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Title: Independent origins of loss-of-function mutations conferring oxamniquine resistance in a Brazilian schistosome population

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Abstract: Molecular surveillance provides a powerful approach to monitoring the resistance status of parasite populations in the field and for understanding resistance evolution. Oxamniquine (OXA) was used to treat Brazilian schistosomiasis patients (mid-1970s to mid-2000s) and several cases of parasite infections resistant to treatment were recorded. The gene underlying resistance (SmSULT-OR) encodes a sulfotransferase required for intracellular drug activation. Resistance has a recessive basis and occurs when both SmSULT-OR alleles encode for defective proteins. Here we examine SmSULT-OR sequence variation in a natural schistosome population in Brazil ~40 years after the first use of this drug. We sequenced SmSULT-OR from 189 individual miracidia (1-11 per patient) recovered from 49 patients, and tested proteins expressed from putative resistance alleles for their ability to activate OXA. We found nine mutations (four non-synonymous single nucleotide polymorphisms (SNPs), three non-coding SNPs and two indels). Both mutations (p.E142del and p.C35R) identified previously were recovered in this field population. We also found two additional mutations (a splice site variant and 1 bp coding insertion) predicted to encode non-functional truncated proteins. Two additional substitutions (p.G206V, p.N215Y) tested had no impact on OXA activation. Three results are of particular interest: (i) we recovered the p.E142del mutation from the field: this same deletion is responsible for resistance in an OXA-selected laboratory parasite population; (ii) frequencies of resistance alleles are extremely low (0.27-0.8%), perhaps due to fitness costs associated with carriage of these alleles; (iii) that four independent resistant alleles were found is consistent with the idea that multiple mutations can generate loss-of-function alleles.



TEXAS BIOMEDICAL
RESEARCH INSTITUTE

Department of genetics

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March 30th, 2016

To the editor,

We are resubmitting our paper entitled **Independent origins of loss-of-function mutations conferring oxamniquine resistance in a Brazilian schistosome population** to the special Symposium for International Research and Innovations in Schistosomiasis (SIRIS) 2016 issue in International Journal for Parasitology as a research article. We thank the reviewers for their helpful comments on our manuscript. We have revised it in the light of these comments.

Please find below our answers to the reviewers' comments. The reviewers' comments are shown in italic, our responses are shown in plain blue text, while changes to the manuscript are shown in tracking mode in the revised manuscript. Line numbers indicated in the answers refer to the tracking mode version of the manuscript.

We think that the revised manuscript is now improved and hope that it will now be suitable for publication in International Journal for Parasitology.

We look forward to hearing from you,

Yours sincerely,

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Reviewer #1:

The article describes a systematic analysis of field-isolated schistosome miracidia for the presence of mutations in the gene encoding the sulfotransferase necessary for the activation of the schistosomicidal drug oxamniquine. In addition, the effect of mutations detected within exon sequences was tested by determining the capacity of the corresponding recombinant enzyme to activate oxamniquine in vitro. The article addresses and answers some important questions about the frequency of resistance mutations in a human population no longer exposed to oxamniquine and the capacity for the evolution of resistance in the event of the deployment of oxamniquine or new derivatives currently under development. Overall, the paper is well presented, the methodology is solid and the results obtained are significant. I have no major criticisms concerning the study and my only remarks concern a few points meriting explanation and some textual errors.

Remarks

1. L.101: *Was the emergence of resistance due to oxamniquine due to the fact that it has the same target as hycanthone, or rather that the resistance mechanism is the same?*

Both the drug target and the mechanisms of resistance are the same for OXA and hycanthone. To clarify this sentence now reads: "This rapid emergence of resistance was most likely due to the previous treatment of the same populations with the related drug hycanthone (Katz1968, Coura2010), as both OXA and hycanthone have the same target (Jansma1977,Pica-Mattoccia1993) and the same mechanism of resistance (Pica-Mattoccia1992b)." (line 102)

2. L.166: *Taq polymerase, used for exon amplification, can introduce random errors in the amplified sequence. How were these detected and distinguished from SNPs, some of which occurred only once in the analyzed sequences?*

This is a concern only when cloned PCR products are sequenced. In this particular case, individual sequences generated during PCR are sequenced and these sequences may contain errors introduced by the enzyme. In our experiment, we used direct sequencing of PCR products to avoid this problem. Errors introduced by Taq polymerase are not an issue with direct sequencing, because PCR products are generated from multiple independent template sequences.

3. L.199: *I assume that the accession numbers will be completed in proof.*

The accession number are now included in the text (line 203).

4. L.298: *"High frequency" SNPs present in 96% of the analyzed sequences seem rather more like a sequence change in the Brazilian strain compared to the strain used for the original genomic sequencing. Are these strictly "SNPs"?*

All nucleotide sites that are variable in the Brazilian population examined are SNPs and are detailed here. We now show the ancestral state at these non-coding SNP sites in Table 2.

5. *Suppl. Movie: The version I downloaded (twice!) was viewable but the lower half of the image trembled a great deal, notably when text was included. The authors should check this and upload a new version.*

We thank the reviewer for pointing this problem out. Indeed, the movie cannot be seen properly on Windows Media Player which we did not previously test. We modified the encoding parameters and tested the movie on several players.

Textual errors

Only a corrected version of the text is given below. Some "corrections" may be considered by the authors to be stylistic whims of the reviewer. I make the suggestions nevertheless.

1. L.58: *"Four independent ..."*

Done

2. L.83: *"only possible when resistance..."*

Done

3. L.101: *"Later genetic studies..."*

Done

4. L:111: *"the fact that the Brazilian government...."*

Done

5. L.115: *"alleles encoding a functional enzyme.."*

Done

6. L.140: *"in plastic bags..."*

"in plastic bag" was changed to "in a plastic bag"

7. L.146: *"in a 1.5mL sterile..."*

Done

8. L.290: *"three in exon 1 and three in exon 2..."*

Done

9. L.299: *"in exon 1 while one deletion was identified in exon 2,..."*

Done

10. L.320: *"truncated protein with no active site..."*

Done

11. L.324: *"and derived from mutations.." But this is too strong. Rather, "non-synonymous and identical to sequences of the gene in the outgroup..."*

It seems that the reviewer misread this section: the mutations identified are derived when compared to the sequence of the outgroup, which represent the ancestral state of the site (i.e. they are different from the outgroup). We have not made any modifications.

12. L.331: *this may have a detrimental effect...*"

Done

13. L.337: *"Both indels have detrimental..."*

Done

14. L.359: *"distant from the region from which our samples..."*

"a different geographic state" was changed to *"the neighboring state"*

15. L.360: *"the allele has been segregating..."*

Done

16. L.386: *"The number of origins of resistance..."*

Done

17. L.391: *"resistance, a high mutation rate..."*

Done

18. L.411: *"In silico analysis, using predictions based on Drosophila, did..."*

Done

19. L.429: *"OXA sensitive populations..." (or susceptible).*

Done

20. L.431: *"different genetic backgrounds..."*

Done

21. L.451: *"and evolved de novo..."*

Done

22. L.644: *"(counts per minute)..." (or c.p.m.).*

Done

23. L.652: *"number of samples sequenced..."*

Done

24. Table 2, right-hand column, line 4: *"Impaired oxamniquine..."*

Done

Reviewer #2:

Overall the manuscript is well written and addresses an interesting problem. The mechanisms that determine the likelihood of the development of resistance have been postulated, but there are not too many examples where there is experimental evidence. The authors provide population genetic analysis, crucially backed by experimental verification of allele function.

*Loss of function, leading to resistance, is perhaps the most likely situation in which recurrent mutation leading to resistance is observed. The authors refer to Redman 2015 as an example of multiple recurrence of resistance mutations. The study by Rufener (Rufener, L., Maser, P., Roditi, I., Kaminsky, R. (2009) *Haemonchus contortus* acetylcholine receptors of the DEG-3 subfamily and their role in sensitivity to monepantel. *PLoS Pathogens* 5:e1000380. doi: 10.1371/journal.ppat.1000380), although using artificial selection for resistance, identified multiple independent mutations leading to monepantel resistance and these also included mutations at splice consensus sequences and aberrant transcripts.*

This reference is now included in our introduction (line 79).

The authors identify physically distinct mutations that must represent independent origins. Since exon sequencing was used, along with substitution rate analysis for positive selection, I am surprised there was no phylogenetic analysis comparing the lab and wild resistant forms. The polymorphism surrounding the resistance mutations could be used to verify the origins of these mutations. If the laboratory and field mutations are identical in causing resistance, but had independent origins, this would be important information to add to their story.

Unfortunately we do not have sufficient variation in the region sequenced to conduct a phylogenetic analysis. However, linkage disequilibrium between two variant provides some information relevant to inferring origins of mutations. We observed the p.E142del in both the laboratory derived HR population (which originated from Puerto Rico) and in the field in Brazil. However, the HR p.E142del is found on an allele in combination with a p.L256W mutation, whereas this is not seen in the Brazilian field samples. A similar situation is observed with p.C35R mutation found with the p.P67L and p.G206V mutations in the MAP strain but with only p.P67L in our Brazilian field samples. One possible interpretation is that the p.E142del and p.C35R have multiple independent origins. We have now included a paragraph in the discussion to mention this possibility (line 377-384).

The way the authors examine the non-synonymous / synonymous substitution rates is a little unusual. The standard methodology would be to use either PAML (phylogenetic analysis using maximum parsimony) or HyPhy (as implemented on the Datamonkey.org website).

Unfortunately, standard use of PAML or HyPhy is not possible when there are NO synonymous mutations, because this results in denominator of 0, and makes calculation of a ratio impossible. We therefore used a Fisher test to compare number of non-synonymous and synonymous changes to

number of non-synonymous and synonymous sites instead of a dN/dS ratio. We now describe why conventional dN/dS ratio cannot be calculated (line 311).

The authors discuss a deviation from Hardy-Weinberg equilibrium and an excess of homozygotes. One possible explanation for this would be that an allele exists in the sample for which the PCR amplification fails. If so, this would mimic an excess of homozygotes. It might be important to summarize details of the cases where DNA amplification was unsuccessful and the attempts to make sure it was a sample failure, rather than failure of the specific diagnostic PCR for some systematic reason.

In our study, we never observed PCR failure of *SmSULT-OR*. The only failed amplifications were observed during the WGA step (probably due to the presence of polymerase inhibitors) but are unrelated to our *SmSULT-OR* amplifications. Hence we do not believe that null alleles are the explanation for the observed deficit. We have included the following sentence in the results to clarify this point “Null alleles are not likely to explain the deficit: while we had small numbers of samples for which WGA failed, *SmSULT-OR* was successfully amplified from all samples for which WGA was successful.” (lines 308-310)

Minor editions were done to correct and improve the manuscript. Here is the list of editions:

- Lines 4 and 20: The “M.” initial was removed from the author name of Winka Le Clec’h at her request.
- Lines 199 to 200: We define the non-reference alleles.
- Lines 215, 217, 238 to 239, 333: Typo corrections.
- Line 314: rewording for clarification.
- Lines 335 to 337 and 352: We modified the sentences to correct an omission.
- Lines 493 to 485: We updated the acknowledgement section.
- Lines 486 to 781: Bibliography was updated.
- Line 803: We replaced alternative with non-reference for clarity. We modified the table accordingly.
- Table 1: We added a column corresponding to the expected amplicon size for each primer pair.
- Table 2: We corrected some errors in numbers in addition of the corrections suggested by the reviewers.
- Lines 813, 816 and 817: We replaced “Supplementary” table, figure or movie by “Table S”, “Fig. S” and “Movie S” respectively. Modifications were done accordingly through the text.
- Table S1: Patient and sample ID were updated.

Independent origins of loss-of-function mutations conferring oxamniquine resistance in a Brazilian schistosome population¹

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¹ Note: Supplementary data associated with this article

31 **Abstract**

32 Molecular surveillance provides a powerful approach to monitoring resistance status of parasite
33 populations in the field and for understanding resistance evolution. Oxamniquine (OXA) was used to
34 treat Brazilian schistosomiasis patients (mid-1970s to mid-2000s) and several cases of parasite infections
35 resistant to treatment were recorded. The gene underlying resistance (*SmSULT-OR*) encodes a
36 sulfotransferase required for intracellular drug activation. Resistance has a recessive basis and occurs
37 when both *SmSULT-OR* alleles encode for defective proteins. Here we examine *SmSULT-OR* sequence
38 variation in a natural schistosome population in Brazil ~40 years after the first use of this drug. We
39 sequenced *SmSULT-OR* from 189 individual miracidia (1-11 per patient) recovered from 49 patients, and
40 tested proteins expressed from putative resistance alleles for their ability to activate OXA. We found
41 nine mutations (4 non-synonymous SNPs, 3 non-coding SNPs, and two indels). Both mutations
42 (p.E142del and p.C35R) identified previously were recovered in this field population. We also found two
43 additional mutations (a splice site variant and 1 bp coding insertion) predicted to encode non-functional
44 truncated proteins. Two additional substitutions (p.G206V, p.N215Y) tested had no impact on OXA
45 activation. Three results are of particular interest: (i) we recovered the p.E142del mutation from the
46 field: this same deletion is responsible for resistance in an OXA-selected laboratory parasite population
47 (ii) frequencies of resistance alleles are extremely low (0.27-0.8%), perhaps due to fitness costs
48 associated with carriage of these alleles (iii) that four independent resistant alleles were found is
49 consistent with the idea that multiple mutations can generate loss-of-function alleles.

