**A new perspective on urogenital schistosomiasis in Malawi: Schistosome interactions within the *Schistosoma haematobium* group**

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**Abstract** Genetic interactions occurring between human (*Schistosoma haematobium*) and livestock (*S. mattheei* and *S. bovis*) schistosome species were revealed by molecular analysis of atypical schistosome eggs retrieved from Malawian children. Detection of hybrid schistosomes adds an important new perspective to the epidemiology and control of urogenital schistosomiasis in Central Africa.

Urogenital schistosomiasis is a high-ranking waterborne disease, transmitted by certain freshwater snails, that occurs throughout much of sub-Saharan Africa; a disease once commonly held attributable to *Schistosoma haematobium* alone which has very limited zoonotic potential [1]. This appraisal is being repeatedly revised as new evidence accrues upon genetic analysis of natural infections with non-invasive larval sampling [2]. In West Africa, for example, species interactions with hybrid combinations of *Schistosoma haematobium* and bovine/ovine species of *S. bovis* and *S. curassoni* are being commonly encountered in humans and snails [3]. Whilst key biological features of hybrids may not always be apparent, the risk of zoonotic transmission alongside enhanced definitive and intermediate host compatibilities needs study [2,3]. The public health impact of such genetic introgression is best evidenced by the recent emergence and persistent transmission of *S. haematobium*-*bovis* hybrids on the Mediterranean island of Corsica [4].

With regard to Central and Southern Africa, genetic analysis of *Schistosoma haematobium* group species is priority. Based on atypical egg morphologies, a capacity for natural hybridization of *S. haematobium* with the bovine species *Schistosoma mattheei* was suggested long ago and was later confirmed by biochemical markers and experimental infections demonstrating viable progeny [3]. During ongoing surveillance of urogenital schistosomiasis in Chikhwawa District, Malawi, we have encountered atypical *S. haematobium* eggs in urine samples from several infected children [5]. We report here on the further genetic characterization of atypical eggs collected from epidemiological surveys of children within Chikhawa, Nsanje and Mangochi Districts that has revealed the presence of both *S. haematobium-mattheei* and *S. haematobium-bovis* hybrids, Figure.

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Schistosome eggs were filtered from the urine of infected children, photographed and measured before being stored on Whatman FTA cards for molecular analysis [2]. The DNA from individual eggs was alkaline eluted and genotyped using both the mitochondrial cytochrome oxidase subunit 1 (*cox*1) and the nuclear ribosomal internal transcribed spacer (rITS) DNA regions [2]. Additionally, for the samples from Mangochi District a partial region (300 bp) of the nuclear ribosomal 18S DNA was analysed to confirm the presence of *S. mattheei* nuclear DNA [2,6]. For more detailed methods *see* Supplemental File. From Chikhwawa, 6 atypical eggs were examined all of which had a pure *S. haematobium* genetic profile, see Figure. From Nsanje, a total of 19 eggs were examined with 3 having atypical morphologies, 18 eggs had a pure *S. haematobium* genetic profile but one atypical egg presented with a discordant genetic profile (i.e. *cox*1 *S. bovis* and rITS *S. haematobium*). From Mangochi, 16 typical *S. haematobium* shaped eggs had a pure *S. haematobium* genetic profile whilst 4 atypical eggs presented identical but discordant genetic profiles (*cox*1 *S. mattheei* and rITS *S. haematobium-mattheei*). Inspection of the partial 18S confirmed *S. haematobium-mattheei* hybrids. All sequence data can be obtained from GenBank Accession Numbers: MK358841-MK358858.

Our genetic analysis has clearly demonstrated the presence of *S. haematobium* group hybrids in Malawi as introgressed forms of *S. haematobium-mattheei* and *S. haematobium-bovis*. It is worthy to note that an unusual egg morphology may not always correspond with the ability to detect introgression with the current combination of genetic markers used [6]. For further referencing *see* Supplemental File. As noted previously in Senegal, successive back crossings of hybrid progeny may obscure our ability in the detection of ancestral introgression, the development of a wider panel of nuclear genetic markers is needed. Nonetheless, detection of these two hybrid schistosomes strongly suggests interactions of *S. haematobium* with two ungulate schistosomes *S. mattheei* and *S. bovis*.The latter schistosome has not been reported in Malawi which implies a changing species dynamic with a suspicion of zoonotic transmission along the drainage basin of Lake Malawi. This adds an important new dimension to the epidemiology and control of urogenital schistosomiasis in Malawi [7].

As miracidial hatching was not attempted during this study, we cannot confirm that these hybrids or introgressed forms are fully viable in natural transmission autochthonously. However, the process of ancestral introgression with subsequent natural selection may help to explain in part unexpected shifts in local snail-schistosome relationships, e.g. the changing compatibility of *Bulinus nyassanus* in Lake Malawi with *S. haematobium* [8]. Further studies are therefore needed to better characterise schistosomes involved in human infection, investigate more thoroughly any zoonotic potential and assess all possible combinations of inter-species introgressions. Concerning the latter, ancestral hybridization between *S. haematobium* and *Schistosoma mansoni* has been noted [9] and given autochthonous transmission of intestinal schistosomiasis in Lake Malawi [10], there may be sufficient epidemiological opportunity for this to further arise. Heightened co-surveillance of urogenital and intestinal schistosomiasis is advised, entailing molecular vigilance for any inter-species interactions, alongside phenotypic assessments for any altered host pathogenicity or susceptibility to praziquantel treatment. The detection of hybrid schistosomes adds an important new perspective to the epidemiology and control of urogenital schistosomiasis in Central Africa.

