Characterisation of the proteomic composition of *Schistosoma haematobium* extracellular vesicles: towards the development of vaccine and diagnostic candidate antigens

PhD Thesis

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Abstract

Schistosoma haematobium is the leading cause of urogenital schistosomiasis, which affects over 100 million people in tropical developing countries, and it is recognised as a class 1 carcinogen due to the robust association of infection with bladder cancer. Proteins and other components, such as extracellular vesicles (EVs), present in the tegument and/or secreted by schistosomes enable the worms to survive in the host for years and studies documenting the proteomic composition of different helminths EVs have revealed many proteins involved in host-parasite interactions. Interestingly, crude EVs and EV membrane proteins, including tetraspanins (TSPs), of helminths can also be recognised by samples from infected animals, suggesting the potential usefulness of EVs for the development of novel diagnostic strategies. Moreover, EVs from helminths also contain vaccine candidate antigens, including TSPs, and immunisation of animals with EVs can reduce parasite burdens and pathogenesis in some models, demonstrating the vaccine potential of EV proteins. Herein, I have characterised the proteomic composition of *S. haematobium* adult worm EVs and assessed the vaccine and diagnostic potential of some of the characterised proteins.

In chapter two, *S. haematobium* EVs, comprising both small EVs (sEVs) and medium/large EVs (m/lEVs), were purified from adult worms excretory/secretory products (ES). The size of each vesicle population was determined by qNano analysis; *S. haematobium* sEVs ranged from 136 nm ± 12.4 to 191 nm ± 27.4 while the average size of m/lEVs was 249 nm ± 22.7. Following proteomic analysis, the most represented domains from sEVs were homologues of other helminths vaccine and drug targets, such as proteasome subunit, TSP family, ferritin-like and cytosol aminopeptidase domains. The most represented domains in *S. haematobium* m/lEVs were proteins involved in EVs biogenesis and release, including EF-hand, Ras family, TCP-1/cpn60 chaperonin family and TSP family domains. In addition, *S. haematobium* EVs

contained homologues of other helminth vaccine candidates, such as glutathione S-transferase (GST), TSPs, Saposin B domain-containing protein and calpain.

In chapter three, six *S. haematobium*-TSPs (*Sh*-TSPs) (*Sh*-TSP-2, MS3_09198, MS3_01153, MS3_01370, MS3_05226 and MS3_05289) were selected from the proteomic analysis and further characterised. Based on their phylogenetic analysis, *Sh*-TSP-2, MS3_09198 and MS3_01370 grouped in the CD63 lineage of TSPs whereas MS3_05289, MS3_05226 and MS3_01153 clustered under the uroplakin family of TSPs. The expression levels of these *Sh*-TSPs were determined and all molecules were expressed throughout all life stages tested with different expression levels. The large extracellular loop (LEL) of each *Sh*-TSP was expressed in a bacterial expression system and polyclonal antibodies were raised to each molecule and used in localisation studies to determine the sites of anatomic expression. *Sh*-TSP-2 and MS3_05289 were identified on the tegument of the worms, whereas MS3_01370, MS3_01153, MS3_09198 and MS3_05226 were identified both on the tegument and internal tissues of adult worms.

In chapter four, the vaccine efficacy of three *Sh*-TSPs (*Sh*-TSP-2, MS3_09198 and MS3_01370) that clustered with known *S. mansoni, S. japonicum* and *O. viverrini* vaccine candidates was assessed using a homologous (hamster/*S. haematobium* challenge) model of infection. Furthermore, the cross-species protective efficacy of these *Sh*-TSPs was assessed in a heterologous (mouse/*S. mansoni* challenge) model of infection. Vaccination with *Sh*-TSPs did not significantly reduce adult worms burdens in the hamster model but significantly reduced the liver egg burden in both MS3_01370 and *Sh*-TSP-2 vaccinated groups (77.8% - $P<0.01$ and 52.27% - $P<0.05$, respectively). In two independent mouse trials, only the MS3_01370 vaccinated group (trial 1) displayed a significant reduction in adult worm burden (22% - $P<0.05$). Liver egg burden decreases were observed in groups vaccinated with *Sh*-TSP-2 (trial 1, 32% - $P<0.01$; trial 2, 49% - $P<0.001$), MS3_01370 (trial 1, 39% - $P<0.05$; trial 2,

54% - P<0.001) and MS3_09198 (trial 1, 49% - P<0.001; trial 2, 27% - P<0.05). Similarly, intestinal egg burden decreases were observed in groups vaccinated with *Sh*-TSP-2 (trial 1, 54% - P<0.001; trial 2, 27% - P<0.05), MS3_01370 (trial 1, 57% - P<0.01; trial 2, 36% - P<0.01) and MS3_09198 (trial 1, 51% - P<0.01; trial 2, 39% - P<0.05)

In chapter five, the diagnostic efficacy of these *Sh*-TSPs was assessed by ELISA using serum from mice experimentally infected with *S. haematobium* and urine from infected individuals from an *S. haematobium*-endemic area of Zimbabwe. *Sh*-TSP-2, MS3_01370 and MS3_09198 were recognised by the serum of experimentally infected mice compared to serum from uninfected mice. To assess cross-reactivity with *S. mansoni*, an indirect ELISA was performed using the serum of mice experimentally infected with *S. mansoni*. Only MS3_09198, MS3_01370, MS3_05226 and MS3_01153 were recognised by antibodies from *S. mansoni* infected mice. The diagnostic efficacy of *Sh*-TSPs was further tested by their recognition of antibodies in urine from infected human subjects from an endemic area in Zimbabwe and all *Sh*-TSPs, each tested in isolation or combination, were recognised by infected individuals, including those with very low levels of infection (those positive for circulating anodic antigen but negative for eggs).

Publication relevant to this thesis

Mekonnen GG, Pearson M, Loukas A, and Sotillo J (2018) Extracellular vesicles from helminths and their potential utility as vaccines. Expert Review of Vaccines, 17 (3): 197-205.

Publication not directly related but relevant to this thesis

Sotillo J, Pearson MS, Becker L, **Mekonnen GG,** Amoah AS, van Dam G, et al. (2019) In-depth proteomic characterisation of *Schistosoma haematobium*: Towards the development of new tools for elimination. PLoS Neglected Tropical Diseases, 13(5): e0007362.

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Chapter 1: Introduction and literature review

1. 1. *Schistosoma haematobium* and extracellular vesicles from helminths

Schistosomiasis is a parasitic disease caused by blood dwelling trematodes from the genus *Schistosoma*. Six species of schistosomes can infect humans, including *Schistosoma haematobium, Schistosoma japonicum, Schistosoma mansoni, Schistosoma mekongi, Schistosoma intercalatum* and *Schistosoma guineensis* [1, 2]. The three most important species, in terms of morbidity and prevalence that can infect humans are *S. haematobium,* which causes urogenital schistosomiasis, and *S. mansoni* and *S. japonicum,* both causing intestinal schistosomiasis [2, 3]. While *S. mansoni* is prevalent in Africa, South America and the Middle East, *S. japonicum* occurs in Asia (primarily the Philippines and China) and *S. haematobium* infection is endemic in Africa and the Middle East (Fig 1.1).

Schistosomiasis is the second most important parasitic disease, only after malaria, in terms of social, economic and public health impact [4]. Two hundred and fifty two million people are already infected, and 700 million people live in areas at risk [5]. Most of the schistosomiasis burden is found in Sub-Saharan Africa (SSA) [6], where around 280,000 people die annually due to the disease, particularly school-age children, adolescents and young adults [5], having important effects in school performance and social and economic development [7].

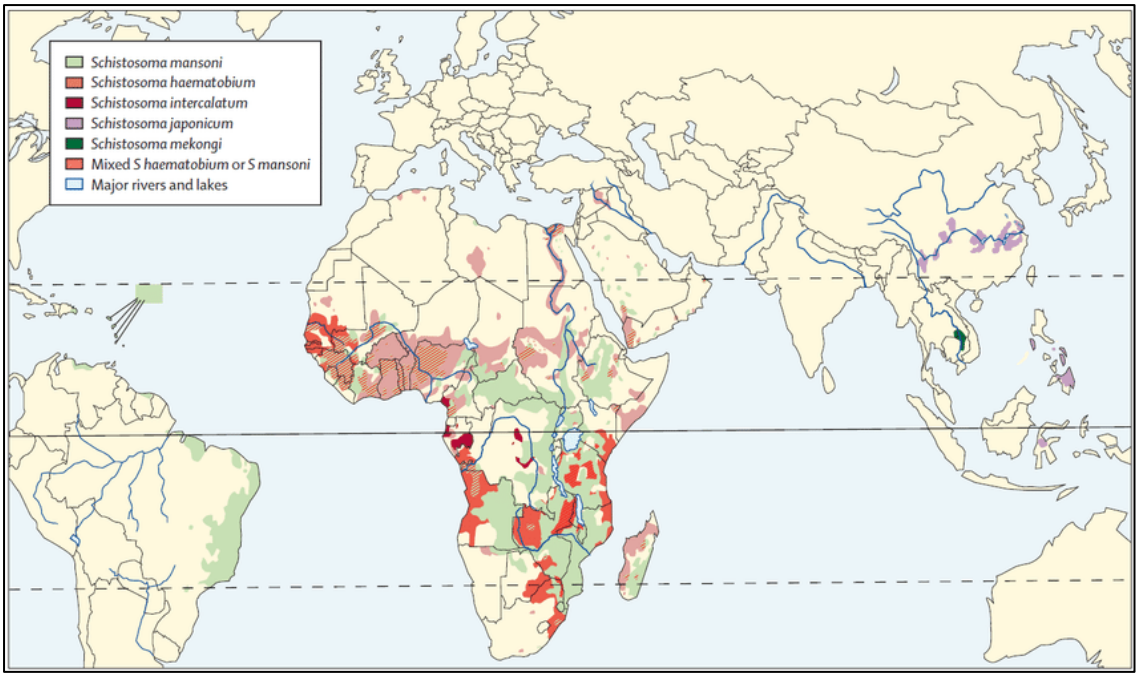

Figure 1.1. Distribution of *Schistosoma* species. Adapted from [8].

Each schistosome species relies on a specific snail as intermediate host to complete its life cycle, and their dissemination is demarcated by their snail's host territory [9]. The aquatic freshwater *Biomphalaria* and *Bulinus* snails are intermediate hosts for *S. mansoni* and *S. haematobium,* respectively, while *Oncomelania* species are the intermediate hosts for *S. japonicum* [8, 9]. The snails harbour different stages of the life cycle and liberate the infective stage (cercariae); while humans and other definitive hosts get infected when they come into contact with water containing cercariae during fishing, farming, swimming, washing and bathing [9].

Praziquantel is the only available drug for the treatment of schistosomiasis; however, it has a high cure rate by acting against the mature worms, but it is not effective against juvenile stages. A dose of 40 mg/kg is effective for the treatment of *S. mansoni* and *S. haematobium* infections, whereas 60 mg/kg is the recommended dose to treat *S. japonicum* and *S. mekongi* infection [10].

S. haematobium adult worms live in the perivesicular veins, whereas *S. mansoni* and *S. japonicum* adult worms live in the intestinal mesenteric veins [2, 11]. These worms can survive

in the host for years, using evasion strategies to remain undetected by the host immune system [12, 13], including the production of immunomodulatory proteins, which are present on their tegument and/or secreted by the worms. Due to the crucial role that proteins of the tegument and excretory/secretory (ES) products play in evading the host immune system, they have been the focus of different studies aimed at understanding host–parasite interactions and developing new therapeutic strategies [14-22]. The secretory products are substances that are secreted from parasite cells or glands and have defined biological roles whereas the excretory products are metabolic wastes that are released from the body [23]. Since schistosomes have a blind gut, the metabolic products are eliminated by regurgitation, therefore, the ES products consist of a mixture of secreted worm proteins and metabolic products occurring from physiological processes within the parasite [18].

It has been recently shown that the ES products released by schistosomes, are not only a mixture of soluble proteins, lipids and glycans, but also contain extracellular vesicles (EVs) [24, 25], similarly to what happens in other parasites [26, 27]. EVs are membrane-bound structures of variable size (from 30 nm to 1,000 nm) that are released into the extracellular space by many different types of cells. They contribute to the transmission of bioactive molecules including proteins, lipids, DNA, and RNA between cells [28]. In mammals, they play a role in the maintenance of physiological processes and are also involved in the pathophysiology of diseases [29, 30]. Diverse parasites produce EVs, which can be internalised by host cells and can modulate the host immune response [26, 31]. These EVs are also involved in the pathology of parasitic disease and have great potential as new diagnostic tools and therapeutic agents against different parasitic pathogens [32].

1.2. *Schistosoma haematobium*

1.2.1. Epidemiology of *Schistosoma haematobium* infection

S. haematobium, the causative agent of urogenital schistosomiasis, is highly prevalent in 53 Middle East and African countries [2] and it is also sporadically seen in India [33] and France [34]. Urogenital schistosomiasis affects more than 90 million people, mostly in SSA, and 180 million inhabitants live in endemic areas and are at risk [35]. Of these, around 70 million, 18 million, 9.6 million and 32 million people suffer from schistosomal haematuria, bladder wall pathology, hydronephrosis, and dysuria, respectively; but, more importantly, urogenital schistosomiasis kills around 150,000 people per year [35].

1.2.2. Transmission and life cycle of *Schistosoma haematobium*

Humans acquire urogenital schistosomiasis when they come into contact with water containing cercariae. Cercariae are shed by the snail host and once shed, cercariae survive for up to 1-3 days [36]. They attach to and penetrate the human skin within 3–5 min [2], losing their tail and developing into schistosomula, which migrate through the bloodstream via the lungs to the liver and develops into mature worms in the portal vein. Finally, the adult worms migrate to the perivesicular veins and, after 4–7 weeks of infection, they start laying eggs [2]. The average survival time of the adult parasite in the host is 3–5 years, but they can survive for up to 30 years [37]. Some of the eggs get excreted through urine while the rest of the produced eggs are carried away with the bloodstream and trapped in the tissues of different organs [8]. These trapped eggs in the tissues are the primary cause of pathology in humans. When the eggs are excreted into fresh water, they release the free-swimming miracidium stage, which locates a snail host using external stimuli such as snail-derived chemicals and sunlight. After they penetrate snails, miracidia develop into sporocysts, which undergo several rounds of asexual reproduction to finally develop into cercariae (Fig 1.2) [8].

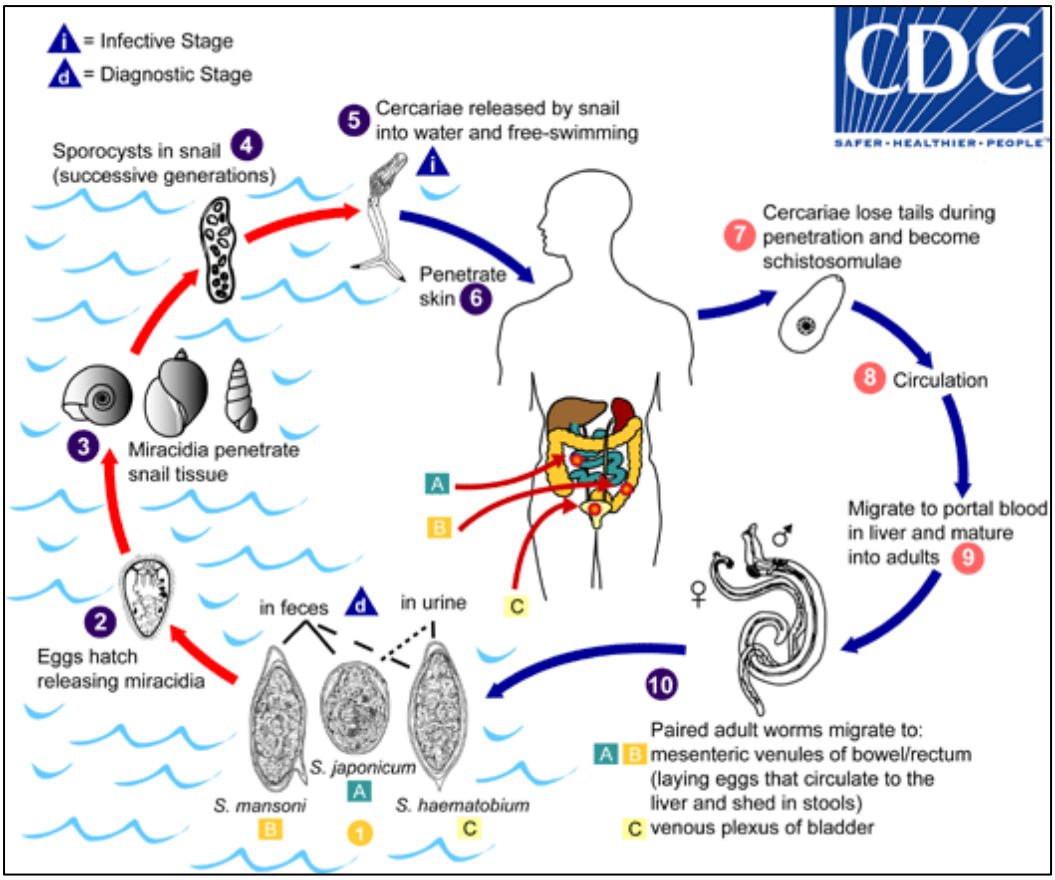

Figure 1.2. Life cycle of *Schistosoma* species. Adapted from. https://www.cdc.gov/parasites/schistosomiasis/biology.html

1.2.3. Immunopathology of *Schistosoma haematobium* infection

Cercariae enter into the human host and develop into schistosomula, which migrate through the bloodstream via the lungs to the liver and develop into mature worms in the portal vein [2]. During this time the host immune system is exposed to many antigens, however, in *S. haematobium* infections, the host immune response against cercariae, schistosomula and adult worm is limited compared to the response raised against the egg stage [38]. In non-human schistosomes, a hypersensitivity response against cercariae can cause swimmer's itch [39] and in *S. mansoni*, the immune response against schistosomula can cause Katayama syndrome [40]. However, both symptoms are rare in *S. haematobium* infections, which is usually asymptomatic until egg production [41]. Cercariae activate the complement cascade when they enter the host.

Specifically, the membrane attack complex (C5–C9) acts against the cercariae, but they escape the immune response by rapidly discarding the glycoprotein cover (glycocalyx) from its membrane [42]. In *S. mansoni*, the schistosomulum covers itself with a new membrane, becoming resistant to the host's immunological attack while developing into adult flukes [43]. Adult *S. haematobium* stimulates the host's immune system eliciting an IgE antibody response, which is important against re-infection but cannot remove the parasite from the infected individuals [44]. Antibody response against *S. haematobium* infection is enhanced by drug treatment because *S. haematobium* worms killed by drugs such as praziquantel release antigens that motivate long-lived plasma cells to produce antibodies that can reduce *S. haematobium* fertility [45]. However, antibody production in *S. haematobium* infection is age-dependent and higher serum levels of IgM, IgG2 and IgG4 isotypes in children younger than 13 years block the protective effect of IgE [46].

Unlike the acute stage of the infection, eggs produced by adult *S. haematobium* stimulate a robust immune response within 24 h from local and recruited innate immune cells [47]. In an experimental animal model, at the early stage of egg production, the character of the initial immune response is mixed, with inflammatory mediators such as tumour necrosis factor (TNF-α) as well as cytokines involved in T helper type 2 (Th_2) responses [48]. Another mouse model of *S. haematobium* showed that cercariae infection combined with egg bladder wall injection stimulates local and systematic immune responses that are distinct from responses to egg injected bladder wall alone [49]. After 4-7 days of oviposition, eggs are engulfed by multinucleated macrophages, which are in turn surrounded by epithelioid macrophages and mixtures of eosinophils and neutrophils. Immune cells surrounding the eggs form a granuloma, which separates eggs from the surrounding tissue [48]. Then, a T helper type 1 (Th_1) immune response ensues to eliminate the trapped egg, but the alteration of the immune response from Th_1 to Th_2 restricts this process [50]. In *S. mansoni*, IPSE/alpha-1 glycoprotein and omega-1

are responsible for the driving of a Th_2 response [51-55]. The *S. haematobium* homolog of IPSE/alpha-1 glycoprotein has also been found in the mature eggs and it infiltrates HTB-9 bladder cells and translocate to the nucleus [56] where it is thought to drive a Th_2-biased immune response. IL-4 shifts the immune response by suppressing the differentiation of naive T cells to IFN-γ producing cells and by stimulating differentiation of naïve T cells into IL-4-producing cells [57]. IL-13 production stimulates activation of myofibroblast-like cells and accumulation of collagen, which ends up in tissue fibrosis [58]. Later on, IL-10 and regulatory T cells suppress the progression of chronic bladder fibrosis but in individuals with a polymorphism in the promoter gene for ficolin-2 the modulatory effect of IL-10 is suppressed [59], which may indicate that people with a polymorphism in the promoter gene for ficolin-2 can suffer from bladder fibrosis during *S. haematobium* infection.

The eggs trapped in capillary beds of urogenital organs stimulate the immune response. These immune responses against eggs trapped in the tissue lead to the formation of a granuloma that can lead to tissue fibrosis [48].Granulomas and sandy patches (small fibrotic nodules) cause inflammation (specially in the bladder) that lead to haematuria in some infected individuals [60]. The granuloma formed affects the bladder wall, leading to secondary urinary tract infection, renal infections, hydronephrosis, obstruction and general urinary dysfunction, which can progress to obstructive renal pathology [60, 61]. Furthermore, infected individuals can also develop squamous cell carcinoma [62].

In female patients urogenital schistosomiasis can increase the risk of acquiring HIV infection [63]. Urogenital schistosomiasis has been suggested to be a risk factor for HIV infection for the following reasons: firstly, macroscopic tissue damage in female genital organs allows the virus to enter easily and facilitates the acquisition of HIV [64]; secondly, the inflammatory cells surrounding the eggs express CD4 T cell receptors, which are the target of HIV virus [65]; thirdly, the recruitment of immune cells to the male genital tract increases the viral load in

semen, which increases the risk of HIV transmission from male to female [66]; and, lastly, Th_1 cytotoxic responses are important in the initial control of HIV infection, and shifting of the immune response from Th_1 to Th_2 by schistosomiasis increases the risk of HIV infection [67].

1.2.4. Diagnosis of *Schistosoma haematobium* infection

Microscopy examination of urine is the gold standard test for diagnosis of *S. haematobium* infection [68] and is necessary to determine the intensity of infection [41]. It is simple to perform and inexpensive [69] but cannot detect the acute stage of the disease (when no eggs are released) and consecutive examination of samples is required in light infections [11].

Detecting antibodies produced against the different developmental stages of *S. haematobium* is another diagnostic method for *S. haematobium* infections [70]. Antibody detection can help in the diagnosis of patients with a light egg load (or acute infection when microscopy examination is still negative) in low-level endemic areas [71], and can help determining re-emergence of schistosomiasis in certain areas and in the diagnosis of travellers [72]. However, one of the main limitations of this method is that it cannot differentiate between past and active infections [11, 41, 73].

The circulating cathodic antigen (CCA) rapid diagnostic test is an immunochromatographic dipstick that detects the presence of this schistosome glycan in patient urine [74]. For diagnosis of urogenital schistosomiasis, the sensitivity and specificity of this method is 88.2% and 96.4%, respectively [75]. However, the sensitivity of this technique is low in areas where *S. mansoni* and *S. haematobium* are co-endemic [76]. Similarly, a urine-based up-converting phosphor-lateral flow circulating anodic antigen (UCP-LF CAA) assay is a highly sensitive and specific diagnostic method for *S. haematobium* infections in low endemic settings [77]. This technique is important to map distribution of schistosomiasis and assess drug efficacy [78]. In addition, the ability of the UCP-LF CAA test to detect a single pair of worms and its correlation with egg burden makes UCP-LF CAA the ideal measure of schistosomiasis infection [78]. However,

since it involves a centrifugation step, its applicability in field settings is limited [78]. Moreover, the CAA test is costly and not yet commercialised [77].

Molecular techniques like PCR are highly specific and sensitive for *S. haematobium* diagnosis from patient serum, plasma or urine [79]. PCR is crucial to detect the parasite in the acute and chronic stage of the disease [80], essential to evaluate the efficacy of anti-schistosome drugs [81] and it is not affected by sample collection time, as is the case for urine samples [82]. However, DNA based diagnosis of helminths requires expensive equipment and reagents [83]. *S. haematobium* infection can also be diagnosed by taking cytology Papanicolaou (Pap) smears from infected female patients. This test method is simple to perform and cheap; however, its specificity is lower than real-time PCR for *Schistosoma*-specific DNA in vaginal lavage and urine samples, and urine microscopy [84].

1.2.5. Current status of *Schistosoma haematobium* vaccines

The insufficient attention given to *S. haematobium* infection contributes to the widespread occurrence of the disease. Praziquantel treatment remains the cornerstone of control strategies; however, high reinfection rate requires frequent re-treatment and, in some infected people with high worm load, severe and irreversible pathology is usually diagnosed too late [85]. Furthermore, low efficacy of praziquantel has been reported from Egypt [86] and Senegal [87] and continuous use of praziquantel may lead to the development of resistance of *S. haematobium* to this drug. Hence, a vaccine is urgently needed for the control and elimination of urogenital schistosomiasis [85]. To alleviate this problem a number of trials have been undertaken to assess experimental vaccines.

Irradiated cercariae and schistosomula have been tested as potential vaccine candidates [88]. Different groups of baboons were immunized repeatedly with 3 kRad- and 20 kRad-irradiated cercariae and schistosomula [88]. All vaccinated groups had reduced worm burden and faecal egg production rates (64-89% and 56-79%, respectively). The protective efficacy of cercariae

was higher than schistosomula, and 20 kRad-irradiated cercariae were more protective than 3 kRad-irradiated cercariae [88]. Repeated immunisation of baboons with 20 kRad-irradiated schistosomula intramuscularly delayed and decreased faecal and urine egg production, reduced the size of granulomas, the gross pathology and the severity of inflammatory responses in the bladder and ureters; however, the proportion of tissue eggs in the liver of vaccinated animals didn't decreased [89]. In another study 20- and 60- kRad-irradiated schistosomula protected baboons from *S. haematobium* infection by 85-90% and 56-50%, respectively [90]. The protective capacity of 20 kRad-irradiated schistosomula decreased over time [90].

A schistosome glutathione S-transferase (Sh28GST) found in the schistosomula and adult stages of the parasite is the only vaccine candidate that has reached phase 3 trials against *S. haematobium* infection [91]. The vaccine efficacy of this antigen has been assessed in different *Schistosoma* species and this will be discussed later in this chapter.

Calpain, a protein located on the tegument and underlying musculature of adult schistosomes [92], and its large subunit (Sm-p80) have been tested as recombinant vaccine candidates against *S. mansoni* infections in mice and non-human primates [93]. This *S. mansoni* vaccine also confers protection against *S. haematobium* infection [94] and this will be discussed later in this chapter.

1.3. Extracellular vesicles

EVs are membrane-bounded vesicles discharged from different cells. Based on their size and origin, three types of EVs can be differentiated: MVs, exosomes, and apoptotic bodies (Fig 1.3) [95]. Exosomes are EVs produced from the endosomal system and they are formed as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) [96]. Initially, endocytic vesicles fuse with early endosomes; contents to be recycled are then organized into recycling endosomes, which transform into late endosomes. Then, ILVs accumulate on the late endosomes forming the larger MVBs. Finally, ILVs become exosomes and are released when

the MVBs fuse with the plasma membrane [97]. Their sizes range from 30–150 nm in diameter [98].

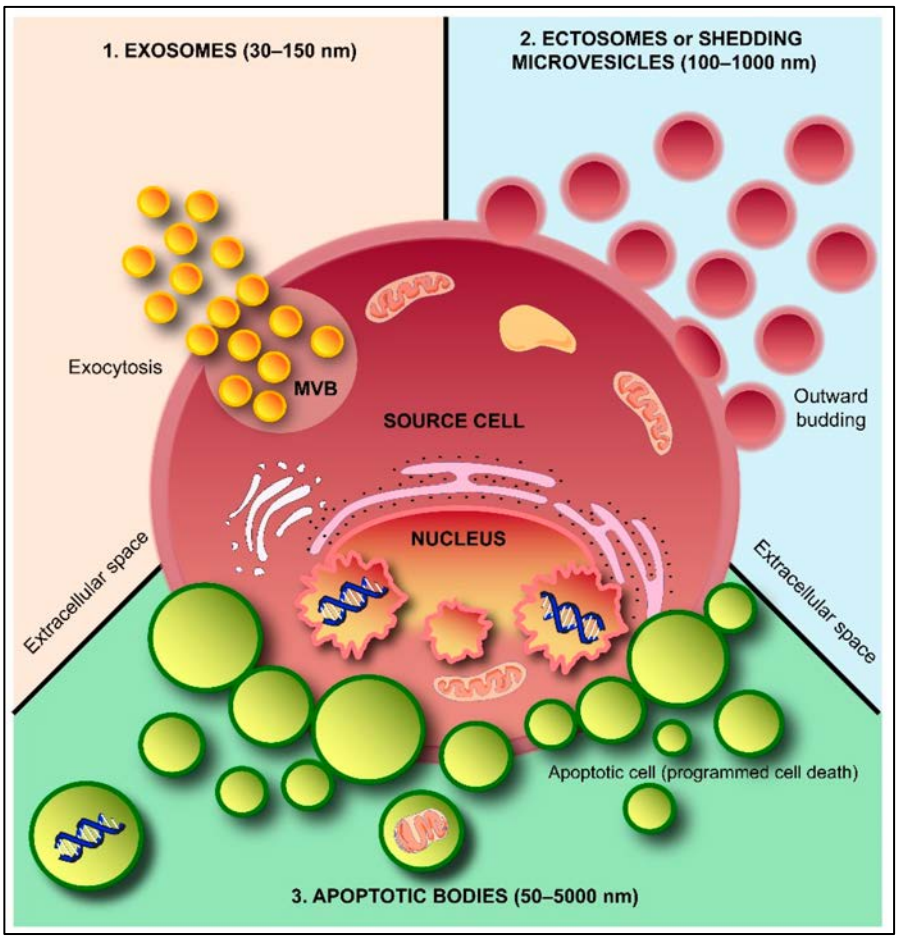

Figure 1.3. Types of extracellular vesicles. Adapted from [98]**.**

MVs are EVs that are released from cell membranes of healthy cells. They are also called ectosomes, shedding vesicles or microparticles and their size ranges from 100-1,000 nm [99]. MVs are formed by direct external budding and fission of the cell membrane. The external budding is initiated by the dynamic interaction between phospholipid redistribution and cytoskeletal protein contraction [100]. The distribution of protein and phospholipid in the cell membrane is controlled by aminophospholipid translocase [100, 101] and this translocase transfers phospholipids from the inner leaflet to the outer leaflet, which induces membrane budding/vesicle formation. Finally, contraction of cytoskeletal structures by actin-myosin interactions completes vesicle formation [100]. External factors like calcium influx and

hypoxia promotes MV discharge [97]. Apoptotic bodies are released by dying cells and their size ranges from 50–5,000 nm [98]. During apoptosis cell and chromatin becomes shrieked and condensed, respectively. Then, extensive plasma membrane blebbing and nuclear fragmentation lead to the formation of apoptotic bodies [102].

EVs play an important role in cell to cell communication [95]. EVs can also be involved in different pathological processes [103-105]. They are also involved in maintenance of homeostasis [106, 107] and can also serve as therapeutic agents [108].

1.3.1. Extracellular vesicles from helminths

The characterisation of helminth proteomes is shedding light on the molecular basis of host–pathogen interactions, with a view to discovering new diagnostic biomarkers and vaccine targets [109, 110], most of which are found at the external surface of the parasite and in the ES products [21, 111, 112]. Traditionally, the ES products were thought to comprise only soluble proteins, glycans, lipids, and nucleic acids [113]; however, recent literature has revealed that helminth ES products also contain EVs [26, 31]. Different parasites (including protozoans and helminths) have been shown to produce and secrete EVs which can be internalised by host cells and modulate the host immune response [26, 31]. These EVs are also involved in the pathology of parasitic disease and have great potential as new diagnostic tools and therapeutic agents against different parasitic pathogens [114, 115].

The field of parasitic EVs attracted the attention of the scientific community very recently, when Marcilla et al. showed the first evidence of EV release from helminths in 2012 [116]. These EVs were identified from the tegument and ES products of the flukes *Echinostoma caproni* and *Fasciola hepatica* [116], and were shown to be internalised by intestinal cells, suggesting a role for EVs in host-parasite interaction and the development of infection [116]. Since then, EVs have been characterised in other parasitic helminths, including nematodes,

trematodes and cestodes, suggesting an important role for these vesicles in host-parasite interactions [24, 25, 117-126].