50

51 **Keywords:** *Schistosoma mansoni*, oxamniquine resistance, sulfotransferase, loss-of-function,
52 biochemical assay, soft selective event

53

54 **Highlights:**

- 55 • We surveyed allelic variation in a schistosome drug resistance gene
- 56 • Four independent origins of alleles encoding loss-of-function proteins
- 57 • Resistance mutations are at very low frequency (< 0.0080)
- 58 • Loss-of-function mutations tested using an *in vitro* oxamniquine activation assay

59

60 **Abbreviations:**

- 61 • Indel: insertion/deletion
- 62 • OXA: oxamniquine
- 63 • PCR: polymerase chain reaction
- 64 • PZQ: praziquantel

- 65 • SmSULT-OR: *Schistosoma mansoni* sulfotransferase oxamniquine resistance
- 66 • SNP: single nucleotide polymorphism
- 67 • UTR: untranslated region
- 68 • WGA: whole genome amplification

69

70

71 1. Introduction

72 Surveys of drug resistance alleles using molecular markers provide a powerful approach to
73 identify pathogen populations in which resistance is emerging, to map resistance spread, and
74 for evidence-based resistance management. Molecular approaches are now widely used for
75 tracking resistance in malaria (Ashley et al., 2014; Pearce et al., 2009), HIV (Panichsillapakit et
76 al., 2015) and bacterial diseases (Bhembe et al., 2014), and for managing insecticide resistance
77 (Djègbè et al., 2014; Essandoh et al., 2013). Such methods are also now actively used for
78 monitoring resistance in helminths of veterinary importance such as *Haemonchus contortus*
79 (Chaudhry et al., 2015; Redman et al., 2015; Rufener et al., 2009)
80 , and have enormous potential for monitoring resistance in helminth parasites infecting
81 humans, because existing phenotypic screening methods based on reductions in production of
82 eggs or larval stages are insensitive for detection of low frequency resistance alleles, and
83 cannot detect recessive resistance alleles present in heterozygous worms. However, molecular
84 surveillance is only possible ~~only~~ when resistance genes have been identified, which is rarely
85 the case for human helminth infections such as schistosomiasis.

86 Schistosomiasis, caused by three major species of blood flukes of *Schistosoma* genus (Colley
87 et al., 2014; Dye et al., 2013), is the second most important tropical parasitic disease after
88 malaria (Steinmann et al., 2006), affecting an estimated 260 million people across Africa, Asia,
89 and South America, and killing over 200,000 people per year. Two drugs are available for
90 treating schistosomiasis. Praziquantel (PZQ) is currently used as a monotherapy in expanding
91 mass drug administration programs in Africa (Dye et al., 2013) making resistance evolution a
92 major concern. A second drug, oxamniquine (OXA), is the focus of this paper. OXA acts
93 specifically against *S. mansoni*, which is found in Africa, together with *S. haematobium*, and in
94 South America, where it is the only species present. OXA was manufactured in Brazil by Pfizer
95 (Cheetham, 1994) and widely used to treat *S. mansoni* infections ~~(~~from the mid-1970s to the
96 mid-2000s (Coura and Amaral, 2004), but has now been replaced by PZQ as the first line drug.

97 Schistosomes resistant to OXA were first identified in Brazil in 1973 (Naftale Katz et al.,
98 1973), around the same time as the first clinical study of OXA treatment (N. Katz et al., 1973).
99 This rapid emergence of resistance was most likely due to the previous treatment of the same
100 populations with the related drug hycanthone (Coura and Conceição, 2010; Katz et al., 1968), as
101 both OXA and hycanthone have the same target (Jansma et al., 1977; Pica-Mattoccia et al.,
102 1993) and the same mechanism of resistance (Pica-Mattoccia et al., 1992a)(~~;-~~). ~~Latter.~~ Later
103 genetic studies showed that resistance is a recessive single locus trait (Cioli and Pica-Mattoccia,
104 1984; Pica-Mattoccia et al., 1993), most likely involving the absence of a sulfotransferase
105 activity necessary for drug activation in resistant schistosomes (Pica-Mattoccia et al., 2006). The
106 gene encoding the *S. mansoni* sulfotransferase involved in OXA resistance (*SmSULT-OR*) was

107 recently identified by classical quantitative trait mapping in concert with crystallographic and
108 functional analyses (Valentim et al., 2013). This work identified an amino acid deletion
109 (p.E142del) in the laboratory selected resistant parasite (HR), while an independent loss-of-
110 function mutation (p.C35R) was identified in a field-collected resistant parasite line (MAP).

111 The identification of the gene involved in OXA resistance now allows us to examine
112 distribution of resistance alleles of *SmSULT-OR* in natural populations. Despite the fact that the
113 Brazilian government has switched from OXA to PZQ during the last decade (Utzinger et al.,
114 2003), the recessive nature of OXA resistance allows the persistence of these alleles, because
115 alleles encoding non-functional enzyme are not counter-selected as long as they segregate with
116 alleles encoding a functional ~~alleles~~enzyme. We collected miracidia larval stages from a village
117 in Minas Gerais, Brazil, and sequenced the *SmSULT-OR* gene in these samples. We sought to
118 answer several questions: How common are resistance alleles? How many times have OXA
119 resistance alleles arisen? Are the OXA resistance alleles selected in the laboratory and identified
120 using linkage mapping actually present in nature? More broadly, our goal is to better
121 understand the evolution of drug resistance in schistosomes and to demonstrate the utility of
122 molecular screening approaches in anticipation that the gene(s) underlying PZQ resistance will
123 soon be identified.

124

125

126 **2. Material and methods**

127 *2.1 Ethics statement*

128 Stool samples were collected in accordance to the Research Ethics Committee of UNIFESP
129 procedures (process number CAAE: 15567313.8.0000.5091)

130

131 *2.2 Sampling of Schistosoma mansoni miracidia*

132 We collected stools from school children from Ponto dos Volantes (Minas Gerais, Brazil, GPS
133 coordinates: S 16°45'3.301", W 41°30'13.755") and shipped these at 4°C by ground
134 transportation overnight to Universidade Federal de Minas Gerais in Belo Horizonte. We
135 processed samples as follows: several grams of stools were filtered through three layers of
136 sieves (mesh size: 250 to 45 µm) to obtain schistosome eggs. Eggs were transferred from the third
137 sieve grid to a Petri dish and exposed under artificial light for at least one hour. All filtering
138 steps and egg transfer were performed with locally available bottled mineral water.

139 Washed eggs were observed under a stereomicroscope. For each patient, 1 to 11 living
140 miracidia were sampled individually in ~2 µL of water and spotted onto CloneSaver FTA cards
141 (GE Healthcare Life Sciences). Spotted samples can be easily located on the card thanks to the
142 card pink dye turning white after water contact. Full cards were allowed to dry one hour at
143 room temperature on the bench before being stored in a plastic bag and finally shipped to San
144 Antonio, Texas, USA.

145

146 *2.3 Preparation of FTA samples for whole genome amplification*

147 For each sample, we removed a 2 mm diameter disc from the FTA card using a 2 mm Harris
148 Micro-punch (GE Healthcare Life Sciences). The 2 mm disc corresponds to the entire spot
149 containing the whole miracidium. Each punch was placed individually in a 1.5 mL sterile tube.
150 Punches were washed 3 times with FTA Purification Reagent (GE Healthcare Life Sciences) then
151 rinsed 2 times with TE⁻¹ buffer (10 mM Tris, 0.1 mM EDTA, pH 8). Washing and rinsing steps
152 were performed by adding 200 µL of solution in each tube followed by 5 minutes of incubation
153 on a nutating mixer (24 RPM) at room temperature and then by discarding the solution while
154 minimizing contact between the pipette tip and the punch. Punches were finally dried in tubes
155 for 10 minutes at 56°C on a dry bath incubator.

156

157 *2.4 Whole genome amplification*

158 We performed whole genome amplification (WGA) on each punch using the illustra GenomiPhi
159 V2 DNA Amplification kit (GE Healthcare Life Sciences). Punches were transferred in 0.2 mL
160 sterile tubes using a sterile tip. We performed reactions following the manufacturer's
161 instructions, immersing each punch in 9 μL of sample buffer and keeping tubes at all times on
162 ice after the denaturation step. After amplification, we quantified DNA using the Qubit dsDNA
163 BR assay (Invitrogen).

164

165 *2.5 Sequencing of SmSULT-OR exons*

166 The two exons of the *SmSULT-OR* gene were amplified and sequenced independently. Each PCR
167 reaction was performed using the TaKaRa Taq kit (Clontech) and composed of 9.325 μL of
168 sterile water, 1.5 μL of 10 \times buffer, 1.2 μL of dNTP (2.5 mM each), 0.9 μL of MgCl_2 (25 mM),
169 0.5 μL of each primer (10 μM ; Table 1), 0.075 μL of Taq polymerase (5 $\text{U}\cdot\mu\text{L}^{-1}$) and 1 μL of DNA
170 template. Amplifications were done using GeneAmp PCR system 9700 thermocycler (Applied
171 Biosystems) with the following program: 95°C for 5 minutes, [95°C for 30 seconds, 60°C for 30
172 seconds, 72°C for 45 seconds] \times 35 cycles, 72°C for 10 minutes.

173 Exon 1 required a nested PCR in order to obtain a specific product. Products from the first
174 PCR were cleaned up by adding 4 μL of ExoSAP-IT (Affymetric USB products). Tubes were then
175 incubated at 37°C for 30 minutes and at 80°C for 15 minutes with a thermocycler. Cleaned PCR
176 products were then used as templates for the second PCR following the above protocol.

177 Sequencing reactions were performed using BigDye[®] Terminator v3.1 cycle sequencing kit
178 (Applied Biosystems) on final PCR products. PCR products were cleaned up using ExoSAP-IT as
179 described above. Sequencing reactions were performed using 2.59 μL of sterile water, 1 μL of
180 5 \times running buffer, 0.25 μL of BigDye Terminator ready reaction mix (Applied Biosystems),
181 0.16 μL of forward or reverse primer used in the final PCR step, and 1 μL of final PCR product.
182 Sequencing fragments were generated using GeneAmp PCR system 9700 thermocycler (Applied
183 Biosystems) with the following program: 96°C for 1 minute, [96°C for 10 seconds, 50°C for 5
184 seconds, 60°C for 4 minutes] \times 25 cycles. Sequencing reactions were cleaned up using BigDye
185 XTerminator[®] purification kit (Applied Biosystems). In each reaction, 20.45 μL of SAM[™] solution
186 and 4.55 μL of XTerminator[™] solution were added. Reactions were then vortexed for 30
187 minutes and run on a 3730xl DNA Analyzer (Applied Biosystems).

188 Sequencing files were first screened using FinchTV (v1.4.0; Geospiza Inc.) to identified failed
189 sequencing reactions. In case of failure, sequencing reactions were performed a second time.

190

191 2.6 Variant identification and functional impact evaluation

192 We scored variants using PolyPhred software (v6.18) (Nickerson et al., 1997) which relies on
193 Phred (v0.020425.c), Phrap (v0.990319), and Consed (v29.0) software, analyzing each exon
194 independently. We identified single nucleotide polymorphisms using a minimum phred quality
195 score (-q) of 40, a minimum genotype score (-score) of 90, and a reference sequence that
196 includes *SmSULT-OR* gene and surrounding regions (position 1519500 to 1525200 of
197 chromosome 6 of *S. mansoni* reference genome v5.0,
198 [ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/genome/Assembly-](ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/genome/Assembly-v5/sma_v5.0.chr.fa.gz)
199 [v5/sma_v5.0.chr.fa.gz](ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/genome/Assembly-v5/sma_v5.0.chr.fa.gz)). Variant sites were labeled as non-reference alleles if they were
200 different from the reference sequence. We identified Insertion/deletion (indel) polymorphisms
201 using a minimum phred quality score (-q) of 40, a minimum genotype score (-score) of 80.
202 Polymorphisms were visually validated using Consed. All the sequences were submitted to
203 GenBank (GenBank accession no ~~XXX-XXX~~[KU951903-KU952091](#)).

