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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

Frédéric D. Chevalier, Ph.D.

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 population[1]

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[1] Note: Supplementary data associated with this article

Abstract

Molecular surveillance provides a powerful approach to monitoring 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 (4 non-synonymous SNPs, 3 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.

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

Highlights:

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

Abbreviations:

- Indel: insertion/deletion
- OXA: oxamniquine
- PCR: polymerase chain reaction
- PZQ: praziquantel

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

1. Introduction

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

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

Schistosomes resistant to OXA were first identified in Brazil in 1973 (Naftale Katz et al., 1973), around the same time as the first clinical study of OXA treatment (N. Katz et al., 1973). This rapid emergence of resistance was most likely due to the previous treatment of the same populations with the related drug hycanthone (Coura and Conceȷcão, 2010; Katz et al., 1968), as both OXA and hycanthone have the same target (Jansma et al., 1977; Pica-Mattoccia et al., 1993) and the same mechanism of resistance (Pica-Mattoccia et al., 1992a)~~(;). Latter~~. Later genetic studies showed that resistance is a recessive single locus trait (Cioli and Pica-Mattoccia, 1984; Pica-Mattoccia et al., 1993), most likely involving the absence of a sulfotransferase activity necessary for drug activation in resistant schistosomes (Pica-Mattoccia et al., 2006). The gene encoding the ***S****. **m**ansoni* **sul**fo**t**ransferase involved in **O**XA **r**esistance (*SmSULT-OR*) was

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

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

2. Material and methods

2.1 Ethics statement

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

2.2 Sampling of Schistosoma mansoni *miracidia*

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

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

2.3 Preparation of FTA samples for whole genome amplification

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

2.4 Whole genome amplification

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

2.5 Sequencing of SmSULT-OR exons

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

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

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

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

2.6 Variant identification and functional impact evaluation

We scored variants using PolyPhred software (v6.18) (Nickerson et al., 1997) which relies on Phred (v0.020425.c), Phrap (v0.990319), and Consed (v29.0) software, analyzing each exon independently. We identified single nucleotide polymorphisms using a minimum phred quality score (-q) of 40, a minimum genotype score (-score) of 90, and a reference sequence that includes *SmSULT-OR* gene and surrounding regions (position 1519500 to 1525200 of chromosome 6 of *S. mansoni* reference genome v5.0, 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 different from the reference sequence. We identified Insertion/deletion (indel) polymorphisms using a minimum phred quality score (-q) of 40, a minimum genotype score (-iscore) of 80. Polymorphisms were visually validated using Consed. All the sequences were submitted to GenBank (GenBank accession no ~~XXX-XXX~~)**KU951903-KU952091**).

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

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

2.7 Population genetics analysis

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

2.8 Recombinant SmSULT-OR protein production

The *SmSULT-OR* sequence from the reference genome (Smp_089320; GenBank accession no. **HE601629.1**) was used to create a codon optimized synthetic gene (GenScript) which was subcloned into the pAG8H vector derived from pKM260 (Melcher, 2000). We introduced mutations using a Phusion site-directed mutagenesis protocol (ThermoFisher Scientific). Transformed *Escherichia coli* strain BL21 pLysS (Promega) were grown at 37°C until the A_{600} reached 0.7, we decreased the temperature to 18°C and expression induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM. The cells were washed and resuspended in 50 mL 50 mM Tris pH 8.0, 500 mM NaCl (column buffer) containing 250 µL Sigma protease inhibitor cocktail per liter of culture and lysed by sonication on ice. The clarified supernatant was loaded onto a Ni^{2+}-NTA affinity chromatography column (GE Healthcare), washed with 5 volumes of column buffer, and eluted using a 10-500 mM imidazole gradient. We pooled fractions identified as the Smp_089320 via SDS-PAGE and added His-tagged TEV protease at a Smp_0893230•protease ratio of 15•1. The resulting solution was dialyzed ~~the resulting solution~~ overnight at 4°C against 50 mM Tris pH 8.0, 100 mM NaCl, 2 mM dithiothreitol (DTT). We passed the dialysate over the Ni^{2+}-NTA column again to remove the His tag and the His-tagged TEV protease, while the cleaved target protein flowed through. The sample was loaded onto a GE-pre-packed Q anion exchange column and eluted with a 0.1-1.0 M NaCl gradient. We pooled fractions containing Smp_089320 as identified by SDS-PAGE and dialyzed overnight at 4°C against 25 mM Tris pH 8.5, 50 mM NaCl, 2 mM reducing agent tris-(carboxyethyl)-phosphine (TCEP) to prevent formation of intermolecular disulfide bonds. The product was ~98% pure as estimated by SDS-PAGE. We concentrated the purified Smp_089320 protein to 10 $mg.mL^{-1}$ using the calculated extinction coefficient ε = 39,880 $mol^{-1}.cm^{-1}$.

