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# Adaptive radiation within the vaccine target tetraspanin-23 across nine *Schistosoma* species from Africa

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ABSTRACT

High levels of polymorphism in DNA sequences of tetraspanin-23 (TSP-23) were revealed within and between nine different species of *Schistosoma* from Africa including *Schistosoma mansoni, Schistosoma rodhaini, Schistosoma margrebowiei, Schistosoma mattheei, Schistosoma intercalatum, Schistosoma haematobium, Schistosoma guineensis, Schistosoma curassoni* and *Schistosoma bovis*. The greatest levels of diversity coincided with evidence of positive selection ($d_N/d_S > 1$) within regions that code for extracellular loops of TSP-23 believed to interact with the host immune system. Kolaskar and Tongaonkar antigenicity predictions of protein sequences were compared across species and high levels of variation in antigenicity were also identified with each species which possessed their own unique antigenic profile. Phylogenetic analysis of TSP-23 proteins suggested evidence of convergent evolution in antigenic lineages as no true interspecies phylogenetic relationships were seen. This could be indicative of host-specific evolution of antigens in different species of schistosomes, a factor that should be considered carefully when developing vaccine targets.



## 1. Introduction

Human schistosomiasis is a neglected tropical disease caused by *Schistosoma* blood flukes and is estimated to affect over 200 million people in developing tropical and subtropical countries (Steinmann et al., 2006). Much research has focused on the development of a multi-species schistosome vaccine (McManus and Loukas, 2008) to contribute towards controlling schistosomiasis. Morbidity control is currently centred on the wide-scale distribution of praziquantel (Fenwick and Webster, 2006) but the threat of a heritable reduction in the parasite's susceptibility to praziquantel remains (Fallon and Doenhoff, 1994; Ismail et al., 1999; Melman et al., 2009).

Several tegument-associated proteins have been identified as potential schistosome vaccine targets with a particular focus on the tetraspanins (TSPs), a large superfamily of membrane bound proteins (McManus and Loukas, 2008). There are currently at least 29 members of the TSP superfamily known in schistosomes, although more are predicted to be present. Tetraspanins are characterised by the presence of four conserved hydrophobic transmembrane domains, intracytoplasmic N- and C-terminal regions, and a small and large extracellular loop (Jiang et al., 2010; Yuan et al., 2010; Cupit et al., 2011; Wu et al., 2011; Zhang et al., 2011). The expression profiles of TSP members are diverse with some being stage-specific and others being universally expressed throughout the schistosome life cycle, indicating that TSPs might have different functions in different life stages (Jiang et al., 2010; Wu et al., 2011). Several authors have suggested that schistosome TSPs play a role in immune evasion with the characteristic large extracellular loop being directly exposed to the host immune system; TSP-1 and TSP-2 appear similar to surface receptors on B and T cells, and other members of the TSP superfamily are thought to be involved in molecular mimicry by incorporating host molecules on the tegument surface to suppress immune detection (Kolvalenko et al., 2005; Jiang et al., 2010; Yuan et al., 2010; Wu et al., 2011). Several TSP proteins have been suggested as potential vaccine candidates including TSP-1, TSP-2, and TSP-29 due to high levels of protective specific antibodies to these proteins found in infected hosts (Cai et al., 2008; Sepulveda et al., 2010).

TSP-23, the first TSP to be identified (Harn et al., 1985), is among the better characterised members of the TSP superfamily. Due to its immunological properties and its expression on the tegument throughout the schistosome life cycle, TSP-23 has been subject to vaccine trials in buffalo (Da'Dara et al., 2008) and mice (Jiang et al., 2010; Wu et al., 2011) but these have achieved variable success. These studies reveal a lack of sustained immune memory or protection against infection/reinfection even after

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immunisation with TSP-23 (Da'Dara et al., 2008; Wu et al., 2011). In the light of such findings, the value of TSPs as vaccine targets has recently been questioned as studies on *Schistosoma mansoni* and *Schistosoma japonicum* have shown the large extracellular loop of other members of the TSP family, especially TSP-2, to be variable relative to the rest of the protein (Cai et al., 2008; Cupit et al., 2011; Zhang et al., 2011). The extracellular loop of *S. japonicum* TSP-2 has been shown to possess at least seven variable forms, which has been suggested to reduce prospects of effective vaccine efficacy (Cai et al., 2008). Conversely, Cupit et al. (2011) showed limited variability in TSP-2 from *S. mansoni* collected from a small cohort of patients from western Kenya, but did highlight that some of the amino acid substitutions could affect protein structure and, potentially, vaccine efficacy. The extent of protein variation, especially antigens, and polymorphism in schistosomes is not well understood (Walker, 2011).

Development of a "silver bullet" multi-species schistosome vaccine that could be used in the control of schistosomiasis is an important goal (McManus, 2005; Wilson and Coulson, 2006; Cai et al., 2008; McManus and Loukas, 2008; Cupit et al., 2011; Zhang et al., 2011). Due to the significant co-evolutionary relationships that exist between schistosomes and their definitive hosts, structural and/or biochemical changes are likely to occur in tegumental antigens of the parasite that prevent antigen recognition by the host, a process also seen on surface antigens of other blood-dwelling pathogenic organisms (Dunne and Cooke, 2005; Lewis-Rodgers et al., 2008). Therefore, it is hypothesised that tegumental antigens will show high levels of variability within and between species driven by the selective pressures from the host immune system, thus allowing the parasite to survive and continue to evade the host immune response.