204 Nucleic sequences showing mutations were translated *in silico* into protein sequences using
205 Translate tool from ExpASy portal (Artimo et al., 2012).

206 We evaluated the potential functional impact of identified polymorphisms on RNA features
207 and on protein structure *in silico*. Modification of RNA motifs and sites were analyzed using
208 RegRNA2.0 website (Chang et al., 2013) for all available features with *Drosophila melanogaster*
209 as reference species when required. Modifications in protein structure were assessed using the
210 mutagenesis function of PyMol software (v1.7.2.0, Schrödinger, LLC) using the structure of
211 *SmSULT-OR* determined previously (PDB code 4MUB, Valentim et al., 2013).

212

213 2.7 Population genetics analysis

214 We evaluated population structure by testing for Hardy-Weinberg equilibrium, measuring
215 fixation indices (F_{st} and the F_{is}) of parasites using Genepop (Rousset, 2008) with default options
216 and considering schistosomes from a given patient as belonging to a same population. To
217 identify positive selection, we calculated the number of synonymous (s) and non-synonymous
218 (n) sites (Nei and Gojobori, 1986) and compared these values to synonymous (S) and non-
219 synonymous (N) changes. We also performed a McDonald-Kreitman test using MKT server
220 (<http://mkt.uab.es>; Egea et al., 2008). The *SmSULT-OR* homolog sequence in *S. rodhaini* was
221 used as an outgroup and was obtained using tblastx from the NCBI server (Coordinators, 2015)
222 on the *S. rodhaini* genome assembly (GenBank accession no. **GCA_000951475.1**) using *SmSULT-*
223 *OR* sequence (GenBank accession no. **KF733459.1**) as query.

224

225 2.8 Recombinant SmSULT-OR protein production

226 The *SmSULT-OR* sequence from the reference genome (Smp_089320; GenBank accession no.
227 **HE601629.1**) was used to create a codon optimized synthetic gene (GenScript) which was
228 subcloned into the pAG8H vector derived from pKM260 (Melcher, 2000). We introduced
229 mutations using a Phusion site-directed mutagenesis protocol (ThermoFisher Scientific).
230 Transformed *Escherichia coli* strain BL21 pLysS (Promega) were grown at 37°C until the A_{600}
231 reached 0.7, we decreased the temperature to 18°C and expression induced by the addition of
232 isopropyl- β -D-thiogalactoside (IPTG) at a final concentration of 1 mM. The cells were washed
233 and resuspended in 50 mL 50 mM Tris pH 8.0, 500 mM NaCl (column buffer) containing 250 μ L
234 Sigma protease inhibitor cocktail per liter of culture and lysed by sonication on ice. The clarified
235 supernatant was loaded onto a Ni²⁺-NTA affinity chromatography column (GE Healthcare),
236 washed with 5 volumes of column buffer, and eluted using a 10-500 mM imidazole gradient.
237 We pooled fractions identified as the Smp_089320 via SDS-PAGE and added His-tagged TEV
238 protease at a Smp_089320•protease ratio of 15•1. The resulting solution was dialyzed ~~the~~
239 ~~resulting solution~~ overnight at 4°C against 50 mM Tris pH 8.0, 100 mM NaCl, 2 mM
240 dithiothreitol (DTT). We passed the dialysate over the Ni²⁺-NTA column again to remove the His
241 tag and the His-tagged TEV protease, while the cleaved target protein flowed through. The
242 sample was loaded onto a GE-pre-packed Q anion exchange column and eluted with a 0.1-
243 1.0 M NaCl gradient. We pooled fractions containing Smp_089320 as identified by SDS-PAGE
244 and dialyzed overnight at 4°C against 25 mM Tris pH 8.5, 50 mM NaCl, 2 mM reducing agent
245 tris-(carboxyethyl)-phosphine (TCEP) to prevent formation of intermolecular disulfide bonds.
246 The product was ~98% pure as estimated by SDS-PAGE. We concentrated the purified
247 Smp_089320 protein to 10 mg.mL⁻¹ using the calculated extinction coefficient $\epsilon = 39,880 \text{ mol}^{-1}$
248 $\cdot \text{cm}^{-1}$.

249 2.9 Oxamniquine activation assay

251 This assay utilizes the fact that OXA is a prodrug that is enzymatically converted into a highly
252 reactive molecule that covalently binds intracellular components such as DNA (Pica-Mattocchia
253 et al., 1989). Because the original assay employs worm extracts which require time for
254 preparation and introduce unpredictable variations in SmSULT-OR concentration (Pica-
255 Mattocchia et al., 1992a; Valentim et al., 2013), we developed an improved *in vitro* assay that
256 uses purified recombinant SmSULT-OR enzyme. This *in vitro* assay measures the enzymatic
257 sulfonation of tritiated OXA molecules which then bind gDNA: The resultant radioactive DNA-
258 OXA complexes can then be quantified by a scintillation counter. Enzymes produced by non-
259 functional *SmSULT-OR* alleles cannot sulfonate OXA so no DNA-OXA complexes are formed.

260 Recombinant proteins were expressed and purified commercially by GenScript (p.C35R,
261 p.P67L, p.E142del; Valentim et al., 2013) or by the Hart laboratory (p.G206V and p.N215Y). To
262 determine which recombinant protein has the ability to activate OXA, 1 nM from each
263 recombinant protein was added to 90 μL of a protease inhibitor cocktail (PIC) consisting of
264 0.1 M HEPES pH 7.4, 0.1mM leupeptin, 2 μM E-64, 2 μM pepstatin A, 0.1 U of aprotinin, and
265 10 $\text{ng}\cdot\mu\text{L}^{-1}$ sheared *S. mansoni* gDNA as a final target. For each reaction 100 μCi of ^3H -OXA (Pica-
266 Mattoccia et al., 1989) was solubilized in 2 μL DMSO and added to 10 μL of a mixture containing
267 the enzyme cofactors ATP and MgCl_2 at 50 mM each, and PAPS at 1 mM. The radiolabeled OXA
268 and co-factor mix was then added to the PIC mix containing the recombinant protein. The
269 resulting reaction was incubated for 2.5 hours at 37°C and stopped by adding 3 volumes of 0.1%
270 SDS in 0.1 M sodium bicarbonate. The reaction was then extracted 3 times with 2 volumes of
271 dichloromethane and the aqueous phase was counted in a liquid scintillation spectrometer
272 (Beckman LS 6500 Scintillation Counter, USA) during 10 minutes. We also measured a blank
273 solution (water) and the background scintillation. Blank and background values were subtracted
274 to sample values. We performed three independent reactions for each recombinant protein.

275

276 *2.10 Statistical analysis*

277 Statistical analyses were done using R (v3.1.3) (R Core Team, 2015). For synonymous and non-
278 synonymous changes, data were compared using a Fisher's exact test. For the OXA activation
279 assay, the data were compared with a Welch t-test after testing for normality (Shapiro test, $p >$
280 0.05).

281 3. Results

282 3.1 Brazilian samples

283 We collected 232 FTA preserved miracidium samples from 51 patients (range: 1-12, mean±sd:
284 4.55±3.65). We successfully amplified DNA from 204 samples (87.93%) from 50 patients (range:
285 1-11, mean±sd: 4.08±3.26). Among the amplified samples, 189 from 49 patients (range: 1-11,
286 mean±sd: 3.86±3.18) contained schistosome DNA (92.65% of amplified samples, 81.47% of
287 total samples). Among the 189 samples, we sequenced exon 1 from 183 samples and exon 2
288 from 188 samples. All samples had at least one exon sequenced (~~Supp. table 4~~Table S1).

289

290 3.2 SmSULT-OR variants in a Brazilian schistosome population

291 We scored nine mutations: four non-synonymous single nucleotide polymorphisms (SNPs), one
292 insertion and one deletion in the coding region, and three non-coding SNPs (Table 2, ~~Supp.~~
293 ~~table 4~~Table S1). The number of mutations in each exon (three in ~~the~~exon 1 and three in~~the~~
294 exon 2) is not different regarding the length of the exon (327 bp and 447 bp, respectively)
295 (Fisher's exact test, $p = 0.7$).

296 Among the seven SNPs, five (71.43%) were transitions and two (28.57%) were
297 transversions. Four of the SNPs were located in the exonic region, two in exon 1 and two in
298 exon 2. Three were present at very low frequency (0.0027-0.0053) while one was present at
299 very high frequency (0.95). One SNP was identified at the first position of the intron at a low
300 frequency (0.0056). The two remaining SNPs were found in the 3' UTR, one at high frequency
301 (0.96), and one at low frequency (0.03).

302 One insertion was identified in ~~the~~exon 1 while one deletion was identified in~~the~~ exon 2,
303 both at very low frequency (0.0027 and 0.0080, respectively).

304 Low frequency mutations were found in heterozygous or in homozygous states among the
305 samples. Observing rare SNPs present as homozygotes was surprising, suggesting population
306 structure. The test for Hardy-Weinberg equilibrium showed a global deficit in heterozygosity
307 ($p < 0.0001$). This is likely due to deficit of heterozygous genotypes within host ($F_{is} = 0.3251$)
308 rather than due to population differentiation between infections ($F_{st} = 0.0153$). Null alleles are
309 not likely to explain the deficit: while we had small numbers of samples for which WGA failed,
310 SmSULT-OR was successfully amplified from all samples for which WGA was successful.

311 Because of the absence of the synonymous mutations which precludes dN/dS calculation,
312 we compared synonymous (S) and non-synonymous (N) changes to synonymous (s) and non-
313 synonymous (n) sites to evaluate evidence for selection on *SmSULT-OR*. This comparison

314 | reveals no differences (Fisher's exact test, $p = 1$) indicating that no ~~particular pressure~~evidence
315 | for selection. We also tested directional evolution by performing a McDonald-Kreitman test
316 | using the homolog sequence of *SmsULT-OR* from *S. rodhaini* as outgroup. We found no
317 | evidence for directional evolution ($\chi^2 = 1.466$, $p = 0.225$).

318

319 | 3.3 Functional impacts of mutations

320 | 3.3.1 Non-coding variants

321 | We evaluated the functional impacts of the polymorphisms identified based on RNA features
322 | such as binding sites or splicing or regulatory motifs (Table 2). The mutation g.649G>A was
323 | predicted to modify the unique splice donor site at the end of the exon 1. The disruption of the
324 | splicing site leads to translation of the beginning of the intron which ends four codons later due
325 | to the introduction of a stop codon. This results in a truncated protein with ~~absence of no~~
326 | site (Fig. 1, ~~Supp. movie~~Movie S1). Mutations present on the 3' UTR were not predicted to be in
327 | any regulatory sites.

328 | 3.3.2 Coding variants

329 | All SNPs identified in the coding sequence of the gene were non-synonymous, and derived
330 | mutations relative to the outgroup *S. rodhaini* (Table 2). c.103T>C induced a substitution of
331 | cysteine to arginine (p.C35R) leading to a misfolded protein as shown previously (Valentim et
332 | al., 2013) (Fig. 1). c.200C>T induced a substitution of proline to leucine (p.P67L) and was
333 | previously found in the OXA-sensitive strain (Valentim et al., 2013) (Fig. 1); ~~);~~ this mutation
334 | therefore does not reduce enzyme activity. c.617G>T induced a substitution of glycine to valine
335 | (p.G206V) which occurs close the binding site of the PAPS co-factor and was previously
336 | observed in field-collected OXA-resistant strain (Valentim et al., 2013); ~~);~~ but was never tested .
337 | We postulated that this mutation may have a potential detrimental effect on co-factor binding
338 | and finally enzyme activity (Fig. 1, ~~Supp. movie~~Movie S1). The mutation c.643A>T induced a
339 | substitution of asparagine to tyrosine (p.N215Y) which occurs on a helix connected to a loop
340 | involved in co-factor binding. This mutation is predicted to have little impact on protein
341 | structure: We therefore postulated that this will not change protein function (Fig. 1, ~~Supp.~~
342 | ~~movie~~Movie S1).

343 | 3.3.3 Indels

344 | ~~The two~~Both indels have ~~both~~ detrimental effects on the enzyme (Table 2). The insertion
345 | c.214_125insA induces a frame shift leading to an early stop codon six codons after the
346 | mutation. This frame shift is predicted to produce a truncated enzyme with no active site. The

347 deletion c.424_426delGAA is known to disrupt oxamniquine binding from previous functional
348 analyses (Valentim et al., 2013).