1.3.1.1 Nucleic acid and proteomic composition of helminth extracellular vesicles

Since 2012, the number of "omic" resources to study helminth EVs has increased significantly, mainly due to the advancement of genomics and proteomics. The proteomes and genomes of several helminth EVs have been characterised [24, 25, 117-126], allowing for the identification of proteins and nucleic acids that could play an important role in host-parasite interactions. The nucleic acid composition of trematode EVs is complex, and different small RNAs and mRNAs have been characterised. Bernal et al. showed that EVs from *Dicrocoelium dendriticum* contains microRNAs (miRNAs) (eg: sma-let-7, sma-miR-2a-3p, emu-miR-71, emu-miR-190 and sma-miR-61), demonstrating for the first time the presence of miRNAs in helminth EVs [124]. Similarly, molecular characterisation of *S. mansoni* schistosomula revealed 20 transfer RNAs (tRNAs) and 205 miRNAs [25], some of which (sma-bantam, sma-miR-10, sma-miR-3479 and sma-miR-n1) have been detected in sera obtained from chronically infected hosts [127, 128] or within EVs from the sera of *S. mansoni* infected travelers (bantam and miR-2c-3p) [129]. Similarly, Samoil *et al.* characterised the *S. mansoni* adult worm EVs and found 143 miRNAs (eg: sma-miR-125b_R-1, sma-bantam, sma-miR-71a). Some of these miRNA were also detected in EVs isolated from mice serum suggesting a role of helminth EVs in host-parasite interactions [130]. Likewise, EVs isolated from *F. hepatica* were shown to contain 42 miRNAs, some of which were hypothesized to play a role in host manipulation [131]. In another study, EVs from *S. japonicum* adult worms were shown to contain a population of small RNAs associated with host gene regulation [119]. These EVs were internalised by murine liver cells *in vitro* and could downregulate the expression of the murine genes *Gins4*, *Tysnd1* and *Utp3* [119]. *S. japonicum* egg EVs have also been analysed, revealing different

types of RNAs such as rRNAs, small nuclear RNAs, repeat associated small RNAs, tRNAs and miRNAs [132].

Similarly, the nucleic acid compositions of cestode EVs have been characterised [133]. The miRNA analysis of *Taenia crassiceps* EVs revealed 7 miRNAs (let-7-5p, miR-61-3p, miR-190-5p, miR-219-5p, miR-4989-3p, miR-71-5p and miR-277-3p) whereas only one miRNA (let-7-5p) was detected in *Mesocestoides corti* EVs [133]. Based on an *in silico* miRNA target prediction approach, the most putatively regulated pathways in the mouse were those related to Wnt signalling, cadherin signalling, gonadotropin-releasing hormone receptor, inflammation mediated by chemokine and cytokine signalling and angiogenesis [133].

Like trematode parasite EVs, the nucleic acid composition of nematode EVs is also complex. For example, *Heligmosomoides polygyrus* EVs contain subpopulations of small RNAs miRNAs and yRNAs) that are involved in host gene regulation [125]; and *Brugia malayi* EVs contain miRNAs that down regulate the expression of host Let-7 [134], a gene involved in macrophage polarization and response to infection [135]. *Trichuris muris* EVs contain 475 full-length mRNA transcripts involved in different roles such as signalling and signal transduction, transport, protein modification and biosynthetic processes, as well as in RNA processing and DNA integration [123]. Similarly, EVs from the same parasite also contained 56 miRNAs predicted to interact with genes involved in signalling, transcriptional regulation, metabolic and disease pathways and genes related with the host immune system [123]. In another study, the small RNA content of *Nippostrongylus brasiliensis* EVs were characterised and revealed 52 miRNA (eg: miR-ev1, miR-ev2, miR-ev3, miR-ev4, miR-ev5) [122]. Based on a computational target prediction, these miRNAs were predicted to interact with genes involved with the immune system as well as in different processes such as signalling, metabolism and disease [122]. Analysing *Ascaris suum* EVs revealed 51, 40, 29, 39 and 42 miRNAs from L3 larvae, L4 larvae, adult worm, adult worm body fluid and adult worm intestinal sections,

respectively [126]. These miRNAs were predicted to interact with genes involved in immunity such as IL-13, 1L-25 and IL-33 [126].

The proteomes of different trematode EVs have been characterised [24, 25, 117-119, 124, 125, 134]. Proteomic analysis of *E. caproni* EVs revealed 51 different proteins including cytoskeletal proteins (actin, tubulin, myosin, paramyosin, tropomyosin), glycolytic enzymes (enolase, aldolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Phosphoenolpyruvate carboxykinase (PEPCK), calcium-binding proteins (calmodulin, calponin), nuclear proteins (histones and elongation factors), stress-related proteins (eg: Heat Shock Proteins (HSPs)) and detoxifying enzymes (eg: peroxiredoxin). In addition to parasite proteins, vesicles from *E. caproni* adult worms also contained 36 host-derived proteins (including immunoglobulins, histones, partial sequences of mucins and metabolic enzymes) [116]. The presence of host proteins in the EV preparation is surprising and could be related to the method used for EV isolation, since only an ultracentrifugation was performed with no further gradient purification. In addition, the database used for matching peptides was not specific for the *E. caproni* genome (due to its unavailability), which could also affect the number and accuracy of identifications [116].

Characterisation of the proteomes of *S. mansoni* adult worms and schistosomula EVs revealed 83 and 109 proteins, respectively, including markers of exosomes such as TSPs, HSP, annexins and Rab11 as well as other proteins such as 14-3-3, cytoskeletal proteins and metabolic enzymes [24, 25]. Several proteins from *S. mansoni* adult worm EVs were homologous to proteins identified in the EVs from *E. caproni* (17, 20%), *F. hepatica* (24, 29%), *D. dendriticum* (17, 20%) and *Opisthorchis viverrini* (19, 23%), which allows researchers to speculate that there is a conserved mechanism for protein packaging inside trematode EVs. Interestingly, a total of 26 (31%) proteins found in *S. mansoni* adult worm EVs are homologues of previously described vaccine candidates [24] and they will be discussed later in this chapter.

The proteome of the *S. mansoni* adult worm EVs has been further characterised and 130 proteins [130] were revealed. Of these 23 proteins including enolase, GAPDH, GST, calpain, leucine aminopeptidase, Sm20.8 and Sm22.6 were previously identified proteins from the adult worm EVs [24] and 25 of them such as taurocyamine kinase, enolase, GST, calpain, 14-3-3 epsilon and Sm20.8 were reported from schistosomula EVs [25]. Similarly, *S. japonicum* EVs contain 403 proteins from which 78 are homologous to exosomal proteins in other parasites. Proteomic analysis of the EVs secreted by the carcinogenic liver-fluke *O. viverrini* revealed 108 proteins including typical exosomal markers (TSPs, HSP-70), cytoskeletal proteins, regulatory proteins and trafficking proteins [117]. This study also showed the first evidence of exosome proteins in the tissues of an infected host (bile duct of infected hamsters and humans) [117]. EVs from *F. hepatica* were shown to be of a larger size range (30-200 nm in diameter) than other trematode EVs and contained some unique proteins, including helminth defense molecules (HDM), as well as known exosomal markers such as HSP-70, ALIX and TSPs [118]. In another study, the proteomic composition of the surface of adult *F. hepatica* EVs was characterised by using biotin to label the EVs surface [136] and 380 proteins were identified including membrane transport proteins (pumps, channels and transporters), proteases (Cysteine peptidase, serine peptidase and metallo-peptidase), protease inhibitors (eg: Serpin7, serpin B6 and serpin B) and proteins involved in EVs biogenesis and release (ESCRT components, small GTPases, SNAREs and membrane structure and remodelling) [136]. Using a lectin microarray, the suface oligosaccharides were characterised and high mannose *N*-linked structures were present as well as *N*-or *O*-linked protein glycosylation and glycolipid structures [136]. Treatment of EVs with glycosidase blocked the internalisation of EVs by macrophages but treatment of EVs with serum from *F. hepatica* infected mice enhanced the internalisation of EVs by macrophages. Similarly, treatment of EVs with antibodies produced against surface proteins (CD63 receptor, DM9-containing protein

and myoferlin) enhanced the internalisation [136]. In another study, the proteomic composition of *D. dendriticum* revealed 84 proteins like metabolic enzymes (eg: catalase, GAPDH, enolase and aldolase), chaperons (HSP-70, annexins), metal-binding proteins (myoglobin-1 and MF6p/HDM-1), nuclear proteins (eg: histones, elongation factors and enzymes involved in RNA synthesis and processing) and signalling molecules (kinases, suppressor of tumorigenicity homologs and serpin homologs) [124].

The proteomic composition of the different cestode parasites EVs has also been characterised. For instance, a proteomic analysis of *Taenia crassiceps* and *M. corti* EVs revealed 48 and 39 proteins, respectively [133]. The most abundant proteins in the EVs secreted by *Taenia crassiceps* and *M. corti* were the immunodiagnostic proteins (H17g protein and a tegumental antigen) and proteins involved in EV transport such as receptor-mediated endocytosis family member and Ras GTPases [133]. In addition to these parasite proteins, host proteins such as immunoglobulins and complement factors were also found [133]. The proteomic content of EVs derived from *Echinococcus granulosus* hydatid cysts was characterised and 663 proteins were found [137]. Some of the most abundant proteins were antigen 5, alpha-mannosidase, malate dehydrogenase, gelsolin, lipid transport protein, antigen B subunit 4, expressed protein, syndecan binding protein syntenin, EG10 and antigen B 4/1 [137]. Among these proteins antigen 5, antigen B and EG10 are diagnostic antigens [138-140]. Proteins that are markers of EVs in mammalian systems like TSPs, transporters and channels (eg: ATPase and chloride channels and multidrug resistance associated protein) and proteins involved in EVs biogenesis and transport (eg: annexins, Rabs, ALIX and ubiquitin) were also identified [137]. Similarly, the proteome of EVs secreted by the protoscolex and the metacestode of *E. granulosus* were characterised by treating with loperamide. From the metacestode, 13 proteins were obtained including, gelsolin, HSP-70, TSPs and 14-3-3 protein, which are usually present in exosomes and 298 proteins were identified from the protoscolex including, exosomal markers (eg:

TSG101, TSPs, ALIX and annexin A6) [121]. The proteomic composition of EVs released by *E. multilocularis* metacestodes revealed 433 proteins including proteins involved in EVs biogenesis and release (eg: annexin, 14-3-3, tetraspanin, Rab and HSPs), cytoskeleton (actin and tubulin) and metabolism (eg: thioredoxin peroxidase, enolase and GAPDH) [120].

In the case of parasitic nematodes, different proteomic studies have been performed [125, 134]. For example, EVs from *H. polygyrus* contained exosomal markers such as HSPs, TSPs, Rab proteins and ALIX [125], which are proteins involved in exosome biogenesis [97]. In another study, EVs from *B. malayi* were found to contain 32 proteins including markers of exosomes such as HSP-70, elongation factor-1α, elongation factor-2, actin, and Rab-1. Furthermore, more than 80% of these proteins were orthologous to mammalian exosome proteins [134]. The L3 larvae and the adult male and female of *B. malayi* EVs were characterised and 31, 20 and 74 proteins were identified [141]. Characterisation of *Teladorsagia circumcincta* EVs revealed 85 proteins including proteins involved in EVs transport like Rab GTPases. EVs from this parasite were recognised by serum samples collected from *T. circumcincta* infected hosts, suggesting the usefulness of EVs for the diagnosis of helminth infection [142]. The proteome analysis of *T. muris* EVs revealed 364 proteins and the most abundant proteins were trypsin domain-containing protein, sperm-coating protein (SCP)-like extracellular proteins, poly-cysteine and histidine-tailed protein and GAPDH. In addition, proteins typically found in EVs from helminths like 14–3–3, HSPs, GSTs and TSPs were found [24]. The proteomic analysis of *N. brasiliensis* EVs revealed a total of 81 proteins including proteins frequently present in exosomes like TSPs, enolase, 14-3-3 protein, HSPs and histones [122]**.** Characterising the protein composition of adult *A. suum* ES EVs revealed 268 proteins including, proteinases, peptidases, oxidases, reductases, kinases and HSPs [126]. Similarly analysing the proteomic composition of *A. suum* adult worm body fluid EVs revealed 125 proteins including HSPs,

peptidases, kinases and proteinases. Among these, 101 proteins were common to adult worm ES EVs [126].

In addition to exosome markers and vaccine candidates, it is worth noting the presence of other classes of proteins in the EVs of some helminths. For instance, saposin-like proteins have been detected in the EVs from *S. mansoni* and *F. hepatica* [24, 118]. These proteins are proposed to be haemolytic and play a role in the nutrient acquisition process of haematophagous parasites by disrupting red blood cell membranes to liberate haemoglobin [143]. EVs from different helminths also contain proteins that have been associated with immunomodulation, like annexins [24, 116, 117]. *F. hepatica and B. malayi* EVs contain cathepsin L [118, 134] that degrades host collagen to help parasite migration through host tissues [144] and peroxiredoxin, which shifts the host immune response from Th_1 to Th_2 by stimulation of M2 macrophages [118, 145]. The presence of these proteins in helminth EVs might indicate that the vesicles could be helping the parasite acquire nutrients, evade host immune responses and establish chronic infections and contribute to the associated pathogenesis.

1.3.1.2. Vaccine candidates presented in helminth extracellular vesicles

In addition to their role in pathogenesis and immunomodulation, EVs from helminths also contain vaccine candidate antigens (Table 1.1), which can be present in the cargo and membrane of EVs (Fig 1.4). The following vaccine candidates have been found in the EVs of different helminths.

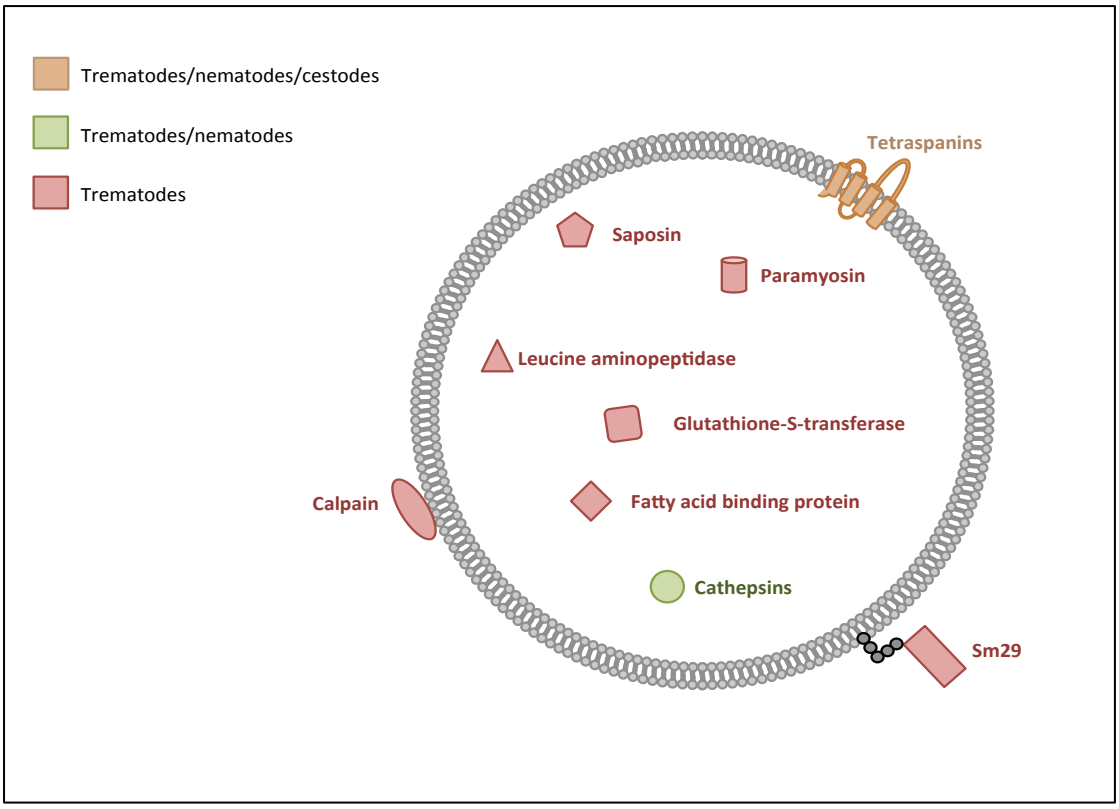

Figure 1.4. Schematic representation of the most important vaccine candidates found in extracellular vesicles secreted by helminths [146].

1.3.1.2.1. Membrane proteins

TSPs are membrane-spanning proteins that serve principally as membrane protein organizers [147]. The first members of this family were identified in humans [148] and in schistosomes [149]. Since then, this group of proteins has been found in both multicellular and unicellular organisms [150-152]. This family of proteins consists of four transmembrane domains, a small extracellular loop (SEL) and a LEL [147]. The LEL contains a conserved Cys-Cys-Gly motif (CCG motif), as well as other cysteine residues that facilitate the formation of disulfide bridges [153] and mediate specific protein-protein interactions with laterally associated proteins and a few known ligands [147]. The cytoplasmic regions provide links to cytoskeletal and signalling molecules. The four transmembrane domains stabilize individual TSPs during biosynthesis, and they promote associations between TSPs and other proteins, which are

crucial for the assembly and maintenance of the tetraspan web [147]. In addition, juxtamembrane cysteine residues in the cytoplasmic domains provide sites for palmitoylation, contributing to the clustering of TSP microdomains. In general, the tetraspan web provides a scaffold by which membrane proteins are laterally organized to coordinate the intracellular transmission of external stimuli for the activation of signalling cascades [147].

TSPs contribute to cellular physiology by organizing molecules within the plasma membrane into microdomains. Indeed, the proposed function of TSPs is to organise the plasma membrane by facilitating the formation of what are termed tetraspanin-enriched microdomains (TEMs) [154]. TEMs consist of homophilic and heterophilic interactions amongst TSPs, interactions between TSPs and other membrane proteins, as well as interactions between TSPs and proteins at the membrane/cytoplasm interface [154, 155]. Furthermore, TSPs are involved in many cellular activities such as differentiation, adhesion and division [156].

In some helminths, TSPs play an important role in the formation of the tegument, the outermost membranous surface of the parasite that is in intimate contact with host tissues, and seem to be key molecules for the survival of the parasites [157-160]. TSP LELs have been shown to be efficacious vaccine antigens in a range of helminth infection models. Immunisation of mice with the LELs of two *S. mansoni* TSPs (*Sm*-TSP-1 and *Sm*-TSP-2) significantly decreased adult worm and liver egg burdens after infection with schistosomes [161] and *Sm*-TSP-2 has completed phase I clinical trials [162]. Similarly, vaccination of hamsters with the LEL of *O. viverrini* (*Ov*-TSP-2, *Ov*-TSP-3 and *Ov*-TSP-2 + *Ov*-TSP-3) significantly reduced adult worm burden and only *Ov*-TSP-2 reduced the fecal egg burden significantly [160]. In other studies, immunisation of mice with a fusion of the *Sj*23 TSP with other vaccine candidates significantly reduced the worm burden and liver eggs in a subsequent *S. japonicum* challenge [163-165]. Analysing the proteomes of different helminth EVs revealed several TSPs [24, 25, 117-123].

Cwiklinski et al. provided the only in-depth characterisation of the proteins present in the membrane of *F. hepatica*-secreted EVs [118], confirming the presence of TSPs (as well as other proteins) in the membrane of *F. hepatica* EVs. Moreover, antibodies produced against a TSP present in *O. viverrini* EVs blocked the internalisation of EVs by cholangiocytes and decreased both cell proliferation and production of cytokines that stimulate tumorigenesis in this carcinogenic infection [117, 160]. These kind of studies characterizing the proteins present in the membrane of EVs and analysing their importance in cell contact and uptake will be key for the development of helminth EV-derived vaccines [136].

Sm29 is a tegumental antigen, present in the tegument of adult worms and schistosomula of *S. mansoni* [166] and was recently found in *S. mansoni* adult worm EVs [24]. DNA immunisation of mice with this vaccine candidate resulted in 17-22% adult worm reduction [167], while immunisation with recombinant protein decreased adult worm, intestinal egg and liver egg burden by 51%, 60% and 50%, respectively [168]. Fusing Sm29 with *S. mansoni* fatty acid binding protein (FABP) decreased adult worm, liver egg and intestinal egg burdens by 40.3%, 68.2% and 57.9%, respectively [169], whereas fusion with *Sm*-TSP-2 resulted in 24-35% adult worm reductions [167, 170]. In another study, mice were immunised with Sm29 formulated with alum or monophosphoryl lipid A adjuvants and Sm29 formulated with alum significantly protected mice by 29%-37% in two independent trials against *S. mansoni* reinfection. This protection was associated with high levels of antibodies against the Sm29 + alum formulation [171].

Calpain is a calcium-activated neutral cysteine protease [172] located on the tegument and underlying musculature of adult schistosomes [92], and has also been found in the EVs of different helminths [24, 25, 117, 120, 121]. Sm-p80 is the large subunit of calpain and has been tested as a vaccine candidate against *S. mansoni* infections [93]. In mice, administration of Sm-p80 DNA vaccine, boosted with recombinant protein, reduced worm burden by 49%, whereas

immunisation with proteins provided 50% protection [173]. This vaccine candidate reduced worm and egg burden by 70% and 75%, respectively, using oligodeoxynucleotide as adjuvant [174]. The vaccine efficacy of Sm-p80 (rSm-p80) emulsified with glucopyranosyl lipid A + alum was assessed in mice in three independent trials. In trials one and two, vaccination of mice with rSm-p80 emulsified with glucopyranosyl lipid A + alum reduced adult worm burden significantly by 33.3% and 53.1%, respectively following *S. mansoni* challenge. In the third trial, worm reduction was not significant but intestinal egg burden was significantly reduced by 75.5% [175]. The same vaccine candidates reduced adult worm burden by 38.5% in baboons but there was no significant reduction in the tissue egg burden [175]. Similarly, vaccination of baboons with rSm-p80 formulated with glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) significantly reduced liver egg burden and egg-hatching rate of *S. mansoni* by 67.7% and 85.6%, respectively, which indicates the potential usefulness of this vaccine candidate in blocking disease transmission [176]. Moreover, this *S. mansoni* vaccine confers partial protection against *S. haematobium* and *S. japonicum* infection [94, 177]. Vaccination of mice with rSm-p80 emulsified with GLA-SE intramuscularly resulted in 46.75% reduction in adult worm burden but no significant reduction in tissue egg burden following *S. japonicum* challenge was observed [177]. Administration of recombinant Sm-p80 emulsified with GLA-SE in hamsters reduced *S. haematobium* adult worm and tissue egg burden by 48% and 64%, respectively [94]. Similarly, in baboons, the Sm-p80/GLA-SE vaccine produced a 25%, 64% and 53% reduction in adult worms, urinary bladder egg load and in urine egg output, respectively following *S. haematobium* challenge [94].

Paramyosin is a protein present in the thick muscle myofilaments of invertebrates [178]. This protein is located on the granules of the post-acetabular glands of cercariae, within the tegument matrix and surface of schistosomula of *S. japonicum* [179] and on the teguments of adult *S. mansoni* [180]. Paramyosin is considered a promising vaccine candidate against *S. japonicum*

[181]. Immunisation of pigs with recombinant paramyosin resulted in 33-34% reduction in *S. japonicum* adult worm burden [181], whereas immunisation of mice with parasite-derived paramyosin resulted in 62%-86% reduction in adult worm burden following *S. japonicum* infection [182]. The vaccine efficacy of paramyosin emulsified with ISA206 adjuvant was assessed in three independent trials in water buffalos [183]. In the first trial, immunisation of water buffalos with recombinant paramyosin protein reduced worm burden by 51.5 % but the reduction was not statistically significant [183]. Increasing the dose in the second and third trial significantly reduced the adult worm burden by 57.8% and 57.8%, respectively [183]. Interestingly, this protein has been found in the EVs of *O. viverrini* [117].

1.3.1.2.2. Proteases and peptidases

Leucine aminopeptidases are metallopeptidases that cleave N-terminal residues from proteins and peptides [184], and their vaccine efficacy against *F. hepatica* has been evaluated in animal models [185, 186]. Immunisation of sheep with native and recombinant leucine aminopeptidase resulted in 49-89% adult worm reduction in subsequent *F. hepatica* challenges [185, 186]. Furthermore, leucine aminopeptidase was shown to be abundant in the EVs from *S. mansoni* and *F. hepatica* [24, 118].

Cathepsin L is a protease that has been found in the EVs of *F. hepatica* and *B. malayi* [118, 134]. Immunisation of cattle and sheep with parasite-derived cathepsin L resulted in 42-69% and 34% reduction in *F. hepatica* adult worm burdens, respectively [186, 187], while in another study, immunisation of cattle with recombinant cathepsin L resulted in a 48% reduction in adult worm burden [188]. Immunisation of mice with recombinant cathepsin B resulted in 59%, 56% and 54% reductions in adult worm, liver egg and intestinal egg burdens, respectively, following *S. mansoni* challenge [189]. Furthermore, cathepsin B has also been found in *F. hepatica* and *S. japonicum* EVs [118, 119, 142].

1.3.1.2.3. Cytosolic proteins

Saposins are activators of sphingolipid hydrolases [190] that have been used in vaccine trials against different helminths [143, 191]. For instance, immunisation of rabbits with a recombinant saposin-like protein reduced adult worm burden and faecal and bile egg loads by 81.2%, 83.8% and 73%, respectively, after *F. hepatica* challenge [191]. Similarly, vaccination of mice with recombinant saposin-like protein 1 (rFgSAP-1) from *Fasciola gigantica* emulsified with aluminium hydroxide gel (alum) stimulated the production of antibody responses that resulted in 73.2% and 74.3% protection when compared with non-vaccinated infected and adjuvant-vaccinated infected controls, respectively. Importantly, this vaccine candidate reduced levels of liver damage [192]. Saposin domain-containing proteins have been revealed in the EVs of several helminths, including *S. mansoni*, *F. hepatica, N. brasiliensis* and *T. circumcincta* [24, 118, 122, 142].

GSTs are group of enzymes that detoxify endogenous compounds and foreign chemicals [193]. This protein has been tested as a vaccine candidate against different helminth infections [194-196] and has recently been found in the EVs of different helminths [24, 117-119, 121]. Immunisation of mice and baboons with Sm28GST as a recombinant protein affects both worm viability and fecundity [197] and the antibody produced against this enzyme blocks its activity, leading to a reduction in female worm fecundity and egg viability [194]. The *S. haematobium* homologue, Sh28GST, is a promising vaccine candidate against *S. haematobium* infections. In the animal model, the antibody produced against this vaccine candidate can deactivate Sh28GST action and downgrade egg-production, which decreased urinary tract pathology and the spread of the disease [198]. Clinical trials using Sh28GST indicated that this vaccine has a good safety profile and stimulates the production of IgG3 [199]. In naturally infected individuals, the cellular immune response against Sh28GST is boosted by praziquantel treatment, increases in younger age groups and increases with schistosome infection status

[200]. A Phase 3 trial was conducted from 2009 to 2012 in *S. haematobium* infected children to assess the efficacy of the vaccine candidate after treatment with praziquantel, but the protective efficacy was not significant [91]. In another study, administration of the *S. japonicum* homologue as a DNA vaccine (Sj26GST) with IL-18 significantly decreased worm burdens, liver and faecal eggs counts in a subsequent *S. japonicum* challenge in mice [195]. Furthermore, *F. hepatica* GST has been evaluated in different animal models with the mean protective capacity reported as 29% and 43% in sheep and cattle, respectively [196, 201].

FABP is a cytosolic protein that aids the parasite with absorption, transportation and sorting of host fatty acids [202]. This molecule has long been considered a potential vaccine candidate in different helminths [203-205] and it has recently been found in *S. mansoni* and *F. hepatica* adult worm EVs [24, 118, 133]. Immunisation of cattle with parasite-derived FABP from *F. hepatica* (FhFABP) resulted in a 55% adult worm reduction [203], while immunisation of sheep decreased adult worm, intestinal egg and bile fluid egg burdens by 24%, 40.3% and 51.8%, respectively [206]. Immunisation of sheep with recombinant FhFABP resulted in a 43% adult worm reduction [207]. Similarly, immunisation of mice, rats and sheep by fusing SjFABP with GST decreased *S. japonicum* adult worm burdens by 34.3%, 31.9% and 59.2%, respectively [204], whereas administration of SjFABP DNA vaccine + IL-18 resulted in 38% and 45% *S. japonicum* adult worm and egg burden reduction [208]. Immunisation of mice with recombinant *Sm*FABP resulted in a 67% *S. mansoni* adult worm reduction [205].

1.3.1.2.4. Extracellular vesicles as vaccines against helminths

Since helminth EVs contain a significant number of vaccine candidates, using these EVs to immunize the host might be a good strategy for the development of vaccines against helminth infections. For instance, EVs from *H. polygyrus* stimulated the production of antibodies that significantly reduced faecal egg counts and intestinal worm burdens after subsequent *H. polygyrus* challenges [209]. In another study, immunisation of mice with EVs from *E. caproni*

produced both humoral and cellular immune responses, which reduced symptom severity and mortality induced by infection [210]. *T. muris*- and *O. viverrini*-derived EVs have also been shown to induce immunity and confer protection against subsequent infection [160, 211].

While protective immunity has been shown with numerous helminth EVs in animal models, the specific proteins responsible for the induction of protective antibodies have not been characterized. Moreover, the immune response against EVs might differ between helminths, so further work is required to characterize the immunogenic proteins present in helminth EVs.

These findings suggest that helminth EVs are important in host–parasite interactions, and they can also be used in the discovery of new vaccine candidates. Since the isolation and purification of EVs is time consuming and might not be feasible at a larger scale, the characterisation of the proteomic composition of EVs will be key for the development of new vaccines. In addition to well-characterised molecules, there is a large number of hypothetical proteins in helminth EVs, which, arguably play parasitism-specific roles and might be good vaccine targets [212]. For this purpose, a better annotation of genomes using different approaches such as *in silico* analyses and proteogenomic studies will be of invaluable help [110].

1.3.1.3. Role of helminth extracellular vesicles in immunomodulation

EVs secreted by different helminths can modulate the host's immune response. For instance, EVs secreted by the murine intestinal nematode *H. polygyrus* contain miRNAs and yRNAs, which suppress the host immune response [125]. Intranasal administration of *H. polygyrus* EVs together with the extracts of the allergenic fungus *Alternaria* decreased bronchoalveolar eosinophilia, suppressed the expression of the Th_2 cytokines IL-5 and IL-13 by innate lymphoid cells and also decreased the expression of the IL-33 receptor in mice [125]. Internalisation of EVs by mouse cells transferred miRNAs which resulted in suppressed expression of *Dusp1* and *Il1rl1* [125]. Dusp1 is a key regulator of mitogen-activated protein kinase signalling and

inhibits type 1 pro-inflammatory reactions. IL1rl1 is, the ligand-specific subunit of the receptor for IL-33, a key alarmin cytokine required for protection against multicellular parasites, which is produced by innate cells to drive early Th_2 immune responsiveness [213]. The above finding correlates with previous studies using ES products [214]. Intranasal co-administration of the ES products of the same parasite with *Alternaria* extracts suppressed IL-33 release, production of IL-4, IL-5, and IL-13, and localized eosinophilia [214]. Thus, this parasite targets IL-33 production as part of its suite of suppressive effects, and thereby preventing the development of the Th_2 immune response to infection and allergic sensitization [214]. Furthermore, EVs from the same parasite were internalised by macrophages and supressed their activation [209]. These studies showed that the ES products from *H. polygyrus* contains exosomes that facilitate the parasite's ability to escape the host's immune response. Similarly, EVs from *N. brasiliensis* prevent colitis induced by trinitrobenzene sulfonic acid (TNBS) in a mouse model of inflammatory bowel disease by suppressing pro-inflammatory cytokines (IL-6, IL-1β, IFN-γ and IL-17a) and inducing the expression of the anti-inflammatory cytokine IL-10 [122].