Dr Bonnie Webster has specific expertise in medical helminthology with a longstanding interest in studies of schistosome hybridization in both natural and experimental settings.

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**Figure A**. Diagrammatic map of Malawi with sampled Districts indicated (GPS of sampled village where egg-patent *S. haematobium* urines were collected): Mangochi (Samama 14°417465S, 35°217580E), Chikhwawa (Mpangani 16°036299S, 34°841063) and Nasanje (Kastiano 16°906398S, 35°266578E). About 10% of sampled children had atypical eggs in their urine in an approximate ratio of 1 (atypical):25 (typical) egg shapes. Note that the Shire River flows southward from Lake Malawi, linking all three sampled locations within the same drainage basin. **Figure B**. Photomicrographs of a representative atypical egg from each location with corresponding genotypes assigned for the mitochondrial *cox*1 and nuclear rITS loci. **Figure C**. Bar chart histogram of length measurements for 83 typical *S. haematobium* eggs collected from Nsanje, the associated density distribution is shown (solid line). The mean length of this sample of typical eggs was 135 ± 28µm (1 standard deviation), with minimum (86 µm) and maximum (180 µm). The length measurements of the 3 atypical eggs are indicated (arrowed dashed lines), indicating they each fall well outside the range of length variation of the 83 eggs [inset photomicrograph of a characteristic *S. haematobium* egg].



SUPPLEMENTAL INFORMATION

**Protocol**

DNA preparation from FTA stored schistosome eggs / larvae

# Add 14μl of **Solution 1** **(0.1M NaOH, 0.3mM EDTA, pH13.0)** to the punch out 2mm FTA disc

# Incubate at room temperature for 5 mins

# Add 26μl of **Solution 2** **(0.1M Tris-HCl, pH7.0)**

# Pulse vortex 3 times

1. Incubate at room temperature for 10 mins
2. Pulse vortex 10 times
3. 3μl of the DNA elution is used in a 25μl PCR

**Table.** The PCR primers used to amplify each DNA region and their associated PCR thermal cycle

|  |  |  |  |
| --- | --- | --- | --- |
| **DNA region** | **Forward Primer** **(name)** | **Reverse Primer** **(name)** | **PCR thermal cycle** |
| ITS1+2 rDNA | TGCTTAAGTTCAGCGGGT (ITS1) | AACAAGGTTTCCGTAGGTGAA (ITS2) | 5 min at 95°C:40 cycles of 30 sec at 95°C, 30 sec at 58°C, 1.30 min at 72°C:10 min at 72°C.  |
| Partial 18S rDNA | GCGAATGGCTCATTAAATCAG (WA) | TCCGGAGAGGGAGCCTGA (300R) | 5 min at 95°C:40 cycles of 30 sec at 95°C, 30 sec at 60°C, 1 min at 72°C:10 min at 72°C. |
| Partial *cox*1 mt DNA | TAATGCATMGGAAAAAAACA (cox1Schisto5’) | TCTTTRGATCATAAGCG (cox1Schisto3’) | 5 min at 95°C:40 cycles of 30 sec at 95°C, 30 sec at 40°C and 1.30 min at 72°C10 min at 72°C. |

**PCR analysis and sequencing**

DNA was eluted as described above from the schistosome eggs stored on the FTA cards. In separate PCR’s, run on a Perkin Elmer 9600 Thermal Cycler, the *cox*1, ITS and 18S DNA regions were amplified (*see* Table above with PCR primers). A 25 µl PCR reaction was each performed using illustraTM puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK) and 10 pmol of each primer and 3μl of the DNA elution.

All PCR reactions were checked for positive amplification of the correct band size by gel electrophoresis (0.8% Gelred agarose gels). PCR amplicons were purified and Sanger sequencd in both directions using a dilution of original PCR primer. All sequence data was visualised and manually edited using Sequencher V5.1.

Mitochondrial *cox*1 sequence identity was confirmed using the Basic Local Alignment Search Tool (BLAST) (Genbank). The ITS and 18S sequence identity was analysed by visual comparison to personal reference sequences for each species (*S. haematobium*, *S. bovis* and *S. mattheei*). Known inter-species SNP regions (see figure below) were visually checked to identity homogenous or heterogenous ITS and 18S DNA.

Genetic profiles, mitochondrial and nuclear, were inspected to identify hybrids (and any discordance of mitochondrial and nuclear DNA data).

**Figure.** Species specific SNP variation which differentiate the three species at 18S and ITS loci.

|  |  |  |
| --- | --- | --- |
| **Marker** | **18S (300bp)** | **ITS (906bp)** |
| **SNP** | **138** | **163** | **210** | **18** | **26** | **50** | **51** | **91** | **92** | **120** | **170** | **225** | **490** | **877** |
| *S. haematobium* | T | C | T | C | T | G | T | C | G | C | G | C | T | T |
| *S. bovis* | C | T | C | C | T | A | T | C | G | T | A | T | T | C |
| *S. mattheei* | C | T | T | T | A | A | C | T | A | T | A | T | A | T |

**Further bibliography on significance of atypical helminth eggs**

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