this respect, a recent study described immunological profiles in previously exposed and re-challenged water buffaloes in China and showed that the intense type-2 immune response at the site of cercarial penetration was significantly different from that seen in naive and permissive animal models, such as mice, suggesting a possible immune mechanism (McWilliam et al., 2013). This study also revealed a reduced and delayed immune response in water buffaloes given a high cercarial challenge infection compared with a moderate infection, particularly in the skin and, overall, the study provided new insights of the immunobiology of schistosomiasis in a natural host (McWilliam et al., 2013).

Of the current leading *S. japonicum* vaccine candidates, a number include membrane proteins, enzymes and muscle components (Table 1). Detailed information of the characteristics and vaccine efficacy of these and other vaccine candidates can be found elsewhere (McManus & Loukas, 2008). One of the encouraging vaccine targets is paramyosin, a 97-KD protein (Sj97), which can induce a reduction in worm numbers of 52% in mice and 50% in water buffaloes against *S. japonicum* infection. Sj97 is expressed on the schistosomular tegument and in the acetabular glands. It appears to have similar function as a Fc receptor (Loukas et al., 2001) and an exogenous form inhibits activation of the terminal pathway of complement, suggesting a key involvement in host immune evasion (Gobert & McManus, 2005). Currently, Sj97 is in early preclinical process development and in further proof-of-concept studies in mice and water buffaloes (Mo et al., 2014). Other important vaccine candidates are Sj26GST and Sj28GST which have shown encouraging protective efficacy against *S. japonicum* in different mammalian hosts (Table 1). A phase II clinical trial of Sm28GST (*S. mansoni* 28GST) has been carried out (Li et al., 2005) and phase I and II trials with Sh28GST (*S. haematobium* 28GST) were completed recently (Mo et al., 2014). Three of the leading vaccine candidates against *S. mansoni* - fatty acid binding protein (Sm14), tetraspanin (Sm-TSP-2) and

calpain (Sm-p80) - have been subjected to animal proof-of-concept studies and preclinical process development (Mo et al., 2014). Notably, none of these molecules from *S. japonicum* were able to generate effective protection against challenge infection (Liu et al., 2004; Wu et al., 2005; Zhu, 2005).

Renewed efforts have been made recently to characterize molecules that control schistosome survival, growth and reproduction in order to identify new targets for vaccine development. Accordingly, a series of recentely discovered and tested components (including Sj23LHD-GST, Sj-F1, SjCYPB, SjCE-2b, SjTGR, SjAR, SjTPx-2, SjTP22.4, SjEsRRBL1, SjPSMA5, SjB04, SjMF, SjGALE, MLP/HsP70) have been tested as vaccines, details for which are shown in Table 2.

The recent availability of genomic, transcriptomic and proteomic information has allowed the research community to gain new insights into the highly adapted relationship between schistosomes and their mammalian hosts and for identifying novel therapeutic and vaccine targets (2009; Berriman et al., 2009; Young et al., 2012). As a result, an insulin receptor was first shown present in *S. japonicum* (2009). It is striking that schistosomes absorb their dry weight of glucose from their hosts every 5 hours (Bueding, 1950), but as they are unable to synthesize insulin (Hu et al., 2003), they thus depend on host insulin to facilitate glucose uptake for metabolism, growth and fecundity (You et al., 2009). Accordingly, we have isolated two types of insulin receptors from *S. japonicum* (SjIRs) that can bind human insulin and which may be involved in modulating the process of glucose uptake in a manner similar to that in *C. elegans* and mammalian cells [56]. Most recently, we showed in a murine vaccine/challenge model that immunization with the L1 subdomain (major insulin binding domain) of the SjIR (SjLDs) fusion proteins expressed in *E. coli*, conferred highly significant reductions in faecal eggs (56-67%), in liver granuloma density (45-55%) and stunting of adult worms (12-42%), and a reduction in the numbers of mature intestinal eggs (75%) (You et al., 2012). Although we did not find a reduction in adult worm burden, our results strongly suggested that the SjLD vaccines were able to induce a significant retardation in the growth of worms and depress fecundity (egg production), emphasising their potential as encouraging transmission blocking vaccine candidates. Furthermore, we also found the SjLD vaccines could depress long-term female growth and egg production (unpublished data), thereby inducing long-lived protection against *S. japonicum* infection in the murine model, reinforcing their potential as encouraging transmission blocking vaccines. Vaccination against schistosomes can be targeted towards the prevention of infection and/or reduced parasite fecundity. A significant reduction in worm burden is regarded as the “gold standard” for development of anti-schistosome vaccines. However, as schistosome eggs are responsible for pathology and transmission, and the alteration of immune responses in disease progression in schistosomiasis, a vaccine targeting parasite fecundity and/or egg viability may perhaps represent a more realistic strategy for vaccine development (McManus & Loukas, 2008). In order to obtain optimum vaccine efficacy, one logical approach is to design multivalent vaccines targeting 2 or more antigens which promote the depression of adult parasite growth and egg production and a reduction in worm numbers.