The third stage larvae of *B. malayi* secrete EVs, which can be internalised by J774A.1 cells (murine macrophages), activating them and significantly increasing the production of G-CSF, MCP-1, IL-6 and MIP-2 levels. This indicated that the EVs produced by *B. malayi* generate a classical pro-inflammatory macrophage phenotype [134]. EVs from *S. japonicum* contain miRNAs and potential virulence factors [119]. Internalisation of these EVs transferred miRNAs to mice that downregulate expression of the *Gins4*, *Tysnd1*, and *Utp3* genes [119]. The EVs released from *S. japonicum* modulate cytokine production in RAW264.7 cells. Treatment of RAW264.7 cells with EVs increased iNOS expression, TNF-α secretion and CD16/32 expression *in vitro*. In contrast, the expression of CD206, Arg-1, and IL-10 by RAW264.7 cells was decreased in cells treated with EVs. The increase in the surface expression of CD16/32, iNOS expression, TNF-α secretion and the decrease in the expression

of CD206, Arg-1, and IL-10 indicated that *S. japonicum* EVs modulate the phenotype of macrophages and skew macrophages to M1 polarization [215]. *E. granulosus* EVs can be internalised by murine dendritic cells and affecting expression of activation markers such as CD86 and MHCII, indicating that *E. granulosus* EVs could interfere with the antigen presentation pathway [121]. Similarly, EVs released by *E. multilocularis* metacestodes decreased nitric oxide (NO) secretion by down regulating inducible nitric oxide synthase (iNOS) in RAW264.7 macrophages [120]. These studies show that EVs from helminths can modulate the host's immune response to escape the host mediated immune attack.

Table 1.1. Efficacy of different vaccine candidates present in the extracellular vesicles (EVs) from helminths. Table summarizing the most important proteins found in EVs from helminths that have been tested as vaccine candidates against helminth infections. Ns: not significant; RP: recombinant protein; GST: glutathione S-transferase; *As: Ascaris suum; Bm: Brugia malayi; Dd: Dicrocoelium dendriticum; Ec: Echinostoma caproni; Eg: Echinococcus granuloses; Em: Echinococcus multilocularis; Fh: Fasciola hepatica; Hc: Haemonchus contortus; Hw: hook worm; Ov: Opisthorchis viverrini; Sh: Schistosoma haematobium; Sj: Schistosoma japonicum; Sm: Schistosoma mansoni; Tm: Trichuris muris*

Vaccine candidates	Vaccine form	Animal model	Helminth in which vaccine trial was done	Adult worm reduction	Egg reduction	Antigen present in EVs from	Ref
TSPs	RP (*Sm*-TSP-2)	Mouse	*Sm*	57%	64%	*Sm, Ov*, *Fh*, *Sj*, *Em*, *Eg*, *Hw* and *Tm*	[161]
	RP (*Sm*-TSP-1)	Mouse	*Sm*	34%	52%		
	RP (*Ov*-TSP-2)	Hamster	*Ov*	34%	41%		[160]
	RP (*Ov*-TSP-3)	Hamster	*Ov*	30%	Ns		
Calpain	DNA	Mouse	*Sm*	70%	75%	*Sm, Ov*, *Sj* and *Eg*	[174]
	RP	Hamster	*Sh*	48%	64%		[94]
Saposin containing protein	RP	Rabbit	*Fh*	81.2%	83.8% (fecal) and 73% (bile)	*Sm* and *Fh*	[191]
	RP	Mouse	*Sm*	Ns	-		[143]
Sm29	DND	Mouse	*Sm*	17-22%	-	*Sm*	[167]
	RP	Mouse	*Sm*	51%	60% (intestinal) 50%(liver)		[168]
	RP (Sm29+Sm FABP)	Mouse	*Sm*	40.3%	68.2(liver) 57.9 % (intestinal)		[169]

	DNA (Sm29+Sm -TSP-2)	Mouse	*Sm*	24-32%	-		[167] [170]
	RP (Sm29+Sm -TSP-2)	Mouse	*Sm*	35%	-		
22.6 kDa tegument antigen	RP	Mouse	*Sm*	34.5%	-	*Sm* and *Sj*	[216]
Thioredoxin peroxidase	RP	Mouse	*Sj*	37.02%	56.52%	*Sm*	[217]
Syntenin	RP	Mouse	*Sm*	30-37%	-	*Sm, Ov* and *Fh*	[218]
Dynein light chain (DLC 12 and DLC 13)	RP	Mouse	*Sm*	43% and 51%	-	*Sm* and *Sj*	[219]
Antigen Sm21.7	DNA	Mouse	*Sm*	56%	41.53% (liver) and 55.63% (intestine)	*Sm*	[220]
HSP-70	RP	Mouse	*Sj*	35.98	31.18%	*Sm, Ov, Dd, Ec* and *Fh*	[221]
Leucine amino peptidase (M17 family)	RP	Sheep	*Fh*	49-86%	-	*Sm* and *Fh*	[185]
	Native protein	Sheep	*Fh*	89%	-		[186]
14-3-3 protein	RP		*Sm*	25-46%	-	*Sm, Ov, Ec* and *Fh*	[222]
Fatty acid binding protein	Native protein	Cattle	*Fh*	55%	-	*Fh* and *Sm*	[203]
		Sheep	*Fh*	24%	40.3% (fecal) and 58.1% (bilefluide)		[206]
	RP	Sheep	*Fh*	43%	-		[207]
	RP +GST	Mouse	*Sj*	34.3%,	-		[204]
		Rats	*Sj*	31.9%,	-		[204]
		Sheep	*Sj*	59.2%	-		[204]

	DNA + IL-18	Mouse	*Sj*	38%	45%		[208]
	RP	Mouse	*Sm*	67%	-		[205]
Cathepsin L	Native	Cattle	*Fh*	42-69%	-	*Fh* and *Bm*	[187]
		Sheep	*Fh*	34%	-		[186]
	RP	Cattle	*Fh*	48%	-		[188]
		Goat	*Fh*	Ns			[223]
Cathepsin B	RP	Mouse	*Sm*	59%	56% (liver) 54% (intestinal)	*Fh* and *Sj*	[189]
Kunitz type molecule	Native	Sheep	*Fh*	Ns	-	*Fh*	[201]
Enolase	RP	Mouse	*As*	61.13% (larvae)	-	*Sm, Sj, Dd, Ec,* and *Fh*	[224]
Glyceraldehyde-3-phosphate dehydrogenase	DNA	Goat	*Hc*	37.73%,	34.9%	*Sm, Sj, Fh, Ec* and *Fh*	[225]
Paramyosin	RP	Pig	*Sj*	33-34%	-	*Ov*	[181]
	Native	Mouse	*Sj*	62%-86%,	-		[182]

Introduction to the study

There is no licenced and effective vaccine to control the transmission of *S. haematobium* infection. Similarly, there are also limitations within the current diagnostic modalities of *S. haematobium* infection. However, EVs from helminths contain vaccine candidate antigens and vaccination of animals with helminths EVs stimulates production of antibodies that significantly reduce egg counts and worm burdens. Furthermore, EVs from helminths also contain diagnostic marks of infection and can also be recognised by antibodies produced from infected animals. These observations demonstrated the usefulness of EVs in vaccine and diagnostic strategies. Hence, the main aim of my thesis will be characterising the proteomic composition of *S. haematobium* adult worm EVs and testing selected antigens as vaccine and diagnostic candidates**.**

Aims of this thesis:

1. Characterisation of the proteomic composition of *S. haematobium* adult worm EVs
2. Characterisation of TSPs from *S. haematobium* EVs
3. Assessment of the vaccine efficacy of *S. haematobium* TSPs
4. Evaluation of *S. haematobium* TSPs as potential novel diagnostic markers

Chapter 2: Characterisation of the proteomic composition of *Schistosoma haematobium* adult worm extracellular vesicles

2.1. Introduction

Adult *Schistosoma* worms survive in the host for years and proteins on the worm tegument and in ES products are vital to this immune evasion strategy. ES products are molecules released by worms and comprise different proteins, glycans, lipids, and nucleic acids [113]. They play a crucial role in evading the host immune system and have been the focus of different studies aiming at understanding host-parasite interactions and the development of novel therapeutics [18-20, 22]. Because of a blind gut, schistosomes metabolic products are removed by vomiting; therefore, schistosome ES products consist of a mixture of secreted worm molecules and metabolites [18].

Recently, it has been documented that the ES products from different helminths (including schistosomes) contain EVs [26, 115, 146]. EVs are membrane-bound organelles released by cells that can act as mediators of intercellular communication by transferring molecular signals mediated by proteins, lipids, metabolites, mRNAs, microRNAs and other non-coding RNA species [226, 227]. In addition to the transmission of information, EVs are also involved in the maintenance of normal physiology [228, 229] and in the pathological process [230].

The first evidence of EV release from helminths was reported from the tegument and ES products of *E. caproni* and *F. hepatica* [116]. Since then, EVs have been characterised in many other helminths, including nematodes, trematodes and cestodes [24, 25, 117-126]. Studies have shown that EVs from helminths are heavily involved in host-parasite interactions [24, 25, 117-126]. For instance, EVs from trematodes and nematodes can be internalised by host cells, modulate host immune responses [125, 134, 215] and are involved in pathogenesis [117]. In addition, EVs also have enormous potential as novel diagnostic tools to detect parasitic infections [142] as proteins on the surface of helminths EVs have been found to be potential

diagnostic candidates [159, 231]. Furthermore, EVs from helminths also contain vaccine candidate antigens. For example, EVs from *S. mansoni* contain molecules that have shown vaccine efficacy in animal models of schistosomiasis [24]. Vaccination of mice with helminth EVs stimulates the production of protective immune responses that significantly reduce faecal egg counts; worm burdens, symptom severity and mortality induced by infection in subsequent parasite challenges [31, 160, 210, 211]. Moreover, antibodies produced against recombinant forms of *O. viverrini* EV surface proteins hinders the uptake of EVs by cholangiocytes and suppresses the immune response that fuels pathogenesis [117, 160].

In addition to their roles in pathogenesis and diagnostic and vaccine potential, EVs from helminths are also useful in immunotherapeutic applications [122, 125, 232]. For example, administration of EVs from *H. polygyrus* suppresses type 2 innate responses and lung allergen-induced eosinophilia and asthma in mice [125]. Similarly, EVs from *N. brasiliensis* prevents colitis induced by trinitrobenzene sulfonic acid (TNBS) in a mouse model of inflammatory bowel disease [122]. Furthermore, administration of *F. hepatica* EVs to mice decreases the expression of pro-inflammatory cytokines, reducing disease severity in dextran sulfate sodium (DSS)-induced colitis [232].

These observations demonstrate the importance of EVs in helminth-host interactions and their usefulness in vaccine, diagnostic and therapeutic applications. This chapter aims to characterise the proteome composition of different EV populations obtained from *S. haematobium* adult ES products. Using a proteomic approach, combined with newly available *S. haematobium* genome and transcriptome resources, I have defined the protein composition of *S. haematobium* adult worm EVs.

2.2. Materials and methods

2.2.1. Parasite materials and experimental animals

Bulinus truncatus snails infected with *S. haematobium* (Egyptian strain) were provided by the Biomedical Research Institute, MD, USA. Snails were maintained in aquaria in a 27°C incubator.

Male BALB/c mice were purchased from the Animal Resource Centre, Canningvale, Western Australia and maintained at the AITHM animal facility in cages under controlled temperature and light with free access to pelleted food and water. All experimental procedures performed on animals in this study were approved by the James Cook University (JCU) animal ethics committee (A2391). All experiments were performed in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

2.2.2. Cercariae shedding and mice challenge

Snails were removed from the tank with a pair of forceps and washed several times with water to remove debris and rotifers, transferred to a Petri dish and incubated without water at 27°C in the dark for 2 h. Water was added and the snails were placed under light for 1.5 h at 28°C. Cercariae were concentrated using a 20 μm pore size sieve and finally, each BALB/c mouse (6 week-old) was infected with 1,000 cercariae by tail penetration [233].

2.2.3. Adult worm culture and ES collection

S. haematobium adult worms were obtained by perfusion of mice at 16 weeks post-infection and parasites were washed several times with serum-free modified Basch media supplemented with 4× antibiotic/antimycotic (SFB) and then incubated in SFB (50 pairs/5 ml) at 37°C in with 5% CO_2 [24]. Dead worms were removed and media containing ES products was harvested

every 24 h for 2 weeks. Every day, ES products were differentially centrifuged at 4°C (500 ×*g*, 2,000 ×*g* and 4,000 ×*g* for 30 min each) to remove large parasite material such as eggs and tegumental debris and stored at -80°C.

2.2.4. Purification of extracellular vesicles

Stored supernatants were thawed on ice, concentrated at 4°C using a 10 kDa spin concentrator (Merck Millipore, USA) and centrifuged for 1 h at 15,000 ×*g* at 4°C. The resultant pellet (containing m/lEVs) was washed with 1 ml of PBS, centrifuged at 15,000 ×*g* for 1 h at 4°C, resuspended in 200 µl PBS and stored at -80°C. The supernatant was ultracentrifuged at 120,000 ×*g* for 3 h at 4°C using an MLS-50 rotor (Beckman Coulter, USA) to collect sEVs. The resultant pellet was resuspended in 70 µl of PBS and subjected to Optiprep® density gradient (ODG) separation. The ODG was prepared by diluting a 60% Iodixanol solution (Optiprep®, Sigma-Aldrich, USA) with 0.25 M sucrose in 10 mM Tris-HCl pH 7.2 to make 40%, 20%, 10% and 5% iodixanol solutions, and 1.0 ml of these solutions was layered in decreasing density in an ultracentrifuge tube. The resuspended sEVs were added to the top layer and ultracentrifuged at 120,000 ×*g* for 18 h at 4°C. A control tube was similarly prepared using PBS instead of sEVs to measure the density of the different sEV fractions recovered from the gradient. sEV fractions obtained from the ODG were diluted with 8 ml of PBS containing 1 × EDTA-free protease inhibitor cocktail (Santa Cruz, USA), and concentrated using a 10 kDa spin concentrator to remove the excess of Optiprep® solution. All sEV fractions were kept at -80°C until use.

The density of different sEV fractions obtained from the ODG was determined as follows. A total of 12 fractions each were collected from the sample and control tube. Then, 100 µl from each fraction obtained from the control tube was diluted (1:1) with water (twice). The absorbance of each fraction was measured at 340 nm using a POLARstar Omega (BMG

Labtech, Australia) and the density of each sEV fraction was determined by interpolating the absorbance in a standard curve as previously shown [24]. The protein concentration of sEVfractions and m/lEVs was quantified using the Quick Start™ Bradford Protein Assay Kit (Bio-Rad Laboratories, Inc. Life Science Research, USA) following the manufacturer's instructions.

2.2.5. Determination of the size and concentration of extracellular vesicles

The size distribution and particle concentration of the different sEV fractions recovered after ODG and m/lEVs was measured using tunable resistant pulse sensing **(**TRPS) using a qNano instrument (Izon, New Zealand) following an established protocol [122]. A Nanopore NP150 and NP400 (Izon, New Zealand) was used to measure sEVs and m/lEVs, respectively. Thirty-five µl of measurement electrolyte (Izon, New Zealand) was added to the upper fluid well and maximum pressure was applied; the shielding lid was clicked 5 to 10 times to wet the Nanopore. Then, 75 µl of measurement electrolyte was added to the lower fluid well, maximum pressure and an appropriate voltage (0.1 V) was applied and Nanopore current was checked for stability. Thirty-five and 75 µl of filtered coating solution (Izon, New Zealand) was loaded in the upper and lower fluid well, respectively, and maximum pressure was applied for 10 min followed by maximum vacuum for another 10 min. The coating solution was flushed out of the upper and lower fluid wells two to three times with measurement electrolyte, maximum pressure was applied for 10 min and the voltage was increased until the current reached between 120 and 140 nA and the baseline current was stable. Then, 35 µl of calibration particles (CP200 carboxylated polystyrene calibration particles) (Izon, New Zealand) was loaded to the upper fluid well at a 1:200 dilution for sEVs and 1: 1,500 for m/lEVs, incubated for 2 min at maximum pressure and the stretch was reduced and the calibration particles were measured at 2 different pressures (P10 and P5). The *S. haematobium* sEV fractions and m/lEVs were diluted 1:5-1:10 and applied to the Nanopore and measured similarly to the calibration particles. The

size and concentration of particles were determined using the software provided by Izon (version 3.2).

2.2.6. Proteomic analysis of extracellular vesicles

2.2.6.1. In- gel trypsin digestion

All *S. haematobium* sEV fractions and m/lEVs were resuspended in 1x loading buffer (10% glycerol, 80 mM Tris-HCl, 2% SDS, 0.01% bromophenol blue and 1.25% beta-mercaptoethanol, pH 6.8), boiled at 95°C for 5 min and electrophoresed in a 15% SDS-PAGE at 100 V. The gel was stained with 0.03% Coomassie Brilliant Blue (40% methanol, 10% acetic acid and 50% water) for 30 min at room temperature (RT) with gentle shaking and destained using destaining buffer 1 (60% water, 10% acetic acid and 30% methanol) for 1 h at RT with gentle shaking. Each lane was sliced into 6 pieces with a surgical blade and placed into a fresh Eppendorf tube. Then, slices were further destained 3 times using destaining buffer 2 (50% acetonitrile (ACN), 20% ammonium bicarbonate and 30% milliQ water) by adding 200 µl of buffer to the gel slice, and incubating at 37°C for 45 min. Supernatants were discarded and, finally, gel slices were dried in a speedivac (LabGear, Australia) on low/high medium heat (< 45°C). One hundred (100) µl of reduction buffer (20 mM dithiothreitol, 25 mM ammonium bicarbonate) was added to each dried slice, incubated at 65°C for 1 h and supernatants were discarded. Alkylation was achieved by adding 100 µl of alkylation buffer (50 mM iodoacetamide, 25 mM ammonium bicarbonate) to each gel slice, which were further incubated in darkness for 40 min at RT. Gel slices were washed with 200 µl of wash buffer (25 mM ammonium bicarbonate) and incubated at 37°C for 15 min twice after the gel slices were dried in a speedivac. For trypsin digestion, a total of 2 µg of trypsin (Sigma-Aldrich, USA) was added to each gel slice and incubated for 5 min at RT. Finally, 50 µl of trypsin reaction

buffer (40 mM ammonium bicarbonate, 9% ACN) was added to gel slices and incubated overnight at 37°C.

2.2.6.2. Peptide extraction, cleaning and purification

Supernatants containing digested peptides were obtained from each sample and placed in new Eppendorf tubes. Then, 50 µl of 0.1% trifluoroacetic acid (TFA) in 50% ACN was added to gel slices to recover extra peptides following an incubation at 37°C for 45 min. Supernatants were combined with their corresponding fractions containing the digested peptides obtained before, dried in a speedivac and kept at -20°C for desalting. Before mass spectrometry analysis, samples were desalted as follows. Peptides were resuspended with 10 µl of equilibration buffer (0.1% TFA), applied to a C18 ZipTip® column (Merck Millipore, USA), washed several times using 0.1% TFA and eluted with 5 µl of elution solution (0.1% TFA, 50% ACN). Eluted peptides were dried in a speedivac and kept at -20°C until needed.

2.2.6.3. Mass spectrometry

Each sEV fraction and m/lEVs were reconstituted in 10 µl of 5% formic acid, added onto a 50 mm 300 µm C18 trap column (Agilent Technologies, USA). The samples were then desalted for 5 min at 30 µl/min using 0.1% formic acid and the peptides were then eluted onto an analytical nano-HPLC column (150 mm × 75 µm 300SBC18, 3.5 µm, Agilent Technologies, USA) at a flow rate of 300 nL/min. Peptides were separated using a 95 min gradient of 1–40% buffer B (90/10 ACN/0.1% formic acid) followed by a steeper gradient from 40 to 80% buffer B in 5 min. A 5600 ABSciex mass spectrometer operated in information-dependent acquisition mode, in which a 1 s TOF-MS scan from 350–1400 m/z was used, and for product ion ms/ms 80–1400 *m/z* ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion. Analyst 1.6.1

(ABSCIEX, Framingham, MA, USA) software was used for data acquisition. Due to scarcity of the *S. haematobium* ES materials, EVs samples were analysed by mass spectrometry once

2.2.6.4. Database search and protein identification

All .wiff files acquired by Analyst 1.6.1 were converted into .mgf format files using MSConvert [234] and default settings. For database search and protein identification, a database was built using a concatenated target/decoy version of the *S. haematobium* predicted proteome downloaded from Parasite Wormbase (www.parasite.wormbase.org) [235, 236] and concatenated to the common repository of adventitious proteins (cRAP, https://www.thegpm.org/crap/), 116 sequences). Database search was performed using a combination of four search engines (X! Tandem version X! Tandem Vengeance (2015.12.15.2) [237], MS-GF+ version Release (v2018.04.09) [238], OMSSA [239] and Tide [240]) using SearchGUI version v3.3.3 [241]. The identification settings were as follows: Trypsin, Specific, with a maximum of 2 missed cleavages 10.0 ppm as MS1 and 0.2 Da as MS2 tolerances; fixed modifications: Carbamidomethylation of C (+57.021464 Da), variable modifications: Deamidation of N (+0.984016 Da), Deamidation of Q (+0.984016 Da), Oxidation of M (+15.994915 Da). Peptides and proteins were inferred from the spectrum identification results using PeptideShaker version 1.16.38 [241]. Peptide Spectrum Matches (PSMs), peptides and proteins were validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy hit distribution. Only proteins having at least two unique peptides were considered as identified.

2.2.6.5. Bioinformatic analysis of extracellular vesicles of *Schistosoma haematobium* proteomic data

Protein family (Pfam) domains were classified using HMMER v3.1b1 [242] as follows. The full sequences of the proteins were obtained using the accession numbers of proteins from the PeptideShaker. The identified proteins were analysed by HMMER v3.1b1 using the Pfam

database (https://pfam.xfam.org/help) [243]. Then, proteins containing Pfam domains with an E-value < 1E-05 were selected. The most predominant protein domains were identified for both sEVs and m/lEVs by counting the numbers of proteins containing one or multiple copies of a specific domain.

Protein gene ontology (GO) categories were classified using Blast2GO v5.2 [244] as follows. A fasta file containing identified proteins was loaded into Blast2GO and blast was performed against NCBI non-redundant database [245]. Functional analysis of protein sequences was performed using InterPro. Mapping was used to retrieve GO terms associated to the hits obtained by the BLAST. GO terms were selected from the GO pool obtained from mapping and query sequences were assigned using annotation. The relationship between GO terms from different GO categories was performed using Annex. Finally, integrated annotation result was produced by merging GO terms with GO annotations. ReviGO was used to visualise GO terms using semantic similarity-based scatterplots [246] as follows. The GO terms from Blast2GO were exported and parent GO terms were filtered out to avoid redundancy. Children terms with their Nodescore were loaded to http://revigo.irb.hr/ and analysed using default parameters.

Fasta files of each amino acid sequence were interrogated with TMHMM software [247] for the transmembrane domain prediction and SignalP 4.1 [248] to predict putative signal peptides.

2.3. Results

2.3.1. Density, protein concentration, particle concentration and purity of small EVs and medium/large EVs from *Schistosoma haematobium*

S. haematobium adult worm sEVs were purified using ODP. The density of the 12 sEVfractions obtained after gradient ranged from 1.039 to 1.4 g/ml (Table 2.1). Protein concentration of each sEV fraction and m/lEVs was measured by Bradford assay and the size and concentration were determined using a qNano. The protein and particle concentration of the 12 sEV fractions ranged from 1.6 to 25.35 μg/ml and 3.72×10^{6} to 1.90×10^{8} particles/ml, respectively, while the protein and particle concentration of m/lEVs was 18 μg/ml and 1.08×10^{7} particles/ml, respectively. The size of the 12 sEV fractions ranged from 135 nm ± 19.3 to 342 nm ± 113.9 and size of m/lEVs was 249 nm ± 22.7. sEV fractions having an appropriate purity and density [24] (fractions 5–9) were selected for further analysis (Fig. 2.1). sEV fractions 6 and 9 contained the highest number of sEVs (1.9×10^{8} and 1.21×10^{8} particles/ml, respectively).

Table 2.1. Density, protein concentration, particle concentration and purity of *Schistosoma haematobium* adult worm medium/large EVs and small EV fractions after purification by Optiprep® density gradient.

sEV Fractions and m/lEVs	Density (g/ml)	Protein concentration (µg /ml)	Particle concentration (particles/ml)	Purity of vesicles (particles/µg)	Size
1	1.039	5.29	2.26×10^{7}	4.27×10^{6}	190 nm ± 27.2
2	1.04	1.6	5.31×10^{7}	3.32×10^{7}	216 nm ± 14.4
3	1.05	1.8	6.96×10^{7}	3.72×10^{7}	135 nm ± 19.3
4	1.06	3.4	3.72×10^{6}	1.09×10^{6}	150 nm ± 29.9
5	1.07	3.29	9.13×10^{7}	2.77×10^{7}	136 nm ± 12.4
6	1.08	8.4	1.90×10^{8}	2.26×10^{7}	146 nm ± 11.3
7	1.08	8.09	9.58×10^{7}	1.18×10^{7}	153 nm ± 10.2
8	1.13	8.69	8.91×10^{7}	1.02×10^{7}	173 nm ± 19.3
9	1.16	11.64	1.21×10^{8}	1.04×10^{7}	191 nm ± 27.4
10	1.24	18.86	1.53×10^{8}	7.95×10^{6}	342 nm ±113.9
11	1.29	25.35	6.20×10^{7}	2.44×10^{6}	310nm ± 103.2
12	1.4	23.35	1.02×10^{7}	4.36×10^{5}	233 nm ± 77.5
m/lEVs	-	18	1.08×10^{7}	6×10^{5}	249 nm ± 22.7

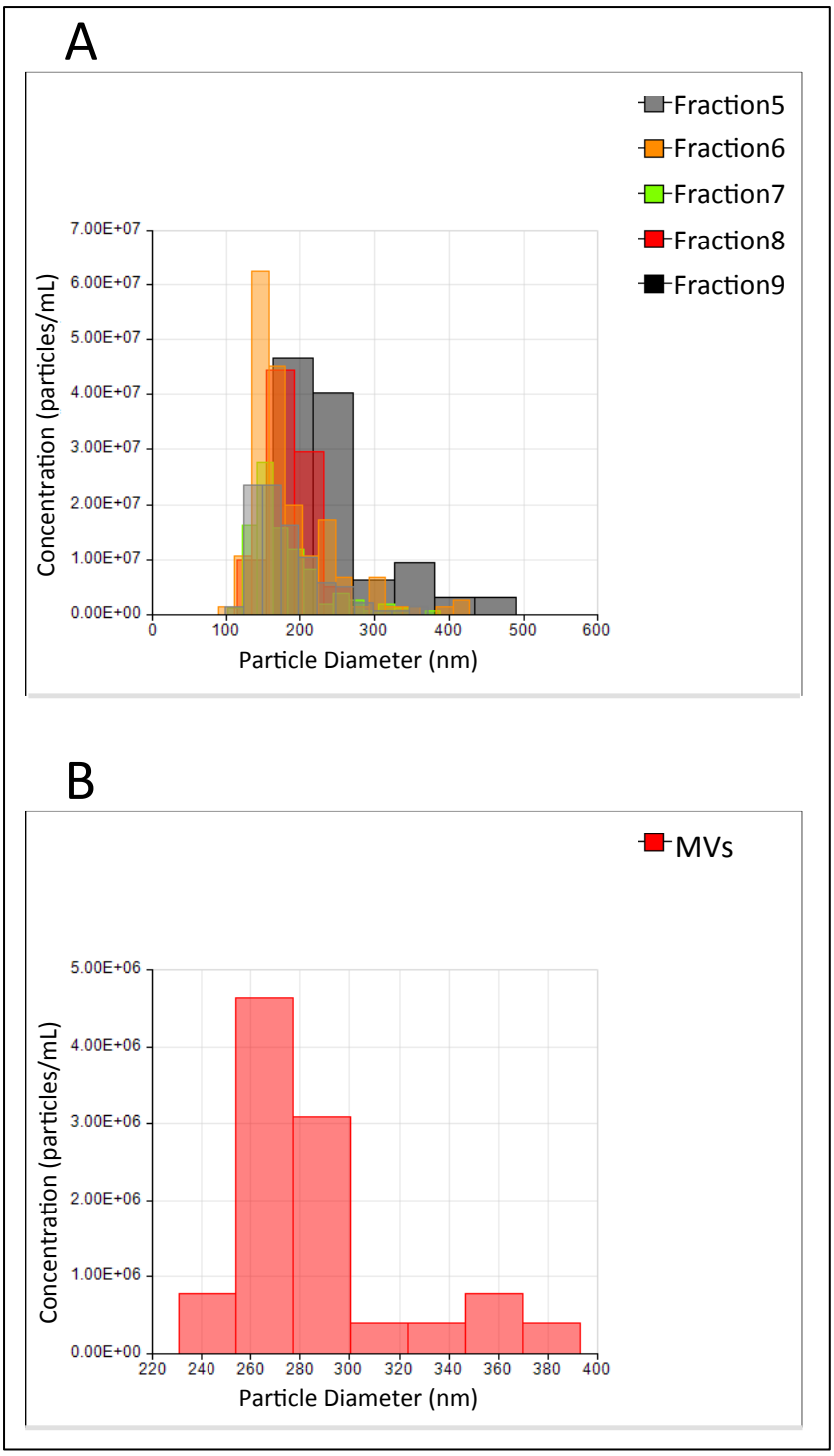

Figure 2.1. Tunable resistant pulse sensing analysis of small EVs and medium/large EVs from *Schistosoma haematobium*. Size and number of EVs secreted by *S. haematobium* was analysed by qNano (iZon). A) Size and concentration of particles in sEV-containing fractions (5-9). B) Size and particle concentration of *S. haematobium* m/lEVs.

2.3.2. Proteomic analysis of small EVs and medium/large EVs from *Schistosoma haematobium*

The proteome composition of *S. haematobium* adult worm EVs was characterised by LC-MS/MS. After combining the results from fractions 5-9 (fractions containing the highest purity of sEVs), a total of 133 proteins matching *S. haematobium* proteins and common contaminants from the cRAP database were identified. From these, 80 proteins were identified with at least two validated unique peptides and 57 of them matched *S. haematobium* proteins. From the 57 identified proteins, 8 (14%) contained a transmembrane domain and 7 (12%) had a signal peptide. Similarly, 509 proteins were identified from m/lEVs. From these, 346 proteins were identified with at least two validated unique peptides and 332 matched *S. haematobium* proteins. From these identified proteins, 54 (16%) contained a transmembrane domain and 26 (8%) had a signal peptide. Forty proteins were identified in both sEVs and m/lEVs. The identity of the most abundant proteins and proteins typically found in other helminth EVs are shown in table 2.2.

Table 2.2. List of proteins found only in each type of *Schistosoma haematobium* extracellular vesicle.