2.9 Oxamniquine activation assay

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

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

2.10 Statistical analysis

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

3. Results

3.1 Brazilian samples

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

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

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

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

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

Low frequency mutations were found in heterozygous or in homozygous states among the samples. Observing rare SNPs present as homozygotes was surprising, suggesting population structure. The test for Hardy-Weinberg equilibrium showed a global deficit in heterozygosity ($p < 0.0001$). This is likely due to deficit of heterozygous genotypes within host ($F_{is} = 0.3251$) rather than due to population differentiation between infections ($F_{st} = 0.0153$). 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.

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

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

3.3 Functional impacts of mutations

3.3.1 Non-coding variants

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

3.3.2 Coding variants

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

3.3.3 Indels

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

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

3.4 *Oxamniquine activation assay*

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

4. Discussion

4.1. Identical mutations in laboratory and field selected parasites

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

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

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

4.2. Multiple origins of OXA resistance

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

4.3. Importance of functional assays for testing gene function

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

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

4.4. Low frequency of resistance mutations

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

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

4.5. Implications for schistosome biology and control

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

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

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

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Figures

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

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

Tables

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	AGTCCATTCATTCAATGTTTCAA		PCR and sequencing

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

	Nucleic mutation	*Schistosoma rodhaini* 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	-	-

808

Supplementary material

~~**Supp. fig. 1**~~

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 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; ~500 km and ~3,000 km, respectively).

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

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

*Graphical Abstract (for review)

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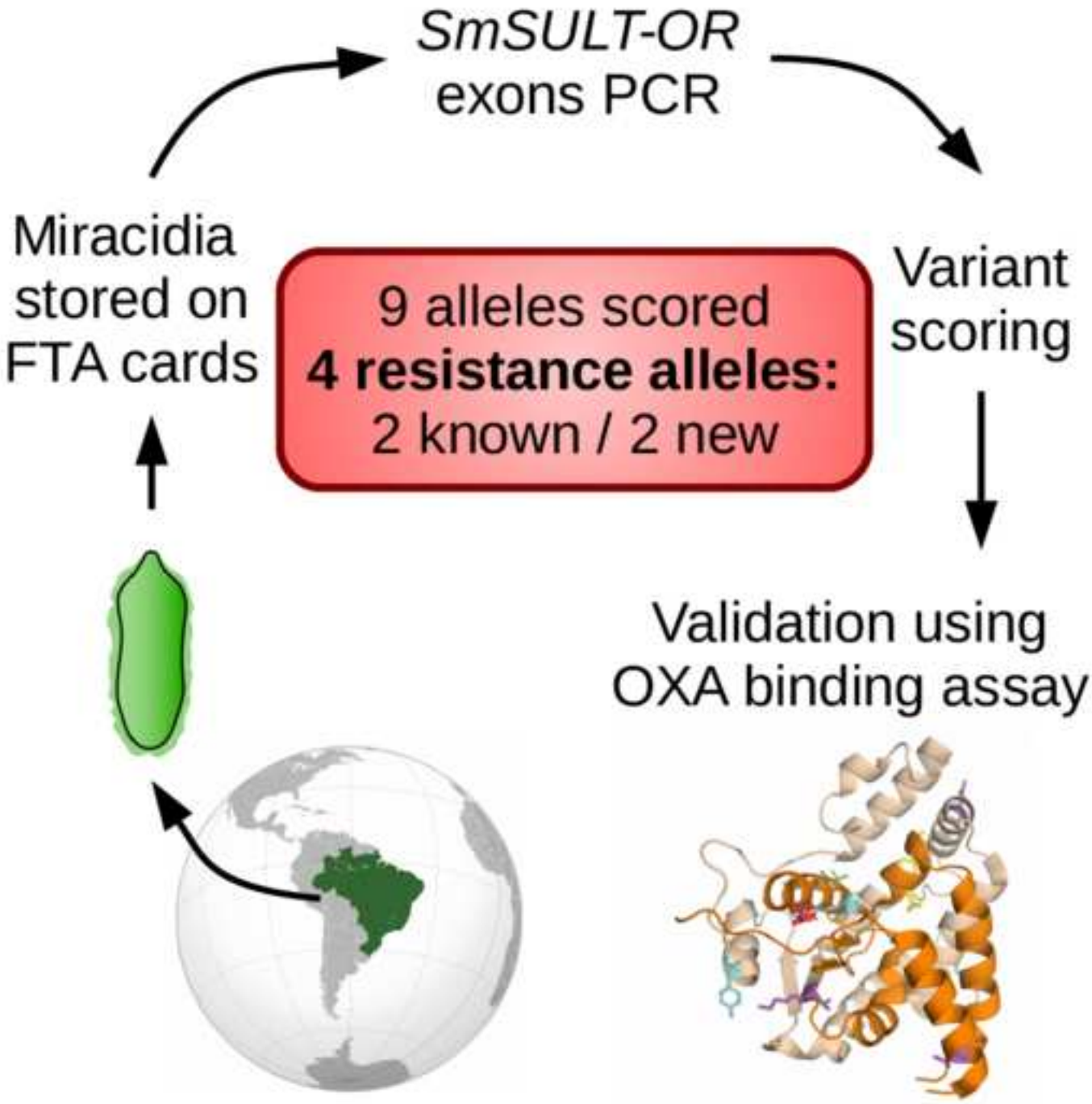