Another lead vaccine candidate against *S. japonicum* infection that is involved in glucose metabolism is triose-phosphate isomerase (SjCTPI), which reduces worm burdens in mice (27.9%) (Zhu et al., 2002), pigs (48%) (Zhu et al., 2006) and water buffaloes (48-52%) (Da'dara et al., 2008; Yu et al., 2006). This enzyme is able to convert glyceraldehyde-3-phosphate to dehydroxyacetone phosphate, which is a key step in the glycolytic pathway, whereby a cell breaks down glucose into energy. TPI is located in most cells of schistosome worms and on the surface membranes of newly transformed schistosomula, the stage most likely to be the target of an anti-schistosome vaccine. Vaccination with a SjCTPI DNA vaccine had a significant effect in reducing female worm burdens (53.6-59.6%) and liver egg numbers (49.4%-65.8%) in the pig model of schistosomiasis japonica; in addition, the granuloma sizes in vaccinated animals were reduced by 42% (Zhu et al., 2012). The efficacy of SjCTPI and the tetraspanin, SjC23, on their own, and as fusions with the heat-shock protein 70 (Hsp70) were assessed as DNA vaccines in water buffaloes in China against *S. japonicum* challenge (Da'dara et al., 2008).The most encouraging vaccine was the SjCTPI-Hsp70 construct, that generated 51.2%, 61.5% and 52.1% reduction in worm burden, hepatic eggs and faecal eggs respectively (Da'dara et al., 2008). The SjCTPI-Hsp70 vaccine is currently undergoing field testing in bovines in China and the Philippines.

It is now clear that several *S. japonicum* vaccine candidates are able to induce levels of between 50 and 70% protective efficacy in vaccination/challenge experiments with mice and larger mammalian species ,with further immunization boosts increasing these levels so that their further development for veterinary use is a realistic proposition (McManus & Loukas, 2008). However, further study is necessary on the development of multivalent vaccines and novel adjuvants to improve on these levels of protection ~~levels~~ (Siddiqui et al., 2011; You et al., 2014). Furthermore, ~~Nevertheless,~~ there are some challenges that will need to be overcome, including the possible risk of atopic IgE responses generated by the various vaccines, the use of appropriate adjuvant/vaccine formulations, whether vaccine efficacy is reduced due to co-infection with other pathogens in schistosomiasis-endemic areas, and the practical requirement of developing a vaccine that can be given as a single dose, ideally orally, without the requirement of boosting.

DEVELOPMENT AND TESTING OF NEW DIAGNOSTICS FOR SCHISTOSOMIASIS

To date, selective chemotherapy with PZQ is one of the components of schistosomiasis control programs so that correct diagnosis of infected individuals is important. However, a sensitive and specific assay for field diagnosis of schistosomiasis japonica that is simple and affordable is not currently available. This poses a barrier to control efforts leading to schistosomiasis elimination, especially when the schistosome prevalence drops to low levels, as is now occurring in China [41]. Hence, the search for novel diagnostic tools for schistosomiasis is recognised as an urgent priority. Generally, *S. japonicum* infections can be detected by three approaches: parasitological, immunological and molecular.