Protein location	Protein identity	Protein accession numbers
sEVs	Proteasome subunit	MS3_10249.1, MS3_05734.1, MS3_01483.1, MS3_06009.1, MS3_04526.1, MS3_08808.1, MS3_07240.1, MS3_02807.1, MS3_09236.1 and MS3_03070.1
	GAPDH	MS3_10141.1
	Papain family cysteine protease	MS3_08498.1
	C-terminal domain of 1-Cys peroxiredoxin	MS3_08460.1
	Ferritin-like domain	MS3_08059.1
	S-adenosyl-L-homocysteine hydrolase	MS3_04449.1
	Cytosol amino peptidase	MS3_01749.1
	Trefoil (P-type) domain-containing protein	MS3_00004.1
sEVs and m/lEVs	TSPs	MS3_09198, *Sh*-TSP-2, MS3_05226, MS3_05289 and MS3_01153
	Ferritin-like domain	MS3_07972.1 and MS3_07178.1
	14-3-3 protein	MS3_03977.1 and MS3_00047.1
	Elongation factor Tu C-terminal domain	MS3_08479.1
	EF hand	MS3_08446.1
	Actin	MS3_07374.1
	GST, N-terminal domain	MS3_06482.1
	Cytosol aminopeptidase family, catalytic domain	MS3_08450.1
	Lipocalin / cytosolic fatty-acid binding protein family	MS3_04307.1
	Immunoglobulin domain	MS3_03208.1
	Saposin-like type B, region 2	MS3_02805.1
	Enolase, N-terminal domain	MS3_02425.1
m/lEVs	EF hand	MS3_05735.1, MS3_00180.1, MS3_09846.1, MS3_05877.1, MS3_05317.1, MS3_04536.1, MS3_10043.1, MS3_05959.1, MS3_05150.1, MS3_04275.1, MS3_05958.1 MS3_05952.1, MS3_00361.1 and MS3_02003.1

	Ras family	MS3_10193.1, MS3_05953.1, MS3_05910.1, MS3_05976.1, MS3_07854.1, MS3_11139.1, MS3_02375.1, MS3_01653.1, MS3_04355.1, MS3_09110.1, MS3_09593.1 and MS3_03443.1
	TCP-1/cpn60 chaperonin family	MS3_03054.1, MS3_06928.1, MS3_01627.1, MS3_10572.1, MS3_06669.1, MS3_07556.1, MS3_08399.1, MS3_00785.1 and MS3_08926.1
	TSPs	MS3_01905.1 and MS3_01370
	Heat-like repeat	MS3_08696.1, MS3_01642.1, MS3_09658.1, MS3_10590.1, MS3_05814.1, MS3_02928.1 and MS3_06293.1
	Calponin homology (CH) domain	MS3_07481.1, MS3_05505.1, MS3_01744.1, MS3_00852.1, MS3_00361.1, MS3_03766.1 and MS3_10701.1
	Dynein light chain type 1	MS3_05351.1, MS3_08569.1, MS3_05345.1, MS3_01173.1, MS3_05342.1, MS3_04412.1 and MS3_05960.1
	Actin	MS3_07374.1, MS3_04014.1, MS3_00351.1, MS3_02465.1, MS3_04907.1 and MS3_01922.1
	HSP-70 protein	MS3_10713.1, MS3_11293.1, MS3_11411.1, MS3_10049.1, MS3_02688.1 and MS3_02787.1
	Immunoglobulin domain	MS3_03027.1, MS3_01271.1, MS3_03208.1, MS3_07594.1 and MS3_01223.1
	Annexin	MS3_08725.1, MS3_08723.1, MS3_04598.1, MS3_01964.1 and MS3_01952.1
	AAA domain	MS3_03802.1, MS3_02581.1, MS3_01139.1, MS3_01650.1 and MS3_07031.1
	14-3-3 protein	MS3_03977.1, MS3_05219.1, MS3_00047.1, MS3_01871.1 and MS3_03976.1

2.3.3. Protein families present in *Schistosoma haematobium* small EVs and medium/large EVs

Identified proteins were subjected to a Pfam analysis using default parameters in HMMER v3.1b1 and proteins containing an identified Pfam domain with a E< 1E-05 were selected. A total of 70 and 387 domains were identified from sEVs and m/lEVs, respectively. In sEVs, the three most abundant domains were proteasome subunit domains (PF00227) (14%), TSP family domains (PF00335) (7%) and ferritin-like domains (PF12902) (4%) (Fig. 2.2A). The most abundant protein domains from m/lEVs were EF-hand domains (PF00036) (3%), Ras family domains (PF00071) (3%), TCP-1/cpn60 chaperonin family domains (PF00118) (2%) and TSP family domains (PF00335) (2%) (Fig. 2.2B). From these, TSP family domains, ferritin-like domains and 14-3-3 protein domains were common to sEVs and m/lEVs.

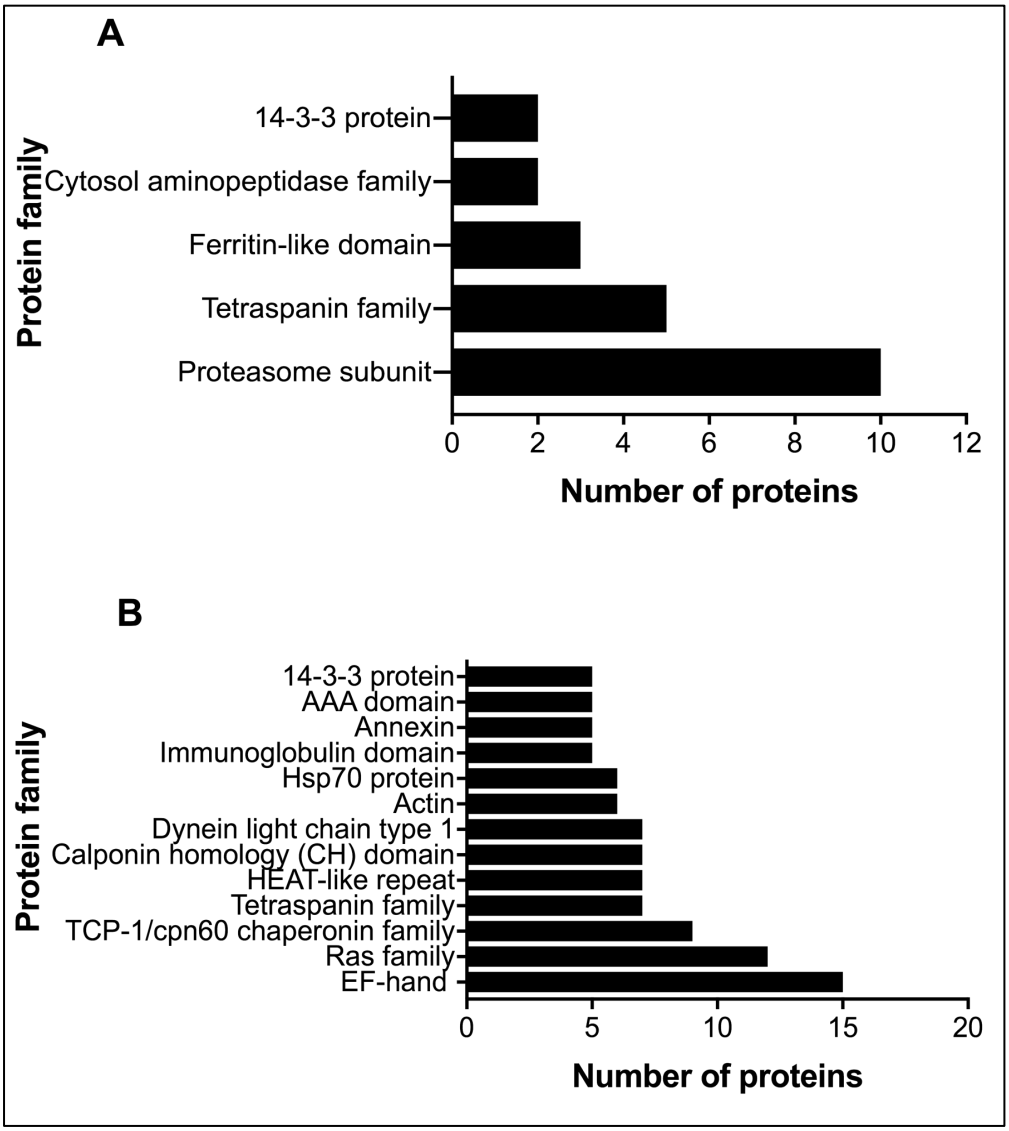

Figure 2.2. Pfam analysis of the most abundant *Schistosoma haematobium* vesicle proteins. X-axis represents the number of proteins containing at least one of those domains. (A) sEVs (B) m/lEVs.

2.3.4. Gene ontology terms of *Schistosoma haematobium* small EVs and medium/large EV proteins

The proteins of adult *S. haematobium* sEVs and m/lEVs were annotated using Blast2GO [244]. In sEVs, Blast2GO returned at least one biological process, molecular function or cellular function term for 70%, 81% and 65% of proteins, respectively. In m/lEVs, Blast2GO returned at least one biological process, molecular function or cellular component term for 52%, 65%

and 48% of proteins, respectively. To avoid redundancy in the analysis and for a better comprehension of the represented GO terms in the sEVs and m/lEVs, the parental GO terms were removed. In sEVs, after removal of parental redundancy, 11, 9 and 7 GO terms belonging to biological processes, molecular function and cellular component, respectively, were identified. Similarly, for m/lEVs, 12, 4 and 8 GO terms belonging to biological processes, molecular function and cellular component, respectively, were identified. These children GO terms were visualised using ReviGO based on semantic similarity-based scatterplots [246]. The GO terms were ranked by the nodescore provided by Blast2GO and plotted using their nodescore and frequency. Semantically similar GO terms plot close together and increasing heatmap score signifies increasing nodescore from Blast2GO. The circle size denotes the frequency of the GO term from the underlying database. In sEVs, several biological processes were highly represented, such as the ubiquitin-dependent protein catabolic process, oxidation-reduction process and gluconeogenesis and glycolytic process (Fig. 2.3A). Similarly, in m/lEVs, several biological processes were highly represented, such as the carbohydrate metabolic process, transport process, organonitrogen compound metabolic process and microtubule-based process (Fig. 2.3B). Oxidation-reduction process was common to both sEVs and m/lEVs. Six sEV proteins and 22 MV proteins were predicted to be involved in this process.

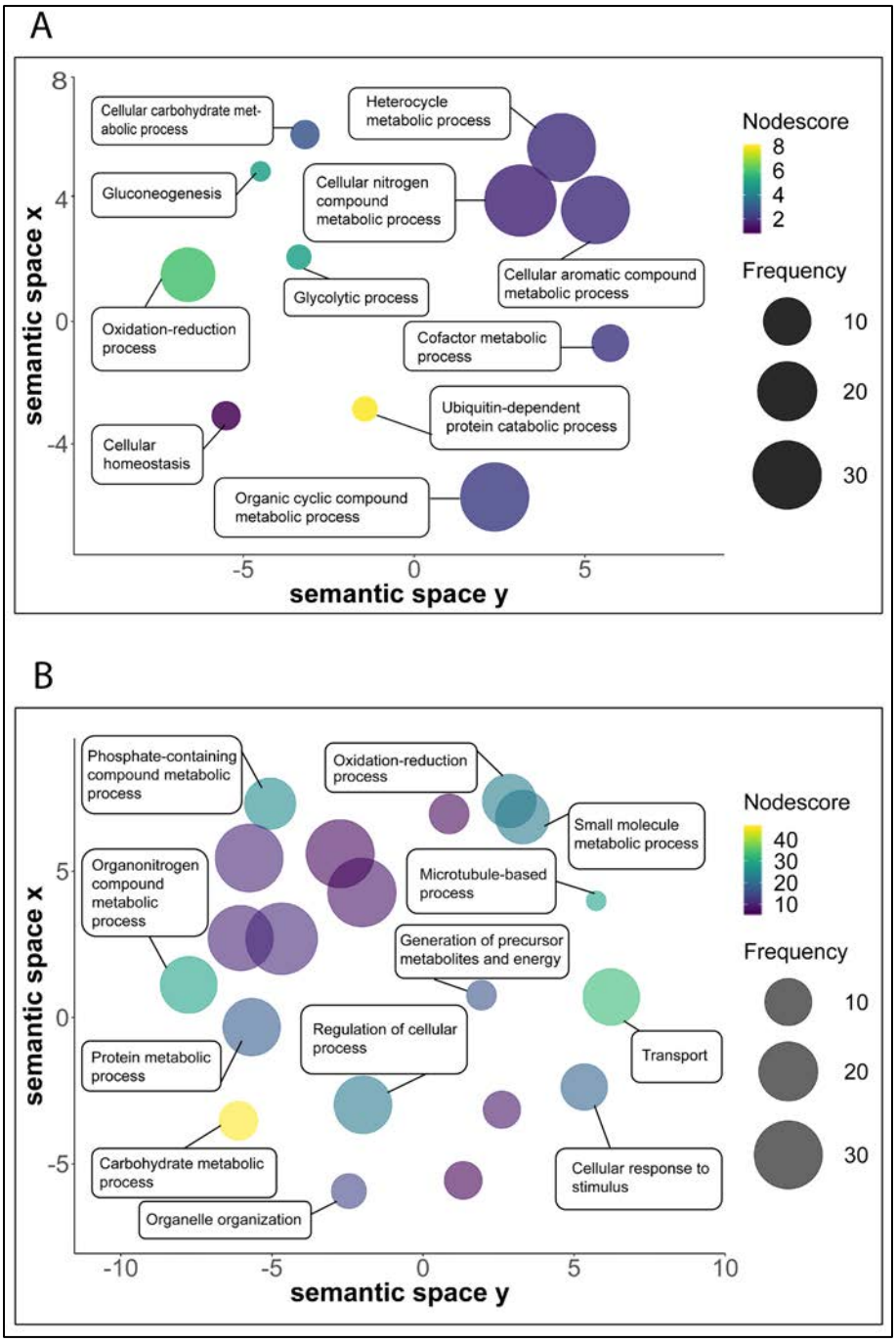

Figure 2.3. Biological process GO term categories of adult *Schistosoma haematobium* vesicle proteins. Biological processes were ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database. (A) sEVs (B) m/lEVs

In sEVs, several molecular functions were highly represented, such as threonine-type endopeptidase activity, protein binding activity, endopeptidase activity and transition metal ion binding activity (Fig. 2.4A). In m/lEVs, molecular functions such as protein binding activity, ATP binding activity, nucleoside-triphosphatase activity and calcium ion binding activity were highly represented (Fig. 2.4B). From these highly represented molecular function terms, protein binding was common to both sEVs and m/lEVs, with 5 proteins from sEVs and 83 proteins from m/lEVs being involved in this process. All 5 sEV proteins involved in protein binding were also involved in protein binding from m/lEVs.

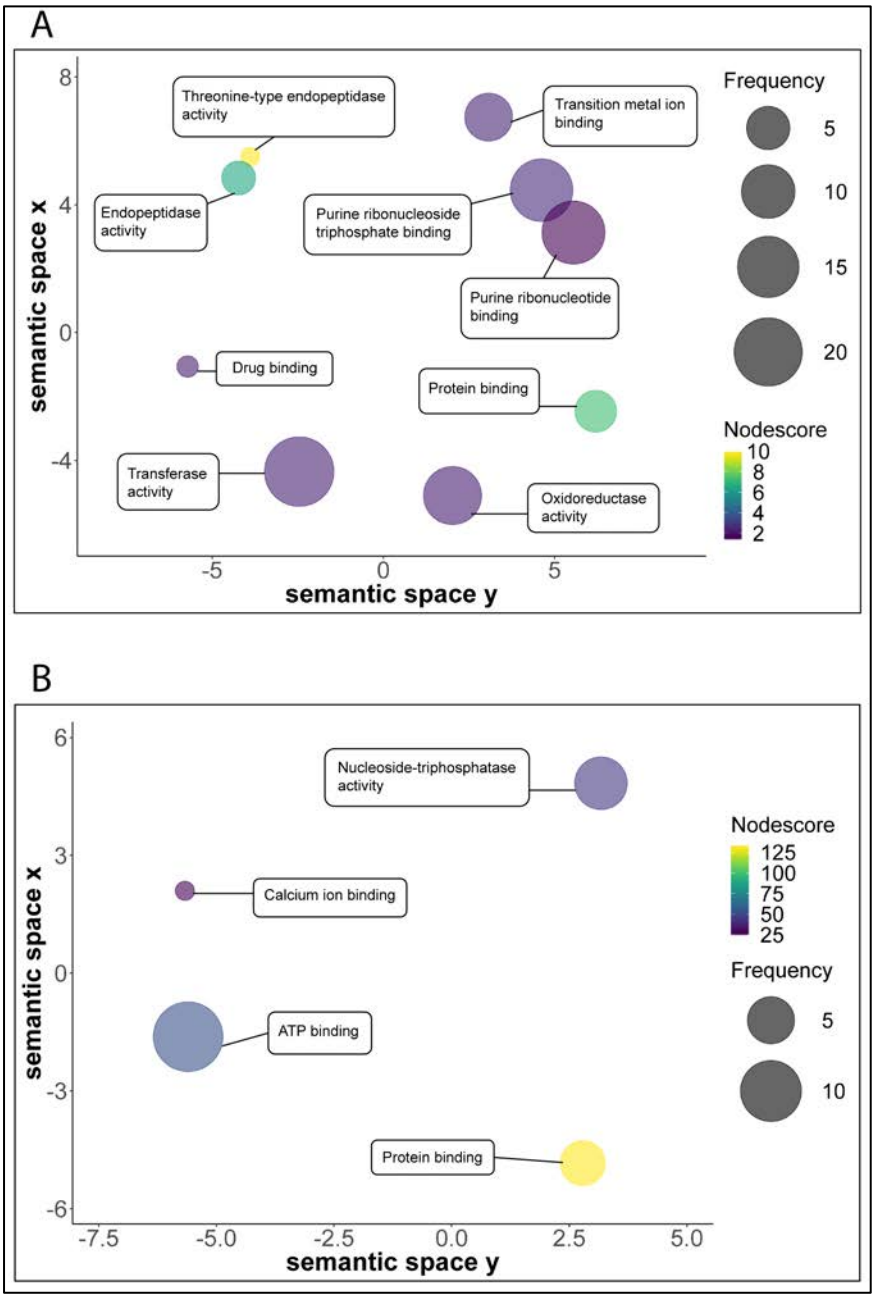

Figure 2.4. Molecular function GO term categories of adult *Schistosoma haematobium* vesicle proteins. Molecular functions were ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database. (A) sEVs (B) m/lEVs.

The most highly represented cellular component terms in sEVs were proteins associated with nucleus, integral component of membrane, non-membrane-bounded organelle and proteasome core complex (alpha-subunit complex) (Fig. 2.5A) and the most abundant cellular component terms in m/lEVs were proteins associated with integral component of membrane, cytoskeletal part, cytoplasmic part and non-membrane-bounded organelle (Fig. 2.5B). Integral component of membrane, cytoplasmic part and non-membrane-bounded organelle terms were common to both sEVs and m/lEVs. In sEVs and m/lEVs, 9 and 83 proteins, respectively, were integral membrane proteins. From sEVs, 5 TSPs are integral membrane proteins whereas in m/lEVs 7 TSPs and calpain large subunit domain are integral membrane proteins. In m/lEVs, 33 proteins including, saposin-like type B protein are located in the cytoplasmic parts of the cell.

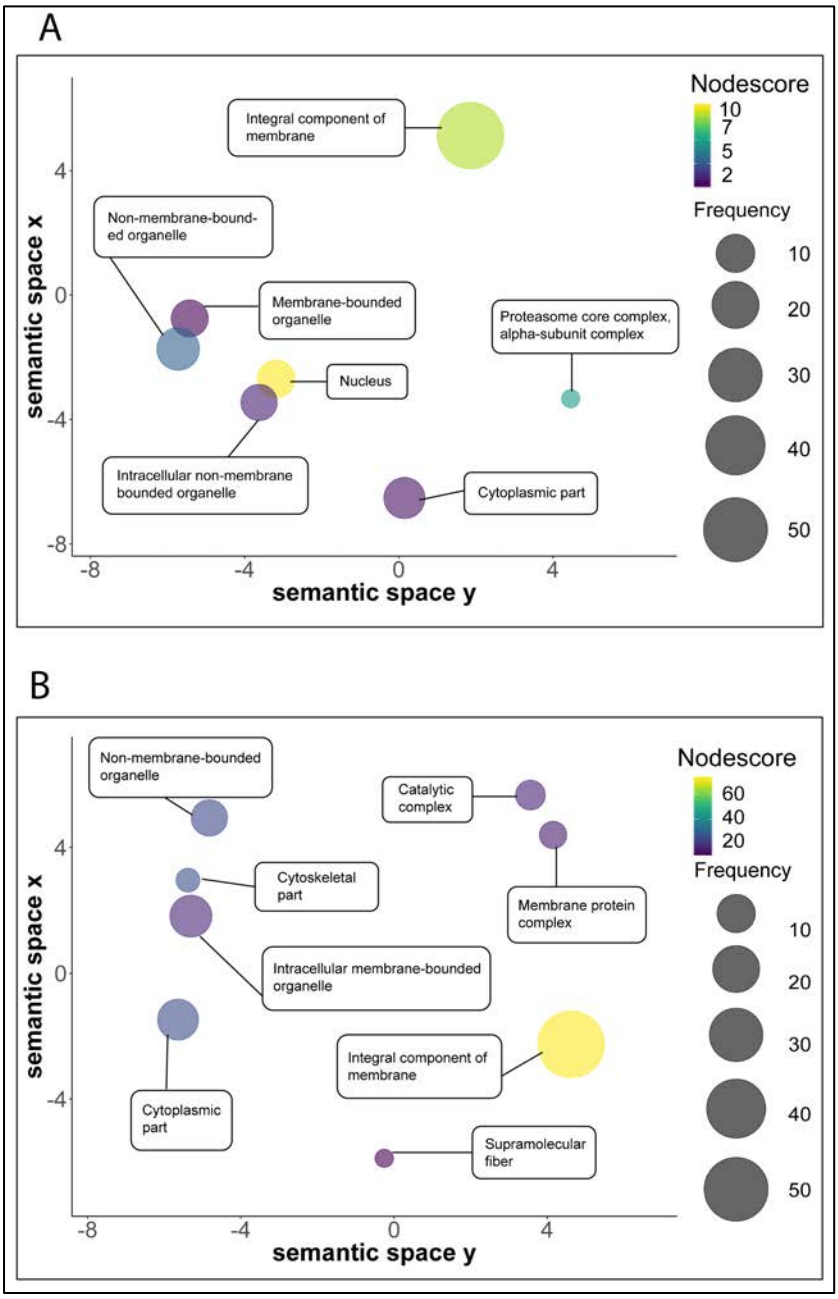

Figure 2.5. Cellular component GO term categories of adult *Schistosoma haematobium* vesicle proteins. Cellular components were ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database. (A) sEVs (B) m/lEVs.

2.4. Discussion

This study showed that *S. haematobium* secretes at least two populations of vesicles (m/lEVs and sEVs) with a clear difference in size and proteomic composition. After separating them from m/lEVs, the sEV fractions were further fractionated by ODP to isolate pure vesicles by reducing contamination from soluble proteins [24]. The size of the purified sEVs ranged from 136 nm ± 12.4 to 191 nm ± 27.4, while in *S. mansoni*, the size of sEVs ranged from 77.4 nm ± 34.8 to 97.9 nm ± 28.5 [24]. The size of *F. hepatica* sEVs ranged from 30-100 nm [118], while in the nematode, *A. suum*, *T. muris* and *N. brasiliensis*, their size ranged from 80 nm to 200 nm, 93 nm ± 41.5 to 165 nm ± 54.9 and 60 nm to 160 nm [122, 123, 126], showing the heterogenicity of EV size between helminths.

Only a fraction of proteins from sEVs and m/lEVs were predicted to contain signal peptides or transmembrane domains, in agreement with similar reports from *O. viverrini*, *S. mansoni, N. brasiliensis and T. muris* [24, 117, 122, 123]. The presence of many proteins without signal peptides and transmembrane domains indicates the role of vesicles in the release of these leaderless proteins into the external environment [249].

The most represented domains contained within sEV proteins were the proteasome subunit domains, TSP domains, ferritin-like domains, cytosol aminopeptidase family catalytic domains and 14-3-3 protein domains. The proteasome is involved in the biogenesis of EVs [250] and also controls protein homeostasis and degradation of damaged proteins [251]. Furthermore, in schistosomes, the proteasome plays an important role in the cellular stress response and survival of the parasite [252]. For example, treating mice with a proteasome inhibitor and infecting with *S. mansoni* cercariae significantly impaired parasite development [253], and treating schistosomula *in vitro* with siRNAs targeting a deubiquitinase subunit of the 19S regulatory particle significantly reduced parasite viability [254]. Proteasomes have also been

targeted in vaccine strategies; immunisation of mice with *S. japonicum* a5-subunit proteasome stimulated a strong antibody response and significantly reduced adult worm and egg burden in the subsequent challenge [255].

Ferritins are iron-storage proteins, involved in maintaining intracellular iron balance [256]. This activity of ferritins minimizes free-radical reactions and prevents cellular damage caused by iron accumulation in the cell [257]. Iron also plays an important role in eggshell formation of schistosomes and *fer-1* is highly expressed in female worms in comparison to males [258]. Since, female worms produce many eggs per day and eggs are the primary cause of pathology, vaccination using ferritins could disrupt the formation of eggs and reduce egg-induced pathology [259]. Indeed, ferritins have been tested as vaccine candidates against schistosomes; immunisation of mice with the recombinant Fer-1 of *S. japonicum* caused 35.5% and 52.1% reduction in adult worm and liver egg burden, respectively [260]. Besides their role as vaccines, ferritins could also be drug targets for iron chelators in parasitic infection [261], and iron chelators have been shown to halt the growth of schistosomes and protozoan parasites *in vitro* [262, 263]. Ferritins have been identified in the proteomic analysis of other blood feeding helminths EVs [24, 118], suggesting a role for EVs in iron acquisition.

In helminths, aminopeptidases cleave peptides to free amino acids before being distributed to the internal tissues of the parasite [264]. They are also involved in the egg hatching process of schistosomes [265] as RNAi-mediated silencing of *S. mansoni* egg aminopeptidase significantly decreased hatching [266]. Moreover, aminopeptidases have been tested as vaccine candidate antigens against *F. hepatica* infection in animal models; immunisation of sheep with native and recombinant leucine aminopeptidases resulted in 49-89% adult worm reduction in subsequent *F. hepatica* challenges [185, 186]. Interestingly, this vaccine candidate was also found in the EVs from other hematophagous helminths like *S. mansoni* [24, 118]. Like ferritin,

the presence of these proteolytic enzymes within the EVs of *S. haematobium* and other helminths suggests the involvement of EVs in nutrient acquisition processes.

From GO analysis, the most represented cellular component of sEV proteins was the nucleus and integral component of membrane. This is consistent with the pfam analysis of sEVs, in which proteasome subunit-containing proteins, the most abundant protein family, is formed from the internal parts of the cell, whereas TSPs, the second most abundant protein family, is an integral component of the EV membrane. This supports the idea that sEVs are formed from endosomes and discharged when the MVB blends with the cell membrane [96] as occurs in other organisms [118].

The second and fourth most abundant protein domains in sEVs and m/lEVs, respectively, were the TSPs. TSPs are involved in EV biogenesis [97] and they are present on the surface membrane of EVs from many different organisms and are considered a molecular marker of EVs [267]. TSPs are also found from the proteomic analysis of other helminth EVs [24, 25, 117-123]. In trematodes, TSPs are involved in tegument development [157-159] and in schistosomes, TSP LELs have been tested as vaccine candidates [160, 161, 268]. Furthermore, antibodies produced against TSPs present in *O. viverrini* EVs blocked the internalisation of EVs by cholangiocytes and decreased pathogenesis [117, 160]. TSPs from other platyhelminths such as *Taenia solium* and *S. japonicum* are immunogenic and have potential as diagnostic candidates [157, 159, 231, 269-271].

The most represented domains in *S. haematobium* m/lEVs were EF-hand, Ras family, TCP-1/cpn60 chaperonin family, TSP family, heat-like repeat, calponin homology (CH), dynein light chain type 1 and actin domains. Proteins containing EF-hand domains are involved in a number of protein-protein interactions for the uptake and release of calcium [272]. The influx of calcium in the cell induces the redistribution of the phospholipids of cell membrane resulting in an increase of the release of m/lEVs [273]. EF-hand domains are the most predominant

protein domains found within other helminths EV proteins [117]. This is also in line with the GO analysis, in which proteins involved in protein binding and calcium ion binding are the highest represented molecular function terms. Ras proteins serve as signalling nodes activated in response to diverse extracellular stimuli [274] and Ras proteins are involved in biogenesis and release of m/lEVs [97]. In *S. mansoni*, Ras proteins are involved in the male-directed maturation of the female worms [275], which could suggest a potential role of EVs in parasite-parasite communication. TCP-1/cpn60 chaperonin family proteins play an important role in the folding of proteins, including actin and tubulin [276], which bind and hydrolyse ATP using magnesium ions [277]. This is consistent with the molecular function GO terms, in which, ATP binding and nucleoside-triphosphatase was the most represented. The calponin homology (CH) domain is an actin-binding protein playing a major regulatory role in muscle contraction [278]. Contraction of cytoskeletal structures by actin–myosin interactions completes vesicle formation [100] and the presence of actin together with calponin helps the contraction of the membrane that facilitates the release of m/lEVs [97]. Dynein is involved in the transport of vesicles and organelles, as well as positioning the mitotic spindle and microtubule organizing centres with respect to the cell cortex [279]. This is also in line with the GO analysis of m/lEVs, in which transport is one the highest represented biological process terms.

The proteomic analysis *S. haematobium* EVs (sEVs and m/lEVs), revealed many proteins involved in host-parasite interactions. Some of these proteins were homologues of other helminths vaccine and diagnostic candidates, including TSPs.

Chapter 3: Characterisation of tetraspanins from *Schistosoma haematobium* extracellular vesicles

3.1. Introduction

In chapter 2, the proteomic composition of *S. haematobium* adult worms EVs was characterised, revealing many different proteins, including TSPs. TSPs are a family of proteins expressed in many organisms [156] and involved in many biological processes, including membrane organisation [147], modulation of immunity [280] and cancer progression [281]. The first member of the TSP family to be identified in humans was CD81 [282]; since then, this family of proteins has been extensively studied in other organisms. To date, a total of 33, 37 and 20 different TSPs have been found in mammals, *Drosophila melanogaster* and *Caenorhabditis elegans,* respectively [151].

In parasitic helminths, TSPs are involved in host-parasite interactions and some of them are being tested as potential vaccines (reviewed in chapter 1.3.1.2.1) and diagnostic candidates [160, 161, 163, 268-270]. *Sm*23 was the first TSP reported from schistosomes [149] and, thereafter, other TSPs from *S. mansoni* and *S. japonicum* have been identified and characterised [161, 283-285]. In addition to their presence in the tegument of helminths [17, 157, 159, 161, 286], TSPs have also been found on the membrane of EVs from many of helminths (including *F. hepatica, T. muris, S. mansoni, O. viverrini, S. japonicum, E. multilocularis, E. granulosus* and *N. brasilensis* [24, 25, 117-123]) and are considered a molecular marker of exosomes [267]. Antibodies produced against TSPs present in the membrane of *O. viverrini* EVs blocked the internalization of EVs by cholangiocytes and decreased the production of cytokines that initiate the pathogenesis [117, 160]. Although TSPs are well characterised in other schistosomes, no studies have been conducted on TSPs from *S. haematobium*. Therefore this

study aimed to characterise *Sh*-TSPs found from the proteomic analysis of adult worm EVs in terms of their potential as vaccine and diagnostic candidates for the control of urogenital schistosomiasis.

There are 19 *Sh*-TSPs encoded in *S. haematobium* genome and, from these, 7 of them were found in *S. haematobium* EVs. Herein, I have selected a total of six *Sh*-TSPs that might be playing key roles in host-parasite interactions based on their presence in *S. haematobium* adult EVs. The expression level of each *Sh*-TSP in different stages of the parasite was analysed by qPCR and the anatomic sites of expression in adult worms was assessed using antibodies raised to recombinant *Sh*-TSPs to broaden understanding of this family of molecules.

3.2. Materials and methods

3.2.1. Experimental animals

Male BALB/c mice were purchased and maintained at the AITHM animal facilities on the Cairns campus as discussed previously (chapter 2.2.1). All experimental procedures performed on animals in this study were approved by the James Cook University (JCU) animal ethics committee (ethics approval number A2391 and A2395).

3.2.2. *Schistosoma haematobium* material

Adult *S. haematobium* worms were obtained as previously described (chapter 2.2.3). Freshly perfused *S. haematobium* adult worms were fixed in paraformaldehyde, embedded in paraffin and cryostatically sectioned into 7.0 µm sections [287].

S. haematobium cercariae were mechanically transformed into schistosomula as described previously [233]. Schistosomula were resuspended to a density of 1,000/ml in modified Basch media supplemented with 4x antibiotic-antimycotic and incubated at 37°C in 5% CO_2. Media was changed daily and schistosomula (1,000) collected at 24 h, 3 and 5 days and immediately stored in 500 µl of TRI reagent (Sigma-Aldrich, USA) at -80°C until further use.

3.2.3. RNA extraction, cDNA synthesis and real time quantitative PCR (RT-qPCR)

Total RNA from *S. haematobium* schistosomula (24 h, 3 and 5 days) was extracted using TRI reagent as per manufacturer's instructions. The RNA pellet was finally resuspended in 12 µl of RNAse-free water and incubated for 5 min at 55°C. First strand cDNA was synthesized using superscript III (Invitrogen, USA) and 11 µl of RNA according to manufacturer's instructions. The cDNA of *S. haematobium* adult, miracidia, cercariae and egg stages were provided by BRI (Maryland, USA).

RT-qPCR was performed to determine the expression levels of *S. haematobium* TSP-encoding genes in different life stages (adult, egg, miracidia, cercaria and schistosomula (24 h, 3 days and 5 days)). Each qRT-PCR reaction consisted of 5 µl of SYBR premix EX Taq (2x) (Qiagen, Netherlands), 1 µl (10 mM) of each forward and reverse primer (Appendix Table 1), 1 µl (50 ng) of the first–stand cDNA and sterile water to a final volume of 10 µl. The reactions were performed on a Rotor-Gene Q (Qiagen, Netherlands) using the following conditions: initial denaturation at 95°C for 10 min (1 cycle) followed by 40 cycles of denaturation (95°C for 10 sec), annealing (50°C for 15 sec) and extension (72°C for 20 sec). *S. haematobium tsp* expression was normalised to a housekeeping gene (*α-tubulin*, accession number XM_012938434.1) as described before [288] and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method using eggs as a reference group [289].