349

350 3.4 Oxamniquine activation assay

351 We produced two recombinant SmSULT-OR enzymes carrying the mutations p.G206V and
352 p.N215Y in order to experimentally test whether these two ~~new~~ mutations impact OXA-
353 activation resulting in OXA resistance. We also tested known resistance alleles (p.C35R and
354 p.E142del) as controls. The newly identified alleles are able to activate OXA as well as the
355 reference allele (wild type) which does not carry any of these mutations (Welch t-test, $t < -1.55$,
356 $p > 0.18$) while the known resistance alleles showed no activation as expected (Welch t-test, $t >$
357 5.58 , $p < 0.021$). Therefore the two mutations tested (p.G206V and p.N215Y) did not disrupt co-
358 factor binding or otherwise interfere with enzymatic activity.

359

360 **4. Discussion**

361 *4.1. Identical mutations in laboratory and field selected parasites*

362 *SmsULT-OR* sequences from 189 miracidia collected from 49 Brazilian patients revealed nine
363 mutations, including both mutations previously implicated in OXA resistance (Valentim et al.,
364 2013). We found the p.C35R mutation, previously identified in the MAP strain. MAP was
365 sampled from a patient living in a city of São Paulo state (Fig. S1) (Pica-Mattoccia et al.,
366 1992b) ~~Supp. fig. 1) (, a different geographic, the neighboring~~ state from which our samples
367 were collected. This suggests that the allele ~~have~~has been segregating in Brazilian parasite
368 populations for more than 25 years. The second variant identified in the field, p.E142del, was
369 previously found in the HR laboratory strain, which was initially sampled from a Puerto-Rican
370 patient and subsequently selected with hycanthon in the laboratory (Fig. S1) (Cioli and Pica-
371 Mattoccia, 1984) ~~Supp. fig. 1) (,). This mutation could have arisen spontaneously in the~~
372 laboratory. However, given that laboratory schistosome lines are maintained as outbred
373 populations, the simplest explanation is that this mutation was segregating within the parasite
374 population originally established in the laboratory. That we located this same deletion in two
375 miracidia from Brazil and a parasite line collected from Puerto-Rico suggests that this allele may
376 be widespread in schistosome populations from the New World.

377 It is not clear from our data whether the p.E142del seen in HR and Brazilian field samples
378 arose independently or has a single origin. The HR p.E142del is found together with another
379 mutation (p.L256W) (Valentim et al., 2013), which is absent from the p.E142del allele found in
380 the Brazilian miracidia, providing some evidence for independent origins. Similarly, MAP differs
381 from the Brazilian miracidia carrying the p.C35R mutation: while both also carry p.P67L, MAP
382 carries an additional p.G206V mutation. Additional flanking SNP data will be required to
383 critically test whether the p.E142del and p.C35R OXA resistance mutations have arisen a single
384 time, or have multiple independent origins.

385 Laboratory selection is commonly used to explore the genetics of pathogen resistance, but a
386 concern with this approach is that the mutations selected in the laboratory may poorly
387 represent those occurring in nature. That we identify the same mutations in laboratory and
388 field selected parasites is extremely encouraging, because ongoing work to identify mutations
389 involved in PZQ resistance also utilizes laboratory selected parasites (Couto et al., 2011). We
390 note that laboratory selection experiments with *Plasmodium* also tend to identify the same
391 genes and often the same mutations that are observed in the field (Anderson et al., 2011),
392 further validating this approach.

393

394 *4.2. Multiple origins of OXA resistance*

395 Besides the two known alleles, two of these new mutations, c.214_215insA and g.328G>A, are
396 predicted to confer oxamniquine resistance: both introduce premature insertion of a stop
397 codon producing a protein without an active site. These mutations therefore add two additional
398 loss-of-function mutations that were probably selected during the OXA treatment. That four
399 independent mutations are found in a single sampled parasite population is remarkable.
400 Multiple origins have previously been observed in the evolution of resistance to benzimidazole
401 drugs in the gastrointestinal nematode *Haemonchus* (Redman et al., 2015)~~(1)~~. ~~The numbers. The~~
402 ~~number~~ of origins of resistance alleles is expected to depend on the size of the parasite
403 population and the rate at which mutation generates resistant alleles (Messer and Petrov,
404 2013). In the case of *H. contortus*, the enormous size of parasite populations is likely to be the
405 main driver, as only several specific mutations within β -tubulin can confer resistance. In the
406 case of OXA resistance, a high mutation rate may be expected as the main driver, because
407 multiple different mutations within *SmSULT-OR* can generate non-functional proteins. It is also
408 possible that OXA-resistant alleles were present within Brazilian *S. mansoni* populations prior to
409 hycanthone or OXA treatment. Such standing variation may even have been present prior to
410 introduction of *S. mansoni* into South America. Analysis of *SmSULT-OR* in African populations,
411 where OXA was not used extensively, will help answer this question.

412

413 4.3. Importance of functional assays for testing gene function

414 Work on OXA resistance is simplified because we have an effective *in vitro* functional assay for
415 screening allelic variants. The assay we used is an improvement on those previously described
416 (Pica-Mattocchia et al., 1992a; Valentim et al., 2013), because worm homogenates are replaced
417 by recombinant SmSULT-OR proteins. We identified two additional exon 2 substitutions
418 (p.G206V, p.N215Y): Structural analyses suggested that one of these mutations (p.G206V) may
419 disrupt OXA activation, by interfering with binding of the co-factor (PAPS), while the other
420 (p.N215Y) is likely to have minimal impact on function. We were able to directly test these
421 predictions by performing OXA activation assays. These assays demonstrated that neither
422 mutation prevents OXA activation, allowing us to reject our prediction for p.G206V and confirm
423 our prediction for p.N215Y. Hence, while structural studies are useful for formulating
424 hypotheses about the consequences of mutations, functional assays are essential for critical
425 testing of these hypotheses.

426 The two last mutations were found in the 3' UTR of the cDNA. *In silico* analysis, using
427 predictions based on *Drosophila*, did not reveal regulatory sites in these regions, suggesting
428 that these mutations do not impact function. However, *Drosophila* may be a poor model to use:
429 when more information on *SmSULT-OR* regulatory regions become available, new analyses of
430 these two mutations may reveal potential effects on mRNA stability or translation rate.

431

432 4.4. Low frequency of resistance mutations

433 The allele frequency of all 4 OXA resistance alleles combined is 3.8%, and only two parasites of
434 183 sampled (1%) are homozygous and therefore expected to be phenotypically resistant. The
435 low frequency of the OXA resistance alleles can be explained by two non-exclusive hypotheses.
436 First, resistance alleles may have remained at low levels even when OXA was the first line drug
437 in Brazil. Second, fitness costs associated to these alleles may have driven reductions in allele
438 frequency after the abandon of OXA treatment. However OXA resistance alleles still persist
439 within the populations in heterozygous state, the cost being present only when worms are
440 homozygous for the defective alleles.

441 Whether there is a cost associated with OXA resistance is questionable because miracidia
442 homozygous for resistance alleles were found in the field from the present study. A previous
443 study showed reduction in infectivity and egg production in OXA resistant parasites relative to
444 the OXA ~~resistant~~susceptible populations from which they were isolated (Cioli et al., 1992).
445 However, this study suffers from a methodological limitation, because resistant and susceptible
446 laboratory populations had different ~~genetics~~genetic backgrounds, complicating interpretation.
447 Comparison of isogenic wildtype and genetically manipulated resistant parasites, or analysis of
448 fitness in the progeny of a genetic cross between OXA resistant and sensitive parasites would
449 allow measurement of associated fitness costs, while minimizing confounding background
450 effects.

451

452 4.5. Implications for schistosome biology and control

453 New drugs are urgently needed for schistosome control because treatment currently relies
454 on widespread monotherapy with PZQ. OXA is effective only against *S. mansoni*, but new OXA-
455 derivatives are under active development, with the aim of making compounds that are active
456 against all three major schistosome species infecting human (Taylor et al., 2015). If such
457 derivatives are to be deployed clinically, understanding the capacity for resistance evolution in
458 schistosome populations is of critical importance. For example, surveys of sequence variation in
459 *SmSULT-OR* or the *S. haematobium* homologue would be an important prerequisite for field
460 deployment of an OXA-derivative active against both these species in Africa.

461 Our results have both positive and negative implications for field deployment of OXA
462 derivatives. That multiple resistance alleles are present in a single parasite population suggests
463 that resistance alleles may evolve and spread rapidly. On the positive side, existing resistance
464 alleles are currently at extremely low frequency. It will be essential that OXA derivatives are

465 deployed with appropriate partner drugs to minimize rate of resistance evolution. We note that
466 resistance to the antimalarial atovaquone was observed in the first clinical trial of this drug
467 | (Looareesuwan et al., 1996) ~~(-) and evolves and evolved~~ *de novo* in different treated patients
468 (Musset et al., 2007). Yet this drug is widely and effectively used with proguanil as a
469 combination drug under the trade name Malarone.

470

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782 **Figures**

783

784 **Fig. 1 – Mapping of the mutations on the gene sequence and structure of SmSULT-OR.** Exon 1 and exon
785 2 are represented in orange and beige, respectively. Single nucleotide polymorphisms and
786 insertion/deletion events are represented in cyan and magenta, respectively. Loss-of-function mutations
787 are highlighted in black. **(A)** Linear representation of the *SmSULT-OR* gene showing the relative position
788 of the mutations and their translation in amino acid sequences. **(B)** Positions of mutations on the
789 SmSULT-OR protein. OXA is represented in yellow, PAPS co-factor is represented in green, and spatial
790 distortion are represented by red discs. For a more detail view of the mutations on the structure and
791 their functional impact, see supplementary video.

792

793 **Fig. 2 – Enzymatic activity of recombinant SmSULT-OR expressed from different allelic variants.** This *in*
794 *vitro* OXA activation assay quantifies DNA-OXA complexes by scintillation (~~count~~counts per minutes)
795 (see section 2.9). Bars showed the mean of three replicates, while error bars are standard errors.
796 Enzyme carrying loss-of-function mutations, such as p.C35 or p.E142del, show no OXA activation, while
797 two newly identified alleles (p.G206V and p.N215Y) do not impair OXA activation. * $p < 0.05$.

798 **Tables**799 **Table 1 – Primer sequences used for PCR and sequencing of the two exons of the *SmSULT-OR* gene.**

	Primer type	Primer sequence (5' to 3' orientation)	Expected amplicon size (bp)	Usage
Exon 1	Outer forward primer	GCGAGATTCAAACCCAGGAT	822	PCR
	Outer reverse primer	GCCGTGATATTACTATCAATCCC		PCR
	Nested forward primer	GGGTAAAGGAAGAGGGTTGG	545	PCR
	Nested reverse primer	TAAGAACAGACATATTAGACGAGT		PCR and sequencing
	Sequencing forward primer	TATATATGAAATATTATAACATTAC	-	sequencing
Exon 2	Forward primer	ACTTCAACCAATCCACAAATCC	672	PCR and sequencing
	Reverse primer	AGTCCATTCAATGTTTCAA		PCR and sequencing

800

801 **Table 2 – Mutations scored in the exons and 3' UTR of the *SmSULT-OR* gene.** For each mutation, the corresponding nucleotide found in
802 *Schistosoma rodhaini*, the number of homozygous and heterozygous samples, the number of ~~samples~~ samples sequenced (sample size), the allele
803 frequency of the ~~alternative~~ non-reference allele, the corresponding amino acid mutation, and the functional impacts are shown. The code used
804 for nucleic mutations indicate the sequence type (c = coding, g = gene), the position, and the mutation type (X>Y = substitution of X by Y,
805 insN = insertion of N, delN = deletion of N). The code used for protein mutations are coded with the sequence type (p = protein), the reference
806 amino acid, the position, and finally the alternative amino acid, and when frame shift (fs) occurs, the position of the stop codon (X) after the
807 mutation. For details about the nomenclature, see Ogino et al., 2007.