Parasitological methods

Detection of eggs in stool samples is the customary method for diagnosing schistosome infections. The Kato-Katz thick smear technique (KK) is the most widely used procedure, being the standard method recommended by the World Health Organization (WHO) for both qualitative and quantitative diagnosis of intestinal schistosomiasis. However, the sensitivity of the KK method can vary from 40% to 100%, with the negative predictive values ranging from 52.5% to 100% (Zhou et al., 2007). Furthermore, if only a single KK slide is prepared from a single stool specimen, sensitivity is low (Lin et al., 2011b). This leads to a marked underestimation of the prevalence and intensity of infection, particularly in low prevalence areas (Lier et al., 2008), and this can confound confirmation of cure after PZQ treatment (Berhe et al., 2004). Ideally, multiple faecal examinations (typically 2 faecal samples per individual; 3 KK slides each sample) should be undertaken to reduce the false-negative results, but this is time and labour intensive and compliance drops with the number of stool samples requested. If an endemic population is first screened serologically by indirect hemagglutination assay (IHA) or enzyme-linked immunosorbent assay (ELISA) and seropositives confirmed by the KK method, correlation analysis indicates that the positivity rate with KK rises with the number of faecal specimens and slides used. Those individuals that were egg-positive but negative by IHA or ELISA were mainly cases with low infection intensity (Zhang et al., 2012a).

It is important to obtain purified eggs free from faecal components in order to increase diagnostic sensitivity and improve microscopic visualization of *S. japonicum* eggs. Two novel egg purification methods were recently described, which have potential for use in areas with low infection intensity, or where there is suspected elimination of schistosomiasis japonica following control efforts. The formalin-ethyl acetate sedimentation-digestion (FEA-SD) technique (Xu et al., 2012) is effective for identifying and quantifying *S. japonicum* eggs in faecal samples from infected bovines in endemic areas. FEA-SD removes about 70% of debris from faecal samples and the remaining material is translucent. Another method for *Schistosoma* egg detection has been developed that is based on magnetic fractionation of parasite eggs from faecal matter (Fagundes Teixeira et al., 2007). With this approach, termed Helmintex, magnetic microspheres are mixed with faecal samples to make parasite egg-magnetic microsphere conjugates. The magnetic microspheres and eggs are then co-purified from other faecal material using a magnetic field and field gradient. The concentrated and purified eggs are more easily detectable by microscopy. This method is able to screen larger sample volumes, resulting in improved diagnostic sensitivity, although the mechanism of formation of the conjugates is unknown but may reflect the specific surface features of eggs and microspheres, or to their magnetic properties (Karl et al., 2013).

Immunological methods

Patent schistosome infections are generally highly immunogenic, and anti-schistosome antibodies can be readily detected in subjects using a wide range of immunodiagnostic techniques. Many variations of indirect immunological approaches in schistosomiasis diagnosis include the circumoval precipitin test (COPT) and

the indirect hemagglutination assay (IHA) which have been widely used historically, and ELISA and Dipstick Dye Immunoassays (DDIA), which have been used more frequently in the last 20 years. ELISA, using soluble egg antigen (SEA) as the source of target antigen, is the most widely used technique currently (Doenhoff et al., 2004). Heat shock protein 70 (HSP70) and 78 kDa glucose-regulated protein, both present in SEA, may have diagnostic value for detecting early *S. japonicum* infections (Wang et al., 2012). The modified fast ELISA (F-ELISA), which combines the 2-step routine ELISA into a single step making the assay process faster and simpler without sacrificing diagnostic accuracy (Hua et al., 2011), may provide a rapid and practical method for schistosomiasis diagnosis in the field. Recently, more rapid, sensitive and portable diagnostic assays to detect human antibodies against *S. japonicum*, have been developed and tested in areas of low endemicity for schistosomiasis japonica in China (Table 3). These methods include: 1) DDIA (Xu et al., 2011) which is commercial available in China, and the dipstick with latex immunochromatographic assay (DLIA) (Yu et al., 2011); 2) Magnetic affinity enzyme-linked immunoassays (MEIAs), based on the signal transduction protein 14-3-3 (Sj14-3-3), recombinant 26kDa glutathione-S-transferase (rSj26GST) (Yu et al., 2012a), or soluble egg antigens (SEA-MEIA) (Yu et al., 2012b); 3) A time resolved fluoroimmunoassay (TRFIA), established for detecting Sj14-3-3, as a circulating antigen in serum (Qian et al., 2011); 4) An electrochemical immunosensor array (ECISA) assay (Deng et al., 2013) which uses a recombinant *S. japonicum* calcium-binding protein (SjE16), with SEA, co-immobilized on a disposable 16-channel screen-printed carbon electrode array. Antibodies in serum samples can be detected by a portable electrochemical detector; 5) A multiplexed bioanalytical assay which is developed by fusing two types of gold nanorods (GNRs) (Huang et al., 2012). This technique allows the serum-based diagnosis of subjects infected with *S. japonicum* without the need for sample pretreatment and it can diagnose individuals co-infected with schistosomiasis and TB.