3.2.4. Phylogenetic analysis

A phylogenetic analysis was performed on the 19 *Sh*-TSPs and 32 well-characterised TSPs from different organisms (*Bos taurus, Danio rerio, Homo sapiens, Mus musculus, S. japonicum, S. mansoni* and *O. viverrini*) belonging to 3 different groups of TSP families (uroplakin, CD81/CD9 and CD63). The sequences of these TSPs were obtained from the NCBI database. A multiple sequence alignment was carried out using the alignment program MAFFT (v7.271) [290]. Outliers with poor alignment (long unaligned regions) were detected and filtered out using ODSeq v1.0 *(https://bioconductor.org/packages/release/bioc/html/odseq.html*) resulting in the removal of two sequences (XP_012793320 and XP_012797041). PhyML (v20160207) [291] was used for maximum-likelihood (ML) phylogenetic analyses of the amino acid sequences using default parameters. The tree was visualised with The Interactive Tree of Life (iTOF) online phylogeny tool (https://itol.embl.de/) [292] using default parameters.

3.2.5. Cloning of *Schistosoma haematobium* tetraspanins

The cDNAs encoding for the open reading frames of the 5 *Sh-tsps* (*ms3_09198*, *ms3_01370*, *ms3_01153*, *ms3_05226* and *ms3_05289*) were obtained from the cDNA library of *S. haematobium* (www.parasite.wormbase.org), while the cDNA encoding for the open reading frame for *Sh-tsp*-2 was obtained from Genbank (accession number MK238557). The LEL regions from the 6 *Sh*-TSPs were identified using Tmpred (https://embnet.vital-it.ch/software/TMPRED_form.html) and amplified by PCR using oligonucleotide primers flanking these regions and *S. haematobium* adult cDNA as a template. The PCR reaction was performed as follows: 2 µl (50 ng) adult worm cDNA, 3 µl (10 µM) each of forward primer and reverse primer (Appendix Table 2), 10 µl MyTaq red reaction buffer (Bioline, UK), 31.5 µl water and 0.5 µl MyTaq DNA polymerase (Bioline, UK). The PCR cycling conditions followed an initial cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 15 sec and extension at 72°C for 30 sec and a final extension step at 72°C for 7 min. Finally, the PCR products of *ms3_09198*, *ms3_01370*, *ms3_01153*, *ms3_05226* and *ms3_05289* were *Nco*I/*Xho*I cloned into pET-32aΔHis such that they were in frame with the N-terminal thioredoxin (TrX) and C-terminal 6xHis tags. The vector pET-32aΔHis is an in-house modified version of pET-32a (Novagen, USA) which has the N-terminal 6XHis-tag absent to facilitate efficient purification after cleavage of the TrX tag. The *Sh-tsp*-2 PCR product was *Nde*I/*Xho*I cloned into pET41a (Novagen, USA) to facilitate native N-terminal expression without the GST fusion tag but retaining the C-terminal 6xHis tag. Recombinant vectors were transformed into *E. coli* TOP10 strains (ThermoFisher Scientific, USA) and recombination confirmed by sequencing.

3.2.6. Protein expression

Plasmids (pET32a recombinants) were transformed into *E. coli* BL21(DE3) (ThermoFisher Scientific, USA) and resultant colonies were inoculated into 10 ml of Luria broth containing 100 µg/ml ampicillin (LB_{amp}) and incubated overnight at 37°C with shaking at 200 rpm. Overnight culture was seeded (1/100) into 500 ml of fresh LB_{amp} and incubated at 37°C with shaking at 200 rpm until OD_{600} = 0.5-1 (approximately 3 h), whereupon expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Bioline, UK). Cultures continued incubating for 4 h (MS3_09198, MS3_01370, MS3_05226, MS3_01153) or 16 h (MS3_05289) and were then harvested by centrifugation at 8,000 ×*g* for 20 min at 4°C. *Sh*-TSP-2 was expressed as for MS3_01370 except that LB supplemented with 50 µg/ml kanamycin was used instead of LB_{amp}.

Each harvested pellet was resuspended in 50 ml of lysis buffer (50 mM sodium phosphate pH 8, 40 mM imidazole and 300 mM NaCl), freezed/thawed 3 times and then sonicated 10 times (5 s bursts) at 4°C. Then, for the soluble proteins (*Sh*-TSP-2, MS3_09198 and MS3_01370 – determined by a small-scale pilot expression) the bacterial lysate was centrifuged at 20,000 ×*g* for 20 min at 4°C and the supernatant decanted and stored at -80°C. In the case of insoluble proteins (MS3_01153, MS3_05226 and MS3_05289 – determined by small-scale pilot expression), Triton X-100 was added to a final concentration of 3% after sonication, the mixture incubated for 1 h at 4°C with gentle shaking and then pelleted at 20,000 ×*g* for 20 min at 4°C. The supernatant was removed, the pellet washed twice with 30 ml of lysis buffer (with centrifugation at 20,000 ×*g* for 20 min at 4°C after each wash) and the final pellet resuspended in 20 ml of solubilisation buffer (50 mM sodium phosphate, 40 mM imidazole, 300 mM NaCl and 6 M urea). The resuspension was incubated at 4°C overnight with gentle shaking, centrifuged at 20,000 ×*g* for 20 min at 4°C and the supernatant decanted and stored at -80°C.

3.2.7. Protein purification

Recombinant proteins were purified by Ni^{2+} IMAC using an AKTA Prime UPC FPLC (GE Healthcare, USA). Each recombinant protein solution was diluted 1:4 in buffer A (1x PBS, 300 mM NaCl (soluble proteins) or 1x PBS, 300 mM NaCl and 6 M urea (insoluble proteins) pH 8) and filtered through a 0.45 μm filter. The solutions were then applied to a 1 ml His-Trap IMAC column (GE Healthcare, USA), equilibrated with buffer A (1x PBS, 300 mM NaCl (soluble proteins) or 1x PBS, 300 mM NaCl and 6 M urea (insoluble proteins) pH 8), at a flow rate of 1 ml/min. Bound proteins were washed with 10 column volume of buffer A for both soluble proteins or insoluble proteins and then eluted with an increasing linear gradient of imidazole (100-500 mM). Fractions containing purified recombinant proteins were combined and buffer exchanged into PBS (soluble proteins) or (1x PBS, 300 mM NaCl and 6 M urea) (insoluble proteins) using a 3 kDa MWCO Amicon Ultra-15 centrifugal filter. The identity of expressed proteins was confirmed by SDS-PAGE and Western blot using anti-His monoclonal antibodies.

3.2.8. Polyclonal antibody production

Three male BALB/c mice (6 weeks old) were immunised subcutaneously with 50 μg of recombinant protein emulsified with an equal volume of alum adjuvant (Thermo Fisher Scientific, USA) and boosted twice at two weekly intervals using same amount of protein as described previously [287]. Blood was collected from each mouse before immunisation and two weeks after the final immunisation. Sera was collected by allowing the blood to clot followed by centrifugation at 10,000 ×*g* for 10 min and storage at -20°C.

3.2.9. Immunohistochemistry

An immunohistochemistry analysis was performed to determine the anatomic sites of *Sh*-TSP

expression in adult worm sections. Adult worm sections from *S. haematobium* were de-paraffinized using 2 x 3 min washes each of 100% and 50% xylene and rehydrated in an ethanol series. Antigen retrieval was performed by boiling the slides in citrate buffer (10 mM sodium citrate, pH 6) for 40 min followed by Tris buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween, pH 9.0), for 20 min. Subsequently, sections were blocked with 10% goat serum for 1 h at RT. After washing 3 times with TBS/0.05% Tween-20 (TBST), sections were incubated with anti-*Sh*-TSP antisera (diluted 1:50 in 1% BSA/TBST) overnight at 4°C and then washed with TBST (3 x 5 min). Sections were finally probed with goat-anti-mouse IgG-Alexa Fluor 647 (Sigma-Aldrich, USA) (diluted 1:200 in 1% BSA/TBST) for 1 h in the dark at RT. After a final washing step with TBST, slides were mounted with Entellan mounting medium (Millipore, Germany) and covered with coverslips. Images were acquired by Nuance software with an AxioImager M1 fluorescence microscope (ZEISS, Germany).

3.3. Results

3.3.1. General characteristics of *Schistosoma haematobium* tetraspanins

A total of 19 TSPs were found in the genome of *S. haematobium* and out of these 7 of them were found in the *S. haematobium* EVs. Then, six *Sh*-TSPs (MS3_01370, MS3_05289, MS3_05226, MS3_09198, MS3_01153 and *Sh*-TSP-2) were selected based on the results obtained from the proteomic analysis of the adult worm EVs as discussed in chapter 2.3.3. The sequences of all *Sh*-TSPs were inspected and all contained four transmembrane domains, SEL, LEL and three intracellular regions. For *Sh*_TSP-2, MS3_09198 and MS3_01370 LEL contained four cysteine residues (forming two disulfide bonds) whereas MS3_05226, MS3_05289 and MS3_01153 LEL contained six cysteine residues (forming three disulfide bonds). All *Sh*-TSPs contained the CCG motif characteristic of TSPs [281]. Full-length cDNA and predicted amino acid sequences are shown in Table 3.1. The amino acid sequence identities between the *Sh*-TSPs described here and their *S. mansoni* homologs (across the entire ORF and the LEL alone) are shown in Table 3.2. Amino acid homology of *Sh*-TSPs with their respective *S. mansoni* homologs ranged from 83-93% (when entire ORFs were compared) and 69-84% (when just the LEL regions were compared) (Appendix Fig. 1A-F).

Table 3.1. Characteristics of *Schistosoma haematobium* tetraspanins. LEL: large extracellular loop, SEL: small extracellular loop, bp: base pair, kDa: kilodalton.

TSPs	*Sh*-TSP-2	MS3_05226	MS3_09198	MS3_05289	MS3_01370	MS3_01153
cDNA length (bp)	660	882	654	888	675	837
Number of amino acids	219	273	218	295	225	278
Weight of encoded proteins (kDa)	24.4	30.4	24.2	32.9	24.9	30.9
SEL position	34-53	31-79	36-56	32-66	36-54	36-76
Inner loop position	71-88	97-109	72-81	87-95	74-82	100-108
LEL position	106-184	128-239	103-184	118-266	108-190	130-250
Cytoplasmic tail	207-219	265-273	205-218	283-295	212-225	272-278

Table 3.2. The amino acid sequence percentage identity of *Schistosoma haematobium* tetraspanin open reading frames (ORF) and large extracellular loop (LEL) with their respective *Schistosoma mansoni* homolog.

***Sh*-TSPs**	***Sm*-homologs**	% Identity (similarity) with homolog *S. mansoni* TSPs	
		ORF	**LEL**
MS3_05226	Smp_041460	86 (93)	84 (93)
MS3_01370	Smp_173150	90 (96)	80 (91)
MS3_05289	Smp_344440	83 (90)	81 (88)
MS3_01153	Smp_140000	86 (94)	82 (92)
*Sh*_TSP-2	AAN17276	84 (90)	69 (78)
MS3_09198	AAA73525	93 (95)	84 (88)

3.3.2. Phylogenetic analysis of *Schistosoma haematobium* tetraspanins

A phylogenetic analysis between the *Sh*-TSPs and other well-characterised TSPs from related trematodes was performed. *Sh*-TSP-2, MS3_09198, MS3_01370 and MS3_02232 grouped together in the CD63 ancestry of the TSP family and clustered together with other well characterised CD63-like TSPs from *S. mansoni* (AAN17276.1, AAA73525 and XP_018650438), *S. japonicum* (CAX70616.1 and AAW26928) and *O. viverrini* (JQ678707.1 and JQ678708.1) (Fig. 3.1). MS3_05289, MS3_05226, MS3_01153, MS3_07569, MS3_08458, MS3_01905, MS3-03452, MS3_03944, MS3_01557, MS3_03883 and MS3_01094 clustered under the uroplakin family of TSPs, together with other TSPs from *S. mansoni* (XP_018649476 XP_002577444 and XP-002575497) and *S. japonicum* (AAW26326, AAW24822, AAP05954 and AAW27174) (Fig. 3.1). MS3_09789 and MS3_09698 clustered under the CD9/81 family of TSPs.

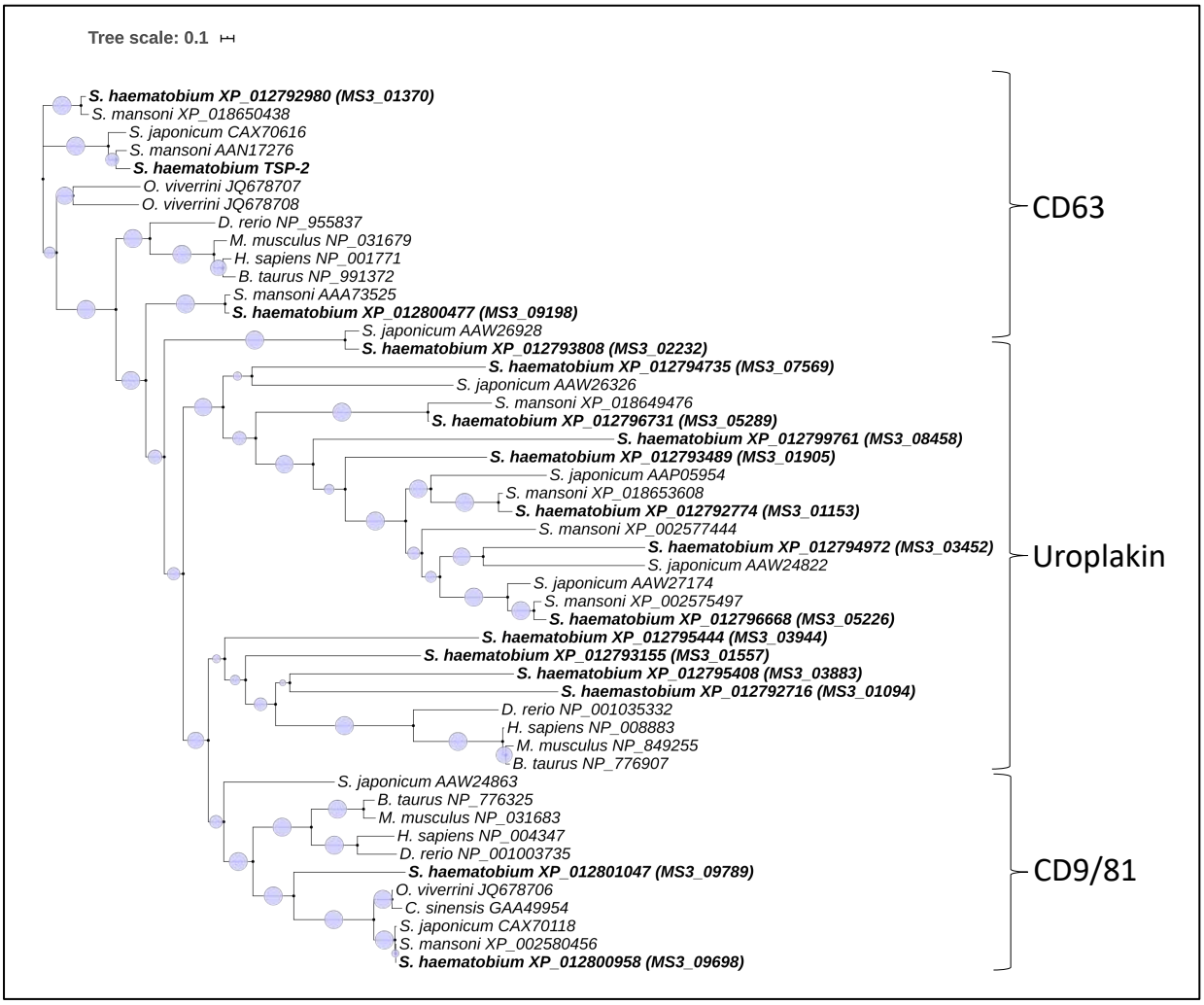

Figure 3.1. Phylogenetic analysis of *Schistosoma haematobium* tetraspanins and homologs from related organisms. A multiple sequence alignment was carried out using the Muscle alignment tool. The tree was visualized with The Interactive Tree of Life (iTOF) online phylogeny tool (https://itol.embl.de/) using default parameters. NP_991372=CD63 antigen, NP_776325.1=CD9 antigen, NP_776907.2=Uroplakin-1b, GAA49954.1=CD9 antigen, NP_955837.1=CD63 antigen,NP_001003735.1=CD81 antigen, NP_001035332.1=Uroplakin-1a, NP_001771.1=CD63 antigen, NP_004347.1=CD81 antigen, NP_008883.2=Uroplakin-1b, NP_031679.1=CD63 antigen, NP_031683.1=CD9 antigen, NP_849255.2=Uroplakin-1b, JQ678707.1=*Ov*-TSP-2,JQ678708.1=*Ov*-TSP-3, JQ678706.1=*Ov*-TSP-1, XP_012800477.1=MS3_09198,XP_012792774.1=MS3_01153,XP_012796731.1=MS3_05289,XP_012796668.1=MS3_05226,XP_012792980.1=MS3_01370,CAX70616.1=CD63 antigen, CAX70118.1=CD9/CD37/CD6, AAW26928.1=*Sj*-TSP-1, AAW24822.1=*Sj*-TSP-2, AAW24863.1= *Sj*-TSP-3, AAP05954.1= *Sj*-TSP-4, AAW27174.1= *Sj*-TSP-5, AAW26326.1=*Sj*-TSP-6, AAN17276.1=*Sm*-TSP-2, XP_002580456.1=*Sm*-TSP-1, XP_002577444.1=Tspan-1,XP_002575497.1=TSP D76, AAA73525.1=*Sm*23, XP_018649476.1= TSP 18, XP_018650438.1= CD63 antigen-like, XP_018653608= Putative TSP, large purple circles= bootstraps (range (0.362 to 1)), small black dots=internal node symbols.

3.3.3. Protein expression and purification

The LEL region from each of the *Sh-tsps* was cloned into a bacterial expression system and sequences were validated by Sanger sequencing. The MS3_09198, MS3_01370 and *Sh*-TSP-2 LELs were expressed as soluble proteins, while MS3_01153, MS3_05226 and MS3_05289 were expressed as inclusion bodies and 6 M urea was used for solubilisation. The expected sizes of the expressed *Sh*-TSPs were confirmed by SDS-PAGE (Fig. 3.2A) and a western-blot using a monoclonal anti-His antibody (Fig. 3.2B).

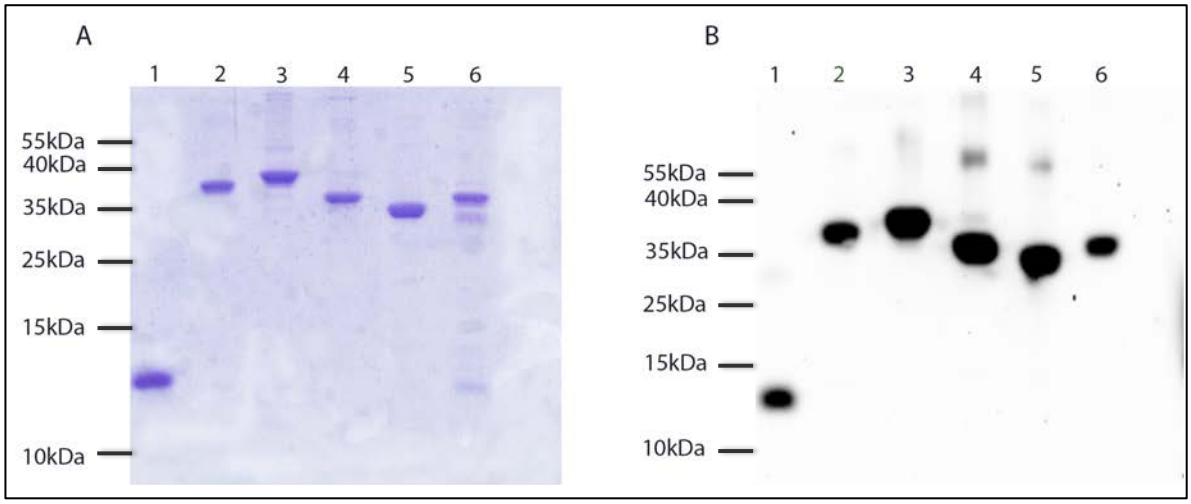

Figure 3.2. Coomassie stained SDS-PAGE gel and Western blot analysis of *Schistosoma haematobium* tetraspanins. One µg of each protein was electrophoresed on an SDS-PAGE gel and stained with Coomassie blue: (A1) *Sh*-TSP-2, (A2) MS3_01153, (A3) MS3_05289, (A4) MS3_05226, (A5) MS3_09198 and (A6) MS3_01370. A western blot was performed using an anti-His monoclonal antibody: (B1) *Sh*-TSP-2, (B2) MS3_01153, (B3) MS3_05289, (B4) MS3_05226, (B5) MS3_09198 and (B6) MS3_01370.

3.3.4. *Schistosoma haematobium* tetraspanins are expressed throughout all life stages

The transcriptional patterns of all *Sh-tsps* were analysed in different life stages of *S. haematobium*: adult, egg, miracidia, cercaria and schistosomula (24 h, 3 and 5 days) by real

time qPCR. All *Sh-tsp* genes were expressed throughout all life stages tested (Fig. 3.3A-F). *Sh-tsp-2* expression peaked at 24 h schistosomula and decreased in subsequent developmental stages (Fig. 3.3A). Similarly, the highest expression levels of *ms3_01370*, *ms3_05226* and *ms3_05289* were identified in the miracidia while the lowest levels of expression were observed in 3 days schistosomula, adult and 24 h schistosomula, respectively (Fig. 3.3B, D, F). In the case of *ms3_09198*, the highest expression level was observed in cercariae and the lowest expression level was observed in miracidia (Fig. 3.3C). The expression level of *ms3_01153* was highest in cercariae and lowest in adult life stages (Fig. 3.3E).

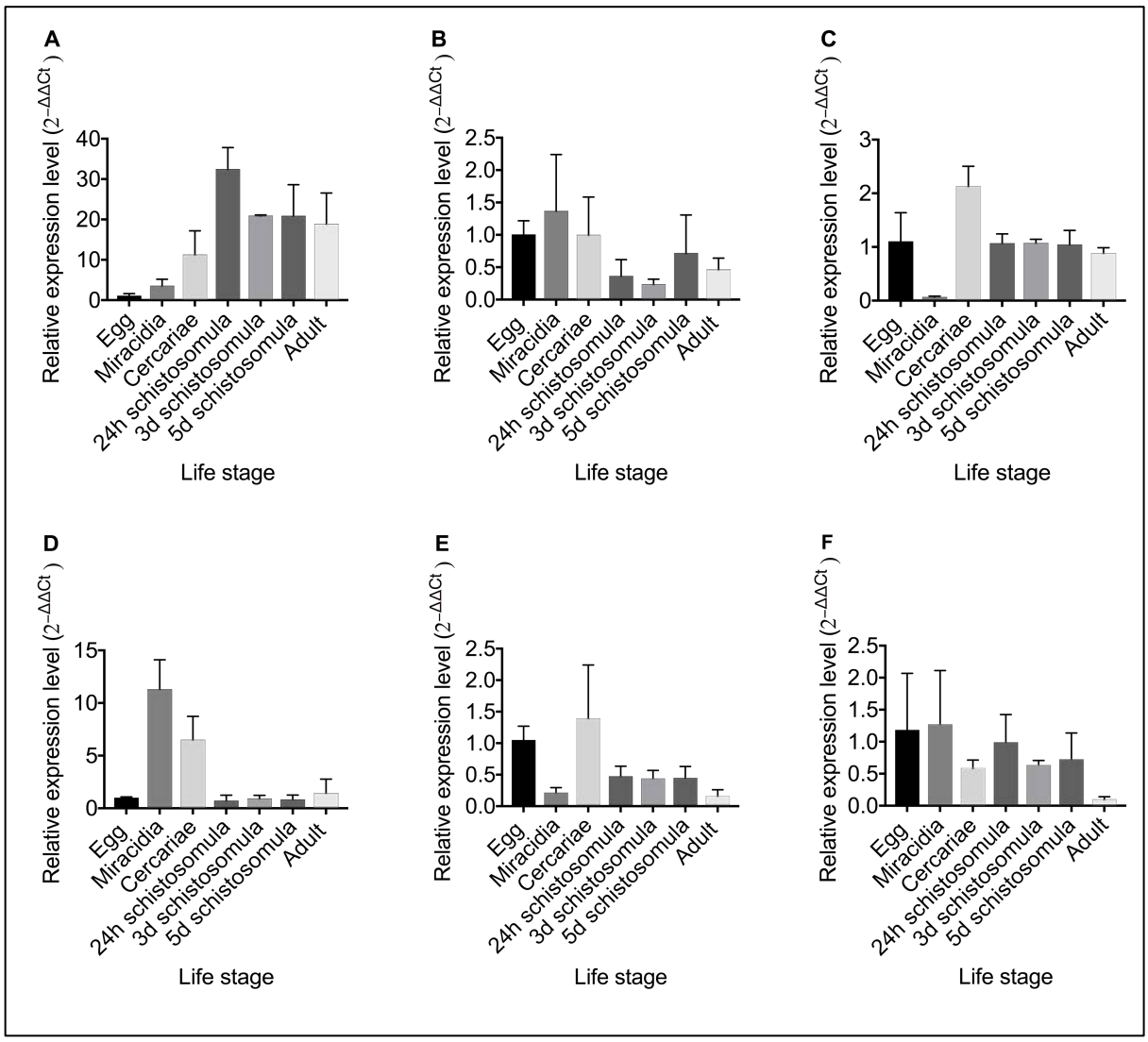

Figure 3.3. Expression levels of *Schistosoma haematobium* tetraspanin mRNAs at different life stages. Relative mRNA expression levels of (A) *Sh-tsp*-2, (B) *ms3_01370*, (C) *ms3_09198*, (D) *ms3_05289*, (E) *ms3_01153* and (F) *ms3_05226* were analysed by qPCR and normalised to a housekeeping gene (*α-tubulin*) using the $2^{-\Delta\Delta Ct}$ method using the egg stage as a reference group. (G) The heat map shows the comparative expression levels of *Sh-tsps* in each life stage.

3.3.5. *Schistosoma haematobium* tetraspanins are expressed in the tegument and internal organs of *S. haematobium* adult worms

To determine the location of *Sh*-TSPs in the adult worms, sections from *S. haematobium* adult worms were probed with mouse polyclonal antibodies produced against each of the selected *Sh*-TSPs (Fig. 3.4A-F). MS3_01153 (Fig. 3.4A) and MS3_09198 (Fig. 3.4C) were identified both on the tegument and gut of the worms, whereas *Sh*-TSP-2 (Fig. 3.4B) and MS3_05289 (Fig. 3.4D) were identified only on the tegument of the worms. MS3_01370 (Fig. 3.4E) had a diffused expression whereas MS3_05226 (Fig. 3.4F) was identified both on the tegument and gastrodermis of adult worms. *S. haematobium* adult worm sections were not recognised by the negative control anti-TrX antibody (Fig. 3.4G).

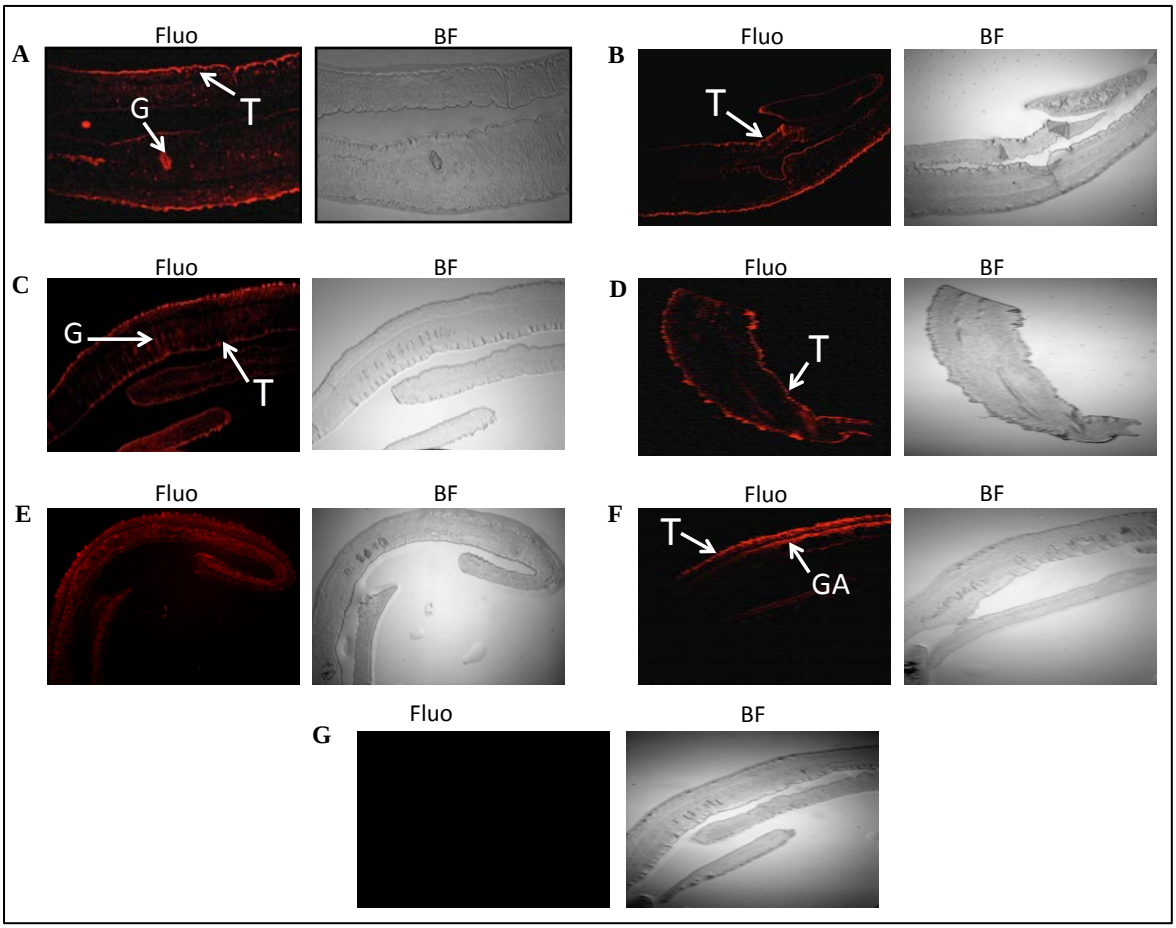

Figure 3.4. Localisation of *Schistosoma haematobium* tetraspanins in adult worms. Immunolocalisations of *Sh*-TSPs in adult worm sections (A) MS3_01153, (B) *Sh*-TSP-2, (C) MS3_09198, (D) MS3_05289, (E) MS3_01370, (F) MS3_05226 and (G) TrX. Sections were probed with anti- *Sh*-TSPs followed by goat-anti-mouse IgG-Alexa Fluor. Fluo: fluorescent, BF: Bright filed, T: tegument, G: gut, GA: gastrodermis.

3.4. Discussion

TSPs are a family of proteins that consist of four transmembrane domains, an SEL and an LEL. TSPs are involved in numerous activities of cells [156] and they can also be involved in host-parasite interactions. Indeed, some of them are being tested as potential vaccine candidates against diverse trematodes [161, 163, 268]. The first TSP identified in *S. mansoni* was *Sm*23 [149] and, since then, other TSPs have been found in the proteome of schistosomes [14, 16, 17, 21]. *Sm*23 is one of the independently tested WHO vaccine candidates [293] and its *S. japonicum* ortholog (*Sj*23) has also been found to be an efficacious vaccine in animal models of schistosomiasis [163, 164]. Recently, the *S. haematobium* ortholog of *Sm*23 (MS3_09198), and 5 other additional TSPs were identified in the proteomic analysis of the EVs from *S. haematobium* adult worms and, in this chapter, we aimed to characterise them to increase our knowledge of this important family of proteins.

Sh-TSP-2, MS3-09198, MS3_02232 and MS3_01370 are grouped together in the CD63 clade of TSPs, together with known *S. mansoni, S. japonicum* and *O. viverrini* vaccine candidates [160, 161, 163, 293], suggesting the usefulness of these *S. haematobium* EV TSPs as vaccine candidates against urogenital schistosomiasis. On the other hand, MS3_05289, MS3_05226, MS3_01153, MS3_07569, MS3_08458, MS3_01905, MS3-03452, MS3_03944, MS3_01557, MS3_03883 and MS3_01094 are clustered under the uroplakin family of TSPs. MS3_09789 and MS3_09698 clustered under the CD9/81 family of TSPs. All *Sh*-TSPs formed a single clade distinct from vertebrate TSPs.