	Nucleic mutation	<i>Schistosoma rodhaini</i> state	Number of homozygous samples for non-ref. allele	Number of heterozygous samples for non-ref. allele	Sample size	Frequency of the alt. allele	Amino acid mutation	Functional impacts
Exon 1	c.103T>C	T	0	1	183	0.0027	p.C35R	Misfolded protein (Valentim et al., 2013)

	c.200C>T	C	169	12	183	0.9563	p.P67L	No effect (Valentim et al., 2013)
	c.214_215insA	-	0	1	183	0.0027	p.T72NfsX5	Truncated protein with no active site
Intron	g.328G>A	G	1	0	179	0.0056	p.V110IfsX3	Splicing site disrupted leading to truncated protein with no active site
Exon 2	c.424_426delGAA	-	1	1	188	0.0080	p.E142del	Impaired oxamniquine binding (Valentim et al., 2013)
	c.617G>T	G	1	0	188	0.0053	p.G206V	No effect (Fig. 2)
	c.643A>T	A	0	1	188	0.0027	p.N215Y	No effect (Fig. 2)
3' UTR	g.4720C>T	C	177	9	188	0.9654	-	-
	g.4741T>C	T	2	8	188	0.0319	-	-

809 **Supplementary material**

810

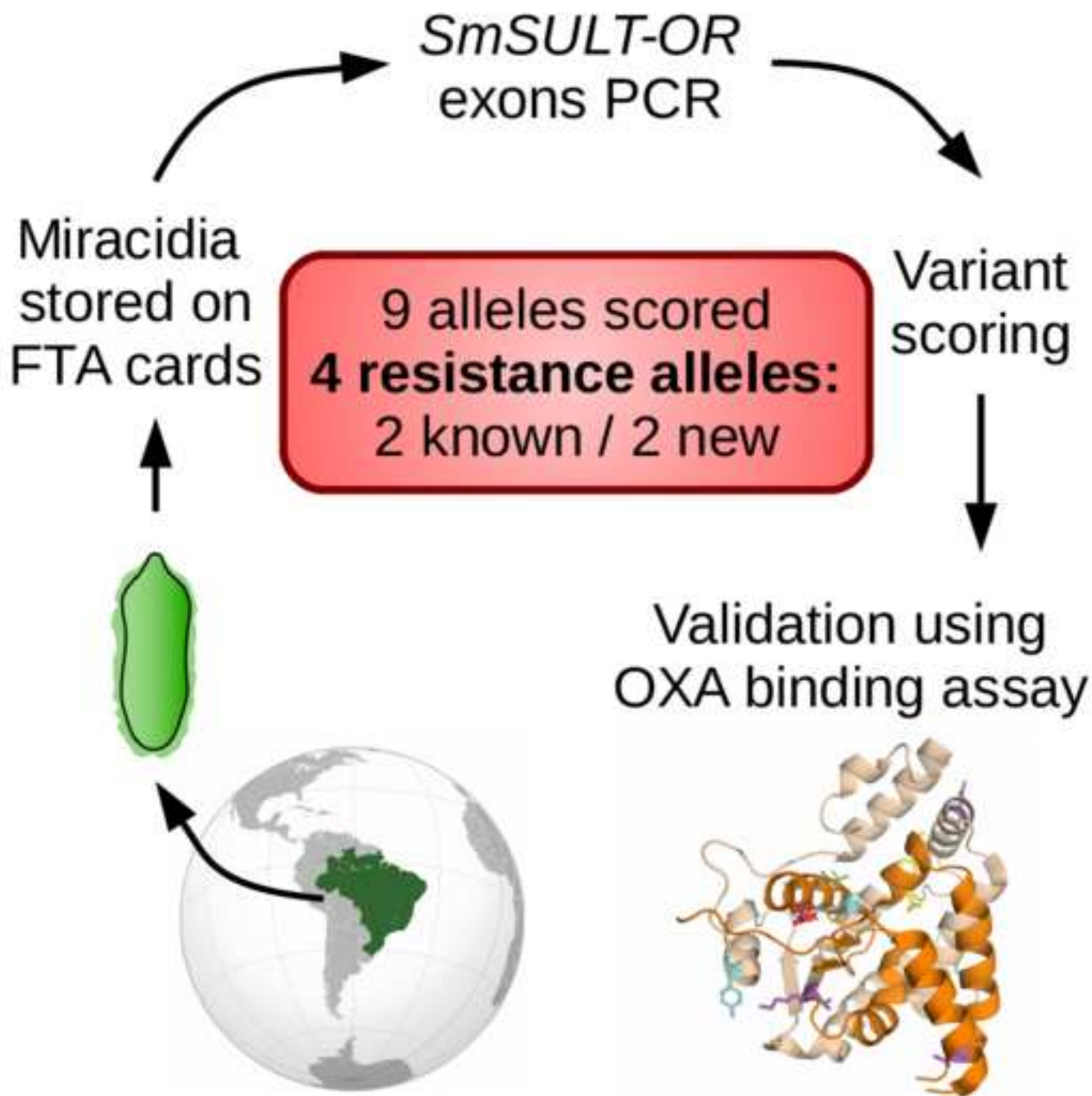
811 **Supp. fig. 1**

812

813 **Fig. S1** – Map of South America showing the places of origin of known and new OXA resistance alleles. Mutation p.C35R was identified in São
814 Paulo state (SP) while p.E142del was identified in Puerto Rico (PR). Both locations are distant from our sample site in Minas Gerais state (MG;
815 ~500 km and ~3,000 km, respectively).

816 **Supp. table 1****Table S1** – Sample summary and genotype details for each sample.

817 **Supp. movie****Movie S1** – Impact of mutation on SmsULT-OR structure and function. This video details the mutations and potential impact on
818 the protein structure and enzyme activity.



Highlights:

- We surveyed allelic variation in a schistosome drug resistance gene
- There were four independent origins of alleles encoding loss-of-function proteins
- Resistance mutations are at a very low frequency (< 0.0080)
- Loss-of-function mutations were tested using an in vitro oxamniquine activation assay

1 **Independent origins of loss-of-function mutations conferring**
2 **oxamniquine resistance in a Brazilian schistosome**
3 **population☆**

4
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20
21 ☆Note: Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL
22 and DDBJ databases under the accession numbers **KU951903 - KU952091**

23 Note: Supplementary data associated with this article

24 **Abstract**

25 Molecular surveillance provides a powerful approach to monitoring the resistance status of
26 parasite populations in the field and for understanding resistance evolution. Oxamniquine
27 (OXA) was used to treat Brazilian schistosomiasis patients (mid-1970s to mid-2000s) and several
28 cases of parasite infections resistant to treatment were recorded. The gene underlying
29 resistance (*SmSULT-OR*) encodes a sulfotransferase required for intracellular drug activation.
30 Resistance has a recessive basis and occurs when both *SmSULT-OR* alleles encode for defective
31 proteins. Here we examine *SmSULT-OR* sequence variation in a natural schistosome population
32 in Brazil ~40 years after the first use of this drug. We sequenced *SmSULT-OR* from 189
33 individual miracidia (1-11 per patient) recovered from 49 patients, and tested proteins
34 expressed from putative resistance alleles for their ability to activate OXA. We found nine
35 mutations (four non-synonymous single nucleotide polymorphisms (SNPs), three non-coding
36 SNPs and two indels). Both mutations (p.E142del and p.C35R) identified previously were
37 recovered in this field population. We also found two additional mutations (a splice site variant
38 and 1 bp coding insertion) predicted to encode non-functional truncated proteins. Two
39 additional substitutions (p.G206V, p.N215Y) tested had no impact on OXA activation. Three
40 results are of particular interest: (i) we recovered the p.E142del mutation from the field: this
41 same deletion is responsible for resistance in an OXA-selected laboratory parasite population;
42 (ii) frequencies of resistance alleles are extremely low (0.27-0.8%), perhaps due to fitness costs
43 associated with carriage of these alleles; (iii) that four independent resistant alleles were found
44 is consistent with the idea that multiple mutations can generate loss-of-function alleles.

45

46 *Keywords:* *Schistosoma mansoni*, Oxamniquine resistance, Sulfotransferase, Loss-of-function,
47 Biochemical assay, Soft selective event

48

49 1. Introduction

50 Surveys of drug resistance alleles using molecular markers provide a powerful approach
51 to identify pathogen populations in which resistance is emerging, to map resistance spread, and
52 for evidence-based resistance management. Molecular approaches are now widely used for
53 tracking resistance in malaria (Pearce et al., 2009; Ashley et al., 2014), HIV (Panichsillapakit et
54 al., 2015) and bacterial diseases (Bhembe et al., 2014), and for managing insecticide resistance (
55 Essandoh et al., 2013; Djègbè et al., 2014). Such methods are also now actively used for
56 monitoring resistance in helminths of veterinary importance such as *Haemonchus contortus* (
57 Rufener et al., 2009; Chaudhry et al., 2015; Redman et al., 2015), and have enormous potential
58 for monitoring resistance in helminth parasites infecting humans, because existing phenotypic
59 screening methods based on reductions in production of eggs or larval stages are insensitive for
60 detection of low frequency resistance alleles and cannot detect recessive resistance alleles
61 present in heterozygous worms. However, molecular surveillance is only possible when
62 resistance genes have been identified, which is rarely the case for human helminth infections
63 such as schistosomiasis.

64 Schistosomiasis, caused by three major species of blood flukes of the genus *Schistosoma*
65 (Dye et al., 2013; Colley et al., 2014), is the second most important tropical parasitic disease
66 after malaria (Steinmann et al., 2006), affecting an estimated 260 million people across Africa,
67 Asia and South America, and killing over 200,000 people per year. Two drugs are available for
68 treating schistosomiasis. Praziquantel (PZQ) is currently used as a monotherapy in expanding
69 mass drug administration programs in Africa (Dye et al., 2013), making resistance evolution a
70 major concern. A second drug, oxamniquine (OXA), is the focus of this paper. OXA acts
71 specifically against *Schistosoma mansoni*, which is found in Africa, together with
72 *Schistosoma haematobium*, and in South America, where it is the only species present. OXA was
73 manufactured in Brazil by Pfizer (Cheetham, 1994) and widely used to treat *S. mansoni*
74 infections from the mid-1970s to the mid-2000s (Coura and Amaral, 2004), but has now been
75 replaced by PZQ as the first line drug.

76 Schistosomes resistant to OXA were first identified in Brazil in 1973 (Naftale Katz et al.,
77 1973), around the same time as the first clinical study of OXA treatment (Katz et al., 1973). This
78 rapid emergence of resistance was most likely due to the previous treatment of the same
79 populations with the related drug hycanthone (Katz et al., 1968; Coura and Conceição, 2010), as
80 both OXA and hycanthone have the same target (Jansma et al., 1977; Pica-Mattoccia et al.,
81 1993) and the same mechanism of resistance (Pica-Mattoccia et al., 1992a). Later genetic
82 studies showed that resistance is a recessive single locus trait (Cioli and Pica-Mattoccia, 1984;
83 Pica-Mattoccia et al., 1993), most likely involving the absence of a sulfotransferase activity
84 necessary for drug activation in resistant schistosomes (Pica-Mattoccia et al., 2006). The gene

85 encoding the *S. mansoni* sulfotransferase involved in OXA resistance (*SmSULT-OR*) was recently
86 identified by classical quantitative trait mapping in concert with crystallographic and functional
87 analyses (Valentim et al., 2013). This work identified an amino acid deletion (p.E142del) in the
88 laboratory-selected resistant parasite (HR), while an independent loss-of-function mutation
89 (p.C35R) was identified in a field-collected resistant parasite line (MAP).

90 The identification of the gene involved in OXA resistance now allows us to examine
91 distribution of resistance alleles of *SmSULT-OR* in natural populations. Despite the fact that the
92 Brazilian government has switched from OXA to PZQ during the last decade (Utzinger et al.,
93 2003), the recessive nature of OXA resistance allows the persistence of these alleles, because
94 alleles encoding non-functional enzyme are not counter-selected as long as they segregate with
95 alleles encoding a functional enzyme. We collected miracidia larval stages from a village in
96 Minas Gerais, Brazil, and sequenced the *SmSULT-OR* gene in these samples. We sought to
97 answer several questions: How common are resistance alleles? How many times have OXA
98 resistance alleles arisen? Are the OXA resistance alleles selected in the laboratory and identified
99 using linkage mapping actually present in nature? More broadly, our goal is to better
100 understand the evolution of drug resistance in schistosomes and to demonstrate the utility of
101 molecular screening approaches in anticipation that the gene(s) underlying PZQ resistance will
102 soon be identified.

103

104 **2. Materials and methods**

105 *2.1. Ethics statement*

106 Stool samples were collected in accordance to the procedures of the Research Ethics
107 Committee of the Universidade Federal de São Paulo, Brazil (process number CAAE:
108 15567313.8.0000.5091). The purpose of the study and the procedures to be followed were
109 explained and written informed consent was obtained from all participants or their legal
110 guardians prior to any collection.