Serodiagnosis of schistosomiasis suffers from a number of drawbacks common to the antibody-based detection of other parasitic infections (Doenhoff et al., 2004; Rabello & Enk, 2006). One difficulty is in identifying an active from a previous infection, as parasite-specific antibodies remain detectable long after cure, and another is the inability to quantify infection intensity. A range of approaches to improve the accuracy of immunodiagnostic assays have been described. These include using specific parasite extracts such as cercarial antigens (Chand et al., 2010), using recombinant proteins as immunodiagnostic targets (Zhou et al., 2010), or using a pool of synthetic peptides selected on the basis of the amino acid sequence of proteins from different antigenic schistosome preparations (de Oliveira et al., 2008). Another approach that has been used with success in China is to combine information from serological surveys with parasitological data and to use a Bayesian statistical approach to develop accurate epidemiological maps of infection prevalence (Wang et al., 2006b). So far, a number of candidate antigens have been tested for diagnosing *S. japonicum* infection; these include Sj23HD(Wang et al., 2011b), rSj26GST-Sj32(Cai et al., 2011), SjEFCAB(Lu et al., 2012b), SjTPx-1 (Angeles et al., 2012) and Sj1TR (Angeles et al., 2012), Sj7TR(Angeles et al., 2011), SjCHGCS19 (Guo et al., 2012), SjLAP (Zhang et al., 2011), whose

characteristics are shown in Table 4. Recentely, Xu et al (2014) undertook a genome-wide survey to discover diagnostic protein markers for *S. japonicum* infection. In the study, 204 *S. japonicum* predicted secreted proteins (SjSPs) were arrayed on glutathione-immobilised microplates and screened with 302 patient serum samples that were diagnosed by the Kato-Katz method as egg-positive. One protein, SjSps-13, was identified as a potential diagnostic protein marker with 90.4% sensitivity and 98·9% specificity and its diagnostic validity was tested in ELISA by using 1371 resident samples in a field study. The current scarcity of effective diagnostic tools is a dominant factor precluding the effective management of schistosomiasis (Colley et al., 2014) so the application of this newly developed sensitive, specific, and affordable rSP13-ELISA method should help reduce schistosomiasis transmission through targeted treatment of individuals, particularly with low intensity infections, and therefore support schistosomiasis control and elimination strategies (Xu et al., 2014).

An early study using immunoassays demonstrated the presence of schistosome-derived antigens in the circulation and/or excreta of hosts with schistosomiasis (Deelder et al., 1976), and this report stimulated considerable research on antigen detection as a means of diagnosing the disease. Indeed, detection of schistosome circulating antigens (CAs) has now been shown to be an efficient method to differentiate between previous exposure and current infection. In one approach, anti-adult worm antigen (AWA) IgY (egg yolk immunoglobulin) was generated by immunization of hyline chicken hens with AWA. The purified IgY was then immobilized onto resin to immune-precipitate CAs in sera from infected subjects. Four proteins including protein BUD31 homolog, ribonuclease, SJCHGC06971 protein and SJCHGC04754 protein were isolated from the precipitated proteins that could be employed as diagnostic biomarkers (Lu et al., 2012a). A novel immunomagnetic bead ELISA, based on IgY, has also been used for detection of circulating CAs in sera or urine of hosts infected with *S. japonicum* (Lei et al., 2012; Lei et al., 2011) (Table 3).