Highlights:

- 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

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

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

Note: Supplementary data associated with this article

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.

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Ethics statement

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

2.2. Sampling of Schistosoma mansoni miracidia

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

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

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

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

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

2.4. WGA

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

2.5. Sequencing of SmSULT-OR exons

The two exons of the *SmSULT-OR* gene were amplified and sequenced independently. Each PCR was performed using the TaKaRa Taq kit (Clontech, USA) and composed of 9.325 µL of sterile water, 1.5 µL of 10× buffer, 1.2 µL of dNTP (2.5 mM each), 0.9 µL of $MgCl_2$ (25 mM), 0.5 µL of each primer (10 µM; Table 1), 0.075 µL of Taq polymerase (5 $U.\mu L^{-1}$) and 1 µL of DNA

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

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

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

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

2.6. Variant identification and functional impact evaluation

We scored variants using PolyPhred software (v6.18) (Nickerson et al., 1997) which relies on Phred (v0.020425.c), Phrap (v0.990319), and Consed (v29.0) software, analyzing each exon independently. We identified single nucleotide polymorphisms using a minimum phred quality score (-q) of 40, a minimum genotype score (-score) of 90, and a reference sequence that includes the *SmSULT-OR* gene and surrounding regions (position 1519500 to 1525200 of chromosome 6 of *S. mansoni* reference genome v5.0, 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 the reference sequence. We identified Insertion/deletion (indel) polymorphisms using a minimum phred quality score (-q) of 40, a minimum genotype score (-iscore) of 80. Polymorphisms were visually validated using Consed. All the sequences were submitted to GenBank (GenBank accession no **KU951903-KU952091**).