All *Sh*-TSPs were expressed throughout all the assessed life stages of the parasite, albeit with differing expression patterns, as has been reported for *S. japonicum* [286] and *S. mansoni* [294]. Taken together with their detected presence in tegumental and ES proteomic studies [22] and in EVs, this data suggests that all *Sh*-TSPs are, upon infection, continuously exposed to the immune system. The highest levels of expression for *ms3_01370*, *ms3_05226*

and *ms3_05289* were observed in miracidia, which implies that these *Sh-tsps* may have specific roles like hatching or host-finding in the intermediate snail. For *ms3_1153*, *ms3_09198* and *Sh-tsp*-2, the highest levels of expression were observed in cercariae, cercariae and 24 h schistosomula, respectively. Similarly, the highest expression level of *Sm-tsp*-2 was detected in egg [158]. On the contrary the highest expression level of *Sj-tsp*-2 and *Sj*23 were observed in adult male worms [268, 295]. Furthermore the highest expression level of CD63 *tsps* (*Ov-tsp*-2 and *Ov-tsp*-3) was observed in the egg stage [159]. This suggested that TSPs likely have different functions in different species of schistosomes.

MS3_05226 is located on the tegumental syncytium as well as on the internal organs while MS3_01370 has a diffuse expression. Similarly, some *S. japonicum* TSPs (AAW27174.1 and AAW26928) are also located on the tegument and internal organs of adult worms whereas others (AAW24863 and AAW26928) are located only in the internal organs of adult *S. japonicum* worms [286]. In contrast, *Sh*-TSP-2 and MS3_05289 are located exclusively on the tegument of adult *S. haematobium* worms and other TSPs from *S. mansoni, O. viverrini* and *S. japonicum* [17, 157, 159, 161, 286] also display this localisation pattern. These tegumental proteins might play an important role in tegument formation, maturation and stability [157-159]. Since the tegument is the most susceptible structure to host-mediated immune attack [296], the LEL regions of TSPs in schistosomes have been tested as vaccine candidates [161], and *Sm*-TSP-2 from *S. mansoni* has already completed phase I clinical trials [162]. MS3_09198 and MS3_01153 are identified on the tegument and gut of adult worms. Similarly, *Sj*-TSP-2 was also located on the tegument and gut of the adult worm [268]. The presence of these EVs TSPs in the gut of adult worms might indicate the involvement of these EVs TSPs in nutrient acquisition process.

The presence of *Sh*-TSPs in the tegument and gut of adult worm suggests the presence of, at least, two distinct populations of EVs, one originating from the tegument and another from the gut as occurs in *F. hepatica* [118].

S. haematobium EVs TSPs were expressed throughout all life stages. Some of the TSPs clustered with known other helminths vaccine and diagnostic candidates and these TSPs were identified on the tegument of the worms, and/ internal tissues of adult worms. This suggests the potential usefulness of characterising these TSPs from *S. haematobium* towards the development of vaccine and diagnostic candidates.

Chapter 4: Assessment of the vaccine efficacy of *Schistosoma haematobium* tetraspanins

4.1. Introduction

The current control program against schistosomiasis is aimed at reducing the morbidity caused by the parasite by regularly treating infected populations with praziquantel [297]. Despite the efforts made to control this devastating disease, schistosomiasis is still spreading to new geographical areas [298]. Furthermore, praziquantel treatment does not prevent reinfection [85] and is not effective against the immature stages of the parasite [299]. Hence, a vaccine that reduces disease severity and/or reduces transmission is needed to control and eliminate schistosomiasis [85]. Despite efforts over decades, there is no licensed and effective vaccine to control the transmission of *S. haematobium* infection [300]. The only vaccine candidate to have progressed into clinical trial is Sh28GST, however, a phase 3 trial conducted from 2009 to 2012 in *S. haematobium* infected children did not report any significant efficacy due to the vaccine [91]. Therefore, it is important to continue identifying new target antigens in the effort to develop a vaccine against *S. haematobium* [300].

Proteins found on the tegument and ES products of schistosomes are integral to the survival of the parasites in the host [301]. The tegument of schistosomes plays an important role in protecting the worm from the host immune response [302] and it also helps the parasite to absorb nutrients and molecules [303]. Therefore, the tegument is crucial for infection, development and worm survival in the host and tegumental antigens could be useful vaccine targets [296]. Indeed, characterisation of the tegument and ES proteomes of adult *S. haematobium* has revealed many proteins of vaccine potential [22]. Helminth EVs are potential vaccine candidates and vaccination of mice with helminths EVs stimulates the production of the protective immune response that significantly reduces fecal egg counts; worm burdens, symptom severity and mortality induced by infection in subsequent parasite challenges [31,

210, 211]. Moreover, EVs from helminths also contain vaccine candidate antigens including TSPs [146]. The TSPs *Sm*23, *Sm*-TSP-1 and *Sm*-TSP-2, all found in the proteomic analysis of *S. mansoni* EVs [24], have been shown to be efficacious vaccines candidates against schistosomiasis [161, 284] and *Sm*-TSP-2 has completed phase I clinical trials [162]. In another study, vaccination of mice with *Sj*-TSP-2e reduced liver egg burden in the first trial but in trial 2 and 3 this vaccine candidate had no effect in liver egg burden [268]. Similarly, *Sj*-TSP-2c had no effect in liver egg burden [283]. Immunisation of mice with *Sj*-TSP-2d decreased the liver egg and fecal egg burdens significantly [304]. Although the vaccine efficacy of TSPs in other schistosomes was well studied, no studies have been conducted on the vaccine efficacy of *Sh*-TSPs. Accordingly, the present study aimed to assess the vaccine efficacy of *Sh*-TSPs as a vaccine target against *S. haematobium* infection. Herein, I have selected three of the soluble *Sh*-TSPs characterised in chapter 3 (MS3_09198, MS3_01370 and *Sh*-TSP-2) and clustered together with known schistosome vaccine candidates [161, 268]. The vaccine efficacy of these *Sh*-TSPs were assessed in both a homologous (hamster; *S. haematobium*) and heterologous (mouse; *S. mansoni*) challenge model of schistosomiasis.

4.2. Materials and Methods

4.2.1. Parasite materials and experimental animals

S. haematobium-infected *B. truncatus* and *S. mansoni*-infected *Biomphalaria glabrata* snails were provided by BRI (Maryland, USA).

Male Syrian hamsters (*Mesocricetus aureus*) were used for the homologous challenge model as hamsters are a more permissive model for *S. haematobium* infection and literature supports the use of these animals [305]. Hamster vaccine trials were undertaken at BRI since hamsters are prohibited in Australia. Experiments were approved by the BRI Institutional Animal Care and Use Committee (protocol #18-01) and recognised by the Office of Laboratory Animal Welfare (assurance #A3080-01). Male Balb/c mice were used for the heterologous challenge model and vaccine experiments (approved under James Cook University Animal Ethics number A2391) were performed at James Cook University.

4.2.2. Vaccine formulation and immunization schedule

For the hamster vaccine trials, four groups of 8 male Syrian hamsters (6-8 weeks) were immunized intraperitoneally on day 1 with either recombinant *Sh*-TSP-2, MS3_09198, MS3_01370 or TrX control protein (50 µg/hamster), each formulated with an equal volume of Imject alum adjuvant (Thermofisher) and 5 µg of CpG ODN1826 (InvivoGen). Immunizations were repeated on day 15 and 29 and each hamster was challenged (abdominal penetration) with 200 *S. haematobiumi* cercariae on day 43. Blood was sampled at day 42 and 126 (12 weeks post-infection) to determine pre- and post-challenge antibody titers.

For the mouse vaccine trials, groups of 10 animals were immunised in the same way as hamsters and mice were challenged (tail penetration) with 120 *S. mansoni* cercariae on day 43. Blood was sampled at day 42 to determine pre-challenge antibody titers.

4.2.3. Necropsy and estimation of parasite burden

Hamsters were necropsied at day 154 (16 weeks post-infection), mice were necropsied at day 91 (7 weeks post-infection) and worms from both hosts harvested by vascular perfusion and counted. Livers were removed, weighed and digested for 5 h with 5% KOH at 37°C with shaking. Schistosome eggs from digested livers were concentrated by centrifugation at 1,000 ×*g* for 10 min and re-suspended in 1 ml of 10% formalin. The number of eggs in a 5 µl aliquot was counted in triplicate and the number of eggs per gram (EPG) of the liver was calculated. Small intestines were removed and cleaned of debris before being weighed and digested as per the livers. Eggs were also similarly concentrated and counted to calculate intestinal EPG.

4.2.4. Serum antibody response to vaccination in hamsters and mice

An ELISA was performed to assess antibody titers to proteins (MS3_09198, MS3_01370 and *Sh*-TSP-2) using the serum collected at different time points during the experiment. Microtiter plate wells (Greiner Bio-One, Austria) were coated with 100 µl (2 µg/ml) of each protein in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed 3 times with phosphate buffer saline/0.05% Tween-20 (PBST) and blocked with skim milk powder (5% in PBST) at 37°C for 1 h. Then, plates were washed 3 times with PBST and 100 µl of serially diluted mouse or hamster serum (1:5,000-1:1,280,000 in PBST) was added and incubated at 4°C overnight. After washing with PBST 3 times, 100 µl of HRP-conjugated anti-hamster or anti-mouse IgG (diluted 1:3,000 in PBST) (Sigma-Aldrich, USA) was added, incubated at 37°C for 1 h and washed 3 times with PBST. Finally, 100 µl of 3,3',5,5-tetramethylbenzidine (TMB, Thermo Fisher Scientific, USA) was added and incubated for 12 min at RT in the dark. The reaction was stopped with 3 M HCl and absorbance was measured at 450 nm using a POLARstar Omega (BMG Labtech, Australia).

4.2.5. Statistics

All statistics were performed using GraphPad Prism 7.0. The worm number reduction and egg number reduction were analysed using a Student's *t* test and results were expressed as the mean ± standard error of the mean. For the hamster and mouse antibody titer, the reactivity cut-off values were determined as the mean + 3SD of the naive serum.

4.3. Results

4.3.1. Antibody response of hamsters and mice following immunisation and parasite challenge

Hamsters immunised with all *S. haematobium* proteins produced specific IgG responses (Table 4.1). Pre-challenge IgG endpoint titers were > 1,280,000 for all hamsters vaccinated with MS3_01370. Pre-challenge IgG endpoint titers for hamsters vaccinated with MS3_09198 ranged from 160,000-1,280,000 except hamster 4, which had no detectable IgG response against the protein. For *Sh*-TSP-2, all hamsters had pre-challenge IgG endpoint titers > 1,280,000, except hamster 8 (320,000). At 12 weeks post challenge, IgG titers ranged from 10,000-640,000 for hamsters immunised with MS3_01370 while the IgG titers for MS3_09198 ranged from 5,000-80,000. For *Sh*-TSP-2, the IgG titers 12 weeks post challenge ranged from 10,000-320,000. Only 1 and 2 hamsters immunised with *Sh*-TSP-2 and MS3_01370, respectively, had antibody titers above 100,000 at 12 weeks post challenge.

Mice vaccinated with all *Sh*-TSPs produced specific IgG responses (Table 4.2). Pre-challenge IgG endpoint titers were > 640,000 for all mice immunised with MS3_01370 in both trials. For *Sh*-TSP-2, all mice had pre-challenge IgG endpoint titers were > 640,000, except hamster 2 in trial 1 (160,000). Pre-challenge IgG endpoint titers for mice vaccinated with MS3_09198 ranged from 40,000-320,000 in both trials.

Table 4.1. Pre-challenge and pre-necropsy serum antibody response of hamsters immunised with *Schistosoma haematobium* tetraspanins

Hamster	IgG titers					
	MS3_01370		**MS3_09198**		***Sh*-TSP-2**	
	Pre-challenge	**12wk post challenge**	**Pre-challenge**	**12wk post challenge**	**Pre-challenge**	**12wk post challenge**
1	>1,280,000	80,000	>1,280,000	10,000	>1,280,000	20,000
2	>1,280,000	40,000	>1,280,000	80,000	>1,280,000	10,000
3	>1,280,000	10,000	>1,280,000	80,000	>1,280,000	80,000
4	>1,280,000	160,000	Not detected	Not detected	>1,280.000	80,000
5	>1,280,000	640,000	>1,280,000	20,000	>1,280.000	10,000
6	>1,280,000	40,000	>1,280,000	5,000	>1,280.000	320,000
7	>1,280,000	40,000	>1,280,000	20,000	>1,280,000	20,000
8	>1,280,000	No sample	160,000	Not detected	320,000	20,000

Table 4.2. Pre-challenge serum antibody response of mice immunised with *Schistosoma haematobium* tetraspanins.

Mouse	Pre-challenge IgG titers					
	MS3_01370		MS3_09198		*Sh*-TSP-2	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
1	>640,000	>640,000	80,000	80,000	>640,000	>640,000
2	>640,000	>640,000	40,000	160,000	160,000	>640,000
3	>640,000	>640,000	80,000	160,000	>640,000	>640,000
4	>640,000	>640,000	160,000	160,000	>640,000	>640,000
5	>640,000	>640,000	160,000	80,000	>640,000	>640,000
6	>640,000	>640,000	160,000	40,000	>640,000	>640,000
7	>640,000	>640,000	40,000	320,000	>640,000	>640,000
8	No sample	>640,000	160,000	No sample	>640,000	>640,000
9	No sample	No sample	No sample	No sample	>640,000	>640,000

4.3.2. Parasite burdens in vaccinated and control hamsters

Vaccination of hamsters with MS3_01370 and *Sh*-TSP_2 decreased worm burden by 22.46% and 8%, respectively. However, these reductions were not statically significant (Fig 4.1A). There was no difference in adult worm burden between hamsters vaccinated with MS3_09198 and the control group. Interestingly, immunisation of hamsters with MS3_01370 and *Sh*-TSP-2 significantly reduced the liver egg burden by 77.8% ($P<0.01$) and 52.27% ($P<0.05$), respectively when compared with the control group (Fig 4.1B). There was no difference in liver egg burden between hamsters vaccinated with MS3_09198 and the control group (Fig 4.1B). Similarly, there was no difference in intestinal egg burden between hamsters vaccinated with *Sh*-TSPs and the control group (Fig 4.1C).

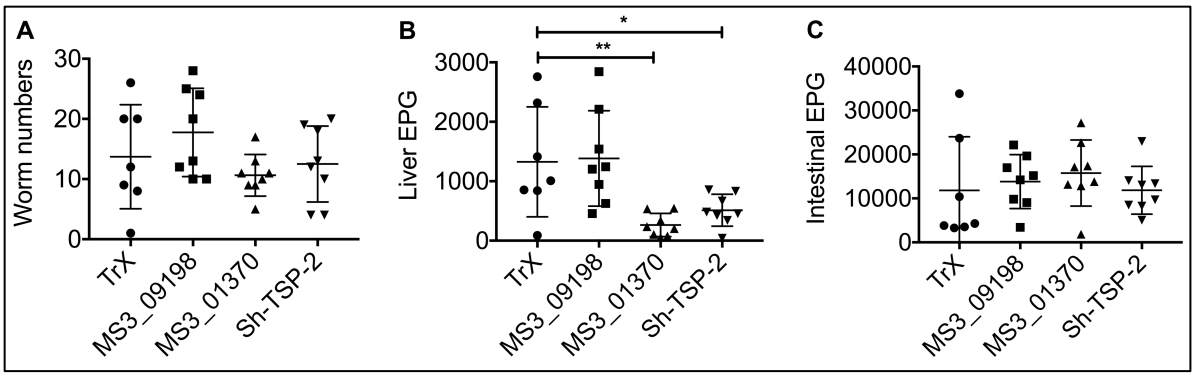

Figure 4.1. *Schistosoma haematobium* worm and egg burden reduction of vaccinated and control hamsters. (A) Adult worm reduction, (B) liver egg reduction, (C) intestinal egg reduction. Differences between groups was analysed with a student's *t*-test. * $P < 0.05$, ** $P < 0.01$.

4.3.3. Parasite burdens in vaccinated and control mice

Vaccination of mice with MS3_01370, MS3_09198 and *Sh*-TSP-2 reduced the adult *S. mansoni* worm burden in trial 1 (trial 2) by 22 (14)%, 12 (5)% and 2% (no reduction), respectively. All these reductions were not statically significant (except MS3_01370 in trial 1 (P<0.05)) (Fig 4.2A,B). In trial 1, vaccination of mice with MS3_01370, MS3_09198 and *Sh*-TSP-2 significantly reduced liver egg burden by 39% (P<0.05), 49% (P<0.001) and 32% (P<0.01), respectively (Fig 4.2C). In trial 2, MS3_01370, MS3_09198 and *Sh*-TSP-2 significantly reduced the liver egg burden by 54% (P<0.001), 27% (P<0.05) and 49% (P<0.001), respectively (Fig 4.2D). Similarly, immunisation of mice with MS3_01370, MS3_09198 and *Sh*-TSP-2 reduced the intestinal egg burden by 57% (P<0.01), 51% (P<0.01) and 54% (P<0.001) in trial 1, respectively (Fig 4.2E). In trial 2, MS3_01370, MS3_09198 and *Sh*-TSP-2 reduced the intestinal egg burden by 36% (P<0.01), 39% (P<0.05) and 27% (P<0.05), respectively (Fig 4.2F).

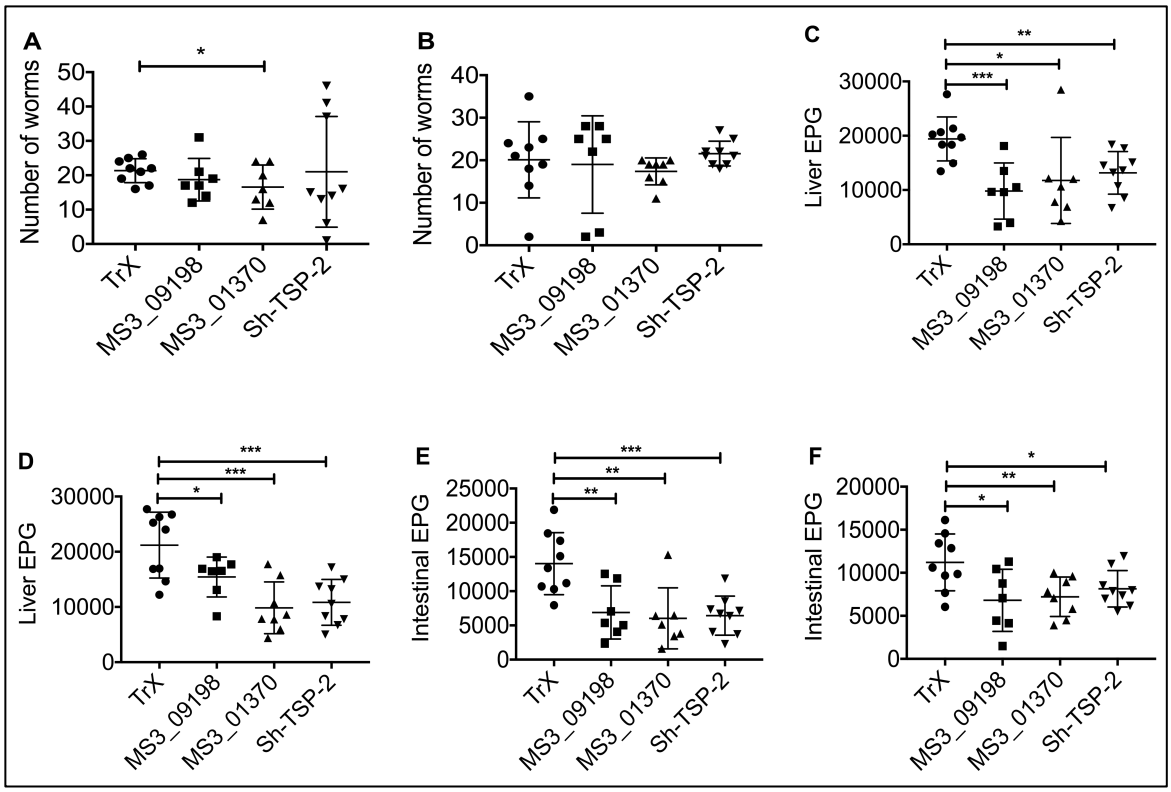

Figure 4.2. *Schistosoma mansoni* worm and egg burden reduction of vaccinated and control mice. (A) Adult worm reduction trial 1, (B) Adult worm reduction trial 2, (C) liver egg reduction trial 1, (D) liver egg reduction trial 2, (E) intestinal egg reduction trial 1, (F) intestinal egg reduction trial 2. Differences between groups was analysed with a student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.4. Discussion

In this chapter, the vaccine protective efficacies of the three *Sh*-TSPs was assessed in a hamster (*S. haematobium*) and mouse (*S. mansoni*) model of infection.

The majority of the vaccinated hamsters and mice produced a strong antibody response against recombinant proteins and the antibody titers observed were in accordance with previous vaccine studies on other schistosome TSPs [161, 268, 287]. In these studies, the antibody titers before challenge showed no correlation with worm burdens or egg counts; similarly, pre-challenge antibody titers of *Sh*-TSPs had no association with worm and egg burdens.

Worm burdens of any group of vaccinated animals were not significantly reduced in either model, compared to controls, except MS3_01370 in mouse trial 1. Similarly, result was obtained in a vaccine efficacy assessessment of a native 28 kDa GST and keyhole limpet haemocyanin (KLH) vaccines from *Schistosoma bovis* [306]. In another study, vaccination of mice with one of the subclass of *Sj*-TSP-2 (*Sj*-TSP-2e) was performed in three independent trials [268]. In the first trial, vaccination of mice with *Sj*-TSP-2e resulted in a 36.4% ($P<0.001$) reduction in worm burden. However, in the second and third trial the adult worm burden reduction was not statistically significant [268]. Similarly, immunisation of mice with *Sj*-TSP-2c subclass showed no protection, while immunisation with a mixture of seven recombinant *Sj*-TSP-2 subclasses resulted in a 16.92% ($P=0.0044$) reduction for the adult worm burden [283]. In another study immunisation of mice with *Sj*-TSP-2d resulted in the reduction of adult worm by 56% ($P<0.0010$) [304]. *Sj*-TSP-2 was found to be highly polymorphic and a total of 9 different subclasses of *Sj*-TSP-2 cDNA sequences have been revealed, which might be responsible for the inconsistent efficacy of *Sj*-TSP-2 in vaccine studies [268, 283]. On the contrary, vaccination of mice with a *S. mansoni* orthologue (*Sm*-TSP-2) resulted in the reduction of the adult worm burden by 57% [161] and 25-27% [287] when administered with Freund's and alum + CPG adjuvants, respectively. *Sm*-TSP-2 has completed phase I clinical

trials [162]. The low efficacy of *Sh*-TSPs might due to the polymorphic nature of *Sh*-TSPs. Vaccination of mice with a DNA plasmid construct encoding *Sm*23 reduced adult worm burden by 21-44% in three trials [284]. In another study, immunisation of mice with plasmid DNA constructs of *Sm*23 by microseeding and gene gun delivery methods resulted in 31-34% and 18% protection, respectively [307]. Furthermore, immunisation of buffaloes with an orthologue of *S. japonicum* (*Sj*23) DNA reduced worm burden by 45.5% and the level of protection increased to 50.9% when the protein was fused with HSP-70 [308]. In another study, immunisation of buffaloes with the same DNA vaccine did not protect against *S. japonicum* challenge while it reduced worm burden in sheep by 41.7% [309]. The difference in the adult worm reduction might be due to the high levels of polymorphism in DNA sequences of MS3_09198 within and between different *Schistosoma* species [310].

In humans infected with *S. haematobium*, eggs trapped in the urogenital organs stimulate the immune response that leads to the formation of granuloma and, ultimately, tissue fibrosis [48]. However, in an animal model, *S. haematobium* fails to establish a urogenital disease [58]; the adult worms are recovered from the portal vein and eggs are trapped in the liver and voided in the feces, similar to animal models of *S. mansoni* and *S. japonicum* infection [81]. Vaccination of hamsters with MS3_01370 and *Sh*-TSP-2 significantly reduced liver egg burden but not the intestinal egg burden and vaccination of mice with MS3_01370, MS3_09198 and *Sh*-TSP-2 significantly reduced the *S. mansoni* liver and intestinal egg burden in both trials. These *S. mansoni* tissue egg reductions imply that these vaccine candidates could be effective in reducing egg-induced pathology and transmission of infection by decreasing the amount of eggs released into the environment. Similarly, vaccination of cattle with 28 kDa GST and KLH significantly reduced liver egg burden following *S. bovis* challenge [306]. Immunisation of mice with *Sm*-TSP-2 reduced the liver and intestinal egg burden by 64% and 65%, respectively [161]. In another study with different adjuvant *Sm*-TSP-2 reduced the liver egg burden by 20–

27% in 2 independent trials [287]. Vaccination of mice with *Sj*-TSP-2e resulted in 26.5% reduction in liver eggs burden in the first trial but in trial 2 and 3 this vaccine candidate had no effect in liver egg burden [268]. Similarly, *Sj*-TSP-2c had no effect in liver egg burden but combination of *Sj*-TSP-2 reduced liver egg burden by 27.04% [283]. Immunisation of mice with *Sj*-TSP-2d decreased the liver egg and fecal egg burdens by 55% (52%), 58% (46%) when compared with TRX (PBS) controls, respectively [304]. Since eggs are the primary causes of pathology egg reduction in hamsters following immunisation with MS3_01370 and *Sh*-TSP-2 might reduce the pathology. Similarly, immunisation of buffaloes and sheep with *Sj*23 had no effect in fecal and tissue egg burden [309]. These difference in the tissue egg burden reduction might be due to the female worm burden reduction by *Sh*-TSPs.

The significant reduction in liver egg burden in both the heterologous and homologous model of infection described here indicates these vaccine candidates are effective against the adult stages of both *S. mansoni* and *S. haematobium* and they could potentially be incorporated into a pan-schistosome vaccine due to the geographical overlap between the two species [1].

Vaccination of mice with MS3_09198, MS3_01370 and *Sh*-TSP-2 significantly reduced the liver and intestinal egg burden. In the case of homologous model of infection only MS3_01370 and *Sh*-TSP-2 significantly reduced the liver egg burden but none of these vaccine candidates reduced the intestinal egg burden.

Chapter 5: Evaluation of *Schistosoma haematobium* tetraspanins as potential novel diagnostic markers

5.1. Introduction

Schistosomiasis is endemic in 54 countries, affecting a round 240 million people and 700 million people are at risk. The World Health Organization designed strategy to control the morbidity by 2020 and to eliminate schistosomiasis by 2025 [311]. To achieve this goal, developing a sensitive, accessible and inexpensive diagnostic screening test is essential [312]. Urine microscopy has been widely used as a standard diagnostic technique for *S. haematobium* infections [68], as this technique is simple to perform, cheap and requires little specialised training [69]. However, the eggs are not frequently voided through urine, which underestimates the prevalence of *S. haematobium* in light infections [68, 69] and the sensitivity is low in HIV infected individuals [313].

CCA and CAA are proteoglycan antigens of schistosomes, can be detected in serum and urine samples [314, 315] and can be used to assess infection intensity and therapeutic responses [316]. However, the accuracy of CCA in detecting *S. haematobium* infection is low in areas endemic to both *S. mansoni* and *S. haematobium* [76].

Molecular detection of parasite-specific DNA, such as the tandem-repeat sequence Dra I, is a sensitive and specific technique for the diagnosis of schistosomiasis [79]. However, it is costly and difficult to apply in the field [83].

Antibodies produced against different life stages of schistosomes can be detected in humans and used for the diagnosis of infection [69]. Despite the inability of antibody detection to distinguish between a current and recent (but cured) infection [11, 41, 73], detecting antibodies

formed against the diverse life stages of *S. haematobium* is a sensitive technique for the diagnosis of infections in areas of low infection intensity [71].

For better control and, eventually, elimination of schistosomiasis, both sensitive and specific diagnostic tests are needed. Recently, the proteomic composition of the egg and adult ES products as well as the tegument [22] from *S. haematobium* adult worms revealed many different proteins of diagnostic potential, including TSPs. TSPs from other helminths such as *T. solium* and *S. japonicum* have been suggested as potential diagnostic candidates [269, 270] and I hypothesize that *Sh*-TSPs might be similarly efficacious. Herein, I test the ability of *Sh*-TSPs characterised in chapter 2 to diagnose *S. haematobium* infection from the urine of individuals from areas endemic for urogenital schistosomiasis.

5.2. Materials and methods

5.2.1. Experimental animals

Male BALB/c mice were purchased and maintained at the AITHM animal facilities on the Cairns campus of JCU as discussed previously (chapter 2.2.1). All experimental procedures performed on animals in this study were approved by the JCU animal ethics committee (ethics approval number A2391).

5.2.2. Human urine samples

A total of 96 urine samples from *S. haematobium* infected individuals from an endemic area of Zimbabwe were collected on three consecutive days for parasitological examinations. *S. haematobium* infection was assessed by microscopic examination of the parasite eggs from 10 ml of filtered urine [317]. These urine samples were kindly provided by Prof Francisca Mutapi from University of Zimbabwe. Fourteen urine samples from Australian volunteer donors that had never travelled to schistosomiasis endemic areas were collected as a negative control. In line with WHO criteria, the infection level of urine samples collected from the endemic area were classified as either high (>50 eggs/10 ml of urine) (n=30), medium (11-49 eggs/10ml of urine) (n=28), low (0.3-10 eggs/10 ml of urine) (n=32) and egg negative (0 eggs/10 ml of urine) (n=17). To confirm the presence or absence of infection, egg negative urines were also tested for the presence of CAA using the UCAA2000 (wet format) as described previously [318]. Of these samples, 6 were positive for CAA and 11 were negative (which were discarded for further analyses). The collected urine samples were aliquoted and placed at -80°C until further use. The collection of urine from Zimbabwean individuals was approved by the Medical Research Council of Zimbabwe; Approval MRCZ/A/1710.

5.2.3. Mouse serum samples

Mice were infected with *S. haematobium* and *S. mansoni* cercariae as previously described (chapter 2.2.2). *S. haematobium* and *S. mansoni* infected mice were necropsied after 14 and 7 weeks post infection, respectively and blood samples were collected. Blood samples from naive mice were collected as negative control. Sera was obtained by allowing the blood to clot followed by centrifugation at 10,000 ×*g* for 10 min and stored at -20°C.

5.2.4. Enterokinase digestion

The N-terminal thioredoxin from the cloned and expressed TSPs was cleaved using enterokinase following the supplier's protocol. Briefly, after purification soluble TSPs were buffer exchanged with PBS followed by (20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8). Then, 2 mg/ml of TSPs were incubated with 2 µl of enterokinase (Biolabs, USA) at RT for 24 h and re-purified as previously described (chapter 3.2.7).

5.2.5. Indirect enzyme-linked immunosorbent assay

An ELISA was performed to assess the diagnostic efficacy of the recombinantly expressed six *Sh*-TSPs using the serum of infected mice (pooled samples from four independent *S. haematobium* infections) and the urine from naturally infected Zimbabwean people. Microtiter plate wells (Greiner Bio-One, Austria) were coated with 50 µl (2 µg/ml) of protein, incubated, washed, blocked and washed again as discussed previously (chapter 4.2.4). Then, 50 µl of human urine (diluted 1:50 in PBST) or mouse serum (diluted 1:3,000 in PBST) was added, incubated and washed as described in chapter 4.2.4. Fifty µl of HRP-conjugated anti-human IgG or anti-mouse IgG (diluted 1:5,000 in PBST) was added, incubated at 37°C for 1 h and washed 3 times with PBST. Finally, 50 µl of TMB (Thermo Fisher Scientific, USA) was added and incubated for 12 min at RT, the reaction was stopped by 3 M HCl and the absorbance was measured as previously described (chapter 4.2.4).

5.2.6. Statistics

All statistics were performed using GraphPad Prism 7.0. The urine diagnostic assay was analysed using a non-parametric Kruskal-Wallis test with multiple comparisons followed by Dunn's post-test when comparing each infection intensity against the negative control at the same time. A non-parametric Mann Whitney test was used when the results from all infected individuals were combined and compared against results from the negative control. For both serum and urine ELISA, the reactivity cut-off points were determined as the average reactivity + 3x standard deviations (SD) of the negative control. The diagnostic accuracy of *Sh*-TSPs was measured by calculating the area under the curve (AUC) of the Receiver Operating Characteristic (ROC) curve generated for each antigen and a frequency of recognition matrix (FoR). ROC curves were used to calculate sensitivity, specificity and the AUC. FoR was determined as a percentage by dividing numbers of OD450 values greater than the reactivity cut-off point to the total infected individuals for each TSP.