Molecular methods

Some of the deficiencies of currently available diagnostic methods for intestinal schistosomiasis are that: 1) Early diagnosis of the disease using egg detection methods is problematical. Generally, egg production within the host takes several weeks as does the passage of eggs through the gut wall, their discharge into the intestinal lumen and their release in faeces; 2) Variability in egg shedding and problems in distribution of eggs in the analysed sample often leads to unreliable results when microscopic examination for eggs is performed; 3) As emphasised earlier, serological tests, based on antibody detection, do not discriminate between active infection and previous exposure. Accordingly, the application of the polymerase chain reaction (PCR) as a tool for the diagnosis of schistosomiasis has been explored for detection of schistosome DNA in human and bovines faeces (Fung et al., 2012), in serum/plasma (Wichmann et al., 2009) and in urine (Obeng et al., 2008) and the approach has proven to be of value (Xu et al., 2013). Some of these

highly sensitive and specific PCR-based methods have been assessed in areas with medium or low intensity of schistosome infection. A combination of real-time PCR (qPCR) and the earlier described FEA-SD technique (Xu et al., 2012) was used in the Philippines to determine the prevalence and intensity of *S. japonicum*, thereby providing a more accurate diagnosis to evaluate the potential role of bovines in the transmission of *S. japonicum* (Gordon et al., 2012). It should be stressed, however, that PCR-based methods give positive results only if the analysed faecal sample contains schistosome DNA and inhibitors present in faeces may affect the PCR assay working optimally.

In another diagnostic approach, Wichmann *et al* (Wichmann et al., 2009) used real-time PCR to detect cell free parasite DNA (CFPD) in human plasma according to the principle that *Schistosoma* worms contain DNA copies, which may be released due to parasite turnover and reach the blood, in stoichiometrical excess over parasite count and that schistosome DNA. This method may provide a new laboratory tool to detect schistosome infection in all phases of clinical disease (Wichmann et al., 2009) .

Another molecular approach is loop-mediated isothermal amplification (LAMP), a highly sensitive DNA detection method that is proving of value for the diagnosis of a number of pathogenic organisms including schistosomes. A LAMP assay has been established that detects *S. japonicum* DNA in faecal and serum samples of rabbits and in sera of infected human subjects. Based on the sequence of a highly repetitive retrotransposon, SjR2, the LAMP method was considerably more sensitive than traditional PCR being able to measure 0.08 fg *S.japonicum* and appropriate for clinical diagnosis and therapeutic evaluation of human schistosomiasis (Xu et al., 2010). LAMP appears suitable for the detection of early *S. japonicum* infection in certain patients including travellers, migrants, military personnel and younger aged subjects but it is likely to be of less value for determining the efficacy of drug treatment in the early stages because of its high sensitivity (Wang et al., 2011a).

Recently, circulating microRNAs (miRNAs) have attracted attention as novel biomarkers for the diagnosis of schistosomiasis and also for further understanding the host-schistosome interaction. Deep sequencing identified five schistosome-specific miRNAs (Bantam, miR-3479, miR-10, miR-3096; miR-0001) in the plasma of *S. japonicum*-infected rabbits, four of which were detectable by real-time RT-PCR in the plasma of mice infected with *S. japonicum* (Cheng et al., 2013). Another miRNA molecule, miR-223, was identified by He *et al* (He et al., 2013) as a potential new biomarker of *S. japonicum* infection and the assessment of the response to PZQ treatment. Parasite-derived miRNAs have also been identified (Anna M. Hoy, 2014) as novel markers of *S. mansoni* infection in mice and humans, some of which could distinguish 'egg-negative' from 'egg-positive' individuals with high specificity and sensitivity with potential diagnostic value.However, this study showed that, whereas several host miRNAs were shown to be dysregulated in the livers of mice during *S. mansoni* infection, they were unable to serve as reliable serum biomarkers of infection in humans (Anna M. Hoy, 2014). These data contrast with those of Han *et al*. (2013) who applied a miRNA microarray to investigate differences in miRNA expression in different tissues, including the liver,

of mice before and 10 days post infection with *S. japonicum* and detected a total of 220 miRNAs in the different tissues whose functions correlated with nutrient metabolism, the immune response, apoptosis, signalling pathways and cell differentiation (Han et al., 2013). As pointed out by Hoy et al. (2014), these conflicting results may have been due to the very early time point (10 days post infection) used in the *S. japonicum* study.