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

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

2.7. Population genetics analysis

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

2.8. Recombinant SmSULT-OR protein production

The *SmSULT-OR* sequence from the reference genome (Smp_089320; GenBank accession no. **HE601629.1**) was used to create a codon optimized synthetic gene (GenScript) which was subcloned into the pAG8H vector derived from pKM260 (Melcher, 2000). We introduced mutations using a Phusion site-directed mutagenesis protocol (ThermoFisher Scientific, USA). Transformed *Escherichia coli* strain BL21 pLysS (Promega) were grown at 37°C until the absorbance (λ = 600 nm) reached 0.7. We then decreased the temperature to 18°C and induced expression by the addition of isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM. The cells were washed and resuspended in 50 mL of 50 mM Tris pH 8.0, 500 mM NaCl (column buffer) containing 250 µL of Sigma protease inhibitor cocktail per liter of culture and lysed by sonication on ice. The clarified supernatant was loaded onto a Ni^{2+}-NTA affinity chromatography column (GE Healthcare), washed with five volumes of column buffer,

and eluted using a 10-500 mM imidazole gradient. We pooled fractions identified as the Smp_089320 via SDS-PAGE and added His-tagged Tobacco etch virus (TEV) protease at a Smp_0893230:protease ratio of 15:1. The resulting solution was dialyzed overnight at 4°C against 50 mM Tris pH 8.0, 100 mM NaCl, 2 mM dithiothreitol (DTT). We passed the dialysate over the Ni^{2+}-NTA column again to remove the His tag and the His-tagged TEV protease, while the cleaved target protein flowed through. The sample was loaded onto a GE-pre-packed Q anion exchange column and eluted with a 0.1-1.0 M NaCl gradient. We pooled fractions containing Smp_089320 as identified by SDS-PAGE and dialyzed overnight at 4°C against 25 mM Tris pH 8.5, 50 mM NaCl, 2 mM reducing agent tris-(carboxyethyl)-phosphine (TCEP) to prevent formation of intermolecular disulfide bonds. The product was ~98% pure as estimated by SDS-PAGE. We concentrated the purified Smp_089320 protein to 10 $mg.mL^{-1}$ using the calculated extinction coefficient ε = 39,880 $mol^{-1}.cm^{-1}$.

2.9. OXA activation assay

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

Recombinant proteins were expressed and purified commercially by GenScript, USA (p.C35R, p.P67L, p.E142del; Valentim et al., 2013) or by the Hart laboratory (University of Texas Health Science Center, USA) (p.G206V and p.N215Y). To determine which recombinant protein has the ability to activate OXA, 1 nM from each recombinant protein was added to 90 µL of a protease inhibitor cocktail (PIC) consisting of 0.1 M HEPES pH 7.4, 0.1mM leupeptin, 2 µM E-64, 2 µM pepstatin A, 0.1 U of aprotinin, and 10 $ng.\mu L^{-1}$ sheared *S. mansoni* gDNA as a final target. For each reaction 100 µCi of ^{3}H-OXA (Pica-Mattoccia et al., 1989) was solubilized in 2 µL of DMSO and added to 10 µL of a mixture containing the enzyme cofactors ATP and $MgCl_2$ at 50 mM each, and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) at 1 mM. The radiolabeled OXA and co-factor mix was then added to the PIC mix containing the recombinant protein. The resulting reaction was incubated for 2.5 h at 37°C and stopped by adding three volumes of 0.1% SDS in 0.1 M sodium bicarbonate. The reaction was then extracted three times with two

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

2.10. Statistical analysis

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

3. Results

3.1. Brazilian samples

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

3.2. SmSULT-OR variants in a Brazilian schistosome population

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

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

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

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

Low frequency mutations were found in heterozygous or in homozygous states among the samples. Observing rare SNPs present as homozygotes was surprising, suggesting population structure. The test for Hardy-Weinberg equilibrium showed a global deficit in heterozygosity ($P < 0.0001$). This is likely due to a deficit of heterozygous genotypes within host ($F_{is} = 0.3251$) rather than due to population differentiation between infections ($F_{st} = 0.0153$). 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.

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

3.3. Functional impacts of mutations

3.3.1. Non-coding variants

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

3.3.2. Coding variants

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

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

3.3.3. Indels

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

3.4. OXA activation assay

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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Figures

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

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

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

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

Reference

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

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

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

	Nucleic mutation	*Schistosoma rodhaini* 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' UTR	g.4720C>T	C	177	9	188	0.9654	-	-
	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