5.3. Results

5.3.1. *Schistosoma haematobium* tetraspanins are recognised by the serum of infected mice

As a first step towards the assessment of TSPs as diagnostic candidates, I performed an indirect ELISA to analyse the immunogenicity of these proteins in infected mice. Antibody levels against *Sh*-TSP-2, MS3_01370 and MS3_09198 were significantly higher in the serum of mice experimentally infected with *S. haematobium* compared to serum from uninfected mice, while MS3_05226 and MS3_01153 were only weakly recognised (Fig. 5.1A). TrX was used as a control and was not recognised by the serum of *S. haematobium* infected mice (Fig. 5.1A). To assess cross-reactivity with *S. mansoni*, an indirect ELISA was performed using the serum of mice experimentally infected with *S. mansoni*. Only MS3_09198, MS3_01370, MS3_05226 and MS3_01153 were recognised by antibodies from *S. mansoni* infected mice (Fig. 5.1B).

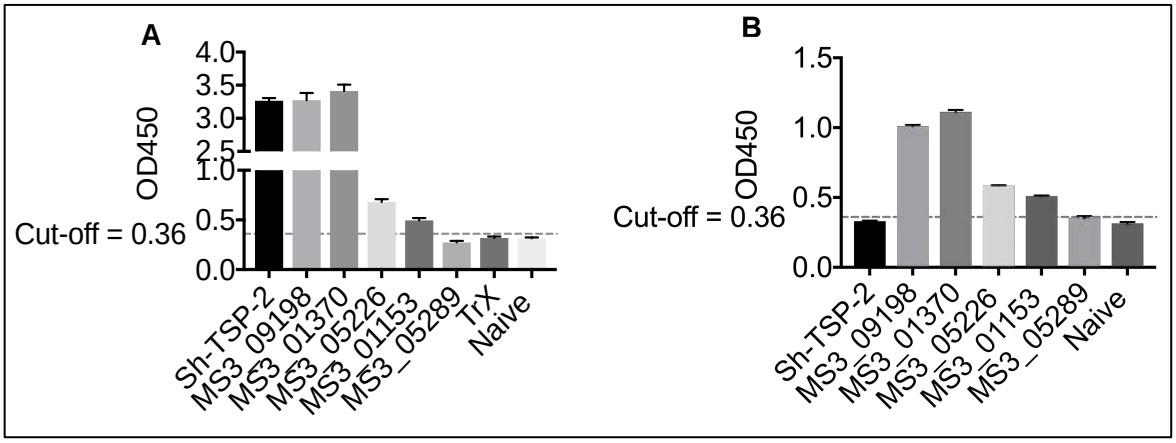

Figure 5.1. Recognition of *Schistosoma haematobium* recombinant tetraspanins by the serum of *S. haematobium* and *Schistosoma mansoni* infected mice. Bar graph showing the detection of *S. haematobium* tetraspanins using (A) *S. haematobium* infected and (B) *S. mansoni* infected mouse sera. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of naive mouse serum.

5.3.2. *Schistosoma haematobium* tetraspanins are recognised by antibodies in the urine of naturally infected individuals from an endemic area

The diagnostic efficacy of *Sh*-TSPs was further tested by their recognition of antibodies in urine from infected human subjects from an endemic area in Zimbabwe. Antibody levels to all *Sh*-TSPs were significantly higher in a pool of infected subjects compared to uninfected non-endemic subjects (Appendix Fig. 2A-F). All *Sh*-TSPs were significantly recognised by high egg burden individuals compared to negative controls (urine samples from a non-endemic area) (Fig. 5.2A-F). Except for MS3_05289, all *Sh*-TSPs were significantly recognised by individuals with a medium egg burden compared to the negative control (Fig. 5.2A-F). Only MS3_01370, *Sh*-TSP-2 and MS3_09198 were significantly recognised by individuals with a low egg burden (Fig. 5.2A-C) and only MS3_01370, MS3_05226 and MS3_09198 were significantly recognised by egg-negative but CAA-positive individuals (Fig. 5.2B, C and E). Since the recombinant LEL domains of MS3_01370, MS3_09198, MS3_01153, MS3_05226 and MS3_05289 were expressed in *E. coli* as fusion proteins with TrX, the effects of TrX on the accuracy of the diagnosis of these TSPs was also confirmed by indirect ELISA. TrX was not recognised by high, medium and low egg burden individuals (Fig. 5.2G). To confirm further the effect of TrX, the N-terminal TrX from TSPs that were strongly recognised by antibodies from infected individuals (MS3_09198 and MS3_01370) was cleaved using enterokinase following the supplier protocol. An indirect ELISA was performed using digested MS3_09198 and MS3_01370. However, there was no significant difference between digested and non-digested TSPs (Appendix Fig. 3A-D). Similarly, enterokinase digested TSPs were significantly recognised when comparing the uninfected non-endemic group with infected individuals as a pool (Appendix Fig. 4A and B).

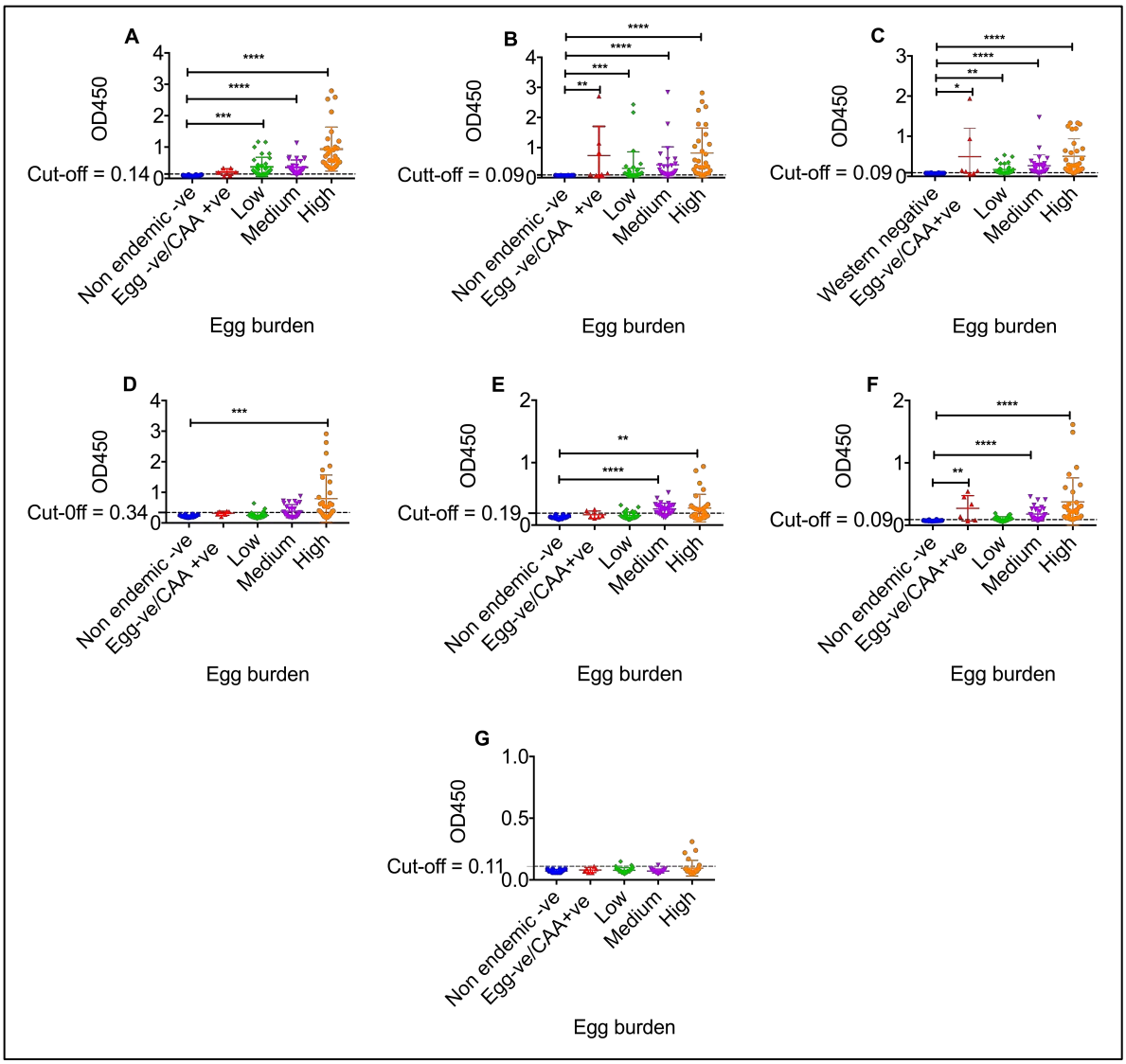

Figure 5.2. Urine IgG recognition of six *Schistosoma haematobium* tetraspanins from Zimbabwean infected individuals with different infection status. The antibody level was measured by indirect ELISA and indicated by OD values: (A) *Sh*-TSP-2, (B) MS3_01370, (C) MS3_09198, (D) MS3_05289, (E) MS3_01153, (F) MS3_05226 and (G) TrX. All the data was entered in GraphPad Prism 7 and analysed using a non-parametric Kruskal-Wallis test with multiple comparisons followed by Dunn's post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0002$. Urine of non-infected individuals from a non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).

5.3.3. Predictive accuracy of *Schistosoma haematobium* tetraspanins for the diagnosis of *S. haematobium* infection

The diagnostic accuracy of *Sh*-TSPs was measured by calculating the AUC of the ROC curve generated for each antigen (Fig. 5.3A-F) and a FoR matrix (Appendix Fig. 5 lane (A-H)). The ROC curves were used to calculate sensitivity, specificity and the AUC. FoR was used to calculate the percentage of OD450 values greater than the reactivity cut-off point. When comparing the uninfected non-endemic group with infected individuals as a pool, the highest accuracy of diagnosis was obtained with *Sh*-TSP-2 (0.98) followed by MS3_01370 (0.97), MS3_09198 (0.95), MS3_05226 (0.89), MS3_01153 (0.78) and MS3_05289 (0.70) (Fig. 5.3A-F). The highest accuracy of diagnosis for individuals with high infection intensity was obtained with *Sh*-TSP-2 (1.0) and MS3-05226 (1.0) followed by MS3_09198 (0.98), MS3_01370 (0.97), MS3_05289 (0.85) and MS3_01153 (0.78) (Fig. 5.3A-F). In the case of medium egg burden individuals, the highest accuracy of diagnosis was obtained with *Sh*-TSP-2 (0.99) followed by MS3_09198 (0.98), MS3_01370 (0.97), MS3_05226 (0.95), MS3_01153 (0.95) and MS3_05289 (0.72) (Fig. 5.3A-F). For individuals with low egg burden, the highest accuracy of detection was obtained with *Sh*-TSP-2 (0.99) followed by MS3_01370 (0.96), MS3_09198 (0.93), MS3_05226 (0.73), MS3_01153 (0.63) and MS3_05289 (0.51) (Fig. 5.3A-F). In the case of egg negative but CAA positive individuals the highest accuracy of detection was obtained from MS3_01370 (0.97) followed by MS3_05289 (0.88), *Sh*-TSP-2 (0.86), MS3_05226 (0.85), MS3_09198 (0.82) and MS3_01153 (0.76) (Fig. 5.3A-F).

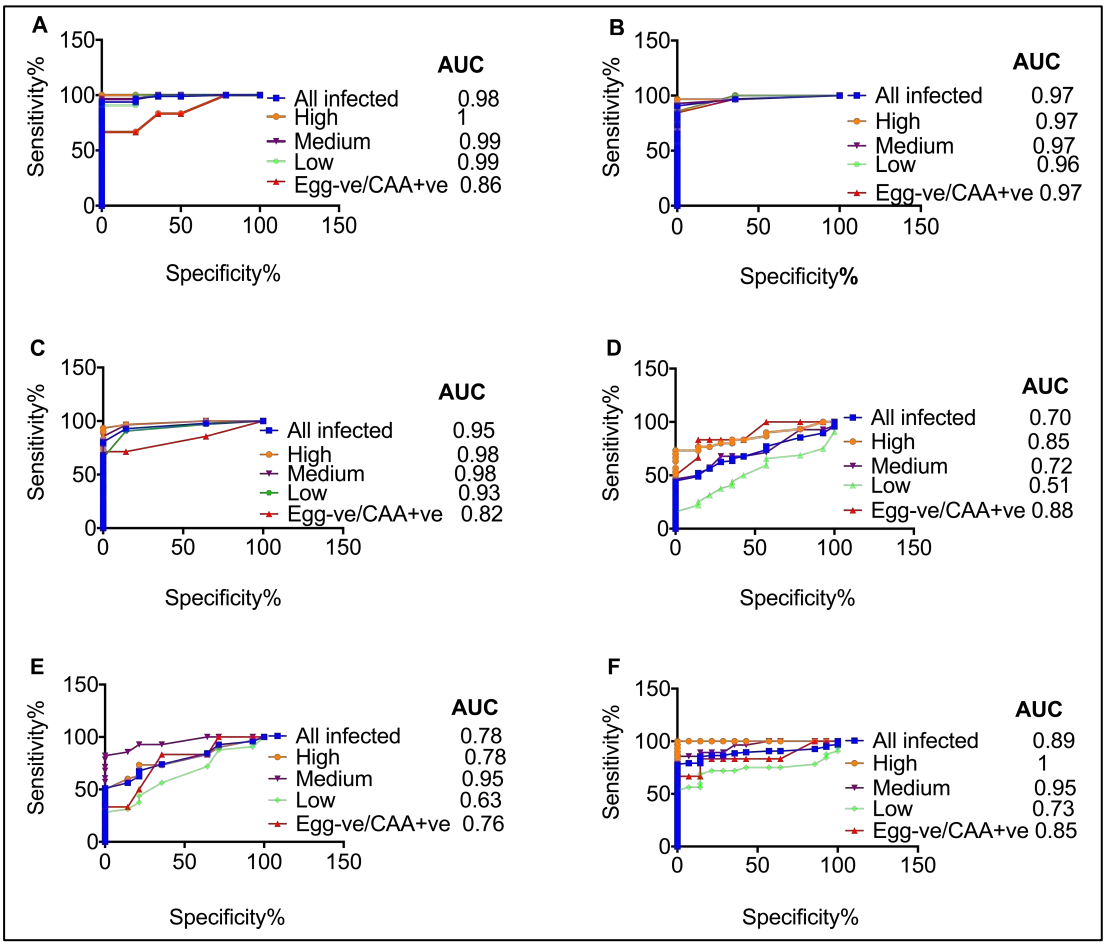

Figure 5.3. Receiver operating characteristic (ROC) curves analysis of six *Schistosoma haematobium* tetraspanins. The diagnostic accuracy of *S. haematobium* tetraspanins to detect antibodies in the urine of infected individuals with differing infection status as well as using pooled urine from all individuals was measured by the area under the ROC curve. (A) *Sh*-TSP-2, (B) MS3_01370, (C) MS3_09198, (D) MS3_05289, (E) MS3_01153 and (F) MS3_05226. The urine from non-infected individuals from a non-endemic area was used as negative control.

The FoR matrix was performed for each TSP (Appendix Fig. 5 lane (A-F)) and the highest values were obtained from *Sh*-TSP-2 (86.5%) (A) followed by MS3_01370 (83.5%) (B), MS3_09198 (80.4%) (C), MS3_05226 (79.2%) (F), MS3_01153 (44.8%) (E) and MS3_05289 (39.6%) (D). For non-fused MS3_01370 and MS3_09198 the frequency of recognition matrix was 82.1% and 73.2%, respectively (Appendix Fig. 5 lane (G, H)). The sensitivity of *Sh*-TSP-2, MS3_01370, MS3_09198, MS3_05226, MS3_01153 and MS3_05289 for the diagnosis of

S. haematobium infection was 86.5%, 83.5%, 80.4%, 79.2%, 44.8, and 39.6%, respectively while the specificity of all *Sh*-TSPs was 100%.

5.3.4. Combining *Schistosoma haematobium* tetraspanins doesn't affect sensitivity and predictive accuracy

An indirect ELISA was performed using combinations of MS3_09198 and MS3_01370, as well as *Sh*-TSP-2 as described above by coating plates with equal amounts (50 ng/well) of each *Sh*-TSP, depending on the combination. All *Sh*-TSPs combinations (fused and non-fused) were also significantly recognised when comparing the uninfected non-endemic group with infected individuals as a pool (Appendix Fig. 6A-F). *Sh*-TSP-2 + MS3_01370, MS3_01370 + MS3_09198 and MS3_09198 + *Sh*-TSP-2 were significantly recognised by patients with a high, medium and low egg burden compared to negative controls (Fig. 5.4A, C and E). *Sh*-TSP-2 + MS3_01370 and MS3_01370 + MS3_09198 were also significantly recognised by egg negative but CAA positive individuals (Fig. 5.4A and E). When comparing uninfected non-endemic group with infected individuals as a pool, the highest accuracy of diagnosis was obtained with *Sh*-TSP-2 + MS3_01370 (0.98) followed by *Sh*-TSP-2 + MS3_09198 (0.97), MS3_01370 + MS3_09198 (0.96) (Fig. 5.4B, D and F). The highest accuracy of diagnosis for individuals having high burden was obtained from the *Sh*-TSP-2 + MS3_01370 combination (1.0) and MS3_09198 + MS3_01370 (1.0) followed by *Sh*-TSP-2 + MS3_09198 (0.98) (Fig. 5.4B, D and F). In the case of patients with a medium egg burden, the highest accuracy of diagnosis was obtained was obtained from TSP-2 + MS3_01370 combination (1.0) followed by MS3_09198 + MS3_01370 (0.98) and *Sh*-TSP-2 + MS3_09198 (0.95) (Fig. 5.4B, D and F). For individuals with a low egg burden, the highest accuracy was obtained from *Sh*-TSP-2 + MS3_09198 (0.99) followed by *Sh*-TSP-2 + MS3_01370 (0.98) and MS3_01370 + MS3_09198 (0.93) (Fig. 5.4B, D and F), whereas for egg negative but CAA positive individuals, the highest diagnostic accuracy was obtained from *Sh*-TSP-2 + MS3_09198 (0.92)

followed by MS3_01370 + MS3_09198 (0.84) and by *Sh*-TSP-2 + MS3_01370 (0.76) (Fig. 5.4B, D and F). Similarly, an indirect ELISA was performed using combinations of digested MS3_09198 and MS3_01370, as well as *Sh*-TSP-2 as described above and there is no difference between fused combinations (Appendix Fig. 7A-F). The frequency of recognition matrix was performed for the combinations of fused TSPs (Appendix Fig. 5 lane (I-K)) and the highest values were obtained for *Sh*-TSP-2 + MS3_01370 (86.6%) followed by MS3_01370 + MS3_09198 (84.2%), Sh-TSP-2 + MS3_09198 (77.2%). From combinations of non-fused TSPs the highest values were obtained for *Sh*-TSP-2 + MS3_01370 (87.6%) followed by MS3_01370 + MS3_09198 (86.6 %), Sh-TSP-2 + MS3_09198 (75.8%) (Appendix Fig. 5 lane (L-N)).

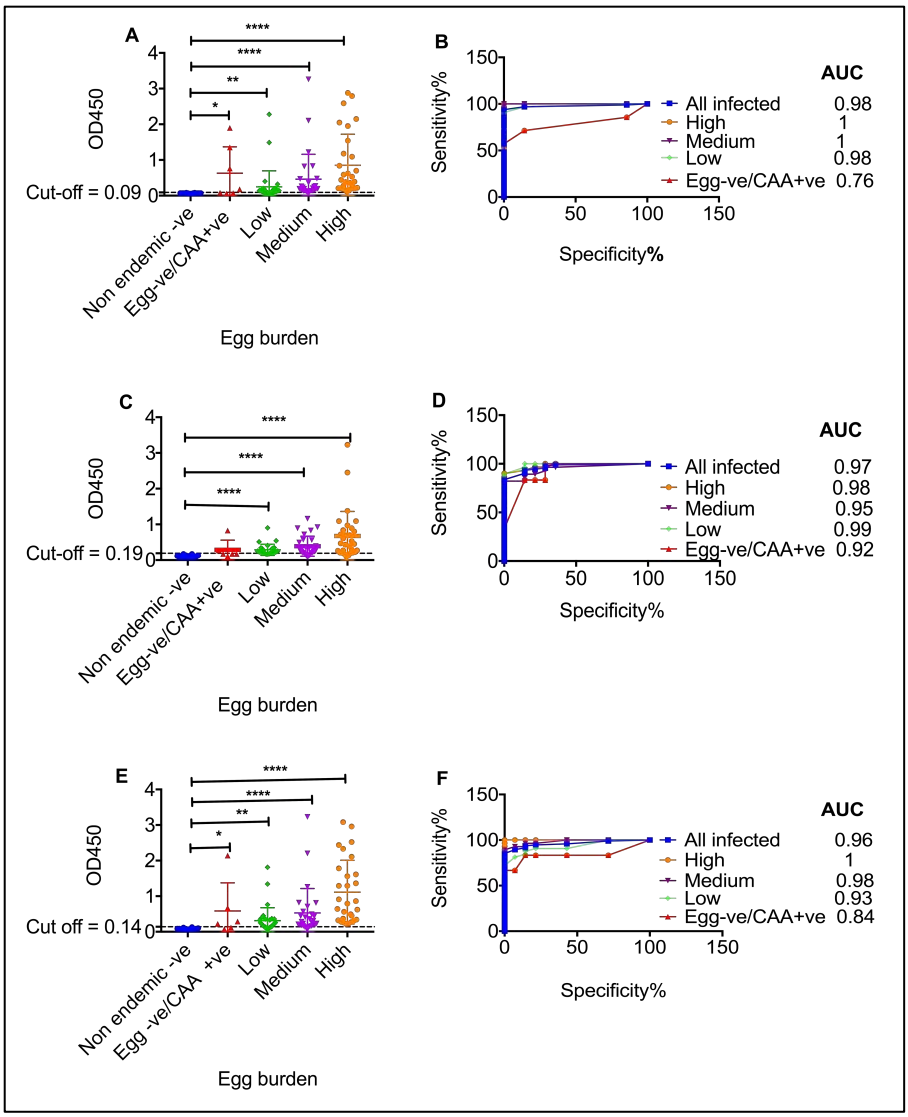

Figure 5.4. Urine IgG recognition and Receiver Operating Characteristic (ROC) curves analysis of the combination of three *Schistosoma haematobium* tetraspanins by Zimbabwean infected individuals with different infection status. The antibody levels were measured by indirect ELISA and indicated by OD values: (A) *Sh*-TSP-2 + MS3_01370, (C) TSP_2 + MS3_09198 and (E) MS3_01370 + MS3_09198. The diagnostic accuracy of the combination of the *S. haematobium* tetraspanins to detect antibodies in the urine of infected individuals with differing infection status as well as using pooled urine from all individuals was measured by the Area Under the Curve (AUC). (B) *Sh*-TSP-2 + MS3_01370, (D) *Sh*-TSP-2 + MS3_09198 and (F) MS3_01370 + MS3_09198. All the data was entered in GraphPad Prism 7 and analysed using a non-parametric Kruskal-Wallis test with multiple comparison followed by Dunn's post-test. * $P < 0.05$ ** $P < 0.01$, **** $P < 0.0002$. The urine from non-infected individuals from a non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).

5.4. Discussion

In most of the sub-Saharan African countries mass drug administration is the only option to control schistosomiasis [319]. However, praziquantel treatment is not helpful in reversing complications caused by schistosomiasis [69] and praziquantel acts only on adult stage worms [299]. For better control and elimination, an effective diagnostic tools that can detect cases from areas with different transmission dynamics and different prevalence intensities are required to provide meaningful assessments of the efficacy of intervention programs [69]. However, urine microscopy, the most common technique for the diagnosis of schistosomiasis, has low sensitivity in HIV infected individuals [313]. HIV-AIDS and schistosomiasis are highly prevalent in sub-Saharan Africa and co-infection is common [320]. Therefore, it is imperative that new diagnostic tools that address these hurdles and accelerate schistosomiasis elimination efforts are developed. In this study the diagnostic potential of antibodies against *S. haematobium* EV TSPs has been evaluated.

The diagnostic accuracy of 6 *Sh*-TSPs was assessed using antibodies from mouse sera and human urine. As a result, *Sh*-TSP-2, MS3_01370 and MS3_09198 were significantly recognised by serum antibodies from experimentally infected mice. *Sh*-TSP-2, MS3_01370 and MS3_09198 were found in the proteomic analysis of adult *S. haematobium* tegument, and a tegument extract of *S. haematobium* was strongly recognized by urine from *S. haematobium* infected human subjects [22]. Similarly, TSPs from other platyhelminths such as *T. solium, O. viverrini, S. mansoni* and *S. japonicum* are also recognised by antibodies of infected humans and experimental animals [149, 157, 159, 231, 269-271]. This, together with the localisation studies and gene expression patterns (chapter (3.3.4 and 3.3.5)), suggests that at least some *S. haematobium* TSPs are accessible to antibodies and are immunogenic during natural

infections. Interestingly, *Sh*-TSP-2 was not recognised by antibodies from *S. mansoni* infected mice, implying the potential usefulness of *Sh*-TSP-2 in the diagnosis of *S. haematobium* infections where both species are co-endemic. Motivated by these results, I decided to test the utility of these antigens for the diagnosis of *S. haematobium* infection by using them to detect antibodies in the urine of individuals infected with the parasite. Urine, not serum, was selected as the diagnostic fluid due to the relative ease of sample collection compared to serum, especially in field conditions [321]. In certain infectious disease states, the use of urine over serum for diagnosis because of the ease of collection may be at the expense of sensitivity, but I posit that, for *S. haematobium* infections, any potential decrease in detection limits may be mitigated by an increased level of IgG present in the urine, relative to that produced by kidney excretion, due to the serum antibody leakage into the bladder as a result of egg-induced damage to the epithelium [322]. Each *Sh*-TSP showed statistically significant recognition by all cohorts, including egg negative but CAA positive urine samples, highlighting the increased diagnostic sensitivity over egg microscopy. Further, the use of *Sh*-TSP combinations in diagnosis doesn't affect the predictive accuracy of infection. The sensitivity of *Sh*-TSPs was less than crude protein mixtures. On the contrary the specificity of all *Sh*-TSPs was higher than crude protein mixtures [323]. With regards to diagnostic use, preparations of defined, recombinant antigens can offer an advantage over crude protein mixtures (such as soluble egg antigen and soluble worm antigen preparation) in that they represent a more standardised and sustainable resource for diagnosis. Indeed, the defined antigen preparations described herein have AUC and FoR values which exceed that of SEA [22], making them a potentially effective, as well as rigorous, tool for the non-invasive diagnosis of *S. haematobium* infection.

MS3_09198, MS3_01370 and *Sh*-TSP-2 are potential diagnostic candidates for the diagnosis of *S. haematobium*. Moreover, *Sh*-TSP-2 is an ideal diagnostic candidate to discriminate *S. mansoni* from *S. haematobium* infection where both species are co-endemic.

Chapter 6: General discussion and Future directions

More than 100 million people are infected by *S. haematobium* [60]. The eggs released by adult female *S. haematobium* worms are associated with pathogenesis and infected individuals suffer from different urogenital abnormalities [35]. The genital ulcers caused by the infection results in sexual dysfunction and infertility in adults [324]. Most importantly, this parasite is responsible for the death of 150,000 people per year [35]. Efforts have been made to reduce the prevalence of schistosomiasis using praziquantel [298] but schistosomiasis is spreading to new areas [34] and is now the second most prevalent of the neglected tropical diseases [325].

The control strategy for schistosomiasis is shifting from targeting morbidity to elimination [326]. To eliminate the disease, effective drugs, a clean water supply, molluscicides to kill the intermediate snail hosts, sensitive diagnostic tools and a vaccine are indispensable [327]; however, the current diagnostic modalities of schistosomiasis have limitations (chapter 1.2.4) and there is no licensed and effective vaccine for this devastating disease. Moreover, praziquantel is the only drug available for schistosomiasis treatment, it doesn't protect against re-infection and reports of resistance are emerging [328]. Therefore, there is an urgent need to develop both sensitive and specific diagnostic candidates, as well as vaccines, to eliminate urogenital schistosomiasis.

Adult schistosomes use ES products and tegumental proteins to modulate the immune response and to escape host-mediated immune attack. [12, 13]. The ES and tegumental proteomes of different schistosomes have been characterised [14, 16-22, 329, 330] and have helped in the identification of molecules that play a key role in host-pathogen interactions. Since EVs are one of the components of ES products, the proteomic compositions of different helminths EVs have also been characterised [24, 25, 117-

126], again revealing many proteins involved in host-parasite interactions. EVs and EV membrane proteins of helminths are also recognised by samples from infected animals [142, 159, 231], suggesting the potential usefulness of EVs for the diagnosis of helminth infection. In addition, immunisation of animals with helminth EVs have reduced parasite burdens and pathogenesis in subsequent parasite challenge [31, 160, 210, 211]. Hence, I have characterised the proteomic composition of *S. haematobium* adult worm EVs to identify and evaluate EV-derived proteins, which could be effective as vaccine and diagnostic candidates.

In chapter 2, *S. haematobium* EVs (sEVs and m/lEVs) were purified from adult worm ES products and their size and concentration were determined using an established procedure [24]. The most represented domains from sEVs were homologues of other helminth vaccine and drug targets such as proteasome subunits, TSPs, ferritin-like proteins and members of the cytosol aminopeptidase family [161, 185, 186, 255, 260, 262]. Similarly, the most represented domains in *S. haematobium* m/lEVs were proteins involved in EV biogenesis and release: EF-hand, Ras family, TCP-1/cpn60 chaperonin family and TSP family proteins [97, 272, 278, 279]. In addition to these most abundant protein families*, S. haematobium* EV proteomics revealed homologues of other helminths vaccine candidates such as GST, saposin B domain-containing protein and calpain [91, 93, 94, 143, 191, 194-196].

TSPs, markers of exosomes, are the second most abundant protein family in *S. haematobium* sEVs and fourth in m/lEVs. Similarly, TSPs are abundant members in different helminth EVs [24, 25, 117-123]. *S. mansoni* EV TSPs (*Sm*-TSP-2 and *Sm*23) have been found to be efficacious vaccine antigens [161]. Moreover, *Sm*23 was also found to be a potential diagnostic candidate [149, 231]. Therefore, six *Sh*-TSPs present in *S. haematobium* EVs were selected and further characterised with regards to their

vaccine and/or diagnostic potential. Based on their expression profile analysis (chapter 3), six *Sh-tsps* were expressed throughout all the assessed life stages, albeit with differing expression levels. Similarly, *tsps* from *S. japonicum* [268, 286, 295], *S. mansoni* [294] and *O. viverrini* [159] were expressed throughout all the assessed life stages with differing expression levels. This result indicated that these *Sh-tsps* may have specific roles in the intermediate snail host and different life stages of the parasite.

In chapter 3, the LEL regions from the *Sh*-TSPs were expressed in *E. coli* and antibodies to each protein were produced in mice to assess the sites of anatomical expression in adult worms. MS3_05226 was located on the tegument surface as well as on the internal organs while MS3_01370 has a diffused expression. Similarly, two *S. japonicum* TSPs were also located on the tegument and internal organs of adult worms while another two TSPs are located only in the internal organs of adult worms [286]. In contrast, *Sh*-TSP-2 and MS3_05289 are located exclusively on the tegument of adult *S. haematobium* worms, which is similar to TSPs from other schistosomes shown to be involved in tegument development [17, 157, 159, 161, 286]. MS3_09198 and MS3_01153 are located on the tegument and gut of adult worms, which is similar to *Sj*-TSP-2 [268]. The presence of EV TSPs in the gut suggests the involvement of EVs in nutrient acquisition and the presence of these tegumental proteins in the EVs indicates that TSPs may facilitate changes in the cell membrane needed for the formation and release of EVs [331].

The tegument is the most susceptible structure to host-mediated immune attack [296] and, as such, different tegumental TSPs from helminths have been effectively tested as vaccine candidates [160, 161, 163, 164, 268]. Herein, I selected three soluble *Sh*-TSPs (from those identified in chapter 2) that clustered together with known schistosome

vaccine candidates [161, 268] and assessed them for vaccine efficacy in both a homologous (hamster/*S. haematobium* challenge) and heterologous (mouse/*S. mansoni* challenge) model of schistosome infection (chapter 4). Immunisation of hamsters with MS3_01370 and *Sh*-TSP-2 significantly reduced the liver egg burden but not adult worm and intestinal egg burden following *S. haematobium* infection. Vaccination of mice with either of the three *Sh*-TSPs significantly reduced tissue egg burdens, suggesting the potential usefulness of developing these *Sh*-TSPs as anti-pathology or transmission-blocking vaccines.