The aim of the current study was to identify adaptive mutational events in TSP-23 as an indicator of the impact of the definitive host immune system in the diversification of schistosomes. The level of diversity of this protein and changes in the structure of TSP-23 between nine schistosome species from Africa with different definitive and intermediate host ranges were explored.

## 2. Materials and methods

### 2.1. Parasite material and DNA extraction

Seventeen isolates representing nine species of *Schistosoma* from various geographical locations across Africa, plus representatives of a Puerto Rican *S. mansoni* (Table 1) were obtained from SCAN (Schistosomiasis Collection at the Natural History Museum (NHM), London; http://www.nhm.ac.uk/research-curation/collections/curation-groups/scan/index.html) (Emery et al., 2012). DNA was extracted from single males using the DNeasy blood and tissue extraction kit (Qiagen, UK) according to the manufacturer's instructions.

### 2.2. PCR and sequencing

Specific TSP-23 primers were designed from alignments of DNA/mRNA sequences of TSP-23 of *S. mansoni* (Accession No. XM_002569532) and *Schistosoma haematobium* (Accession No. U23771) available from the online NCBI databases (http://www.ncbi.nlm.nlh.gov/). Alignments were performed using MUSCLE (http://www.ebi.ac.uk/Tools/muscle/index.html) and visualised/edited in Bioedit (Version 7.0.5.2; Hall, 1999). Once intron and exon boundaries were identified, primers that would amplify the majority of the TSP–23 (~1,600 bp) gene were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

PCR was performed using 1 μM forward primer AfrTSP23_Ex1F (5′-TGGCAACCCTGGGTACTGGT-3′) and reverse primer AfrTSP23_Ex5R (5′-TGGCACCCAAACAACAAGCT-3′), 12.5 μl of Thermo-Start® PCR master mix (0.625 U of Taq DNA polymerase, 1× reaction buffer, 0.2 mM of each dNTP and 1.5 mM $MgCl_2$) and 1–2 ng/μl of DNA. Final reactions were made up to 25 μl with double-distilled pure water and PCRs were performed using a Veriti 96 well thermal cycler (Applied Biosystems™, UK). The initial denaturing and activation step was performed at 95 °C for 15 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. This was followed by a final elongation step at 72 °C for 10 min. Then 5 μl of each PCR product was visualised in 1% agarose gels stained with gel red (Bioline, UK). The remaining 20 μl of PCR products were sequenced at the DNA sequencing facility of the NHM, London, using the same primers used for PCR with Fluorescent Dye Terminator Sequencing Kits (Applied Biosystems™, UK); sequencing reactions were run on an Applied Biosystems 3730XL automated sequencer.

### 2.3. Sequence analysis and bioinformatics

Obtained DNA sequences were assembled and edited manually using Sequencher (Version 4.2; GeneCodes Corp) and Bioedit (Hall, 1999). Sequences were then aligned with those used to design primers, and introns were removed ensuring that only exon coding regions were further analysed; *S. japonicum* TSP-23 (Accession No. **FN316354**) was used as an outgroup for all analysis as it represents one of the basal Asian *Schistosoma* spp. Codons were identified and DnaSP 5 (Librado and Rozas, 2009) was used to measure levels of diversity within and between species and to perform sliding window analysis to identify variable regions of diversity. Window and step sizes of 3 bp were used to measure diversity in each codon contributing to the protein. This allowed variability to be identified by measuring the singleton mutations (S), nucleotide diversity ($\pi$) as described by Nei (1987), number of synonymous ($d_S$) and non-synonymous ($d_N$) mutations and presence of selection and/or neutrality was identified by calculating the $d_N/d_S$ ratio within and between the *S. mansoni* and *S. haematobium* clades.

The aligned DNA sequences were translated into proteins in Bioedit (Hall, 1999) and the antigenicity of each protein was determined using the Kolaskar and Tongaonkar (KT) antibody epitope prediction tool (http://tools.immuneepitope.org/tools/bcell/iedb_input; Kiss et al., 2008). The KT antibody prediction uses the physicochemical properties and the frequency of amino acid residues to determine antigenicity of proteins (Kolaskar and Tongaonkar, 1990) and therefore was used as an indicator of diversity in antigenicity of the TSP-23 protein across the nine species of *Schistosoma*. Exposed regions of the TSP-23 proteins were also identified using the Emini surface accessibility prediction test (http://tools.immuneepitope.org/tools/bcell/iedb_input) and structural information was ascertained from UniProt (http://www.uniprot.org/). Structural characteristics of each TSP-23, including alpha helical regions, extended strand and random coils, were determined using the GOR3 secondary structure prediction method (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gib.html). The association between $d_S$ and protein structure was identified. Further analysis of the effects of amino acid change was carried out by classifying the biochemical properties of amino acids as hydrophobic, hydrophilic or neutral, in order to assess any major evolutionary changes that might have affected the shape and biochemical properties of TSP-23 between species. Finally, phylogenetic analysis of protein sequences was performed using distance-based neighbour joining (NJ) methods as well as model based maximum likelihood (ML) using the JTT+G substitution model in MEGA 5.04 (Tamura et al., 2011). Nodal support was tested using bootstrap values, which were calculated using 500 replicates.