FUTURE DIRECTIONS

The key to eliminating schistosomiasis is to reduce transmission combined with morbidity control, an approach that is more cost effective than treating the clinical outcome of continued reinfection, and which is currently being used successfully in China (Sun et al., 2011). Central to this goal is to integrate traditional control measures–PZQ treatment, the use of molluscicides, environmental modification, health education/promotion, enhanced water supply (Kosinski et al., 2012) and improved sanitation – with an effective vaccine, a tool that is needed to accelerate intervention efforts to eliminate a disease that has existed for many centuries. In this respect, the Chinese experience serves as a good model, whereby a comprehensive control approach based on interventions to block transmission of *S. japonicum* infection from bovines and humans to snails has proven highly effective (Seto et al., 2011). As emphasised earlier, it has been established that bovines are the major animal reservoir host for *S. japonicum* in China and the Philippines (McManus & Loukas, 2008) and this fact underpins efforts to develop a bovine transmission blocking vaccine against *S. japonicum* as an effective and feasible objective. Recent developments in the preclinical and clinical testing of existing anti-schistosome vaccine candidates have been encouraging (Mo et al., 2014). Furthermore, the most recent comprehensive understanding of schistosome genomes and proteomes has equipped us with all the information for antigen choice as novel vaccine targets. Based on the fact that the membrane proteins located on the tegument of the schistosomulum and the adult worm are rational vaccine targets (Loukas et al., 2007), we need to select the best antigen or combined antigens as a schistosomiasis vaccine by using other innovative tools. Defining a clear target product profile (TPP) for an optimum schistosomiasis vaccine and using new technologies and tools which are available for new antigen discovery, clinical research, and vaccine efficacy assessment will all contribute to accelerated progress (Mo et al., 2014). Effective schistosomiasis vaccines and new drugs that act on both adult and larval schistosomes would help to achieve and sustain disease control and eventual elimination. Importantly as well, further research, similar to that described by Xu et al (2014), is required to develop improved diagnostic tests that are able to identify light *S. japonicum* infections so as to determine the extent of the interruption of transmission in an endemic area and to establish whether control efforts have led to schistosomiasis elimination.

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Figure legend

Fig.1 Predicted model of the Th2 immune response induced by schistosome egg antigens. Granulomatous lesions comprise collagen fibres and host cells, (macrophages, eosinophils and CD4+ T cells, coloured in green) around the egg. Dendritic cells (DCs), can induce T helper 2 (Th2) responses by activating naive CD4+ T cells. Toll-like receptor 2 (TLR2) located at the surface of DCs can be activated by egg secreted proteins that influence their functional maturation by inducing regulatory T cells to secrete IL-10 (Kane et al., 2004). IL-10 so generated may suppress IL-12 production, which is a potent inhibitor of Th2 responses, and minimizes the progression of the Th1 response. The interactions of CD40–CD154 and OX40L–OX40 are also important in the induction of Th2 responses to schistosome antigens (de Jong et al., 2002). However, the exposure of DCs to egg antigens does not stimulate their expression of the co-stimulatory molecules CD40, CD80 or CD86. Induction of alternatively activated macrophage populations is a dominant characteristic of Th2 immunity. Development of the Th2 response depends on IL-4 from a source other than DCs and the main Th2 cytokine responsible for fibrosis is IL-13 (Fallon et al., 2000). A schistosome egg glycoprotein can induce the release of IL-4 and IL-13 from basophils and the fibrogenic role of IL-13 seems to be important, together with IL-4, to induce the expression of arginase in macrophages (Hesse et al., 2001). Arginase uses L-arginine to make proline which is an essential amino acid associated with collagen production and fibrosis. Mediators that are involved in Th1 responses, such as interferon-γ (IFN-γ), IL-12, TNF and NO can hamper Th2-response development and also stimulate the expression by macrophages of inducible nitric oxide synthase (iNOS) which uses arginine for the production of citrulline and nitric oxide (NO). During this process, L-hydroxyarginine is produced which inhibits arginase and reduces the level of expressed proline, thereby reducing collagen synthesis. The Th2 response results in an increase in the level of serum IL-5, tissue fibrosis accompanied by massive blood and bone eosinophilia. Natural killer T (NKT) cells, eosinophils and mast cells are all potential sources of IL-4 (Sabin et al., 1996). As a signature cytokine of Th17 cells, IL-17, induced by *S. japonicum* infection in mouse pulmonary lymphocytes, contributes to granuloma formation and fibrosis in the liver (Chen et al., 2013a). Th17 cells express more IL-4 and IL-5 than IFN-γ, but less IL-10 (Chen et al., 2013b; Luo et al., 2012).