The potential of *Sh*-TSPs to diagnose *S. haematobium* infection in individuals from an endemic population was examined in chapter 5 and this was motivated by localisation results (chapter 3) and a recent study documenting the diagnostic potential of molecules present in *S. haematobium* tegument and ES products [22], indicating that these molecules were accessible to the host immune system in a natural and active infection. As a proof of concept, *Sh*-TSPs were assessed for their recognition by sera from mice experimentally infected with *S. mansoni* or *S. haematobium* and three of the six *Sh*-TSPs (*Sh*-TSP-2, MS3_01370 and MS3_09198) achieved a positive result, an observation documented for other helminth TSPs, including the *S. mansoni* and *S. japonicum* orthologues of MS3_09198 [149, 157, 159, 231, 269-271]. Interestingly, only *Sh*-TSP-2 was not recognised by antibodies from *S. mansoni*-infected mice, indicating the potential usefulness of *Sh*-TSP-2 in the diagnosis of *S. haematobium* infections in African and Middle East countries where both species are co-endemic [8]. The diagnostic efficacy of these TSPs was further assessed by testing urine samples from individuals naturally infected with *S. haematobium* for the presence of anti-*Sh*-TSP antibodies. All *Sh*-TSPs were significantly recognised by individuals with a high infection intensity and, except for

MS3_05289, all *Sh*-TSPs were significantly recognised by individuals with a medium infection intensity. Only MS3_01370, *Sh*-TSP-2 and MS3_09198 were significantly recognised by individuals with a low infection intensity and only MS3_01370, MS3_05226 and MS3_09198 were significantly recognised by egg-negative but CAA-positive individuals. The recognition of these TSPs by egg negative but CAA positive urine samples indicates the higher sensitivity of *Sh*-TSPs over egg microscopy for the diagnosis of urogenital schistosomiasis. This might be due to the presence of IgG in urine of individuals infected with *S. haematobium* due to serum antibody leakage into the bladder as a result of bladder pathology caused by parasite eggs [322].

In this thesis, I have generated novel data on the proteomic composition of *S. haematobium* adult worm EVs and proteins found in the membrane of EVs (TSPs). This study provides a framework for characterisation of other proteins found in *S. haematobium* EVs which will undoubtedly play fundamental roles in host-parasite interactions. Furthermore, the compelling vaccine and diagnostic data generated herein provides a focus for intervention targets to help in the control and elimination of urogenital schistosomiasis.

Future directions

In chapter two of this thesis, the proteomic composition of *S. haematobium* EVs was determined. To allow for a better understanding of the role of *S. haematobium* EVs in host-parasite interactions, future studies will characterise the proteomic composition of different compartments as well as the nucleic acid composition of *S. haematobium* EVs. *S. haematobium* EVs were purified by ODG and the size distribution was determined by TRPS. For further confirmation the size of *S. haematobium* EVs, future studies will use transmission electron microscopy images to complement the size distribution analysis.

In chapter 3 of this thesis, an immunohistochemistry analysis was performed to determine the anatomic sites of *S. haematobium* TSPs expression in adult worm sections using AxioImager M1 fluorescence microscope. To allow for a better understanding on the site of production and mechanism of release of EVs from the worms, future studies will perform immunogold labelling to see localisation of *Sh*-TSPs at the ultrastructural level.

In chapter 4 of this thesis, *Sh*-TSPs showed significant vaccine potential, reducing tissue egg burden reduction in both homologues and heterologous models of infection. Additional research will aim at increasing the level of protection by performing vaccine experiments with the use of different adjuvants and a co-formulation of antigens.

Sh-TSPs showed significant intestinal egg burden reduction in heterologous models of infection but not in homologues model of infection. Therefore, future studies will further explore the reasons for the non-reduction of intestinal eggs in homologues model of infection.

Sh-TSPs significantly reduced the liver egg burden in both the heterologous and homologous model of infection. To see the effect of these egg reduction in the pathology, future studies will perform histological examination of liver tissue from these animals to see if the reduced egg count correlated to any difference in pathology. Furthermore, to understand the mode of action of the *S. haematobium* EVs-TSPs in reducing the egg counts will be further studied.

In chapter 4 of this thesis, *Sh*-TSPs were recognised by antibodies from naturally infected human and experimentally infected mice. To determine whether the protective antibodies induced by the *Sh*-TSPs were actually targeting EVs or the tegument where they are also expressed, future studies will use antibodies from infected human/animal

to see if antibodies, from either infected humans or rodents, recognizing *Sh*-TSPs also label isolated *S. haematobium* EVs.

I have assessed the diagnostic and vaccine potentials of EVs TSPs but due to scarcity of EVs materials I couldn't assess the vaccine and diagnostic potentials of *S. haematobium* EVs. Therefore, future studies will assess the vaccine and diagnostic potentials of *S. haematobium* EVs.

I have assessed the diagnostic potentials of *Sh*-TSPs by detecting antibodies from infected individuals. Future studies will assess the diagnostic potentials of these *Sh*-TSPs by detecting antigens using the polyclonal antibodies raised against them.

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Appendices

Table 1. List of oligonucleotide primers used for qPCR analysis of *Schistosoma haematobium* tetraspanins

Proteins	Forward primer	Reverse primer
α-tubulin	5'GGGCGCGTCTAGATCATAAG3'	5'GTCAACACCAACCTCCTCGT3'
MS3_01370	5'TGCTGTGCTGAGAGACGAAG3'	5'GGACGGTTTGTCCAGATGAT3'
MS3_05226	5'AGCCCGAAATGGAAGTGATT3'	5'TGGTTTCAAGCATCCATTATGT3'
MS3_09198	5'GGCCAAACATTCCAGCTTCA3'	5'GCGACCCAAACAACAAGCTA3'
MS3_01153	5'TCCTGTACCGTGTTGCAAAA3'	5'TGTGAATAGAACAACGAGCAACA3'
Sh-TSP-2	5'CACCACCGGAATCCTGTTTC3'	5'CATCATCACCGCGCTTTACA3'
MS3_05289	5'AAATTCCTCCAGCTTGCTGT3'	5'TATGAACGGGAGGGCTGTTT3'

Table 2. List of oligonucleotide primers flanking the large extracellular loop (LEL) region of *Schistosoma haematobium* tetraspanins.

Tetraspanins	Forward primers	Reverse primers
MS3_01370	NcoI-NdeI-F 5'CGCCCATGGGTCATATGAGAGACGAAGTAAAATCTCAG3'	XhoI-R-5'CGCCTCGAGCTTTTCAAAGAAGGAGATTAC3'
MS3_05289	NcoI-NdeI-F 5'CGCCCATGGGTCATATGGGCACACAAAGTTTGTGGAAG3'	XhoI-R-5'CGCCTCGAGTGGAATAGCTATGAACGGGAG3'
MS3_05226	NcoI-NdeI-F 5'CGCCCATGGGTCATATGAGAAAACAAGTCCCTCATACA3'	XhoI-R-5'CGCCTCGAGTTGATGTAATGGTTTCAAGCA3'
MS3_01153	NcoI-NdeI-F -5'CGCCCATGGGTCATATGTCTCGTAAAGATGAGATTGGC3'	XhoI-R-5'CGCCTCGAGATAATCCATATATTTAAGGAA3'
MS3_09198	NcoI-NdeI-F 5'CGCCCATGGGTCATATGGTAGCAGTTGTTTACAAAGAT3'	XhoI-R-5'CGCCTCGAGGTTGCGTTTCAAGAATGCTCC3'
Sh-TSP- 2	NcoI-NdeI-F 5'CGCCCATGGGTCATATGGAAAAGCCAAAGGTAAAAAAA3'	XhoI-R-5'CGCCTCGAGGGTGCATTTTGCTTAGATCAC3'

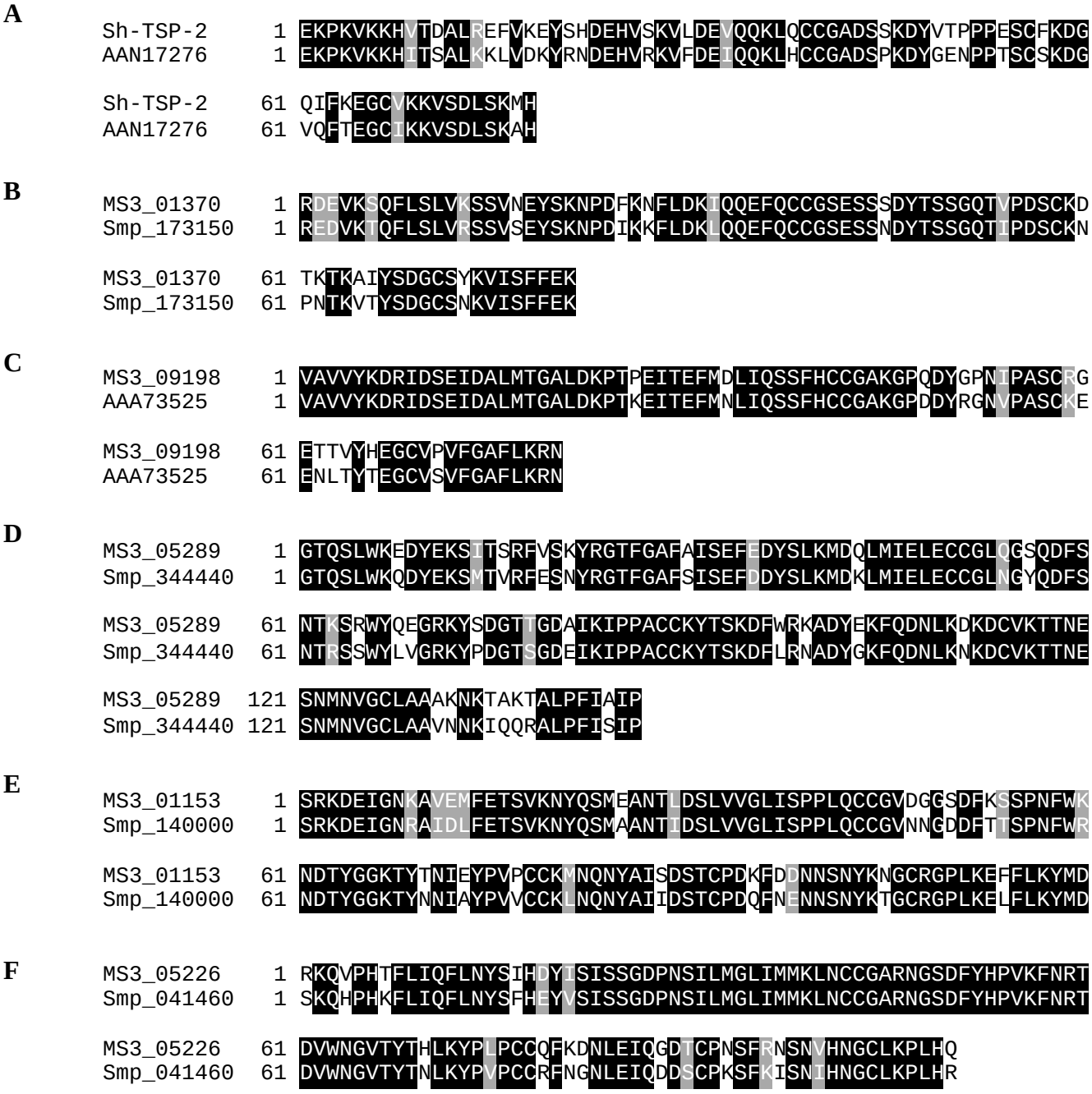

Figure 1. The amino acid sequence alignment of *Schistosoma haematobium* tetraspanins large extracellular loop with their respective *Schistosoma mansoni* homologs. (A) *Sh*-TSP-2 and *Sm*-TSP-2, (B) MS3_01370 and Smp_173150, (C) MS3_09198 and AAA73525, (D) MS3_05289 and Smp_344440, (E) MS3_01153 and Smp_140000 and (F) MS3_05226 and Smp_041460.

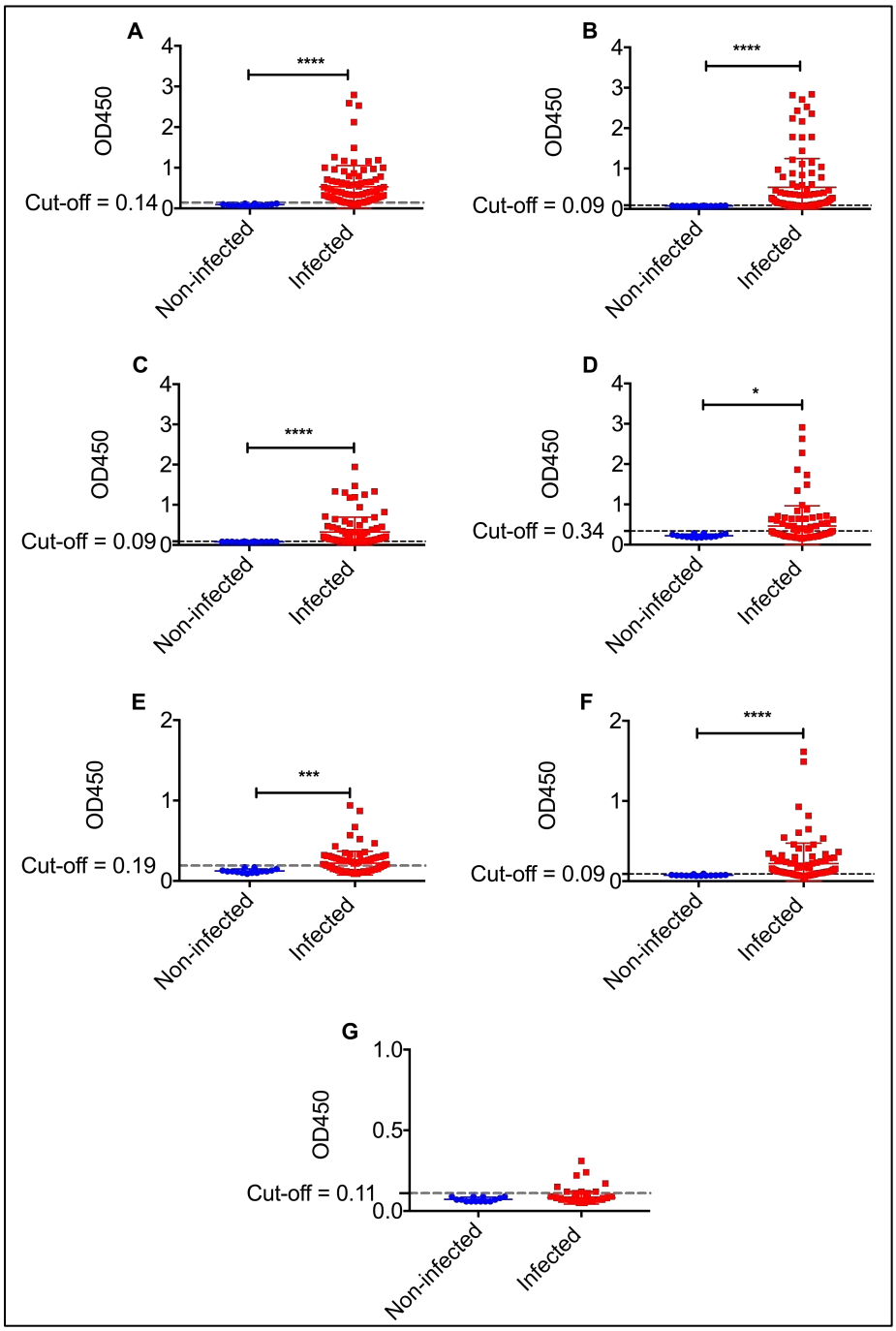

Figure 2. Urine IgG recognition of six *Schistosoma haematobium* tetraspanins from Zimbabwean infected individuals when comparing uninfected non-endemic group with infected individuals as a pool. The antibody level was measured by indirect ELISA and indicated by OD values: (A) *Sh*-TSP-2, (B) MS3_01370, (C) MS3_09198, (D) MS3_05289, (E) MS3_01153, (F) MS3_05226 and (G) TrX. All the data was entered in GraphPad Prism 7 and analysed using a non-parametric Mann Whitney test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0002$. Urine of non-infected individuals from non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).

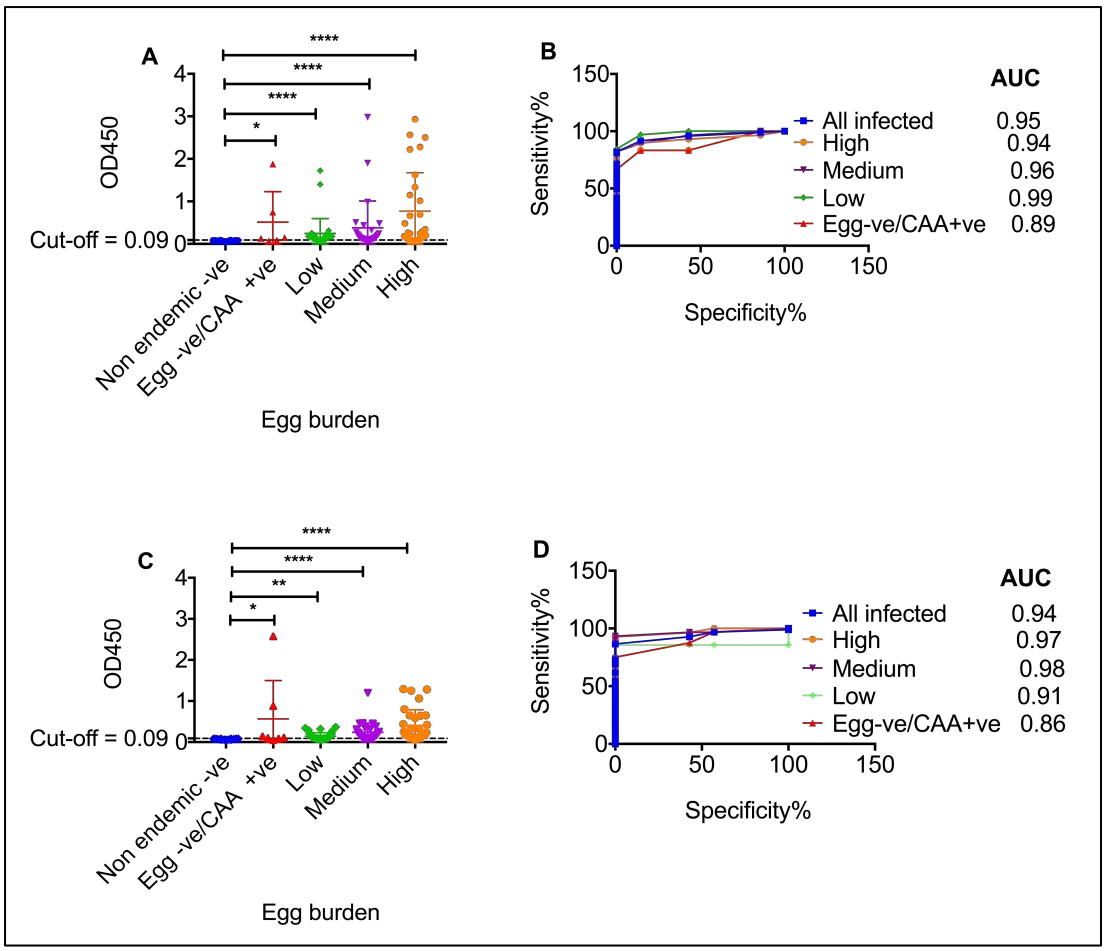

Figure 3. Urine IgG recognition and ROC curves analysis of digested *Schistosoma haematobium* tetraspanins from Zimbabwean infected individuals with different infection status. The antibody level was measured by indirect ELISA and indicated by OD values: (A) MS3_01370, (C) MS3_09198. The diagnostic accuracy of enterokinase digested *S. haematobium* tetraspanins (TSPs) to detect antibodies in the urine of infected individuals with differing infection status was measured by the area under the ROC curve (AUC) (B) MS3_01370, (D) MS3_09198. All the data was entered in GraphPad Prism 7 and analysed with a non-parametric Kruskal-Wallis test with multiple comparison by Dunn's post-test. * $P<0.05$ ** $P<0.01$, **** $P<0.0002$. Urine of non-infected individuals from non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).

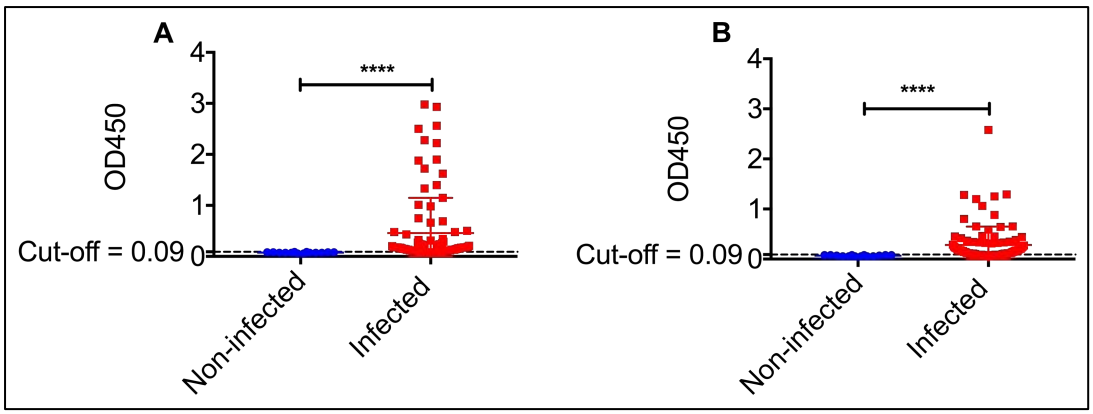

Figure 4. Urine IgG recognition and ROC curves analysis of digested *Schistosoma haematobium* tetraspanins from Zimbabwean infected individuals when comparing uninfected non-endemic group with infected individuals as a pool. The antibody level was measured by indirect ELISA and indicated by OD values: (A) MS3_01370, (B) MS3_09198. All the data was entered in GraphPad Prism 7 and analysed with a non-parametric Mann Whitney test. **** $P < 0.0002$. Urine of non-infected individuals from non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).

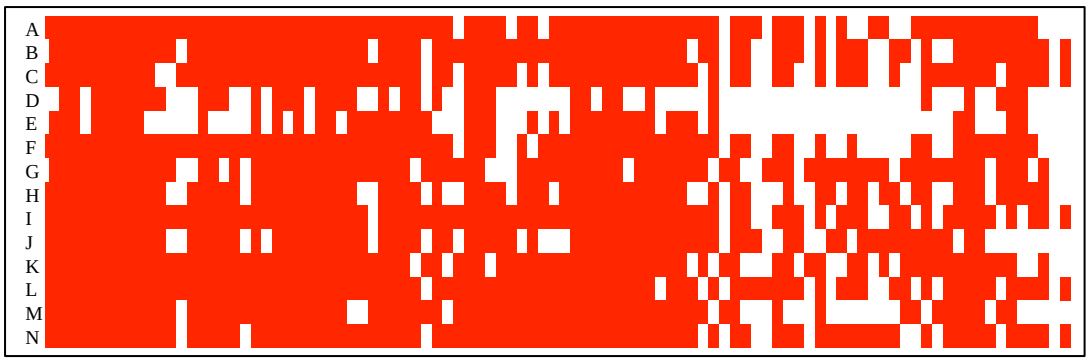

Figure 5. Frequency of Recognition matrix (FoR) analysis of *Schistosoma haematobium* tetraspanins for the detection of *Schistosoma haematobium* infection. The diagnostic accuracy of *S. haematobium* TSPs to detect antibodies in the urine of infected individuals was measured by the Frequency of Recognition matrix (FoR) (lane A) *Sh*-TSP-2, (lane B) MS3_01370, (lane C) MS3_09198, (lane D) MS3_05289, (lane E) MS3_01153, (lane F) MS3_05226, (lane G) MS3_01370 (non-fused), (lane H) MS3_09198 (non-fused), (lane I) *Sh*-TSP-2 + MS3_01370 (fused), (lane J) *Sh*-TSP-2 + MS3_09198 (fused), (lane K) MS3_09198 +MS3_01370 (both fused), (lane L) *Sh*-TSP-2 + MS3_09198 (non-fused), (lane M) *Sh*-TSP-2 + MS3_09198 (non-fused) and (lane N) MS3_09198 +MS3_01370 (both non-fused). Urine of non-infected individuals from non-endemic area was used as negative control and FoR was determined as a percentage by dividing numbers of OD450 values greater than the cut-off point to the total infected individuals for each TSP.

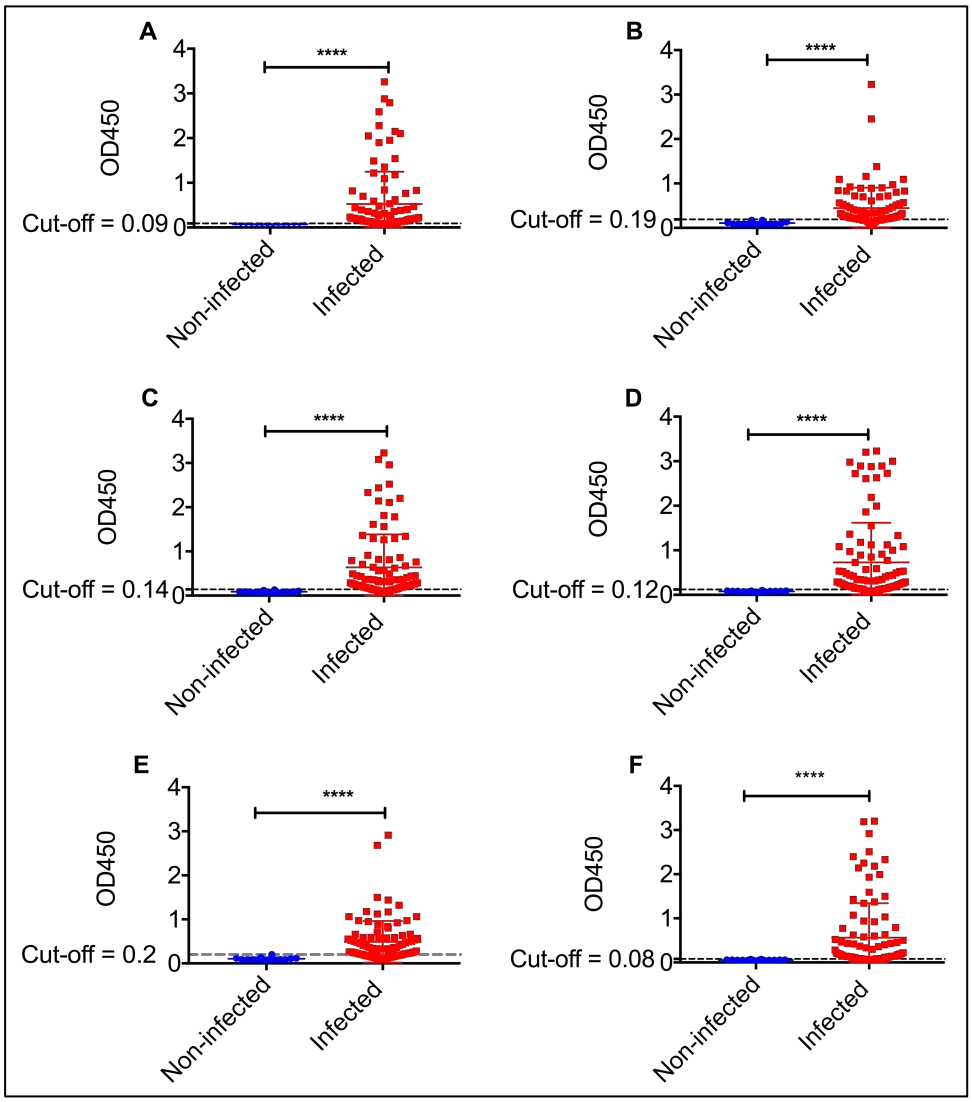

Figure 6. Urine IgG recognition of combinations of fused and non-fused *Schistosoma haematobium* tetraspanins from Zimbabwean infected individuals when comparing uninfected non-endemic group with infected individuals as a pool. The antibody level was measured by indirect ELISA and indicated by OD values: (fused) (A) *Sh*-TSP-2 + MS3_01370, (fused) (B) *Sh*-TSP-2 + MS3_09198, (both fused) (C) MS3_09198 +MS3_01370, (non-fused) (D) *Sh*-TSP-2 + MS3_01370, (non-fused) (E) *Sh*-TSP-2 + MS3_09198 and (both non- fused) (F) MS3_09198 +MS3_01370. All the data was entered in GraphPad Prism 7 and analysed with a non-parametric Mann Whitney test. **** $P < 0.0002$. Urine of non-infected individuals from non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).

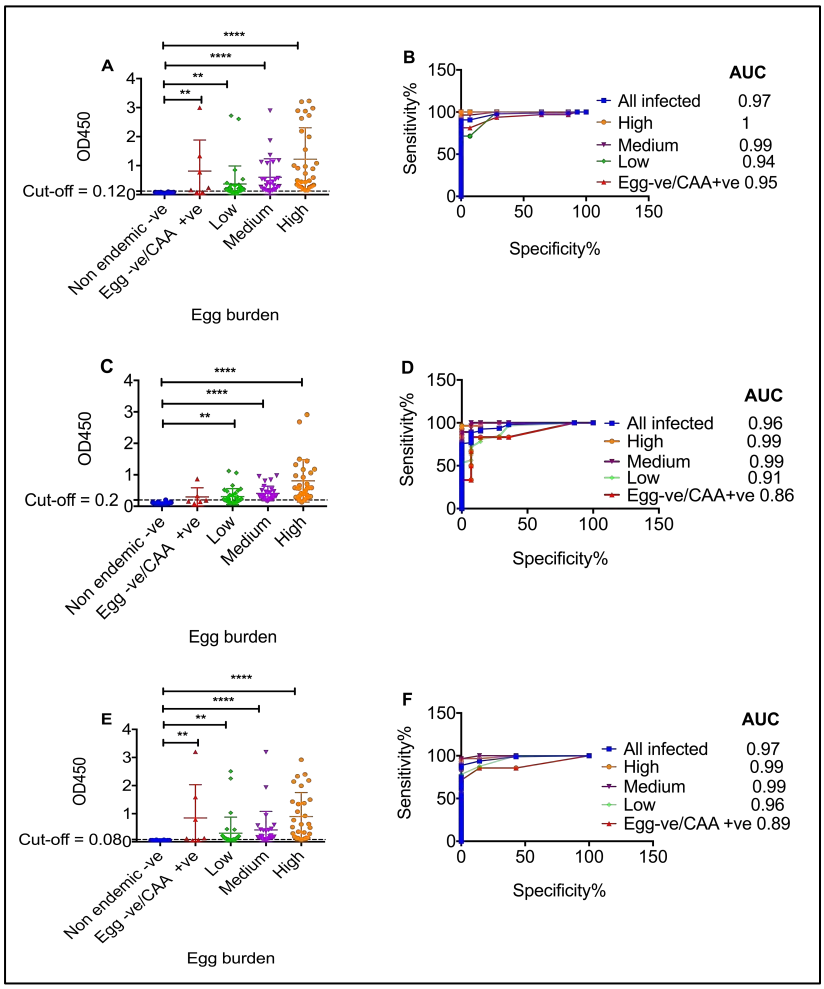

Figure 7. Urine IgG recognition and ROC curves analysis of the combination of three non-fused *Schistosoma haematobium* tetraspanins from Zimbabwean infected individuals with different infection status. The antibody level was measured by indirect ELISA and indicated by OD values: (A) *Sh*-TSP-2 + MS3_01370, (C) TSP_2 + MS3_09198, (E) MS3_01370 + MS3_09198. The diagnostic accuracy of combination of non-fused *S. haematobium* TSPs to detect antibodies in the urine of infected individuals with differing infection status was measured by the area under the ROC curve (AUC) (B) *Sh*-TSP-2 + MS3_01370, (D) *Sh*-TSP-2 + MS3_09198, (F) MS3_01370 + MS3_09198. All the data was entered in GraphPad Prism 7 and analysed with a non-parametric Kruskal-Wallis test with multiple comparison by Dunn's post-test. ** $P < 0.01$, **** $P < 0.0002$. Urine of non-infected individuals from non-endemic area was used as negative control. The reactivity cut-off points were determined as the average reactivity + 3x standard deviation of non-endemic negative individuals (indicated by broken lines).