111

112 *2.2. Sampling of Schistosoma mansoni miracidia*

113 We collected stools from school children from Ponto dos Volantes (Minas Gerais, Brazil,
114 GPS coordinates: 16°45'3.301" S, 41°30'13.755" W) and shipped these at 4°C by ground
115 transportation overnight to the Universidade Federal de Minas Gerais in Belo Horizonte, Brazil.
116 We processed samples as follows: several grams of stools were filtered through three layers of
117 sieves (mesh size: 250 to 45 µm) to obtain schistosome eggs. Eggs were transferred from the third

118 sieve grid to a Petri dish and exposed under artificial light for at least 1 h. All filtering steps and
119 egg transfer were performed with locally available bottled mineral water.

120 Washed eggs were observed under a stereomicroscope. For each patient, 1 - 11 living
121 miracidia were sampled individually in ~2 μ L of water and spotted onto CloneSaver FTA cards
122 (GE Healthcare Life Sciences, USA). Spotted samples can be easily located on the cards because
123 the pink dye on the cards turns white after water contact. Full cards were allowed to dry for 1 h
124 at room temperature on the bench before being stored in a plastic bag and finally shipped to
125 San Antonio, Texas, USA.

126

127 *2.3. Preparation of FTA samples for whole genome amplification (WGA)*

128 For each sample, we removed a 2 mm diameter disc from the FTA card using a 2 mm
129 Harris Micro-punch (GE Healthcare Life Sciences). The 2 mm disc corresponds to the entire spot
130 containing the whole miracidium. Each punch was placed individually in a 1.5 mL sterile tube.
131 Punches were washed three times with FTA Purification Reagent (GE Healthcare Life Sciences)
132 then rinsed twice with TE⁻¹ buffer (10 mM Tris, 0.1 mM EDTA, pH 8). Washing and rinsing steps
133 were performed by adding 200 μ L of solution to each tube followed by 5 min of incubation on a
134 nutating mixer (24 RPM) at room temperature and then discarding the solution while
135 minimizing contact between the pipette tip and the punch. Punches were finally dried in tubes
136 for 10 min at 56°C on a dry bath incubator.

137

138 *2.4. WGA*

139 We performed WGA on each punch using the illustra GenomiPhi V2 DNA Amplification
140 kit (GE Healthcare Life Sciences). Punches were transferred in 0.2 mL sterile tubes using a sterile
141 tip. We performed reactions following the manufacturer's instructions, immersing each punch
142 in 9 μ L of sample buffer and keeping tubes on ice at all times after the denaturation step. After
143 amplification, we quantified DNA using the Qubit dsDNA BR assay (Invitrogen, USA).

144

145 *2.5. Sequencing of SmSULT-OR exons*

146 The two exons of the *SmSULT-OR* gene were amplified and sequenced independently.
147 Each PCR was performed using the TaKaRa Taq kit (Clontech, USA) and composed of 9.325 μ L of
148 sterile water, 1.5 μ L of 10 \times buffer, 1.2 μ L of dNTP (2.5 mM each), 0.9 μ L of MgCl₂ (25 mM),
149 0.5 μ L of each primer (10 μ M; Table 1), 0.075 μ L of Taq polymerase (5 U. μ L⁻¹) and 1 μ L of DNA

150 template. Amplifications were done using a GeneAmp PCR system 9700 thermocycler (Applied
151 Biosystems, USA) with the following program: 95°C for 5 min; 95°C for 30 s, 60°C for 30 s, 72°C
152 for 45 s, for 35 cycles; then 72°C for 10 min.

153 Exon 1 required a nested PCR in order to obtain a specific product. Products from the
154 first PCR were cleaned up by adding 4 µL of ExoSAP-IT (Affymetrix USB products, USA). Tubes
155 were then incubated at 37°C for 30 min and at 80°C for 15 min using a thermocycler. Cleaned
156 PCR products were then used as templates for the second PCR following the above protocol.

157 Sequencing reactions were performed using a BigDye® Terminator v3.1 cycle sequencing
158 kit (Applied Biosystems) on final PCR products. PCR products were cleaned up using ExoSAP-IT
159 as described above. Sequencing reactions were performed using 2.59 µL of sterile water, 1 µL
160 of 5× running buffer, 0.25 µL of BigDye Terminator ready reaction mix (Applied Biosystems),
161 0.16 µL of forward or reverse primer used in the final PCR step, and 1 µL of final PCR product.
162 Sequencing fragments were generated using a GeneAmp PCR system 9700 thermocycler
163 (Applied Biosystems) with the following program: 96°C for 1 min; 96°C for 10 s, 50°C for 5 s,
164 60°C for 4 min, for 25 cycles. Sequencing reactions were cleaned up using a BigDye
165 XTerminator® purification kit (Applied Biosystems). In each reaction, 20.45 µL of SAM™ solution
166 and 4.55 µL of XTerminator™ solution were added. Reactions were then vortexed for 30 min
167 and run on a 3730xl DNA Analyzer (Applied Biosystems).

168 Sequencing files were first screened using FinchTV (v1.4.0; Geospiza Inc.) to identify
169 failed sequencing reactions. In the case of failure, sequencing reactions were performed a
170 second time.

171

172 *2.6. Variant identification and functional impact evaluation*

173 We scored variants using PolyPhred software (v6.18) (Nickerson et al., 1997) which
174 relies on Phred (v0.020425.c), Phrap (v0.990319), and Consed (v29.0) software, analyzing each
175 exon independently. We identified single nucleotide polymorphisms using a minimum phred
176 quality score (-q) of 40, a minimum genotype score (-score) of 90, and a reference sequence
177 that includes the *SmSULT-OR* gene and surrounding regions (position 1519500 to 1525200 of
178 chromosome 6 of *S. mansoni* reference genome v5.0,
179 [ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/genome/Assembly-
180 v5/sma_v5.0.chr.fa.gz](ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/genome/Assembly-v5/sma_v5.0.chr.fa.gz)). Variant sites were labeled as non-reference alleles if they differed from
181 the reference sequence. We identified Insertion/deletion (indel) polymorphisms using a
182 minimum phred quality score (-q) of 40, a minimum genotype score (-iscore) of 80.
183 Polymorphisms were visually validated using Consed. All the sequences were submitted to
184 GenBank (GenBank accession no **KU951903-KU952091**).

185 Nucleic sequences showing mutations were translated in silico into protein sequences
186 using the Translate tool from ExpASy portal (Artimo et al., 2012).

187 We evaluated the potential functional impact of identified polymorphisms on RNA
188 features and on protein structure in silico. Modifications of RNA motifs and sites were analyzed
189 using the RegRNA2.0 website (Chang et al., 2013) for all available features with *Drosophila*
190 *melanogaster* as the reference species when required. Modifications in protein structure were
191 assessed using the mutagenesis function of PyMol software (v1.7.2.0, Schrödinger, LLC) using
192 the structure of *SmSULT-OR* determined previously (PDB code 4MUB, Valentim et al., 2013).

193 194 2.7. Population genetics analysis

195 We evaluated population structure by testing for Hardy-Weinberg equilibrium,
196 measuring fixation indices (F_{st} and the F_{is}) of parasites using Genepop (Rousset, 2008) with
197 default options and considering schistosomes from a given patient as belonging to the same
198 population. To identify positive selection, we calculated the number of synonymous (s) and
199 non-synonymous (n) sites (Nei and Gojobori, 1986) and compared these values to synonymous
200 (S) and non-synonymous (N) changes. We also performed a McDonald-Kreitman (MKT) test
201 using the MKT server (<http://mkt.uab.es>; Egea et al., 2008). The *SmSULT-OR* homolog sequence
202 in *Schistosoma rodhaini* was used as an outgroup and was obtained using tblastx from the NCBI
203 server (Coordinators, 2015) on the *S. rodhaini* genome assembly (GenBank accession no.
204 **GCA_000951475.1**) using the *SmSULT-OR* sequence (GenBank accession no. **KF733459.1**) as a
205 query.

206 207 2.8. Recombinant *SmSULT-OR* protein production

208 The *SmSULT-OR* sequence from the reference genome (Smp_089320; GenBank
209 accession no. **HE601629.1**) was used to create a codon optimized synthetic gene (GenScript)
210 which was subcloned into the pAG8H vector derived from pKM260 (Melcher, 2000). We
211 introduced mutations using a Phusion site-directed mutagenesis protocol (ThermoFisher
212 Scientific, USA). Transformed *Escherichia coli* strain BL21 pLysS (Promega) were grown at 37°C
213 until the absorbance ($\lambda = 600$ nm) reached 0.7. We then decreased the temperature to 18°C
214 and induced expression by the addition of isopropyl- β -D-thiogalactoside (IPTG) at a final
215 concentration of 1 mM. The cells were washed and resuspended in 50 mL of 50 mM Tris pH 8.0,
216 500 mM NaCl (column buffer) containing 250 μ L of Sigma protease inhibitor cocktail per liter of
217 culture and lysed by sonication on ice. The clarified supernatant was loaded onto a Ni²⁺-NTA
218 affinity chromatography column (GE Healthcare), washed with five volumes of column buffer,

219 and eluted using a 10-500 mM imidazole gradient. We pooled fractions identified as the
220 Smp_089320 via SDS-PAGE and added His-tagged Tobacco etch virus (TEV) protease at a
221 Smp_089320:protease ratio of 15:1. The resulting solution was dialyzed overnight at 4°C
222 against 50 mM Tris pH 8.0, 100 mM NaCl, 2 mM dithiothreitol (DTT). We passed the dialysate
223 over the Ni²⁺-NTA column again to remove the His tag and the His-tagged TEV protease, while
224 the cleaved target protein flowed through. The sample was loaded onto a GE-pre-packed Q
225 anion exchange column and eluted with a 0.1-1.0 M NaCl gradient. We pooled fractions
226 containing Smp_089320 as identified by SDS-PAGE and dialyzed overnight at 4°C against 25 mM
227 Tris pH 8.5, 50 mM NaCl, 2 mM reducing agent tris-(carboxyethyl)-phosphine (TCEP) to prevent
228 formation of intermolecular disulfide bonds. The product was ~98% pure as estimated by SDS-
229 PAGE. We concentrated the purified Smp_089320 protein to 10 mg.mL⁻¹ using the calculated
230 extinction coefficient $\epsilon = 39,880 \text{ mol}^{-1}.\text{cm}^{-1}$.

231

232 2.9. OXA activation assay

233 This assay utilizes the fact that OXA is a prodrug that is enzymatically converted into a
234 highly reactive molecule that covalently binds intracellular components such as DNA (Pica-
235 Mattoccia et al., 1989). Because the original assay employs worm extracts which require time
236 for preparation and introduce unpredictable variations in SmSULT-OR concentration (Pica-
237 Mattoccia et al., 1992a; Valentim et al., 2013), we developed an improved in vitro assay that
238 uses purified recombinant SmSULT-OR enzyme. This in vitro assay measures the enzymatic
239 sulfonation of tritiated OXA molecules which then bind genomic DNA (gDNA). The resultant
240 radioactive DNA-OXA complexes can then be quantified by a scintillation counter. Enzymes
241 produced by non-functional *SmSULT-OR* alleles cannot sulfonate OXA, so no DNA-OXA
242 complexes are formed.

243 Recombinant proteins were expressed and purified commercially by GenScript, USA
244 (p.C35R, p.P67L, p.E142del; Valentim et al., 2013) or by the Hart laboratory (University of Texas
245 Health Science Center, USA) (p.G206V and p.N215Y). To determine which recombinant protein
246 has the ability to activate OXA, 1 nM from each recombinant protein was added to 90 μL of a
247 protease inhibitor cocktail (PIC) consisting of 0.1 M HEPES pH 7.4, 0.1mM leupeptin, 2 μM E-64,
248 2 μM pepstatin A, 0.1 U of aprotinin, and 10 $\text{ng}.\mu\text{L}^{-1}$ sheared *S. mansoni* gDNA as a final target.
249 For each reaction 100 μCi of ³H-OXA (Pica-Mattoccia et al., 1989) was solubilized in 2 μL of
250 DMSO and added to 10 μL of a mixture containing the enzyme cofactors ATP and MgCl₂ at
251 50 mM each, and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) at 1 mM. The radiolabeled
252 OXA and co-factor mix was then added to the PIC mix containing the recombinant protein. The
253 resulting reaction was incubated for 2.5 h at 37°C and stopped by adding three volumes of 0.1%
254 SDS in 0.1 M sodium bicarbonate. The reaction was then extracted three times with two

255 volumes of dichloromethane and the aqueous phase was counted in a liquid scintillation
256 spectrometer (Beckman LS 6500 Scintillation Counter, USA) for 10 min. We also measured a
257 blank solution (water) and the background scintillation. Blank and background values were
258 subtracted from sample values. We performed three independent reactions for each
259 recombinant protein.