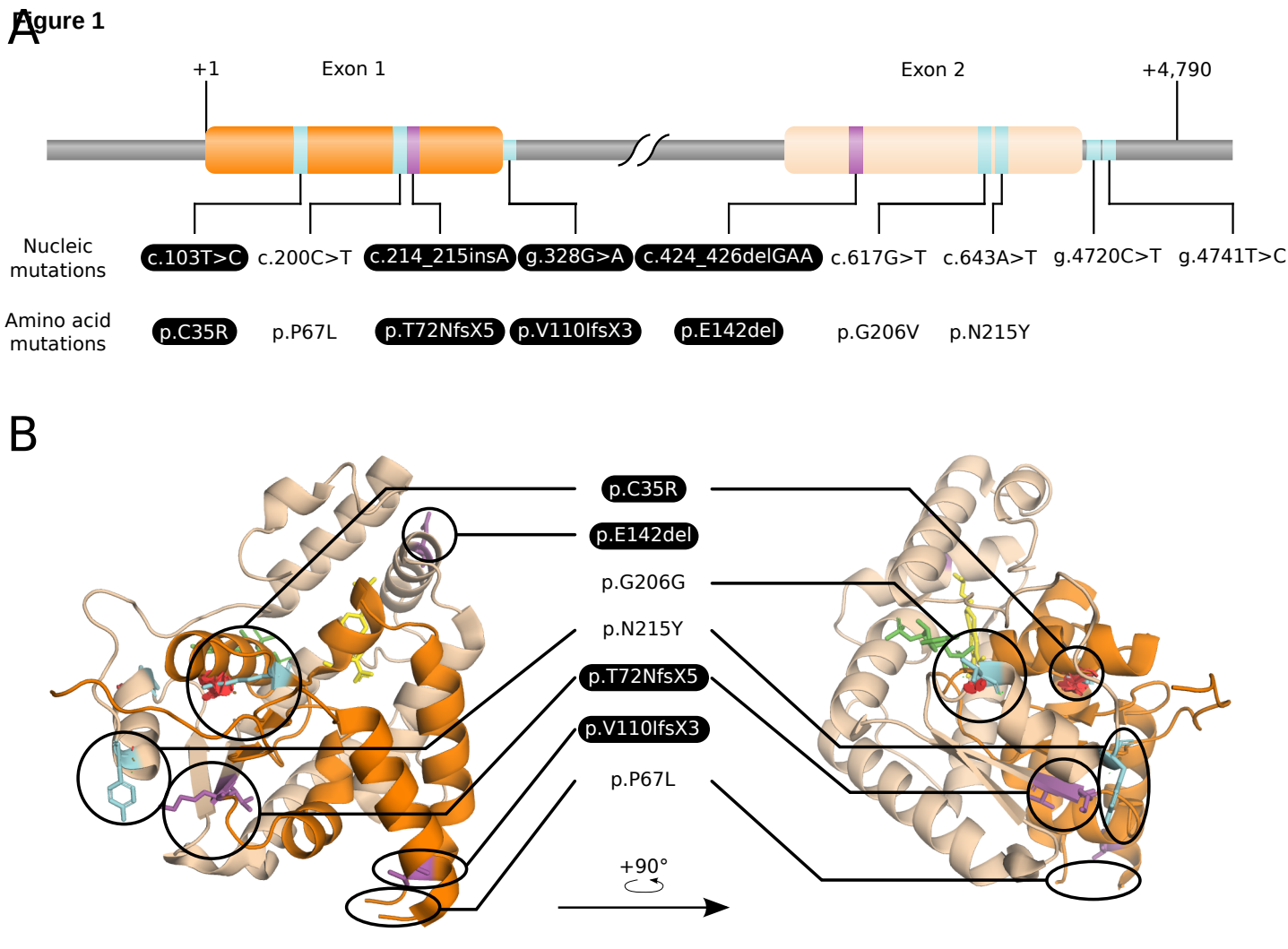
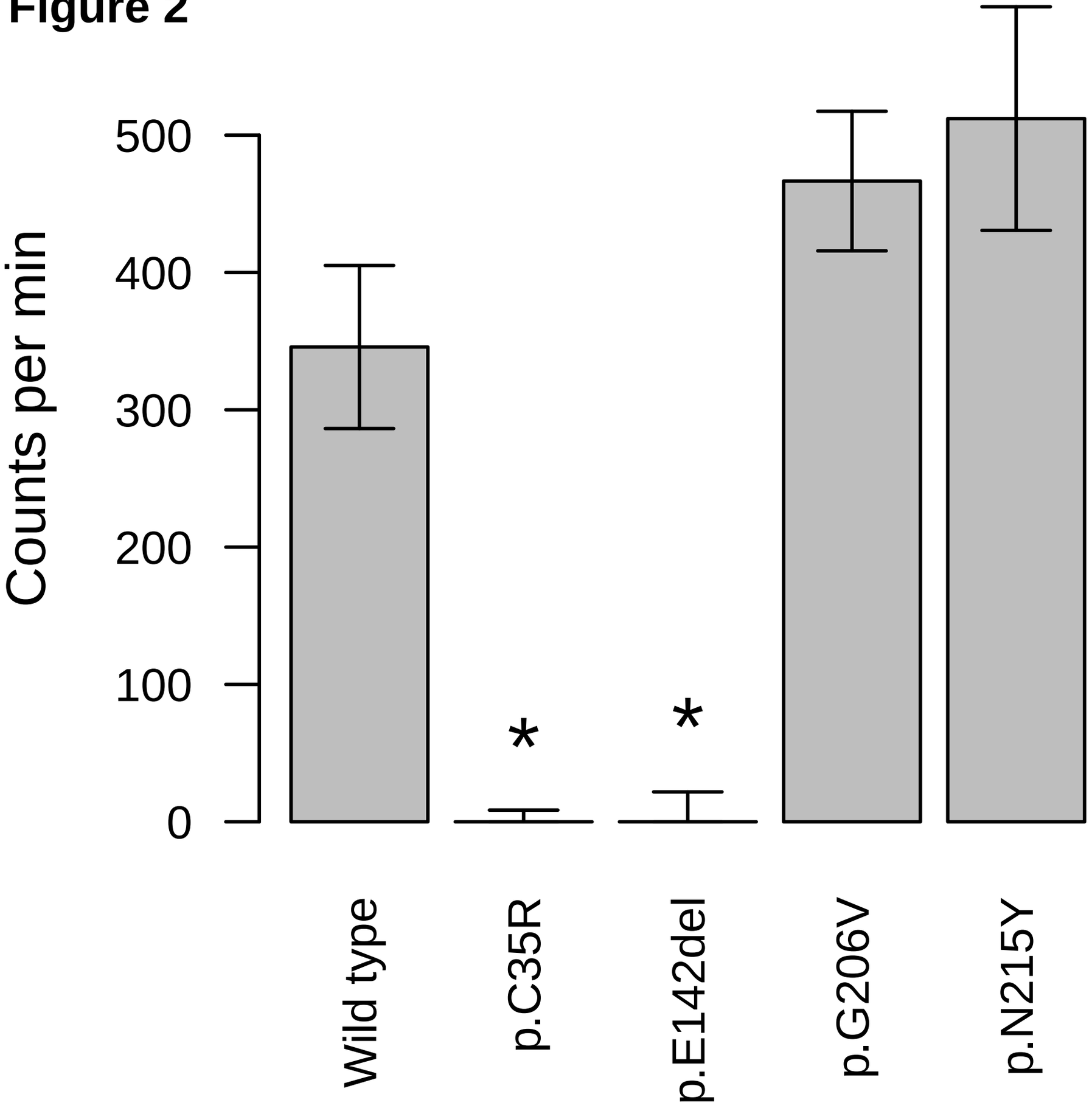

A
+1
Exon 1
Exon 2
+4,790
Nucleic mutations
c.103T>C
c.200C>T
c.214_215insA
g.328G>A
c.424_426delGAA
c.617G>T
c.643A>T
g.4720C>T
g.4741T>C
Amino acid mutations
p.C35R
p.P67L
p.T72NfsX5
p.V110IfsX3
p.E142del
p.G206V
p.N215Y
B
p.C35R
p.E142del
p.G206G
p.N215Y
p.T72NfsX5
p.V110IfsX3
p.P67L
+90°

Figure 2


Counts per min
500
400
300
200
100
0
*
*
Wild type
p.C35R
p.E142del
p.G206V
p.N215Y

Supplementary Table S1

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