260

261 *2.10. Statistical analysis*

262 Statistical analyses were done using R (v3.1.3) (R Core Team, 2015). For synonymous
263 and non-synonymous changes, data were compared using a Fisher's exact test. For the OXA
264 activation assay, the data were compared with a Welch t-test after testing for normality
265 (Shapiro test, $P > 0.05$).

266

267 **3. Results**

268 *3.1. Brazilian samples*

269 We collected 232 FTA preserved miracidium samples from 51 patients (range: 1-12,
270 mean±S.D.: 4.55±3.65). We successfully amplified DNA from 204 samples (87.93%) from 50
271 patients (range: 1-11, mean±S.D.: 4.08±3.26). Among the amplified samples, 189 from 49
272 patients (range: 1-11, mean±S.D.: 3.86±3.18) contained schistosome DNA (92.65% of amplified
273 samples, 81.47% of total samples). Among the 189 samples, we sequenced exon 1 from 183
274 samples and exon 2 from 188 samples. All samples had at least one exon sequenced
275 (Supplementary Table S1).

276

277 *3.2. SmSULT-OR variants in a Brazilian schistosome population*

278 We scored nine mutations: four non-synonymous single nucleotide polymorphisms
279 (SNPs), one insertion and one deletion in the coding region, and three non-coding SNPs (Table
280 2, Supplementary Table S1). The number of mutations in each exon (three in exon 1 and three
281 in exon 2) did not differ regarding the length of the exon (327 bp and 447 bp, respectively)
282 (Fisher's exact test, $P = 0.7$).

283 Among the seven SNPs, five (71.43%) were transitions and two (28.57%) were
284 transversions. Four of the SNPs were located in the exonic region, two in exon 1 and two in
285 exon 2. Three were present at very low frequency (0.0027-0.0053) while one was present at
286 very high frequency (0.95). One SNP was identified at the first position of the intron at a low

287 frequency (0.0056). The two remaining SNPs were found in the 3' untranslated region (UTR),
288 one at high frequency (0.96), and one at low frequency (0.03).

289 One insertion was identified in exon 1 while one deletion was identified in exon 2, both
290 at very low frequency (0.0027 and 0.0080, respectively).

291 Low frequency mutations were found in heterozygous or in homozygous states among
292 the samples. Observing rare SNPs present as homozygotes was surprising, suggesting
293 population structure. The test for Hardy-Weinberg equilibrium showed a global deficit in
294 heterozygosity ($P < 0.0001$). This is likely due to a deficit of heterozygous genotypes within host
295 ($F_{is} = 0.3251$) rather than due to population differentiation between infections ($F_{st} = 0.0153$).
296 Null alleles are not likely to explain the deficit. While we had small numbers of samples for
297 which WGA failed, *SmSULT-OR* was successfully amplified from all samples for which WGA was
298 successful.

299 Due to the absence of the synonymous mutations which precludes dN/dS calculation,
300 we compared synonymous (S) and non-synonymous (N) changes with synonymous (s) and non-
301 synonymous (n) sites to evaluate evidence for selection on *SmSULT-OR*. This comparison
302 reveals no differences (Fisher's exact test, $P = 1$) indicating that there was no evidence for
303 selection. We also tested directional evolution by performing a McDonald-Kreitman test using
304 the homolog sequence of *SmSULT-OR* from *S. rodhaini* as an outgroup. We found no evidence
305 for directional evolution ($\chi^2 = 1.466$, $P = 0.225$).

306

307 *3.3. Functional impacts of mutations*

308 *3.3.1. Non-coding variants*

309 We evaluated the functional impacts of the polymorphisms identified based on RNA
310 features such as binding sites or splicing or regulatory motifs (Table 2). The mutation g.649G>A
311 was predicted to modify the unique splice donor site at the end of exon 1. The disruption of the
312 splicing site leads to translation of the beginning of the intron which ends four codons later due
313 to the introduction of a stop codon. This results in a truncated protein with no active site (Fig. 1,
314 Supplementary Movie S1). Mutations present on the 3' UTR were not predicted to be in any
315 regulatory sites.

316 *3.3.2. Coding variants*

317 All SNPs identified in the coding sequence of the gene were non-synonymous, and
318 derived mutations relative to the outgroup *S. rodhaini* (Table 2). c.103T>C induced a
319 substitution of cysteine to arginine (p.C35R) leading to a misfolded protein as shown previously

320 (Valentim et al., 2013) (Fig. 1). c.200C>T induced a substitution of proline to leucine (p.P67L)
321 and was previously found in the OXA-sensitive strain (Valentim et al., 2013) (Fig. 1); this
322 mutation therefore does not reduce enzyme activity. c.617G>T induced a substitution of glycine
323 to valine (p.G206V) which occurs close the binding site of the PAPS co-factor and was previously
324 observed in field-collected OXA-resistant strain (Valentim et al., 2013) but was never tested .
325 We postulated that this mutation may have a potential detrimental effect on co-factor binding
326 and finally enzyme activity (Fig. 1, Supplementary Movie S1). The mutation c.643A>T induced a
327 substitution of asparagine to tyrosine (p.N215Y) which occurs on a helix connected to a loop
328 involved in co-factor binding. This mutation is predicted to have little impact on protein
329 structure. We therefore postulated that this will not change protein function (Fig. 1,
330 Supplementary Movie S1).

331 3.3.3. *Indels*

332 Both indels have detrimental effects on the enzyme (Table 2). The insertion
333 c.214_125insA induces a frame shift leading to an early stop codon six codons after the
334 mutation. This frame shift is predicted to produce a truncated enzyme with no active site. The
335 deletion c.424_426delGAA is known from previous functional analyses (Valentim et al., 2013) to
336 disrupt OXA binding.

337

338 3.4. *OXA activation assay*

339 We produced two recombinant SmSULT-OR enzymes carrying the mutations p.G206V
340 and p.N215Y in order to experimentally test whether these two mutations impact OXA
341 activation, resulting in OXA resistance. We also tested known resistance alleles (p.C35R and
342 p.E142del) as controls. The newly identified alleles are able to activate OXA as well as the
343 reference allele (wild type) which does not carry any of these mutations (Welch t-test, $t < -1.55$,
344 $P > 0.18$) while the known resistance alleles showed no activation as expected (Welch t-test, $t >$
345 5.58 , $P < 0.021$) (Fig. 2). Therefore the two mutations tested (p.G206V and p.N215Y) did not
346 disrupt co-factor binding or otherwise interfere with enzymatic activity.

347

348 4. Discussion

349 *SmSULT-OR* sequences from 189 miracidia collected from 49 Brazilian patients revealed
350 nine mutations, including both mutations previously implicated in OXA resistance (Valentim et
351 al., 2013). We found the p.C35R mutation, previously identified in the MAP strain. MAP was
352 sampled from a patient living in a city of São Paulo state, Brazil (Supplementary Fig. S1) (Pica-
353 Mattoccia et al., 1992b), the neighboring state of that from which our samples were collected.
354 This suggests that the allele has been segregating in Brazilian parasite populations for more

355 than 25 years. The second variant identified in the field, p.E142del, was previously found in the
356 HR laboratory strain, which was initially sampled from a Puerto-Rican patient and subsequently
357 selected with hycanthone in the laboratory (Supplementary Fig. S1) (Cioli and Pica-Mattocchia,
358 1984). This mutation could have arisen spontaneously in the laboratory. However, given that
359 laboratory schistosome lines are maintained as outbred populations, the simplest explanation is
360 that this mutation was segregating within the parasite population originally established in the
361 laboratory. That we located this same deletion in two miracidia from Brazil and a parasite line
362 collected from Puerto-Rico suggests that this allele may be widespread in schistosome
363 populations from the New World.

364 It is not clear from our data whether the p.E142del seen in HR and Brazilian field
365 samples arose independently or has a single origin. The HR p.E142del is found together with
366 another mutation (p.L256W) (Valentim et al., 2013), which is absent from the p.E142del allele
367 found in the Brazilian miracidia, providing some evidence for independent origins. Similarly,
368 MAP differs from the Brazilian miracidia carrying the p.C35R mutation; while both also carry
369 p.P67L, MAP carries an additional p.G206V mutation. Additional flanking SNP data will be
370 required to critically test whether the p.E142del and p.C35R OXA resistance mutations have
371 arisen a single time, or have multiple independent origins.

372 Laboratory selection is commonly used to explore the genetics of pathogen resistance,
373 but a concern with this approach is that the mutations selected in the laboratory may poorly
374 represent those occurring in nature. That we identified the same mutations in laboratory and
375 field selected parasites is extremely encouraging, because ongoing work to identify mutations
376 involved in PZQ resistance also utilizes laboratory selected parasites (Couto et al., 2011). We
377 note that laboratory selection experiments with *Plasmodium* also tend to identify the same
378 genes and often the same mutations that are observed in the field (Anderson et al., 2011),
379 further validating this approach.

380 Besides the two known alleles, two of these new mutations, c.214_215insA and
381 g.328G>A, are predicted to confer OXA resistance; both introduce premature insertion of a stop
382 codon producing a protein without an active site. These mutations therefore add two additional
383 loss-of-function mutations that were probably selected during the OXA treatment. That four
384 independent mutations are found in a single sampled parasite population is remarkable.
385 Multiple origins have previously been observed in the evolution of resistance to benzimidazole
386 drugs in the gastrointestinal nematode *Haemonchus* (Redman et al., 2015). The number of
387 origins of resistance alleles is expected to depend on the size of the parasite population and the
388 rate at which mutation generates resistant alleles (Messer and Petrov, 2013). In the case of
389 *Haemonchus contortus*, the enormous size of parasite populations is likely to be the main
390 driver, as only several specific mutations within β -tubulin can confer resistance. In the case of

391 OXA resistance, a high mutation rate may be expected as the main driver, because multiple
392 different mutations within *SmSULT-OR* can generate non-functional proteins. It is also possible
393 that OXA-resistant alleles were present within Brazilian *S. mansoni* populations prior to
394 hycanthone or OXA treatment. Such standing variation may even have been present prior to
395 the introduction of *S. mansoni* into South America. Analysis of *SmSULT-OR* in African
396 populations, where OXA was not used extensively, will help answer this question.

397 Work on OXA resistance is simplified because we have an effective in vitro functional
398 assay for screening allelic variants. The assay we used is an improvement on those previously
399 described (Pica-Mattocchia et al., 1992a; Valentim et al., 2013), because worm homogenates are
400 replaced by recombinant SmSULT-OR proteins. We identified two additional exon 2
401 substitutions (p.G206V, p.N215Y): Structural analyses suggested that one of these mutations
402 (p.G206V) may disrupt OXA activation, by interfering with binding of the co-factor (PAPS), while
403 the other (p.N215Y) is likely to have minimal impact on function. We were able to directly test
404 these predictions by performing OXA activation assays. These assays demonstrated that neither
405 mutation prevents OXA activation, allowing us to reject our prediction for p.G206V and confirm
406 our prediction for p.N215Y. Hence, while structural studies are useful for formulating
407 hypotheses about the consequences of mutations, functional assays are essential for critical
408 testing of these hypotheses.

409 The two last mutations were found in the 3' UTR of the cDNA. In silico analysis, using
410 predictions based on *Drosophila*, did not reveal regulatory sites in these regions, suggesting
411 that these mutations do not impact function. However, *Drosophila* may be a poor model to use.
412 When more information on *SmSULT-OR* regulatory regions become available, new analyses of
413 these two mutations may reveal potential effects on mRNA stability or translation rate.

414 The allele frequency of all four OXA resistance alleles combined is 3.8%, and only two
415 parasites of 183 sampled (1%) are homozygous and therefore expected to be phenotypically
416 resistant. The low frequency of the OXA resistance alleles can be explained by two non-
417 exclusive hypotheses. First, resistance alleles may have remained at low levels even when OXA
418 was the first line drug in Brazil. Second, fitness costs associated with these alleles may have
419 driven reductions in allele frequency after the abandonment of OXA treatment. However OXA
420 resistance alleles still persist within the populations in a heterozygous state, the cost being
421 present only when worms are homozygous for the defective alleles.

422 Whether there is a cost associated with OXA resistance is questionable because
423 miracidia homozygous for resistance alleles were found in the field in the present study. A
424 previous study showed a reduction in infectivity and egg production in OXA-resistant parasites
425 relative to the OXA-susceptible populations from which they were isolated (Cioli et al., 1992).
426 However, this study suffers from a methodological limitation, because resistant and susceptible

427 laboratory populations had different genetic backgrounds, complicating interpretation.
428 Comparison of isogenic wildtype and genetically manipulated resistant parasites, or analysis of
429 fitness in the progeny of a genetic cross between OXA resistant and sensitive parasites would
430 allow measurement of associated fitness costs, while minimizing confounding background
431 effects.

432 New drugs are urgently needed for schistosome control because treatment currently
433 relies on widespread monotherapy with PZQ. OXA is effective only against *S. mansoni*, but new
434 OXA derivatives are under active development, with the aim of making compounds that are
435 active against all three major schistosome species infecting humans (Taylor et al., 2015). If such
436 derivatives are to be deployed clinically, understanding the capacity for resistance evolution in
437 schistosome populations is of critical importance. For example, surveys of sequence variation in
438 *SmSULT-OR* or the *S. haematobium* homologue would be an important prerequisite for field
439 deployment of an OXA derivative active against both these species in Africa.

440 Our results have both positive and negative implications for field deployment of OXA
441 derivatives. That multiple resistance alleles are present in a single parasite population suggests
442 that resistance alleles may evolve and spread rapidly. On the positive side, existing resistance
443 alleles are currently at extremely low frequency. It will be essential that OXA derivatives are
444 deployed with appropriate partner drugs to minimize the rate of resistance evolution. We note
445 that resistance to the antimalarial atovaquone was observed in the first clinical trial of this drug
446 (Looareesuwan et al., 1996) and evolved de novo in different treated patients (Musset et al.,
447 2007). Yet this drug is widely and effectively used with proguanil as a combination drug under
448 the trade name Malarone.

449

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626

627

628 **Figures**

629

630 **Fig. 1.** Mapping of the mutations on the gene sequence and structure of *Schistosoma mansoni* SmSULT-
631 OR sulfotransferase. Exon 1 and exon 2 are represented in orange and beige, respectively. Single
632 nucleotide polymorphisms and insertion/deletion events are represented in cyan and magenta,
633 respectively. Loss-of-function mutations are highlighted in black. (A) Linear representation of the
634 *SmSULT-OR* gene showing the relative position of the mutations and their translation in amino acid
635 sequences. (B) Positions of mutations on the SmSULT-OR protein. Oxamniquine (OXA) is represented in
636 yellow, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) co-factor is represented in green, and spatial
637 distortions are represented by red discs. For a more detailed view of the mutations on the structure and
638 their functional impact, see Supplementary Move S1.

639

640 **Fig. 2.** Enzymatic activity of recombinant *Schistosoma mansoni* SmSULT-OR sulfotransferase expressed
641 from different allelic variants. This in vitro oxamniquine OXA activation assay quantifies DNA-OXA
642 complexes by scintillation (counts per min) (see section 2.9). Bars show the mean of three replicates,
643 while error bars are S.E.M. Enzyme carrying loss-of-function mutations, such as p.C35 or p.E142del,
644 showed no OXA activation, while two newly identified alleles (p.G206V and p.N215Y) did not impair OXA
645 activation. * $P < 0.05$.

646

647 **Supplementary Fig. S1.** Map of South America showing the places of origin of known and new
648 *Schistosoma mansoni* oxamniquine (OXA) resistance alleles. Mutation p.C35R was identified in São Paulo
649 state (SP, Brazil) while p.E142del was identified in Puerto Rico (PR). Both locations are distant from our
650 sample site in Minas Gerais state (MG, Brazil; ~500 km and ~3,000 km, respectively).

651

652 **Supplementary Movie S1.** Impact of mutations on *Schistosoma mansoni* SmSULT-OR sulfotransferase
653 structure and function. This video details the mutations and potential impact on the protein structure
654 and enzyme activity. OXA: oxamniquine, PAPS: 3'-phosphoadenosine-5'-phosphosulfate

655 **Reference**

656 Valentim, C.L.L., Cioli, D., Chevalier, F.D., Cao, X., Taylor, A.B., Holloway, S.P., Pica-Mattocchia, L.,
657 Guidi, A., Basso, A., Tsai, I.J., Berriman, M., Carvalho-Queiroz, C., Almeida, M., Aguilar, H., Frantz, D.E.,
658 Hart, P.J., LoVerde, P.T., Anderson, T.J.C., 2013. Genetic and molecular basis of drug resistance and
659 species-specific drug action in schistosome parasites. *Science* 342, 1385–1389.
660 doi:10.1126/science.1243106

661

662 **Table 1.** Primer sequences used for PCRs and sequencing of the two exons of the *Schistosoma mansoni* *SmSULT-OR* gene in this study.

	Primer type	Primer sequence (5' to 3' orientation)	Expected amplicon size (bp)	Usage
Exon 1	Outer forward primer	GCGAGATTCAAACCCAGGAT	822	PCR
	Outer reverse primer	GCCGTGATATTACTATCAATCCC		PCR
	Nested forward primer	GGGTAAAGGAAGAGGGTTGG	545	PCR
	Nested reverse primer	TAAGAACAGACATATTAGACGAGT		PCR and sequencing
	Sequencing forward primer	TATATATGAAATATTATAACATTAC	-	sequencing
Exon 2	Forward primer	ACTTCAACCAATCCACAAATCC	672	PCR and sequencing
	Reverse primer	AGTCCATTCATTCAATGTTTCAA		PCR and sequencing

663

664

665 **Table 2.** Mutations scored in the exons, intron, and 3' untranslated regions (UTR) of the *Schistosoma mansoni* *SmSULT-OR* gene. For each
666 mutation, the corresponding nucleotide found in *Schistosoma rodhaini*, the number of homozygous and heterozygous samples carrying the non-
667 reference allele, the number of samples sequenced (sample size), the allele frequency of the non-reference allele, the corresponding amino acid
668 mutation, and the functional impact are shown.

	Nucleic mutation	<i>Schistosoma rodhaini</i> state	No. of homozygous samples for non-reference allele	No. of heterozygous samples for non-reference allele	Sample size	Frequency of the non-reference allele	Amino acid mutation	Functional impact
Exon 1	c.103T>C	T	0	1	183	0.0027	p.C35R	Misfolded protein (Valentim et al., 2013)
	c.200C>T	C	169	12	183	0.9563	p.P67L	No effect (Valentim et al., 2013)
	c.214_215insA	-	0	1	183	0.0027	p.T72NfsX5	Truncated protein with no active site
Intron	g.328G>A	G	1	0	179	0.0056	p.V110IfsX3	Splicing site disrupted leading to truncated protein with no active site
Exon 2	c.424_426delGAA	-	1	1	188	0.0080	p.E142del	Impaired oxamniquine binding (Valentim et al., 2013)
	c.617G>T	G	1	0	188	0.0053	p.G206V	No effect (Fig. 2)
	c.643A>T	A	0	1	188	0.0027	p.N215Y	No effect (Fig. 2)
3'	g.4720C>T	C	177	9	188	0.9654	-	-
UTR	g.4741T>C	T	2	8	188	0.0319	-	-

669 The code used for nucleic mutations indicates the sequence type (c = coding, g = gene), the position, and the mutation type (X>Y = substitution
670 of X by Y, insN = insertion of N, delN = deletion of N). The code used for protein mutations indicates the sequence type (p = protein), the
671 reference amino acid, the position, and finally the alternative amino acid, and when frame shift (fs) occurs, the position of the stop codon (X)
672 after the mutation. For details about the nomenclature, see Ogino et al., 2007.
673

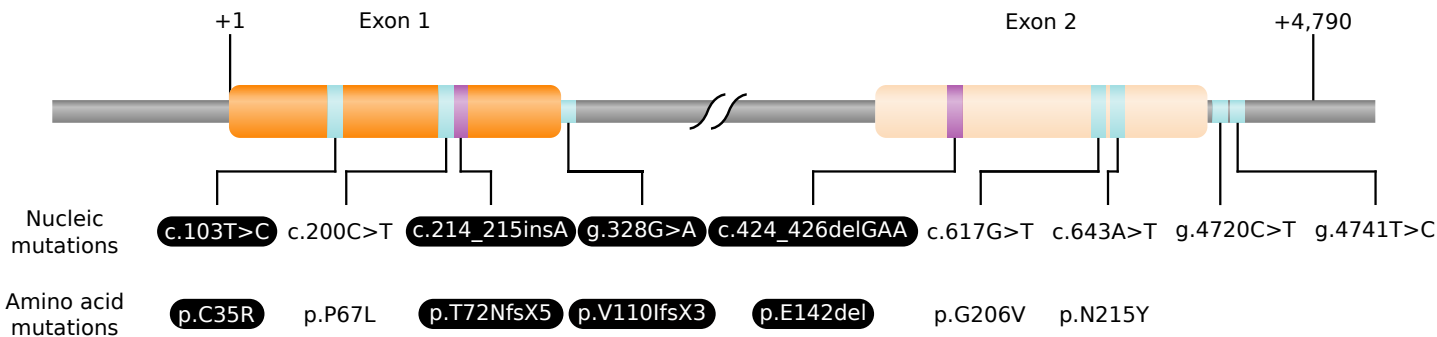
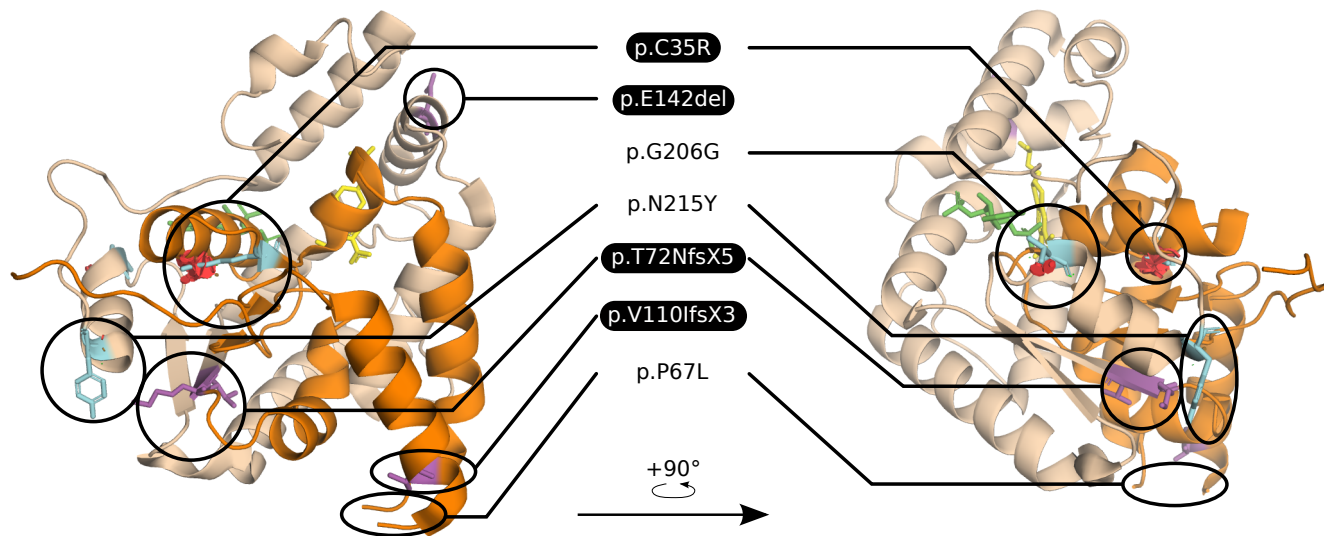
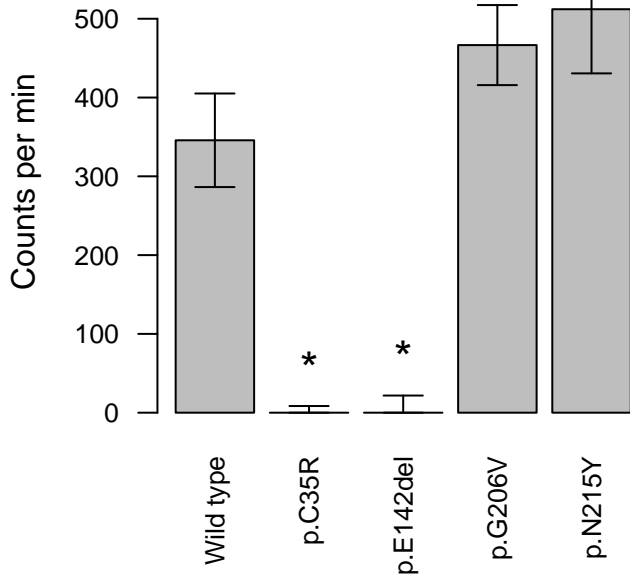
Figure 1**B**

Figure 2



Supplementary Table S1

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Supplementary Fig. S1

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